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EXPLORATION OF FUNCTIONAL NUCLEAR UROTENSIN II RECEPTORS: BIOCHEMICAL AND PHARMACOLOGICAL CHARACTERISTICS, AND SPECIFIC TISSUE DISTRIBUTION

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LIST OF ABBREVIATIONS

Abbreviation	Full name
9CRA	*cis*-retinoic acid
AC	adenylyl cyclase
ACTH	adrenocorticotropic hormone
Ang-II	angiotensin II
AT_1, AT_2	angiotensin II receptor type 1, angiotensin II receptor type 2
ANP	atrial natriuretic peptide
AP-R	apelin receptor
AR	adrenergic receptor
arr	arrestin
BK	bradykinin
B2R	bradykinin 2 receptor
BNP	brain natriuretic peptide
cAMP	cyclic adenosine monophosphate
CaN	calcineurin
CBF	cerebral blood flow
CCR2	chemokine 2 receptor
CKD	chronic kidney disease
CNS	central nervous system
COX-2	cyclooxygenase-2
c-Src	cellular-proto-oncogene
CTGF	connective tissue growth factor
CX43	connexin 43
Cyr61	cysteine-rich angiogenic inducer 61
DNase I	deoxyribonuclease I
DTT	dithiothreitol
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
eNOS	endothelial nitric oxide synthase
EP3-R	prostaglandin E_2 receptor type 3
ER	endoplasmic reticulum
ERK1/2	extracellular regulated kinase 1 and 2
ESRD	end-stage renal disease
ET-1	endothelin 1,
ET_A, ET_B	endothelin receptor type A, endothelin receptor type B
βFGF	β fibroblast growth factor

$GABA_AR\gamma$	γ-aminobutyric acid receptor
GDP	guanosine diphosphate
GnRH	gonadotropin-releasing hormone
GnRH-R	gonadotropin-releasing hormone receptor
GPCR	G protein-coupled receptor
GPR14	G protein-coupled receptor 14
GRK	G protein-coupled receptor kinase
GSK-3β	glycogen synthase kinase-3β
GTP	guanosine triphosphate
HDP	dihydropyridin
i.v.	intravenous
icv	intracerebroventricular
IGF-I	insulin-like growth factor
Imp	importin
iNOS	inducible nitric oxide synthase
IP3	inositol triphosphate
InsP3R	inositol trisphosphate receptor
JNK	C-Jun N-terminal protein kinase
LAM	lymphagioleiomyomatosis
LPA1-R	lysophosphatidic receptor type 1
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase
mGlu5	metabotropic glutamate receptor
MLCK	calmodulin/myosin light chain kinase
moxLDL	mildly oxidized low-density lipoprotein
MW	molecular weight
$n[Ca^{2+}]$	nuclear calcium concentration
NADPH	nicotinamide adenine dinucleotide phosphate
NARFL	nuclear prelamin A recognition factor-like
NDP	nucleoside diphosphate
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLS	nuclear localization signal
NO	nitric oxide
NT	neurotensin
NTS2	neurotensin receptor type 2
Nup62	nucleoporin p62
PACAP	pituitary adenylate cyclase-activating polypeptide
PAF-R	platelet activating factor receptor
PGE2	prostaglandin

PI3K	phosphatidylinositol 3-kinases
PKA	protein kinase A
PKB or AKT	protein kinase B
PKC	protein kinase C
PTH1R	parathyroid hormone receptor type 1
PTX	pertussis toxin
REM	rapid eye movement
RhoA	Ras homolog gene family, member A
RhoK	Ras homolog gene family kinase
RNase	ribonuclease
ROS	reactive oxygen species
SRIF	somatostatin
stt-R	somatostatin receptor
S1P	sphingosine 1 phosphate receptor type 1
SERCA	sarco-endoplasmic reticulum Ca^{2+} ATPase
SHP-2	Src homology-2 containing tyrosine phosphatase
SMC	smooth muscle cell
TGF	transforming growth factor
TM	transmembrane domain
TMEM33	transmembrane protein 33
Trn1	transportin 1
UII, hUII, rUII	urotensin II, human urotensin II, rat urotensin II
URP	urotensin II-related peptide
UT	urotensin II receptor
VIP	vasoactive intestinal peptide
VPAC1	vasoactive intestinal peptide receptor-1

RÉSUMÉ EN FRANÇAIS

L'urotensine II (UII) a été initialement isolée de l'urophyse de gobie et il a été rapidement établi que le peptide possède plusieurs caractéristiques structurales similaires à celles de la somatostatine (SRIF). Ce peptide et son paralogue, l'*urotensin II-related peptide* (URP), provoquent une vasoconstriction chez de nombreuses espèces y compris les poissons, les amphibiens et les mammifères. L'UII et l'URP sont les ligands endogènes du récepteur à 7 domaines transmembranaires nommé UT. En plus de leurs capacités à causer la contraction et la dilatation de tissus vasculaires, l'UII peut induire la prolifération de plusieurs types cellulaires ou encore causer par exemple l'hypertrophie des cardiomyocytes. En fait, le système urotensinergique est associé à de nombreuses pathologies humaines, et plus particulièrement aux maladies cardiovasculaires et rénales. Par contre, malgré la présence marquée du système urotensinergique dans le système nerveux central (SNC), une relation claire entre ce système et des pathologies du SNC n'a pas encore été établie, même si une injection intracérébroventriculaire (icv) d'UII provoque des effets centraux.

L'UT appartient à la classe A de la super famille des récepteurs couplés aux protéines G (RCPGs), une famille de récepteurs ayant comme archétype la rhodopsine. De façon générale, il est admis que ces récepteurs sont localisés à la membrane plasmique mais récemment, une localisation nucléaire a été démontrée pour de nombreux RCPGs. Notamment, les récepteurs de l'angiotensine II (Ang-II) et de l'endothéline 1 (ET-1) qui se retrouvent dans la même famille de récepteurs qu'UT et qui partagent des fonctions similaires, ont été observés au niveau des noyaux. Ces récepteurs nucléaires représentent donc de nouvelles cibles pharmacologiques et une bonne compréhension de leur activité cellulaire est essentielle pour le développement d'outils pharmacologiques adéquats.

Ce projet d'étude visait donc à démontrer la présence de récepteurs UT fonctionnels au niveau du noyau de cellules isolées de divers organes. Nos résultats d'immunobuvardage de type Western ont permis d'établir pour la première fois la présence de ce récepteur aux noyaux de cellules dans les tissus du cœur et du système nerveux central chez le rat et le singe, et aussi dans des lignées cellulaires humaines de neuroblastomes et d'astrocytes. Étonnamment, parmi tous les

tissus évalués, incluant le cœur, le cerveau, la moelle épinière, les reins, les glandes surrénales, le foie, les poumons, le pancréas et les muscles squelettiques qui expriment tous UT au niveau membranaire, la présence de ce récepteur au noyau n'a été observée que dans le cœur et le système nerveux central. De plus, cette régiosélectivité d'expression du récepteur UT aux noyaux des cellules ne semblent pas être une caractéristique propre à une espèce puisque ce phénomène a été observé chez le rat et le singe. Enfin, ces résultats ont été confirmés par immunofluorescence par microscopie confocale avec des coupes de cœur et de cerveau de ces deux espèces animales ainsi qu'avec des lignées cellulaires humaines. Un examen plus détaillé de l'expression de l'UT dans le SNC de rat a révélé que ce récepteur était exprimé dans tous les tissus centraux étudiés, *i.e.* le cortex, l'hypothalamus, l'hippocampe, le cervelet, le pons et la moelle épinière, tant au niveau des protéines totales que nucléaires. Par la suite, des études de liaison sur des noyaux isolés de cœur et de cerveau de rat ont permis de caractériser pharmacologiquement ce récepteur nucléaire. Pour ce faire, les deux ligands endogènes de l'UT, soit l'UII et l'URP, ainsi que l'urantide, un antagoniste de l'UT, et la somatostatine ont été utilisés. Les résultats ont démontré que les ligands de l'UT, soit l'UII, l'URP et l'urantide, étaient capables de déplacer l'UII radiomarqué lié aux sites de liaison situés sur des noyaux isolés alors que la somatostatine n'avait pas cette capacité. L'immunobuvardage 2D a dévoilé trois isoformes de l'UT dans les fractions membranaires et nucléaires de cœur ayant un même poids moléculaire mais des points isoélectriques différents, alors que dans les extraits provenant du cerveau, il semble que des modifications post-traductionnelles distinctes pour les récepteurs aux noyaux et à la membrane génèrent des espèces protéiques de poids moléculaires différents. Des essais d'initiation de la transcription utilisant de l'UII humaine et de rat, de l'URP et de l'urantide sur des noyaux isolés de cœur et de cerveau de rat ont démontré que ces récepteurs nucléaires étaient fonctionnels. Toutefois, les agonistes de l'UT ont modulé différemment la transcription car seule l'UII a été capable d'initier la synthèse *de novo* d'ARN. La question s'est ensuite posée à savoir si les deux peptides pouvaient atteindre leur récepteur nucléaire. Pour ce faire, des dérivés fluorescents des deux ligands endogènes ont été préparés pour évaluer leur capacité de pénétration cellulaire. Les résultats obtenus en microscopie confocale et par cytométrie en flux démontrent que les deux ligands de l'UT peuvent atteindre l'espace intracellulaire, l'UII pénètrant dans la cellule plus rapidement que l'URP, sans impliquer UT. En outre, le mécanisme

d'entrée de l'URP impliquerait principalement les cavéoles alors que l'UII n'utiliserait ce mécanisme que très partiellement.

Comme mentionné ci-dessus, le système urotensinergique est associé à divers états pathologiques, mais une relation claire avec des atteintes centrales n'a pas encore été démontrée. Par ailleurs, dans cette étude, la présence d'UT a été clairement démontrée dans tous les tissus du SNC, tant dans les extraits totaux que nucléaires. Or, ces tissus sont très sensibles aux conditions hypoxiques et/ou ischémiques. Ainsi, l'influence de telles conditions sur l'expression de ce récepteur a été étudiée sur une lignée cellulaire humaine neuroblastomale en présence ou non d'un traitement avec l'UII ou l'URP. Nos résultats indiquent qu'une exposition au chlorure de cobalt (pour induire des conditions hypoxiques *in vitro*) diminue l'expression de l'UT nucléaire tandis que son expression totale augmente. De plus, un traitement avec l'un ou l'autre des deux agonistes de l'UT a ramené l'expression de l'UT à un niveau basal. Clairement, la localisation intracellulaire de ce récepteur est modulée en conditions hypoxiques et l'UII et l'URP pourraient avoir un effet répresseur sur cette modulation.

Contrairement aux connaissances restreintes associées au SNC, le système urotensinergique a été largement étudié en relation avec les maladies cardiovasculaires. Au cours de cette étude, une analyse d'expression génique par micro-réseaux sur des noyaux isolés de cardiomyocytes de rat traités avec l'UII a permis d'élargir ces connaissances en suggérant que le récepteur nucléaire UT serait un médiateur de l'expression de certains gènes liés aux maladies cardiaques, au diabète ainsi qu'à la prolifération cellulaire.

Globalement, notre étude suggère que les récepteurs UT fonctionnels présents au noyau joueraient un rôle physiologique complémentaire à l'activation des récepteurs UT à la membrane plasmique. Ces résultats offrent une nouvelle vision plus complexe du fonctionnement du système urotensinergique et pourraient ouvrir de nouvelles voies pharmacologiques dans le développement de stratégies pour le traitement de maladies cardiovasculaires.

INTRODUCTION

Receptors are proteins mainly located at the cell surface, but also within the cytosol, as well as in the nucleus. Among cell surface receptors, G protein-coupled receptors (GPCRs), containing seven transmembrane domains (TM), belong to the largest protein superfamily. They are expressed in eukaryotes from yeast to humans where they regulate metabolic and cellular functions through signal transductions, *i.e.* generation of second messenger molecules and cellular phosphorylation cascades (Millar and Newton, 2010). To date, GPCRs account for two thirds of current therapeutic targets. They can be activated by different ligands including hormones, peptides, neurotransmitters and other types of molecules. Thus, ligand-GPCR system studies are among the most active and attractive domains in the pharmacological research (Millar and Newton, 2010; Wang and Lewis, 2012).

GPR14 is a protein belonging to the rhodopsin-like receptor family. It was identified in 1999 as the cognate receptor for urotensin II (UII) (Ames et al., 1999; Liu et al., 1999; Mori et al., 1999; Nothacker et al., 1999), a potent vasoconstrictor initially isolated from the goby urophysis (Pearson et al., 1980). This receptor, now renamed urotensin II receptor (UT), is also the receptor of another endogenous ligand isolated in the rat brain and called urotensin II-related peptide (URP) (Sugo et al., 2003). Both peptides exert vasoconstrictive effects on vascular beds from many species including fishes, amphibians and mammals (Vaudry et al., 2010). Biological activities of UII and URP were well studied and in addition to UII ability to cause constriction or dilation of vascular tissues, this peptide also induces cell proliferation, as well as cardiac and vascular hypertrophy (Jarry et al., 2010; Papadopoulos et al., 2008; Watanabe et al., 2001b; Yoshimoto et al., 2004). UII can also cause strong angiogenic effects (Albertin et al., 2009; Guidolin et al., 2010; Spinazzi et al., 2006), induce collagen and fibronectin accumulation (Dai et al., 2007; Zhang et al., 2008) and modulate the inflammatory response (Shiraishi et al., 2008). Besides, it was shown that intracerebroventricular (icv) injection of UII causes central effects including behavioural and endocrine actions (Do-Rego et al., 2005; Do-Rego et al., 2008; Gartlon et al., 2001). However, the physiological role of the urotensinergic system in the central nervous system (CNS), where it is widely distributed, is still unclear. Due to the abovementioned

effects and its up-regulation in many pathophysiological states, the urotensinergic system was linked to numerous diseases including cardiovascular diseases, diabetes, renal and liver diseases, as well as neurological disorders (Ross et al., 2010).

Until recently, GPCRs were only considered as plasma membrane proteins but now, there is growing evidence of their intracellular presence, especially in nucleus. Indeed, angiotensin II receptors (AT_1 and AT_2), endothelin receptors (ET_A and ET_B), apelin receptor and adrenergic receptors (AR) were concomitantly observed at the cell membrane and nucleus (Tadevosyan et al., 2012). Several studies on isolated nuclei demonstrated that these receptors are activated by their agonist(s) thus resulting in an increase of nuclear calcium concentration ($n[Ca^{2+}]$) (Bkaily et al., 2009; Boivin et al., 2003), kinase phosphorylation (Boivin et al., 2003; Boivin et al., 2006), transcription initiation (Boivin et al., 2006; Vaniotis et al., 2011) or gene expression (Eggena et al., 1993; Thomas et al., 2006). Though the origin of these nuclear GPCRs is still speculative, there is evidence suggesting that they may translocate from the cell surface to the nucleus following the recognition of specific nuclear localization signals (NLS) within their amino acid sequence by cytosol-nuclear transporters such as importins (Lee et al., 2004). However, other studies have shown the existence of NLS-independent nuclear translocation mechanisms (Re et al., 2010; Zhu et al., 2006) and it has also been hypothesized that these nuclear GPCRs could arise from *de novo* protein synthesis (Gobeil et al., 2006a).

GPCRs play very important physiological roles since they transfer up to 80% of total cell signaling across the plasma membrane (Millar and Newton, 2010). As such, they are targets for many pharmaceutical products. Thus, a better understanding of GPCR pharmacology represents the basis for the development of new therapeutic drugs. In the present study, we demonstrated for the first time the tissue-specific distribution of nuclear UT. As a matter of fact, nuclear UT was only found in cardiac and CNS tissues isolated from rat and monkey as well as in human neuronal and glial cell lines (Doan et al., 2012b; Nguyen et al., 2012). We also reported that nuclear receptors are functional and that their cellular distribution appeared to be modulated under hypoxic conditions (Nguyen et al., 2012). Finally, our study on gene expression using isolated nuclei from rat cardiac cells suggests that nuclear UT could mediate important processes involved in cardiovascular pathologies.

Antagonists of angiotensin II and endothelin receptors are now used as drugs in cardiovascular risk management (Aronson and Krum, 2012; McLaughlin et al., 2011). UT specific antagonists have also been developed but most of them have failed in terms of clinical efficacy (Maryanoff and Kinney, 2010), a situation that could be attributable to the absence of intracellular evaluation of these molecules. Thus, studies regarding the physiological and pathophysiological role of those intracellular UT receptors are extremely important. The fact that these GPCR systems can have their pharmacological profile complexified by the concomitant existence of signaling pathways arising from the plasma membrane and from intracellular organelles could surely contribute to the development of new pharmacotherapy approaches in the future.

SECTION 1: LITERATURE REVIEW

CHAPTER 1: THE UROTENSINERGIC SYSTEM

Introduction

Peptides, as well as proteins, play critical roles in the regulation of most living organism processes, such as metabolism, pain, reproduction and immunological responses. Peptides are produced within cells and then secreted into the bloodstream to act as endocrine factors or in the surrounding environment where they can act on the same cell (autocrine signaling) or nearby cells (paracrine signaling) (Sewald and Jakubke, 2009). In fact, neurotransmitters, neuropeptides, lipopeptides and peptide hormones transmit their signals to cells via the activation of specific receptors that mediate highly complex chemical signaling systems to achieve intercellular biochemical communication (Sewald and Jakubke, 2009). The involvement of these peptides and their receptors in various biological functions represents an important research field in modern life science.

Urotensin II (UII), a disulfide bridged peptide hormone characterized in various species, encompasses a highly conserved C-terminal cyclic core and a variable N-terminal region. This peptide contains a triad (Phe-Trp-Lys) located in the hexacyclic C-terminal portion that is also present in somatostatin (SRIF), an inhibitory growth hormone secreted from the pituitary gland (Veber et al., 1979). Both SRIF and goby UII possess an Ala-Gly dipeptide in their N-terminal segment. In addition, the two peptides share some structural features such as the presence of a disulfide bridge. This disulfide bond in UII plays a crucial role in the maintenance of its biological activity like the hydrophobic π-stacking interaction between Phe^{6} and Phe^{11} in SRIF (Pearson et al., 1980) (Figure 1-1). However, UII and SRIF differ in terms of their key biological activities.

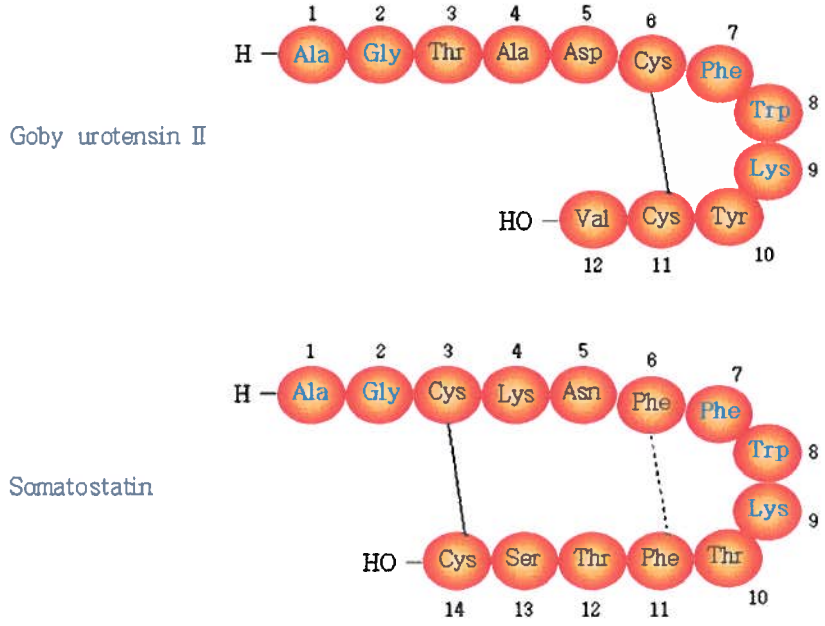

Figure 1-1: Comparison of structures of goby urotensin II (UII) and somatostatin (SRIF). Homologous sequences are in blue letters.—— Disulfide bonds; ---- π-stacking interaction

Briefly, UII can act both as a vasoconstrictor and a vasodilator in mammals and it is also involved in cell proliferation and cardiomyocyte hypertrophy (Vaudry et al., 2010). In the late twentieth century, it was discovered that GPR14, an orphan GPCR, was the cognate receptor of UII (Ames et al., 1999) and also of another endogenous ligand termed urotensin II-related peptide (URP) (Sugo et al., 2003). This octapeptide, possessing like UII the same C-terminal hexacyclic core, is also able to cause constriction along with other UII-associated biological actions. Both peptides, as well as their receptor, are widely distributed in mammals where they exert multiple biological activities directly involved in the control and regulation of physiological functions. As such, the urotensinergic system was linked to the pathogenesis and

the progression of various diseases including cardiovascular and renal diseases, as well as diabetes (Ross et al., 2010). This system is also highly present in the CNS, but up to now, no putative UT-associated neuronal disease has been reported. Therefore, the role of the urotensinergic system in mammals, including humans, still remains to be clarified although this peptidergic system appears to be a potent modulator of the cardiovascular system.

1. Urotensin II and Urotensin II-Related Peptides

1.1. Discovery

Urotensin II was initially isolated in the caudal neurosecretory system of the *Gillichthys mirabilis*, a teleost fish known for its ability to survive in extremely severe conditions, such as prolonged hypoxic periods, huge alterations of temperature and salinity (Pearson et al., 1980). Initially considered as a hormone released from the caudal neurosecretory system of teleost fish, UII was later observed in the CNS of various fish subclasses, such as chondrosteans, elasmobranchs and agnatha (Conlon, 2008). These studies indicated that the UII gene was phylogenetically ancient and had been generated before teleosts. Interestingly, the vascular effects caused by goby UII in mouse and rat smooth muscle tissues suggested its existence in higher vertebrates including mammals (Gibson, 1987; Gibson et al., 1984). Indeed, the peptide was subsequently isolated from a brain extract of an amphibian (European green frog) (Conlon et al., 1992). Later, human UII (hUII) was found to derive from a pre-pro protein encoded by a gene located at chromosome 1p36. The protein contains 124 amino acids that inferred from cDNA first cloned in the spinal cord (Coulouarn et al., 1998). Interestingly, another UII cDNA encoding sequence, which varies mostly at the peptide signal sequence, was also discovered (Ames et al., 1999) (Figure 1-2). To date, from fish to human, the length of this peptide ranges from 11 residues for human to 17 residues for mouse (Vaudry et al., 2010) (Table 1-1).

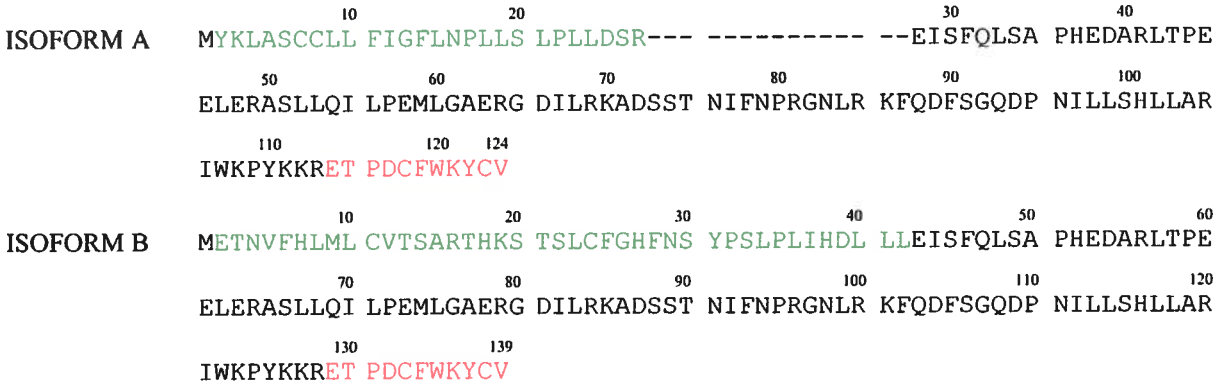

Figure 1-2: Amino acid sequence comparison of hUII propeptide isoform A (Coulouarn et al., 1998) and isoform B (Ames et al., 1999). The putative mature hUII sequence is shown in red and heterologous sequences are shown in green.

Two decades after the first isolation of UII, a peptide paralogue, *i.e.* URP, was isolated in the rat brain as the sole immunoreactive species (Sugo et al., 2003). This peptide was also characterized in human where its gene is located in close proximity to the SRIF gene on chromosome 3q28 (Sugo et al., 2003). The UII gene, located in 1p36, although its peptide shares some structural similarities to somatostatin, does not appear to be related to the SRIF gene but rather to the cortistatin gene (Tostivint et al., 2006), which encodes a neuropeptide belonging to the somatostatin peptide family (Spier and de Lecea, 2000). So far, UII and URP were isolated or predicted in nearly 40 species (Vaudry et al., 2010). However, due to its earlier discovery, publications about UII are still predominant.

Table 1-1: Amino acid sequence of UII and URP in various species

(Adapted from Vaudry et al., 2010)

Species	Sequence
UII	
Goby	H-Ala-Gly-Thr-Ala-Asp-*Cys*-Phe-Trp-Lys-Tyr-*Cys*-Val-OH
Frog	H-Ala-Gly-Asn-Leu-Ser-Glu-*Cys*-Phe-Trp-Lys-Tyr-*Cys*-Val-OH
Chicken	H-Gly-Asn-Leu-Ser-Glu-*Cys*-Phe-Trp-Lys-Tyr-*Cys*-Val-OH
Mouse	Glp-His-Lys-Gln-His-Gly-Ala-Ala-Pro-Glu-*Cys*-Phe-Trp-Lys-Tyr-*Cys*-Ile-OH
Rat	Glp-His-Gly-Thr-Ala-Pro-Glu-*Cys*-Phe-Trp-Lys-Tyr-*Cys*-Ile-OH
Monkey	H-Glu-Thr-Pro-Asp-*Cys*-Phe-Trp-Lys-Tyr-*Cys*-Val-OH
Chimpanzee	H-Glu-Thr-Pro-Asp-*Cys*-Phe-Trp-Lys-Tyr-*Cys*-Val-OH
Human	H-Glu-Thr-Pro-Asp-*Cys*-Phe-Trp-Lys-Tyr-*Cys*-Val-OH
URP	
Chicken	H-Ala-*Cys*-Phe-Trp-Lys-Tyr-*Cys*-Ile-OH
Mouse	H-Ala-*Cys*-Phe-Trp-Lys-Tyr-*Cys*-Val-OH
Rat	H-Ala-*Cys*-Phe-Trp-Lys-Tyr-*Cys*-Val-OH
Chimpanzee	H-Ala-*Cys*-Phe-Trp-Lys-Tyr-*Cys*-Val-OH
Human	H-Ala-*Cys*-Phe-Trp-Lys-Tyr-*Cys*-Val-OH

Conserved hexacyclic sequences of UII and URP are shown in red color; Cys residues that form a disulfide bond in UII and URP are shown in italic letters; green and blue represent adjacent non-polar and acidic residues of the cyclic sequence; Glp: pyroglutamic acid

1.2. Distribution

Because UII was found in a fish caudal neurosecretory organ that does not exist in mammals, the peptide was initially considered as an anomalous neuropeptide in other species. However, as mentioned earlier, it is known that UII is not restrictively distributed to the fish urophysis since it has also been found in the CNS of both teleost and non-teleost fishes (Conlon, 2008). In rodents and human CNS, UII is ubiquitously distributed with highest levels of UII mRNA observed in the medulla oblongata and the ventral horn motoneurons (Conlon, 2008;

Dubessy et al., 2008). The URP expression is rather different to what was described for UII in the CNS (Conlon, 2008; Dubessy et al., 2008). As a matter of fact, UII mRNA was more expressed in this tissue than URP mRNA (Sugo et al., 2003). A similar pattern, observed in both rat and human spinal cord, raised the hypothesis that URP could play a more restricted physiological or pathological role, while UII would be associated with systemic neuroendocrine functions (Sugo et al., 2003).

In human peripheral tissues, although very low UII mRNA was detected in cardiovascular tissues (Matsushita et al., 2001), hUII-like immunoreactivity is mostly observed in the endothelial cells but not in the smooth muscle cells (SMCs) of aorta and epicardial coronary (Maguire et al., 2004). This peptide is also present in non-cardiac vasculatures such as internal mammary and umbilical arteries, capacitance vessels, as well as small resistance vessels within heart, kidney, lung, placenta and adrenal gland (Maguire et al., 2004). The expression of UII and URP mRNA levels in periphery has been shown to be similar in rodent testis and skeletal muscle, in human placenta, ovary, pancreas, prostate, small intestine, spleen and thymus while neither peptide is present in rodent kidneys, in mouse colon, in rat heart and in skeletal muscle in rat and human (Dubessy et al., 2008; Sugo et al., 2003). However, differences in UII/URP distribution are not limited to the CNS but were also observed in the peripheral tissues. For instance, in mouse uterus and pituitary gland where UII is expressed, URP was not observed. In a similar manner, while URP mRNA is observed in the heart, ovary, prostate and adrenal gland, these organs do not express UII mRNA (Dubessy et al., 2008) (Figure 1-3). Similarly, in rat, only UII mRNA is detected in the pancreas, ovary, uterus, pituitary gland whereas only URP mRNA is present in colon, liver and lung. Moreover, the high gene expression of UII is found in the adrenal gland and upper intestinal parts but the spleen and thymus possess higher URP in rat (Sugo et al., 2003). In human, only UII is expressed in leukocytes and lung while very high URP mRNA level is found in the testis, which expresses little UII (Sugo et al., 2003). As mentioned, the distribution of UII and URP is not only variable among tissues but also between species. High levels of both peptides are found in mouse skeletal muscle but absent in rat and human (Dubessy et al., 2008; Sugo et al., 2003). The human kidney and adrenal gland possess significant UII and URP levels while neither UII nor URP mRNA were found in these tissues in mouse and rat (Dubessy et al., 2008; Sugo et al., 2003) (Figure 1-3). This apparent difference in

these peptide distributions may reflect specific roles that could be species restricted. Clearly, UII and URP expression is tissue-dependent which might infer distinct and/or complementary roles for each peptide that could vary among species.

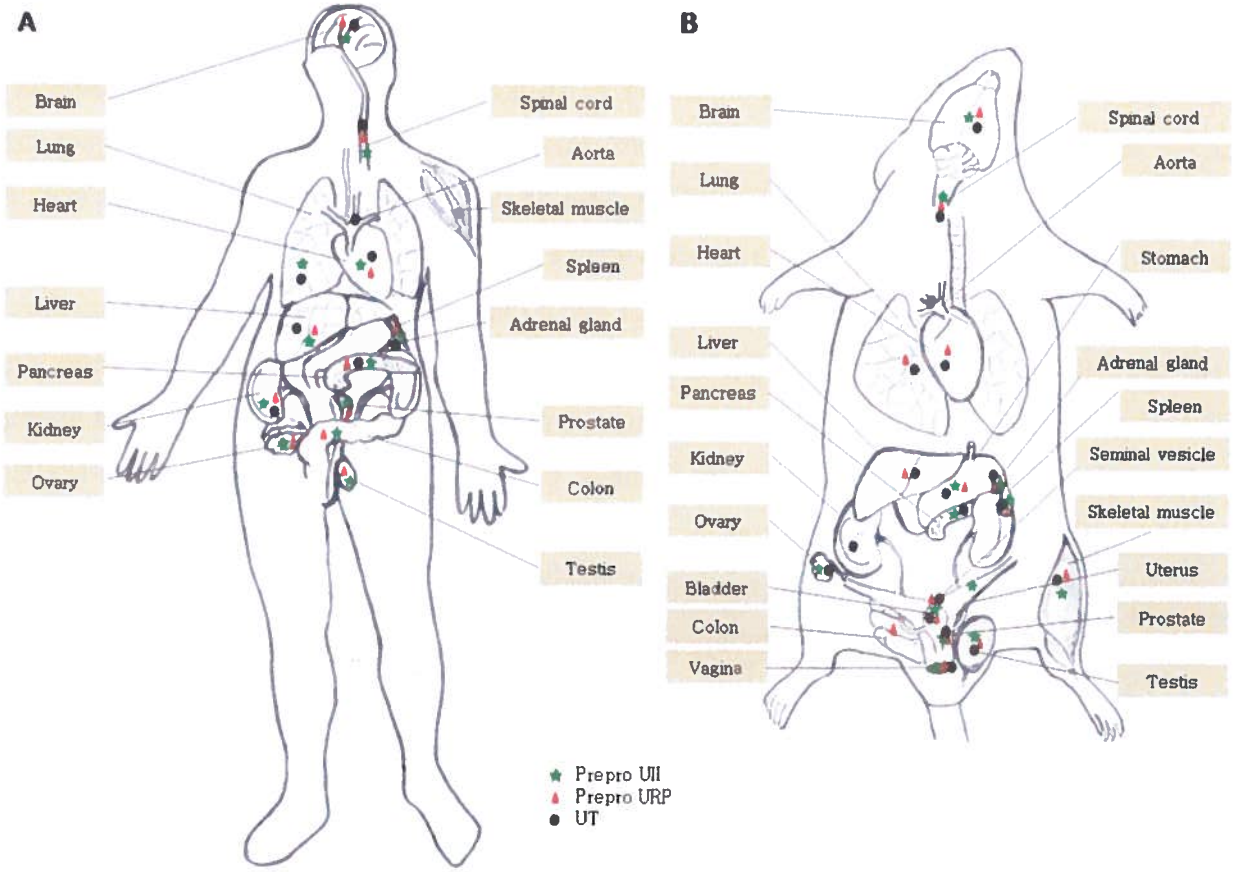

Figure 1-3: Distribution of the urotensinergic system in (A) primates (human and monkey) and (B) rodents (rat and mouse) (Ames et al., 1999; Conlon, 2008; Dubessy et al., 2008; Maguire et al., 2008; Sugo et al., 2003)

1.3. Biological activities

UII exerts various biological activities including both central and peripheral actions in many species. URP, due to its recent discovery, was mainly regarded as a redundant version of UII and therefore only investigated in vasoactive experiments in mammals and some fishes.

1.3.1. Osmoregulatory effects

In fish, UII modulates water and sodium chloride absorption across isolated skin (Marshall and Bern, 1979), urinary bladder (Loretz and Bern, 1981) and intestine (Loretz et al., 1983; Mainoya and Bern, 1982). Recently, some evidence has highlighted a similar role of UII in mammals. Indeed, intravenous (i.v.) bolus administration of rUII to anaesthetized rats caused a reduction in effective renal blood flow, glomerular filtration and urine flow rate as well as urinary sodium excretion (Balment et al., 2005). However, a continuous intra-renal infusion of hUII in rat led to an opposite result (Zhang et al., 2003). At a dose that does not induce any renal haemodynamics, *i.e.* 0.6 pmol/min/100g body weight, rUII exerted direct tubular actions resulting in an increase in fractional excretion of sodium and potassium (Abdel-Razik et al., 2008b). However, observations in children did not show any relationship between UII level and Na^+/K^+concentration (Balat et al., 2005a). Although these effects are complex and inconsistent among species, kidney is a major source of UII in the human body (Matsushita et al., 2001; Nothacker et al., 1999; Shenouda et al., 2002). Altogether, these results suggest that UII could be involved in body fluid homeostasis in higher vertebrates, including humans.

1.3.2. Central effects

While the neurobehavioral actions of endothelin 1 (ET-1) and angiotensin II (Ang-II) have been investigated only recently (Khimji and Rockey, 2010; Nakano-Tateno et al., 2012), early studies demonstrated that UII induces a dose-dependent and long-lasting increase in rat and mouse locomotion (Do-Rego et al., 2008) (Table 1-2). Depression and anxiety-like effects have also been observed following the icv administration of UII (Do-Rego et al., 2008). Besides, UII

induces quite rapidly orexigenic and pro-dipsic effects in mouse (Do-Rego et al., 2005) whereas Ang-II, using the same species, causes a suppressive effect on food intake and a very temporary increase of water intake (Nakano-Tateno et al., 2012; Yoshida et al., 2012). At low doses, UII causes a significant increase of rapid eye movements (REM) in rats probably through the stimulation of brainstem cholinergic neurons. This effect was blocked with a UT antagonist, *i.e.* SB-710411, thus providing evidence that the observed activity occurs through UT activation (Huitron-Resendiz et al., 2005). Moreover, UII also induces norepinephrine release from rat cerebrocortical slices (Ono et al., 2008) suggesting its involvement in the regulation of sleep-wakefulness.

UII icv injection evokes cardiovascular effects in mammals, and fishes as well. Indeed, following UII administrations, an increase of the arterial blood pressure, correlated with a heart rate elevation, was observed in conscious rats (Lin et al., 2003) and sheeps (Hood et al., 2005). In anaesthetized rats, the microinjection of UII into the A1 region of the medulla oblongata induced a bradycardic effect while microinjection into the A2 did not cause any noticeable change. Those results, correlated with the tachycardic and vasopressor effects following UII microinjections into either paraventricular or arcuate nucleus, suggest that this peptide may play different roles in cardiac homeostasis in different cerebral regions (Lu et al., 2002).

Finally, UII can also stimulate the secretion of diverse hormones (Table 1-2). In rats, UII icv injections provoke the release of prolactin along with thyrotropin (Gartlon et al., 2001). In sheeps, UII stimulates the release of adrenaline, a hormone that increases heart rate, constricts blood vessels and regulates ACTH production. Besides, cortisol, a hormone regulated by ACTH, and causes blood sugar increase and inhibition of the immune system, is also secreted upon UII treatment in sheeps (Watson et al., 2003) (Table 1-2). The fact that a high density of binding sites is found in the hypothalamus perfectly correlates with the hormone secretion and feeding behaviour effects observed following icv UII administration (Do-Rego et al., 2005). Since UII regulates the secretion of various hormones, this neuropeptide appears to be involved in the regulation of metabolic, endocrine and immune functions. The complex central actions of UII are in agreement with its widespread receptor distribution in the CNS.

Table 1-2: Effects of UII (icv) in the central nervous system

Effects	Species	References
Behavioral effects		
Hyperlocomotion	Trout, mouse, rat	(Do-Rego et al., 2008)
Depression-like and anxiety-like effects	Mouse	
Orexigenic and dipsigenic effects	Mouse	
Increased REM number	Rat	
Endocrine action		
Prolactin and cortisol release	Sheep	(Vaudry et al., 2010)
Prolactin and thyrotropin release	Rat	
Adrenalin and adrenocorticotropic hormone release	Sheep	
Neurotransmitter release effect		
Norepinephrine, dopamine, serotonin and histamine release	Rat	(Bruzzone et al., 2010; Ono et al., 2008)
Acetylcholine release	Mouse	

1.3.3. Contraction and relaxation effects

The first accounted biological activity of UII was a spasmogenic action in teleost fish (Bern and Lederis, 1969). Then, it was later discovered that UII provokes the relaxation of mouse anococcygeus muscle, a non-vascular tissue found in the urogenital tract of male subjects (Gibson et al., 1984). Several studies indicated that this peptide is able to induce the constriction of various isolated vessels in rat, dog, pig and monkey (Ames et al., 1999; Douglas et al., 2000b; Itoh et al., 1988). Similarly, URP also exerts, but with a lower potency, a vasoconstrictive effect on rat aorta (Chatenet et al., 2004). To date, UII has been considered as the most potent vasoconstrictor because it acted more potently than ET-1 on rat isolated thoracic aorta and cynomolgus monkey arteries (Ames et al., 1999). However, UII biological actions, which vary among species, highly depend on the vascular bed. In rat, though it strongly acted on thoracic aorta, UII did not cause any contraction on abdominal aorta or femoral and renal arteries (Douglas et al., 2000b). Surprisingly, this cyclic peptide had no response on dog thoracic aorta but was a potent vasoconstrictor on rat and rhesus monkey aorta (Douglas et al., 2000b). In human studies, hUII did not generate any vasoactive response on biopsic small subcutaneous resistance arteries and veins (Hillier et al., 2001). In summary, UII contractive action selects large vessels but not small arteries (Table 1-3).

As mentioned earlier, the peptide also induces vasorelaxation that appears to be endothelium-dependent (Bottrill et al., 2000), while vasoconstriction was noticeable in either intact or removed endothelium vessels. In cynomolgus monkeys, low i.v. doses of hUII caused a moderate local vasodilation (Ames et al., 1999) that was also observed in human pulmonary and abdominal resistance arteries (Stirrat et al., 2001). In normal human subjects, UII induced vasodilation in skin microvascular tone but mediated a dose-dependent vasoconstriction in patients with chronic heart failure (Lim et al., 2004) (Table 1-3). Vasodilation was also induced with URP on rat coronary arteries *in vitro* (Prosser et al., 2006) and in unconscious rats following an i.v. administration (Chatenet et al., 2012; Sugo et al., 2003).

The vascular effects caused by UII are undoubted but the irregular changes of activity lead to the lack of firm conclusions about its vasoactive activity. Due to inconsistent *in vivo* activity, UII is actually considered as a high-potency, low-efficacy vasoconstrictor (McDonald et al., 2007). Such variable effects make it difficult to clearly define its vasoactive role.

Table 1-3: *In vitro* biological effects of UII

Effects	Species	Target tissues/cells	References
SpaSmogenic effects	Fish	Urophysis, urinary bladder, intestine, oviduct, sperm duct	(Ames et al., 1999; Douglas et al., 2000a; Gibson et al., 1984; Vaudry et al., 2010)
	Mouse	Anococcygeus muscles	
Vasoconstriction	Mouse	Thoracic aorta, coronary arteries	
	Rat	Thoracic and abdominal aorta, coronary and mesenteric arteries	
	Monkey	Pulmonary, basilar, renal and internal mammary vessels, venous tissues	
Vasodilation	Mouse	Coronary, small mesenteric and basilar arteries	(Ames et al., 1999; Bottrill et al., 2000; Stirrat et al., 2001)
	Monkey	Cardiac vessels	
	Human	Pulmonary and abdominal arteries	
Proliferation	Rat	Aorta, airway and vascular SMCs	(Albertin et al., 2009; Jarry et al., 2010; Spinazzi et al., 2006; Yoshimoto et al., 2004)
	Pig	LLCPK1 cell line	
	Human	SW13, VMRC-RCW carcinoma cell lines, astrocytes	
Angiogenesis	Human	Umbilical vein-isolated cells	
	Rat	Neuromicrovascular endothelial cells	

Abbreviations: LLCPK1: pig kidney epithelial cell line; SW13: human adrenal cortex carcinoma cell line, VMRC-RCW: human renal carcinoma cell line

1.3.4. Mitogenic and angiogenic activities

Similarly to Ang-II and ET-1, two potent vasoconstrictors that cause mitogenic actions (Hynynen and Khalil, 2006; Touyz, 2005), UII is able to induce the proliferation of various cell lines including airway and vascular smooth muscle cells (SMCs), endothelial cells, astrocytes and carcinoma cells (Jarry et al., 2010; Yoshimoto et al., 2004) (Table 1-3). Investigations on human vascular endothelial cells from various vascular beds showed that UII was able to induce the proliferation of cells isolated from the umbilical vein but not from saphena and jugular veins as well as aorta (Albertin et al., 2009). Moreover, UII causes proliferation of vascular SMCs in synergy with serotonin or mildly oxidized low-density lipoprotein (moxLDL), both compounds playing a crucial role in atherosclerosis, vasospasm and thrombosis (Watanabe et al., 2001a, b). Interestingly, UII but not URP induced the proliferation of rat astrocytes in a dose-dependent manner. UII also causes proliferation and vasculogenesis on late endothelial progenitor cells, which play a key role in the neovascularisation of ischemic tissues and re-endothelialization of injured blood vessels (Yi et al., 2012). Moreover, angiogenes is was observed in rat neuro-microvascular endothelial cells treated with UII (Spinazzi et al., 2006). This effect, similar to the cytokine and fibroblast growth factor 2 (FGF-2) angiogenic action, but not associated with the epidermic growth factor (EGF) system, was demonstrated in human vascular endothelial cells isolated from saphena, jugular and umbilical veins, as well as aorta (Albertin et al., 2009). Furthermore, *in vivo* angiogenic action of UII was demonstrated in a chorioallantoic membrane assay from white-leghorn chicken eggs (Spinazzi et al., 2006).

1.3.5. Other activities

In addition to the abovementioned actions, UII exerts fibrogenic, migratory and chemotatic effects. Indeed, in neonatal rat cardiac fibroblasts, UII stimulates collagen synthesis by increasing fibronectin and procollagen mRNA expression (Tzanidis et al., 2003). Moreover, transforming growth factor β1 (TGF-β1), which responds to fibrotic effect, is also up-regulated upon UII treatment (Dai et al., 2007). Besides, collagen I is also increased in the rat aortic adventitial fibroblasts (Zhang et al., 2008). The increase of collagen I and TGF-β1 synthesis is

not only observed in cardiac fibroblasts but also in other cells including human umbilical vein endothelial cells (Wang et al., 2004) and rat proximal renal tubular epithelial cell line NRK-52E (Tian et al., 2008). As mentioned, UII induces chemotaxis, which is observed in monocytes isolated from human heparinized blood and rat spleen where UT is expressed (Segain et al., 2007). In addition, UII-induced cell migration is also observed in cardiac fibroblasts (Zhang et al., 2008) and late endothelial progenitor cells (Yi et al., 2012).

