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Involvement of endogenous antioxidant systems in the protective activity of pituitary adenylate cyclase-activating polypeptide against hydrogen peroxide-induced oxidative damages in cultured rat astrocytes

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Abbreviations used: H_2O_2, hydrogen peroxide; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; NBT, nitrotetrazolium blue chloride; $O_2^{\circ-}$, superoxide anions; PACAP, pituitary adenylate cyclase-activating polypeptide; PKA, protein kinase A; PLC, phospholipase C; ROS, reactive oxygen species; SOD, superoxide dismutase.

Abstract

Astroglial cells possess an array of cellular defense mechanisms, including superoxide dismutase (SOD) and catalase antioxidant enzymes, to prevent damages caused by oxidative stress. Nevertheless, astroglial cell viability and functionality can be affected by signifcant oxidative stress. We have previously shown that pituitary adenylate cyclase-activating polypeptide (PACAP) is a potent glioprotective agent that prevents hydrogen peroxide (H_2O_2)-induced apoptosis in cultured astrocytes. The purpose of the present study was to investigate the potential protective effect of PACAP against oxidative-generated alteration of astrocytic antioxidant systems. Incubation of cells with subnanomolar concentrations of PACAP inhibited H_2O_2-evoked reactive oxygen species accumulation, mitochondrial respiratory burst and caspase-3 mRNA level increase. PACAP also stimulated SOD and catalase activities in a concentration-dependent manner, and counteracted the inhibitory effect of H_2O_2 on the activity of these two antioxidant enzymes. The protective action of PACAP against H_2O_2-evoked inhibition of antioxidant systems in astrocytes was PKA, PKC and MAP-kinase dependent. In the presence of H_2O_2, the SOD blocker NaCN and the catalase inhibitor 3-aminotriazole, both suppressed the protective effects of PACAP on SOD and catalase activities, mitochondrial function and cell survival. Taken together, these results indicate that the anti-apoptotic effect of PACAP on astroglial cells can account for the activation of endogenous antioxidant enzymes and reduction of respiration rate, thus preserving mitochondrial integrity and preventing caspase-3 expression provoked by oxidative stress. Considering its powerful anti-apoptotic and anti-oxidative properties, the PACAPergic signaling system should thus be considered for the

development of new therapeutical approaches to cure various pathologies involving oxidative neurodegeneration.

Key words: PACAP; astroglial cells; apoptosis; catalase; superoxide dismutase; oxidative stress

Running Title: *PACAP prevents astrocytic cell death by activating antioxidant systems*

Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) was first isolated from ovine hypothalamus on the basis of its ability to stimulate cAMP formation in rat anterior pituitary cells (Miyata *et al.* 1989). PACAP is a member of the vasoactive intestinal peptide (VIP) / secretin / growth hormone-releasing hormone / glucagon superfamily (Vaudry *et al.* 2009). Three PACAP receptors have been cloned, the PACAP-selective PAC1-R and the PACAP/VIP mutual receptors VPAC1-R and VPAC2-R (Vaudry *et al.* 2009). All PACAP receptors belong to the seven-transmembrane domain G protein-coupled receptors superfamily (Harmar *et al.* 2012) and modulate several signaling pathways including the cAMP / protein kinase A (PKA), phospholipase C (PLC) / protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) cascade (Masmoudi *et al.* 2003, Ravni *et al.* 2006, Vaudry *et al*. 2009). PACAP sequence has been highly conserved during evolution (Vaudry *et al*. 2009), and it is now established that this peptide is involved in the regulation of important biological functions, such as neurotrophic and neuroprotective activities (Seaborn *et al.* 2011). The antiapoptotic effects of PACAP have been reported on several neuronal cell types *in vitro* and models of neurodegenerative diseases *in vivo* (Seaborn *et al*. 2011). In particular, PACAP has been found to protect dopaminergic neurons (Reglodi *et al.* 2006), cerebellar granule neurons (Vaudry *et al.* 2002) and hippocampal neurons (Stetler *et al.* 2010) from oxidative stress-induced apoptosis. Increasing evidence indicates that the neuroprotective effect of PACAP may be indirect, via the release of neuroprotective factors by glial cells (Dejda *et al.* 2005, Masmoudi-Kouki *et al.* 2007, Miyamoto *et al.* 2014, Yang *et al.* 2006). Moreover,

previous studies have revealed that part of the antiapoptotic effect of PACAP is mediated through an inhibition of reactive oxygen species (ROS) production by astrocytes (Masmoudi-Kouki *et al.* 2011, Miyamoto *et al*. 2014), highlighting their importance in the neuroprotective activity of the peptide against oxidative stress. As astrocytes are in close interaction with neurons, their protection from oxidative insult appears essential to maintain brain function and to prevent neuronal damages (Barreto *et al.* 2011).

It is well established that astrocytes play an important role in the protection of the brain against oxidative stress damage in both physiological and pathological conditions (Fernandez-Fernandez *et al.* 2012, Steele and Robinson 2010, Takuma *et al*. 2001, 2004). Oxidative stress induces an imbalance in ROS production, damages cellular biomolecules, impairs cellular antioxidant defenses and finally triggers cell death by apoptosis (Dasuri *et al.* 2013, Sanders and Greenamyre 2013). Astrocytes contain high levels of antioxidant molecules, such as reduced glutathione, and express high level of antioxidant enzymes such as superoxide dismutases (SODs), catalase, glutathione peroxidase or peroxiredoxin, which strongly contribute to protect neurons from oxidative stress induced injury (Cabezas *et al.* 2012, Takuma *et al*. 2004, Zhu *et al.* 2012). Despite their high antioxidative activities, astrocytes cannot survive and protect neurons under substantial oxidative stress (Chen and Gibson 2008, Emerit *et al.* 2004, Shibata and Kobayashi 2008). In particular, astrocytes are very sensitive to hydrogen peroxide (H_2O_2; Ferrero-Gutierrez *et al.* 2008, Hamdi *et al.* 2011) and astrocytic apoptosis, which is associated with a depletion of antioxidant activities that occurs in brain injuries caused by excessive ROS production (Hsu *et al.* 2009, Ouyang

and Giffard 2004). Reciprocally, stimulation of the activity of the endogenous antioxidant system or over-expression of SOD in astroglial cells is associated with an increased resistance to oxidative injury (Chen *et al.* 2001, Hamdi *et al.* 2012). Altogether, these observations suggest that antioxidant systems play a prominent role in the protection of astrocytes from oxidative assault. As a matter of fact, we have previously reported that PACAP rescues astroglial cells from the deleterious effects of oxidative stress by attenuating H_2O_2-evoked ROS accumulation and glutathione content reduction (Masmoudi-Kouki *et al*. 2011). In contrast, regarding the glioprotective action of PACAP upon oxidative stress injuries, the potential implication of endogenous antioxidant systems are currently unknown. Therefore, the aim of the present study was to investigate the possible effects of PACAP on SOD and catalase activities and to characterize the transduction pathways mediating the antioxidant action of PACAP in astrocytes.

Materials and methods

Animals

Wistar rats (Pasteur Institute, Tunis, Tunisia, and Charles River Laboratories, St 5 Germain sur l'Arbresle, France) were kept in a temperature-controlled room (21 ± 1°C) 6 under an established photoperiod (lights on from 7:00 am to 7:00 pm) with free access to food and water. All experiments have been performed in accordance with the American Veterinary Medical Association. Approval for these experiments was obtained from the Medical Ethical Committee For the Care and Use of Laboratory Animals of Pasteur Institute of Tunis (approval number: FST/LNFP/Pro 152012).

Chemicals

Dulbecco's modified Eagle's medium (DMEM), F12 culture medium, D(+)-glucose, L-glutamine, *N*-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) buffer solution, fetal bovine serum (FBS), trypsin-EDTA and the antibiotic-antimycotic solution were obtained from Gibco (Invitrogen, Grand Island, NY, USA). Lactate dehydrogenase (LDH) assay kit, nitrotetrazolium blue chloride (NBT), bovine liver catalase, DL-epinephrine, chelerythrine, H89, U73122, Triton X-100, insulin and calcein-AM and fluorescein diacetate-acetoxymethyl ester (FDA-AM) were purchased from Sigma-Aldrich (St. Louis, MO, USA). U0126 was commercialized by Promega (Charbonnières, France). The mitochondrial potential sensor JC-10 was obtained from Molecular Probes (Eugene, Oregon, USA). Bovine serum albumin fraction V (BSA) was supplied by Roche Diagnostics (Mannheim, Germany). The 38-amino acid form

of PACAP and PACAP6-38 were synthesized by solid-phase methodology as previously described (Jolivel *et al.* 2009).

Secondary cultures of rat cortical astrocytes

Secondary cultures of rat cortical astrocytes were prepared from newborn Wistar rats of both sexes as previously described (Masmoudi *et al.* 2005). Briefly, cerebral hemispheres were collected in DMEM/F12 (2 : 1; v/v) culture medium supplemented with 2 mM glutamine, 1% insulin, 5 mM HEPES, 0.4% glucose and 1% of the antibiotic-antimycotic solution. The tissues were scattered mechanically with a syringe equipped with a 1-mm gauge needle and filtered through a 100-µm sieve (Falcon, Franklin Lakes, NJ, USA). Dissociated cells were resuspended in culture medium supplemented with 10% FBS, plated in 75-cm^2 flasks (Greiner Bio-one GmbH, Frickenhausen, Germany) and incubated at 37°C in a 5% CO_2/95% air. When cultures were confluent, astrocytes were isolated by shaking overnight the flasks on an orbital agitator. Adherent cells were detached by trypsination and pre-plated for 5 min to discard contaminating microglial cells. Then, the non-adherent astrocytes were harvested and plated on 35-mm Petri dishes at a density of 0.3×10^6 cells/mL. All experiments were performed on 5- to 7-day-old secondary cultures and cell purity was controlled by immunofluorescence as reported below.

Cell immunolabeling

For immunolabeling studies, cells were cultured on poly-L-lysine coated coverslips. After 5 days, cells were fixed with 4% paraformaldehyde in phosphate buffered saline

1M (PBS), and incubated for 1 h in a blocking solution containing 1:50 normal donkey serum, 1% bovine serum albumin, and 10% Triton X-100 (VWR International, Strasbourg, France) in PBS. Cells were then incubated overnight at 4˚C with primary antibodies, i.e. rabbit anti-GFAP (astrocytes labeling; Dako Denmark A/S; at1:400), rabbit anti-CD68 (microglia labeling; Abcam, Paris, France; at 1:200) or sheep anti-olig2 (oligodendrocyte labeling; Abcam, Paris, France; at 1:100). Subsequently, cells were incubated with secondary antibodies for 2 h at room temperature, *i.e.* Alexa 488-conjugated donkey anti-rabbit IgG (Invitrogen, Boulogne-Billancourt, France), Alexa 594-conjugated donkey anti-rabbit IgG (Invitrogen, Boulogne-Billancourt, France) or Alexa 594-conjugated donkey anti-Sheep IgG (Invitrogen, Boulogne-Billancourt, France) at 1:400 in PBS. Cell nuclei were stained with the nuclear marker DAPI (1 µg/mL, 10 min at room temperature) and finally cells were cover slipped with Mowiol®. Immunofluorescence was observed under an Eclipse E600 microscope (Nikon Instrument, Champigny-sur-Marne, France). After counting, it appeared that in our culture conditions, more than 98% of the cells were labeled with GFAP antibodies (Supplementary Figure 1).

