

# Aquaporin 8 is involved in H<sub>2</sub>O<sub>2</sub>-mediated differential regulation of metabolic signaling by $\alpha_1$ - and $\beta$ -adrenoceptors in hepatocytes

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Reactive oxygen species participate in regulating intracellular signaling pathways. Herein, we investigated the reported opposite effects of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) on metabolic signaling mediated by activated  $\alpha_1$ - and  $\beta$ -adrenoceptors (ARs) in hepatocytes. In isolated rat hepatocytes, stimulation of  $\alpha_1$ -AR increases H<sub>2</sub>O<sub>2</sub> production *via* NADPH oxidase 2 (NOX2) activation. We find that the H<sub>2</sub>O<sub>2</sub> thus produced is essential for  $\alpha_1$ -AR-mediated activation of the classical hepatic glycogenolytic, gluconeogenic, and ureagenic responses. However, H<sub>2</sub>O<sub>2</sub> inhibits  $\beta$ -AR-mediated activation of these metabolic responses. We show that H<sub>2</sub>O<sub>2</sub> mediates its effects on  $\alpha_1$ -AR and  $\beta$ -AR by permeating cells through aquaporin 8 (AQP8) channels and promoting Ca<sup>2+</sup> mobilization. Thus, our findings reveal a novel NOX2-H<sub>2</sub>O<sub>2</sub>-AQP8-Ca<sup>2+</sup> signaling cascade acting downstream of  $\alpha_1$ -AR in hepatocytes, which, by negatively regulating  $\beta$ -AR signaling, establishes negative crosstalk between the two pathways.

**Keywords:**  $\beta$ -adrenergic receptors; adrenaline; Ca<sup>2+</sup> mobilization; epinephrine; gluconeogenesis; glycogenolysis; H<sub>2</sub>O<sub>2</sub>; hepatocytes; signaling; ureagenesis;  $\alpha_1$ -adrenergic receptors

Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) is a ubiquitous nonradical oxidant with a main role in redox signaling that regulates metabolism in aerobic organisms [1]. The redox code involves NAD<sup>+</sup>/NADP<sup>+</sup> and thiol/disulfide, as well as O<sub>2</sub>/H<sub>2</sub>O<sub>2</sub> ratios to maintain redox homeostasis [2]. NADPH oxidases (NOX) are a family of seven enzyme isoforms that generate reactive oxygen species (ROS); the main ROS are superoxide (O<sub>2</sub><sup>-</sup>) and H<sub>2</sub>O<sub>2</sub> [3]. In the past, our group reported that adrenaline stimulates H<sub>2</sub>O<sub>2</sub> generation *via* NOX [4]. This stimulation proceeded through  $\alpha_1$ -adrenergic receptor (AR) activation, exhibited an adrenaline dose and Ca<sup>2+</sup>

dependency, and required Mn<sup>2+</sup> or GTP $\gamma$ S addition [4]. Later on, our group identified NADPH oxidase 2 (NOX2) as the complex responsible for H<sub>2</sub>O<sub>2</sub> synthesis after  $\alpha_1$ -AR stimulation, both systems being functionally linked through Rac1 and G $\alpha_{13}$  proteins, with absolute GTP (or GTP $\gamma$ S) participation [5]. A negative crosstalk between  $\alpha_1$ -ARs and  $\beta$ -ARs for H<sub>2</sub>O<sub>2</sub> synthesis was observed [5]. These facts were in accordance with previous reports showing that isoproterenol, a  $\beta$ -AR agonist, has an inhibitory action on NOX-dependent H<sub>2</sub>O<sub>2</sub> generation in human adipocyte plasma membrane [6]. On the other hand, it is well

## Abbreviations

AR, adrenergic receptor; AQP3, aquaporin 3; AQP8, aquaporin 8; AQPs, aquaporins; ER, endoplasmic reticulum; GPCRs, G protein-coupled receptors; H<sub>2</sub>O<sub>2</sub>, Hydrogen peroxide; HPM, hepatocyte plasma membranes; NOX, NADPH oxidases; NOX2, NADPH oxidase 2; PKA, protein kinase A; ROS, reactive oxygen species;  $\alpha_1$ -adr-ckt,  $\alpha_1$ -adrenaline cocktail;  $\beta$ -adr-ckt,  $\beta$ -adrenaline cocktail.