UII and URP may however exert some cardioprotective functions in cardiac ischemia-reperfusion injury by increasing coronary circulation flow and reducing cardiac contractility (Prosser et al., 2008). UII is also able to reduce apoptotic cell death in smooth muscle-derived A10 cells, a protective effect abolished by either urantide or EGFR antagonist AG1478 (Esposito et al., 2011). Recently, UII was proposed to protect cardiomyocytes against doxorubicin-induced apoptosis (Chen et al., 2012) and hypertrophy by up-regulating myostatin, a negative regulator of cardiac growth (Gruson et al., 2012).

In summary, UII and URP are peptides playing pleiotropic roles in both peripheral and central systems. These peptides, acting as vasoconstrictor and vasodilator, are clearly associated with cardiovascular activities. However, because it also induces proliferation and angiogenesis, as well as multiple central effects, the main physiological role of the urotensinergic system is still unclear.

2. G protein-coupled receptors

According to the GRASF classification system, G protein-coupled receptors (GPCR), also known as seven transmembrane (7TM) receptors, comprise five families (Fredriksson et al., 2003) (Figure 1-4). The rhodopsin-like family with 672 members (63 still being orphan receptors), represents the largest one (Millar and Newton, 2010). In addition to its 7TM domains connected by extracellular and intracellular loops, a GPCR contains an N-terminal domain protruding outside the cell and a C-terminal tail directed toward the cytoplasm. These receptors may be functional as monomers, dimers or oligomers and can undergo multiple modifications including glycosylation, phosphorylation, acetylation, palmitoylation, and ubiquitinylation (Millar and Newton, 2010).

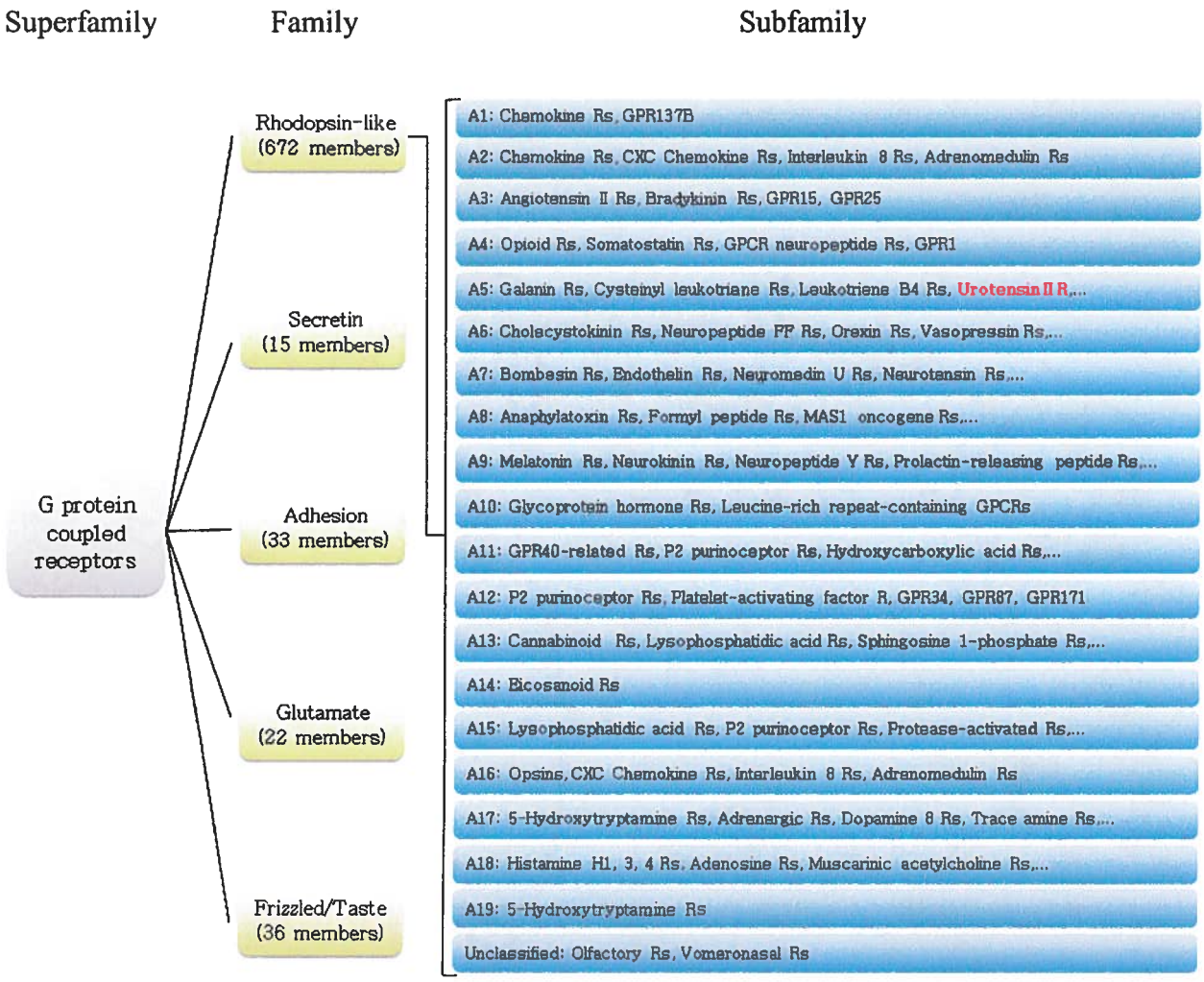

Figure 1-4: Classification of G protein-coupled receptors and the rhodopsin-like family (Joost and Methner, 2002; Millar and Newton, 2010)

GPCRs, activated by various ligands, *i.e.* sensory signals, chemokines, lipid mediators, growth factors and peptide hormones, transfer up to 80% of all signal transduction in cells (Millar and Newton, 2010). The initial interactions between the receptor and its ligand recruit G protein α subunits known as Gs, Gi/o, Gq/11 and G12/13, which in turn stimulate their effector molecules such as cyclic adenosine monophosphate (cAMP) generated from ATP by adenylyl

cyclase (AC), proto-oncogene serine/threonine-protein kinase (RAF), phospholipase C (PLC), and Ras homolog gene family (Rho) GEFs (RhoGEFs) (Park, 2012) (Figure 1-5).

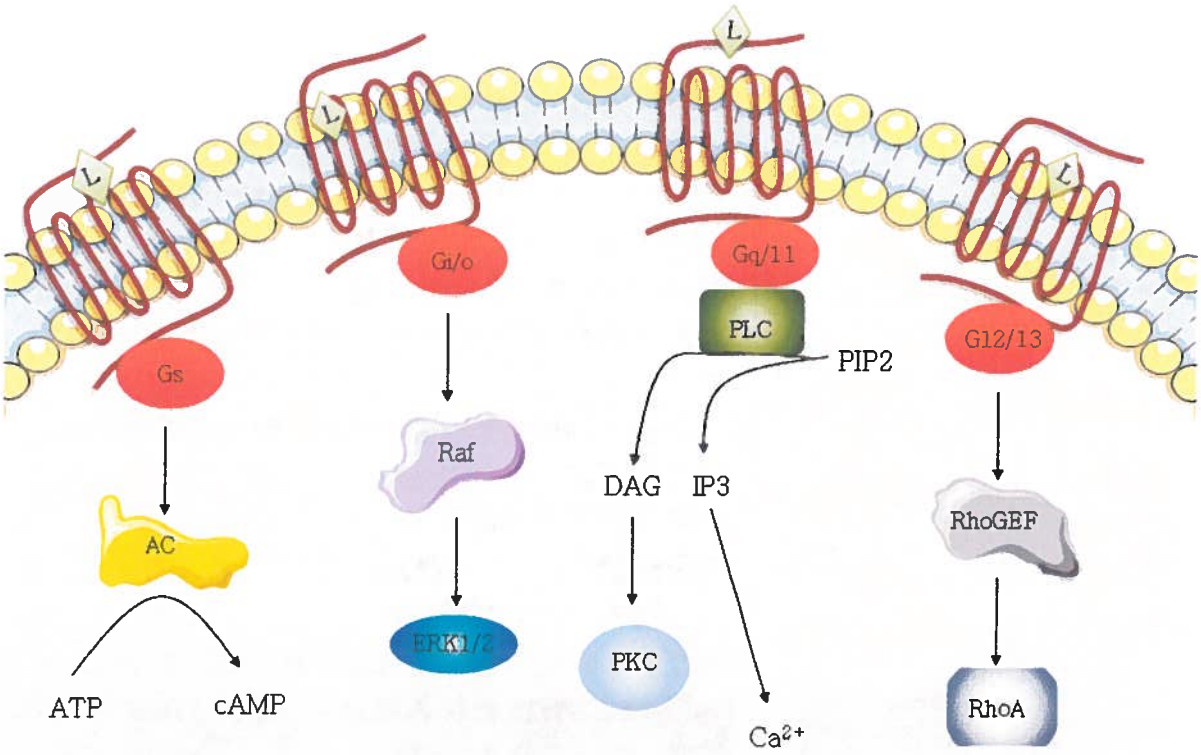

Figure 1-5: Coupling of G protein-coupled receptors to G proteins and their effectors

Abbreviations: L, ligand; Gs, Gi/o, Gq/11, G12/13, G protein α subunits; AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; ATP, adenosine triphosphate; Raf, proto-oncogene serine/threonine-protein kinase; PLC, phospholipase C; ERK1/2, extracellular-signal-regulated kinase 1 and 2; PKC, phosphokinase C; RhoGEF, Ras homolog gene family (Rho) GEF; RhoA, Rho member A; DAG, diacylglycerol; PIP2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol triphosphate

Although this receptor superfamily has been initially defined as G protein-coupled, a growing number of studies have indicated that many GPCRs mediate G protein-independent signaling by involving the β-arrestin (β-arr) pathways as in the case of the AT1 receptor (Millar and Newton, 2010). In general, once exposed to their ligands for a certain time, an active GPCR becomes desensitized through phosphorylation by G protein-coupled receptor kinases (GRKs) that facilitates the anchoring of β-arrestins to the GPCR, thus resulting in either (1) receptor internalization followed by dephosphorylation then recycling to the plasma membrane or trafficking to the lysosome for degradation, or (2) initiation of separate signaling pathways such as Src tyrosine kinases, ERK1/2 or JNK3 MAPKs in which β-arr functions as scaffold for these cascades (Luttrell and Lefkowitz, 2002) (Figure 1-6).

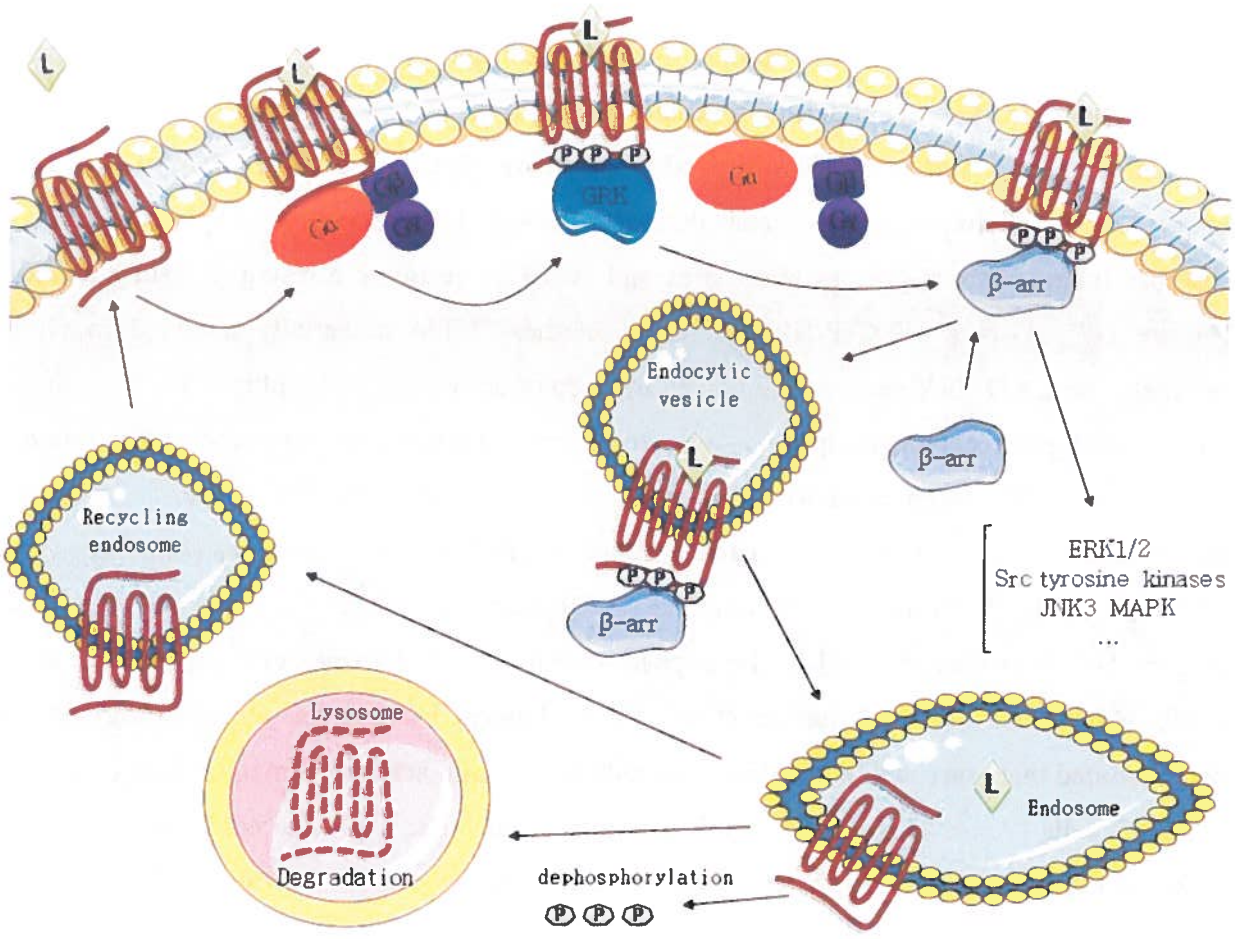

Figure 1-6: Recycling and signal termination of GPCRs (Luttrell and Lefkowitz, 2002).

Abbreviations: L, ligand; G protein-coupled receptor kinase, GRK; Gα, Gβ, Gγ: G protein α, β, γ subunits; β-arr, β-arrestin

Among the 800 members of GPCRs, the rhodopsin-like receptors represent the largest portion of this protein superfamily and therefore, they are of utmost pharmaceutical importance (McNeely et al., 2012).

3. The urotensin II receptor

3.1. Identification

In 1995, two independent studies reported a rat gene encoding a novel GPCR termed sensory epithelium neuropeptide-like receptor (SENR) (Tal et al., 1995) or GPR14 (Marchese et al., 1995). This receptor is characterized, like many GPCRs of the A family, by seven hydrophobic regions (transmembrane domains) linked together by hydrophilic sequences (cellular loops), two N-glycosylation sites and two Cys residues forming a disulfide bridge (Figure 1-7). Also, a NPXXP/NPXXY motif in the 7th TM potentially involved in GPCR internalization, a D/ERY sequence, involved in receptor activation, and multiple Ser/Thr residues corresponding to receptor phosphorylation sites, represent additional conserved motifs found in the rhodopsin-like receptor family (Tal et al., 1995) (Figure 1-7). The sequence of GPR14 indicated much similarity to the somatostatin and δ-opioid receptor sequences (Marchese et al., 1995; Tal et al., 1995). In 1999, this orphan GPCR, classified within the rhodopsin-like family (Figure 1-4), was characterized as the cognate receptor for UII (Ames et al., 1999; Liu et al., 1999; Mori et al., 1999; Nothacker et al., 1999). Indeed, by reversed pharmacology, it was demonstrated that goby and human UII were able to bind and activate human GPR14-expressing HEK293 cells (Ames et al., 1999) by increasing intracellular Ca^{2+} concentrations (Liu et.al., 1999, Nothacker et al., 1999) or releasing arachidonic acid metabolites (Mori et al. 1999). This receptor, now renamed UT, is encoded by an intronless gene located on chromosome 17q25.3 in human (Protopopov et al., 2000). Consisting of 389 residues in human (Figure 1-7), this receptor

shares 98% homology with monkey UT (Elshourbagy et al., 2002) but only 75% with the rat isoform containing 386 amino acids (Ames et al., 1999). Because, as mentioned earlier, UT and some somatostatin receptors share a high similarity in sequence, a crosstalk involving these two systems can be observed, *i.e.* UII and URP can activate the somatostatin receptor subtypes 2 and 5 and somatostatin, in some conditions, can also act on UT (Vaudry et al., 2010).

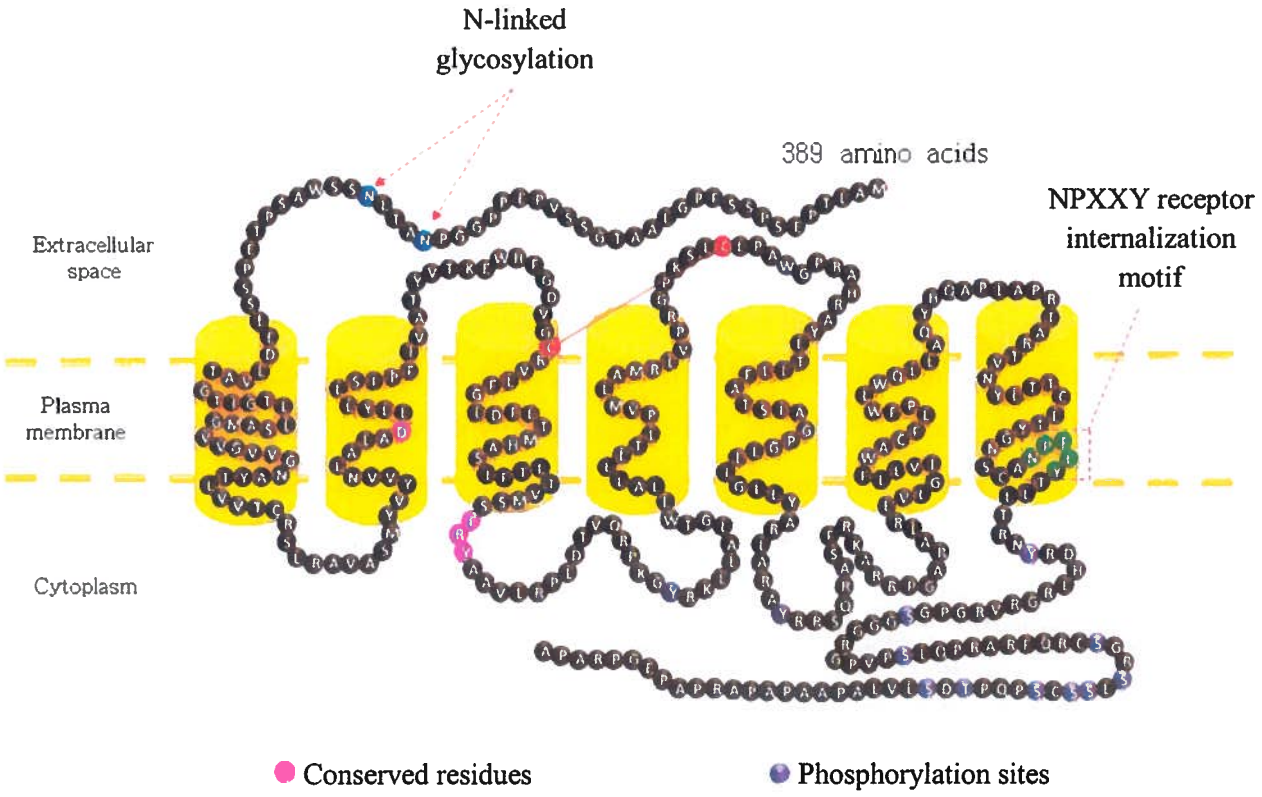

Figure 1-7: Primary structure of human UT (Adapted from Douglas et al., 2000a)

3.2. Distribution

UT receptor was identified in many species from fishes to mammals (Lu et al., 2008). As mentioned earlier, this protein is abundantly distributed in the CNS. In rat, the highest density of UII binding sites was found in the abducens and the lateral septal nuclei. Northern blot

examinations have demonstrated the presence of UT mRNA in various brain regions including the cortex, the olfactory bulb, the amygdala, the thalamus, the dorsal medulla, the pontine nuclei and the cerebellum (Jegou et al., 2006). In human and monkey, UT is highly expressed in the cerebral cortex (Maguire et al., 2000) and spinal cord (Elshourbagy et al., 2002, Totsune et al., 2003) while a low density in the thalamus superior gyrus and the substantia nigra is observed (Ames et al., 1999; Matsushita et al., 2001).

In periphery, human UT was abundantly detected in coronary artery but lower levels were observed in other vascular tissues such as renal, adrenal and pulmonary vessels, as well as umbilical and saphenous veins (Maguire et al., 2000, 2008, Matsushita et al., 2001, Totsune et al., 2003). A similar UT expression was observed in both human (Maguire et al., 2000, Matsushita et al., 2001, Totsune et al., 2003, Bousette et al., 2004) and monkey (Elshourbagy et al., 2002) kidney, pancreas, adrenal gland, skeletal muscle, aorta and heart. However, while UT was not observed in human lung (Maguire et al., 2000), it is expressed in monkey lung (Elshourbagy et al., 2002). Moreover, while UT is highly expressed in monocyte/macrophage population isolated from healthy human blood samples (Bousette et al., 2004), this receptor was not found in monkey spleen, an organ possessing half of the monocyte population (Elshourbagy et al., 2002). In addition, UT is also distributed in the upper gastrointestinal tract and the thyroid gland but not observed in the bladder and the liver in monkey (Elshourbagy et al., 2002). As for rat, aorta and other blood vessels from heart, kidney and skeletal muscle express UT (Maguire et al., 2008). Low levels were detected by autoradiography analysis in heart and skeletal muscle, as well as in the renal cortex. Conversely, higher levels were observed in the kidney medulla (Maguire et al., 2008) while no UT was noticeable in adrenal glands by Northern blotting (Jegou et al., 2006). Interestingly, a common UT distribution was reported in the heart of various species. Indeed, in rat (Gong et al., 2004), human (Marguire et al., 2000) and monkey (Elshourbagy et al., 2002), UT is only present or mainly observed in the normal left ventricle.

The similar distribution of UT and its endogenous ligands within CNS in both rodents and human, especially their co-localization in motor neurons of the spinal cord and the brain, suggests that UII and URP could act as autocrine and/or paracrine factors (Watson and May, 2004). However, due to their expression discrepancies, *e.g.* neither UII nor URP mRNA was detected in rodent kidneys where UT is abundant and the absence of UII mRNA in the heart of

rodents and humans (Dubessy et al., 2008; Sugo et al., 2003), these peptides cannot solely be considered as autocrine/paracrine but also endocrine factors (Gartlon et al., 2001).

3.3. Signal transduction

3.3.1. Constriction and vasodilation

UII is currently identified as the most potent vasoconstrictor (Douglas and Ohlstein, 2000) and several studies have explored the signaling pathways of UII-induced vasoconstriction in smooth muscle cells (SMCs). After the first study of Ames and coworkers (1999) demonstrating UII signaling through UT, the role of this receptor as a vasoconstriction mediator was confirmed *in vivo* using UT-knock-out mice (Behm et al., 2003). Similarly to ET-1 and Ang-II receptors, UT is coupled to a G protein αq/11 subunit and its activation is thus associated with an intracellular Ca^{2+} elevation (Ames et al., 1999) through PLC activation. Indeed, PLC stimulates the accumulation of inositol triphosphate (IP_3) (Saetrum Opgaard et al., 2000) and diacylglycerol (DAG), which in turn activate extracellular Ca^{2+} influx (Watanabe et al., 2006). Such calcium release in rat aorta provokes the modulation of the calmodulin/myosin light chain kinase (MLCK) pathway (Tasaki et al., 2004) and the protein kinase C (PKC), which subsequently phosphorylates RhoA/Rho kinase (RhoK) pathways, resulting in vasoconstriction (Rossowski et al., 2002). The UII-associated vasoconstriction is also dependent on the mitogenic activated-protein kinase (MAPK) pathways including p38MAPK and the extracellular regulated kinase 1 and 2 (ERK1/2) on endothelium-denuded rat aorta (Tasaki et al., 2004) (Figure 1-8).

As abovementioned, UII is also an endothelium-dependent vasodilator. This peptide acts on endothelial cells in an autocrine manner, through UT, to induce the release of nitric oxide (NO) and prostanoids, which cause the vasodilation on some small arteries (Bottrill et al., 2000; Stirrat et al., 2001) (Figure 1-8).

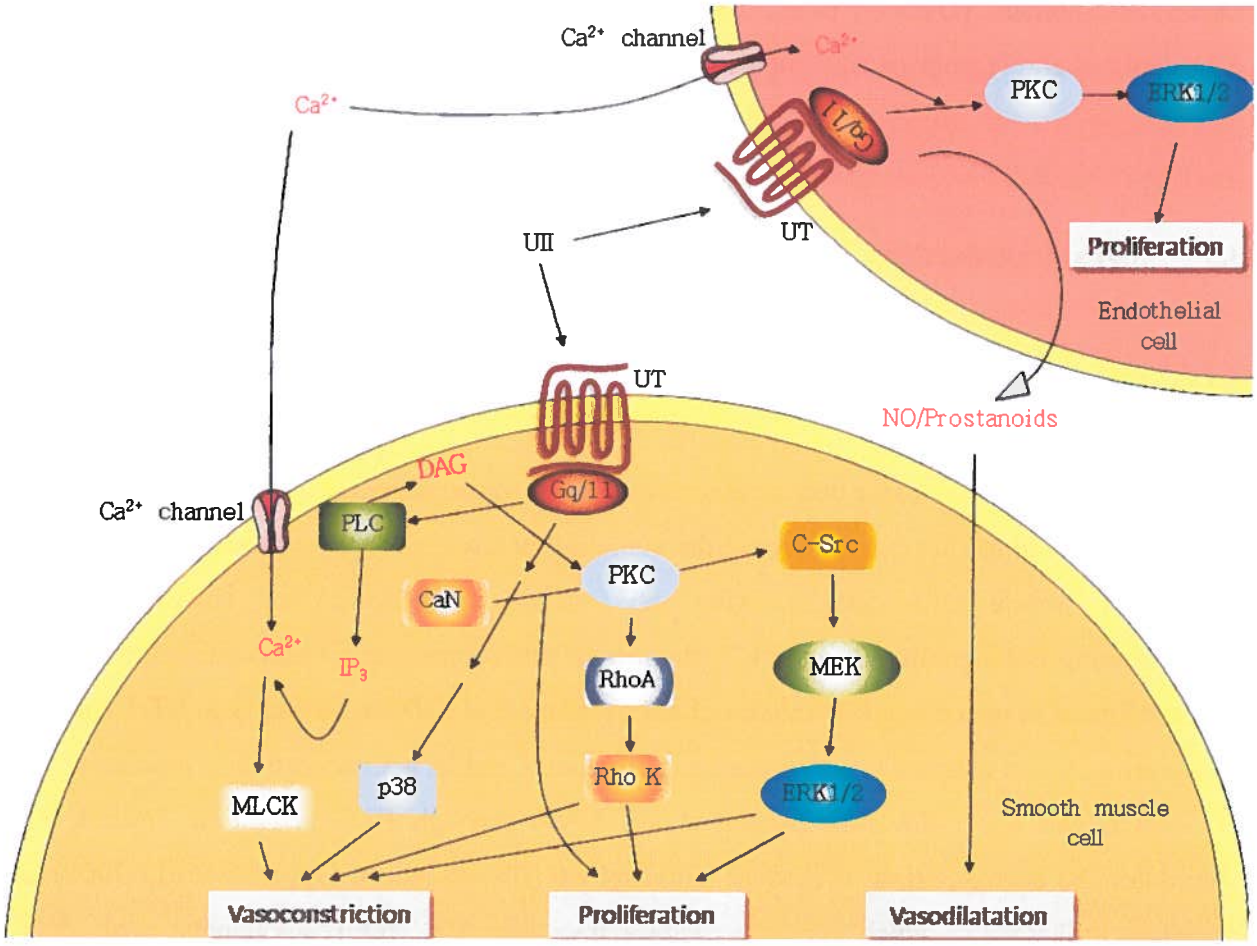

Figure 1-8: UII-associated signal transduction involved in vascular effects and proliferation in smooth muscle and endothelial cells

Abbreviations: Gq/11, G protein subunit αq/11 ; PLC, phospholipase C; PKC, protein kinase C; CaN, calcineurin; MLCK, myosin light-chain kinase; p38, p38-mitogen-activated protein kinase; RhoA, Ras homolog gene family (Rho) member A; RhoK, Rho kinase; c-Src, cellular-proto-oncogene; MEK, mitogen-activated protein kinase kinase; ERK1/2, extracellular-signal-regulated kinase 1 and 2; DAG, diacylglycerol; IP_3, inositol triphosphate; NO, nitric oxide

3.3.2. Proliferation and hypertrophy

UII induces proliferation through the recruitment of Gq/11, which in turn phosphorylates various cell type-dependent signal transduction pathways such as ERK1/2, a common mitogenic regulation pathway, as described in transfected CHO cell line (Ziltener et al., 2002), in native SMCs (Tamura et al., 2003; Watanabe et al., 2001b), as well as in endothelial cells (Matsushita et al., 2003). However, in aortic and airways SMCs, UII acts through the RhoA/RhoK (Sauzeau et al., 2001) and the PKC/calcineurin (CaN) pathways (Chen et al., 2004), respectively, which are independent from ERK. In addition, the proliferative activity also occurs as a consequence of UII synergistic effect with other agents including mildly oxidized LDL and serotonin. This action triggers PKC-activated cellular-proto-oncogene (c-Src) tyrosine kinase, which finally phosphorylates ERK1/2 in vascular SMCs (Watanabe et al., 2001b) (Figure 1-8)

Interestingly, Ca^{2+} mobilization, often associated with vasoconstriction, is also involved in UII proliferative effect through PKC and dihydropyridin (HDP)-sensitive Ca^{2+} channels, as suggested in the porcine renal epithelial cell line LLCPK1 (Matsushita et al., 2003) (Figure 1-8), or PLC/IP_3, as observed in rat cortical astrocytes (Jarry et al., 2010). Although the mitogenic activity of UII is well documented, a recent report has shown that UII also inhibits the proliferation of a population of mouse cardiac cells through C-Jun N-terminal protein kinase (JNK), a MAPK pathway element. Noteworthy, UII does not influence the differentiation of this cardiac cell population (Gong et al., 2011). These results contribute to the functional complexity of this peptide in mammalian species.

In cardiomyocytes, UII provokes hypertrophy by transactivation of the epidermal growth factor (EGF) receptor (EGFR). Using neonatal UT-overexpressing rat cardiomyocytes, it was shown that hypertrophy induced by UII is significantly decreased when blocking EGFR (Liu et al., 2009a; Onan et al., 2004) and treatments with the UT antagonists abolished or reduced EGFR phosphorylation confirming the involvement of UT-EGFR in UII hypertrophic activity (Gruson et al., 2010). The transactivation process is initiated by a UT-transmitted signal to the nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase, a membrane-bound enzyme complex that generates reactive oxygen species (ROS). Simultaneously, the activation of UII via UT causes the interaction of EGFR with Src homology-2 containing tyrosine phosphatase (SHP-

2). This would normally lead to EGFR dephosphorylation but in this case, ROS will oxidize the active site of SHP-2 leading to the suppression of EGFR dephosphorylation. As a consequence, EGFR is transactivated in order to induce hypertrophy through ERK1/2 and P38MAPK (Liu et al., 2009a; Onan et al., 2004) (Figure 1-9). Besides, phosphatidylinositol 3-kinases (PI3K)/protein kinase B (PKB or AKT), which partially mediates glycogen synthase kinase-3β (GSK-3β) phosphorylation along with the formation of its downstream effector β catenin, also mediates UII-induced hypertrophy in rat cardiomyocytes (Gruson et al., 2010) (Figure 1-9).

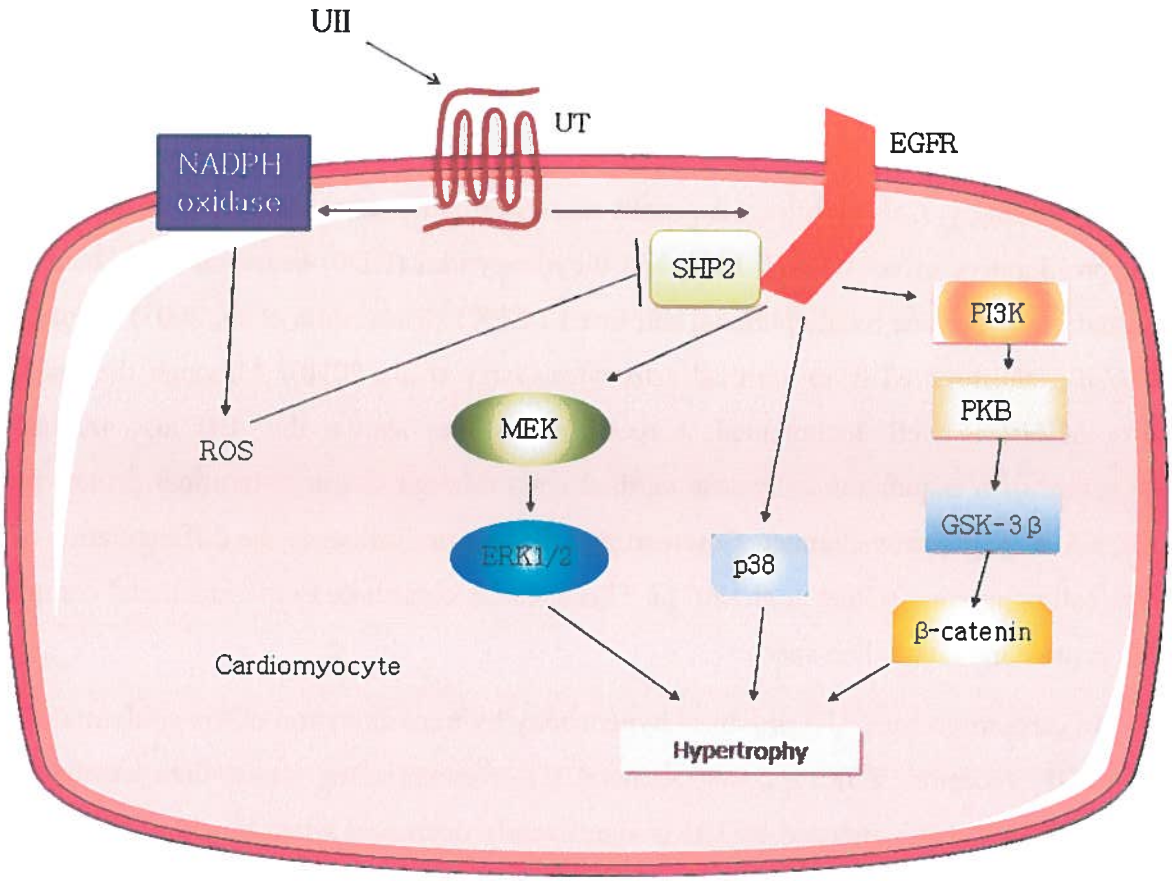

Figure 1-9: Signal transduction triggered by UII in hypertrophic cardiomyoctyte

Abbreviations: NADPH, nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species; EGFR, epidermal growth factor receptors; SHP2, Src homology-2 containing tyrosine

phosphatase; MEK, mitogen-activated protein kinase kinase; ERK1/2, extracellular-signal-regulated kinase 1 and 2; p38, p38-mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinases; PKB, protein kinase B; GSK-3β, glycogen synthase kinase-3β

In summary, the cell signaling triggered by UII involved in vascular, mitogenic and hypertrophic effects is mainly mediated through UT coupled with Gq/11, Gi/o or G12/13, subsequently activating various pathways.

3.4. The physiological and pathological relevance of the urotensinergic system

The UII system appears to be critically involved in various pathological states, *e.g.* cardiorenal pathologies (Table 1-4) while its physiological role is still not completely understood. Aside from clear cellular observations of this system in various pathological states, reports have highlighted that high UII plasma level generally correlated with pathological conditions forming statistical evidence for a role of the urotensinergic system in the pathogenesis and the progression of human diseases.

3.4.1. Cardiovascular functions

In physiological conditions, UII provokes haemodynamic effects. In anaesthetized rats, bolus injection of UII induced a decrease in cardiac contractility, left ventricle systolic pressure (Hassan et al., 2003), as well as arterial pressure (Gendron et al., 2005; Hassan et al., 2003). However, in conscious rats, systemic UII injection increased tachycardia and vasodilation with a slow development of blood flow velocity (Gardiner et al., 2006). Although UII obviously exerts haemodynamic effects in rat, hUII does not seem to play *in vivo* cardiac or vasoactive roles in human. Indeed, intra-arterial infusion of urotensin II had no effect on forearm blood flow ratio. Although there is a significant increase in systolic blood pressure in healthy men, no *in vivo* effect on human forearm resistance was observed (Cheriyan et al., 2009). This study is in line with previous observations, in which UII did not stimulate any significant change in heart rate,

augmentation index, pulse wave velocity and forearm blood flow (Affolter et al., 2002; Wilkinson et al., 2002). However, a vasodilatory response was indicated in normotensive human subjects (Sondermeijer et al., 2005).

Despite unclear physiological roles, UII vascular actions have been clearly observed in pathological states. The high hypotensive response to hUII is documented in spontaneous hypertension rats (SHRs), especially at 8 weeks of age when UT, which mediates the effect, is up-regulated in these animals (Gendron et al., 2005). In this rat strain, rUII causes a change in the mean arterial pressure, as well as in renal haemodynamics (Abdel-Razik et al., 2008b; Shi et al., 2008). In hypertensive patients, in opposition to normotensive men, UII induces a vasoconstrictive response (Sondermeijer et al., 2005) and an increase in heart rate and mean arterial pressure (Cheriyan et al., 2009).

Among human diseases, heart failure is one of the most deadly clinical conditions, arising from various pathological states such as hypertension, myocardial infarction, ischemic heart disease, valvular heart disease and cardiomyopathy, including cardiac hypertrophy (McMurray and Pfeffer, 2005). A clear overexpression of the urotensinergic system has been generally demonstrated in heart failure, as well as in all its related diseases (Table 1-4). High levels of plasma UII and/or URP were shown in patients with chronic and acute heart failure (Jani et al., 2012; Kruger et al., 2005), as well as in patients suffering from hypertension (Cheung et al., 2004; Sondermeijer et al., 2005; Thompson et al., 2003; Watanabe et al., 2006), coronary heart disease (Chai et al., 2010) and systemic sclerosis (Pehlivan et al., 2011). However, in patients with acute coronary symptom, the circulating UII is decreased (Joyal et al., 2006), especially in the patients with the high risks. Thus, the decrease of plasma UII in these patients may be related to a more severe cardiac injury (Babinska et al., 2012) suggesting a cardioprotective activity of this peptide in this condition.

These studies clearly demonstrated the correlation between the plasma UII/URP levels and various cardiovascular diseases. It is worth to pinpoint that the methodologies used until recently to measure plasma UII, such as radioimmunoassays and immunoluminometric assays could not discriminate between UII and URP meaning that the data are reflecting the possible combination of both peptides (Jani et al., 2013). Nonetheless, by using a solid phase extraction

technique for each peptide, it is clear now that not only plasma UII but also plasma URP levels are increased in heart diseases (Jani et al., 2013).

Initial report has indicated the over-expression of UII and UT in cardiomyocytes and to a lesser extent in inflammatory, endothelial and smooth muscle cells of patients with advanced heart failure (Douglas et al., 2002). In rat suffering from myocardial infarction, cellular expression of UII and UT was increased in both infarct and non-infarct zones of the left ventricle. However, the highest receptor expression was found in the right ventricle and may result from a decrease of myocyte population in the left ventricle in this pathological condition (Tzanidis et al., 2003). Another investigation carried out in SHRs showed that UT mRNA was up-regulated in all heart tissues and aorta whereas UII and URP mRNA were significantly elevated only in the atria and aorta, respectively. The different expression patterns of these two ligands in the cardiovascular system were thought to be related to their distinct roles in hypertension (Hirose et al., 2009). The up-regulation of the different components of this system was also noticeable in human carotid plaque (Bousette et al., 2004), in atherosclerotic human coronary arteries (Hassan et al., 2005) and in the aorta of apolipoprotein E (apoE)-knockout mice (Wang et al., 2006b). Together with the proliferation effects on endothelial cells, which is an early event during atherosclerosis, UII seems to participate to the pathogenesis of this disease (Yoshimoto et al., 2004). Indeed, plasma UII level was increased in diabetic associated atherosclerosis patients and mice (Watson et al., 2013). Moreover, the deletion of UII alone or in combination with apoE, a risk factor for atherosclerotic premature development, or treatments with the UT antagonist SB657510, resulted in a significant decrease of atherosclerosis progression (You et al., 2012), thus demonstrating a clear involvement of the urotensinergic system in this pathology.

In addition, a lot of evidence indicated an *in vitro* hypertrophic effect for UII on UT-overexpressing (Onan et al., 2004; Tzanidis et al., 2003) and wild type primary cardiomyocytes (Liu et al., 2009a) and this activity is inhibited by urantide (Gruson et al., 2010). *In vivo*, UII and UT expressions were increased in the heart of isoproterenol (ISO)-treated rats suggesting that this system is involved in the development of cardiac fibrosis and hypertrophy. However, the use of UT specific antagonists (urantide and SB-657510) did not alter the left ventricular weight/body weight ratio *in vivo* in mouse and rat pressure overload-induced hypertrophy models

(Esposito et al., 2011; Kompa et al., 2010). The reason for the ineffectiveness of these antagonists *in vivo* is still unclear, although the increase of UII and its receptor expression in ventricles of cardiac hypertrophy animal models (Kompa et al., 2010; Zhang et al., 2007) strongly suggests an association between the UII system and this pathological condition.

The urotensinergic system is thus involved in cardiac disease and also in the modulation of vascular effects and the expression of pathological markers. Indeed, vasoconstriction in response to UII stimulation was observed in a dose-dependent manner in chronic heart failure patients, whereas this peptide caused vasodilation in normal subjects suggesting the contribution of UII to peripheral vasoconstriction in this disease, which correlated with UII and UT up-regulation (Lim et al., 2004). Moreover, in rat hearts undergoing an ischemia reperfusion, both UII and URP reduce perfusion pressure prior to and following ischemia, dilate cardiac coronary arteries and reduce the level of creatine kinase, a marker of damaged cardiomuscular tissue. However, only UII can diminish the post-ischemia left ventricular contractility and has the ability to decrease the release of atrial natriuretic peptide (ANP), a hormone secreted by cardiomyocytes in response to high blood pressure in isolated hearts. This study suggests a potential cardioprotective effect of both peptides from ischemia (Prosser et al., 2008). The difference observed between them was proposed to be due to the possibility of UII/URP to bind to another receptor rather than UT in cardiac tissues. Notably, a potential UII/UT and URP/UT distinct modulation, based on allosteric control, was recently proposed (Chatenet et al., 2012).

Taken together, with the over-expression in damaged tissues, as well as in plasma, the urotensinergic system clearly plays an important role in cardiovascular pathophysiology including hypertension, atherosclerosis, myocardial infarction, ischemic heart disease and heart failure.

3.4.2. Renal diseases and diabetes

Renal impairment often accompanies cardiovascular diseases because kidneys play a key role in the control of cardiovascular homeostasis (Douglas et al., 2004). This organ is an important source of urinary, as well as circulating UII in the body (Ashton, 2006). In humans, a study involving patients with end-stage renal disease (ESRD) indicated that circulating UII levels

were directly associated with left ventricular systolic function but inversely correlated with left atria volume and left ventricle muscular component (Zoccali et al., 2008). Also in the same cohort, plasmatic UII was remarkably elevated while lower levels of norepinephrine and neuropeptide Y, two cardiovascular stress hormones, were noticeable (Mallamaci et al., 2006). Moreover, circulating UII concentration was significantly lower in patients with shorter survival and fatal cardiovascular events in chronic kidney disease (CKD) making UII an inverse predictor of cardiovascular mortality (Ravani et al., 2008). Agreeing with these reports, medical exams of patients with ESRD or CKD indicated low plasma UII in ESRD; the lowest levels were shown in patients with CKD (Mosenkis et al., 2011). Thus, in addition to an osmoregulatory role taking place in the kidneys, the urotensinergic system also plays a role in the pathophysiology of renal diseases.

The expression in renal tissue of this system is also changed in various pathological conditions. Observations in a SHR population showed that UII, URP and UT expressions were significantly higher than in Wistar-Kyoto rats. As a matter of fact, UII and UT were up-regulated in the renal medulla but not in the cortex (Abdel-Razik et al., 2008a) while URP mRNA was mainly distributed in the renal tubules, vascular SMCs and vascular endothelial cells (Mori et al., 2009). These observations raised the possibility of an autocrine/paracrine osmoregulatory role of URP in the mammalian renal tubular cells similar to the role of UII in fish urophysis. In addition, an important pathophysiological role was also suggested for this system in renal diseases because URP, UII and their receptor were shown to be up-regulated in rats suffering from chronic renal failure (Mori et al., 2009). Observations in children with chronic glomerulonephritis have shown that UII immunoreactivity levels were highly expressed in various renal tissues. Briefly, people with normal kidneys possess low UII in the glomerulus whereas children with membranoproliferative glomerulonephritis (MPGN), focal segmental glomeruloscrelosis (FSGS) or membrane nephropathy (MGN) have high UII levels in the glomerular basement membrane and Bowman capsule. Moreover, the up-regulation of UII also appeared very significant in different renal tissues during these states, such as in the glomerular mesangium of MPGN patients, in the sclerotic area of FSGS patients and in the distal tubules, endothelium and mesangium of children with MGN. Furthermore, a positive correlation between blood pressure and UII expression in endothelium and mesangium in MPGN and in Bowman capsule in MGN,

were also recorded. Although the observations of abnormal UII expressions could not point out its specific role in these diseases, it suggests that this peptide could contribute to the pathophysiology of human kidney and play an important role in the regulation of blood pressure in these renal diseases (Balat and Buyukcelik, 2012).