Cell treatment

To examine the potential protective action of PACAP against H_2O_2-provoked astrocytes assault (LDH release, cell death, caspase-3 activation and expression, SOD and catalase activities.), cultured cells were treated with a control solution or PACAP 10 min before starting incubation with H_2O_2 for 1 h (Supplementary Figure 2A). When

PACAP antagonist or transduction pathway inhibitors were used, they were added 30 minutes before H_2O_2 exposure. To block catalase activity, ATZ was added 3 h before H_2O_2. In one subset of experiments, PACAP was administered 10 min after the beginning of the incubation with H_2O_2 (Supplementary Figure 2B).

Measurement of cell cytotoxicity

Cultured cells were incubated at 37°C with fresh serum-free medium in the absence or presence of test substances. Membrane integrity was assessed as a function of the amount of cytoplasmic LDH released into the medium. The amount of LDH released into the medium was quantified using a cytotoxicity detection kit (MAK066, Sigma-Aldrich) according to the manufacturer's instructions. LDH activity was measured at 450 nm with a spectrophotometric microplate reader (Flexstation 3, Molecular Devices Sunnyvale, CA, USA).

Assessment of cell survival

Cultured cells were incubated at 37°C with fresh serum-free culture medium in the absence or presence of the test substances. Qualitative visualization of cell survival was conducted by using the LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (Invitrogen) according to the manufacturer protocol, as previously reported (Vaudry *et al*. 2002). Briefly, cells were incubated for 20 min at 37°C with a solution of 1.2 µg/mL calcein-AM (producing green fluorescence in living cells) and 3.4 µg/mL ethidium homodimer-1 (EH-1, producing red fluorescence in dead cells) and

rinsed twice with PBS. Images of astroglial cells were randomly acquired on an inverted microscope (IRE2; Leica Microsystems, Nanterre, France). For quantification of surviving astrocytes, cells were incubated for 8 min with FDA-AM, rinsed twice with PBS and lysed with a Tris/HCl solution containing 1% sodium dodecyld sulfate. Fluorescence was measured with excitation at 485 nm and emission at 538 nm using a fluorescence microplate reader FL800TBI (Bio-Tek Instruments, Winooski, VT, USA). Pilot experiments have shown that the fluorescence intensity is proportional to the number of cells (in the range 75 X 10^4 to 1 X 10^6 cells/mL).

Quantitative PCR analysis

The effect of PACAP on caspase-3 mRNA levels was performed by quantitative RT-PCR. Total RNA was isolated from astrocytes using the NucleoSpin kit (Macherey-Nagel, Hoerd, France) and 3-4 µg were used for cDNA synthesis using ImProm II Promega kit (Promega). PCR amplifications were performed with an ABI PRISM 7500 Sequence Detection System (Applied Biosystems) using 5 ng cDNA, 1X Fast SYBR Green universal PCR Mastermix (Applied Biosystems, Courtaboeuf, France) and 300 nM forward (5'-CTGACTGGAAAGCCGAAACTCT-3′) and reverse (5'-CATCGTCAGTTCCACTGTCTGTCT-3′) caspase-3 primers, under standard running conditions as suggested by the manufacturer. The amount of cDNA in each sample was calculated by the comparative quantification cycle (Cq) method and expressed as $2^{\exp(-_{\Delta\Delta}Cq)}$ using glyceraldehyde-3-phosphate dehydrogenase as an internal control.

Measurement of mitochondrial activity

Mitochondrial membrane potential was quantified using the JC-10 probe. Cells seeded into 96-well plates were subjected to various treatments, incubated in the presence of the JC-10 probe (10 μg/mL) at 37°C for 1 h and then washed twice with PBS. In healthy astrocytes, the intact membrane potential allows the lipophilic dye JC-10 to enter into the mitochondria where it accumulates and aggregates producing an intense orange signal. In dying cells, where the mitochondrial membrane potential collapses, the monomeric JC-10 remains cytosolic and stains cell cytoplasm in green. Fluorescence was measured with excitations at 485 (monomer) and 510 nm (aggregates), and emissions at 534 (green) and 610 nm (orange), respectively.

Superoxide radical generation assay

The intracellular production of superoxide anion was detected by the reduction of nitroblue tetrazolium (NBT) to dark blue formazan deposits. Treated cells in 6-well plates were incubated for 2 h in the dark with reaction mixture containing NBT (1 mg/mL) and BSA (1 mg/mL). To visualize the formation of blue deposits, cells were examined and images were acquired with an eclipse E-600 microscope (Nikon, Champigny-sur-Marne, France) equipped with a 3 CCD Sony DXC950 camera interfaced with the Visiolab computerized program (Biocom, Les Ulis, France). To quantify cellular superoxide radical levels, the deposits were dissolved with (2 M) KOH/DMSO (v:v, 1:1.15) solution and the absorbance of the mixture was measured at 645 nm with a spectrophotometer (Jenway, Philadelphia, USA).

Measurement of antioxidant enzyme activities

Cells were incubated at 37°C with fresh serum-free medium. At the end of the incubation, cells were rinsed twice with PBS, rubber scraped and centrifuged at 3000 g for 10 min at 4°C. The cell pellet was resuspended in 50 µL of ice-cold lysing buffer containing 50 mM Tris–HCl (pH 8), 10 mM EDTA, 100 µM phenylmethyl-sulfonylfluoride and 1% Triton X-100 before centrifugation at 16,000 g for 20 min at 4°C. The supernatant was stored at -20°C until enzyme activity determination.

SOD activity was assessed using a spectrophotometric assay, which consists of measuring epinephrine autoxidation induced by superoxide anions. Samples were incubated for 3 min with a mixture containing bovine catalase (0.4 U/µL), DL-epinephrine (5 mg/mL) and Na_2CO_3/$NaHCO_3$ buffer (62.5 mM, pH 10.2). The oxidation of epinephrine was measured at 480 nm using a Bio-Rad spectrophotometer (Bio-Rad Laboratories, Philadelphia, PA, USA).

Catalase activity was determined on the basis of the disappearance of H_2O_2. Samples, prepared as described above, were mixed with 30 mM H_2O_2 in PBS. The decrease of H_2O_2 was followed at a wavelength of 240 nm for 3 min at 30 s intervals. Catalase activity was expressed using the extinction coefficient of 40 mM^{-1} cm^{-1} for H_2O_2.

Statistical analysis

Data are expressed as the mean ± SEM from three independent experiments. Statistical analysis of the data was performed by using ANOVA, followed by Bonferroni's test. A *p* value of 0.05 or less was considered as statistically significant.

Results

Effect of PACAP on H_2O_2-induced caspase-3 activation and astrocyte cell death

We have previously shown that incubation of cultured astrocytes with graded concentrations of PACAP (10^{-14} M to 10^{-8} M), dose-dependently prevented cell death induced by 300 μM H_2O_2. Here we show that the addition of PACAP (10^{-9} M) to the culture medium almost completely abolished the effect of moderate concentrations of H_2O_2 (150 to 300 μM) on LDH release and markedly attenuated the stimulatory effect of higher concentration of H_2O_2 (500 μM; Fig. 1A). Pre-incubation of astrocytes for 30 min with the PACAP receptor antagonist PACAP6-38 (10^{-6} M), which had no effect by itself, totally abolished the action of PACAP on H_2O_2-induced LDH release (Fig. 1B). Incubation of astrocytes with the selective protein kinase C (PKC) inhibitor chelerythrine (10^{-7} M), the PKA inhibitor H89 (2×10^{-5} M) or the mitogen-activated protein kinase kinase (MEK) inhibitor U0126 (10^{-6} M) also blocked the effect of PACAP on H_2O_2-induced LDH release (Fig. 1C). Furthermore, the fact that addition of PACAP (10^{-9} M) to the culture media 10 min after 300 μM H_2O_2 pre-incubation rescues asrocytes from cell death (97% ; $P< 0.001$), provides evidence that the beneficial effects of PACAP are still present when the peptide is added after oxidative injuries (Supplementary figure 3A).

To determine whether PACAP reduced H_2O_2-induced cell death, astrocytes were stained with calcein-AM (green color) and EH-1 (red color), markers of living and dead cells, respectively. We observed very few cells labeled with EH-1 in control- and PACAP-treated cells (Fig. 2Aa, 2Ac and 2B), while incubation of astrocytes with 300

μM H_2O_2 induced a significant increase in the number of EH-1-labeled cells ($P < 0.001$) which mirrored a decrease in the number of calcein-positive cells (Fig. 2Ab and 2B). Pretreatment of the cells with PACAP (10^{-9} M; 10 min) counteracted 86% ($P < 0.001$) of H_2O_2-induced decrease in the number of calcein-positive cells (Fig. 2Ad, 2B). Quantification of fluorescein diacetate-acetoxymethyl ester fluorescence intensity incorporated by astrocytes with a microplate reader gave similar results (data not shown).

When looking at GFAP labeling, cells in control conditions exhibited a flat polygonal morphology (Fig. 2Ca), while in cells exposed to 300 μM H_2O_2, processes were significantly retracted (Fig. 2Cb). Treatment of the cells with PACAP totally prevented the morphological alterations induced by H_2O_2 (Fig. 2Cd).

To further explore the mechanisms involved in the glioprotective action of PACAP, the effect of the peptide on caspase-3 activity and gene expression were investigated. In agreement with its trophic action (Ravni *et al*. 2006; Vaudry *et al*., 2000), PACAP exhibited a transient intrinsic inhibition of caspase-3 gene expression (-35.8% $\pm$ 2.23; $P < 0.05$) within 5 to 10 min after peptide treatment and a concomitant decrease of basal caspase-3 activity (-35% $\pm$ 7.05; $P < 0.01$). On the opposite, exposure of cultured astrocytes to H_2O_2 for 30 min produced a significant increase of caspase-3 activity (+49.9 $\pm$ 8.5; $P < 0.01$) and mRNA levels (+40.6% $\pm$ 2.8; $P < 0.001$; Fig. 3A and 3B) which lasted at least 30 min. Pretreatment with PACAP (10^{-9} M) of cells exposed to H_2O_2 induced a decrease of caspase-3 activity to a similar level to the one observed in cells that have not been exposed to H_2O_2 (Fig. 3A) and a decrease of caspase-3 mRNA which remained significant for all the duration of the treatment (Fig. 3B). Addition of

graded concentrations of PACAP (10^{-14} M to 10^{-6} M) to the culture medium suppressed dose-dependently the stimulatory effect of H_2O_2 (300 µM; 30 min) on caspase-3 gene expression (Fig. 3C).