known that H<sub>2</sub>O<sub>2</sub> is involved in insulin signaling in fat tissue and adipocytes, as well as in the heart [7–10]. In this regard, catecholamines acting through  $\beta$ -ARs produce lipolysis and contraction in those tissues, respectively; both pathways are impaired by insulin-induced H<sub>2</sub>O<sub>2</sub> generation by NOX activation [8,9]. In addition,  $\alpha_1$ -ARs and  $\beta$ -ARs seem to be involved in the regulation of metabolic/contraction events through H<sub>2</sub>O<sub>2</sub> signaling [5,11,12]. The present study was undertaken to advance understanding on the physiological role of NOX2-generated-H<sub>2</sub>O<sub>2</sub> in liver cells, in particular the constituents of their signaling pathways and their metabolic responses activated by either  $\alpha_1$ -AR or  $\beta$ -AR. Based on our results, we propose an expanded signaling pathway for adrenaline in hepatocytes that includes  $\alpha_1$ -AR-NOX2-H<sub>2</sub>O<sub>2</sub>-aquaporin 8 (AQP8)-Ca<sup>2+</sup> mobilization, in which NOX2-derived H<sub>2</sub>O<sub>2</sub> is a second messenger that coordinates spatiotemporal actions of the canonical cascades for both ARs. NOX2-derived H<sub>2</sub>O<sub>2</sub> is the second messenger that must be transported through cell membranes by AQP8, which is necessary for  $\alpha_1$ -AR-mediated Ca<sup>2+</sup> mobilization to activate glycogenolysis, gluconeogenesis, and ureagenesis, whereas for the  $\beta$ -AR, H<sub>2</sub>O<sub>2</sub> inhibits the same three metabolic pathways.

## Materials and methods

### Chemicals

Rabbit polyclonal antibody raised against amino acids 1–80 at the N terminus of aquaporin 3 (AQP3) of human origin (sc-20811; Santa Cruz Biotechnology, Dallas, TX, USA), rabbit polyclonal antibody raised against amino acids 1–85 at the N terminus of AQP8 of human origin (sc-28624; Santa Cruz), and peroxidase–goat anti-rabbit IgG (Cat. No. 656120; Zymed Laboratories, Santiago, Chile) were used. Soluble extracts of rat kidney and AQP8 transfected 293T cells (sc-126431; Santa Cruz) were used as positive control of AQP3 and AQP8, respectively. BAPTA-AM (1,2-Bis(2-aminophenoxy)ethane-N,N',N'-tetraacetic acid, tetrakis (acetoxymethyl ester), Cat. No. 14510), and collagenase type IV from *Clostridium histolyticum* (Cat. No. C5138) were from Sigma-Aldrich (St. Louis, MO, USA); peptides gp91ds-tat and scrambled-tat [13] were synthesized by New England Peptide (Gardner, MA, USA); AEBBSF (hydrochloride (4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride) was from MP Biomedicals, Inc. (Solon, OH, USA); A61603, N-[5-(4,5-dihydro-1H-imidazol-2-yl)-2-hydroxy-5,6,7,8-tetrahydronaphthalen-1-yl]methanesulfonamide hydrobromide, a selective  $\alpha_{1A}$ -AR agonist, was from Tocris (Cat. No. 1052, Ellisville, MO, USA); all other reagents were analytical grade from local sources.

### Cell isolation

All animal experiments were conducted according to the Federal Guidelines for Animal Care and Use (NOM 062-ZOO-1999, Secretariat of Agriculture, Livestock, Rural Development, Fisheries and Food, Mexico). The study protocol was approved by the Ethics Committee of Facultad de Medicina, Universidad Nacional Autónoma de México (UNAM).

Hepatocytes were isolated by collagenase digestion from male Wistar rats by the method of Berry and Friend [14] using slight modifications as described by Guinzberg *et al.*, [15]. Due to lot-to-lot variability between different batches of collagenase, in some experiments 16  $\mu$ M protease inhibitor AEBBSF was added to improve isolated cell viability. The trypan blue exclusion test was used to measure cell viability; experiments were conducted when > 90% of cells excluded the dye.