As mentioned earlier, UII, present in pancreatic extracts, plays a regulatory role in insulin secretion through UT (Silvestre et al., 2004) suggesting a potential role of this system in diabetes. Indeed, patients with type II diabetes had a significant increase of plasma and urinary UII, an observation similar to the ones obtained under renal failure (Totsune et al., 2004). UII and UT mRNA were also drastically elevated in renal tissues of diabetic patients suffering from kidney damage (diabetic nephropathy) (Langham et al., 2004). Likewise, both mRNA and protein levels of UII and its receptor were found to be elevated in the glomeruli and tubular epithelial cells of diabetic rat kidneys along with renal profibrotic factor transforming growth factor TGF-β1 (Tian et al., 2008) (Table 1-4). Polymorphisms of the prepro-UII gene (UTS2) were observed among population with high fasting plasma insulin and type II diabetes development, such as Japanese (Suguro et al., 2008; Suzuki et al., 2004; Wenyi et al., 2003) Chinese (Ong et al., 2005; Tan et al., 2006; Zhu et al., 2002) and European populations (Okumus et al., 2012; Saez et al., 2011). Therefore, single nucleotide polymorphisms (SNP) can represent early candidate diagnostic markers, as well as therapeutic targets for the treatment of diabetes mellitus (Okumus et al., 2012). In addition, SNP examinations of both UTS2 and UT gene (UTS2R) showed that UTS2 influences the relative amount of saturated fatty acids whereas UTS2R polymorphisms affect the relative amount of saturated, as well as monosaturated fatty acid Δ9 desaturase activity and fat deposition in skeletal muscle (Jiang et al., 2008). Altogether, these data clearly indicate the involvement of this system in the pathophysiology of type II diabetes mellitus.

The effect of UT antagonists on kidney function in various pathological states has been studied. Infusion of urantide in SHRs induced a significant increase of the renal blood flow and the glomerular filtration rate (Abdel-Razik et al., 2008a). Palosuran, a non-peptide antagonist, improved survival, increased insulin secretion and delayed the renal damage in diabetic rats (Clozel et al., 2006). Moreover, this derivative is also able to decrease urinary albumin excretion rate in macroalbuminuric, diabetic patients who tend to develop renal diseases (Sidharta et al.,

2006). Altogether, these studies suggested that targeting the urotensinergic system could represent a new alternative for the treatment of diabetes-nephropathy. However, studies have so far also pointed out the inefficiency of UT antagonists, *i.e.* palosuran and urantide on insulin release and blood glucose level in type II diabetic patients (Sidharta et al., 2009) and kidney function in lipopolysaccharide-endotoxaemic rats, respectively (Nitescu et al., 2010). The involvement of the urotensinergic system is undoubtedly, but there may be unknown facts, such as the failure of these antagonists to reach intracellular UT, thus preventing these UT-selective antagonists to exert their activity.

3.4.3. Other diseases

The urotensinergic system is also involved in many other pathological states including lung and liver diseases, eclampsia and cancer (Table 1-4). Women with pre-eclampsia and eclampsia present an increased concentration of circulating UII (Balat et al., 2005b; Liu et al., 2010) along with an increase of neurokinin B level, a marker of pre-eclampsia (Liu et al., 2010). In children with liver disease, the level of UII in plasma directly correlated with the seriousness of the condition (Pawar et al., 2011). The urotensinergic system is also altered in lymphagioleiomyomatosis (LAM), a rare lung disease characterized by SMC growth throughout the lungs. Indeed, UII, URP and UT expression was increased in the lung lesions and co-localized with HMB45, a marker of LAM (Kristof et al., 2010). Rats under induced chronic hypoxia showed an increased mRNA level of UII and its cognate receptor in the right ventricle that correlated with pulmonary hypertension and right ventricular hypertrophy (Huang et al., 2006) (Table 1-4). Another study on rat damaged and reperfused brains demonstrated that UII icv injection strongly increased the post-reperfusion hyperaemia, the cortical cerebral blood flow (CBF), as well as the hemispheric infarction volume in ischemic animals (Chuquet et al., 2008). Interestingly, under physiological condition, UII contributed to a long-lasting increase of intravenous pulse pressure but with no change in CBF. Therefore, in opposition to the study demonstrating a cardioprotective function for UII in the ischemic heart disease (Prosser et al., 2008), this peptide exacerbated ischemic brain lesions while inducing no histological damage in normal subjects.

Because UII is also able to induce the *in vitro* proliferation of carcinoma cells such as adrenocortical cell line (SW13), renal cell carcinoma cell line (VMRC-RCM) (Yoshimoto et al., 2004) and lung adenocarcinoma cells (A549) (Wu et al., 2010), it was suggested that UII promotes tumor growth. In fact, this peptide affects the tumor development of nude mice xenograft (Wu et al., 2010). Moreover, the UII imunoreactivity is increased in human adrenocortical tumor where its cognate receptor is also localized (Morimoto et al., 2008). In human prostate cancer, both UT mRNA and protein levels were also highly present in well-differentiated carcinomas. Noteworthy, the UT antagonist urantide can significantly decrease the motility and invasion of androgen dependent cells (LNCaP), which is disadvantageous for tumor development (Grieco et al., 2011).

Table 1-4: Involvement of the urotensinergic system in various pathological states

Disease	Expression location	Urotensinergic system regulation	References
Cardiovascular diseases			
Advanced heart failure	Human cardiomyocytes, endothelial cells and SMCs	↑UT	(Douglas et al., 2002)
Chronic heart failure	Human hearts	↑UII, UT	(Lim et al., 2004)
Myocardial infarction	Rat left infarct ventricle	↑UII	(Tzanidis et al., 2003)
	Rat infarct and non-infarct ventricle	↑UT	
Hypertension	Rat atria	↑UII, UT	(Hirose et al., 2009)
	Rat ventricles	↑UT	
	Rat aorta	↑URP	
	Human plasma, cerebrospinal fluid	↑UII	(Thompson et al., 2003)
Coronary heart disease	Human plasma	↑UII	(Chai et al., 2010)
Systemic sclerosis	Human plasma	↑UII	(Pehlivan et al., 2011)
Ventricular hypertrophy	Rat ventricles	↑UII, UT	(Kompa et al., 2010; Zhang et al., 2007)
Renal disease and diabetes			
Hypertension	Rat kidneys	↑URP, UT	(Mori et al., 2009)
ESRD	Human plasma	↓UII	(Ravani et al., 2008)
CKD	Human plasma	↓UII	(Mosenkis et al., 2011)
Diabetes type II	Human plasma, urine	↑UII	(Totsune et al., 2004)
Renal dysfunction diabetes	Rat kidneys	↑UII, UT	(Tian et al., 2008)

Diabetic nephropathy	Human kidneys	↑UII, UT	(Langham et al., 2004)
Other diseases			
Pre-eclampsia	Human plasma	↑UII	(Liu et al., 2010)
Liver disease	Human plasma	↑UII	(Pawar et al., 2011)
LAM	Human lungs, interstitial nodular lesions, pulmonary epithelium, vasculature and inflammatory cells	↑UII, URP and UT	(Kristof et al., 2010)
Pulmonary hypertension	Right ventricle	↑UII, UT	(Huang et al., 2006)
Cancer	Human adrenocortical tumor	↑UII	(Morimoto et al., 2008)
	Human prostate tumor	↑UII, UT	(Grieco et al., 2011)

Abbreviations: SMCs: smooth muscle cells; ESRD: end-stage renal disease; CKD: chronic kidney disease, LAM: lymphagioleiomyomatosis; ↑: up-regulated; ↓: down-regulated

In summary, the urotensinergic system is clearly involved in various pathological states including cardiovascular and renal diseases, as well as diabetes. However, no convincing evidence for a role of this system in the CNS was brought into light although CNS tissues in all species possess a high level of both UII/URP and UT. Therefore, a better understanding of its physiological and pathophysiological role in human is crucially needed.

CHAPTER 2: NUCLEAR G PROTEIN-COUPLED RECEPTORS

Introduction

It was considered that "all GPCRs are located within the plasma membrane" (Millar and Newton, 2010). However, in recent years, growing evidence indicated that GPCRs are also present in the perinuclear/nuclear membranes, in many cell types (Table 1-5). For instance, the Ang-II receptors (AT_1 and AT_2) were shown in the cell nuclei of liver, spleen, cardiac and brain tissues, as well as vascular SMCs. Similarly, nuclear ET receptors (ET_A and ET_B) were found in cardiac ventricular myocytes, human aortic vascular SMCs and liver as well as brain tissues (Gobeil et al., 2006a). Other nuclear GPCRs were also found and among them, functionality was demonstrated through induction of nuclear Ca^{2+} influx, mediation of some nuclear signaling pathways, transcription initiation, as well as gene expression. The presence of these nuclear receptors was observed independently of any ligand stimulation (Boivin et al., 2003; Boivin et al., 2006; Eggena et al., 1993).

Because GPCRs are "some of the most promising targets for drug development" (Bkaily et al., 2009), a better understanding of their roles, not only at the cell membrane but also at the nuclear level, in healthy and disease states, will for sure provide new avenues for therapeutic intervention.

1. Detection of GPCRs at cell nuclei

An initial study in 1971 reported the presence of Ang-II binding sites on the nucleus of rat vascular and cardiac muscle cells (Robertson and Khairallah, 1971). Then, in addition to the several reports confirming the existence of nuclear binding sites in hepatic cells, AT_1 receptor was observed by immunological methods and functional assays in the nuclei of many cell types including brain cortex cells, neurons, cardiomyocytes and vascular SMCs (Gobeil et al., 2006a). This receptor, like UT, belongs to the rhodopsin-like receptor family of GPCRs. In this family, other receptors, comprising endothelin, bradykinin, apelin and β-adrenergic receptors, were also

found in nuclei (Boivin et al., 2003; Boivin et al., 2006; Lee et al., 2004). For instance, upon ET-1 stimulation, nuclear Ca^{2+} is increased in human vascular SMCs (Bkaily et al., 2000) and in cardiomyocytes isolated from chicken embryo, with a concomitant accumulation of ET-1 probes (Bkaily et al., 2002), hence suggesting the existence of the cognate receptors, ET_A and ET_B, at the nucleus. These two receptor subtypes were observed in nuclei of rat ventricular myocytes via ligand binding and immuno-procedures (immunoblot and immunofluorescence with specific antiseras of ET-Rs) (Boivin et al., 2003). Following the identification of functional ET-Rs in ventricular myocytes (Boivin et al., 2003), the same group demonstrated the existence of βARs in these cells in rat and mouse (Boivin et al., 2006). Confocal immunofluorescence microscopy was also used to reveal the presence of other receptors from the same GPCR family, including bradykinin and apelin receptors (Lee et al., 2004) (Table 1-5). Increasing evidence clearly demonstrates the existence of nuclear rhodopsin-like receptors, which indicates a putative physiological role for these receptors. Apart the rhodopsin-like GPCRs, the secretin-like GPCRs, including the parathyroid hormone receptor type 1 (PTH1R) and vasoactive intestinal polypeptide receptor 1 (VPAC-1), were also localized in the cell nuclear fraction of osteosarcoma cells (Pickard et al., 2006) and human breast tissues (Valdehita et al., 2010), respectively. Moreover, pituitary adenylate cyclase-activating polypeptide (PACAP) receptors were recently shown in nuclei of rat testis cells (Doan et al., 2012a). Finally, a member of the metabotropic glutamate receptor family, *i.e.* the metabotropic glutamate receptor subtype 5 (mGlu5), is also present in the nucleus of neuronal and HEK293 cells (O'Malley et al., 2003) (Table 1-5).

Table 1-5: Nuclear GPCRs in various cell lines and tissues
(modified from Tadevosyan et al., 2012)

GPCR	Cell/Tissue	Methods of detection	Signalings and actions	References
		Rhodopsin-like family		
AT-Rs	Rat vascular endothelial cells (ECs), VSMCs, ventricular cardiomyocytes, hepatocytes; human adrenal tissue, transfected HEK-293 cells, transfected cerebellum D293 Med cells	PL, LBA, GFP-CM, ICC, WB, IHC	↑ n(Ca^{2+}), ERK and p38, G protein coupling, tyrosine phosphorylation, transcription, proliferation	(Cook et al., 2006; Lee et al., 2004; Re and Parab, 1984; Tadevosyan et al., 2010)
ET-Rs	Rat heart, brain and liver; human	LBA,	↑ n(Ca^{2+}) via IP3 mediation,	(Boivin et al., 2003;

	aortic VSMCs, endocardial ECs; chick embryo ventricular myocytes	ICC,WB, PL, IFCM	CaMKII phosphorylation, transcription	Hocher et al., 1992; Jacques et al., 2005; Merlen et al., 2013)
B2R	HEK-293T, rat hepatocytes	IHC, GFP-CM, WB	↑ n(Ca^{2+}) via R-type Ca channel and/or IP4/IP3Rs	(Lee et al., 2004; Savard et al., 2008)
NK3R	HEK-293T, rat ventral tegmental area neuron	IFCM, EM	Unknown	(Hether et al., 2013; Lessard et al., 2009; Misono and Lessard, 2012)
AP-R	Human cerebellum, hypothalamus, transfected cerebellum D283 Med cells	IHC, GFP-CM, WB	Unknown	(Lee et al., 2004)
α-AR	Rat neonatal ventricular cardiomyocytes; mouse adult ventricular cardiomyocytes α1ARKO	LBA, GFP-CM	ERK, proliferation, survival	(Buu et al., 1993; Huang et al., 2007)
β-AR	Rat and mouse ventricular cardiomyocytes	LBA, WB, ICC	↑ n(cAMP), G protein coupling, PKB, transcription	(Boivin et al., 2006; Buu et al., 1993; Vaniotis et al., 2011)
CysLT1	Human colon tissue, colorectal carcinomas, Int 407 cells, CaCo-2 cells	IHC, ICC, GFP-CM, WB	↑ n(Ca^{2+}), ERK, DNA replication, proliferation?	(Nielsen et al., 2005)
CXCR4	Human hepatoma, colorectal cancer HT-29 cells, adenocarcinoma, (NSCLS), HeLa	IHC, ICC, FCM	Unknown	(Shibuta et al., 2002; Spano et al., 2004)
EP-R	Newborn pig brain, porcine cerebralmicrovessel ECs; adult rat liver, murine Swiss 3T3; transfected HEK293	PL, ICC, IHC, WB	↑ n(Ca^{2+}), ERK and PKB, ↑ n(cAMP) and n(IP3), G-protein coupling, eNOS mRNA, transcription, proinflammatoryresponse?	(Bhattacharya et al., 1999; Bhattacharya et al., 1998; Gobeil et al., 2002; Provost et al., 2010)
GnRHR	*C. elegands* germline, intestine, HEK293, HTR-8/SVneo	IHC	Acetylation and phosphorylation of histone H3	(Re et al., 2010; Vadakkadath Meethal et al., 2006)
LPA1R	Rat hepatocytes, PC12 cells; CHO; newborn pig cerebral microvessel ECs, human brochial epithelial cells HBEC	WB, LBA, IHC, ICC	↑ n(Ca^{2+}), ERK and PKB, eNOS-derived NO, iNOS activation and transcription	(Gobeil et al., 2003; Gobeil et al., 2006b; Waters et al., 2006)
OPR1/2	Rat-mouse neurohybrid cells NG 108-15; hamster cardiomyopathic adult ventricular myocytes	IHC, ICC, LBA	G protein coupling, PKC, Transcription	(Belcheva et al., 1993; Ventura et al., 1998)
PAF-R	Rat liver; transfected CHO; HEK293, COS1 cells, porcine cerebral ECs	FCM, ICC, IHC, LBA, GFP-CM, WB	↑ n(DAG) and n(Ca^{2+}), ↓ n(cAMP), ERK, Gprotein coupling, PLC, Transcription, pro-inflamatory response	(Marrache et al., 2002; Miguel et al., 2001; Zhu et al., 2006)
mAChR	Rabbit cornea, corneal epithelial and endothelial cells	PL, LBA	↑ DNA and RNA Pol II activity, ↑ nuclear cGMP	(Lind and Cavanagh, 1993, 1995)
Secretin family				

VPAC	HT29 cells, breast cancer cells; rat testis	LBA, WB, PL	cAMP production, ↑ n(Ca^{2+}), transcription	(Doan et al., 2012a; Valdehita et al., 2010)
		Metabotropic family		
PTH1R	Rat kidney, liver, gut, uterus, ovary, osteoclast cell lines of mouse MC3T3-E1, rat ROS17/2.8, UMR106 and human SaOS-2; dear antler osteoclasts, LLC-PK1cells	IHC, ICC, WB, GFP-CM	DNA synthesis, mitosis	(Pickard et al., 2006; Watson et al., 2000a; Watson et al., 2000b)
mGluR	Mouse neuronal cells; rat neonatal mesencephalic neurons; transfected HEK293 cells	IHC, ICC, WB	↑ n(Ca^{2+}), transcription, CREB phosphorylation	(Jong et al., 2005; O'Malley et al., 2003)

Abbreviations: AT-Rs, angiotensin receptors; ET-Rs, endothelin receptors; AP-R, apelin receptor; α-AR, alpha-adrenergic receptor; β-AR, beta-adrenergic receptor; CysLT1, cysteinyl leukotriene receptor 1; CXCR4, chemokine receptor type 4; EP-R, prostaglandin receptor; GnRHR, gonadotropin-releasing hormone receptor; LPA1R, lysophosphatidic acid-1-receptor; OPR1/2, opioid peptide receptors 1 and 2; PAF-R, platelet activating factor receptor; PRL-R, prolactin receptor; mAChR, muscarinic acetylcholine receptor; VPAC, vasoactive intestinal peptide receptor; PTH1R, parathyroid hormone receptor type 1; mGluR, metabotropic glutamate receptor; GFP-CM, green fluorescent protein-confocal microscopy; ICC, immunocytochemistry; IHC, immunohistochemistry; WB, Western blot; LBA, ligand binding assay; PL, photolabelling; FCM, flow cytometry; IFCM, immunofluorescence confocal microscopy; EM, electron microscopy

2. Functional nuclear G protein-coupled receptors

The nucleus is now perceived as a cell within a cell because many biochemical processes taking place in plasma membrane and in cytoplasm are also observed in the nuclear envelope and in the nucleoplasm (Bkaily et al., 2009). In fact, numerous studies have indicated that nuclei possess the signaling machinery related to the control of intracellullar ionic homeostasis, gene expression, cell cycle and apoptosis (Bkaily et al., 2009; Bootman et al., 2009). Such nuclear machines including nuclear tubules, formed by the invagination of the nuclear envelope inside

the nucleus, which permits faster, deeper and higher transport of ions and signaling between cytosol and nucleus through an increase of the membrane surface, can influence gene expression, as well as nuclear import and export. Also, ionic transporters, present in the nuclear envelope, are involved in Ca^{2+} influx through R-type channels, Ca^{2+}ATPase pumps and Na^{+}/Ca^{2+} exchangers (Bkaily et al., 2009). Moreover, the nuclear translocation of some proteins, such as the cAMP response element-binding protein (CREB), is known to regulate Ca^{2+} influx, and the activation/repression of gene transcription in high/low nuclear Ca^{2+} level can be regulated by the transcriptional regulator downstream regulatory element antagonist modulator (DREAM) (Bootman et al., 2009). The translocation of calmodulin into the nucleus also implicates an increase of Ca^{2+}, crucial for nuclear signaling, and the activation of the inositol trisphosphate receptors (InsP3Rs) located in both inner and outer nuclear membranes (Bootman et al., 2009).

Several nuclear GPCRs located at the nuclear membrane, *i.e.* ET-R, AT-R, B2R, mGlu5, EP3-R, PAF-R and LPA1-R are able to stimulate an increase of intranuclear Ca^{2+} level (Bkaily et al., 2002; Boivin et al., 2003; Jong et al., 2005; O'Malley et al., 2003; Savard et al., 2008; Zhu et al., 2006). However, this modulation might be mediated indirectly through R-type channels, as for ET-1, Ang-II and B2K receptors (Bkaily et al., 2009) or through an interaction with a nuclear calcium-activated potassium (K_{Ca}) channel, as in the case of EP3-R (Gobeil et al., 2002). In addition to the activation of ionic channels and transporters (Bkaily et al., 2009), the elevation of intranuclear Ca^{2+}can result from the release of Ca^{2+} from nuclear storages including the nuclear envelope lumen (Boivin et al., 2003). Other factors involved in GPCR-mediated nuclear Ca^{2+} signaling were poorly described, except for nuclear LPA1-R and PAF-R, which are coupled to Gi/o and may directly bind to Gβ/γ subunits in order to activate nuclear membrane ion channels (Zhu et al., 2006). Both receptors mediate Ca^{2+}-dependent ERK1/2 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) binding to DNA, which appears to modulate gene transcription (Zhu et al., 2006). Although the mechanism responsible for Ca^{2+} increase inside isolated nuclei by direct stimulation of GPCR ligands is not yet understood, these studies clearly demonstrate the functionality of these nuclear GPCRs.

As mentioned, Ca^{2+} plays a major role in various signaling pathways associated with gene expression/modulation (Bootman et al., 2009). Accordingly, some nuclear GPCRs, mediating nuclear Ca^{2+}, seem to be involved in the regulation of gene transcription such as AT_1

(Tadevosyan et al., 2010), EP3-R, PAF-R and LPA1-R (Zhu et al., 2006). Indeed, dot blot RNA hybridization on isolated rat liver nuclei treated with Ang-II showed an elevation of renin and angiotensinogen expression (Eggena et al., 1993). Moreover, this treatment caused an increase of cyclooxygenase-2 (COX-2) expression in AT_1-trasfected HEK293 cells in a dose-dependent manner and this elevation was directly correlated with nuclear AT_1-R accumulation (Thomas et al., 2006). Other nuclear GPCRs, including β3AR (Boivin et al., 2006), prostaglandin (PGE2), PAF and LPA receptors were shown to induce the synthesis of various transcription factors including c-Fos, the endothelial nitric oxide synthase (eNOS), COX-2 and the inducible nitric oxide synthase (iNOS) (Gobeil et al., 2006a).

So far, some mechanisms related to nuclear GPCR-mediated gene expression were investigated. The transcription initiation *via* nuclear β3AR was shown to be mediated by Gi/o with the signal being transmitted to all three MAPK pathways (ERK, p38 and JNK), as well as PKB (Vaniotis et al., 2011). The synthesis of some transcription factors induced by PGE2, PAF and LPA was found to follow a Ca^{2+}-MAPK-NF-κB-dependent signaling pathway. In addition, LPA receptors were also found to be coupled to Gi/o proteins and to induce the phosphatidylinositol 3-kinases (PI3K)/AKT pathway (Gobeil et al., 2003). PI3K/AKT-induced phosphorylation can also be elicited by B2K in isolated rat hepatocyte nuclei, thus resulting in the regulation of nucleoplasmic Ca^{2+} homeostasis and histone H3 acetylation, which leads to the transcription of iNOS (Savard et al., 2008). As a matter of fact, the involvement of nuclear GPCRs in histone H3 modification was observed not only with acetylation but also with phosphorylation, as seen following stimulation of the gonadotropin-releasing hormone receptor (GnRH-R) (Re et al., 2010). This phenomenon was most probably associated to chromatin modifications necessary for transcription initiation (Savard et al., 2008). In opposition to mediation via signaling cascades, a direct gene regulation was observed through the interaction of PTH1R with DNA, since the receptor was only present in the nucleus at the phases of cell cycle when DNA was ready for transcription (Pickard et al., 2006).

Along with studies demonstrating that nuclear GPCRs are linked to Ca^{2+} increase in the nucleus, the abovementioned evidence of gene transcription regulation strongly supports the functionality of the intracellular receptors. However, to date, their key role is still not well understood and remains consequently to be discovered.

3. Nuclear G protein-coupled receptors: translocation versus direct nuclear addressing

How nuclear GPCRs locate in cellular nucleus is not fully understood. An explanation for this intracellular localization can come from a translocation from plasma membrane. Indeed, some receptors accumulate in nuclei after ligand treatment, as reported for AT_1-R (Chen et al., 2004; Lu et al., 1998; Thomas et al., 2006), VPAC1 (Valdehita et al., 2010) and S1P receptors (Estrada et al., 2009). As demonstrated, the presence of AT_1-R in the nucleus is associated with nucleoporin complex protein p62 phosphorylation, in a ligand-dependent manner (Lu et al., 1998). Nonetheless, Lee et al. (2004) also observed an agonist-independent nuclear existence of AT_1-R in live transfected-HEK 293 cells. Translocation of the AT_1-R from the plasma membrane to the nucleus is mediated by a nuclear localization signal (NLS) recognized by importin, *i.e.* KKFKKY present in the carboxy-terminal region (Thomas et al., 2006). Similar NLS sequences playing a role in nuclear import were also found for other nuclear GPCRs, including KRFRK in the C-terminal segment of B2R and RKRRR in the third intracellular loop of the apelin receptor (Lee et al., 2004) (Figure 1-10, pathway 1). Surprisingly, for the GnRH-R, the deletion of the hypothetical NLS sequence, conserved throughout species and found in the first intracellular loop of GnRH-R, did not alter the distribution of this receptor at the nuclear envelope. Therefore, there must be other mechanisms, not involving NLS-importin, that mediate the nuclear location of GPCRs. Even though firm evidence is still lacking, some mechanisms explaining how these receptors may directly translocate from the trans-Golgi network or cell membrane, in an NLS-independent pathway, were hypothesized (Re et al., 2010). Because neoglycoproteins can NLS-independently be imported from the cytosol into the nucleus through an N-glycosylation mechanism, it was suggested that GPCRs could be synthesized in the rough endoplasmic reticulum (ER), then glycosylated in the Golgi compartment that influences their localization (Gobeil et al., 2006) (Figure 1-10, pathway 2). Indeed, the Golgi apparatus is an organelle where modulation of the cellular compartmentalization takes place, along with receptor functionalization through post-translation modification including glycosylation (Re et al., 2010). Supporting this postulate, the presence of glycosylated EP3-R was shown at the cell surface while a non-glycosylated form was observed in the ER (Gobeil et al., 2003). In addition to

glycosylation, other NLS-independent trafficking mechanisms for nuclear GPCRs were mentioned, such as the caveolar pathway, because LPA1-R co-localized with caveolin in nuclear preparations (Zhu et al., 2006) (Figure 1-10, pathway 3) or direct interaction with transportin 1 (Trn1), as observed for the chemokine 2 receptor (CCR2). In the latter case, the internalization of CCR2 is followed by an interaction with Trn1 and subsequently, it is localized at the outer edge of the nuclear envelope where the complex is associated (Favre et al., 2008) (Figure 1-10, pathway 4).

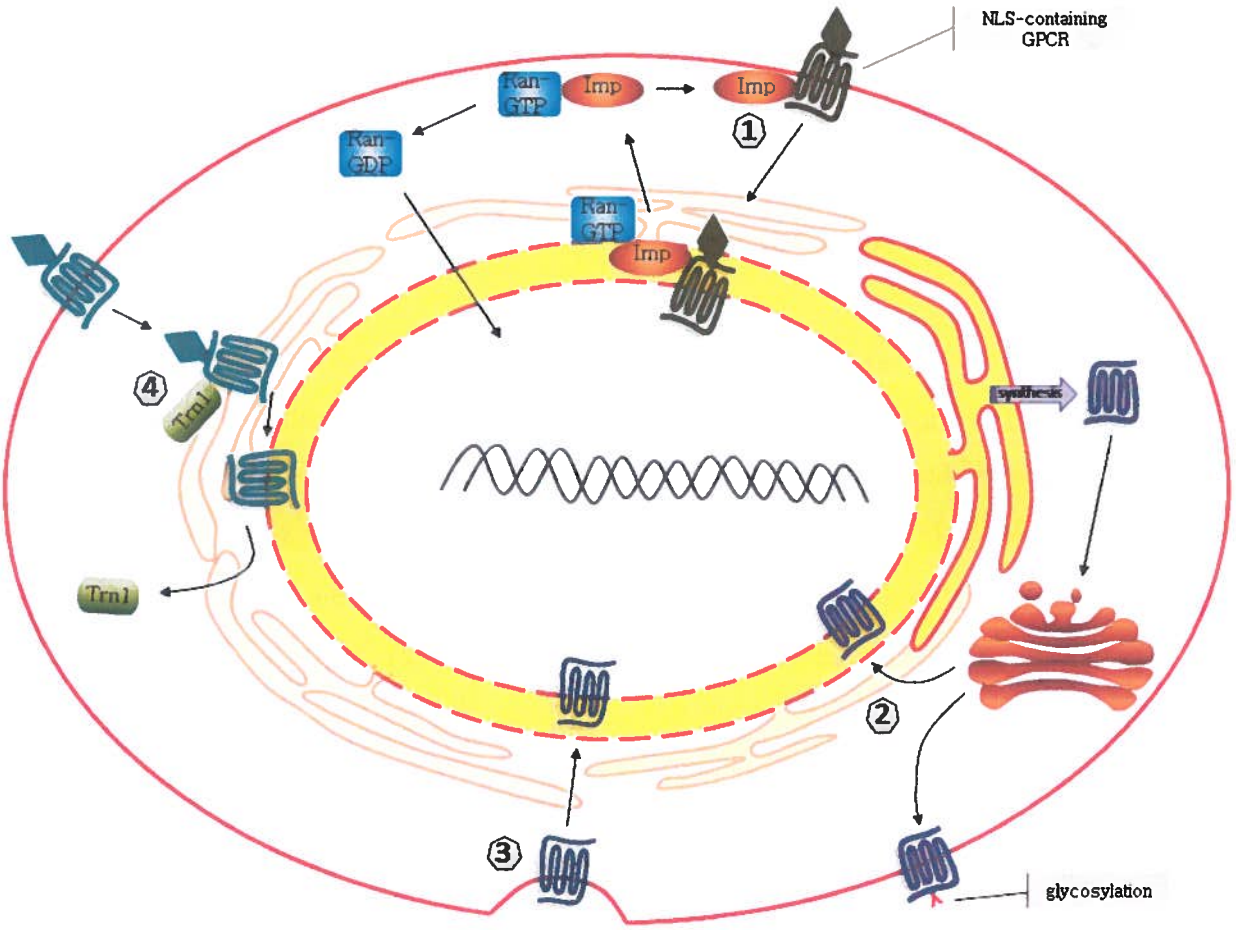

Figure 1-10: Possible origins of nuclear GPCRs

(1) Ligand-dependent translocation from plasma membrane via NLS recognition by importin (Imp), (2) *De novo* synthesis in ER with different glycosylation in the Golgi apparatus, leading to

nuclear or plasma membrane localization, (3) Involvement of caveolae in nuclear translocation of GPCRs, (4) Ligand-dependent translocation of plasma membrane GPCRs into the nucleus through Trn1.

Besides these mechanisms, it has been suggested that *de novo* synthesis could be a source of nuclear GPCRs (Boivin et al., 2003; Merlen et al., 2013). Though it is a matter of argument (Dahlberg and Lund, 2004; Iborra et al., 2004), a hypothesis suggested that translation could occur inside the nucleus (Bkaily et al., 2009). Thus, some nuclear GPCRs would be a protein newly synthesized in the nucleus, either waiting to be addressed at the nuclear envelope or directly interacting with DNA, as suggested for PTH1R in the nucleoplasm of brain cells (Pickard et al., 2006).

4. Ligand uptake mechanisms

A receptor is activated upon ligand binding. So, for a receptor located inside the cell, the ligand must find a way to reach it in order to trigger signaling pathways associated with biological functions. Several studies about how ligands can reach their nuclear receptors have been reported (Figure 1-11).

It was believed that ligands reach intracellular compartments through binding to their cell surface receptors, which elicited endocytosis (Posner et al., 1982) (Figure 1-11, pathway 1). This process is not only a mean to modulate activity of the receptor through signal ending, desensitization and down regulation but also plays a role in some intracellular signaling pathways. Indeed, the neurotensin (NT) receptor type 2 (NTS2) can activate ERK1/2 following NT treatment, an effect abolished in the presence of an endocytosis inhibitor (Mazella and Vincent, 2006). However, it appears that some ligands can cross the plasma membrane without their receptors. Peptidic ligands are frequently considered as cell impermeable molecules because of their hydrophilic nature. Yet, some small cationic peptides (containing less than 20 amino acids) can reach the cytoplasm (Alves et al., 2009; Drin et al., 2003) through multiple pathways including pore or inverted micelle formation, diffusion through the lipid bilayer (Figure 1-11, pathway 2) or endocytotic internalization (Madani et al., 2011).

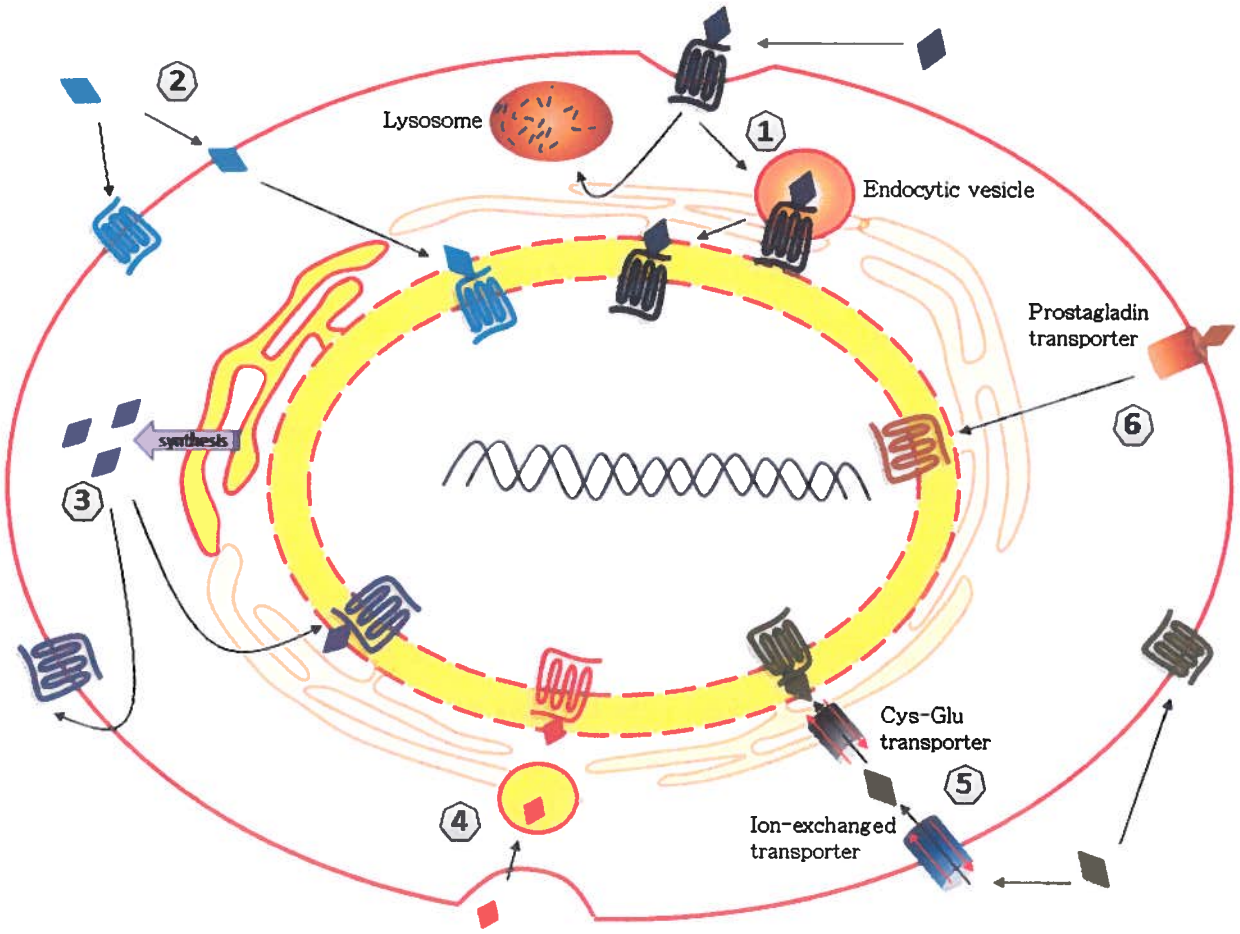

Figure 1-11: Proposed uptake mechanisms for the delivery of endogenous ligands to their nuclear GPCRs

(1) Ligand-receptor complex translocates into the nucleus or goes directly to the lysosome for degradation, (2) Extracellular ligand is directly diffused through the phospholipid bilayer into the cytosol, (3) Ligand is locally synthesized in the ER then reaches its nuclear/membrane receptor in the same cell, (4), Ligand is transported into the nucleus via macropinocytosis, (5) Ligand goes across plasma membrane via ion-exchange transporter then enter the nuclear envelope lumen through a Cys-Glu transporter, (6) Ligand uptake via prostagladin transporter.

Moreover, besides the receptor-dependent internalization mechanism mentioned above, Ang-II was found to be produced and retained inside the cells before being redistributed at the nuclei of neonatal rat ventricular myocytes (Singh et al., 2007). Such ligands, synthesized inside the cell are also known as intracrine ligands (Figure 1-11, pathway 3). In addition to Ang-II, PACAP was characterized as a peptide also able to act as an intracrine factor. Indeed, it was shown that the PACAP precursor sometimes lacks the peptide signal sequence that ensures peptide secretion (Tominaga et al., 2010). Hence, the peptide remains inside the cell where it can reach nuclear PACAP receptors present in the tissue. Moreover, our group showed recently that the two PACAP isoforms (PACAP 27 and 38) are able to quickly and efficiently go across the plasma membrane at physiological concentration via clathrin-dependent endocytosis and macropinocytosis (Doan et al., 2012a) (Figure 1-11, pathway 4). GnRH is also considered as a possible intracrine factor because it was described as co-localized with its receptor in the trophoblast cell line HTR-8/SVneo (Re et al., 2010). In line with these studies, non-peptide ligands of prostanoid and PAF receptors were also shown to be synthesized intracellularly at the nuclear compartment of the brain cells (Bhattacharya et al., 1998; Marrache et al., 2002; Vazquez-Tello et al., 2004). Another mechanism was proposed by Jong et al. (2005) for the mGlu5-R/ligand interaction. Since the N-terminal domain of the nuclear receptor resided between the two nuclear leaflets, agonists such as glutamate and quisqualate must cross the plasma membrane and outer nuclear membrane to activate the nuclear receptors. These extracellular ligands can penetrate within the cell *via* a sodium-dependent plasma membrane transporter and then use the cysteine-glutamate exchanger to reach the inner nuclear membrane (Figure 1-11, pathway 5). Finally, for EP3-R, a prostagladin transporter inhibitor, *i.e.* bromocresol green, prevents the receptor agonists from inducing eNOS up-regulation thereby suggesting that the prostagladin transporter is involved in PGE2 uptake (Gobeil et al., 2002) (Figure 1-11, pathway 6). Altogether, it can be seen that multiple mechanisms are involved in ligand cellular penetration and that even a particular ligand can utilize more than one pathway to reach its intracellular GPCR.

In summary, GPCRs undoubtedly play very important physiological roles making them attractive pharmacological targets. The growing list of observed intracellular/nuclear GPCRs has raised questions about their cellular functions. Even though strong evidence indicated the

functionality of many intracellular GPCRs, so far, there has not been any study unveiling their physiological role, not to mention their involvement in pathology. Finally, whether they independently or synergistically act with receptors of the plasma membrane remains unknown, putting forward the need for investigation in this particular field of pharmacology.

SECTION 2: HYPOTHESIS AND OBJECTIVES

As previously mentioned, several functional GPCRs have been observed in the nucleus of various cell lines and tissues. Interestingly, Ang-II and ET-1 receptors, sharing multiple biological activities with UT, have been observed at the nuclei where they retain some biological activities. The fact that rUT-GFP expressed in HEK cells were not only found at the plasma membrane but also intracellularly without ligand stimulation, suggested newly synthesized proteins waiting to be transported to the plasma membrane or constitutively internalized proteins already in a perinuclear compartment (Giebing et al., 2005). Since UT is widely distributed in the body, **the putative intracellular UT could be present in some specific tissues or in every tissue** where membrane UT distribution has been reported. Additionally, **the intracellular localization of UT could be species dependent** since interspecies differences in tissue distribution have been observed for this receptor. Altogether, **these results prompted us to evaluate the existence of nuclear UT in various tissues and species.** Moreover, since the URP sequence contains the active segment of UII and exhibits an equal binding affinity towards UT, it was thought that it exerts identical activities as those of UII (Sugo et al., 2003). However, other evidence showed that these two ligands induced differently vasoconstriction and vasodilation effects, *i.e.* URP caused a less potent vasoconstriction than UII on the rat aorta and it induced a 10-fold less potent effect than rUII on rat coronary arteries (Chatenet et al., 2004; Prosser et al., 2006). Furthermore, both ligands induced an elevation in Ca^{2+} concentration on astrocytes but URP and UII cause a mono- and biphasic increase, leading to short and long Ca^{2+} mobilization, respectively. These distinct mechanisms were believed to be involved in mitogenic activity induced by only UII (Jarry et al., 2010). In addition, their expression in pathological states, such as hypertension, was also contrasting. In SHRs, while UII was significantly up-regulated in the atrium in 11-12 weeks old animals, URP mRNA level in this tissue was not changed compared to normal rats, whereas in the kidney, opposite expressions were observed (Hirose et al., 2009). Hence, it appears that these two peptides act in a more complex fashion on UT than what it was believed so far, and this might be related, at least in part, to the putative presence of functional intracellular UT receptors.

Like the Ang-II and ET-1 receptor, UT mediates many processes such as vascular effects, cell proliferation and angiogenesis. These receptors, *i.e.* AT_1, AT_2, ET_A and ET_B, are deemed functional in the nuclei since they regulate nuclear Ca^{2+}mobilization, kinase phosphorylation and gene expression upon ligand stimulation (Boivin et al., 2003; Eggena et al., 1993; Thomas et al., 2006). **In the second aspect of this work, we investigated the functionality of this putative nuclear UT.**

If nuclear UT receptors are functional and consequently play a physiological role, they must be activated by UII and/or URP. In the case that functional UT exists in the nucleus, then its cognate ligands must either cross the plasma membrane to reach the receptor or be retained in the cell after biosynthesis. This latter phenomenon was already demonstrated for Ang-II in neonatal rat ventricular myocytes (Singh et al., 2007). As for the ability of peptides to enter cells by crossing the plasma membrane, in the past decade, various cell-penetrating peptides have been reported; the majority being cationic and smaller than 20 amino acids. In this section, **we have thus evaluated the propensity of UII and URP to cross the plasma membrane by a receptor-independent mechanism.** As abovementioned, the urotensinergic system is actually involved in many pathological states. Indeed, in cardiovascular and renal diseases, this system is clearly up-regulated making it relevant for therapeutic intervention (Ross et al., 2010). However, the pathological role of the urotensinergic system, as well as its regulation in response to pathological conditions in the CNS, which possesses a marked UT level, is unknown. Interestingly, in a rat model of cerebral ischemia, UII exacerbates brain lesions (Chuquet et al., 2008). **Therefore, UT endogenous ligands could play a role in hypoxia/ischemia by modulating the expression of UT and influencing its subcellular localization/expression in brain cells.**

From these hypotheses, specific investigation goals have been set.

1. Examine the presence of nuclear UT in several rat and monkey tissues including heart, brain, spinal cord, kidney, adrenal gland, lung, pancreas, liver and skeletal muscle.
2. Demonstrate the functionality of nuclear UTs through transcription initiation assay upon UII and URP stimulation, in the presence or not of a UT antagonist (urantide).

3. Investigate UII and URP cellular uptake as a mechanism for the ligands to reach nuclear UTs.

4. Examine whole cell and nuclear UT expression in neuroblastoma cells under *in vitro* hypoxic/ischemic conditions and evaluate the effects of UII and URP on UT expression in these conditions.

These specific objectives are aimed at broadening our knowledge of the urotensinergic system and its relevance in pathological conditions.

SECTION 3: RESULTS

PUBLICATION 1

BIOCHEMICAL AND PHARMACOLOGICAL CHARACTERIZATION OF NUCLEAR UROTENSIN II BINDING SITES IN RAT HEART

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Running Title: Intracellular UII binding sites

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Contribution of the authors: A significant part of the work carried out in the laboratory was achieved by Thi Tuyet Mai Nguyen (TTMN). The study was in the follow-up of an MSc research program performed by Kathy Turcotte. Ngoc-Duc Doan (NDD) also participated, especially in the development of strategies for studying the peptide cellular uptake. It was estimated that NDD and TTMN contributed equally to this study. Myriam Létourneau (ML), David Chatenet (DC) and Alain Fournier (AF) participated in the choice of the scientific orientations of the research program focussing on urotensin II, and supervised the work. TTMN and NDD wrote the first draft of the manuscript while ML, DC and AF revised the paper. It was published in British Journal of Pharmacology (**166**:243-257, [2012] – 2011 impact factor: 4,409).