Effect of PACAP on H_2O_2-generated superoxide anions

Considering the major effect of oxidative stress in the induction of respiratory burst and superoxide anion ($O_2^{\circ-}$) production, we examined the effect of PACAP on H_2O_2-induced $O_2^{\circ-}$ generation in astrocytes. Control and PACAP (10^{-9} M)-treated astrocytes exhibited very few blue precipitates (reflecting reduction of NBT by $O_2^{\circ-}$ production) in the cell bodies (Fig. 4Aa and 4Ac). Treatment of astrocytes with 300 µM H_2O_2 resulted in the labeling of most cell bodies in blue, indicating that large amounts of $O_2^{\circ-}$ are produced by respiratory burst (Fig. 4Ab). When PACAP 10^{-9} M was added to H_2O_2-treated cells, only a few cells were labeled in blue (Fig. 4Ad), suggesting that the peptide could abolish $O_2^{\circ-}$ generation. Quantitative analysis indicated that H_2O_2 induced a significant increase (+518.9 % $\pm$ 14.0; $P < 0.001$) of $O_2^{\circ-}$ production which was reduced by a factor of 2 in the presence of PACAP (Fig. 4B).

Effect of PACAP on the activity of antioxidant enzymes in cultured astrocytes

To determine if the endogenous antioxidant system was involved in the protective action of PACAP, the activity of antioxidant enzymes SOD and catalase in astrocytes was monitored. Incubation of cultured astrocytes with increasing concentrations of PACAP (10^{-14} M to 10^{-6} M) for 10 min, induced a dose-dependent stimulation of SOD and catalase activities with EC_{50} values of 1.11 x 10^{-9} M and 5.32 x 10^{-9} M,

respectively (Fig. 5A and 5B). Time-course experiments revealed that PACAP (10^{-9} M) significantly enhanced SOD and catalase activities within 5 and 10 min (SOD and catalase) of incubation. The stimulatory effect of PACAP on SOD and catalase activities reached a maximum after 10 and 20 min, respectively (Fig. 5C and 5D). Thereafter the activity of the two antioxidant enzymes gradually returned to control values within 60 min of treatment.

Time-course experiments revealed that exposure to 300 μM H_2O_2 significantly reduced SOD and catalase activities within 30 min, with a maximum effect after 1 h of incubation (Fig 6A and 6B, inset: SOD -42.75 % $\pm$ 2.08, P <0.001 and catalase -40.69 % $\pm$ 2.07; P <0.001). After 1 h of treatment, addition of graded concentrations of PACAP (10^{-14} M to 10^{-6} M) totally blocked in a dose-dependent manner the inhibitory action of H_2O_2. Nanomolar concentrations of PACAP even significantly stimulated SOD and catalase activities above control levels (Fig. 6A and 6B).

Receptor and signal transduction pathways involved in the effects of PACAP on endogenous antioxidant systems

Administration of the PACAP receptor antagonist PACAP6-38 (10^{-6} M) to cultured astrocytes did not induce any modification of SOD and catalase activities by itself, but totally abolished the stimulatory effect of PACAP (10^{-9} M) on SOD and catalase activities in the absence (Fig. 7A and 7B) or presence (Fig. 7C and 7D) of H_2O_2 exposure.

Incubation of astrocytes with the PLC inhibitor U73122 (10^{-7} M), the PKC inhibitor chelerythrine (10^{-7} M) or the PKA inhibitor H89 (2 X 10^{-5} M) which had no impact by

themselves on antioxidant enzyme activities, totally abrogated the stimulatory effect of PACAP on SOD and catalase activities. In addition, blockage of ERK phosphorylation with the MEK inhibitor U0126 (1 μM) suppressed the stimulatory action of PACAP on both enzyme activities in the absence of H_2O_2 (Fig. 8A and 8B). In the presence of H_2O_2, incubation of astrocytes with U73122, chelerythrine, H89 or U0126 also abolished the ability of PACAP to stimulate the activity of endogenous antioxidant systems SOD and catalase (Fig. 8C and 8D).

Addition of 2 X 10^{-2} M NaCN, a SOD inhibitor, significantly reduced basal enzymatic activity and fully suppressed the stimulatory effect of PACAP (Fig. 9A). In a very similar manner, exposure of astrocytes to 10^{-2} M 3-aminotriazole, a specific catalase inhibitor, strongly decreased basal and PACAP-enhanced catalase activity (Fig. 9B). Exposure of the cells to 2 X 10^{-2} M NaCN and 10^{-2} M 3-aminotriazole, which did not affect cell survival by themselves, fully blocked the protective effect of PACAP against H_2O_2-evoked alteration of SOD and catalase activities (Fig. 9A and 9B), mitochondrial integrity (Fig. 9C and inset) and cell death (Fig. 9D).

Discussion

It has been clearly established that oxidative stress causes apoptosis in various cell types, notably in astrocytes (Sun *et al.* 2014, Wang *et al.* 2014). We have previously reported that, like in neurons, PACAP protects astrocytes against H_2O_2-induced cell death (Masmoudi-Kouki *et al*. 2011). Here, we show that PACAP, through the activation of its receptors and the PKA, PKC and MAP-kinases signaling pathways, counteracts superoxide anion accumulation and prevents the inhibition of SOD and catalase activities induced by oxidative stress in cultured astrocytes. Furthermore, blocking SOD and catalase activation reduces the protective effect of PACAP. Thus, our results show that the glioprotective action of PACAP against H_2O_2-induced apoptotic cell death can be accounted for activation of endogenous antioxidant systems (Fig. 10).

In agreement with the well-known cytoprotective effect of PACAP (Bourgault *et al.* 2011), we found that nanomolar concentrations of PACAP exert a protective effect against oxidative stress-induced cell death in cultured astrocytes. Indeed, PACAP was able to prevent the deleterious action of graded concentrations of H_2O_2 (100 to 500 µM) on astrocytes. Visualization of living cells by calcein-AM and GFAP staining revealed that the cytotoxic effect of H_2O_2 was associated with modifications in astrocyte morphology, such as cell shrinkage and appearance of thin processes that are suggestive of apoptotic cell death. These morphological changes were also prevented by the addition of subnanomolar concentrations of PACAP to the culture medium. In agreement with these observations, it has already been shown that H_2O_2-treated astrocytes exhibit the characteristic features of apoptotic cells with increased caspase-3

activity, nuclear condensation and DNA fragmentation (Peng *et al.* 2013, Ramalingam and Kim 2014, Zhou *et al.* 2015), which are all blocked by PACAP treatment. Furthermore, it has been reported that in cerebellar granule cells PACAP is able to prevent ROS-induced mitochondrial dysfunction, the stimulation of caspase-3 activity and LDH leakage, which together induce apoptotic cell death (Tabuchi *et al.* 2003, Vaudry *et al*. 2002). These data suggest that PACAP, which is released by both glial cells and neurons in the brain (Vaudry *et al*. 2009), may act as an autocrine and/or paracrine factor to enhance the resistance of astrocytes to H_2O_2.

The cytotoxic effect of H_2O_2 via production of highly reactive species inside the cell has been well documented in numerous cell types, including astrocytes (Feeney *et al.* 2008, Hamdi *et al.* 2011, Liu *et al.* 2013). Various studies indicate that an excess of H_2O_2 can provoke cell apoptosis via multiple mechanisms, including the stimulation of some pro-apoptotic genes of the Bcl-2 family responsible for the formation of mitochondrial permeability transition pores, a collapse of the mitochondrial membrane potential and a decrease of ATP generation (Gyulkhandanyan *et al.* 2003, Kaddour *et al*. 2013, Liu *et al*. 2013). Consistent with these notions, the present study shows that H_2O_2 severely impaired mitochondrial integrity, increased respiration rate and mitochondrial $O_2^{\bullet -}$ generation, which were significantly reduced by PACAP. Measurement of mitochondria activity by means of the membrane potential-sensitive probe JC-10 revealed that H_2O_2 decreased the proportion of active mitochondria, and that PACAP prevented this deleterious action of H_2O_2. The inhibitory effect of PACAP on H_2O_2-evoked burst of superoxide generation is probably a key mechanism in its glioprotective action. Indeed, we have previously reported that, in H_2O_2-treated

astrocytes, PACAP increases the cellular content of GSH, the major intracellular free radical scavenger in the brain (Masmoudi-Kouki *et al*. 2011), and enhances cell resistance to oxidative injury. The involvement of GSH in the protective effect of PACAP is supported by recent data showing that PACAP increases uptake of the GSH precursor, cysteine, in cultured astrocytes (Resch *et al.* 2014). Furthermore, inhibition of GSH synthesis in neuroblastoma cells enhances intracellular ROS levels and increases cell sensitivity to oxidative damages (Miyama *et al.* 2011).

It has been reported that intracellular ROS overproduction and GSH depletion impair endogenous antioxidant defences, notably by decreasing SOD and catalase activities (Dokic *et al.* 2012, Lopez *et al.* 2007). The present study reveals that PACAP at subnanomolar concentrations induced a dose-dependent increase of both SOD and catalase activities in astrocytes and simultaneously block H_2O_2-evoked inhibition of the activity of these two enzymes. We have previously shown that, in cultured astrocytes, PACAP in the same range of concentrations protects cells against apoptosis provoked by oxidative assault (Masmoudi-Kouki *et al*. 2011). The fact that PACAP increases Mn-SOD and peroxiredoxins (Prx-1 and Prx-6) mRNA levels to promote neuronal survival after spinal cord injury (Fang *et al.* 2010), provides evidence for the implication of antioxidant enzymes in the antiapoptotic action of PACAP. Consistent with these observations, it has already been reported that a reduction in the activities of the antioxidant enzyme suppresses the beneficial effect of protective molecules against oxidative-stress induced astrocyte apoptosis (Gaspar *et al.* 2008, Hamdi *et al*. 2011, Smith *et al.* 2007). Besides, *in vivo* studies have demonstrated that Mn-SOD overexpression improves resistance to oxidative injuries in mouse models of ischemia

or Alzheimer's disease (Dumont *et al.* 2009, Saito *et al*. 2003) and deficiency of SOD enhances oxidative damages, in a mouse model of Alzheimer's disease (Schuessel *et al.* 2005). Together, these data indicate that the protective effect of PACAP against H_2O_2-provoked oxidative stress and cell death in astrocytes is attributable to the activation of the antioxidant enzymes that act as H_2O_2 and ROS scavengers. It is noteworthy that in H_2O_2-treated astrocytes PACAP was still effective in maintaining SOD and catalase activities above control values after 1 h of treatment. This indicates that the neuropeptide exhibits a sustained antioxidant action under stress situations. The increase of PACAP receptors in cultured astrocytes under stress assault (Suzuki *et al*. 2003, Stum *et al*., 2007) may contribute to the extended activation of the endogenous antioxidant system and to prolonged cell protection.