Résumé en français

Caractérisation biochimique et pharmacologique des sites de liaison nucléaires de l'urotensine II dans le cœur de rat

Au cours de la dernière décennie, quelques récepteurs couplés aux protéines G (RCPG) ont été observés à la membrane nucléaire où ils exercent vraisemblablement des fonctions physiologiques complémentaires à celles des récepteurs membranaires. Dans cet article, nous avons démontré (1) la présence de récepteurs de l'urotensine II fonctionnels (UT) dans des extraits nucléaires de cœur de rat et (2) la capacité de l'urotensine II (UII) et de l'*urotensin II-related peptide* (URP) à traverser la membrane plasmique en procédant par un mécanisme indépendant de la présence des récepteurs. Des méthodes biochimiques et pharmacologiques, y compris des analyses de liaison compétitive, du photomarquage, de l'immunobuvardage ainsi que des essais de synthèse *de novo* d'ARN ont été utilisées pour caractériser la présence d'UT fonctionnel dans des noyaux de cœur de rat. En outre, la microscopie confocale et l'analyse par cytométrie en flux ont été utilisées pour étudier la translocation intracellulaire de dérivés fluorescents d'UII et d'URP. Ainsi, la présence de sites de liaison spécifiques de l'UII a été démontrée dans des extraits nucléaires cardiaques de rat. De plus, cette localisation subcellulaire a aussi été observée dans des extraits de cœur de singe. Des essais d'initiation de transcription *in vitro* sur des noyaux fraîchement isolés de cœur de rat suggèrent que les récepteurs UT nucléaires sont fonctionnels et que l'UII, mais pas l'URP, participe à l'expression de gènes associée à l'UT nucléaire. Étonnamment, l'hUII et l'URP traversent efficacement la membrane plasmique par un mécanisme récepteur-indépendant impliquant une endocytose médiée par les cavéolines; cette absorption de l'hUII, mais pas de l'URP, est également influencée par le pH extracellulaire.

Nos résultats suggèrent (1) que l'UII et l'URP peuvent moduler différemment l'activité des récepteurs UT nucléaires et notamment l'expression des gènes et (2) que les deux ligands peuvent atteindre l'espace cellulaire interne par un mécanisme indépendant du récepteur.

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RESEARCH PAPER

Biochemical and pharmacological characterization of nuclear urotensin-II binding sites in rat heart

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Keywords
urotensin-II; urotensin II-related peptide; nuclear receptors; intracrine; cardiovascular; endocytosis

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BACKGROUND AND PURPOSE

During the past decade, a few GPCRs have been characterized at the nuclear membrane where they exert complementary physiological functions. In this study, we investigated (1) the presence of a functional urotensin-II (U-II) receptor (UT) in rat heart nuclear extracts and (2) the propensity of U-II and U-II-related peptide (URP) to cross the plasma membrane in a receptor-independent manner.

EXPERIMENTAL APPROACH

Biochemical and pharmacological methods including competitive binding assays, photoaffinity labelling, immunoblotting as well as *de novo* RNA synthesis were used to characterize the presence of functional UT receptors in rat heart nuclei. In addition, confocal microscopy and flow cytometry analysis were used to investigate the cellular uptake of fluorescent U-II and URP derivatives.

KEY RESULTS

The presence of specific U-II binding sites was demonstrated in rat heart nuclear extracts. Moreover, such subcellular localization was also observed in monkey heart extracts. *In vitro* transcription initiation assays on rat, freshly isolated, heart nuclei suggested that nuclear UT receptors are functional, and that U-II, but not URP, participates in nuclear UT-associated gene expression. Surprisingly, hU-II and URP efficiently crossed the plasma membrane in a receptor-independent mechanism involving endocytosis through caveolin-coated pits; this uptake of hU-II, but not that of URP, was dependent on extracellular pH.

CONCLUSION

Our results suggest that (1) U-II and URP can differentially modulate nuclear UT functions such as gene expression, and (2) both ligands can reach the internal cellular space through a receptor-independent mechanism.

Abbreviations

Ahx, L-2-aminohexanoic acid; BOP, benzotriazol-1-yl-oxy-*tris*(dimethylamino)-phosphonium hexafluorophosphate; Bpa, *para*-benzoyl-phenylalanine; DCM, dichloromethane; DIEA, *N*,*N*-diisopropylethylamine; DMF, dimethylformamide; FITC, fluorescein isothiocyanate; MALDI-TOF, matrix-assisted laser desorption/ionization – time-of-flight; MEM, minimum essential medium; MFI, mean fluorescence intensity; Nup62, nucleoporin 62; PACAP(28-38), C-terminal segment 28–38 of pituitary adenylate cyclase-activating polypeptide; PTX, pertussis toxin; TFA, trifluoroacetic acid; U-II, urotensin-II; URP, urotensin-II-related peptide; UT, urotensin-II receptor

Introduction

Urotensin-II (U-II), initially isolated from the teleost urophysis, was later found to be also expressed in mammalians where it was characterized as a potent vasoconstrictor (Vaudry *et al.*, 2010). While all U-II isoforms contain a conserved C-terminal cyclic hexapeptide, the N-terminal segment is highly variable (Leprince *et al.*, 2008). A paralogue peptide, the U-II-related peptide (URP), was isolated from rat brain extracts, and mature forms from other mammalian species, including humans, were predicted from prepro-URP cDNA sequences (Sugo and Mori, 2008). So far, all mammalian URP isoforms are strictly identical (Leprince *et al.*, 2008). Structurally related, U-II and URP possess the conserved cyclic region encompassing the biologically active triad Trp-Lys-Tyr (Leprince *et al.*, 2008). Both peptides are endogenous ligands of a GPCR known as UT, belonging to subclass 1A of GPCRs (Lavecchia *et al.*, 2005; Proulx *et al.*, 2008). The urotensinergic system is not only widely distributed within the CNS but also in the peripheral nervous system and more precisely within the cardiovascular system where it exerts inotropic effects, vascular smooth muscle cell proliferation, remodelling changes as well as vasoconstriction or vasodilatation depending on the vascular bed (Dubessy *et al.*, 2008; Russell, 2008). Because U-II was isolated more than 10 years before its peptide paralogue, it has always been considered as the peptide of interest, and most of the *in vitro* and *in vivo* studies are related to its putative effects and pathophysiological relevance. In fact, a high expression of U-II and its receptor has been found to be associated with several pathological states including hypertension, atherosclerosis, heart failure, pulmonary hypertension, diabetes mellitus and renal failure (Ross *et al.*, 2010).

Once bound to its receptor, U-II gives rise to the generation of second messengers (i.e. inositol triphosphate and diacylglycerol), leading to intracellular calcium release and activation of different pathway systems such as p38MAPK, ERK1/2 and Rho A/ROCK (Proulx *et al.*, 2008). However, the intracellular signalling of URP has not been thoroughly studied, most probably because of its structural homology with U-II and similar potencies at triggering calcium release in UT-transfected cells (Dubessy *et al.*, 2008) and inducing hypotension in anaesthetized rats (Sugo and Mori, 2008). However, the differential distribution of U-II and URP mRNAs suggests that these peptides could have complementary biological functions (Vaudry *et al.*, 2010). Corroborating this assertion, two studies have demonstrated that these two peptides exert different biological effects on astrocyte cell proliferation (Jarry *et al.*, 2010) and cardiac contractility (Prosser *et al.*, 2008).

In many species, including chicken, rodent and human, it has been shown that the U-II gene is closely related to the cortistatin gene, whereas the URP gene is located on the same chromosome as somatostatin (Tostivint *et al.*, 2008). Interestingly, cortistatin shares many biological activities with somatostatin and is not only able to bind and activate all its receptors but also possesses unique functional activities (Ferone *et al.*, 2006). An undiscovered receptor subclass and/or a receptor dimerization (homo or hetero) phenomenon could explain this kind of atypical pharmacological profile. Nevertheless, an increasing amount of evidence shows that the localization of GPCRs is not limited to the plasma membrane, and that functional GPCRs can be found at the nuclear membrane. In particular, functional β-adrenergic, endothelin and angiotensin receptors were characterized at the cell nucleus (Boivin *et al.*, 2006). Moreover, it has been suggested that these intracellular receptors can trigger different signalling pathways in relation to their localization (Vaniotis *et al.*, 2011).

In this study, we investigated the presence of functional UT receptors on the nuclear membrane and probed the ability of both endogenous ligands (i.e. U-II and URP) to cross the plasma membrane. Our results demonstrated the propensity of both peptides to enter, in a receptor-independent manner, the intimal compartment and to differentially activate the receptor found in nuclei extracted from the rat heart. This study establishes the presence of functionally coupled UT receptors in nuclei isolated from heart tissue and raises the question about its physiological and pathophysiological role upon U-II and/or URP activation.

Methods

Materials

Fmoc-protected amino acids, Wang polystyrene resin and BOP reagent [benzotriazol-1-yl-oxy-*tris*(dimethylamino)-phosphonium hexafluorophosphate] were purchased from Chem-Impex (Wood Dale, IL, USA). Common solvents for solid phase peptide synthesis and purification were obtained from Fisher Scientific (Nepean, ON, Canada), whereas trifluoroacetic acid (TFA) was from PSIG (Montreal, QC, Canada). Isotopes (i.e. $Na^{125}I$ and ^{32}P-labelled uracil) were purchased from Perkin Elmer (Montreal, QC, Canada). Antifade reagent (ProLong), streptavidin-Alexa Fluor 568 conjugate, Alexa Fluor 448 conjugated anti-rabbit and Alexa Fluor 568 conjugated anti-mouse secondary antibodies as well as dNTPs were supplied by Invitrogen (Burlington, ON, Canada). Other chemicals, including chloramine-T, sodium bisulfite, nocodazole, nystatin, amiloride, sucrose, pertussis toxin (PTX), ammonium chloride, chloroquine, maleimide, sodium azide, fluorescein isothiocyanate (FITC), D-biotin, propidium iodide (PI), bacitracin, iodoacetamide, dithiothreitol (DTT), PMSF, the protease inhibitor cocktail, secondary antibodies as well as cell culture media and additives were ordered from Sigma Aldrich (Mississauga, ON, Canada). Rabbit anti-UT receptor antibodies were from GeneTex (San Antonio, TX) or from Alpha diagnostic International (San Antonio, TX), whereas rabbit anti-nucleoporin 62, anti-caveolin-3, anti-cytochrome C and anti-lamin A antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). DRAQ5™ nuclear staining reagent was from BioSatus Ltd. (Leicestershire, UK). The biotinylated anti-rabbit secondary antibody was obtained from Vector Laboratories (Burlington, ON, Canada). SuperSignal West Pico chemoluminescent substrate and monomeric avidin columns were from Pierce Biotechnology (Rockford, IL, USA), while the Nuclear Extract kit was from Active Motif (Carlsbad, CA, USA). The DC Protein Assay kit, used for protein quantification, and PVDF membranes were purchased from Bio-Rad (Montreal, QC, Canada).

Peptide synthesis

Human (h) and rat U-II, URP, N-biotin-[Ahx^0, Bpa^3]-hU-II, TAT(48–60) and PACAP(28–38), as well as their fluorescent derivatives, were synthesized using Fmoc chemistry with a BOP coupling strategy as previously described (Brkovic *et al.*, 2003). In order to obtain FITC-conjugated peptides, peptidyl resins were coupled with an ε-amino acid (Fmoc-Ahx-OH) and then were reacted overnight with FITC (1.2 equiv) and triethylamine (20 equiv) in a DMF/DCM mixture (1:1) (Jullian *et al.*, 2009). TFA-mediated cleavage (TFA/ethanedithiol/phenol/water; 92/2.5/3/2.5; 2 h) afforded the expected fluorescent peptides. Cyclization of hU-II, URP and their fluorescent analogues (FL-hU-II and FL-URP) was mediated by iodine (10% in methanol) in a mixture of acetic acid (70% in water) for 30 min and stopped by the addition of ascorbic acid (Erchegyi *et al.*, 2005). Preparative RP-HPLC was carried out using a Phenomenex Luna C_{18} column (250 × 21.2 mm), and fractions were analysed using matrix-assisted laser desorption/ionization – time-of-flight (MALDI-TOF) mass spectrometry (Voyager DE system from Applied Biosystems in linear mode using the α-cyano-4-hydroxycinnamic acid matrix, Carlsbad, CA, USA) and analytical RP-HPLC with a Phenomenex Jupiter C_{18} column (250 × 2.4 mm). Fractions corresponding to the desired product with purity greater than 98% were pooled and lyophilized. The physicochemical characteristics of all peptides are shown in Figure S1 (see supporting information).

Calcium mobilization assay

Activation of UT receptors was evaluated with a functional cell line co-expressing human UT receptors and a mitochondrial apo-aequorin protein from Euroscreen in accordance with the manufacturer's protocol (Gosselies, Belgium). A detailed experimental procedure is provided in the supporting information (Appendix S1).

Peptide iodination

Synthetic hU-II, URP and N-biotin-[Ahx^0, Bpa^3]-hU-II were radiolabelled with 0.5 mCi $Na^{125}I$ using the chloramine-T technique, as previously described (Doan *et al.*, 2011). Iodinated peptides were purified using a C_{18} Sep-Pak cartridge (Waters Corp., Milford, MA, USA), collected and stored at –20°C until use.

Nuclear receptor binding assay

Radioligand binding assays were performed with 150 µg of proteins in a binding buffer containing 50 mM Tris-base (pH 7.4), 100 mM NaCl, 10 mM $MgCl_2$, 0.1% BSA, 1 mM PMSF for a reaction volume of 500 µL. For competition studies, 0.2 nM [^{125}I]-hU-II was incubated with unlabelled hU-II, URP, urantide or somatostatin (10^{-6} M). After a 2 h incubation, the reaction was stopped by rapid filtration under reduced pressure through Whatman glass microfibre filters (GF/C) pre-soaked in 5% skim milk with a 1225 Sampling Manifold from Millipore (Bedford, MA, USA). Filters were rinsed three times with cold 25 mM Tris–HCl (pH 7.5) and then counted using a γ-counter to quantify residual radioactivity. Data were plotted in a displacement histogram using Prism 4.0 (GraphPad Software, San Diego, CA, USA).

Protein isolation

Male Sprague–Dawley rats (200–250 g) were obtained from Charles River (St-Constant, QC, Canada). All experiments were performed according to the recommendations of the ethics committee of this research centre and were supervised by authorized investigators. First, rats were killed by carbon dioxide asphyxiation, and then fresh heart tissues were isolated and placed in cold PBS. Hearts from male cynomolgus monkeys were a generous gift from Pr. Veronika von Messling (INRS-IAF, Laval, QC, Canada). Tissues were weighed, diced, pulverized in liquid nitrogen to obtain a fine powder and finally homogenized with a Polytron. Whole cell and nuclear proteins were isolated according to the supplier's procedure described in the Nuclear Protein Extract kit. Protein samples were quantified following the Bio-Rad DC Protein Assay.

Isolation of membrane and nuclei from heart

A procedure described by Boivin *et al.* (2003) was followed. The nuclei enriched pellet was re-suspended and stored at –80°C until use or directly re-suspended in transcription buffer for transcription initiation assay (Appendix S1).

Electrophoresis and immunoblotting

Protein extracts were separated either by 1D- or 2D-PAGE. Experimental details are presented in the supporting information (Appendix S1).

Purification and characterization of photolabelled complexes

Transfected CHO cells overexpressing the UT receptor were used as a positive control for the photolabelling and purification processes applied to membrane preparations and nuclei isolated from rat heart tissues. Radiolabelled N-biotin-[Ahx^0, Bpa^3]-hU-II (5 nM) was incubated for 90 min at room temperature with 200 µg of proteins from either rat heart (whole nuclei or enriched membrane fraction) or CHO-UT transfected cells (Chatenet *et al.*, 2004) in a buffer composed of 50 mM Tris–HCl (pH 7.4), 100 mM NaCl, 10 mM $MgCl_2$, 0.1% (w/v) BSA, 1 mM PMSF and 0.01% (v/v) of protease inhibitor cocktail. Then, the total volume (200 µL) was transferred into a Millipore-Microcon centrifugal device (molecular cut-off: 10 kDa), which was used in accordance with the manufacturer's recommendations to remove unbound peptide. Resulting peptide–protein complexes were washed four times with 500 µL of binding buffer in these centrifugal devices before being transferred to a well of 96-well plate placed on ice. Volumes were adjusted to 100 µL, and samples were irradiated for 1 h with a UV lamp (100 W, 365 nm) in order to activate the Bpa residue of the U-II analogue and to obtain a covalent bond between the biotinylated peptide probe and the bound protein. Proteins were solubilized by adding 150 µL of a lysis buffer [50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.5% (v/v) Igepal, 0.1% (w/v) SDS, 0.1% (v/v) Triton X-100 and 0.1% (v/v) of the protease inhibitor cocktail] to each well. Following a 30 min incubation at 4°C, insoluble proteins were removed by centrifugation (45 min, 13 000× *g*, 4°C). Supernatants were kept for purification of the biotinylated photolabelled complex with monomeric avidin columns from Pierce. As recommended by the manufacturer, columns were treated with a 2 mM D-biotin/PBS solution to block irreversible binding sites,

regenerated with a 100 mM glycine–HCl buffer (pH 2.8) and washed four times with PBS. Proteins to be purified (150 µg) were loaded onto the column along with 1.8 mL of PBS and let to stand for 1 h at room temperature. Then, the column was washed four times with PBS and eluted with 2 mM D-biotin/PBS. Specifically bound photolabelled complexes were collected as 2 mL fractions and counted with a γ counter. Fractions with the highest radioactivity counts were lyophilized and kept at −20°C until further use. The photolabelled receptors were solubilized in water and then analysed using a 10% SDS-PAGE performed during 2 h at 100 V. Gels were dried on a cellophane membrane and revealed onto an X-ray film, in the presence of an intensifying screen.

Transcription initiation assay

Transcription initiation was evaluated in freshly isolated nuclei from rat heart, as previously described with small modifications to the protocol (Boivin *et al.*, 2006); a detailed description is provided in the supporting information (Appendix S1).

Binding assay

The presence of specific binding sites at the cellular membrane was assessed by competitive binding assays performed on CHO-K1, HEK-293 and HeLa cells. Cells were seeded at a density of 500 000 cells per well in six-well plates and cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After 24 h, medium was removed, and cells were washed three times with binding buffer [25 mM Tris–HCl, 25 mM $MgCl_2$, 1% (w/v) BSA, and 5 µg·L^{-1} bacitracin] and then exposed to 0.2 nM of [^{125}I]-hU-II or [^{125}I]-URP in the presence or not of hU-II or URP (10^{-5} M), respectively. Following a 2 h incubation at room temperature, cells were washed twice, lysed with NaOH (0.1 M), and the cell-bound radioactivity was quantified using a γ-counter (1470 Automatic Gamma Counter, Perkin Elmer).

Confocal microscopy

To monitor receptor-independent uptake, CHO-K1, HeLa and HEK-293 cells were plated on an eight-well chamber slide (Lab-Tek, Nalge Nunc International, Rochester, NY, USA) and incubated for 24 h at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Subcellular localization was assessed by incubating adherent cells with fluorescent peptides (FL-hU-II and FL-URP, 10^{-6} M) for 1 h at 37°C in HEPES–Krebs–Ringer (HKR) buffer (5 mM HEPES, 137 mM NaCl, 2.68 mM KCl, 2.05 mM $MgCl_2$, 1.8 mM $CaCl_2$ and 1 g·L^{-1} glucose, pH 7.4) (Holm *et al.*, 2006). Following careful washes with an acidic aqueous solution containing 0.2 M glycine and 0.1 M NaCl (pH 4) and then PBS, cell nuclei were stained with DRAQ5™ (5 µM) for 15 min. For HeLa cells, an additional staining step was added before the nuclear staining. Following incubation with fluorescent peptides and acid washes, non-specific binding was blocked with 10% goat serum in PBS for 1 h at room temperature. Cells were then incubated with anti-caveolin-3 primary antibody (1:200) at 4°C overnight. Coverslips were washed several times with PBS and incubated with a biotinylated anti-rabbit secondary antibody (1:200) for 1 h at room temperature. Unbound secondary antibodies were removed by washing, and cells were then incubated with 1 µg·mL^{-1} of Streptavidin-Alexa Fluor 568 conjugate in PBS for another hour. The peptide subcellular localization was directly analysed with an oil immersion Nikon Plan Apo 100 objective mounted onto a Nikon Eclipse E800 microscope (Nikon, Melville, NY, USA) equipped with a Bio-Rad Radiance 2000 confocal imaging system (Bio-Rad Laboratories, Hercules, CA, USA).

For studies using pharmacological inhibitors, living CHO-K1 cells were pre-incubated, for 30 min at 37°C, with one of the following agents: nocodazole (20 µM), nystatin (25 µg·mL^{-1}), sucrose (0.25 M) or amiloride (2.5 mM). Other inhibitors such as NH_4Cl (10 mM), chloroquine (50 µM) and maleimide (10 µM) were introduced into the culture medium (without FBS) 10 min before FITC-labelled peptide treatment (10^{-6} M). Finally, PTX, a $G_{i/o}$-protein blocker, was added (200 ng·mL^{-1}) to cells in serum-free medium 6 h before addition of the fluorescent peptides (10^{-6} M).

In order to evaluate the effect of low temperature on the cellular uptake, CHO-K1 cells were cooled to 4°C in serum-free medium 15 min before the incubation with fluorescent peptides (10^{-6} M) and maintained at this temperature for 1 h. It should be noted that all solutions were also cooled to 4°C before use.

Left ventricles from rat heart tissue were isolated, fast frozen, sliced into serial 6 µm-thick sections and mounted on slides. Frozen sections were fixed in methanol : acetone (1:1) for 10 min at room temperature and then rehydrated with 100% ethanol (5 min, 3 times) and 95% ethanol (1 min) respectively. Slides were incubated for 45 min at room temperature in a blocking buffer containing 1% BSA, 10% goat serum and 0.1% Triton X-100 in PBS. Double labelling was performed by incubating sections in PBS (1% BSA) containing rabbit anti-rat UT (1:50) and mouse anti-lamin A (a protein related to inner nuclear envelop; 1:50) for 2 h at room temperature. Secondary antibodies (i.e. Alexa Fluor 488-conjugated anti rabbit and Alexa Fluor 568-conjugated anti mouse) were diluted (1:2000 each) in PBS containing 5% goat serum and then applied to the slides for 1 h at RT. After each incubation, sections were rinsed with PBS at least three times. Nuclei were stained for 15 min at room temperature with DRAQ 5 (5 µM). After the final washing with PBS, the sections were air-dried and then mounted with ProLong to prevent photobleaching. Negative controls (i.e. non-specific binding of the secondary antibodies obtained by omitting primary antibodies in the staining protocol) were included for each experiment. Microscopic analysis was performed with the confocal microscope described above.

Flow cytometry

As previously mentioned, cell lines lacking UT receptors were used to monitor receptor-independent uptake. Cells seeded in 12-well plates were incubated with fluorescent peptides as described for confocal microscopy. After acid washes to remove unbound and surface-bound ligand, cells were then enzymatically removed from the surface with trypsin/EDTA (5 min at 37°C), and the reaction was quenched by adding 10% FBS/PBS. After centrifugation (5 min at 1000× *g*), pelleted cells were re-suspended in 500 µL of PBS containing 0.5 µg·mL^{-1} of PI and kept on ice until analysis. A minimum of 10 000 cells per sample were then analysed on a FACScan (BD Biosciences, San Jose, CA, USA). PI staining positive cells

were excluded from the analysis, and the mean fluorescence intensity (MFI) of the living cell population was used for statistical analysis. Data interpretation was achieved with WinMDI software (Windows Multiple Document Interface for Flow Cytometry).

Kinetics of entry was investigated by incubating CHO-K1 cells with FITC-conjugated peptides (10^{-6} M) for various periods of time at 37°C in HKR buffer while following the protocol described above. Maximal uptake was calculated as the amount of internalized peptide at 37°C after 1 h.

To study the effect of pH on the cellular uptake of FL-hU-II and FL-URP, an HKR buffer for which the pH was adjusted at 6.5, 7.0, 7.5 and 8.0 was used following the same protocol.

Experiments investigating the effects of pharmacological inhibitors or of temperature on uptake were performed as described in the confocal microscopy section, except that DRAQ5™ was replaced with PI.

Data analysis

Data are expressed as mean ± SEM. Statistical analysis was assessed using Prism version 4.0 (GraphPad) with a one-way ANOVA followed by a Dunnett's post-test, a two-way ANOVA followed by a Bonferroni's post-test or Student's unpaired *t*-test.

Results

Identification and biochemical characterization of nuclear U-II binding sites

A biotinylated photoactivable peptide probe, N-biotin-[Ahx^0, Bpa^3]hU-II, was used to pull down U-II binding sites from nuclear proteins. The identity of the derivative was assessed by mass spectrometry and RP-HPLC, and the probe was found to have a similar calcium mobilization potency and efficacy as that of hU-II (see Figure S1). Photolabelled complexes, obtained with radiolabelled N-biotin-[Ahx^0, Bpa^3]-hU-II and three different preparations, that is, CHO-UT transfected cells (positive control), as well as membrane and nuclear heart extracts were isolated on a column of monomeric avidin and migrated on SDS-PAGE (Figure 1A). Nuclear heart extracts were characterized by verifying the presence of specific known subcellular marker proteins including caveolin-3 (rafts-plasma membrane), cytochrome C (mitochondria) and Nup62 (nuclear envelope) (Figure 1A). Immunoreactivity against UT and Nup62, but not for caveolin-3 and cytochrome C, was detected in the nuclear fraction, demonstrating the high purity of this nuclear extract. The results from the photolabelling experiments showed the presence of only one protein specifically and covalently labelled

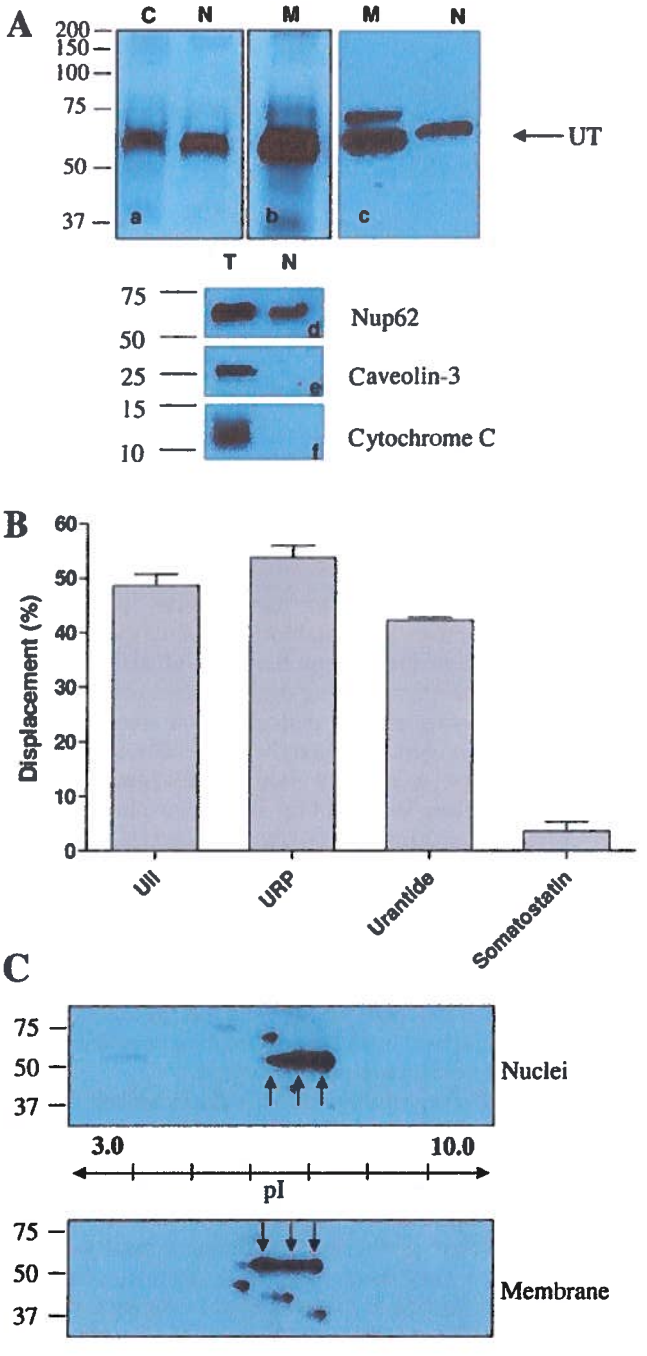

Figure 1

(A) Purified photolabelled complexes from CHO-UT total protein, rat heart nuclear extracts and rat heart membrane proteins characterized by autoradiography (a,b). UT detection in rat heart extracts by immunoblotting using GeneTex anti-UT antibody (c). C: total proteins from CHO-UT transfected cells (positive control), N: rat heart nuclear extracts and M: rat heart membrane proteins extract, T: rat heart total proteins. Proteins isolated from rat heart tissues were resolved using 10% SDS-PAGE and analysed by Western blot using anti-Nup62 (d), anti-caveolin 3 (e) and anti-cytochrome C (f) as described in the experimental section. (B) [^{125}I]-hU-II binding to rat isolated heart nuclei and displacement by non-radioactive peptides. Results shown are the amount of bound [^{125}I]-hU-II displaced when incubated in the presence of the unlabelled peptide indicated. Data are mean ± SEM of at least three independent experiments, and each determination was performed in duplicate. (C) 2D immunoblots revealed with anti-UT antibody performed on membrane and nuclear proteins extracted from rat heart. Proteins were isoelectrically focused in a pH gradient of 3 to 10 and then resolved through a 10% SDS-PAGE as described in the Methods section. Immunoreactive species corresponding to the UT receptor are located between 50 and 75 kDa at a pI value of 6–7.

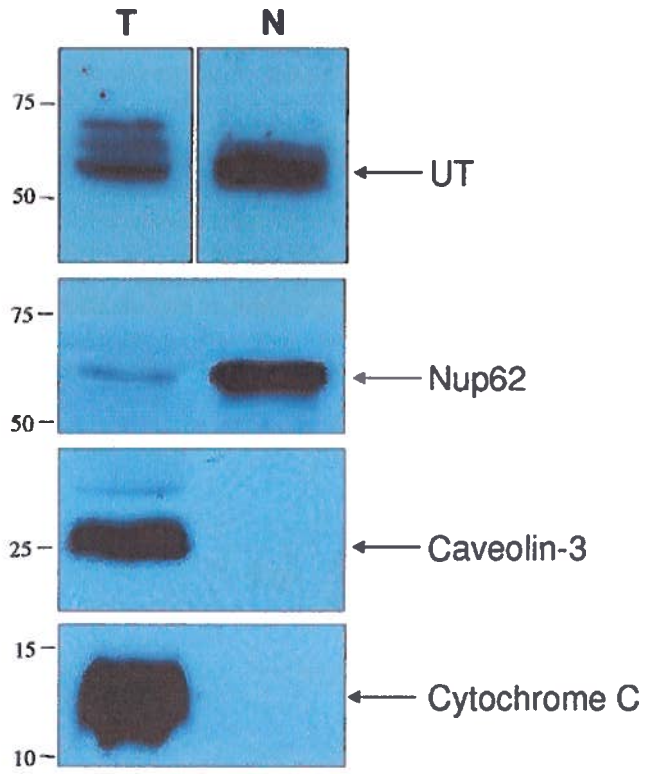

Figure 2

Total (T) and nuclear (N) protein extracts isolated from cynomolgus monkey heart. Total and nuclear proteins were resolved using 10% SDS-PAGE and analysed by Western blot using anti-Nup62, anti-caveolin 3 and anti-cytochrome C, as described in the Methods section.

with the U-II analogue in these three preparations (Figure 1A). Moreover, immunoblotting, using specific antisera against UT, also identified a band of ~60 kDa in the rat heart nuclear extract supporting the abovementioned results. Radioligand binding assays, performed on nuclei isolated from the rat heart, demonstrated the propensity of hU-II and URP to displace specifically bound radiolabelled hU-II (Figure 1B). Nuclear U-II binding sites were pharmacologically characterized using urantide, an UT-selective antagonist, and somatostatin, a peptide sharing sequence homology with the U-II peptides. Like U-II and URP, urantide was able to displace, in a similar manner, bound [^{125}I]-hU-II, whereas somatostatin was unable to displace it (Figure 1B). These observations suggest (1) that the receptor expressed in the isolated nuclei is probably the UT receptor, (2) that it specifically binds its cognate ligands and specific antagonist and (3) that it is not a somatostatin-like receptor.

Nuclear UT receptors were further characterized through 2D-PAGE immunoblotting. Nuclear and membrane proteins extracted from heart tissues expressed three major UT-immunoreactive spots with an apparent molecular weight of 60 kDa at a pI value of 6–7 (Figure 1C). Finally, UT receptors were also detected in the nuclear proteins extracted from cynomolgus monkey heart (Figure 2), showing that this subcellular localization is not restricted to rodents. Interestingly, the anti-UT antibody (GeneTex) used in Figures 1 and 2 was raised against a human C-terminal receptor fragment, and species cross-reactivity had not been tested by the manufacturer. Our results show that it also specifically recognizes rat UT receptors since the immunoreactive species detected in rat tissue preparations (Figure 1Ac) corresponded to the band observed with the photolabelling experiments (Figure 1Aa,Ab) and with Western blot results from cynomolgus monkey tissues (Figure 2).

Nuclear localization of the UT receptor by confocal microscopy

The subcellular localization of UT receptors was further studied in rat ventricular tissue section by confocal immunofluorescence microscopy using a rabbit anti-rat UT selective antibody (Alpha Diagnostic). Specificity of this antibody was previously demonstrated (Gong *et al.*, 2004). Of note, and as previously reported, no specific staining was observed in right ventricular sections (data not shown) (Gong *et al.*, 2004). Left ventricular sections were double-stained for UT and lamin A and imaged with confocal microscopy. Nuclei were counterstained with DRAQ5™ (Figure 3A,E). As expected, immunostaining associated to lamin A, a nuclear envelope protein, was mostly surrounding nuclei (Figure 3B), while the UT staining was more diffused (Figure 3C), probably reflecting the presence of receptors across cardiomyocyte cell membranes exposed on the tissue cryosection. Moreover, co-localization of UT and lamin A was observed under immunofluorescence double staining (Figure 3D), suggesting that UT might be localized at, or in the close vicinity of, the nuclear membrane. An enlargement of a nucleus can be found in the supporting information (Figure S2). Control experiments using only the secondary antibodies detected minimal non-specific fluorescence under the imaging conditions employed (Figure 3F–H).

Transcription initiation

Transcriptional activity of U-II isoforms and URP on rat heart isolated nuclei was assessed by *in vitro* transcription initiation assays (Figure 4). Purity of nuclei used for this assay is shown in Figure 1 and demonstrated no important contamination of membrane or mitochondrial protein. In addition, this fractionation protocol was previously shown to yield a negligible amount of endoplasmic reticulum elements (Boivin *et al.*, 2003). As shown, total transcription was increased in rU-II- and hU-II-, but not URP-, treated nuclei. Interestingly, a typical bell-shaped curve was observed with hU-II but not rU-II. Pretreatment of nuclei with urantide completely abolished the rU-II- and hU-II-associated transcriptional activity, while urantide alone was unable to stimulate transcription.

Cellular uptake of fluorescein-conjugated hU-II (FL-hU-II) and URP (FL-URP)

In these experiments, the well-established cell-penetrating peptide TAT(48–60) (Dennison *et al.*, 2007) and the PACAP(28–38) segment that has no cell-penetrating properties were used as positive and negative controls, respectively. It is important to mention that cell lines (CHO-K1, HeLa and HEK-293) used in those experiments are not known to endogenously express UT receptors. Moreover, the presence of UT receptors on these cell types was assessed through binding

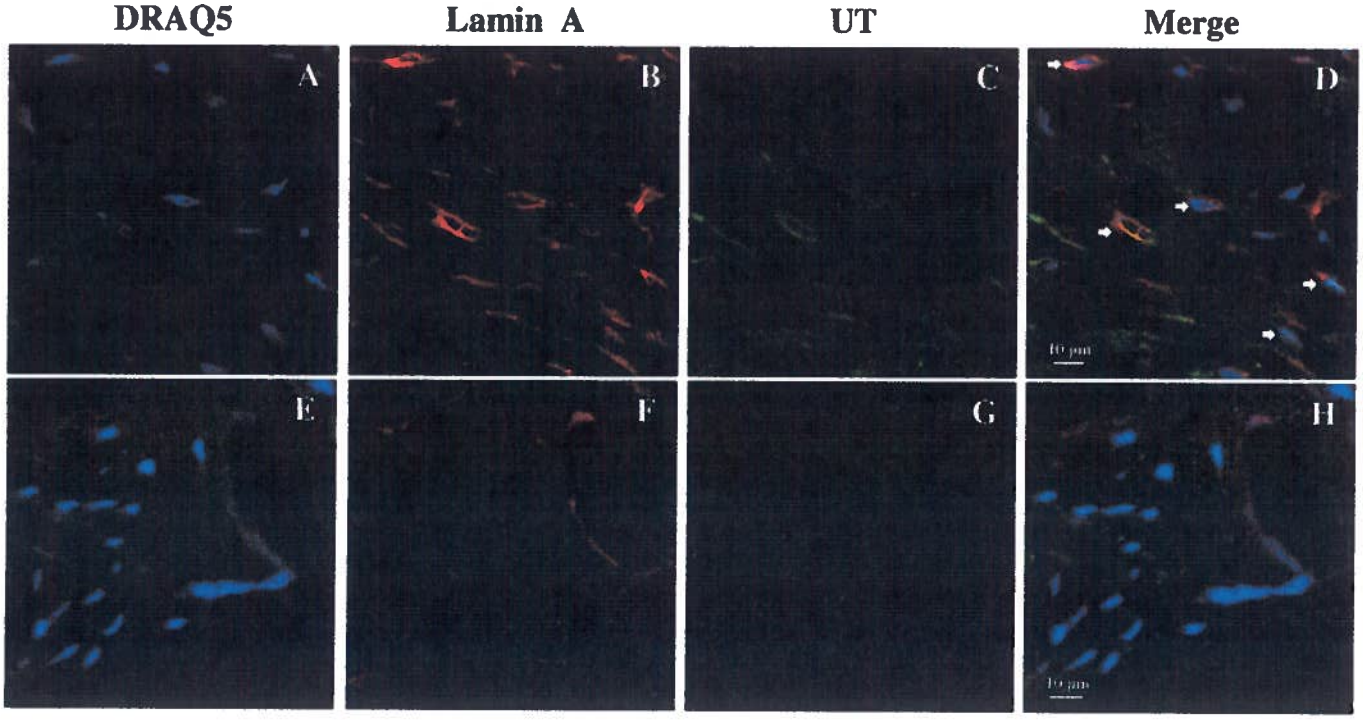

Figure 3

Immunofluorescence double staining with anti-UT and anti-lamin A antibodies show positive signals for UT protein on the cardiac myocytes imaged with a confocal microscope. Nuclei are stained with DRAQ5™ (A,E, blue signal). Left ventricular sections are double-stained for lamin A (B, red signal) and UT (C, green signal). (D) is the superimposition of (A), (B) and (C), showing the co-localization of UT (yellow signal, arrowhead) and the nuclear envelope. (F) and (G) represent negative control sections using 1% BSA–PBS instead of primary antibodies. (H) is the superimposition of (E), (F) and (G), showing no co-localization of the non-specific labelling.

experiments using ^{125}I-labelled peptides (0.2 nM) in the presence or not of cold peptide (10^{-6} M). As shown in Figure 5, no significant displacement was observed with either hU-II or URP (data presented in Figure 5 are representative of the CHO-K1 cells, but similar results were obtained with HEK-293 and HeLa cells). These results suggest that UT receptors are probably not present (or at a very low density) on the cellular membrane of these cell types. Using confocal microscopy analysis on fixed (Figure S3) or living (Figure 6A) CHO-K1 cells, we demonstrated the propensity of FL-hU-II and FL-URP to cross the plasma membrane in order to be located not only in the cytoplasm but also in the nucleus. However, for both peptides, the signal recorded at the nucleus in living cells was weak but still representative of a nuclear localization of the peptides. Similar distributions were also observed in other cell types such as HEK-293 and HeLa cells, demonstrating that hU-II and URP can enter into different cell types (see Figure S4). Interestingly, incubation with FL-URP resulted in a punctuate cytoplasmic distribution that may represent endocytic vesicles (Figure 6A).

Quantification of cellular uptake confirmed the ability of both peptides to efficiently penetrate inside the intracellular compartment but with a lower potency than TAT(48–60) (Figure 6B–D). Even if no uptake variation was observed between cell lines for FL-hU-II or FL-URP, a significant difference in the uptake between both peptides was observed in each cell line (Figure 6B). Both peptides were also shown to be taken up by cells in a concentration-dependent manner, and a good uptake, represented by a significant increase in fluorescence, was obtained at a concentration of 10^{-7} M for both peptides (Figure 6E).

Penetration kinetics of fluorescein-conjugated hU-II and URP

Characterization of FL-hU-II and FL-URP translocation into living CHO-K1 cells revealed that the cellular uptake rate of both ligands was not significantly different (Figure 7A). After 10 min, about 41% (MFI: 31 ± 9) of the maximum FL-hU-II uptake (the maximal uptake of FL-hUII or FL-URP being the MFI value obtained after 1 h of incubation with cells; MFI: 72 ± 6) had entered inside cells. After the same period of time, only 28% (MFI: 12 ± 3) of maximal FL-URP (MFI: 46 ± 10) was found in the cytoplasm (Figure 7A). This significant difference in cellular uptake, not uptake kinetics, between FL-hU-II and FL-URP was observed as soon as 2 min after the beginning of the experiment and up to the end. Moreover, using radiolabelled peptides (i.e. [^{125}I]-hU-II and [^{125}I]-URP) we were able to observe an efficient cellular uptake at a physiological concentration (0.2 nM) (Figure S5).

Influence of extracellular pH on cellular uptake of FL-hU-II and FL-URP

Following incubation of cells (CHO-K1) with FL-hU-II or FL-URP in media at different pH (6.5–8), cellular uptake

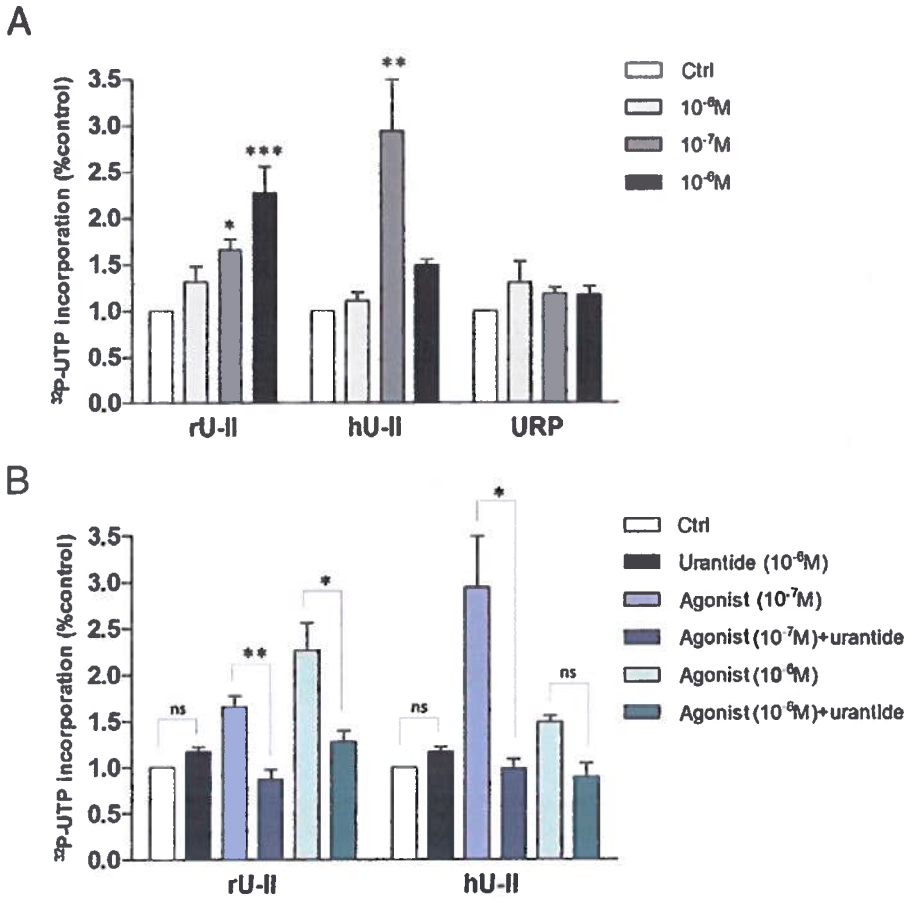

Figure 4

Regulation of UT transcriptional responses in nuclei isolated from rat heart. (A) Isolated nuclei were treated with increasing concentration of endogenous UT ligands (i.e. rat U-II, human U-II or URP). (B) Incorporation of [^{32}P]-UTP was measured in isolated nuclei either untreated or pretreated with UT-specific antagonist (i.e. urantide). Data, collected from at least three separate experiments, represent mean ± SEM. Significant differences (*P < 0.05; **P < 0.01; ***P < 0.001) were determined by one-way ANOVA followed by Dunnett's multiple comparison test or Student's unpaired t-test.

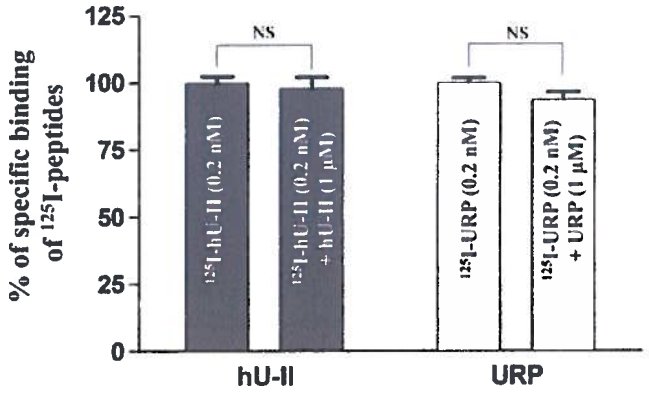

Figure 5

Displacement of bound [^{125}I]-hU-II or [^{125}I]-URP in CHO-K1 cells by hU-II or URP (10^{-6} M), respectively. Data are expressed as a percentage of the specific binding of [^{125}I]-hU-II or [^{125}I]-URP in the absence of the competitive ligands. Data represent the mean ± SEM of at least three independent assays performed in duplicate.

analysis was assessed by flow cytometry. Results showed no pH-dependent changes in the fluorescence signal of FL-URP, whereas at acidic pH (6.5), a significantly higher cellular uptake was recorded for the FL-hU-II-treated cells (Figure 7B). Noteworthy, at pH 6.5, but also at other pH values, the uptake of FL-hU-II compared to FL-URP was significantly higher. It should be noted that no difference in cell viability was observed (data not shown) in this experiment, and all cells positive for PI staining were excluded from the flow cytometry analysis.

Distinct uptake mechanism for hU-II and URP

As mentioned above, no specific U-II or URP binding sites were found on CHO-K1, HEK-293 or HeLa cells (Figure 5). Supporting these observations, pre-incubation of CHO-K1 cells with PTX, a $G_{i/o}$-protein inhibitor (0.2 µg·mL^{-1}, 6 h), did not affect the cellular uptake of either peptide (Figure 8A,C). Overall, these results suggest that both hU-II and URP can enter into cells by a receptor-independent mechanism such

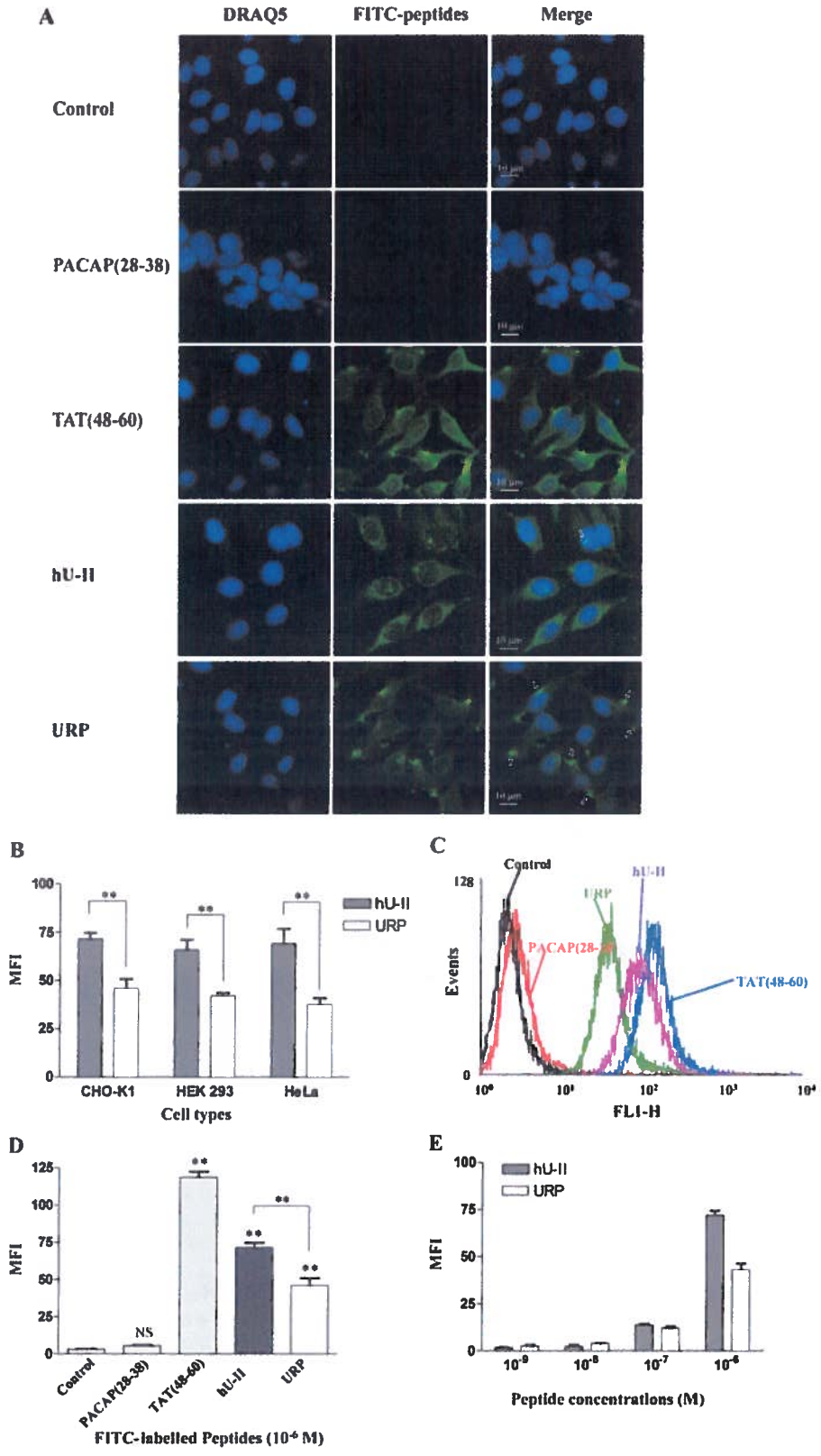

Figure 6

(A) Distribution of FITC-conjugated hU-II and URP in living untransfected CHO-K1 cells. Nuclei were stained with DRAQ5™. In these experiments, FL-TAT(48–60) and FL-PACAP(28–38) were used as positive and negative control, respectively. (B) Cellular uptake efficiency of FL-hU-II and FL-URP in various cell lines. (C,D) Flow cytometry analysis of cell penetration of FL-hU-II, FL-URP, FL-PACAP(28–38) and FL-TAT(48–60) at a concentration of 10^{-6} M in CHO-K1 cells. (E) Effect of peptide concentration on cellular uptake. CHO-K1 cells were incubated with various concentrations of fluorescent peptides ranging from 10^{-9} to 10^{-6} M.

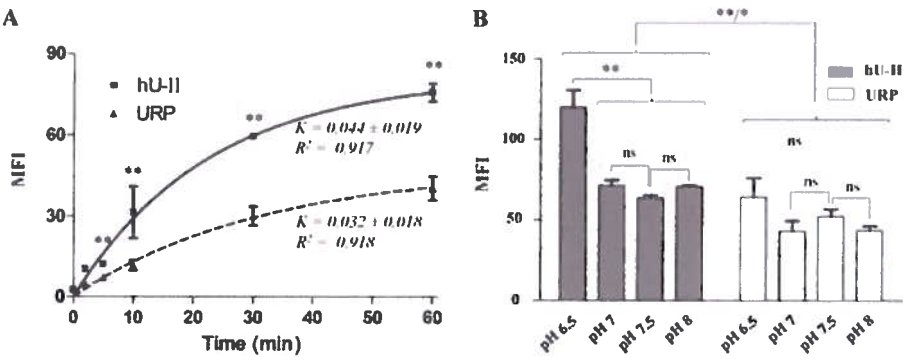

Figure 7

(A) Cellular uptake kinetics of FL-hU-II and FL-URP in CHO-K1 cells. CHO-K1 cells were incubated with FITC-conjugated peptides (10^{-6} M) for various periods of time. Maximal uptake was calculated as the amount of internalized peptide at 37°C after 1 h. (B) Effect of extracellular pH on the cellular uptake of FL-hU-II and FL-URP in CHO-K1 cells. Living cells were incubated for 1 h with fluorescent peptides (10^{-6} M) in HKR buffer for which the pH was adjusted at 6.5, 7.0, 7.5, and 8.0, respectively. Statistical significance was assessed by one-way or two-way ANOVA (*$P < 0.05$; **$P < 0.01$).

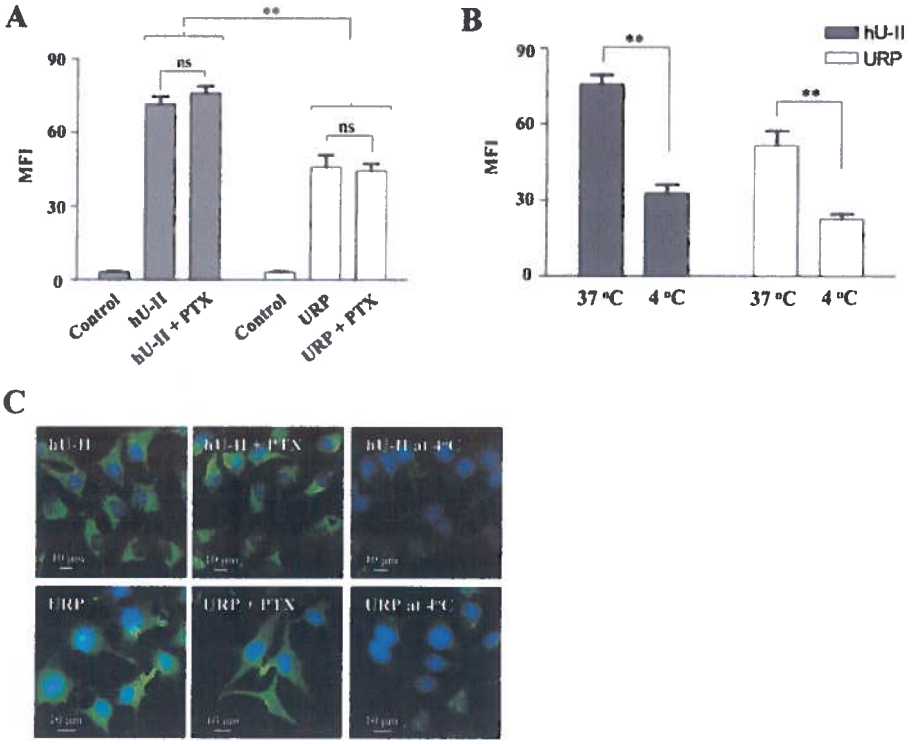

Figure 8

(A) Effect of pertussis toxin (PTX) on the cellular uptake of FL-hU-II or FL-URP in CHO-K1. PTX was added to cells placed in medium without FBS 6 h before addition of the fluorescent peptides (10^{-6} M). (B) Influence of low temperature on the cellular uptake of FL-hU-II or FL-URP in CHO-K1 cells. CHO-K1 cells were cooled to 4°C in serum-free medium for 15 min before a 1 h incubation with fluorescent peptides. (C) Confocal fluorescent images of the distribution of the fluorescent peptides following incubation with pertussis toxin or at low temperature.

as endocytosis, and/or direct translocation. Using confocal microscopy and flow cytometry experiments, after incubation of CHO-K1 cells at 4°C, we observed a significant reduction of the cellular uptake of both peptides (Figure 8B,C).

Cellular uptake of the peptides in the presence of NH_4Cl or chloroquine, known to inhibit endosomal acidification and consequently slow endocytosis (Zaro *et al.*, 2009), was decreased, with the reduction being more severe for FL-URP (27 ± 3% and 37 ± 9%, respectively) than for FL-hU-II (67 ± 14% and 76 ± 10%, respectively) (Figure 9A,B).

Pretreatment of cells with amiloride (macropinocytosis inhibitor), nocodazole (a microtubule-disrupting reagent), maleimide (vesicular transport blocker) or hypertonic sucrose

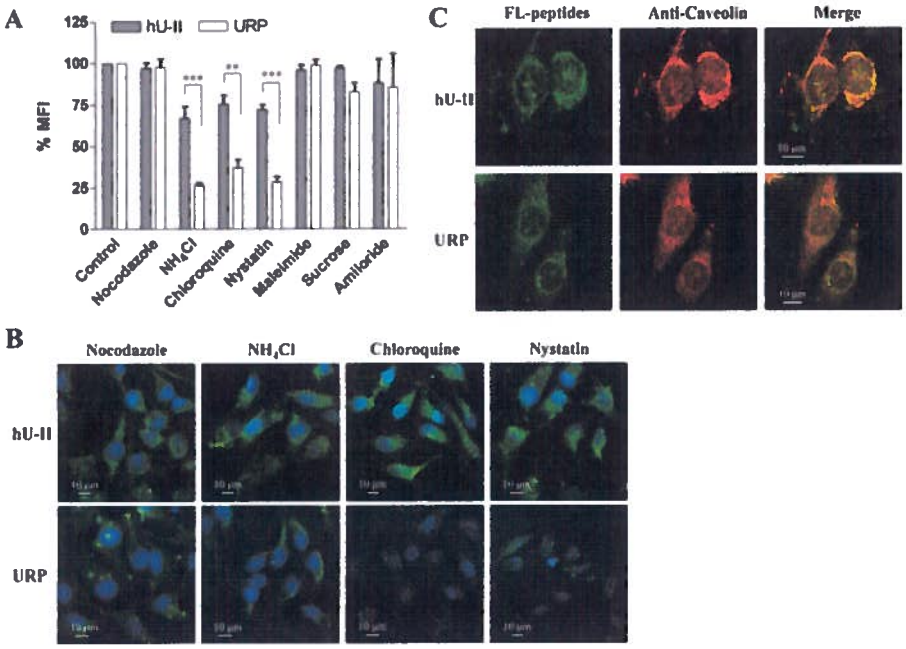

Figure 9

Effects of various endocytosis inhibitors on the cellular uptake of FL-hU-II and FL-URP in CHO-K1 cells. (A) For flow cytometry analysis, CHO-K1 cells were treated with different inhibitors as detailed in the Methods section. Values of MFI were normalized to that of the control experiments. Each point is the mean ± SEM of three separate determinations. Statistical significance was assessed by ANOVA (**$P < 0.01$; ***$P < 0.001$). (B) Confocal fluorescent images of the distribution of the fluorescent peptides following treatment with endocytosis inhibitors. (C) Co-localization of hU-II or URP with caveolin in HeLa cells by confocal immunofluorescent microscopy. Cells were double-stained with FL-hU-II or FL-URP and anti-caveolin-3 as described in the Methods section.

(a blocker of clathrin-coated pits formation) had no significant effect on the cellular uptake of FL-hU-II or FL-URP (Figure 9A,B). Hence, it is unlikely that macropinocytosis or clathrin-mediated endocytosis are involved in the cellular uptake of FL-hU-II or FL-URP. However, pretreatment with nystatin, which binds cholesterol, disrupts lipid rafts along with caveolae structures and blocks caveolae function, significantly decreased the cellular uptake of FL-URP to 29 ± 6% (Figure 9A). Interestingly, FL-hU-II cellular uptake was less affected, suggesting that the hU-II uptake mechanism only partly involves a caveolin-dependent pathway. However, FL-hU-II and FL-URP were co-localized with caveolin-3 in the segregated caveolae compartment (Figure 9C). These results further indicate that, even though hU-II and URP share similar structural and physicochemical properties, they are translocated within the cell through different endocytic uptake mechanisms.

Discussion

By combining photolabelling experiments, Western blot analyses and radioligand binding assay, we were able to demonstrate the presence of the UT receptor in rat heart nuclear extracts. Interestingly, the presence of multiple immunoreactive spots in 2D-gel experiments was observed, which could be ascribed to either a single receptor subtype undergoing various forms of post-translational modification or multiple UT receptor isoforms simultaneously co-existing within cells regardless of protein localization. Whether or not these patterns are involved in the variable U-II-associated biological activity will need further investigation. The presence of UT receptors in nuclei isolated from the cynomolgus monkey heart was demonstrated, suggesting that this particular pattern might also be found in other species including humans. Further, using characteristic proteins of specific cellular organelles, both nuclear preparations (i.e. rat and monkey heart nuclear extracts) were found to be free of membrane or mitochondrial contaminants. These results were supported by confocal microscopy that highlighted the presence of UT receptors in left ventricle heart tissue sections where it co-localized with lamin A, a protein related to the inner nuclear envelope. No immunostaining was observed in the right ventricle, suggesting the absence of UT receptors at both the membrane and the nuclei in this heart section. Such results have been observed previously in paraffin-embedded heart sections, where positive immunohistochemical staining was also observed in the left ventricle but not in the right ventricle and atria (Gong *et al.*, 2004). A perinuclear localization of the rat UT receptor was reported in UT-transfected HEK-293 cells in the absence of stimulation by its cognate ligand (Giebing *et al.*, 2005). Several vasoactive GPCRs, including endothelin, angiotensin and β-adrenoceptors, have been shown to be present in the nuclear/perinuclear environment (Boivin *et al.*, 2008). These intracellular receptors may have the capacity to regulate signalling pathways that differ from those of their plasma membrane counterparts, as recently demonstrated for the metabotropic glutamate recep-

tor 5 (Jong *et al.*, 2009) and the renin–angiotensin system (De Mello, 2008). In this study, we observed that U-II and URP, both endogenous ligands of UT, have very distinct roles as regards transcription initiation. Rat U-II dose-dependently increased transcriptional activity, whereas the observed effect of hU-II on [^{32}P]-UTP incorporation was biphasic. Rat U-II and hU-II differ in their composition and the length of their respective N-terminal segments (Vaudry *et al.*, 2010). Although it has been reported that this section is not mandatory for full biological activity (Leprince *et al.*, 2008), it might interact in a specific manner with the receptor, changing its conformation and therefore triggering complementary signalling. Further, rat and human UT receptors share around 75% of sequence homology (Elshourbagy *et al.*, 2002). Thus, a possible explanation for the observed effect could be an impaired interaction of the N-terminal domain of hU-II with the rat UT receptor, leading to a rapid desensitization of the receptor. Even though it was previously demonstrated that hU-II induces chemotaxis of peripheral human blood mononuclear cells (Segain *et al.*, 2007) and plasma extravasation in mice (Vergura *et al.*, 2004) with a typical bell-shaped dose–response curve associated with a receptor-mediated effect, further experiments are needed to clarify the difference between hU-II and rU-II.

There is no doubt that U-II and URP are endogenous agonists of UT (Vaudry *et al.*, 2010). Surprisingly, URP had no effect with regards to transcription initiation. Signalling pathways associated with UT activation have been studied, but mostly in relation to U-II and not URP as U-II was discovered almost 10 years before URP, and because both peptides have similar affinity on transfected cells system and potency on rat aortic ring (Vaudry *et al.*, 2010). The main transduction pathway associated with UT activation by U-II involves the recruitment of Gαq/11, Gαi/o, and $G_{12/13}$ subtypes of G-proteins with a subsequent increase in inositol triphosphates (IP_3) (Proulx *et al.*, 2008), ERK1/2 and RhoA activation (Guidolin *et al.*, 2010). However, recent studies have demonstrated that U-II and URP regulate astrocyte activity (Jarry *et al.*, 2010) and cardiac contractility (Prosser *et al.*, 2008) differently. Thus, it is conceivable that U-II, which structurally differs from URP due to the presence of an extended N-terminal domain that varies in length and composition, might induce a different conformational change in UT upon binding and activation. Involvement of the N-terminal peptide region in the differences in biological activity between U-II and URP has already been suggested (Prosser *et al.*, 2008). Hence, as U-II and URP share common functions, the concept of URP being an endogenous biased agonist of the urotensinergic system is proposed. The concept of biased agonist has recently emerged from various studies, resulting in the hypothesis that specific ligand-induced conformational changes can lead to precisely directed signalling (Patel *et al.*, 2010). It is thus conceivable that a similar pattern is taking place, and that hU-II and URP are able to trigger not only common but also different second messengers, leading to divergent physiological actions.

As mentioned earlier, the nuclear preparation used in this experiment is devoid of membrane or mitochondrial contaminants. Further, as exemplified in a previous report (Boivin *et al.*, 2003), the protocol used for heart nuclei isolation results in the presence of negligible amounts of endoplasmic reticulum elements. Therefore, the observed effect on transcriptional activity can definitely be ascribed to nuclear activity. It is well established that cell calcium signalling affects nuclear activity, and that the amplitude and frequency of global cellular calcium can regulate gene transcription (Dolmetsch *et al.*, 1998). The machinery required for the generation of calcium mobilizing messengers, such as the ADP-ribosyl cyclase enzyme and phosphoinositide-specific PLC, exists in nuclei (Bootman *et al.*, 2009). Moreover, it has been shown that nuclei possess phosphoinositide signalling mechanisms that lead to IP_3 production (Ye and Ahn, 2008). Interestingly, UT-mediated calcium mobilization has been linked to IP_3 production and Gq activation (Jarry *et al.*, 2010). Thus, in a similar fashion to the signalling taking place at the plasma membrane, activation of the UT receptor found on isolated nuclei could be associated with phosphoinositide activation, leading to the accumulation of calcium in nucleoplasm. Noteworthy, such nuclear calcium signals have been shown to stimulate gene transcription following nuclear angiotensin receptor activation in isolated cardiomyocytes (Tadevosyan *et al.*, 2010). Overall, these observations, which support a physiological and/or pathophysiological role for nuclear GPCRs, suggest that nuclear UT receptors might represent new or complementary therapeutic targets that should be taken into account during drug development.

A key question still remains regarding how these intracellular UT receptors are activated by their endogenous ligands. In this study, FITC-conjugated hU-II and URP were both able to reach the internal cell compartment through receptor-independent mediated endocytosis in cell lines not expressing the UT receptor. However, higher levels of FL-hU-II were found in the cytoplasm compared to FL-URP. Because the only difference found between hU-II and URP is in the sequence and length of the N-terminal region, the higher propensity of hU-II compared to URP to cross the plasma membrane might be ascribed to the specific physicochemical characteristics of this domain. Pretreatment of the cell with ammonium chloride or chloroquine, known to reduce the acidification of the endosome–lysosome system and consequently slow endocytosis, only dramatically reduced the cellular uptake of URP, characterized by a punctuate cytoplasmic distribution. These results suggest that URP, and to a lesser extent hU-II, might be trapped inside endocytic vesicles. Moreover, the fact that hU-II is less sensitive to ammonium chloride and chloroquine pretreatment suggests that hU-II can also escape more efficiently from the endosomal/lysosomal compartments. In addition, MALDI-TOF analysis of cell lysates, incubated for 1 h with native peptides, revealed that the integrity of hU-II and URP, at least in terms of amino acid sequence, is conserved, indicating that they might be stable enough in the cytoplasm to activate the intracellular receptor (data not shown). In a cell system, co-expressing membrane and nuclear receptors, the receptor-mediated endocytosis might be complemented by the receptor-independent translocation we describe in this study. Following internalization of peptide–receptor complexes, acidification of the endosome will induce dissociation of complexes and recycling of the receptor to the outer membrane (Giebing *et al.*, 2005). In this particular case, the fate of the peptide is not known, but based on our results, we assume that it might be able to leak from the vesicle and ultimately

activate the intracellular receptor. Also, the intracellular production of U-II and/or URP, as demonstrated for the angiotensin system (Singh *et al.*, 2007), is another possibility to be considered. To this extent, it is worth mentioning that the human U-II precursor is synthesized as two isoforms differing only in their peptide signal (Coulouarn *et al.*, 1998; Ames *et al.*, 1999). Comparison of peptide signal amino acid composition revealed a poor sequence homology that might reflect the fact that one sequence contains a specific signal associated with trafficking to the nuclei, as demonstrated recently for the pituitary adenylate cyclase-activating polypeptide (PACAP) gene (Tominaga *et al.*, 2010). Altogether, these observations indicate that hU-II, but not URP, is a potential intracrine factor.

The demonstrated ability of hU-II and URP to cross the plasma membrane, by a receptor-independent endocytic mechanism including the caveolin-dependent pathway, has provided new insights into the pseudo-irreversible binding characteristics often described. The inability to desensitize UT through classic mechanisms (acid wash or trypsin treatments) was thought to reflect the strong, pseudo-irreversible nature of U-II binding (Douglas and Ohlstein, 2000). Based on our results, it is conceivable that part of the pseudo-irreversible character is due to the ability of both endogenous peptides to translocate within the internal compartment of the cell. To support this hypothesis, Castel and colleagues demonstrated that temperature reduction affected the irreversible nature of the binding (Castel *et al.*, 2006). In the same way, the pseudo-irreversibility, as well as the slow internalization process of UT receptors (half-life: 15 min) account for the unusual sustained cellular responses, leading to potent vasoconstriction (Douglas and Ohlstein, 2000; Giebing *et al.*, 2005; Du *et al.*, 2010). However, it is well established that intracrine factors can form intracellular positive feedback loops, thereby maintaining a specific cellular state (Petersen *et al.*, 2006).

Identification of the precise role of this new intracellular urotensinergic system will need further experiments especially in order to ascertain if the two systems work in synergy or independently of each other. U-II and its receptor were found to be up-regulated in the failing heart (Nakayama *et al.*, 2008). In addition, elevated plasma levels of U-II have been demonstrated in numerous disease conditions, including hypertension, atherosclerosis, heart failure, pulmonary hypertension, diabetes, renal failure and the metabolic syndrome (Ross *et al.*, 2010). As observed, hU-II and URP are both able to efficiently enter the cell, this uptake being increased for hU-II at lower pH. Many pathological conditions, such as cancer, ischaemic stroke, inflammation and atherosclerotic plaques, are associated with increased metabolic activity and hypoxia resulting in an elevated extracellular acidity (Andreev *et al.*, 2010). For instance, the contractility of heart muscle is sensitive to small, physiological changes in the extracellular pH. A reduction in contractility associated with acidosis has been involved in a number of pathological conditions, most dramatically during myocardial ischaemia (Crampin *et al.*, 2006). Therefore, in such conditions where U-II is able to enter the cell more easily than URP, specific gene transcription associated with U-II activation of the nuclear receptor will be triggered. In fact, isolated ischaemic heart experiments revealed that both U-II and URP are able to decrease myocardial damage by reducing creatine kinase, but only U-II reduced atrial natriuretic peptide (ANP) production, and hence blocked the production of VEGF and inhibited angiogenesis (Prosser *et al.*, 2008). Thus, following a decrease in membrane pH during hypoxic conditions, it is reasonable to assume that the inhibition of ANP production by hU-II might be the result of the nuclear receptor activation.

In conclusion, we report here, for the first time, a specific nuclear/perinuclear expression of the U-II receptor in the rat and monkey heart, which upon activation by U-II or URP results in different modulatory effects on transcription. Although the physiological role of this nuclear GPCR remains to be established, our results suggest that nuclear UT receptors are associated with a specific biological role and that U-II, which is able to specifically activate nuclear UT receptors, but not URP, should be considered as an intracrine factor. This study also highlighted a complementary receptor-independent mediated endocytosis that might be, at least in part, involved in the intracellular presence of UT ligands. Most antagonists developed so far have failed in clinical trials for several reasons including a lack of efficacy in pathological *in vivo* models, low potency, low selectivity or concomitant agonist/antagonist behaviour (Maryanoff and Kinney, 2010). However, the lack of efficacy of such compounds might also be related to their inability to reach and block the action of the nuclear UT receptor. Critical questions still remain such as 'Are the pleiotropic effects within the cardiovascular system, including modulation of cardiac contractility, vascular tone, cell proliferation and cell growth, equally modulated by U-II and URP?' and 'Are these actions directly associated with the activation of the nuclear and/or the membrane receptor?' Nevertheless, the presence of functional UT receptors at the cell membrane and at the nucleus should be taken into account during the development of new therapeutic compounds for the treatment of pathologies associated with the urotensinergic system.

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Conflicts of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 (A) Amino acid sequence of synthesized peptides. (B) Analytical data obtained by MALDI-TOF spectrometry and by RP-HPLC. [a]Percentage of purity determined by HPLC using a buffer system [A = H_2O (pH 2.5) and B = 100% CH_3CN] with a gradient slope of 1% B per minute, at a flow rate of 1 mL·min^{-1} on a Vydac C_{18} column (5 µm particle size, 300 Å pore size). Detection at 214 nm. [b]Observed *m/z*-value compared with the calculated $[M + H]^+$ monoisotopic mass. (C) Effect of hU-II or N-biotin-[Ahx[0], Bpa[3]]-*h*U-II on Ca^{2+} mobilization using CHO cells co-expressing human UT receptors and a mitochondrial apo-aequorin protein. Data are expressed as a percentage of the response (bioluminescence) obtained with digitonin (50 µM) added to the same 96-well culture plate. Data represent the mean ± SEM of at least four independent assays performed in duplicate.
Figure S2 Enlargement of a nucleus from Figure 4D. Nucleus was stained with DRAQ5™ (blue colour). Left ventricular sections are double-stained for lamin A (red colour) and UT (green colour). This enlargement demonstrates the co-localization of UT (yellow colour, arrowhead) and lamin A.
Figure S3 Distribution of FITC-conjugated hU-II and URP in fixed untransfected CHO-K1 cells. Nuclei were stained with PI.
Figure S4 Distribution of FITC-conjugated hUII and URP in living (A) HeLa and (B) HEK-293 cells. Nuclei were stained with DRAQ5™.
Figure S5 Internalization of ^{125}I-hU-II and ^{125}I-URP in CHO-K1 cells. CHO-K1 cells in 12-well plates were incubated for various periods of time in FBS-free media containing 0.2 nM ^{125}I-hU-II or ^{125}I-URP. Internalization was stopped by washing, and cells were then solubilized with 1 M NaOH, and the radioactivity was quantified using a γ-counter. Membrane-bound radioactivity was evaluated by incubation of CHO-K1 cells with ^{125}I-labelled peptide (0.05 nM) for 30 s.
Appendix S1 Methods.

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Supplemental information

Biochemical and Pharmacological Characterization of Nuclear Urotensin-II Binding Sites in Rat Heart

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Methods

Cell Culture. CHO-K1 and HEK-293 cells were maintained respectively in Ham-F12 and DMEM medium supplemented with 2 mM L-glutamine, 100 UI/ml each of penicillin and streptomycin, and 10% fetal bovine serum (FBS). HeLa cells were grown in MEM media containing 10% fetal bovine serum, 100 UI/ml each of penicillin and streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate. Cell line co-expressing human UT receptors and a mitochondrial apo-aequorin protein (Euroscreen, Gosselies, BE) were cultured in HAM-F12 medium supplemented with 10% fetal bovine serum, 400 µg/ml G418, 100 IU/ml penicillin, 100 IU/ml streptomycin, 2.5 µg/ml amphoterin B and 5 µg/ml puromycin as recommended by the supplier. Cells were maintained as monolayer at 37°C in a humidified atmosphere of 5% CO_2 and 95% air and passages were performed by trypsinization once cells reached 70-80% confluence.

Calcium Mobilization Assay. Activation of UT receptors was evaluated with a functional CHO-UT transfected cell line from Euroscreen (Gosselies, BE). These cells stably express UT receptors and a mitochondrially-targeted aequorin responsible for generating bioluminescence upon receptor activation. Cells in midlog phase were detached with 5 mM EDTA/PBS, centrifuged twice (1500 g, 2 min) and resuspended in assay medium (DMEM/HAM-F12 with HEPES but without phenol red and containing 0.1% BSA, 100 UI/ml penicillin, 100 UI/ml streptomycin and 2.5 µg/ml amphoterin B) at a final concentration of 5 x 10^6 cells/ml. Coelenterazine H (0.5 mM solution in methanol) was added to cells to obtain a final concentration of 5 µM. Cells were then incubated at room temperature and protected from light during 4 h with gentle agitation. Incubation was prolonged for another 30 min after diluting cells 10 times with the assay buffer. A volume of 50 µl (*i.e.* 25000 cells) was then individually added to each well of a white 96-well plate containing 50 µl of the ligand (concentration varying from 10^{-10} M to 10^{-5} M) and the emitted light was recorded with a BioTek Instruments Synergy HT microplate reader (Winooski, VT, USA) for 20 sec immediately following cell injection. Individual responses were expressed as a percentage of the effect generated by 5 µM of digitonin and plotted according to ligand concentration to generate concentration-response curves.

Electrophoresis and Immunoblotting. Protein extracts were separated either by 1D or 2D-polyacrylamide gel electrophoresis (PAGE). For 2D-PAGE, samples (25 µg) were loaded onto 7 cm Immobiline DryStrip Gels (IPG strips, pH 3-10) from GE Healthcare (Baie D'Urfé, QC, Canada) in 100 µl of rehydration solution (8M urea, 20 mM Tris-HCl, 2% CHAPS (w/v), 0.5% IPG buffer (pH 3-10), 40 mM DTT, 0.005% bromophenol blue (w/v). Rehydration was performed at room temperature for 7 h and isoelectric focusing was achieved at 600V for 10h on a GE Ettan IPGPhor system. Strips were then

placed successively for 10 min in 1% DTT (w/v) and 2.5% iodoacetamide solutions (w/v) (prepared from a stock solution of 6M urea, 75 mM Tris-HCl pH 8.8, 2% SDS (w/v), 29% glycerol (v/v), 0.002% bromophenol blue (w/v)) and mounted at the top of a 10% SDS-polyacrylamide gel. Electrophoresis was performed at constant amperage (15mA) until the migration front reached the bottom of the gel. As for 1D-PAGE, proteins were also resolved through a 10% SDS-polyacrylamide gel but at a constant voltage (100V) for 2h. Separated proteins were transferred at 100V, 4°C for 90 min onto a PVDF membrane in a buffer containing 25 mM Tris base, 192 mM glycine and 20% methanol. At room temperature, membranes were then blocked for 1h in TBST (25 mM Tris-HCl, 150 mM NaCl and 0.05% Tween 20 (v/v)) supplemented with 5% skim milk (w/v). Incubation with primary antibodies (rabbit anti-human UT, anti-Nup62, anti-caveolin 3 or anti-cytochrome C) diluted in TBST containing 1% BSA (w/v) was performed overnight at 4°C. Membranes were then incubated for 1h at room temperature with horseradish peroxidase-conjugated secondary antibody in TBST containing 5% skim milk (w/v). After each blocking and incubation, membranes were washed 3 times with TBST. Finally, immunoreactive species were visualized by exposing membranes to an X-ray film using the Super Signal West Pico Chemiluminescent Substrate kit from Pierce.

Isolation of membrane and nuclei from heart. Briefly, tissues were diced and pulverized in liquid nitrogen. Cold PBS was added and homogenization was achieved with a Polytron. Homogenates were then centrifuged for 15 min at 500 g at 4°C to remove non-homogenized tissues and supernatants were diluted 1:1 in 10 mM K-HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT supplemented with a protease inhibitor cocktail. Following a 10 min incubation on ice, a centrifugation was performed (2000 g, 15 min). The supernatant, containing enriched membrane fraction, was aliquoted and stored at -80°C until use. The resulting pellet was resuspended in 300 mM K-HEPES (pH 7.9), 1500 mM KCl, 30 mM $MgCl_2$ containing a protease inhibitor cocktail. This solution was incubated on ice for 10 min and centrifuged at

2000 g for 15 min. The pellet, containing the nuclei, was resuspended in 20 mM Na-HEPES (pH 7.9), 25% (v/v) glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM EGTA, 0.5 mM PMSF, 0.5 mM DTT with a protease inhibitor cocktail and stored at -80°C until use or directly re-suspended in transcription buffer for transcription initiation assay.

Transcription Initiation Assay. Transcription initiation was evaluated on freshly isolated rat heart nuclei, as previously described with small modifications to the protocol (Boivin et al., 2006). Briefly, nuclei were incubated at 30°C for 30 min with increasing concentrations of rUII, hUII or URP (10^{-8}M-10^{-6}M) and urantide (10^{-6}M) in a transcription buffer containing 50 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 0.15 M KCl, 1 mM $MnCl_2$, 2 mM DTT and 1 UI/µl RNAse inhibitor. Prior to incubation with agonists, 1µCi of [^{32}P]-labelled uracil (3000 Ci/mmol) and 1 mM adenine were added (guanine and cytosine were omitted to prevent RNA chain elongation). Following nuclear lysis with 10 mM Tris-HCl (pH 8), 10 mM EDTA and 1% SDS, total RNA was extracted using phenol/chloroform/isoamyl alcohol (25:24:1), precipitated with alcohol, and then re-suspended in water. Concentration was estimated based on UV absorbance at 260 nm and the radioactivity counted using a Packard TriCarb 2100TR Liquid Scintillation Analyzer. In a second set of experiments, isolated nuclei were either pre-treated at room temperature with urantide (10^{-6}M) for 15 min before addition of [^{32}P]-labelled Uracil and stimulation with rUII or hUII (10^{-7}M or 10^{-6}M).

Confocal Microscopy. Cells were first plated in 24-well plates (80,000 cells/well) over coverslips for 24h at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Following incubation with FITC conjugated peptides (10^{-6}M, 1h, 37 °C) and careful washes as described above, cells were fixed with methanol/acetone (1:1) at -20°C for 10 min before nuclear staining with propidium iodide (PI, 1.5 µg/ml) for another 15 min. Coverslips were then washed several times with PBS and mounted with ProLong to

prevent photobleaching. The subcellular localization was directly analyzed on the previously described confocal microscope.

Cellular Uptake Studies. Cellular uptake kinetics was investigated at physiological concentration by incubating CHO-K1 cells (250,000 cells/well) in FBS-free media with radiolabelled peptides, *i.e.* ^{125}I-hUII and ^{125}I-URP (0.2 nM), for various periods of time at 37 °C. Internalization was stopped by ice-cold PBS and acid buffer (pH 4) washings. Cells were then lysed with NaOH (0.1M), and the radioactivity was quantified using a γ-counter. Membrane-bound radioactivity was evaluated by incubating CHO-K1 cells with radiolabelled peptides for 30 sec.

FIGURE LEGENDS

Figure S1. Analytical data obtained by MALDI-TOF spectrometry and by RP-HPLC. [a]Percentage of purity determined by HPLC using a buffer system (A = H_2O (pH 2.5) and B = 100% CH_3CN) with a gradient slope of 1% B/min, at a flow rate of 1 mL/min on a Vydac C18 column (5 μm particle size, 300 Å pore size). Detection at 214 nm. The observed m/z of the monoisotope compared with the calculated $[M+H]^+$ monoisotopic mass. Effect of hUII or N-biotin-[Ahx^0, Bpa^3]*h*UII on Ca^{2+} mobilization using CHO cells co-expressing human UT receptors and a mitochondrial apo-aequorin protein. Data are expressed as a percentage of the response (bioluminescence) obtained with digitonin (50 μM) added to the same 96-well culture plate. Data represent the mean ± S.E.M. of at least 4 independent assays performed in duplicate.

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A

hUII Glu[1]-Thr[2]-Pro[3]-Asp[4]-Cys[5]-Phe[6]-Trp[7]-Lys[8]-Tyr[9]-Cys[10]-Val[11] (S—S: Cys[5]–Cys[10])

URP Ala[1]-Cys[2]-Phe[3]-Trp[4]-Lys[5]-Tyr[6]-Cys[7]-Val[8] (S—S: Cys[2]–Cys[7])

N-biotin-[Ahx[0], Bpa[3]]hUII Biotine-Ahx[0]-Glu[1]-Thr[2]-Bpa[3]-Asp[4]-Cys[5]-Phe[6]-Trp[7]-Lys[8]-Tyr[9]-Cys[10]-Val[11] (S—S: Cys[5]–Cys[10])

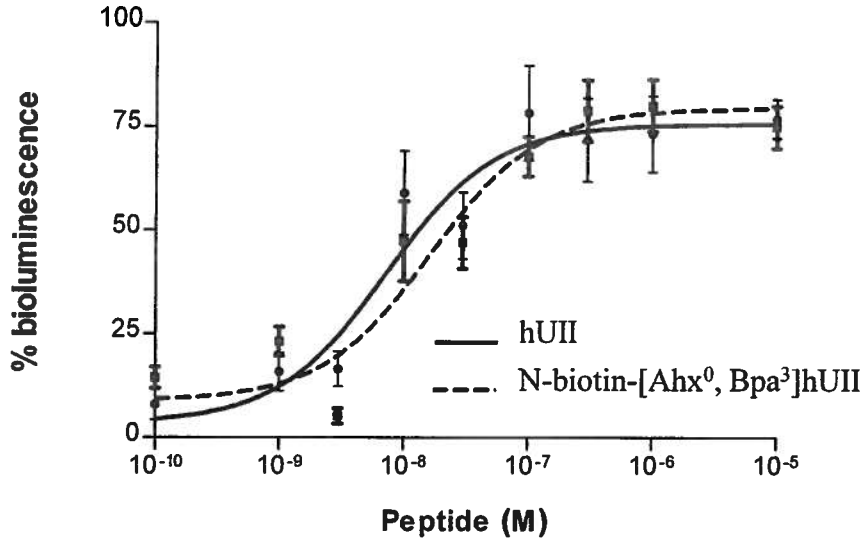
FL-hUII FITC-Ahx[0]-Glu[1]-Thr[2]-Pro[3]-Asp[4]-Cys[5]-Phe[6]-Trp[7]-Lys[8]-Tyr[9]-Cys[10]-Val[11] (S—S: Cys[5]–Cys[10])

FL-URP FITC-Ahx[0]-Ala[1]-Cys[2]-Phe[3]-Trp[4]-Lys[5]-Tyr[6]-Cys[7]-Val[8] (S—S: Cys[2]–Cys[7])

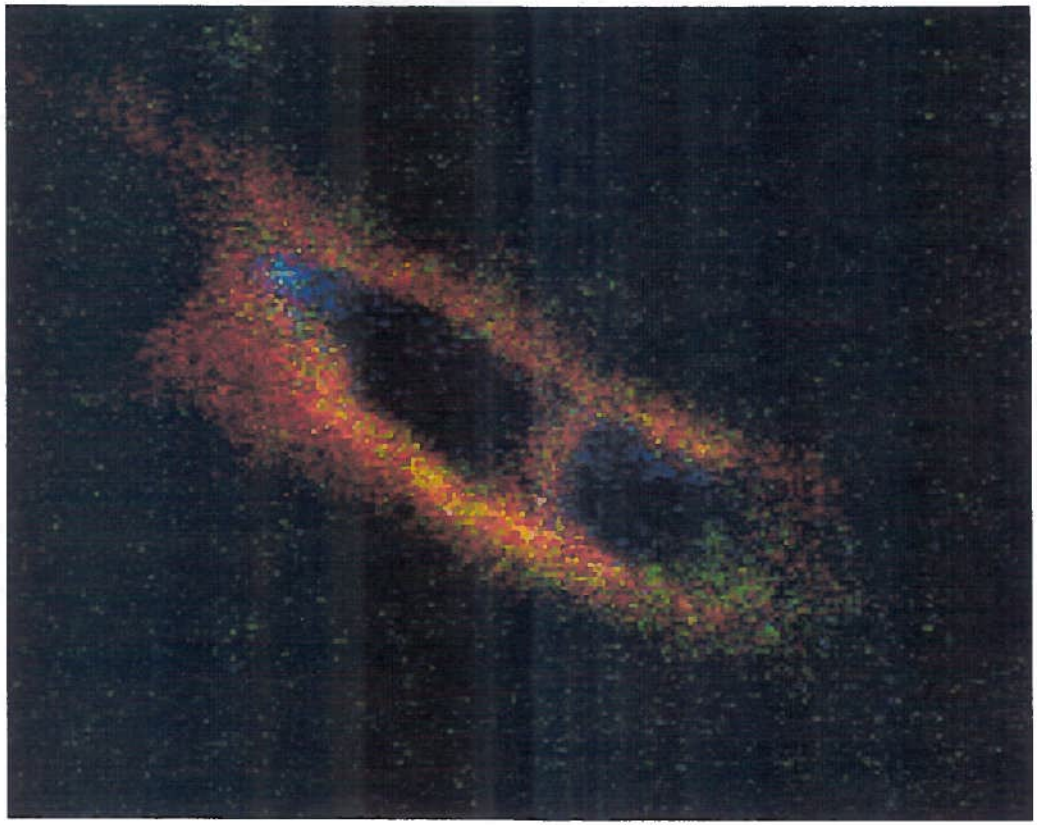
B

Compounds	HPLC[a]	MS calculated[b]	MS found[b]
hUII	> 98%	1387.6	1388.1
URP	> 98%	1016.4	1016.4
N-biotin-[Ahx[0], Bpa[3]]hUII	> 98%	1879.9	1880.9
FL-hUII	> 98%	1888.9	1889.7
FL-URP	> 98%	1518.6	1519.6