Previous studies have shown that the glioprotective action of PACAP upon H_2O_2-evoked cell death is mediated through activation of PAC1 receptor coupled to the PKA, PKC and MAP-kinase extracellular signal-regulated kinase (ERK) transduction pathways (Masmoudi-Kouki *et al*. 2011). Indeed, blocking the activity of these kinases abrogated the cell survival-promoting effect of PACAP under oxidative stress. Furthermore, we demonstrated that like cell survival, activation of SOD and catalase activities were PKA and PLC / PKC dependent. It has also been shown that stimulation of SOD and catalase activities or expression of the enzymes in a mouse model of Alzheimer's disease (Leem *et al.* 2009), in the rat inferior colliculus (Mei *et al.* 1999) and in cultured glial cells (Huang *et al.* 2001) depend on PKA and/or PKC activation (Kupershmidt *et al.* 2011, Leem et al. 2009, Mao *et al.* 2014, Priyanka *et al.* 2013). Downstream of PKA and PKC, PACAP stimulates phosphorylation of ERK

(Seaborn *et al*. 2011), a kinase which alone or together with PKA induces c-fos gene expression (Mullenbrock *et al.* 2011, Vaudry *et al.* 1998) an immediate early gene known to stimulates SOD and catalase protein expression (Radjendirane V. and Anil, Mikawa *et al*. 1995). Concomitantly, PACAP promotes the expression and activity of the antioxidant protein peroxyredoxin-2 and glutathione peroxidase 1 through the AC/PKA, PLC/PKC and MAPK/ERK pathways (Miyamoto *et al*. 2014) and inhibition of peroxyredoxin-2 with siRNA reduces the antiapoptotic activity of the peptide (Botia *et al.*, 2008). Taken together, these data highlight the importance of the AC/PKA/PKC/MAPK/ERK pathways in the antioxidant action of PACAP. However, further investigations are needed to definitely establish the functional contribution of PKA/PKC/ERK transduction pathways in the glioprotective action of PACAP, for example by using siRNA against PKA, PKC and/or MAPK/ERK, as previously done with SH-SY5Y cell lines (Quesada *et al*. 2011) or granule neurons (Botia *et al.*, 2008).

The protective effect of PACAP against H_2O_2-reduced antioxidant enzyme activities might have pathophysiological significance in the context of brain injuries *i.e.* in neurodegenerative diseases or stroke. The central nervous system is very vulnerable to oxidative damage due to its high metabolic rate and high levels of unsaturated lipids. Furthermore, the up-regulation of antioxidant enzymes in astroglial cells would prove beneficial against cellular dysfunction, biomolecule oxidation and neuronal apoptosis observed in brain injuries (Baraibar *et al.* 2012, Negre-Salvayre *et al.* 2010). In agreement with this hypothesis, it has been shown that PACAP exerts potent glioprotective and neuroprotective effects against oxidative stress-provoked apoptosis (Seaborn *et al*. 2011). The fact that the anti-apoptotic action of PACAP is likely

mediated through PAC1 receptor is of particular interest. Recent data indicate that a synthetic PACAP analog, Ac-[Phe(pI)6, Nle17]PACAP(1-27), mimics the neuroprotective action of PACAP against 1-methyl-4-phenylpyridinium (MPP+) toxicity on SH-SY5Y neuroblastoma cells (Lamine *et al.* 2015). Since intravenous injections of the analog induce potent neuroprotection in a mouse model of Parkinson's disease (Lamine *et al*. 2015), the development of specific PACAP analogs, that would selectively promote the glioprotective and anti-oxidative proprieties of PACAP, might prove useful for the treatment of ischemia and neurodegenerative diseases.

In conclusion, the present study demonstrates that the neuropeptide PACAP rescues cultured astrocytes against cell death provoked by oxidative stress. This glioprotective action of PACAP is attributable, at least in part, to the activation of endogenous antioxidant enzymes and reduction of ROS formation, which preserve mitochondrial membrane integrity and prevent caspase-3 activation.

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Legends to figures

Fig. 1 Glioprotective effect of PACAP on H_2O_2-induced cell death. (A) Cultured rat astrocytes were pre-incubated for 10 min in the absence or presence of 10^{-9} M PACAP and then incubated for 1 h with medium alone (□) or with graded concentrations of H_2O_2 (100 – 500 µM) in the absence (■) or presence of 10^{-9} M PACAP (◪). (B) Cells were pre-incubated for 20 min in the absence or presence of PACAP6-38 (10^{-6} M) and then incubated for 1 h with medium alone (□) or with H_2O_2 (300 µM) in the absence (■) or presence of 10^{-9} M PACAP (◪). (C) Cells were pre-incubated for 30 min in the absence or presence of chelerythrine (10^{-7} M; Chel), H89 (2 x 10^{-5} M), or U0126 (10^{-6} M), and then incubated for 1 h with medium alone (□) or with 300 µM H_2O_2 in the absence (■) or presence of PACAP (◪). Membrane integrity was assessed by measuring LDH activity in culture media. - Each value is the mean (± SEM) of at least 12 different wells from three independent cultures. ANOVA followed by the Bonferroni's test. ** $P < 0.01$, *** $P < 0.001$, NS, not statistically different *vs*. untreated cells (absence of PACAP and absence of H_2O_2, open bars). $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, ns, not statistically different *vs*. cells treated with H_2O_2 in the absence or presence of transduction pathway inhibitors (black bars). $^{\S\S\S}P < 0.001$ *vs*. cells treated with PACAP plus H_2O_2 (vertical hached bars).

Fig. 2 Representative images illustrating the effect of PACAP on H_2O_2-induced cell death. (A) Cells were pre-incubated for 10 min in the absence or presence of 10^{-9} M PACAP, and then incubated for 1 h with medium alone (a), PACAP (c), 300 µM

H_2O_2 without (b) or with (d) 10^{-9} M PACAP. Living cells were labeled with calcein-AM (green fluorescence) and dead cells were labeled with EH-1 (red fluorescence). Scale bar = 50 µm. (B) Quantification of green (calcein) and red (EH-1) fluorescence from 12 fields in each conditions. ANOVA followed by the Bonferroni's test. *** $P < 0.001$ *vs*. calcein labeled untreated cells. $^{§§§}P < 0.001$, $^{§}P < 0.05$,*vs*. EH-1 labeled untreated cells. $^{\#}P < 0.05$ *vs*. calcein labeled H_2O_2-treated cells. $^{\&}P < 0.05$ *vs*. EH-1 labeled H_2O_2-treated cells. (C) Cells were pre-incubated for 10 min in the absence or presence of PACAP 10^{-9} M, and then incubated for 1 h with medium alone (a), PACAP (c), H_2O_2 300 µM without (b) or with (d) PACAP 10^{-9} M. Control and treated cells were labelled with GFAP (green) antibodies and Dapi (blue). Scale bar = 100 µm.

Fig. 3 Effect of PACAP on H_2O_2-induced stimulation of caspase-3 expression and activity in astroglial cells. (A, B) Cultured rat astrocytes were pre-incubated for 10 min without or with PACAP (10^{-9} M) and then incubated for the indicated time with medium alone (●), PACAP 10^{-9} M (▲) or H_2O_2 300 µM without (■) or with PACAP 10^{-9} M (▼). (C) Cells were pre-incubated for 10 min in the absence or presence of graded concentrations of PACAP (10^{-14} M – 10^{-6} M) and then incubated for 30 min with medium alone (□) or with H_2O_2 300 µM in the absence (■) or presence of PACAP (◪). Caspase-3 mRNA levels were measured by quantitative RT-PCR. Data were corrected using the glyceraldehyde-3-phosphate dehydrogenase signal as an internal control and the results are expressed as percentages of control. Caspase-3 activity was assessed by measuring clivage of the profluorescent caspase-3/7 substrate, Z-DEVD-R110. Each value is the mean (± SEM) of at least four different wells from

three independent cultures. ANOVA followed by the Bonferroni's test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, NS, not statistically different *vs*. control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, ns, not statistically different *vs*. H_2O_2-treated cells. $^{\S\S\S}P < 0.001$, Ns, not statistically different *vs*. PACAP-treated cells.

Fig. 4 Effect of PACAP on H_2O_2-induced superoxide radical production. (A) Cultured rat astrocytes were pre-incubated for 10 min in the absence (a, b) or presence of PACAP 10^{-9} M (c, d), and then incubated for 1 h with medium alone (a), PACAP 10^{-9} M (c), H_2O_2 300 µM without (b) or with PACAP 10^{-9} M (d). Phase-contrast images illustrating the generation of superoxide radicals visualized with the presence of a blue-dark precipitate inside cells. Scale bar, 20 µm. (B) Cellular superoxide radical levels were quantified by measuring nitrotetrazolium blue chloride (NBT) reduction induced by superoxide anion and the results are expressed as percentages of control. Each value is the mean (± SEM) of at least three different wells from three independent experiments. ANOVA followed by the Bonferroni's test. ***$P < 0.001$; NS, not statistically different *vs*. control. $^{\#\#\#}P < 0.001$ *vs*. H_2O_2-treated cells.

Fig. 5 Effect of PACAP on SOD and catalase activities in cultured rat astrocytes. Effect of graded concentrations of PACAP on SOD (A) and catalase (B) activities. Cells were incubated for 10 min in the absence or presence of graded concentrations of PACAP (10^{-14} M – 10^{-6} M). Time-course of the effect of PACAP on SOD (C) and catalase (D) activities. Cells were incubated in the absence or presence of PACAP 10^{-9} M for the times indicated. The activity of SOD was measured using a spectrophotometric assay which consists in measuring epinephrine autoxidation

induced by superoxide anion, and catalase activity was determined on the basis of the decomposition of H_2O_2. The results are expressed as a percentage of SOD or catalase activity with respect to control. Each value is the mean (± SEM) of at least four different dishes from three independent experiments. ANOVA followed by the Bonferroni's test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, NS, not statistically different *vs*. control.

Fig. 6 Effect of PACAP against H_2O_2-evoked inhibition of SOD and catalase activities in cultured rat astrocytes. Cells were pre-incubated for 10 min in the absence or presence of graded concentrations of PACAP (10^{-14} M – 10^{-6} M) and then incubated for 1 h with medium alone (□) or with 300 µM H_2O_2 in the absence (■) or presence of PACAP (◪). Inset, cells were treated with 300 µM H_2O_2 for durations ranging from 10 to 60 min. The activity of SOD and catalase were quantified as described in figure 5. The results are expressed as a percentage of SOD (A) or catalase (B) activity with respect to control. Each value is the mean (± SEM) of at least four different dishes from three independent experiments. ANOVA followed by the Bonferroni's test. *$P <$ 0.05, **$P < 0.01$, ***$P < 0.001$, NS, not statistically different *vs*. control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, ns, not statistically different *vs*. H_2O_2-treated cells.

Fig. 7 Pharmacological characterization of the receptor involved in the stimulatory effect of PACAP on SOD and catalase activities in cultured rat astrocytes. Cells were pre-incubated for 20 min in the absence or presence of PACAP6-38 (10^{-6} M) and then incubated for 10 min or 1 h in the absence (£) or presence of 10^{-9} M PACAP (▥ ; A and B) or 300 µM H_2O_2 without (■; C and D) or with PACAP (◪). The activity of

SOD and catalase were quantified as described in figure 5. The results are expressed as a percentage of SOD or catalase activity with respect to control. Each value is the mean (± SEM) of at least four different dishes from three independent experiments. ANOVA followed by the Bonferroni's test. $^{**}P < 0.01$, $^{***}P < 0.001$, NS, not statistically different *vs*. control. $^{\#\#\#}P < 0.001$, ns, not statistically different *vs*. H_2O_2-treated cells. $^{\S\S\S}P < 0.001$ *vs*. PACAP + H_2O_2-cotreated cells.