C



Doan et al.
Figure S1



Doan et al.
Figure S2

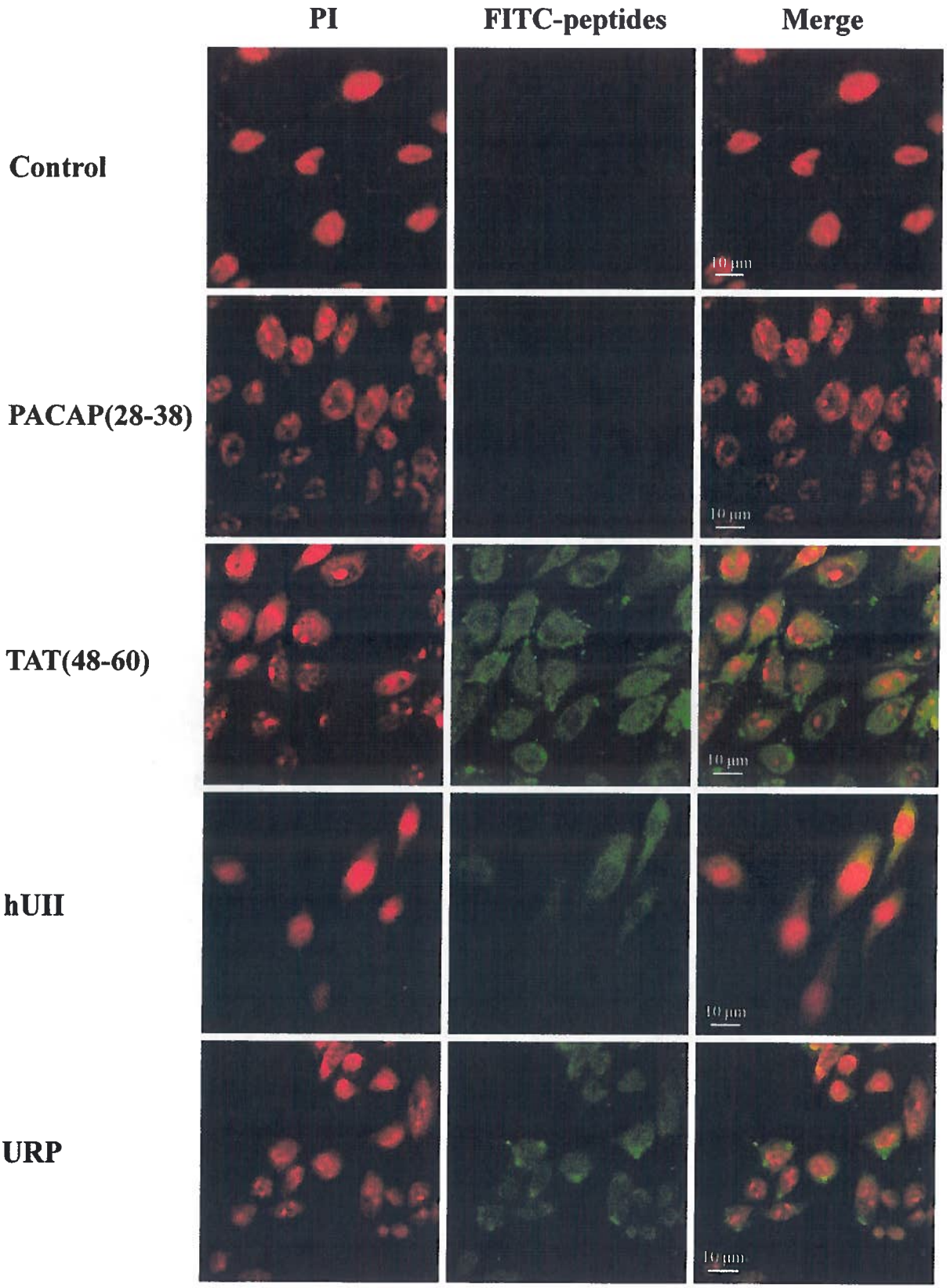


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Figure S3

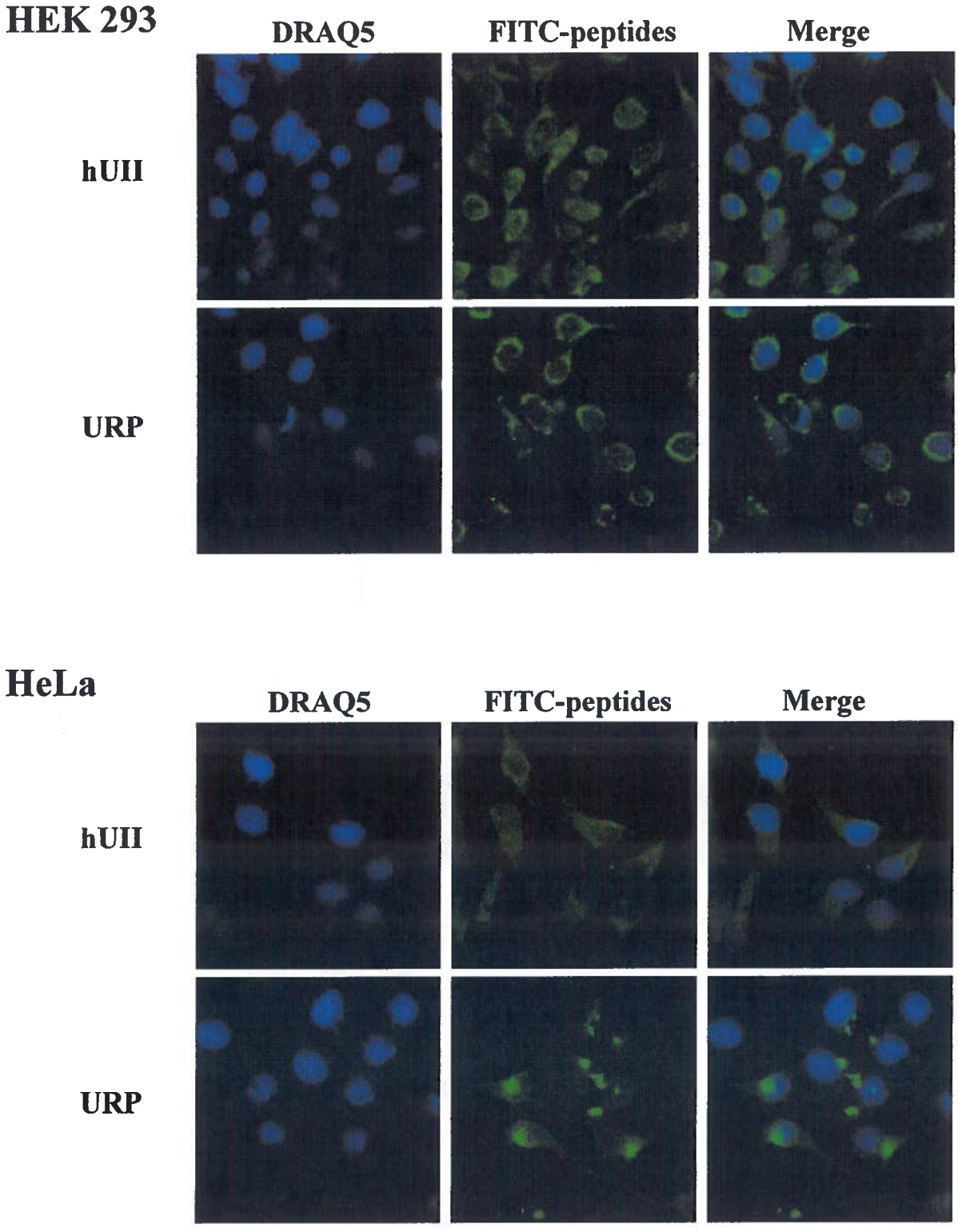

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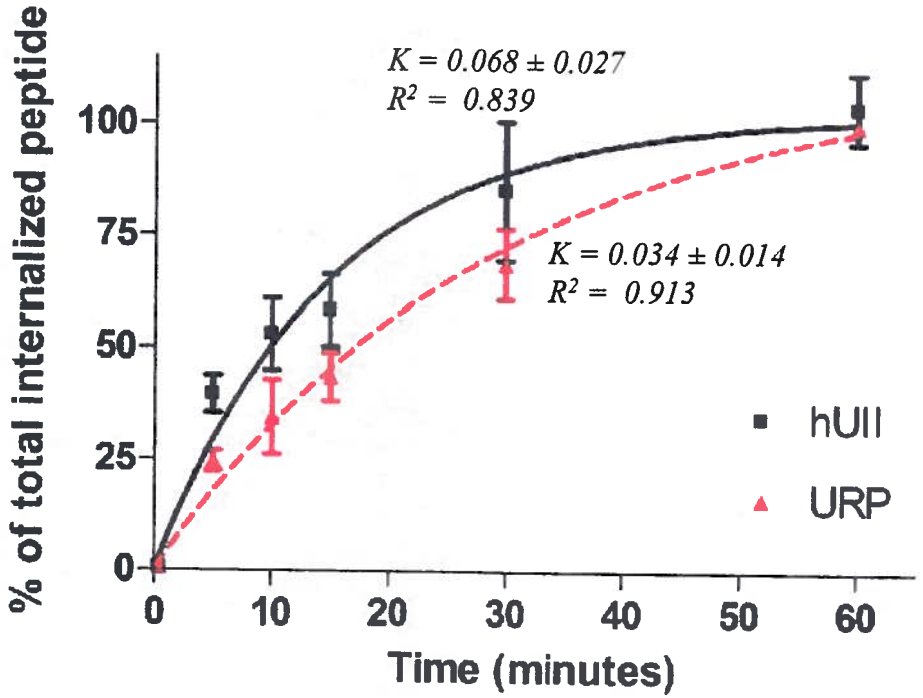
Figure S4



Doan et al.
Figure S5

PUBLICATION 2

PRESENCE OF UROTENSIN-II RECEPTORS AT THE CELL NUCLEUS: SPECIFIC TISSUE DISTRIBUTION AND HYPOXIA INDUCED MODULATION

T-T-Mai Nguyen[a,b], Myriam Létourneau[a,b], David Chatenet[a,b], and Alain Fournier[a,b,*]

[a]Laboratoire d'études moléculaires et pharmacologiques des peptides, Université du Québec, INRS – Institut Armand-Frappier, Ville de Laval, Qc, Canada; [b]Laboratoire International Associé Samuel de Champlain (INSERM – INRS – Université de Rouen).

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Contribution of the authors: The work carried out in the laboratory, as well as the data analysis, were achieved by Thi Tuyet Mai Nguyen (TTMN). Myriam Létourneau (ML), David Chatenet (DC) and Alain Fournier (AF) participated in the choice of the scientific orientations of the research program focussing on urotensin II, and supervised the work. TTMN wrote the first draft of the manuscript while ML, DC and AF revised the paper. It was published in The International Journal of Biochemistry & Cell Biology (**44**:639-647, [2012] – 2011 impact factor: 4,634).

Résumé en français

Présence de récepteurs de l'urotensine II dans le noyau de cellules: Distribution tissulaire spécifique et modulée par l'hypoxie

L'urotensine II (UII) et son récepteur UT, tous deux abondamment présents dans le système nerveux central et le système cardiovasculaire, exercent des actions dominantes, vraisemblablement tant dans des conditions physiologiques que pathologiques. Notre programme de recherche, visant entre autres à montrer la présence du récepteur UT fonctionnel au niveau du noyau de cellules de divers tissus de ratte de singe, ainsi que danses lignées cellulaires humaines, a démontré pour la première fois, par immunobuvardage de type Western et par immunofluorescence en microscopie confocale, une expression nucléaire tissu-spécifique pour ce récepteur (cœur et système nerveux central). Celui-ci a aussi été caractérisé au moyen d'études de liaison en utilisant des ligands spécifiques du système urotensinergique. De plus, lors d'expériences d'électrophorèse sur gel en 2D, nous avons observé des différences post-traductionnelles entre les récepteurs UT des membranes plasmiques et nucléaires provenant d'extraits de cerveau. Des essais d'initiation de la transcription ont montré que l'UII et l'*urotensin II-related peptide* (URP) sont capables d'induire la synthèse *de novo* d'ARN et que celle-ci peut être inhibée par l'urantide, un antagoniste de l'UT. Dans des conditions hypoxiques/ischémiques, les récepteurs UT sont apparus comme étant modulés différemment, en ce qui concerne leur localisation subcellulaire. Par conséquent, la régiospécificité du récepteur UT nucléaire, jumelée à sa modulation particulière dans des conditions hypoxiques, suggèrent un rôle physiologique spécifique et notamment, une participation à l'action pro-angiogène et neuromodulatrice de l'UII, dans les systèmes nerveux central et cardiovasculaire.

Cet article a dû être retiré en raison de restrictions liées au droit d'auteur.

Nguyen TT, Létourneau M, Chatenet D, Fournier A. Presence of urotensin-II receptors at the cell nucleus: specific tissue distribution and hypoxia-induced
modulation. Int J Biochem Cell Biol. 2012 Apr;44(4):639-47. doi:10.1016/j.biocel.2011.12.022. Epub 2012 Jan 9. PubMed PMID: 22245063.

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Supplemental information

PRESENCE OF UROTENSIN-II RECEPTORS AT THE CELL NUCLEUS: SPECIFIC TISSUE DISTRIBUTION AND HYPOXIA INDUCED MODULATION

T-T-Mai Nguyen[a,b], Myriam Létourneau[a,b], David Chatenet[a,b], and Alain Fournier[a,b,*]

[a]Laboratoire d'études moléculaires et pharmacologiques des peptides, Université du Québec, INRS – Institut Armand-Frappier, Ville de Laval, Qc, Canada; [b]Laboratoire International Associé Samuel de Champlain (INSERM – INRS – Université de Rouen).

Materials

Isotopes, *i.e.* $Na^{125}I$ and ^{32}P-labelled uracil, were purchased from Perkin Elmer (Montreal, QC, CAN). Anti-fade reagent (ProLong), FITC-conjugated anti-rabbit, Alexa Flour 488-conjugated anti-rabbit secondary antibodies, Streptavidin Alexafluor 568 conjugate as well as NTPs were supplied by Invitrogen (Burlington, ON, CAN). Rabbit anti-human urotensin II receptor (hUT) antibody and horseradish peroxidise-conjugated anti-rabbit secondary antibody were from GeneTex (San Antonio, TX, USA) whereas rabbit anti-nucleoporin 62, anti-Sp3, anti-caveolin-3, anti-cytochrome C, and mouse anti-lamin A antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). DRAQ5™ nuclear staining reagent was from BioSatus Ltd. (Leicestershire, UK). The biotinylated anti-rabbit secondary antibody was obtained from Vector Laboratories (Burlington, ON, CAN). SuperSignal West Pico chemoluminescent substrate was from Pierce Biotechnology (Rockford, IL, USA) while the Nuclear Extract kit was from Active Motif (Carlsbad, CA, USA). Mouse anti-beta actin antibody, DC Protein Assay kit and polivinylidene difluoride (PVDF) membranes were purchased from Bio-Rad (Montreal, QC, CAN). Horseradish peroxidise-conjugated anti-mouse secondary antibody was ordered from Sigma Aldrich (Mississauga, ON, CAN). Human and rat UII and URP peptides were synthesized in house following a Fmoc chemistry-based protocol as previously described (Brkovic et al., 2003). Finally, urantide and somatostatin were from Bachem (Torrance, CA, USA) and all other products were from Sigma-Aldrich (Oakville, ON, CAN).

Total protein isolation

Male Sprague-Dawley rats (200-250g) were obtained from Charles River (St-Constant, QC, CAN). All experiments were performed according to the research center ethical committee recommendations and under the supervision of authorized investigators. First, rats were killed by carbon dioxide asphyxiation

and then fresh brain, kidney, lung, skeletal muscle, spinal cord, pancreas, liver and adrenal gland tissues were isolated and placed in cold PBS. Tissues from cynomolgusmonkeys were a generous gift from Pr. Veronika von Messling (INRS-IAF, Laval, QC, CAN). Tissues were weighted, diced, pulverized in liquid nitrogen to obtain a fine powder and finally homogenized with a Polytron in ice-cold complete lysis buffer (3 ml/g of tissue) containing dithiothreitol (DTT) and protease inhibitor cocktail supplied in the Nuclear Extract kit. For the human neuroblastoma SH-SY5Y and astrocyte U87-MG cell lines, cells were harvested when the cultures reached 70-80% confluence. Whole cell and nuclear proteins were isolated according to the supplier's procedure described in the Nuclear Protein Extract kit. Protein samples were quantified following the Bio-Rad DC Protein Assay.

Isolation of membrane and nuclei from heart.

A procedure described by Boivin *et al.* was followed (Boivin et al., 2003). The nuclei enriched pellet was resuspended and stored at -80°C until use or directly re-suspended in transcription buffer for transcription initiation assay.

Electrophoresis and Immunoblotting

Protein extracts were separated either by 1D or 2D-polyacrylamide gel electrophoresis (PAGE). For 2D-PAGE, samples (25 µg) were loaded onto 7 cm Immobiline DryStrip Gels (IPG strips, pH 3-10) from GE Healthcare (Baie D'Urfé, QC, Canada) in 100 µl of rehydration solution (8M urea, 20 mM Tris-HCl, 2% CHAPS (w/v), 0.5% IPG buffer, 40 mM DTT, 0.005% bromophenol blue (w/v). Rehydration was performed at room temperature for 7h and isoelectric focusing was achieved at 600V for 10h. Strips were then placed successively for 10 min in 1% DTT (w/v) and 2.5% iodoacetamide solutions (w/v) (prepared from a stock solution of 6M urea, 75 mM Tris-HCl pH 8.8, 2% SDS (w/v), 29% glycerol (v/v), 0.002%

bromophenol blue (w/v)) and mounted at the top of a 10% SDS-polyacrylamide gel. Electrophoresis was performed at constant amperage (15mA) until the migration front reached the bottom of the gel. As for 1D-PAGE, proteins were also resolved through a 10% SDS-polyacrylamide gel but at a constant voltage (100V) for 2h. Separated proteins were transferred at 100V, 4°C for 90 min onto a PVDF membrane in a buffer containing 25 mM Tris base, 192 mM glycine and 20% methanol. At room temperature (RT), membranes were then blocked for 1h in TBST (25 mM Tris-HCl, 150 mM NaCl and 0.1% Tween 20 (v/v)) supplemented with 5% skim milk (w/v). Incubation with primary antibodies (anti-UT, anti-actin, anti-Sp3, anti-Nup62, anti-caveolin 3 or anti-cytochrome C) diluted in TBST containing 1% BSA (w/v) was performed overnight at 4°C. Membranes were then incubated for 1h at room temperature with horseradish peroxidase-conjugated secondary antibody in TBST containing 5% skim milk (w/v). After each blocking and incubation, membranes were washed 3 times with TBST. Finally, immunoreactive species were visualized by exposing membranes to an X-ray film using the Super Signal West Pico Chemiluminescent Substrate kit from Pierce.

Peptide Iodination.

Synthetic human urotensin II (hUII) was radiolabeled with 0.5 mCi $Na^{125}I$ using the chloramine-T technique, as previously described (Doan et al., 2011).Iodinated peptide was purified using a C_{18} Sep-Pak cartridge (Waters Corp., Milford, MA, USA), collected and stored at -20°C until use.

Nuclear Receptor Binding Assay.

Radioligand binding assays were performed with isolated nuclei (150 µg of protein) in a binding buffer containing 50 mM Tris-base (pH 7.4), 100 mM NaCl, 10 mM $MgCl_2$, 0.1% BSA, 1 mM PMSF for a reaction volume of 500 µl. For competition studies, 0.2 nM ^{125}I-hUII was incubated with unlabeled

hUII, URP, urantide or somatostatin (10^{-6}M). After a 2h incubation, the reaction was stopped by rapid filtration under reduced pressure through Whatman glass microfiber filters (GF/C) pre-soaked in 5% skim milk with a 1225 Sampling Manifold from Millipore (Bedford, MA, USA). Filters were rinsed three times with cold 25 mM Tris-HCl (pH 7.5) and then counted using a γ-counter to quantify residual radioactivity. Data were plotted in a displacement histogram using Prism 4.0 (GraphPad Software).

Confocal Microscopy

Monkey cortex and cerebellum were isolated, fast frozen, sliced into serial 6 µm-thick sections and mounted on slides. Frozen sections were fixed in methanol:aceton (1:1) for 10 min at room temperature and then rehydrated with 100% ethanol (3 min, 2 times) and 90% ethanol (1 min). Non specific binding was then blocked with 10% goat serum in PBS containing 0.2% Triton X100 for 1h at RT. The sections were incubated in PBS (10% goad serum) containing rabbit anti-human UT antibody (1:100) overnight at 4°C. The sections were then incubated with 1:1000 Alexa Fluor 448-conjugated anti-rabbit secondary antibody for 1h at RT. Cell nuclei were stainedwith the DNA dye DRAQ5™ (5µM) for 30 min at RT and after 3 washes, the sections were dried at room temperature in the dark, and then covered with the anti-fading solution before being visualized by an oil immersion Nikon PlanApo 100 objective mounted onto a Nikon Eclipse E800microscope (Nikon, Melville, NY, USA) equipped with a Bio-Rad Radiance 2000 confocal imagingsystem (Bio-Rad Laboratories, Hercules, CA, USA).

Human glioblastoma U87-MG and neuroblastoma SH-SY5Y cells were seeded on coverslips for 24h, fixed in 4% paraformaldehyde and permebilized with 0.1% Triton X100 for 10 min on ice. Cells were blocked in 10% goat serum in PBS, and then concomitantly incubated with a rabbit-raised anti-hUT(1:100) and a mouse-raised anti-lamin A (1:200) overnight at 4°C. Coverslips were then incubated with a solution containing aFITC-conjugated anti-rabbit (1:100) and abiotinylated anti-mouse antibody (1:200). Finally, cells were incubated in PBS with a streptavidin-Alexa Fluor 568-conjugate (10 µg/ml)

for 15 min at RTfollowed by DRAQ5 ™ nuclear staining.Coverslips then were washed, mounted with ProLong and analyzed as described for confocal microscopy.

Transcription Initiation Assay

Transcription initiationwas evaluated on freshly isolated rat brain nuclei, as previously described with small modifications to the protocol (Boivin et al., 2006). Briefly, nuclei were incubated at 30°C for 30 min with increasing concentrations of rat UII (rUII), hUII or URP (10^{-8}M-10^{-6}M) and urantide (10^{-6}M) in a transcription buffer containing 50 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 0.15 M KCl, 1 mM $MnCl_2$, 2 mM DTT and 1 UI/µl RNAse inhibitor. Prior to incubation with agonists, 1µCi of [^{32}P]-labelled uracil (3000 Ci/mmol) and 1 mM adenine were added (guanine and cytosine were omitted to prevent RNA chain elongation). Following nuclear lysis with 10 mM Tris-HCl (pH 8), 10 mM EDTA and 1% SDS, total DNA was extracted using phenol/chloroform/isoamyl alcohol (25:24:1), precipitated with alcohol, and then re-suspended in water. Concentration was estimated based on UV absorbance at 260 nm and the radioactivity counted using a Packard TriCarb 2100TR Liquid Scintillation Analyzer. In a second set of experiments, isolated nuclei were either pre-treated at room temperature with urantide (10^{-6}M) for 15 min before addition of [^{32}P]-labelled Uracil and stimulation with rUII or hUII (10^{-7}M or 10^{-6}M).

Effect of Cobalt chloride on UT receptor expression

The human neuroblastoma (SH-SY5Y) cell line was cultured in MEME/F12 (1:1) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 0.5 mM sodium pyruvate, 100 UI/ml of penicillin, 100 UI/ml streptomycin until the culture reached 70–80% confluence. Cells were then treated with cobalt chloride (100 µM). This metal can produce oxidative stress and may trigger signalling pathways resulting in the activation of the HIF-1 transcription factor and upregulation of hypoxia-related genes

(Poomthavorn et al., 2009). After 6h, 24h and 48h, cells were washed twice with PBS and recuperated by gentle scraping. In parallel experiments, following incubation with cobalt chloride (24h), cells were then treated with either hUII or URP (10^{-6}M). Total and nuclear proteins were extracted as described above and analyzed by Western blots after 1D-PAGE using a rabbit raised human anti-UT. Quantification of the level of expression, by comparison with non treated cells, was done using Alpha easy® FC software version 6.0.0 (Alpha Innotech). Normalization was achieved with actin and sp3 for total and nuclear proteins, respectively.

Data Analysis. Data are expressed as mean ± SEM. Statistical analysis was assessed using Prism version 4 (GraphPad, San Diego, CA, USA) with a one-way ANOVA followed by a Dunnett's post-test.

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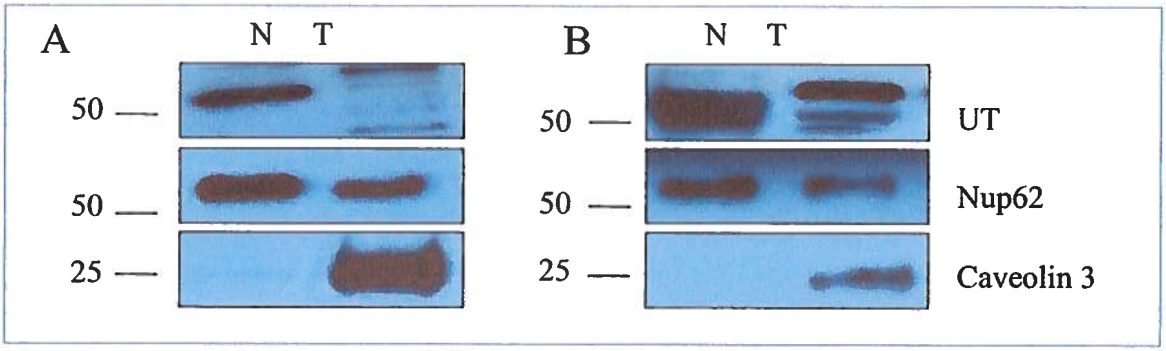

Figure S1. Nuclear UT receptor in human neuroblastoma SH-SY5Y **(A)** and astrocyte U87-MG **(B)** cell lines. Total (T) and nuclear (N) protein extracts were resolved in 10% SDS-PAGE and analyzed by Western blot using anti UT, anti-Nup62, and anti-Caveolin 3 antibodies.

SECTION 4: GENERAL DISCUSSION AND CONCLUSIONS

A growing number of nuclear GPCRs were reported in many mammalian cell types including human cells. These receptors are able to modulate second messenger levels and gene expression following activation by their cognate ligand. While plasma membrane receptors are proactive in response to stimulation, nuclear GPCRs likely play major roles in the prolongation of specific ligand effects (Tadevosyan et al., 2012). Indeed, the expression of several nuclear GPCRs is increased in cardiovascular diseases and cancer cells (Gobeil et al., 2006a). Therefore, these receptors could play an important role in controlling physiological as well as pathological processes and may thus represent new therapeutic targets.

In this project, we first demonstrated the presence of UT in various rat and monkey tissues. Previous studies indicated that in human, UT is distributed in skeletal muscles (Maguire and Davenport 2002), cardiovascular tissues including coronary arteries, saphenous vein, pulmonary vessels and cardiomyocytes (Ames et al., 1999; Maguire et al., 2000; 2008; Nothacker et al., 1999; Totsune et al., 2001) as well as in the central nervous system including brain cortex, hypothalamus and medulla oblongata (Maguire et al., 2000; Totsune et al., 2001). Moreover, it is also found in endocrine glands such as the pituitary, the adrenal glands and the placenta (Morimoto et al., 2008; Totsune et al., 2001).

In the rat heart, UT is localized in the left ventricle. More specifically, the presence of UT in cardiac myocytes and SMCs was detected by various methods including binding assay, immunocytochemistry, and confocal immunofluorescence (Maguire et al., 2008). Surprisingly, under physiological conditions, this receptor was only observed in the left ventricle, but not in the atria or the right ventricle of rat heart (Gong et al., 2004). In the present study, in addition to confirming the presence of this receptor in rat heart by immunoblotting, the presence of UT was also detected in the left but not the right ventricular sections of rat heart, thus suggesting a specific role for this receptor in relation with left ventricular functions (Doan et al., 2012b).

In the rat CNS, UT was previously observed in the spinal cord as well as in most brain areas (Gartlon et al., 2001; Jegou et al., 2006; Spinazzi et al., 2006). In agreement with these

studies, we detected the presence of UT in the spinal cord, cerebral cortex, hypothalamus, hippocampus, cerebellum and pons by Western blotting ((Nguyen et al., 2012), Figure 2A).

In addition to the heart and CNS, in our study, UT immunoreactive species appeared in all investigated tissues as previously described by other groups (Elshourbagy et al., 2002; Maguire et al., 2008). However, in the liver and lung of rat and monkey, while the expression of UT was observed in very low amounts by RT-PCR in physiological conditions (Elshourbagy et al., 2002; Totsune et al., 2003), our results showed a remarkable protein expression of this receptor. Likewise, in rat adrenal gland, the presence of UT was not detected by Northern blotting (Jegou et al., 2006) but UT was shown in the immunoblots agreeing with previous RT-PCR examination (Albertin et al., 2006). UT is also present in pancreas of both rat and monkey, which consolidate the hypothesis that the urotensinergic system plays a role in the modulation of insulin release (Marco et al., 2008; Silvestre et al., 2004).

As indicated in section 1 (Table 1-5), a number of GPCRs, including AT_1 and ET receptors (ET_A and ET_B) (Boivin et al., 2008), were detected in cell nuclei. The existence of nuclear UT, as demonstrated in this study, increases the number of nuclear GPCRs belonging to the rhodopsin-like receptor family. Through competitive binding assays using nuclei isolated from rat heart and brain tissues, we were able to observe the presence of UII and URP binding sites. Indeed, the significant displacement of labelled UII by UT ligands, combined with the failure of somatostatin to alter (^{125}I)-UII binding, demonstrated the presence of UT and the absence of sst-R in the nuclei ((Doan et al., 2012b), Figure 1B; (Nguyen et al., 2012), Figure 1D). Photolabeling experiments, which are used to covalently link a ligand and its receptor, revealed a 60 kD band corresponding to a glycosylated UT form ((Doan et al., 2012b), Figure 1A). Two dimension-gel immunoblots performed with rat heart and brain nuclear and membrane proteins showed that in heart, three main species exist in both protein extracts with a pI ranging from 6 to 7, and potentially representing different isoforms ((Doan et al., 2012b), Figure 1C). However, in brain, apparent molecular weight (MW) varied between the membrane and nuclear proteins ((Nguyen et al., 2012), Figure 1E). Such variation could be attributed to specific post-translational modifications such as glycosylation for each subcellular compartment. It was well demonstrated that glycosylation can modulate the subcellular addressing and biological activity of various receptors (Boivin et al., 2006; Duverger et al., 1995; Rondanino et al., 2003). Hence,

nuclear UT could result from *de novo* synthesis following a particular glycosylation in the Golgi apparatus associated with nuclear compartmentalization whereas higher glycosylated forms of the receptors are directed to the plasma membrane. The presence of an immunoreactive species at 37 kDa is in agreement with the observation of a PNGase F insensitive-truncated UT corresponding to the receptor lacking its N-terminal portion (Boucard et al., 2003), a phenomenon previously demonstrated for ET_B (Grantcharova et al., 2002).

Furthermore, immunoblotting and confocal immunofluorescence assays in rat and monkey tissues as well as in human cell lines supported the existence of nuclear UT ((Doan et al., 2012b), Figure 1A, 2, 3; (Nguyen et al., 2012), Figure 1B, 2B, 3B, 4, 5, 6, S1; chapter 3, Figure 1). Thus, UT clearly localizes not only at the plasma membrane but also at the cellular nucleus.

As mentioned earlier, previous studies have demonstrated the presence of GPCRs at cell nuclei, such as AT_1 and ET receptors, but without any indication regarding a particular tissue distribution. As a matter of fact, AT_1 was shown in nuclei of liver, spleen, heart and brain along with vascular SMCs, and nuclear ET receptors were found in cardiac ventricular myocytes, human aortic vascular SMCs as well as liver and brain (Boivin et al., 2003, Gobeil et al., 2006). Interestingly, nuclear UT receptors are specifically present in the heart and CNS tissues. This tissue-dependent distribution of nuclear UT is not consistent with the cellular expression of UT and could reflect an important role in cardiovascular and cerebral function. Moreover, the strictly identical regiospecificity observed in rat and monkey suggests that this particular distribution is a key characteristic of the urotensinergic system and might be common in various mammalian species, including humans.

Based on the biological activities of UII and URP in the cardiovascular and central nervous systems, we can postulate that some of these effects are mediated by the activation of nuclear UTs. Therefore, in these tissues, nuclear receptors would play a complementary physiological role in regards to the plasma membrane receptors.

The presence of UT in brain and spinal cord nuclei prompted us to investigate in more detail the nuclear UT localization in the CNS. Interestingly, nuclear UT was observed in all examined tissues belonging to the CNS ((Nguyen et al., 2012), Figure 2B). Moreover, nuclear

UT are also present in human neuroblastoma and astroglial cell lines, their localization being similar to other GPCRs of the same family such as the AT (Lu et al., 1998) and apelin receptors (Lee et al., 2004) that are also observed at nuclei of human neuronal cells. In fact, neurons and glia are the main cell types in the CNS. Neurons, the core components of the CNS, are electrically excitable cells functioning in all the crucial electrical communications by processing and transmitting information through nerve impulses and neurotransmitters (Epp et al., 2013). Glial cells, the non-neuronal cells including astrocytes, which are the most abundant of macroglial cells, provide a structural and metabolic support for neuronal networks, by anchoring neurons to their blood supply. Moreover, glial cells also act as repairmen in the regulation of neuronal injury (Baumann and Pham-Dinh, 2001).

Nuclear UTs, following the activation by UII or URP, can induce an elevation of transcription initiation ((Doan et al., 2012b), Figure 4; (Nguyen et al., 2012), Figure 7). Such action of nuclear GPCRs was also reported for AT_1 in isolated hepatic nuclei (Re and Parab, 1984) and PGE2, PAF, LPA receptors (Gobeil et al., 2006) as well as β3AR in cardiac tissues (Vaniotis et al., 2011). As shown in our two publications (section 3), UII and URP did not exert the same activity in total RNA synthesis in heart and brain nuclei preparations. While UII induced a concentration-dependent *de novo* RNA synthesis in nuclei of both tissues, URP was only able to mediate this effect in brain nuclei. A difference in the actions of these two UT ligands was also observed in rat ischemic hearts (Prosser et al., 2008) although both peptides caused mainly similar effects on cardiac tissues. For instance, only UII had the ability to reduce left ventricular contractility post ischemia and to decrease the release of the high blood pressure marker ANP. Together with our results, it can be hypothesized that URP fails to exert all UII observed effects in the heart due to its inability to activate the cardiac nuclear UTs. However, a recent study from our group suggested that UII and URP might have a different binding mechanism. In fact, the existence of a different binding site for URP within UT might result in functional selectivity (Chatenet et al., 2012) causing the recruitment of different messengers and thus, different activities in the cardiac tissue (Prosser et al., 2008) and isolated heart nuclei (Doan et al., 2012b). In contrast to the heart nuclei, similar transcription initiation activities were observed for both peptides in isolated brain nuclei ((Nguyen et al., 2012), Figure 7), though only UII was able to increase intracellular Ca^{2+} in astrocytes (Jarry et al., 2010). We therefore

hypothesized that the putative glycosylation states of nuclear and membrane UT isoforms in the brain cells ((Nguyen et al., 2012), Figure1E) could lead to different interactions with UII and URP resulting in distinct modulations of Ca^{2+} influx in the astrocytes. Altogether, while UII exerts its UT activity at the membrane level, as well as at the nucleus, in both heart and brain cells (Doan et al., 2012b; Jarry et al., 2010; Nguyen et al., 2012; Prosser et al., 2008), URP fails to activate the nuclear receptor mediating transcription initiation in the heart (Doan et al., 2012b) but is able to induce this activity when interacting with the cerebral nuclear UT (Nguyen et al., 2012). Consistent with its widespread distribution in the CNS (Nguyen et al., 2012), this functional nuclear receptor might be necessary for a specific physiological role in this system, in mammals.

Although there is a study demonstrating that UII decreases neuronal activity by potentiating γ-aminobutyric acid receptor ($GABA_AR\gamma$)-mediated Cl^- current in hippocampal neurons (Wu et al., 2006), the physiological relevance of this receptor in the brain is presently poorly understood. As mentioned in section 1, in ischemic conditions, UII increases ischemic lesions in damaged brains after reperfusion (Chuquet et al., 2008). In our study, the influence of hypoxic/ischemic conditions on UT expression in the neuronal cell line SH-S5Y5 was evaluated. While the global protein expression of UT was increased, the nuclear receptor was significantly down-regulated after only 6 hours ((Nguyen et al., 2012), Figure 8A, B). Undoubtedly, UT is modulated under *in vitro* hypoxic conditions, which could demonstrate its involvement in this pathological state. Interestingly, applying either UII or URP re-establishes basal state receptor expression ((Nguyen et al., 2012), Figure 8B, C) suggesting a possible protective role for these peptides in hypoxia/ischemia.

The decrease of UT in the nucleus following chemical hypoxia could result from its degradation or could be related to a trafficking of the receptor from this organelle to the cell membrane. In fact, the presence of UT at the nucleus can be linked to a putative NLS-like sequence found in the third intracellular loop (KRARR in human and monkey UT; KQTRR in mouse and rat UT) and composed of basic amino acid residues, as observed in AT_1 and apelin receptors (Lee et al., 2004). However, our data do not support a trafficking mechanism. The 2D-gel analysis revealed that different UT isoforms are present in the membrane and nucleus fractions of brain tissues. This might reflect different post-translational modifications ((Nguyen

et al., 2012), Figure 2E). Moreover, confocal microscopy experiments in the cerebral cortex and cerebellum tissues have detected some fluorescence signal associated to UT antisera, not only in the nuclear envelope but also in the nucleoplasm ((Nguyen et al., 2012), Figure 4), that might be due to nascent receptors within the nucleus waiting to be addressed to the nuclear membrane. Worth to mention, it is believed that coupling of transcription and translation occurs inside the nucleus (Bkaily et al., 2009). Besides, the fluorescence signals might also reflect a direct interaction of UT with DNA such as described for PTH1R (Pickard et al., 2006). Thus, nuclear UTs in brain tissue would not be translocated from the membrane but *de novo* synthesized, yet further studies are needed to clarify this aspect.

Another question arises. "*If nuclear UTs can be activated by endogenous ligands, how UII and URP can reach the nucleus?*"

In section 3, we have shown the agonist-independent presence of nuclear UT in both rat and monkey heart and brain, as well as in human cell lines. UII and URP must be able to interact with their receptor in order to induce cellular functions, such as transcription. Internalization of the ligand after binding to the membrane UTs could be a way for UII and URP to enter within the cytosol. However, penetration without the receptor must also be considered. Using confocal microscopy and flow cytometry, we were able to clearly demonstrate the ability of UII and URP to penetrate in the cytosol of various cell lines known to be unable to express UT ((Doan et al., 2012b), Figure 5, 6, S3, S4). Moreover, through the use of various inhibitors ((Doan et al., 2012b), Figure 8), we demonstrated that these agonists were able to cross the plasma membrane in a receptor-independent manner by involving a caveolin-dependent pathway. Interestingly, both peptides revealed an unequal capability to enter cells because hUII cellular uptake was significantly higher than that of URP. Furthermore, the fluorescence signal of URP appeared as punctual in the cytoplasm, which suggested an endocytic entrapment of this peptide. In fact, our study demonstrated that the caveolin-dependent pathway was mostly responsible for URP uptake mechanism while it could only account for a part of the intracellular presence of UII ((Doan et al., 2012b), Figure 9). Though these peptides are able to penetrate into cells by a receptor-independent mechanism, a possible local biosynthesis of UII and URP must also be considered.

Nuclear GPCRs were first mentioned more than 40 years ago (Robertson and Khairallah, 1971), but only a few studies investigated signal transduction associated with their activation.

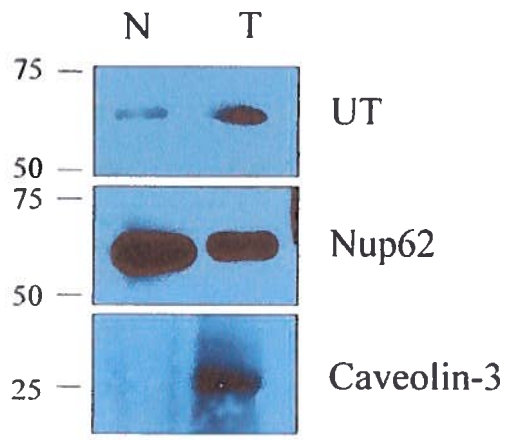

Figure 4-1: Nuclear UT in rat cardiomyocyte H9C2 cell line

N: Nuclear protein extract, T: total protein extract. Proteins extracted from H9C2 cells were resolved with SDS-PAGE and analyzed by Western blot using anti-rat UT, anti-Nup62 and anti-caveolin-3 antibodies.

For instance, in cardiomyocytes, nuclear AT_1 can activate the PLC/IP3 pathway, leading to Ca^{2+} mobilization, and induce the phosphorylation of ERK, as well as P38 (Tadevosyan et al., 2012). For ET receptors, evidence of nuclear Ca^{2+} increases have been published (Bkaily et al., 2002, Boivin et al., 2003) and recently, the Ca^{2+} signaling regulated by ET_B has been shown to be mediated via IP3R activation and then followed by a CaMKII autophosphorylation (Merlen et al., 2013). Nuclear G protein-dependent signaling is poorly understood. However, studies on β1AR suggested that this receptor is coupled to the Gs/AC/cAMP pathway while the β2AR is associated with the Gi/o/ERK1/2 and P38/Akt pathways (Vaniotis et al., 2011). UT is known to be coupled to Gq/11, but it may also bind to Gi/o and trigger ERK phosphorylation (Proulx et al., 2008). The UT/ERK pathway, in addition to its involvement in the constriction and the proliferation of SMCs, also mediates hypertrophy in cardiomyocytes (Onan et al., 2004, Liu et al., 2009). Therefore, in the present study, to study pathways involved in nuclear signaling of cardiomyocytes, we first examined the presence of nuclear UT in rat cardiomyocytes. In agreement with our previous results, Western blot analysis using nuclear extracts from the rat cardiomyocyte H9C2 cell line showed that a specific UT immunoreactive band is observed (Figure 4-1).

Because 10^{-7}M rUII activated transcription initiation on nuclei isolated from rat heart, this concentration was used to stimulate H9C2 nuclei. The results showed that rUII produced a

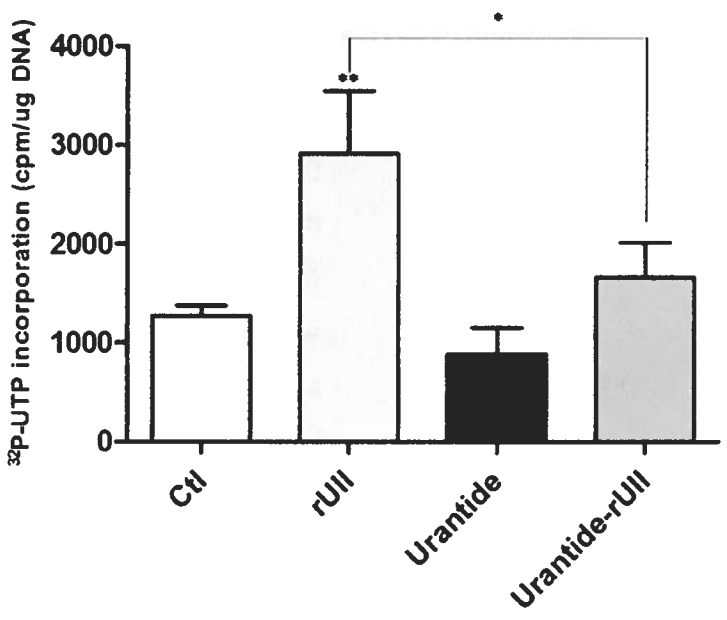

Figure 4-2: rUII mediates transcription initiation on isolated H9C2 nuclei

Ctl: No treatment, rUII: nuclei treated with rat UII (10^{-7}M), Urantide: nuclei treated with urantide (10^{-6}M), Urantide-rUII: nuclei treated with urantide (10^{-6}M) before stimulation with rat UII (10^{-7}M). *, $p<0.05$;**, $p<0.01$

significant increase of labelled uracil incorporation into DNA as it did on rat cardiac nuclei. The UT antagonist urantide, which alone does not induce any effect in this assay, abolished the activity of rUII in initiating transcription initiation (Figure 4-2).

In order to identify the transduction signals involved in the transcription initiation activity taking place once nuclear UTs are stimulated by rUII, isolated nuclei from H9C2 cardiomyocytes were examined following a pre-treatments with PTX (Gi/o inhibitor), or PD98059 (MEK inhibitor).

As shown in Figure 4-3, PTX and PD98059 were unable to inhibit rUII-induced transcription initiation. Hence, the Gi/o and ERK1/2 do not appear to be involved in the transcriptional activity of UII in the rat cardiomyocyte nucleus. Further transcription initiation assays using other inhibitors need to be performed in order to determine which second messenger is involved in this biological activity. As mentioned in section 1, several nuclear GPCRs are able to mediate nuclear Ca^{2+} mobilization, which in turn modulates gene transcription (Bkaily et al., 2009; Zhu et al., 2006). UII increases intracellular Ca^{2+} mobilization via the activation of PLC and Ca^{2+} channels (Figure 1-8), two components known to be present in the nucleus (Bootman et al., 2009; Manzoli et al., 2005). Therefore, the involvement of the PLC and Ca^{2+} channels

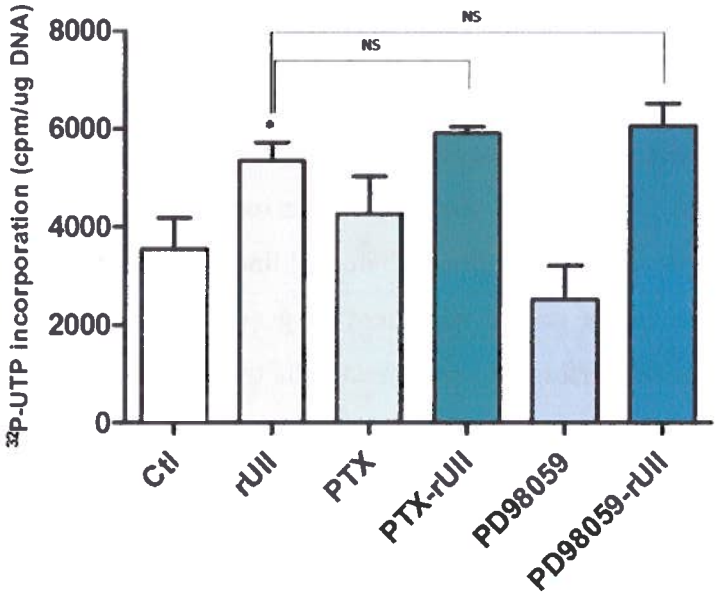

Figure 4-3: Transcription initiation induced by rUII in H9C2 is not prevented by Gi/o and MEK inhibitors

Ctl: No treatment, rUII: nuclei treated with rat UII (10^{-7}M), PTX or PD98050: nuclei incubated with 5µg/mL PTX (Gi/o inhibitor) or 1mM PD98059 (MEK inhibitor), respectively. PTX-rUII or PD98059-rUII: nuclei incubated with 5µg/mL PTX or 1mM PD98059, respectively before stimulation with rat UII (10^{-7}M). *, $p<0.05$, NS: non-significant.