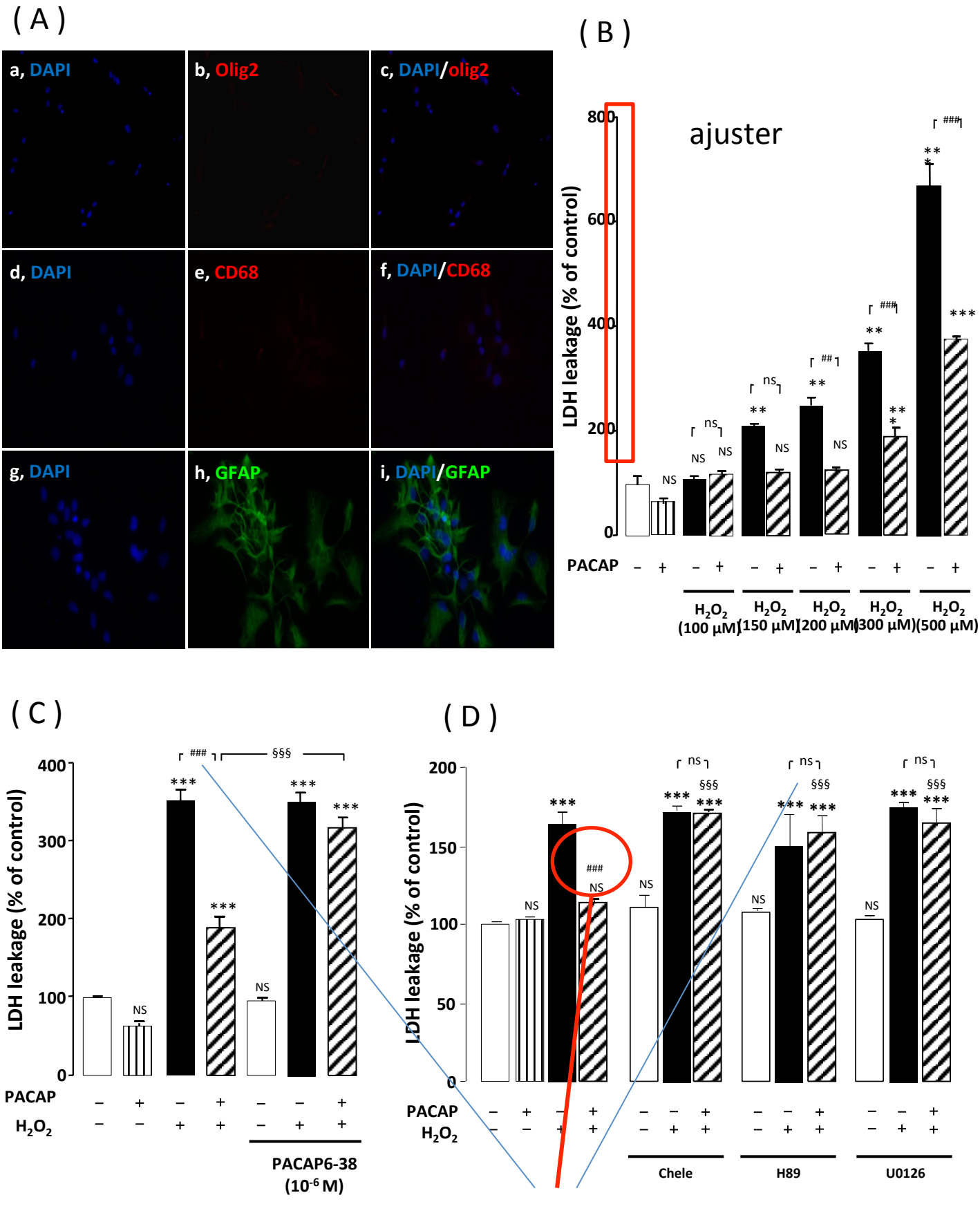
Fig. 8 Identification of intracellular transduction pathways involved in the effects of PACAP on the activities of SOD and catalase in cultured rat astrocytes. (A, B) Cells were pre-incubated for 30 min in the absence or presence of U73122 (10^{-7} M), chelerythrine (10^{-7} M; Chel), H89 (2 x 10^{-5} M), or U0126 (1 µM) and then incubated for 10 min with medium alone (£) or in the presence of PACAP 10^{-9} M (▥). (C, D) Cultured rat astrocytes were pre-treated for 30 min in the absence or presence of the same blockers and then incubated for 1 h with medium alone or with H_2O_2 300 µM, in the absence (■) or presence of PACAP 10^{-9} M (◪). The activity of SOD and catalase were quantified as described in figure 5. The results are expressed as a percentage of SOD or catalase activity with respect to control. Each value is the mean (± SEM) of at least four different dishes from three independent experiments. ANOVA followed by the Bonferroni's test. $^{***}P < 0.001$, NS, not statistically different *vs*. control. $^{\#\#\#}P < 0.001$, ns, not statistically different *vs*. H_2O_2-treated cells. $^{\S\S}P < 0.01$, $^{\S\S\S}P < 0.001$ *vs*. PACAP + H_2O_2-cotreated cells.

Fig. 9 Effects of SOD and catalase inhibitors on the protective action of PACAP against H_2O_2-induced oxidative damage and astrocyte cell death**.** Cultured rat

astrocytes were pre-incubated for 10 min or 3 h in the absence or presence of the SOD inhibitor NaCN (2 x 10^{-3} M) or the catalase inhibitor 3-aminotriazole (AZT, 10^{-2} M), incubated for 10 min with medium alone or in the presence of PACAP 10^{-9} M and then incubated for 1 h with medium alone (£) or with H_2O_2 300 µM, in the absence (■) or presence of PACAP 10^{-9} M (◪). The results are expressed as a percentage of SOD (A) and catalase (B) activities, cellular superoxide radical levels (C) or LDH activity (D) with respect to control. The activity of SOD and catalase were quantified as described in figure 5. Respiratory burst was measured by quantification of cellular superoxide radical levels (C) and cell death was determined by measuring LDH activity in culture media (D). Inset, effect of ATZ on the protective action of PACAP on H_2O_2-induced alteration of mitochondrial membrane potential. Mitochondrial transmembrane potential was determined by using the JC-10 probe, and the ratio of fluorescence emissions 610 nm *vs*. 534 nm was measured as an index of the mitochondrial activity. The results are expressed as percentage of control. Each value is the mean (± SEM) of at least four different wells from three independent cultures. ANOVA followed by the Bonferroni's test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, NS, not statistically different *vs*. control. $^{\#}P < 0.05$, $^{\#\#} P < 0.01$, $^{\#\#\#}P < 0.001$ *vs*. H_2O_2-treated cells. $^{\S} P < 0.05$, $^{\S\S}P < 0.01$, $^{\S\S\S} P < 0.001$ *vs*. NaCN- or ATZ-treated cells.

Fig. 10 Schematic representation of the signaling mechanisms likely involved in the protective effect of PACAP against H_2O_2-induced astroglial cell apoptosis. PACAP, acting through PAC1-R, activates both adenylyl cyclase (AC) and phospholipase C (PLC) in astrocytes, and stimulates phosphorylation of extracellular regulated kinase (ERK) in a PKA and PKC-dependent manner. Downstream, PACAP stimulates

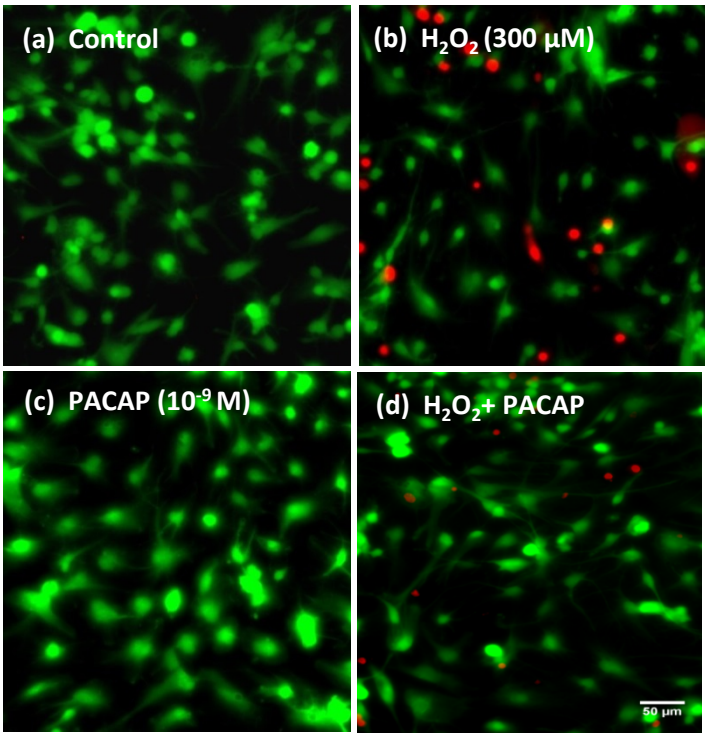
activity of enzymatic antioxidant systems (SOD and catalase) and abolishes H_2O_2-induced decrease of mitochondrial potential (ψm) via the formation of highly reactive oxygen species (ROS) and superoxide anion ($O_2^{\circ -}$) generation. This cascade triggered by PACAP blocks the activation of caspase-3 expression and thus cell death provoked by H_2O_2. ATZ, catalase inhibitor; Casp-3, caspase-3; Chel, chelerythrine and protein kinase C inhibitor; Cyt C, cytochrome C; H89, protein kinase A inhibitor; NaCN, SOD inhibitor; Per, peroxysome; U0126, MAP kinase kinase inhibitor. ↓ activation; ⊥, inhibition.



Mettre comme les autres

Figure 1
Douiri S. et al.

(A)



(B)

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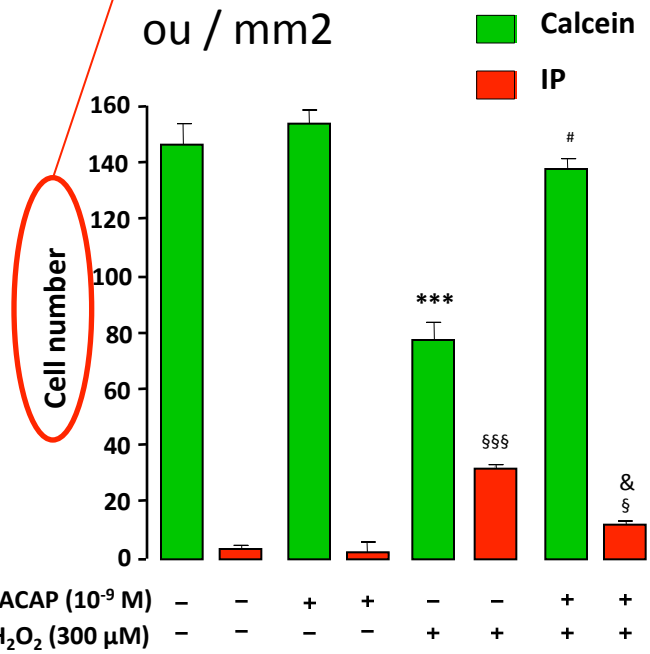
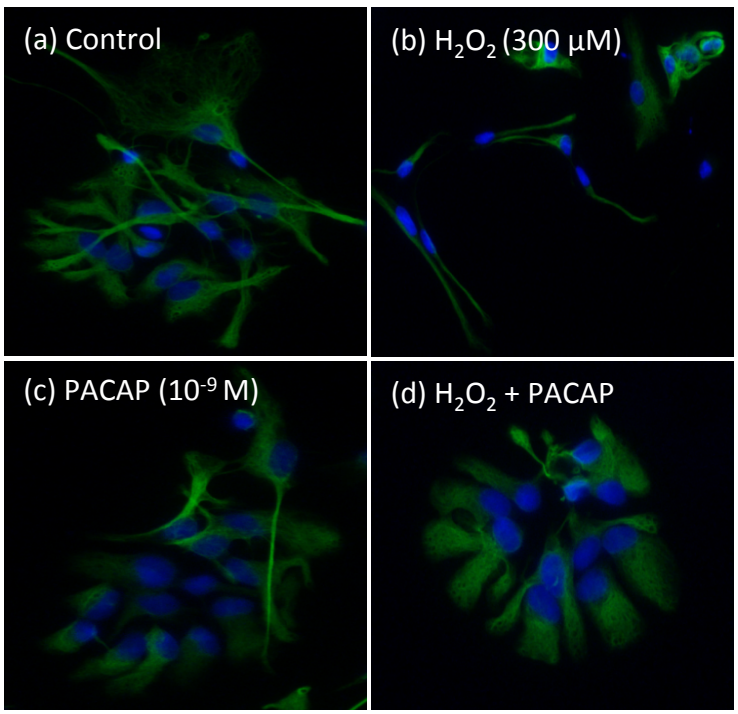


(C)



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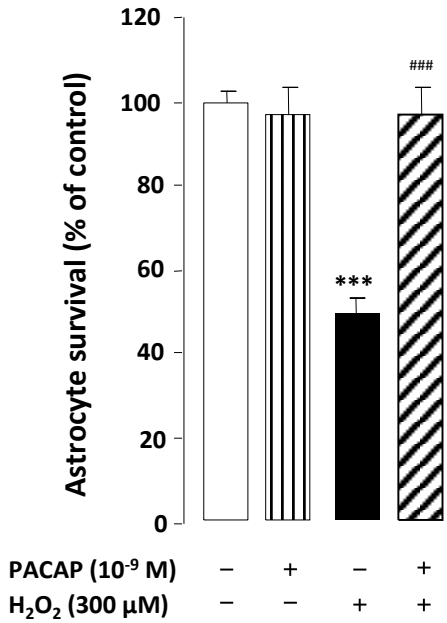
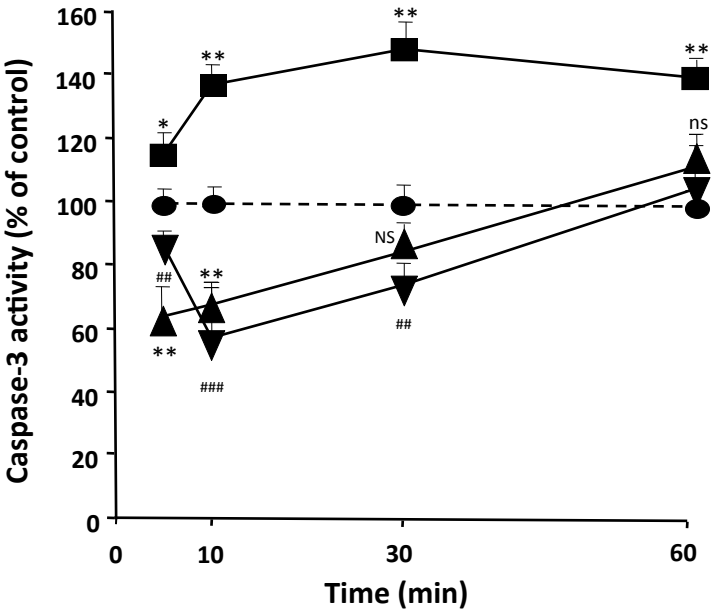


Figure 2
Douiri S. et al.

(A)



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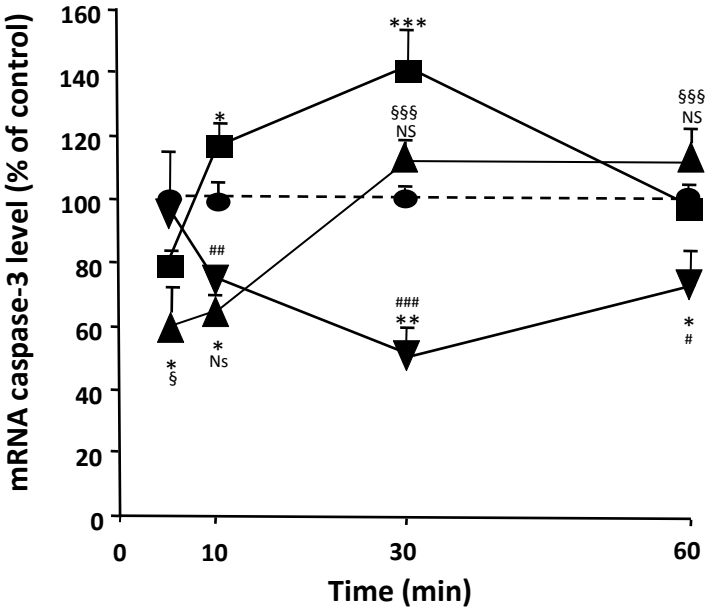

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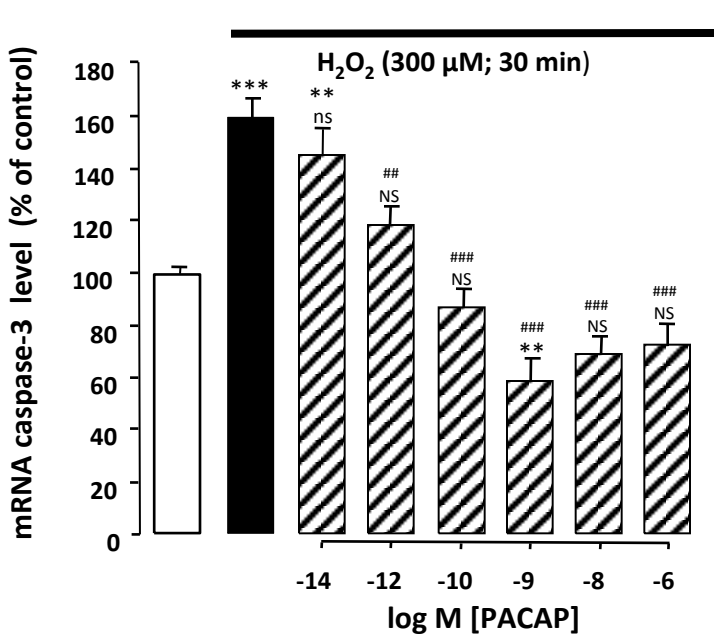

A 60 min, le PACAP+H2O2 doit être significatif par rapport au PACAP

Figure 3
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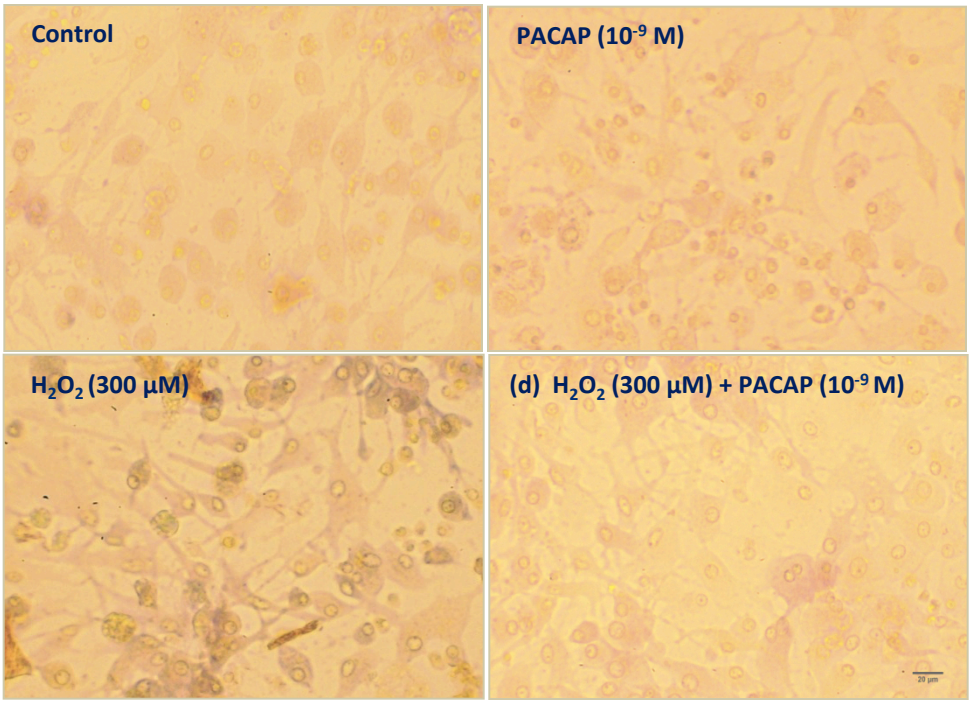
(A)



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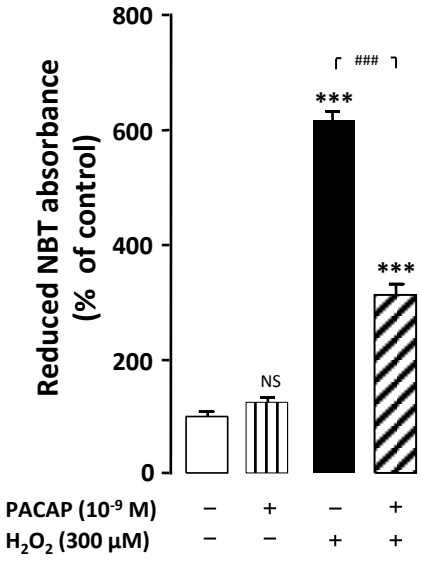