(ryanodin-sensitive and IP3-sensitive calcium channels), in UT-mediated transcription should be carried out.

Because nuclear UTs do not appear to be coupled to Gi/o, whether Gq/11 mediates UII nuclear signaling should also be verified by using an immunoprecipitation assay with antibodies against Gq/11.

In this study, heart nuclei were isolated from both atria and ventricles, which contain mainly cardiac myocytes and fibroblasts. These cell types play a key role in cardiac remodelling and thus heart failure (Dai et al., 2007). UII is known to play a role in regulation of genes playing an important role in heart pathologies (Table 1-4). For instance, both atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), two peptide hormones able to decrease blood volume and cardiac output, were up-regulated in response to UII-induced cardiomyocyte hypertrophy (Zou et al., 2001). Besides, rat cardiac fibroblasts, the non-myocyte cells occupying two third of cardiac cell number (Banerjee et al., 2007), are known to positively regulate major fibrosis factors including TGFβ1, and collagen I and III *via* UT activation (Dai et al., 2007). So, the role of nuclear UT could be related to the expression of genes associated to heart pathologies. Moreover, the similarity of physiological actions between this peptide and

Ang-II or ET-1 led us to postulate that UII might also be involved in the control of specific nuclear functions. Since UII is able to activate transcription in the nuclei isolated from rat cardiomyocytes (Figure 4-2), we have performed a DNA microarray analysis on these nuclei upon UII treatment. As shown in Table 4-1, the modulation of gene expression upon UII treatment is not very marked. The small fold changes observed in our results may be related to the low density of endogenous UT receptors in this cardiomyocyte cell line (Onan et al., 2004) or the short UII exposure (30 min) which might not be sufficient to generate a clear response. Indeed, some studies about gene expression performed with intact cells treated with UII showed that RNA production peaked at 3h and even at more than 24h, depending of gene types. For instance, after 30 min, gene expression was just starting to be significantly increased with a small fold change in endothelial cells (Cirillo et al., 2008; Wang et al., 2004). Similarly, in SMCs, at least 1h was necessary for UII to induce strong RNA changes (Diebold et al., 2011; Djordjevic et al., 2005; Tsai et al., 2009). Moreover, with cardiac fibroblasts or cardiomyocytes, 100 nM UII was used to stimulate the cells during 4h - 48h to obtain clear responses (Dai et al., 2007; Gruson et al., 2012). Although our conditions were not fully optimized, this initial study was mainly designed to screen several genes presenting significant changes ($p<0.05$, Student's t test) in their expression following a short UT activation.

Table 4-1: Modulated genes following U-II stimulation of H9C2 isolated nuclei

Gene name	Gene symbol	Control (Mean ± SD) n = 6	Treatment (Mean ± SD) n = 6	Fold change
kalirin, RhoGEF kinase	*Kalrn*	39.1 ± 3.2	33.1 ± 1.9	↓1.181*
cAMP dependent regulatory, type II β subunit	*Prkar2b*	45.2 ± 2.9	38.1 ± 2.5	↓1.186**
similar to phosphoseryl-tRNA kinase	RGD1564300_pre	47.7 ± 3.2	40.3 ± 5.2	↓1.184*
mitogen activated protein kinase kinase 4	*Map2k4*_pre	113.7 ± 12.6	98.0 ± 6.3	↓1.160*
aarF domain containing kinase 5	*Adck5*	89.8 ± 5.8	108.0 ± 11.3	↑1.203*
dual specificity phosphatase 12	*Dusp12*	44.0 ± 3.4	52.3 ± 3.4	↑1.189*
similar to guanine nucleotide binding protein 13, γ	LOC682159	77.2 ± 5.1	92.4 ± 10.8	↑1.197*
CREB binding protein	*Cbp*	77.6 ± 5.9	63.6 ± 8.1	↓1.220*
Pro-Ser-Thr phosphatase-interacting protein 2	*Pstpip2*	39.2 ± 3.6	34.1 ± 3.0	↓1.150*
growth factor receptor binding protein 10 (Grb10) interacting GYF protein 1	*Perq1*_pre	56.3 ± 4.8	66.9 ± 5.8	↑1.188*
retinoid X receptor beta	*Rxrb*	42.9 ± 1.7	51.9 ± 4.7	↑1.210**
family with sequence similarity 126, member A	*Fam126a*	44.4 ± 2.9	38.4 ± 2.7	↓1.156*
fatty acid desaturase 1	*Fads1*	256.2 ± 23.3	217.1 ± 16.2	↓1.180*
superoxide dismutase 3, extracellular	*Sod3*	175.5 ± 15.1	148.4 ± 17.8	↓1.183*
WD repeat and SOCS box-containing 1	*Wsb1*	106.9 ± 11.1	90.9 ± 9.6	↓1.176*
diacylglycerol O-acyltransferase homolog 2	*Dgat2*	98.4 ± 6.7	85.2 ± 7.9	↓1.155*
poly (A) polymerase alpha	*Papola*_pre	59.8 ± 7.8	48.6 ± 5.7	↓1.230*
Rab geranylgeranyl transferase, α subunit	*Rabggta*	41.3 ± 2.8	35.9 ± 3.7	↓1.150*
F-box only protein 33	*Fbxo33*_pre	49.3 ± 6.5	40.6 ± 5.7	↓1.214*
peroxisomal trans-2-enoyl-CoA reductase	*Pecr*	71.6 ± 9.8	87.4 ± 9.7	↑1.221*
Narfl nuclear prelamin A recognition factor-like	*Narfl*	52.3 ± 5.1	62.8 ± 3.3	↑1.201*

sorting nexin 13	*Snx13*	40.8 ± 2.9	34.9 ± 2.9	↓1.172*
karyopherin (importin) alpha 1	*Kpna1*	79.6 ± 8.9	69.0 ± 4.8	↓1.154*
vacuolar protein sorting 18	*Vps18*_PRE	104.5 ± 11.3	90.4 ± 7.2	↓1.156*
golgi autoantigen, golgin subfamily a, 7	*Golga7*	65.5 ± 6.3	55.6 ± 7.0	↓1.178*
similar to CG13957-PA (*Drosophila melanogaster*)	RGD1309995_pre	52.0 ± 5.5	42.1 ± 3.3	↓1.235*
sorting nexin 1	*Snx1*	175.4 ± 15.8	207.2 ± 20.9	↑1.181*
similar to Chain A, Nuclear Transport Factor 2 (Ntf2) H66a mutant	RGD1565456_pre	40.0 ± 3.0	48.1 ± 5.6	↑1.203*
zinc finger protein 655	*Znf655*	106.9 ± 16.2	86.8 ± 6.4	↓1.232*
zinc finger protein 426-like 2	*Zfp426l2*	37.5 ± 2.1	32.6 ± 1.9	↓1.150*
zinc finger protein 386 (Kruppel-like)	*Znf386*	49.0 ± 8.1	40.1 ± 1.2	↓1.222*
zinc finger, MYND-type containing 10	*Zmynd10*	59.8 ± 4.2	73.3 ± 4.5	↑1.226**
similar to 60S ribosomal protein L27a	RGD1562402_pre	775.6 ± 24.0	947.4 ± 119.2	↑1.222*
similar to 60S ribosomal protein L7a	RGD1559149_pre	37.8 ± 2.2	46.1 ± 5.8	↑1.220*
hypothetical LOC362000	RGD1566183	33.0 ± 3.6	28.5 ± 0.4	↓1.158*
similar to IKEN cDNA 6720480D16	LOC502282	43.2 ± 4.7	35.3 ± 4.2	↓1.224*
transmembrane protein 33, transcript variant 1	*Tmem33*	39.0 ± 3.0	33.8 ± 2.2	↓1.154*
similar to RIKEN cDNA 1810029B16	RGD1305222_pre	57.1 ± 6.4	67.5 ± 6.5	↑1.182*

↑, up-regulated; ↓, down-regulated; *, $P < 0.05$; **, $P < 0.005$ (student's T test)

Kinases/phosphatases, G protein, co-activators, receptor, other signaling proteins, other enzymes, transporters, zinc-finger proteins, ribosomal proteins, unknown function proteins

Thirty eight genes, presenting a fold change >1.15 were identified. Among them, some kinases that have been never linked before to UT activation in cardiomyocytes. Interestingly, PKA type II-R subunit and MAPK kinase 4 were recently pointed-out for their clear involvement in cardiac functions (Liu et al., 2009b; Manni et al., 2008). Indeed, the phosphorylation of the former protein is elevated in basal state in cardiomyocytes and reduced in heart failure (Manni et al., 2008). This subunit may play a role in maintaining cardiac function because it modulates the negative expression of sarco-endoplasma reticulum Ca^{2+} ATPase (SERCA), a protein whose abundance and activity are reduced in heart failure (Manni et al., 2008) and diabetic cardiomyopathy (Isfort et al., 2013). Moreover, in heart failure, both protein and autophosphorylation levels of RII subunit is decreased (Zakhary et al., 2000a; Zakhary et al., 2000b). Maybe the down-regulation of this subunit decreases the downstream interaction of PKA catalytic subunit, thereby leading to a decrease in the PKA activation. Therefore, in the cardiomyocyte nuclei, UII may decrease the PKA signaling by down-regulation of the PKA type IIb regulatory subunit, which subsequently antagonizes cardiac functions. As for MAPK kinase 4 (MAPKK4 or MKK4), a specific activator of JNK in the heart that is required for cardiomyocyte differentiation (Wang et al., 2012), this kinase was also decreased by UII treatment. MKK4 was known to be involved in pathological but not physiological hypertrophy (Liu et al., 2009b). In fact, a decrease in the expression of MKK4 which is not phosphorylated was observed in TAC-induced-hypertrophic mice. Moreover, patients with heart failure also showed a remarkable decrease in MKK4 expression while it is not changed in mice with exercise-induced physiological hypertrophy (Liu et al., 2009b). Recently, it was shown that MAPK kinase 4-deficient myocardium mice negatively regulated the expression of connexin 43 (CX43) (Zi et al., 2011), a gap junction protein that plays a vital role in working myocardium. Indeed, sudden cardiac death from spontaneous ventricular arrhythmias was correlated in mice with CX43-depleted hearts (Gutstein et al., 2001; van Rijen et al., 2004). The modulation of this particular gene by nuclear UT suggests a possible role for this receptor in the pathogenesis of hypertrophy-associated ventricular arrhythmias. Another protein that is down-regulated following nuclear UT activation was identified as the cAMP-response element-binding protein (CREB)-binding protein (CBP), a coactivator of p53, a tumor repressor playing a key role in cardiomyocyte apoptosis (Komarova and Gudkov, 2000; Long et al., 1997). By participating in p53-induced gene

activation, CBP regulates this transcription factor in response to genotoxic stress. Thus, prevention of CBP from binding to p53 results in a cardiomyocyte protection (Borah et al., 2011).

Other enzymes, including extracellular superoxide dismutase 3 (SOD3) and fatty acid desaturase 1 (FADS1), which not only play a role in cardiovascular diseases but also in diabetes (Faraci and Didion, 2004; Merino et al., 2010) were found to be modulated by UII treatments of isolated nuclei. SODs, which are antioxidant enzymes, when inactivated by glycation lead to the impairment of the myocardial antioxidant defence (Ansley and Wang, 2013). SOD3 isoform expression can be altered by many elements including exercise, growth factors, cytokine, Ang-II and NO (Faraci and Didion, 2004). SOD3 overexpression, mostly beneficial, causes an increase of total cellular SOD activity that elongates the replicative lifespan of human fibroblasts in hyperoxia as well as normoxia and slows down telomere shortening (Serra et al., 2003). Hence, its down-regulation by UII may be deleterious to this tissue. As abovementioned, FADS1 plays a role in cardiovascular diseases (Erkkila et al., 2008) and in type II-diabetes (Merino et al., 2010). A study on primary rat liver cells demonstrated that cells treated with glucose or insulin showed an increased expression of this enzyme (Wang et al., 2006a), thus suggesting its involvement in glucose homeostasis. Moreover, it was shown in non-diabetic individuals with different insulin sensitivity that FADS1 was down-regulated in the group with insulin resistance versus insulin-sensitive subjects, in both adipose and skeletal muscle tissues (Elbein et al., 2011). Recently, a study carried out on patients with type II-diabetes indicated that low FADS1 activity is inversely associated with risk of this disease (Kroger et al., 2010) bringing a direct evidence for a clear involvement of this enzyme in type II-diabetes. Furthermore, glucose-induced insulin release which is a normal protective effect of the body in response to insulin resistance, is inhibited by UII (Vaudry et al., 2010), so the decrease of FADS1 level by UII could be associated to this pathological condition. Another gene found to be up-regulated in our results is RXRB, a gene encoding the nuclear retinoid X receptor. Interestingly, there is a cross-talk between RXRs and Gq proteins without ligand stimulation. This interaction is enhanced upon stimulation with the RXR ligand, cis-retinoic acid (9cRA), and inhibits Gq signaling. So, it was suggested that the Gq family represents a novel set of compounds that interact and co-regulates with nuclear receptors (Moraes et al., 2007). Therefore, the regulation of RXRB level by UII may involve a cross-talk

between this protein and nuclear UT-Gq signaling. This nuclear RXRB receptor, regarding to insulin secretion, also weakens the glucose-induced insulin release in mouse β cells *in vitro* and *in vivo* (Miyazaki et al., 2010). Hence, nuclear UT receptors could likely participate in the regulation of insulin secretion. Moreover, our results also indicated the down-regulation of an insulin resistance promoting gene known as diacylglycerol O-acyl transferase homolog 2 (DGAT2) (Jornayvaz et al., 2011; Levin et al., 2007; Wakimoto et al., 2003). As a matter of fact, chronic high blood glucose and diabetes lead to an increase in the risk of developing cardiovascular diseases (Ansley and Wang, 2013; Brown et al., 2006; Fowlkes et al., 2013; Isfort et al., 2013). Indeed, ventricular hypertrophy along with myocardial remodelling, was also observed in the early stages of diabetic patients (Isfort et al., 2013). Diabetes is also related to an increase in cardiomyocyte necrosis, an initial damage in ischemia-reperfusion injury (Ansley and Wang, 2013). Moreover, hyperglycemia gives rise *in vivo* to an alteration of the cardiac fibroblast phenotype and function, as well as a widespread collagen areas, thereby causing cardiac fibrosis in the diabetic ventricles (Fowlkes et al., 2013). Finally, death rate in patients with diabetic-heart disease is higher than with non diabetic-heart disease (Isfort et al., 2013). Altogether, these specific gene modulations generally suggest the involvement of nuclear UT in glucose homeostasis, a factor related to cardiovascular diseases.

Our study also points out the participation of nuclear UT in cell proliferation. From the modulated genes identified in our DNA microarray analysis that are involved in proliferation, the growth factor receptor-bound protein 10 interacting GYF protein 1 (PERQ1) can interact with growth factor receptor-bound protein 10 (GRB10), a protein binding to the insulin-like growth factor (IGF-1) receptor in order to regulate receptor signaling associated with IGF-1 stimulation, and promotes cell growth and proliferation (Giovannone et al., 2003). Furthermore, it was also observed that PERQ1-knockdown in breast cancer cell lines significantly reduced cell proliferation induced by epidermal growth factor (EGF) stimulation (Ajiro et al., 2010). Thus, a positive regulation of PERQ1 by UII, as observed, could enhance cell proliferation. RXRB, besides its involvement in the regulation of insulin release, is also associated with the proliferation and the survival of various cell types including cardiomyocytes (Stuckmann et al., 2003), as well as the growth and the differentiation of cardiac cells during embryonic and post-development periods (Guadix et al., 2011; Pan and Baker, 2007). The up-regulation of RXRB in

our experiment supports even more the role of nuclear UT in mitogenic effects. However, CBP, which was decreased in the present study, demonstrated that its down-regulation or silencing inhibits proliferation of endothelial cells and vascular SMCs induced by thrombin and Ang-II, respectively (Yang et al., 2010). In fact, it was suggested that maintaining proper CBP level may be a potential therapeutic target for Ang-II-induced cardiovascular disorders (Yang et al., 2010). Such opposite effects in regards to proliferation following UII stimulation were previously observed. Indeed, UII was shown to induce mitogenic actions on various cell types including SMCs, endothelial cells, astrocytes and carcinoma cells (Jarry et al., 2010; Xu et al., 2012; Yoshimoto et al., 2004) while it inhibits proliferation of some cardiac cells (Gong et al., 2011). However, adult cardiomyocytes were well demonstrated to permanently withdraw from cell cycle (Soonpaa and Field, 1997; Walsh et al., 2010). Hence, the regulation by nuclear UT of genes involved in proliferation could be related to abnormal states of cardiomyocytes.

Our results also revealed changes in the modulation of the expression pattern of genes encoding transporters. Some of these proteins are involved in endosomal-lysosomal trafficking such as sorting nexin 1 (SNX1), sorting nexin 13 (SNX13) and vascuolar protein sorting 18 (VPS18). Consequently, nuclear UT could participate in the regulation of protein degradation via the modulation of these transporters. SNX1, which was increased by UII stimulation, is known to enhance the trafficking of EGFR from endosomes to lysosomes (Zhong et al., 2002). Interestingly, this receptor can be transactivated by UT, thus conferring cardioprotection in cardiac hypertrophy (Esposito et al., 2011). SNX13, known to delay the degradation of EGFR (Zheng et al., 2001) was down-regulated in this study. Therefore, nuclear UT may be involved in EGFR desensitization pathway by regulating these transporters. Nuclear UT can also modulate the expression of the nuclear transport factor 2 and karyopherin α1, an isoform of importin α involved in the classical nuclear import pathway of many proteins including nuclear GPCRs. Besides, the expression of the Golgin subfamily A member 7 (GOLGA7), involved in the vesicular transport from the Golgi to cell surface (Ohta et al., 2003), was also significantly altered following nuclear UT activation, a process involved in intracellular GPCR location after glycosylation. Although it is not possible to give any firm conclusion based on these observations, we could at least hypothesize that nuclear UT might be involved in some plasma-cytoplast-nuclear trafficking.

Activation of this nuclear receptor mediates the elevation of ribosomal proteins (60S ribosomal protein L27a and L7a) necessary for the translation agreeing with the transcription initiation elevation triggered by UII in this cell line, as well as in isolated rat heart nuclei. Accordingly, ribosome regulation was observed with peptide hormones, known as intracrine ligands like UII (Re and Cook, 2008).

Finally, a last group of genes encoding for zinc finger proteins was modulated in our experiment. Among them, zinc finger protein 426-like 2 (Znf426l2) and zinc finger protein 386 (Znf386), known as Kruppel-associated box containing proteins, plays a role in transcriptional repression (Bellefroid et al., 1993; Fredericks et al., 2000). Another protein named zinc finger protein 655 (Znf655) was shown to be involved in the inhibition of the G1 (growth phase)/S (synthesis phase) transition during cell cycle progression (Houlard et al., 2005). Those three Znf proteins are down-regulated by UII stimulation supporting the idea that this peptide evokes intracellular mitogenic action.

Altogether, the microarray results indicate some regulation trends for UII through nuclear UT activation. However, more studies are needed to clarify the precise roles of this nuclear GPCR. To pursue this study, real-time PCR with longer UII treatments could be performed on isolated nuclei in order to obtain higher responses in terms of fold changes in gene expression. Moreover, from our results, we hypothesize that UII plays a critical role as a promoter of hypertrophy and/or cardiac remodelling through activation of its nuclear receptor, leading to the regulation of specific genes (*Map2k4, Prkar2B, Sod3, Fads1, Rxrb*, *Perq* and *Cbp*) related to heart diseases, diabetes and proliferation. Hence, these genes would be good candidates for real-time PCR experiments carried out on isolated nuclei from H9C2 cells and rat heart tissue. Moreover, several potential kinases mediating these gene expressions could be examined, including p38-MAPK and PKB, two kinases taking part in UII-induced cardiac hypertrophy pathways (Figure 1-9), JNK, which regulates cardiac hypertrophy through Gq signaling in cardiomyocytes (Choukroun et al., 1999; Minamino et al., 2002) and PKA, which is important in cardiac protection in heart failure (Mahaney et al., 2005; Sipido and Eisner, 2005; Wehrens et al., 2004), myocardial no-reflow and reperfusion injury (Li et al., 2012), and in ischemic preconditioning in oxidative stress-induced H9C2 cells (Qu et al., 2010), as well as in hyperglycemia-induced cardiomyocyte apoptosis (Younce et al., 2013).

In conclusion, this study allowed us to confirm the existence of a novel functional nuclear GPCR, the nuclear UT, with a tissue-dependent expression that is limited to heart and CNS tissues in both rat and monkey. In both tissues, nuclear UT mediates transcription initiation by UII and/or URP. These two peptides can receptor-independently enter cells, but only UII can trigger nuclear UT receptors in the heart tissue. Once activated, a signal pathway that does not involve Gi/o and ERK allows the modulation of gene expression. These genes can be associated to heart diseases, insulin release and proliferation. Moreover, under hypoxic/ischemic conditions, this receptor is regulated at both nuclear and membrane levels while either UII or URP restores initial protein levels. Thus, in our understanding, this is the first study to have a global insight about a nuclear GPCR function with a tissue-specific distribution that surely relates to a patho- and/or physiological role for the urotensinergic system in mammals. However, a question remains as to why nuclear UTs are only expressed in heart and CNS tissues. Answering this question would certainly enhance our understanding of the physiological role of UII.

Recently, the concept of intracrine signaling has gained much interest. Intracrine networks are known to participate in many biological processes including cellular differentiation, hormone responsiveness and memory. Especially, all intracrine molecules identified so far and trafficking to the nucleus are regulators of angiogenesis (Re and Cook, 2007, 2008). Regarding cardiac physiology, many hormones were pointed out as intracrine ligands including angiotensin, angiogenin, endothelin, isoproterenol, PDGF, VEGF, beta fibroblast growth factor (βFGF) and PTHrP (Tadevosyan et al., 2012). Among them, Ang-II, the principal effector of the renin-angiotensin system, promotes cell proliferation in an intracrine manner (Cook et al., 2006). Although many antagonists and blockers related to Ang-II were developed, many compounds failed to inhibit angiogenesis and proliferation caused by intracellular Ang-II (Re and Cook, 2008). Therefore, the study of intracrine factors, in terms of their biological actions, trafficking and biochemical mechanisms, is one of key aspects in modern pharmacology. In this study, we not only showed the functionality and distribution of UT receptor but also demonstrated a novel intracrine system. Because of the seemingly important role played by intracrine ligands, our work along with future results may open new therapeutic strategies.

APPENDIX

1. Supplemental materials and methods

1.1. Materials

The rabbit anti-rat UT antibody and horseradish peroxidase-conjugated anti-rabbit secondary antibody were obtained from Alpha diagnostic International (San Antonio, TX, USA) and GeneTex, respectively. The rabbit anti-nucleoporin 62 (Nup62) and the rabbit anti-caveolin-3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The SuperSignal West Pico chemoluminescent substrate was from Pierce Biotechnology (Rockford, IL, USA) while the Nuclear Extract kit was from Active Motif (Carlsbad, CA, USA). Polyvinylidene difluoride (PVDF) membranes were obtained from Bio-Rad (Montreal, QC, Canada). All UII derivatives were synthesized following an Fmoc chemistry-based protocol as previously described (Brkovic et al., 2003). Urantide was purchased from Bachem (Torrance, CA, USA) and RNase inhibitor was from Invitrogen (Burlington, ON, Canada). Finally, GeneElute™ mammalian total RNA miniprep kit, Deoxyribonuclease I (DNase I), pertussis toxin (PTX), PD98059, culture medium and other chemicals were purchased from Sigma-Aldrich (Sain Louis, Missouri, USA).

1.2. Methods

1.2.1. Cell culture

The rat cardiomyocyte cell line H9C2 was cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum as well as Penicillin/Streptomycin (100 unit/ml) at 37°C until 80% confluence was reached.

1.2.2. Isolation of nuclei and nuclear protein extract

H9C2 cells were harvested and subjected to nuclear isolation and nuclear protein extraction following the experimental procedures already described in Nguyen et al. (2012).

1.2.3 Western blot (WB)

Total and nuclear proteins extracted from H9C2 cells were subjected to WB as previously described (Doan et al., 2012b; Nguyen et al., 2012).

1.2.4 Transcription Initiation Assay

Transcription initiation assays were performed on freshly isolated H9C2 nuclei as previously described (Doan et al., 2012b; Nguyen et al., 2012). Isolated nuclei were either pre-treated at room temperature with 10^{-6}M urantide (UT antagonist) for 15 min, 1mM PD98059 (MEK inhibitor) for 1h or 5µg/mL Pertussis toxin (PTX) (Gi/o inhibitor) for 2h before addition of (^{32}P)-labelled uracil and stimulation with rUII (10^{-7}M).

1.2.5 RNA extraction

RNA was extracted with 4M Guanidium thiocyanate and phenol (pH4), precipitated with isopropanol and finally resuspended in RNase free water. RNA extracts were treated with DNase I before purification with the total RNA miniprep kit following the manufacturer's (Sigma-Aldrich).

1.2.6 Microarray assay

Isolated nuclei from ~10^7 H9C2 cells resuspended in storage buffer (50 mM Tris-Cl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$ and 0.1 mM EDTA) were combined with 2X reaction buffer (10

mM Tris-Cl, pH 4, 5 mM MgCl2, 0.3 M KCl) supplemented with 1 mM NTP, 50 mM DTT and RNase inhibitor. Nuclei were then stimulated with rUII (10^{-7}M) for 30 min at 30°C before RNA extraction. Purified RNA extracts including 6 untreated (control) and 6 rUII-treated samples were used for a microarray assay. 50-500 ng total RNA of each sample were provided to the Genome Quebec Innovation Centre (McGill University) to perform a genome-wide expression analysis using the Illumina RatRef-12 Expression BeadChip which contains 21,910 probes primarily selected from the National Center for Biotechnology Information (NCBI) RefSeq database.

1.2.7 Data analysis.

Data are expressed as mean ± SEM. Statistical analysis for transcription initiation assays was assessed using Prism version 4 (GraphPad, San Diego, CA, USA) with a one-way ANOVA followed by a Dunnett's post-test. As for microarray analysis results, FlexArray software version 1.6.1 provided by Genome Quebec was used to determine gene expression changes between control and rUIl treated groups. Student's t test was used to evaluate significant changes ($P<0.05$) from simple variations between experiments.

2. Ligand binding assays on rat brain and heart tissues show the different displacements of labelled UII between the nuclear and membrane fragments.

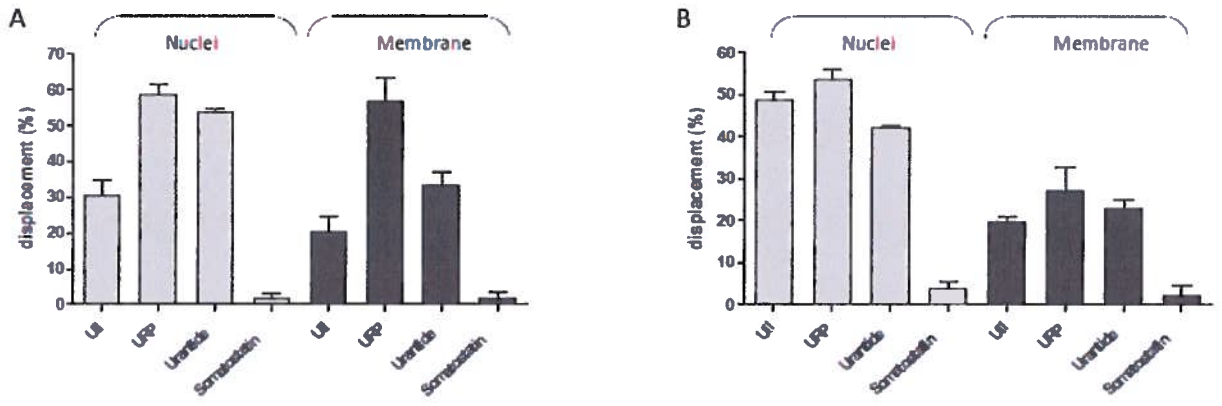

Figure 1: [^{125}I]hUII binding to isolated cardiac and brain nuclei and displacement by nonradioactive peptides. Nuclei isolated from rat brain (A) or heart (B) were incubated with 0.2 nM [^{125}I]hUII and in the absence or presence of non-labeled hUII (1 μM), URP (1 μM), urantide (1 μM), or somatostatin (1 μM). Results shown are the amount of bound [^{125}I]hUII displaced when incubated in the presence of the indicated non-labeled peptide.

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Update on the urotensinergic system: new trends in receptor localization, activation, and drug design

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The urotensinergic system plays central roles in the physiological regulation of major mammalian organ systems, including the cardiovascular system. As a matter of fact, this system has been linked to numerous pathophysiological states including atherosclerosis, heart failure, hypertension, diabetes as well as psychological, and neurological disorders. The delineation of the (patho)physiological roles of the urotensinergic system has been hampered by the absence of potent and selective antagonists for the urotensin II-receptor (UT). Thus, a more precise definition of the molecular functioning of the urotensinergic system, in normal conditions as well as in a pathological state is still critically needed. The recent discovery of nuclear UT within cardiomyocytes has highlighted the cellular complexity of this system and suggested that UT-associated biological responses are not only initiated at the cell surface but may result from the integration of extracellular and intracellular signaling pathways. Thus, such nuclear-localized receptors, regulating distinct signaling pathways, may represent new therapeutic targets. With the recent observation that urotensin II (UII) and urotensin II-related peptide (URP) exert different biological effects and the postulate that they could also have distinct pathophysiological roles in hypertension, it appears crucial to reassess the recognition process involving UII and URP with UT, and to push forward the development of new analogs of the UT system aimed at discriminating UII- and URP-mediated biological activities. The recent development of such compounds, *i.e.* urocontrin A and rUII(1–7), is certainly useful to decipher the specific roles of UII and URP *in vitro* and *in vivo*. Altogether, these studies, which provide important information regarding the pharmacology of the urotensinergic system and the conformational requirements for binding and activation, will ultimately lead to the development of potent and selective drugs.

Keywords: urotensin II, urotensin II-related peptide, allosteric modulation, biased agonist, nuclear receptors

THE UROTENSINERGIC SYSTEM

During the last decade, the urotensinergic system has drawn the attention of the scientific community due to its marked involvement in various pathological states including cardiovascular diseases. Initially isolated from the caudal neurosecretory system of the teleostean fish *Gillichthys mirabilis*, urotensin II (UII), a somatostatin-like peptide, was first characterized as a spasmogenic agent (Pearson et al., 1980). During more than 15 years, this peptide and its unknown receptors were thought to be restricted to fishes until it was demonstrated that UII was able to induce the relaxation of the mouse anococcygeus muscle (Gibson et al., 1984) and provoke the contraction of rat aortic strips (Gibson, 1987). These results, suggesting the presence of an homologous peptide in higher vertebrates, led to the isolation and characterization of UII in the frog *Rana ridibunda* (Conlon et al., 1992). Following this discovery, UII isoforms were either characterized or isolated in various vertebrate species including humans (Vaudry et al., 2010). A few years later, a peptide paralog, termed urotensin II-related peptide (URP), was isolated in rat brain extracts and subsequently identified in other mammalian species (Vaudry et al., 2010). Sequence comparison of all UII and URP isoforms revealed a striking conservation of the C-terminal cyclic hexapeptide (Vaudry et al., 2010). Conversely, the N-terminal region is highly variable both in length, ranging from 11 residues in humans to 17 residues in mice, and sequence composition (**Figure 1**) (Vaudry et al., 2010). In the human genome, UII and URP genes are respectively found at position 1p36 and 3q29 (Sugo et al., 2003). Those two genes are primarily expressed in motoneurons located in discrete brainstem nuclei and in the ventral horn of the spinal cord (Vaudry et al., 2010). However, UII and URP mRNAs have also been detected, although at a much lower level, in various peripheral tissues including the pituitary, heart, spleen, lung, liver, thymus, pancreas, kidney, small intestine, adrenal, and prostate (**Figure 2**) (Vaudry et al., 2010).

Both peptides are endogenous ligands of a G protein-coupled receptor initially identified as the orphan GPR14 receptor (Ames et al., 1999; Liu et al., 1999; Mori et al., 1999; Nothacker et al., 1999). Structural studies of this urotensin II receptor (UT) showed that, in addition to the common features found in the 1A GPCR family, such as the existence of a disulfide bridge between extracellular loops 1 and 2, N-linked glycosylation sites in the N-terminus portion, and phosphorylation sites in intracellular

3. Review

Update on the urotensinergic system: new trends in receptor localization, activation and drug design

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Running Title: Update on the urotensinergic system

Keywords: Urotensin II, Urotensin II-related peptide, allosteric modulation, biased agonist, nuclear receptors.

Species	Sequence
Urotensin II	
Mouse	<Gln-His-Lys-Gln-His-Gly-Ala-Ala-Pro-**Glu**-**Cys**-**Phe**-**Trp**-**Lys**-**Tyr**-**Cys**-**Ile**-OH
Rat	<Gln-His-Gly-Thr-Ala-Pro-**Glu**-**Cys**-**Phe**-**Trp**-**Lys**-**Tyr**-**Cys**-**Ile**-OH
Chimpanzee	H-Glu-Thr-Pro-**Asp**-**Cys**-**Phe**-**Trp**-**Lys**-**Tyr**-**Cys**-**Val**-OH
Human	H-Glu-Thr-Pro-**Asp**-**Cys**-**Phe**-**Trp**-**Lys**-**Tyr**-**Cys**-**Val**-OH
URP	
Mouse	H-**Ala**-**Cys**-**Phe**-**Trp**-**Lys**-**Tyr**-**Cys**-**Val**-OH
Rat	H-**Ala**-**Cys**-**Phe**-**Trp**-**Lys**-**Tyr**-**Cys**-**Val**-OH
Chimpanzee	H-**Ala**-**Cys**-**Phe**-**Trp**-**Lys**-**Tyr**-**Cys**-**Val**-OH
Human	H-**Ala**-**Cys**-**Phe**-**Trp**-**Lys**-**Tyr**-**Cys**-**Val**-OH

FIGURE 1 | Amino acid sequences of UII and URP in mammalian species; <Gln, pyroglumatic acid. Modified from Vaudry et al. (2010).

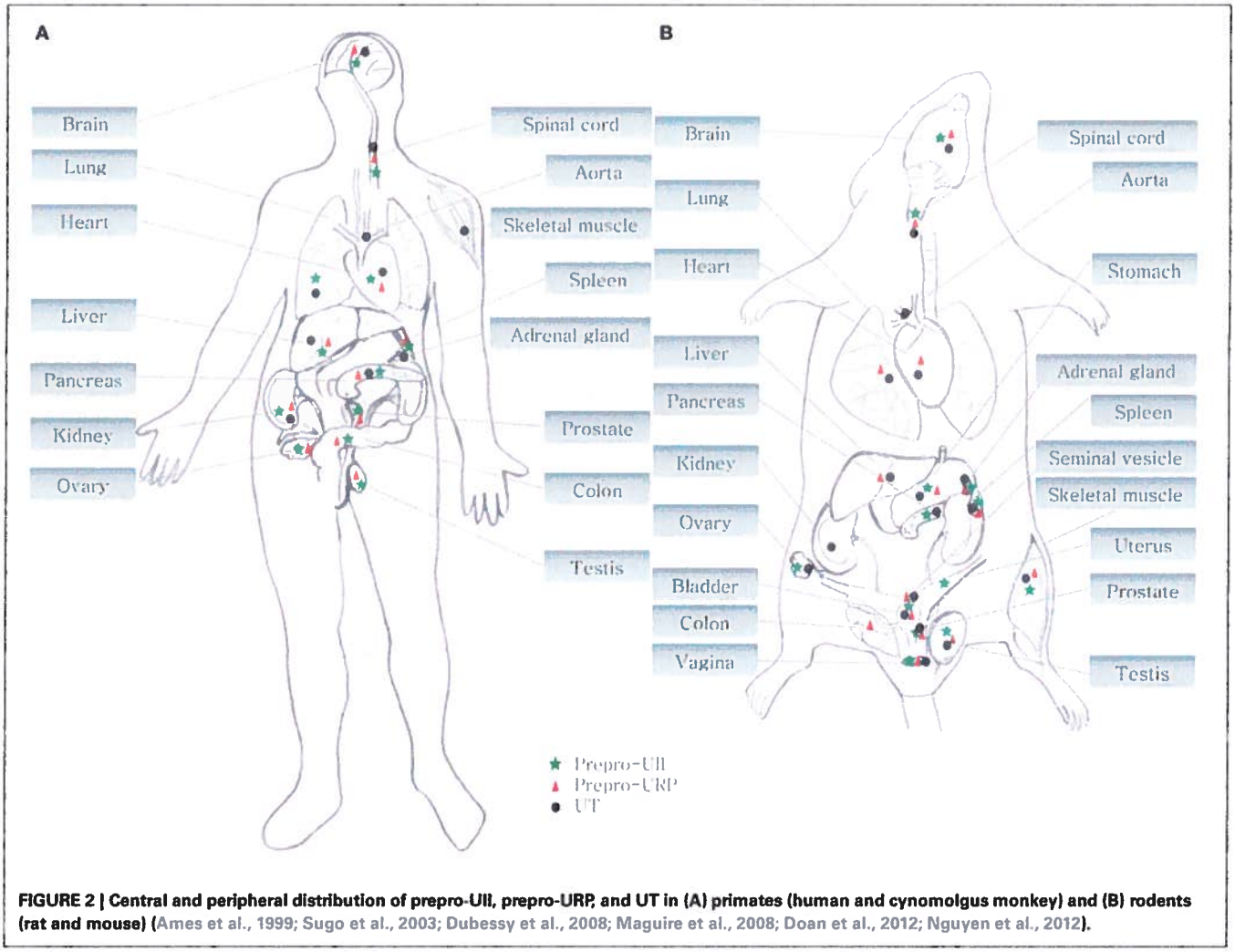

FIGURE 2 | Central and peripheral distribution of prepro-UII, prepro-URP, and UT in (A) primates (human and cynomolgus monkey) and (B) rodents (rat and mouse) (Ames et al., 1999; Sugo et al., 2003; Dubessy et al., 2008; Maguire et al., 2008; Doan et al., 2012; Nguyen et al., 2012).

loops (Douglas et al., 2000), this protein also possesses a palmitoylation site located in the C-terminal segment of the rodent isoform that is not present in the human isoform (**Figure 3**). Worth to mention, the rat UT, consisting of 386 amino acids, shows only 75% homology with the human protein while sequences of human and monkey receptors, comprising 389 residues, are almost identical (Elshourbagy et al., 2002). Like UII and URP, UT is widely expressed in the central nervous system as well as

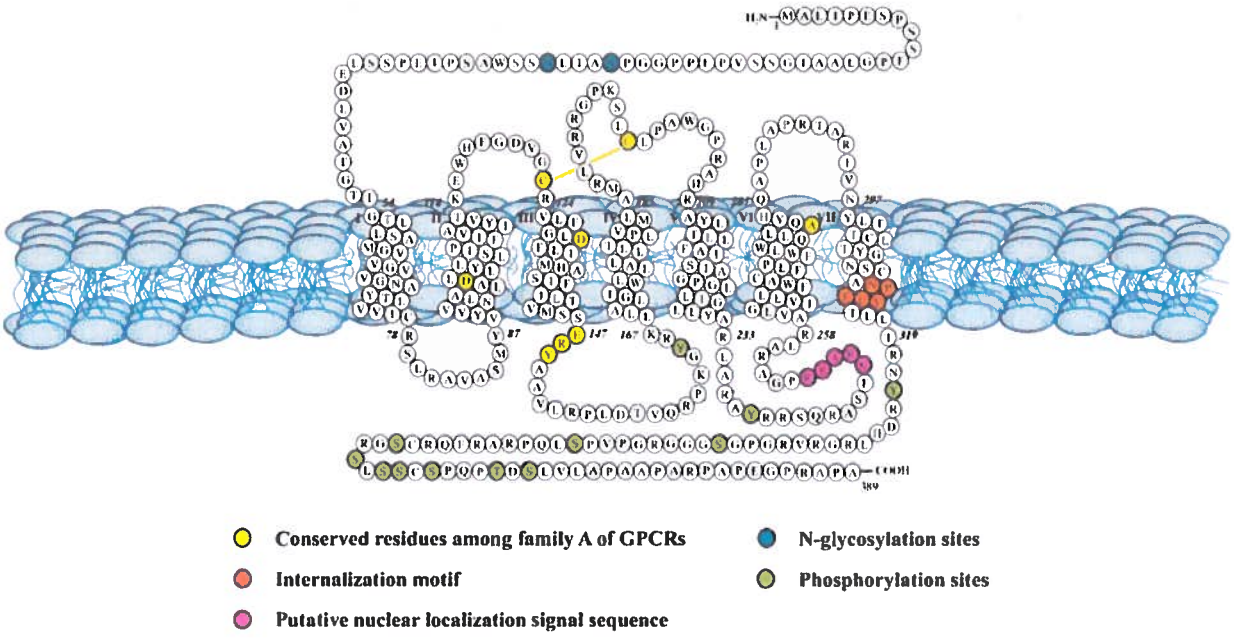

FIGURE 3 | Schematic representation of the human urotensin II (hUT) receptor. Adapted from Douglas et al. (2000).

in various peripheral organs including the cardiovascular system, kidneys, bladder, pancreas, and adrenal gland (**Figure 2**) (Vaudry et al., 2010).

The urotensinergic system plays a seminal role in the physiological regulation of major mammalian organ systems, including the cardiovascular system (Vaudry et al., 2010). As a matter of fact, UII exerts potent haemodynamic effects (Krum and Kemp, 2007), positive inotropic and chronotropic responses (Watson et al., 2003), and osmoregulatory actions (Song et al., 2006), induces collagen and fibronectin accumulation (Dai et al., 2007; Zhang et al., 2008), modulates the inflammatory response (Shiraishi et al., 2008), plays a role in the induction of cardiac and vascular hypertrophy (Papadopoulos et al., 2008), causes a strong angiogenic effect (Guidolin et al., 2010) and inhibits the glucose-induced insulin release (Silvestre et al., 2004). Thus, the urotensinergic system was linked to numerous pathophysiological states including atherosclerosis, heart failure, hypertension, pre-eclampsia, diabetes, renal and liver diseases, variceal bleeding, ulcers, as well as psychological, and neurological disorders (Ross et al., 2010).

The present review focuses on the latest findings about the urotensinergic system in terms of receptor localization and pharmacology as well as receptor activation with the conception of new urotensinergic ligands aimed at discriminating UII- and/or URP-mediated biological actions.