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Figure 3
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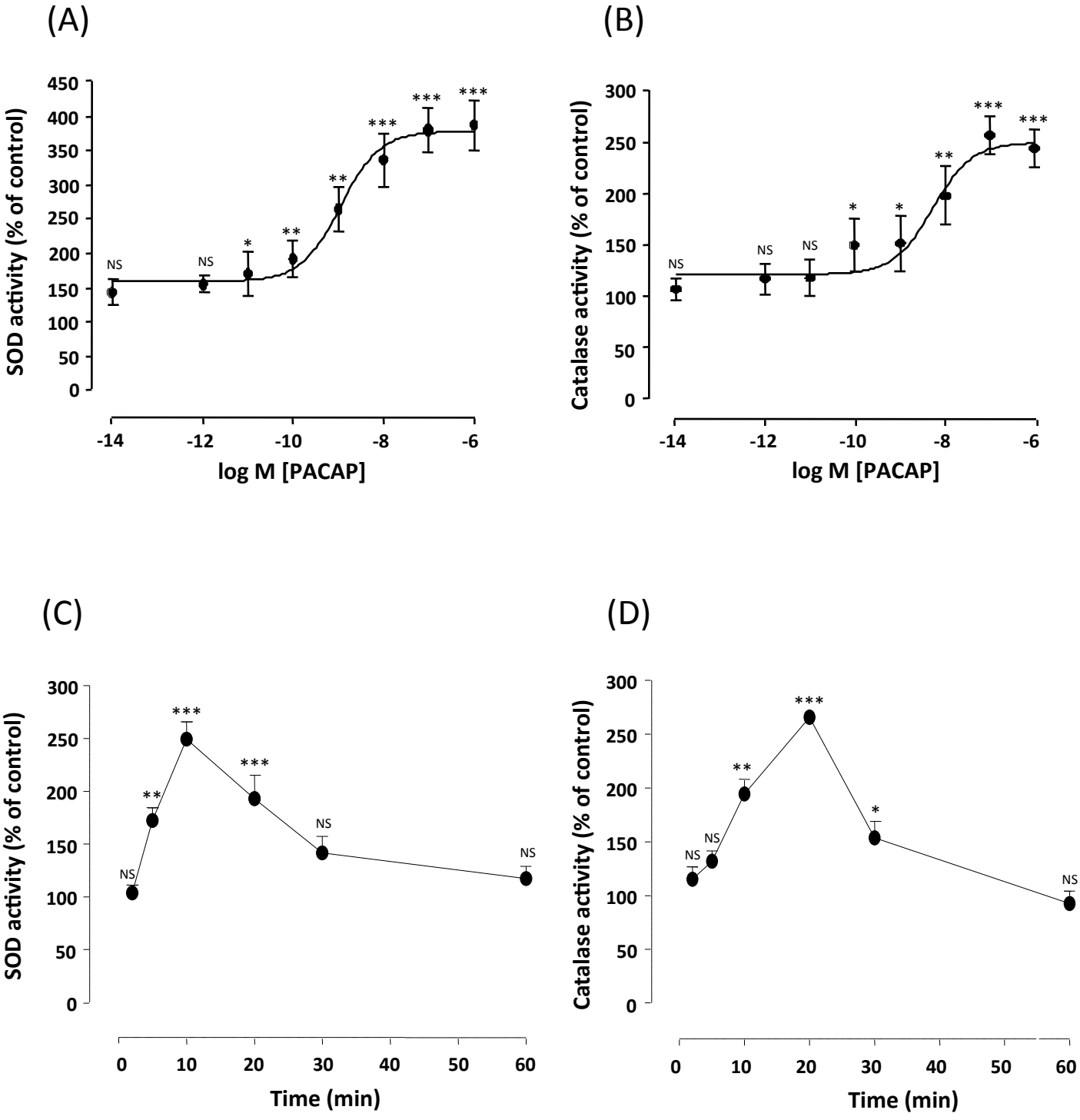
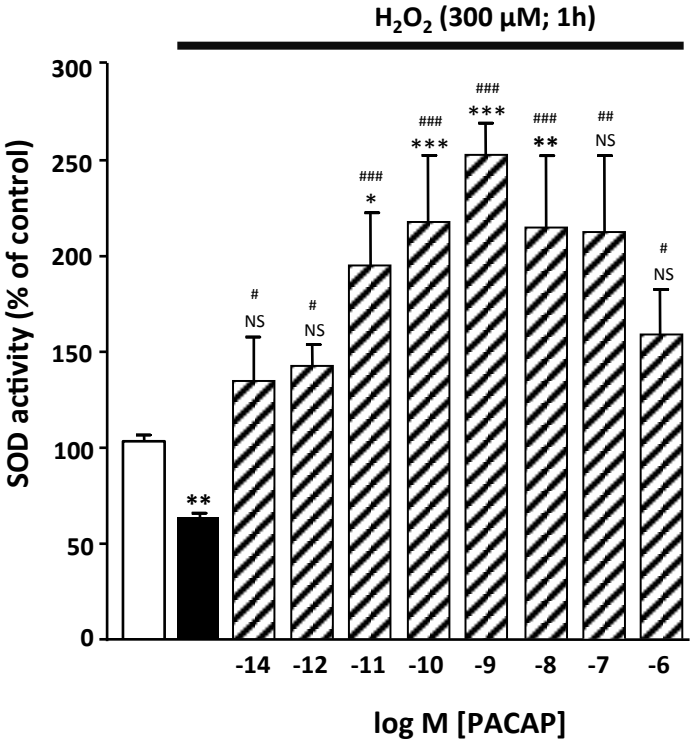
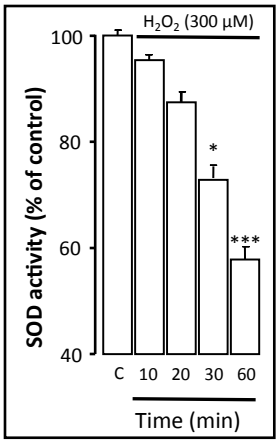


Figure 4
Douiri S. et al.

(A)





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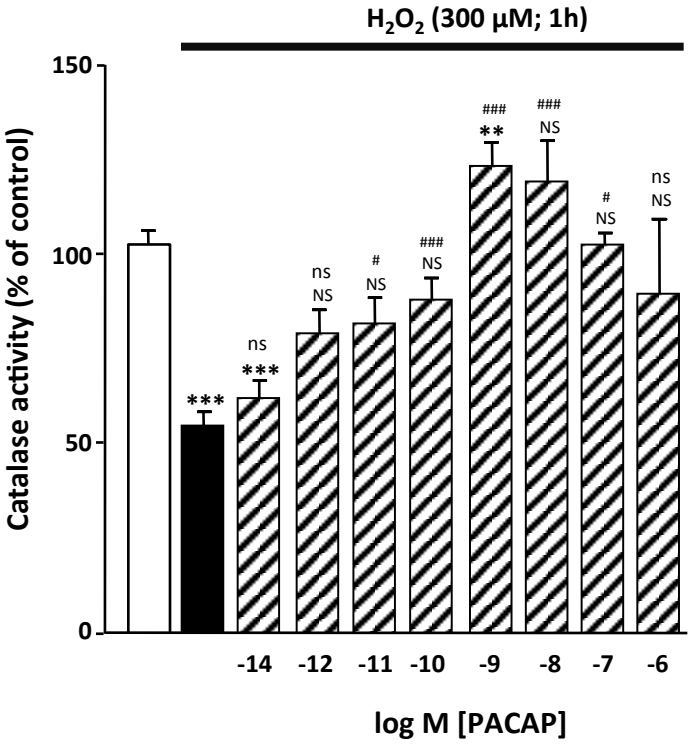

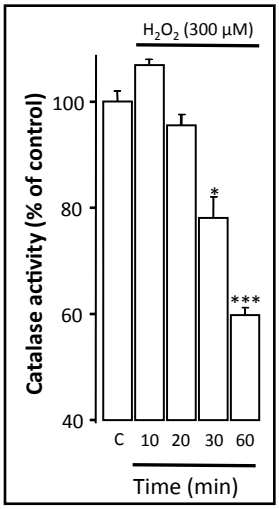

Figure 5
Douiri S. et al.

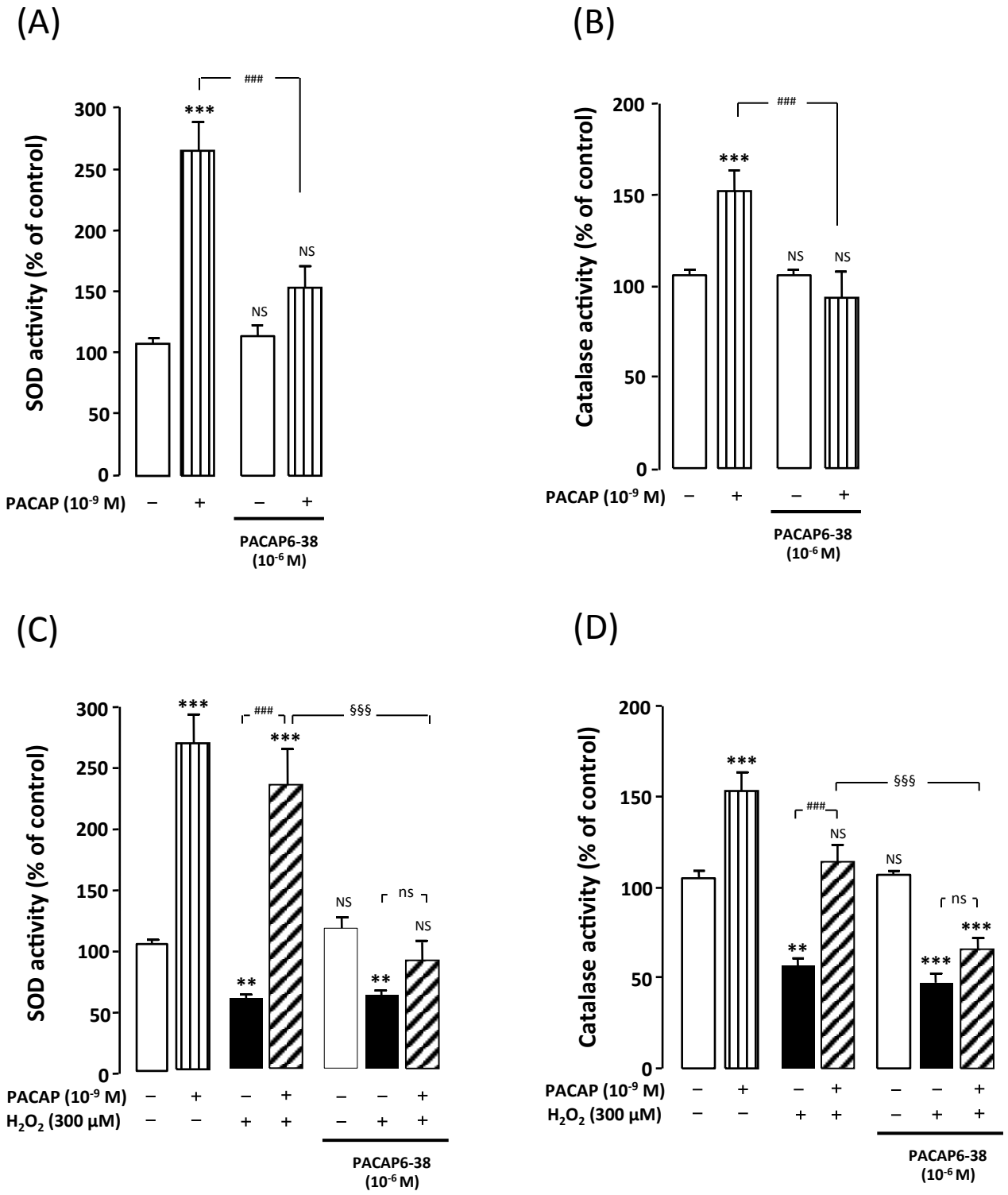

Figure 6
Douiri S. et al.