DISCOVERY OF AN INTRACRINE PHARMACOLOGY OF THE UROTENSINERGIC SYSTEM

PRESENCE OF NUCLEAR UT IN THE HEART AND IN THE CENTRAL NERVOUS SYSTEM

In many ways, UII exhibits actions similar to other key neurohormonal factors, *i.e.* angiotensin II (Ang-II) and endothelin-1 (ET-1), in driving a variety of cardiac and vascular disease processes (Maguire and Davenport, 2002). These include vasoconstriction as well as mitogenic, trophic and pro-fibrotic effects (Vaudry et al., 2010). A clear interaction of the urotensinergic system with the renin-angiotensin-aldosterone and endothelin systems is acknowledged in terms of regulation of systolic and diastolic functions (Fontes-Sousa et al., 2009). However, key differences were observed between these systems. In particular, UII induces a rather weak or absent vasoconstriction in a variety of human vascular beds (Maguire et al., 2000; Hillier et al., 2001) while it can also acts as a vasodilator in some vascular beds, such as those in the pulmonary vasculature (Stirrat et al., 2001). The recent discovery of specific intracellular receptors associated with the physiological and pathophysiological actions of Ang-II and ET-1 highlighted a high level of complexity for these peptidergic systems in the regulation of cardiovascular homeostasis. Traditionally, GPCRs are located at the plasma membrane where they modulate the activity of membrane-associated second messengers. As such, GPCRs can exert their effects through the regulation of ion channels, second messenger production, and protein kinase cascades in order to control cellular activity, gene expression, plasticity, differentiation, morphogenesis, and migration. However, in the recent years, the presence of functional intracellular receptors has almost become "a classic GPCR paradigm" (Boivin et al., 2008). These intracellular GPCRs could be involved in the control of several cellular processes including regulation of gene transcription, ionic homeostasis, cellular proliferation, and remodeling (Boivin et al., 2008). Intracellular GPCRs may be constitutively active, or may be activated by ligands internalized from the extracellular space or synthesized within the cell (**Figure 4**). Besides, they can regulate signaling pathways distinct from those activated by the

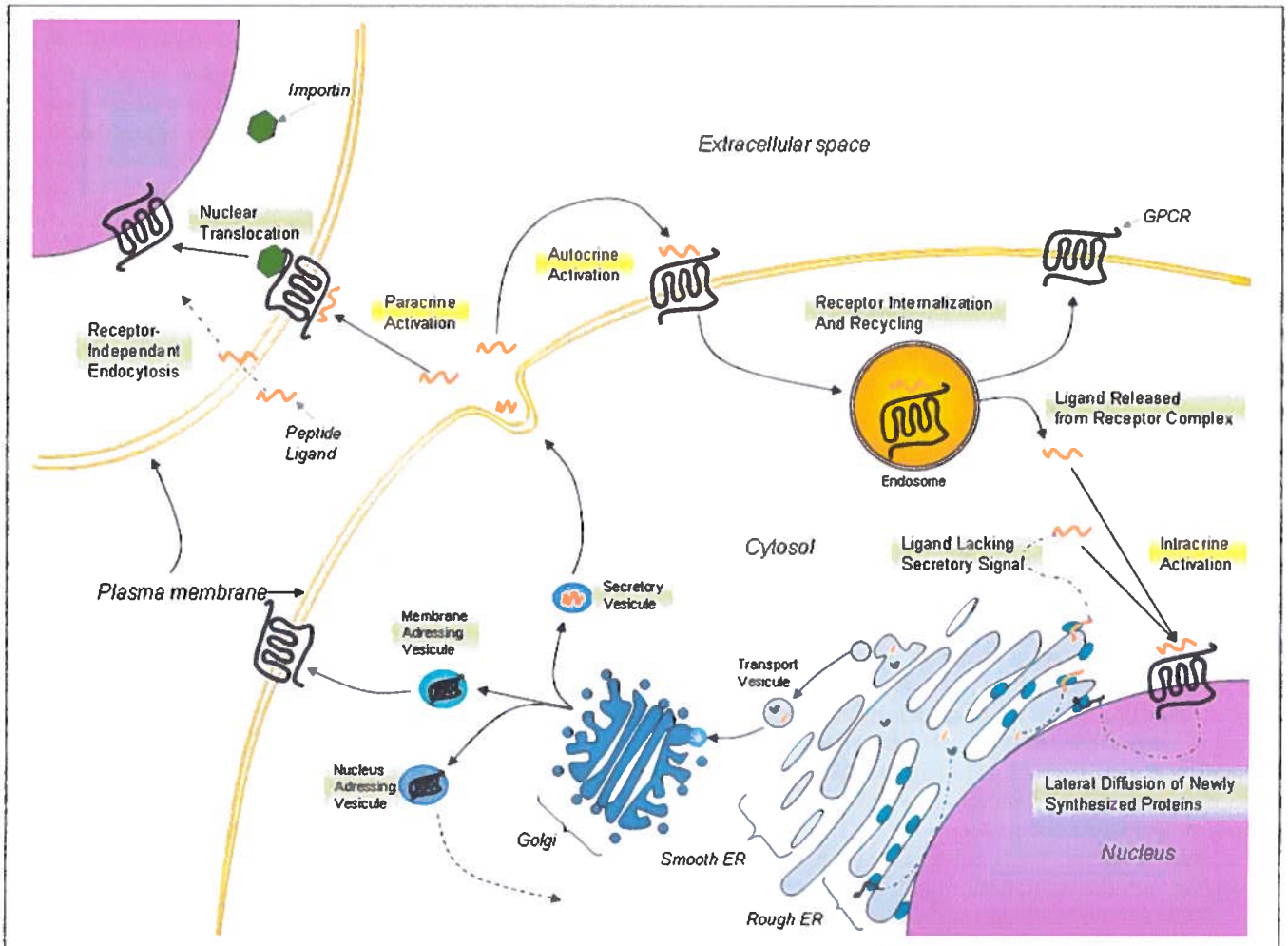

FIGURE 4 | Proposed trafficking of UT and endogenous ligands. UT receptors are located either at the plasma membrane or at the nucleus. The presence of nuclear UTs can be attributed to lateral diffusion of newly synthesized proteins from the rough endoplasmic reticulum surface, which is contiguous with the nuclear shell, to specific nuclear addressing following modification in the Golgi apparatus or importin-assisted nuclear translocation after ligand stimulation. UII or URP binds to UT receptors at the cell surface but can also cross the plasma membrane to stimulate intracellular receptors. From Lee et al. (2004); Giebing et al. (2005); Gobeil et al. (2006); Doan et al. (2012); Tadevosyan et al. (2012).

same receptor at the cell surface (Re, 1999). As such, biological outcomes might result from the integration of extracellular and intracellular signaling events (Terrillon and Bouvier, 2004; Hanyaloglu and von Zastrow, 2008; Sorkin and von Zastrow, 2009). This new paradigm for cellular signaling provides more complexity to study the function and physiological roles of GPCRs.

In a recent report, specific UII binding sites were observed on heart and brain cell nuclei from rat and monkey tissues (Doan et al., 2012; Nguyen et al., 2012). Except those two tissues and the spinal cord, none of the tested tissues including kidneys, lung, and skeletal muscle, all expressing UT at the cell surface, presented a subcellular localization of UT (Doan et al., 2012; Nguyen et al., 2012). Supporting the presence of such nuclear expression also in humans, the presence of nuclear UT was also observed in two human cell lines, *i.e.* SH-SY5Y neuroblastoma and U87 astrocytoma cell lines (Nguyen et al., 2012).

NUCLEAR UT ACTIVATION CAN MODULATE TRANSCRIPTION INITIATION

As previously reported for nuclear Ang-II (Eggena et al., 1993), β3-adrenergic (Boivin et al., 2006; Vaniotis et al., 2011), and ET-1 receptors (Boivin et al., 2003), nuclear UT receptors can initiate transcription (Doan et al., 2012; Nguyen et al., 2012). Although UII and URP stimulated the transcription in isolated brain cell nuclei (Nguyen et al., 2012), only UII was able to trigger a similar effect in rat cardiac nuclei (Doan et al., 2012). Two-dimensional gel electrophoresis clearly indicated the occurrence of different immunoreactive species in both brain and heart membrane and nuclear fractions (Doan et al., 2012; Nguyen et al., 2012). Nuclear and membrane proteins extracted from heart tissues expressed three major UT-immunoreactive spots with an apparent molecular weight of 60 kDa at a pI value of 6–7 (Doan et al., 2012). Interestingly, a different pattern was observed in brain tissue (Nguyen et al., 2012). Since the UT gene is intronless,

the various immunoreactive species were principally ascribed to post-translational modifications (Doan et al., 2012; Nguyen et al., 2012). Whether or not these UT species are involved in distinct UII-associated biological activities will require further investigation. However, it is well-known that glycosylation can modulate the cellular compartmentalization and functionality of the receptor, thereby influencing its intracellular trafficking and biological activity (**Figure 4**) (Duverger et al., 1995; Rondanino et al., 2003; Gobeil et al., 2006).

A growing body of evidence supports the presence of GPCRs at the surface of the nuclear membrane, their orientation within this membrane, however, remains controversial. If they maintain the topology adopted in the endoplasmic reticulum during protein synthesis, the ligand binding site would be located in the lumen of the nuclear envelop (perinuclear space) with the C-terminal of the protein being localized either within or outside the nucleus. In fact, the topology of the nuclear membrane lumen is very similar to the extracellular space, which makes it a favorable environment for a binding site (Jong et al., 2005; Bootman et al., 2009). Since signaling starts with the recruitment of specific proteins to the C-terminal portion of the receptor, signals would be sent toward the cytosol or into the nucleus in accordance with the adopted GPCR orientation within the nuclear shell (**Figure 5**). Hence, the orientation of those nuclear GPCRs would determine the direction in which the signal is transmitted. As recently reported, nuclear UT receptors are able to regulate gene transcription (Doan et al., 2012; Nguyen et al., 2012). Furthermore, it is well-known that calcium ions play an important role in the control of gene expression (Bootman et al., 2009). In isolated nuclei, nuclear calcium levels can regulate gene transcription by interacting with the cyclic AMP response element-binding protein (CREB) and the downstream regulatory element antagonist modulator (DREAM), which are constitutively present in the nucleus. Changes in nucleoplasmic calcium can be achieved by triggering inositol(1,4,5)-triphosphate receptors (IP_3Rs) located on the inner nuclear membrane (Bootman et al., 2009). Because it is generally accepted that UT activation is associated with the recruitment of Gαq/11 proteins to its C-terminal tail resulting in an IP_3 increase (Proulx et al., 2008), it is highly probable that this portion of the receptor is located into the nucleoplasm (**Figure 5**). Interestingly, IP3Rs are concentrated in the nuclear membrane of heart ventricular cells and their activation was shown to initiate a pro-hypertrophic pathway (Arantes et al., 2012). These findings are well-correlated with the UII-induced cardiomyocyte hypertrophy and the presence of nuclear UT receptors in cardiac tissues (Gruson et al., 2010; Doan et al., 2012). Nevertheless, these intracellular UT receptors may have the capacity to regulate signaling pathways that differ from those of their plasma membrane counterparts, as recently demonstrated for the metabotropic glutamate receptor 5 (Jong et al., 2009), and the renin–angiotensin system (De Mello, 2008). As such, this intracrine pharmacology of the urotensinergic system represents a complementary system that could potentially involve the regulation of physiological functions.

INTRACELLULAR TRAFFICKING OF UT

This new intracrine pharmacology clearly highlights the complexity of this peptidergic system where UII and URP can trigger not only common but also different biological activities (Prosser et al., 2008; Jarry et al., 2010; Doan et al., 2012). Previous studies have detected the presence of GPCRs, such as Ang-II receptors, at the nucleus in an agonist-independent manner (Lee et al., 2004). Confocal microscopy of heart and brain tissue sections as well as various non-transfected cell lines clearly revealed a constitutive nuclear localization for UT (Doan et al., 2012; Nguyen et al., 2012). However, it is also possible that following their agonist-stimulated internalization, GPCRs relocate at the nuclear membrane (Lee et al., 2004). In such a case, the translocation is initiated by the presence of a nuclear localization signal (NLS), a short stretch of basic amino acid residues often localized within the intracellular loops that is recognized by importins α and/or β (**Figure 4**). For example, a NLS was observed in the seventh transmembrane domain and the carboxy-terminal segment of the Ang-II receptor subtype1 (Lys-Lys-Phe-Lys-Arg) and the third intracellular loop of the apelin receptors (Arg-Lys-Arg-Arg-Arg) (Lee et al., 2004). Interestingly, a similar sequence, *i.e.* Lys-Arg-Ala-Arg-Arg, is also observed in the third intracellular loop of human and monkey UT isoforms (**Figure 3**) while a Lys-Gln-Thr-Arg-Arg segment is observed in rat and mouse UT. However, it is important to note that many NLS signals are still unknown and that the presence of an obvious NLS motif may mask the existence of still uncharacterized NLS sequences. Specific post-translational modifications such as palmitoylation were reported to be involved in the addressing of the receptor either to the membrane or the nuclei. For instance, it was demonstrated that de-palmitoylation of GRK6A promoted its translocation from the plasma membrane to both the cytoplasm and nucleus (Jiang et al., 2007). Such a putative palmitoylation site is also found within the seventh transmembrane domain (Cys^{339}) of rat and mouse UT isoforms but is absent in the primate (human and monkey) receptor (Marchese et al., 1995; Tal et al., 1995; Ames et al., 1999). Under chemically mediated hypoxic conditions, an increase of total UT expression, was observed suggesting that hypoxia might induce *de novo* synthesis of the peptide receptor. However, a significant decrease in nuclear UT expression was reported that could be interpreted as an increase in translocation of the protein to the membrane or a decrease of internalization with concomitant nuclear translocation (Nguyen et al., 2012). Altogether, it could be noted that the subcellular UT localization could be either attributed to translocation from the cell surface and/or *de novo* synthesis (**Figure 4**).

UII AND URP AS INTRACRINE LIGANDS

UII, and by extension URP, were originally thought to act as autocrine and paracrine modulators rather than as hormones (Yoshimoto et al., 2004). The term "intracrine" ligand relates to intracellular molecules binding to and activating intracellular receptors (**Figure 4**). Such ligands can be synthesized and targeted to the Golgi apparatus for secretion or act intracellularly either before secretion or following reuptake. The intracrine gene product might also arise from an alternative transcription initiation site, differences in mRNA maturation or translation leading to a gene product lacking secretory signals and consequently active only in the intracellular space (**Figure 4**) (Kiefer et al., 1994; Lee-Kirsch et al., 1999; Xu et al., 2009). To this extent, it is interesting

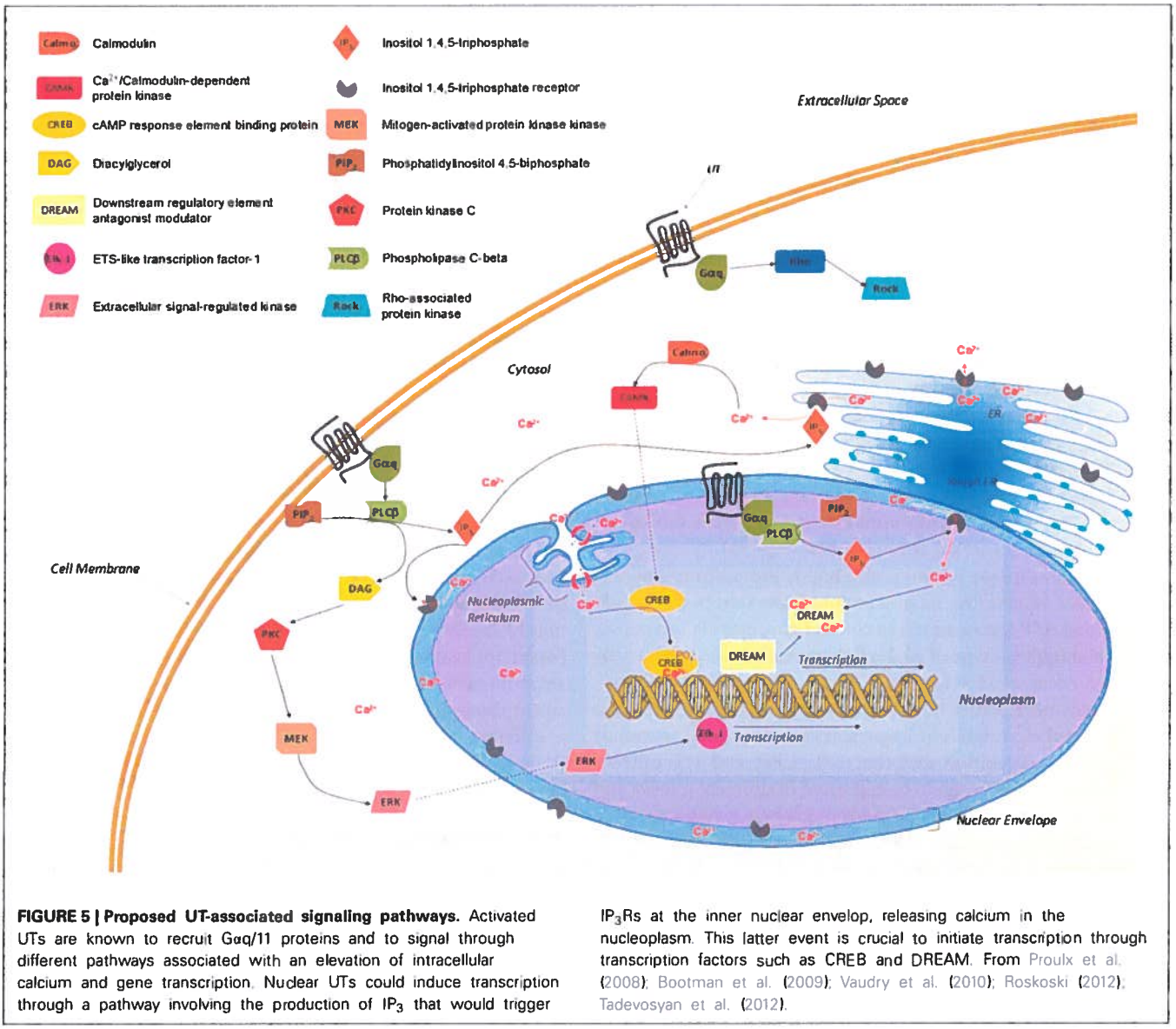

FIGURE 5 | Proposed UT-associated signaling pathways. Activated UTs are known to recruit Gαq/11 proteins and to signal through different pathways associated with an elevation of intracellular calcium and gene transcription. Nuclear UTs could induce transcription through a pathway involving the production of IP_3 that would trigger IP_3Rs at the inner nuclear envelop, releasing calcium in the nucleoplasm. This latter event is crucial to initiate transcription through transcription factors such as CREB and DREAM. From Proulx et al. (2008); Bootman et al. (2009); Vaudry et al. (2010); Roskoski (2012); Tadevosyan et al. (2012).

to note that two isoforms of the human UII precursor, differing mostly by their peptide signal, were discovered (Coulouarn et al., 1998; Ames et al., 1999).

A recent study demonstrated that FITC-conjugated hUII and URP were both internalized in non-expressing UT cell lines through receptor-independent mediated endocytosis (Doan et al., 2012) (**Figure 4**). This receptor-independent endocytic mechanism brought a new perception of the pseudo-irreversible binding characteristics often described for the urotensinergic system. Indeed, the lack of rapid UT desensitization through classic mechanisms (acid wash or trypsin treatments) was thought to reflect a strong, pseudo-irreversible binding of the ligands (Douglas and Ohlstein, 2000). However, this pseudo-irreversible character could also be due to the ability of both endogenous peptides to reach the internal compartment of the cell. Moreover, it is yet possible that following the internalization of ligand-receptor complexes, ligands are subsequently released from internalized endosomes within the cell. As such, internalized peptide-receptor complexes can be dissociated under the acidic environment found in endosomes, giving rise to receptor recycling at the plasma membrane (**Figure 4**) (Giebing et al., 2005). At this point, the fate of the peptide is unknown but based on the results published by Doan et al. (2012), it is conceivable that UII, and to a lesser extend URP, could leak from the vesicle and ultimately activate intracellular receptors.

PROSPECTIVE ROLES OF NUCLEAR UT

The precise role of this new intracrine urotensinergic system has yet to be elucidated both in physiological and pathological conditions. However, as for other GPCRs including Ang-II and ET-1 receptors, these intracellular receptors are important regulators of physiological and pathological functions and could therefore represent new targets for therapeutic interventions (Boivin et al., 2008; Tadevosyan et al., 2012).

Elevated UII plasma levels were observed in numerous disease conditions, including hypertension, atherosclerosis, heart failure, pulmonary hypertension, diabetes, renal failure, and metabolic syndrome (Ross et al., 2010). As demonstrated, the cellular uptake of UII but not URP is increased at lower pH (Doan et al., 2012). Pathological conditions such as cancer, ischemic stroke, inflammation, and atherosclerotic plaques often induce an increase in metabolic activity and hypoxia associated with an elevated extracellular acidity (Andreev et al., 2010). In these conditions, UII would enter more easily than URP inside the cell triggering transcription of UII-associated genes by activating the nuclear receptor. Thus, the elevated concentration of UII observed during the etiology of various diseases could sustain specific cellular responses while an intracellular feedback loop could maintain a particular cellular state (Petersen et al., 2006). Interestingly, known intracrines do not present any structural or chemical similarities but are generally growth regulators that can directly or indirectly modulate angiogenic or anti-angiogenic actions. Therefore, the angiogenic actions of the urotensinergic system, reported both *in vivo* and *in vitro* (Spinazzi et al., 2006), could thus involve the activation of nuclear UT.

The urotensinergic system is also highly expressed in the central nervous system, but its physiological function is still poorly understood. UT was observed in cortical astrocytes (Castel et al., 2006), a ubiquitous type of glial cell that greatly outnumbers neurons and occupies 25% to 50% of brain volume (Bignami et al., 1991). It is noteworthy that glioblastoma multiform (GBM) is characterized by exuberant angiogenesis, a key event in tumor growth and progression and that UII, URP, and UT mRNAs were systematically found to be expressed in different glioma and glioblastoma tumors (Diallo et al., 2007). These results support a role for the urotensinergic system, and in particular nuclear UT, in human brain tumorogenesis possibly via angiogenesis regulation. Finally, in the CNS, UII is able to induce norepinephrine, dopamine, and serotonin release in noradrenergic neurons (Ono et al., 2008). Intracerebroventricular UII administration modulates cardiac homeostasis via β-adrenoreceptor activation (Hood et al., 2005). These observations bring up the idea that the presence of nuclear UT receptors could also be associated to excitatory neurotransmission. In accordance with this hypothesis, various intracrines were reported to act as neurotransmitters within the CNS (Re, 2004).

Whether specific UII or URP biological actions on the CNS and the cardiovascular system are mediated totally or in part by the nuclear UT will need further studies as well as the development of specific nuclear UT probes. Although still poorly understood, the diverse functions exerted by agonists and hormones acting on intracellular GPCRs suggest that intracrine signaling might activate cellular responses distinct from those at the cell surface for a given receptor. In the last decade, biological actions of intracrines in heart and vasculature, including those of the renin–angiotensin-system in cardiac pathology, dynorphin B in cardiac development, as well as endothelin, highlighted the importance of intracrine physiology in pathological processes such as left ventricular hypertrophy, diabetic cardiomyopathy, and arrythmogenesis. So, the presence of functional UT receptors at the cell membrane and at the nucleus will probably be a new aspect to take into account during the development of therapeutic compounds for the treatment of pathologies associated with the urotensinergic system.

NEW INSIGHTS INTO UT ACTIVATION

The precise definition of the (patho) physiological roles of the urotensinergic system *in vivo* was hampered by the absence of potent and selective UT antagonists. Indeed, the lack of efficacy observed with Palosuran (ACT-058362) (Clozel et al., 2004, 2006), the only UT antagonist that reached a phase II clinical trial in patients with diabetic nephropathy, was clouded by its low antagonist potency (Behm et al., 2008). Therefore, drug discovery programs continued to focus on the identification of potent and selective UT antagonists suitable for assessment in both preclinical species and man (Maryanoff and Kinney, 2010). As reported earlier this year, Sanofi launched a phase I clinical trial regarding a long acting UT antagonist, derived from a 5,6-bisaryl-2-pyridine-carboxamide scaffold (European patent application *EP2439193*), for the treatment of diabetic nephropathy. Similarly, GlaxoSmithKline started a phase I clinical trial for the use of an UT antagonist, *i.e.* SB1440115 (United States Patent application *12,373,901*), for the treatment of asthma. Finally, over the past few years, Boehringer-Ingelheim (European patent application *EP2155748*) as well as Janssen Pharmaceutical (United States Patent application *8,193,191*) filled several patents regarding UT antagonists but no phase I clinical trial was yet reported. With the recent discovery that UII and URP could exert common as well as different biological activities (Prosser et al., 2008; Hirose et al., 2009; Jarry et al., 2010), development of selective UT antagonists has become a more complex task.

UT AS SHAPESHIFTING PROTEINS

GPCRs represent the largest and most diverse family of cell surface receptors. These plasma membrane proteins bind their endogenous ligands in order to activate an intracellular signaling cascade that will result in a biological action. Conventional views of ligand-receptor activation considered all components of the signaling cascade to be linearly related, *i.e.* to emanate from the initial activation of the receptor. However, multiple studies pointed out the ability of some ligands to selectively trigger specific signaling pathways, therefore having collateral and not linear efficacy (Roettger et al., 1997; Kohout et al., 2004). As such, GPCRs cannot be considered as pharmacological on/off switches anymore. Their intrinsic nature rather suggests that dynamic changes in the receptor conformation, resulting from ligand binding, are a mean of information transfer (Kenakin and Miller, 2010). Hence, the propensity of GPCRs to assume multiple conformations make them allosteric proteins that are able to select specific subsets of secondary messengers depending on the ligand-induced adopted conformation. As such, various ligands were reported to possess differential functional profiles for a given receptor, as it was initially described for the CCR7 receptor (Kohout et al., 2004).

The URP sequence, strictly conserved throughout species, supports the concept that specific receptor interactions were maintained despite variation in the receptor amino acid sequence (Elshourbagy et al., 2002). Based on the specific expression of

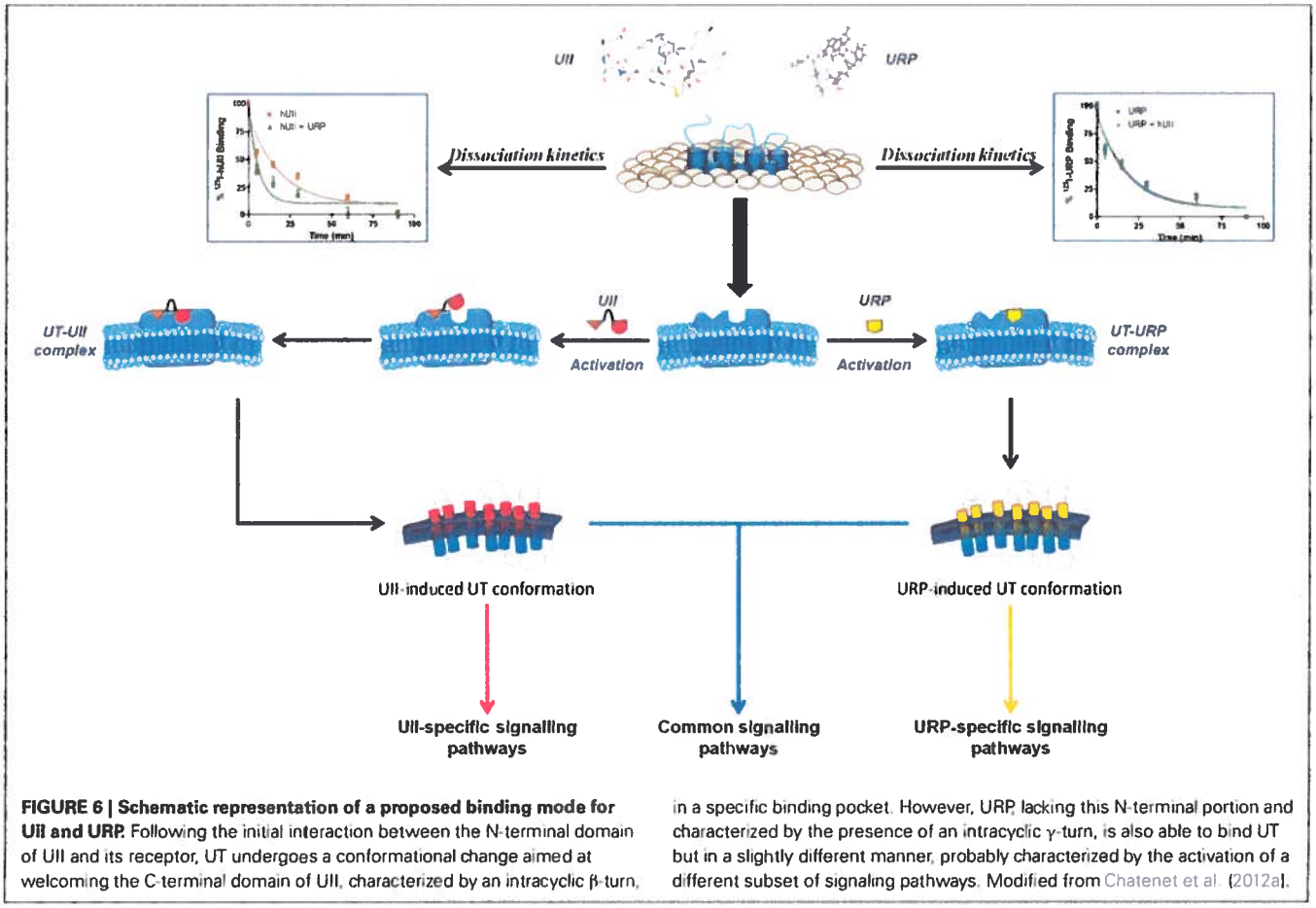

FIGURE 6 | Schematic representation of a proposed binding mode for UII and URP. Following the initial interaction between the N-terminal domain of UII and its receptor, UT undergoes a conformational change aimed at welcoming the C-terminal domain of UII, characterized by an intracyclic β-turn, in a specific binding pocket. However, URP, lacking this N-terminal portion and characterized by the presence of an intracyclic γ-turn, is also able to bind UT but in a slightly different manner, probably characterized by the activation of a different subset of signaling pathways. Modified from Chatenet et al. (2012a).

URP mRNA in several cerebral structures (rostroventrolateral medulla) and tissues (heart, seminal vesicle), it was suggested that URP rather than UII would be the biologically active peptide in the UT-associated regulation of autonomic, cardiovascular and reproductive functions (Dubessy et al., 2008). Moreover, distinct pathophysiological roles for UII and URP in hypertension have been suggested (Hirose et al., 2009). Indeed, mRNA expression of both UII and URP was up-regulated in the atrium of spontaneously hypertensive rats (SHR) when compared with age-matched Wistar Kyoto (WKY) rats. However, the specific up-regulation of URP but not UII mRNA in aorta and kidney of SHR rats supported the idea that these peptides might act individually in various biological systems (Hirose et al., 2009). Accordingly, it was demonstrated that UII and URP were able to exert not only common but also divergent physiological actions clearly suggesting the propensity of these two endogenous ligands to select a specific UT conformation (Prosser et al., 2008; Jarry et al., 2010; Doan et al., 2012). The concept of biased agonism has recently emerged from various studies, putting forward the notion that specific ligand-induced conformational changes can lead to particular signaling (Patel et al., 2010). In isolated ischaemic heart experiments, UII and URP were both able to reduce myocardial damages through creatine kinase reduction but only UII was able to reduce atrial natriuretic peptide (ANP) production (Prosser et al., 2008). Supporting the idea that UII and URP interact with UT in a distinct manner, it was recently demonstrated that URP, an equipotent UII paralog, was able to accelerate the dissociation rate of membrane bound ^{125}I-hUII while hUII had no noticeable effect on URP dissociation kinetics (Chatenet et al., 2012a). Altogether, these results suggest that each ligand is able to select a specific UT conformation that triggers definite biological activities for each of these two peptides.

The peptide N-terminal region was initially pointed out as potentially involved in the observed biological activity differences between UII and URP (Prosser et al., 2008). However, it is only recently that this region was clearly associated with a putative differential binding mode of hUII and URP (Chatenet et al., 2012a). Using exocyclic Ala-derivatives of hUII, acting as very potent ligands of the UT receptor (Brkovic et al., 2003), dissociation kinetics experiments revealed a putative interaction between UT and the glutamic residue at position 1 of hUII. Indeed, it was observed that the replacement of this residue by an alanine moiety, *i.e.* [Ala^1]hUII, provoked an increase of the dissociation rate of hUII but not URP (Chatenet et al., 2012a). In agreement with this view, an important electrostatic interaction between Glu^1 of hUII and its receptor was previously reported in docking studies (Lescot et al., 2008). No other substitution was able to induce such pharmacological changes. Because this compound was reported to exert almost equipotent contractile activity compared to hUII and URP, the lost of a putative specific interaction with UT was

thought to have generated an analog behaving as an URP derivative therefore acting at the URP-associated orthosteric binding site. Supporting this hypothesis, hUII(4–11), considered as the minimal hUII fragment to exert full biological activity, was also able to alter hUII but not URP dissociation rate (Chatenet et al., 2012a). This N-terminal segment could thus be crucial for signaling pathway selection upon activation and its deletion could lead to signaling mechanism misinterpretation, all UII truncated analogs potentially acting as URP derivatives.

Overall, these results support the presence of specific pockets/interactions within UT, aimed at selecting distinct UT conformations that can differentiate UII and URP biological activities (**Figure 6**). Briefly, it is hypothesized that upon the initial UII-UT interaction, involving the N-terminal region of UII, UT undergoes conformational changes aimed at welcoming the C-terminal domain of UII, characterized by an intracyclic β-turn (Carotenuto et al., 2004). To the opposite, URP, lacking this N-terminal portion and characterized by the presence of an intracyclic γ-turn (Chatenet et al., 2004), would bind UT in a slightly different manner; ultimately triggering a slightly different subset of signaling pathways. These observations have clearly highlighted the crucial need to reassess the development of pan UT antagonists, *i.e.* blocking UII- and URP-mediated receptor activation, and to develop new analogs of the urotensinergic system aimed at discriminating UII- or URP-mediated biological activities. Such compounds would allow a better understanding of the pathophysiological roles of the urotensinergic system and also expand our knowledge on allosteric modulation of class A GPCRs.

Allosteric modulation of the urotensinergic system

As demonstrated, the urotensinergic system is far more complex than previously thought with the presence of nuclear receptors and UII/URP specific as well as common actions. For the past two decades, identification of peptidic and non-peptidic agonists and antagonists of the urotensinergic system has gathered much interest for the treatment of various cardiovascular pathologies. An extensive review regarding the various peptidic and non-peptidic ligands of the urotensinergic system, all acting as competitive compounds, is beyond the scope of this review but more details can be found elsewhere (Maryanoff and Kinney, 2010).

Additional biological complexity, but also novel opportunities for drug discovery, has arisen from the fact that many GPCRs possess allosteric binding sites (Christopoulos and Kenakin, 2002). Similar to the initial concept of agonism, *i.e.* linear efficacy, antagonism has been historically viewed as a simple "turning off" of the receptor. As such, this non-accommodating mechanism does not allow any agonist to impart information to the receptor, the orthosteric binding site being occupied by the competitive antagonist. However, an allosteric modulator binds to its own site, different from the orthosteric site, forming a complex characterized by the concomitant presence of the endogenous agonist and the allosteric modulator. Such modulators can alter the biological properties of the endogenous orthosteric ligand either via changing its affinity, its efficacy, or both (Leach et al., 2007; May et al., 2007). This type of antagonism, termed permissive, can modify the reactivity of the receptor toward the agonist probably through conformation selection and stabilization of one or

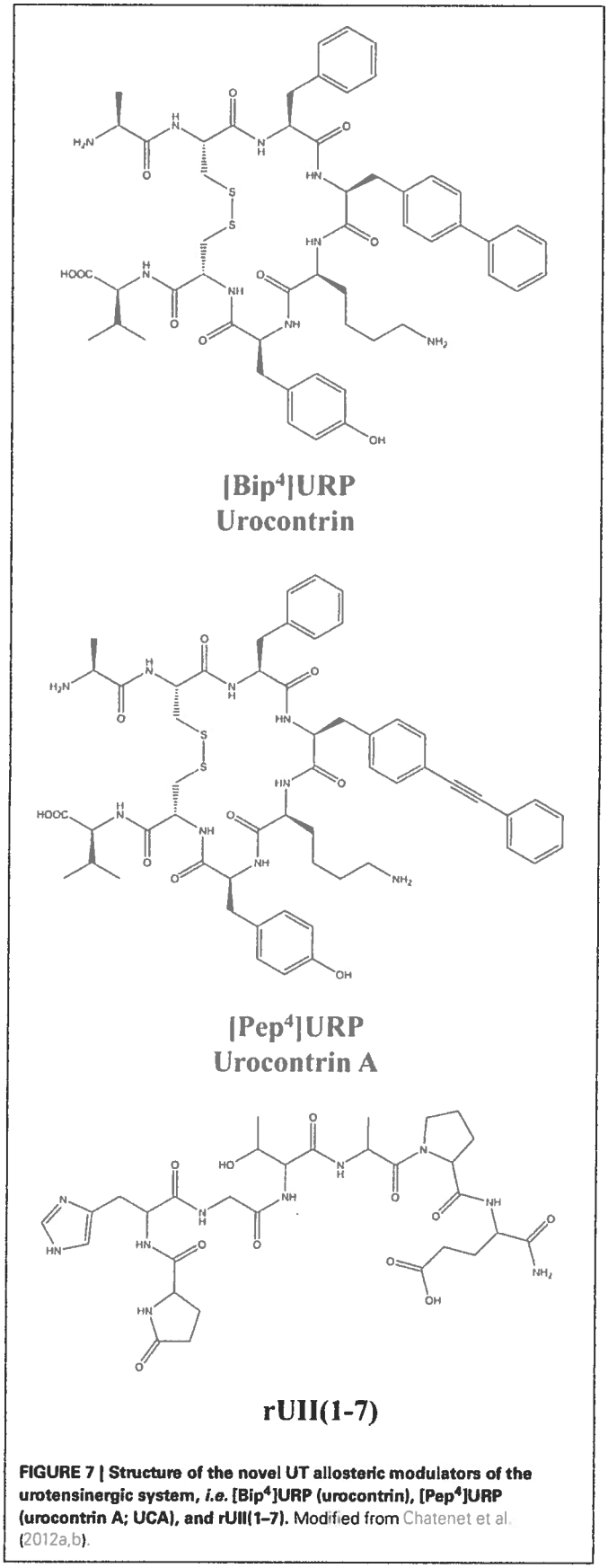

FIGURE 7 | Structure of the novel UT allosteric modulators of the urotensinergic system, *i.e.* [Bip^4]URP (urocontrin), [Pep^4]URP (urocontrin A; UCA), and rUII(1–7). Modified from Chatenet et al. (2012a,b).

part of the receptor states. Targeting receptor allosteric sites can offer the possibility of greater selectivity due to a lower sequence conservation within allosteric pockets across subtypes of a given GPCR, as well as the potential to fine-tune physiological signaling in a more spatial and temporally-selective manner (Kenakin, 2011).

The urotensinergic system, encompassing two endogenous peptides, provides potential for allosteric compounds to differentially modulate individual peptide responses, a behavior termed "probe dependence" (Kenakin, 2008). During the course of structure-activity relationship studies on URP derivatives, two compounds, *i.e.* [Bip^4]URP and [Pep^4]URP, termed urocontrin and urocontrin A (UCA) respectively, showed a specific behavior that has set them apart from known UT antagonists (**Figure 7**) (Chatenet et al., 2012a,b). Indeed, these compounds were able to selectively and significantly reduce hUII-induced contraction without altering URP-mediated vasoconstriction (Chatenet et al., 2012a,b). For instance, the efficacy of hUII-induced rat aortic ring vasoconstriction was significantly reduced (~31%) by a pretreatment with a nanomolar concentration of UCA (**Figure 8**). Interestingly, this ability to selectively and significantly reduce UII-induced contraction was not specie-dependent since a similar effect was observed on cynomolgus monkey aortic rings. To the best of our knowledge, only two other UT ligands exerted insurmountable activity (Herold et al., 2003; Behm et al., 2010). However, none of them could differentially alter hUII and URP biological activity. The insurmountable nature of urocontrin and UCA antagonism was attributed to an allosteric modulation of UT. Indeed, an excess of UCA accelerated the ^{125}I-hUII dissociation rate, thus suggesting that the binding of the antagonist changes the receptor conformation in such a way that the radioligand is released from the receptor. Accordingly, no difference in ^{125}I-URP dissociation kinetics was observed in similar conditions (**Figure 8**). The apparent absence of effect on the URP pharmacological profile by UCA was attributed to its ability to select a receptor conformation through functional allosteric modulation that impairs hUII-associated actions but not URP-mediated biological activities.

As stated above, for a given receptor, an allosteric modulation that depends on the type of orthosteric ligand used is referred to as "probe dependence" (Kenakin, 2005; Keov et al., 2011). This probe dependence phenomenon supports the idea that the two endogenous ligands, despite depicting a high structure homology and recognizing a similar binding pocket, represent chemically distinct entities interacting in different structural environments within the orthosteric pocket. Because hUII and URP differ only by the length and composition of their N-terminal domain (Vaudry et al., 2010), it was postulated that this region could be involved in their putative different binding modes. Corroborating this hypothesis, the hUII counterpart of UCA, *i.e.* [Pep^7]hUII,

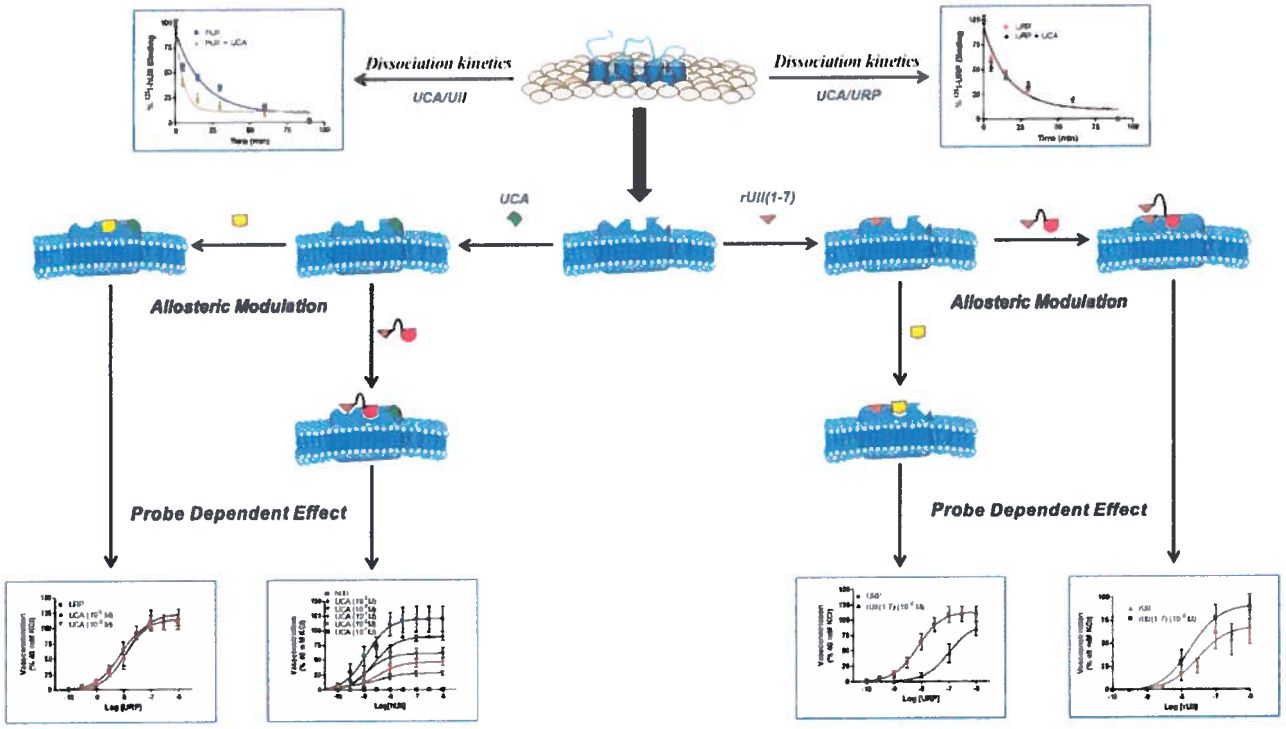

FIGURE 8 | Schematic representation of a proposed allosteric modulation of the urotensinergic system by urocontrin A and rUII(1–7). By acting at an allosteric binding site, UCA is able to modify the receptor topography preventing the proper interaction of UT with the linear UII N-terminal region ultimately leading to an inefficient activation characterized by a reduced efficacy. On the opposite, such a receptor conformational change has no effect on URP-mediated action. Conversely, binding of the rUII(1–7) N-terminal segment, initiates a topographical change that antagonizes the effect of URP, but not UII. Modified from Chatenet et al. (2012a).

acted as a weak but full agonist of the UT receptor (Chatenet et al., 2012a). This N-terminal domain is thus able to modulate the topology of the receptor in such a manner that the C-terminal domain of UII is able to trigger receptor activation. However, could this N-terminal segment be biologically active? As an agonist, the N-terminal domain of rat UII [rUII(1–7)], *i.e.* Pyr-His-Gly-Thr-Ala-Pro-Glu-amide (**Figure 7**), was unable to induce the contraction of rat aortic rings (Chatenet et al., 2012a). Amazingly, pre-treatment of rat aortic rings with rUII(1–7) induced an apparent increase in rUII contractile efficacy while reducing the potency and the efficacy of URP-mediated vasoconstriction (Chatenet et al., 2012a). These results clearly suggested that rUII(1–7) acted as a probe dependant allosteric modulator on rUII- and URP-mediated vasoconstriction (**Figure 8**) (Chatenet et al., 2012a). Since all UII isoforms possess different N-terminal domains, it is hypothesized that these regions could act as specie-selective specific URP modulators but there is currently no clue regarding an endogenous production of those N-terminal UII domains *in vivo*.

CONCLUSIONS

These latest findings about the urotensinergic system will probably generate a considerable interest within the scientific community. First, the discovery of UT on the nuclear membrane and the presence of intracellular ligands open up new avenues in UT signaling physiology. In general, nuclear-localized receptors may regulate distinct signaling pathways, suggesting that biological responses mediated by GPCRs are not only initiated at the cell surface but might result from the integration of extracellular and intracellular signaling pathways. These receptors are therefore well-positioned to play major roles in the physiological and pathophysiological responses associated with their endogenous ligands. Finally, the discovery of allosteric modulators of the urotensinergic systems such as urocontrin, UCA, and rUII(1–7), will surely enable a better understanding of the urotensinergic system by allowing to discriminate *in vitro* and *in vivo* specific biological actions mediated by UII and/or URP. Therefore, these unique derivatives will be useful as chemical templates for the rational design of novel UT receptor ligands, as well as pharmacological tools for *in vitro* and particularly *in vivo* studies aimed at clarifying the role(s) played by the UII/URP/UT receptor system in physiology and pathology.

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