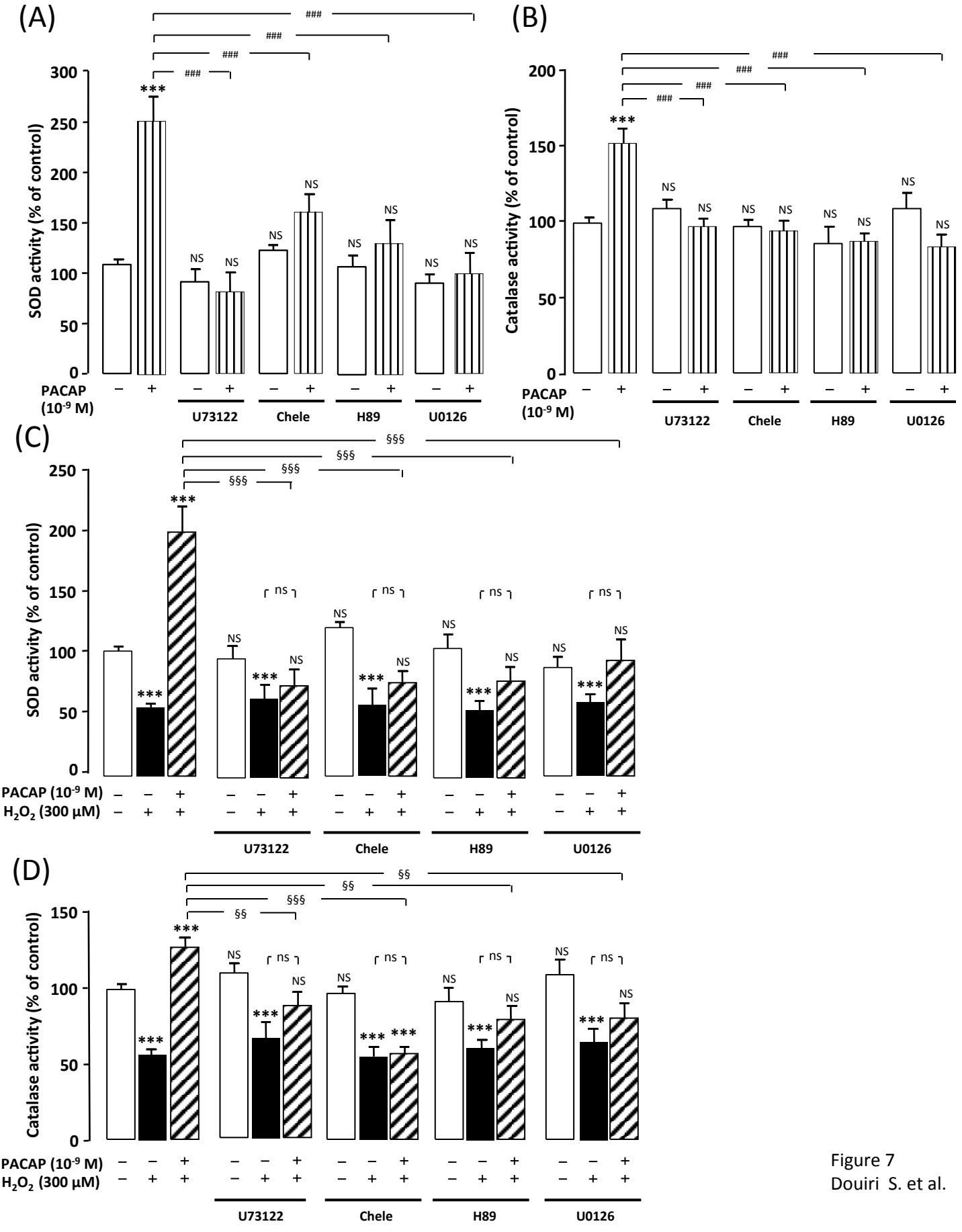

Figure 7
Douiri S. et al.

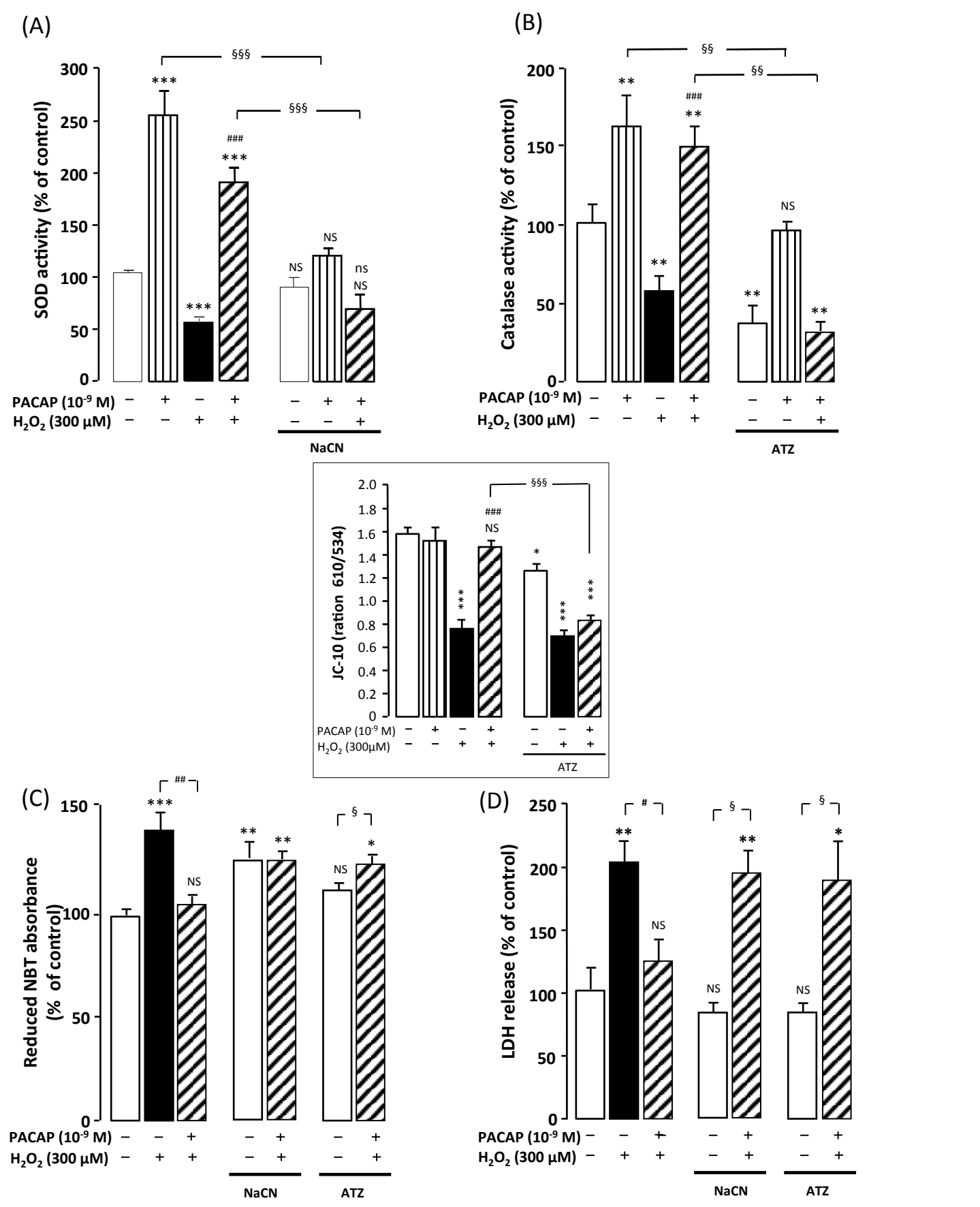

Figure 8
Douiri S. et al.

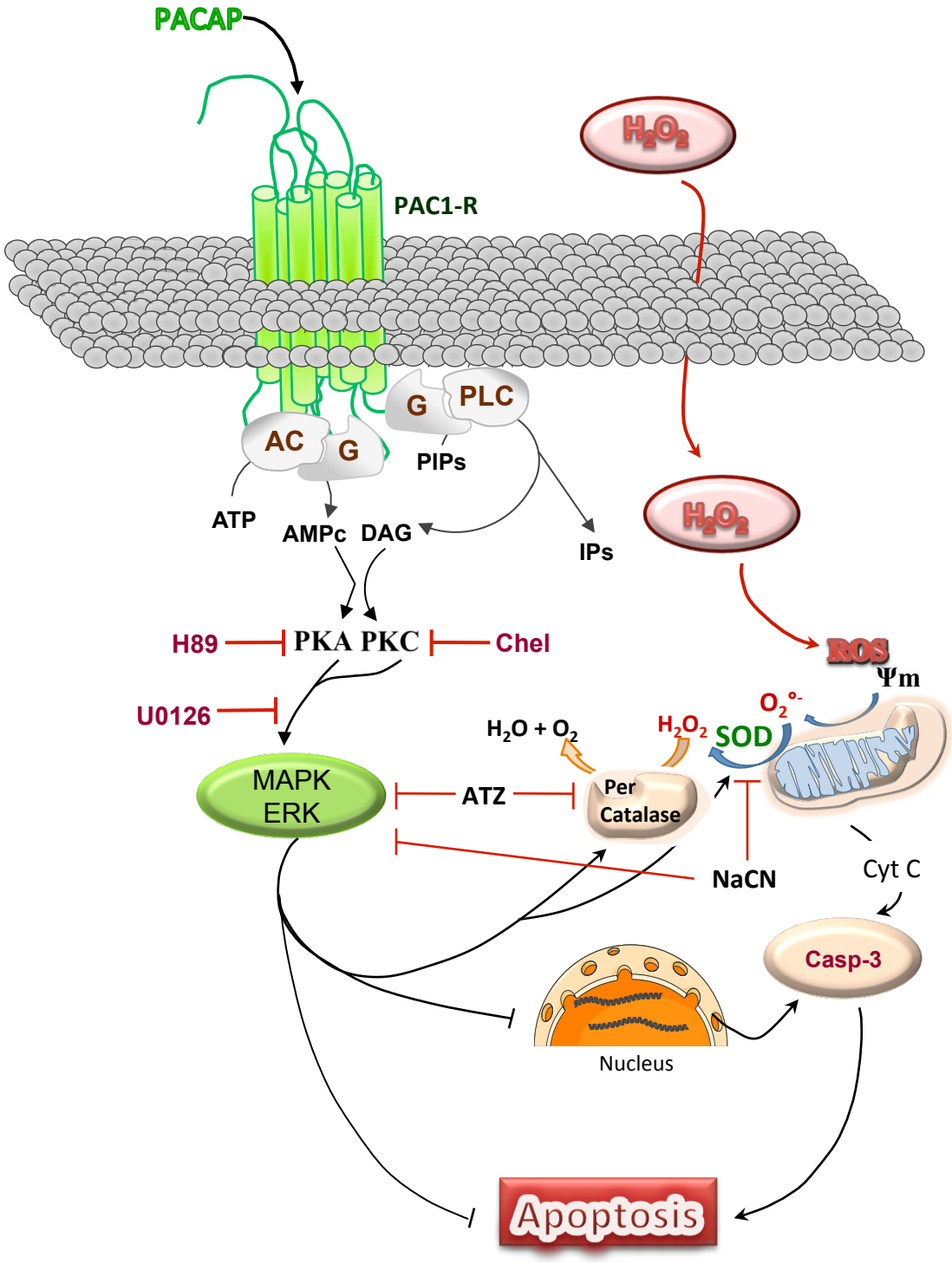

Figure 10
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