### Hepatic cell plasma membrane preparation

Freshly prepared hepatocytes were enriched up to 99% by centrifugal elutriation in order to avoid the presence of Kupffer cells that possess a high NOX activity [5]. This enriched hepatocyte preparation was added with 1 mL protease inhibitor cocktail (cat. no. 158837; MP Biomedicals Inc.) and homogenized in a Teflon-glass Potter-Elvehjem during 2 min at 4 °C and then mixed with 100 mL of lysis buffer [20 mM MES (2-(N-morpholino) ethanesulfonic acid), pH 5.8; 2 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, and 5 mM KCl] during 2 min at 4 °C. Plasma membranes were obtained by centrifugation to obtain highly enriched hepatocyte plasma membranes (HPM) [4,5].

### NOX2 activation in HPM

For NOX2 activation: 20  $\mu$ g protein of HPM was suspended in 1 mL of activation buffer at 4 °C [in mM, NaCl 120, NaHCO<sub>3</sub> 10, CaCl<sub>2</sub> 1.2, MOPS 30 ([4-morpholinepropanesulfonic acid])], pH 7.4, then immediately incubated at 37 °C for 2 min, and as indicated were added: (a) gp91ds-tat or scrambled-tat peptides: gp91ds-tat peptide is a competitive inhibitor specific for NADPH oxidase assembly and scrambled-tat peptide is used as control [13], (b) an  $\alpha_1$ -adrenaline cocktail ( $\alpha_1$ -adr-ckt) composed of 1  $\mu$ M adrenaline and antagonists (propranolol, a  $\beta$ -AR; yohimbine, an  $\alpha_{2A}$ -AR, and rauwolscine, an  $\alpha_{2B}$ -AR, at 0.1  $\mu$ M), to stimulate only  $\alpha_1$ -AR; (c), a  $\beta$ -adrenaline cocktail ( $\beta$ -adr-ckt) composed of 1  $\mu$ M adrenaline and antagonists (prazosin, an  $\alpha_1$ -AR; yohimbine and rauwolscine, at 0.1  $\mu$ M), to stimulate only  $\beta$ -AR; (d) GTP $\gamma$ S 10  $\mu$ M, a nonhydrolysable analog of GTP. Reaction ended by 5-min centrifugation at 10 600 g, and the pellet was suspended in catalysis buffer as enzyme source for the NOX-isoform catalytic step.

### NADPH-dependent H<sub>2</sub>O<sub>2</sub> generation

Immediately after activation, HPM were suspended in 540  $\mu$ L of catalysis buffer (120 mM NaCl, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM NaN<sub>3</sub>, 20 mM MES, and 100  $\mu$ M flavin adenine dinucleotide, pH 6.0) at 37 °C. Enzymatic reactions were started by adding 60  $\mu$ L of 0.25 mM NADPH, incubated 4 min at 37 °C, and stopped with 1.5 M trichloroacetic acid. Quantitation of H<sub>2</sub>O<sub>2</sub> generated was determined by the method of Thurman *et al.* [16]. In brief, samples were centrifuged at 10 600 *g* for 10 min, and 200  $\mu$ L of 10 mM ferrous ammonium sulfate and 100  $\mu$ L of 2.5 M potassium thiocyanate were added to the supernatant, incubated 30 min at room temperature and subsequently read in a spectrophotometer at 480 nm and compared to a standard curve generated from dilution of concentrated H<sub>2</sub>O<sub>2</sub>.

### Metabolic activity assays

Gluconeogenesis was determined in 24-h starved rats derived hepatocytes ( $5 \times 10^5$  cells), incubated in 1 mL glucose-free Ringer–Krebs with 10 mM lactate, and each adrenaline cocktail described, at 37 °C in O<sub>2</sub>/CO<sub>2</sub> (19 : 1) atmosphere for 60 min with shaking. Tubes were placed in ice-water, and glucose was determined as previously described [17]. To determine glycogenolysis, hepatocytes from fed rats were incubated as described, without lactate during 45 min. Glucose was measured as indicated [17]. To determine ureagenesis, hepatocytes from fed rats were incubated 1 h in 1 mL Ringer–Krebs with glucose (10 mM), (NH<sub>4</sub>)<sub>2</sub>CO<sub>3</sub> (5 mM), ornithine (3 mM), and each of the adrenaline cocktail described. At the end of incubation, urea was determined as described [18]. As indicated in the figures, hepatocytes were incubated with AgNO<sub>3</sub>, H<sub>2</sub>O<sub>2</sub>, peptides, or antibodies against aquaporins (AQPs) also.

### Reverse transcriptase polymerase chain reaction (RT-PCR)

Aquaporins expression genes (AQP3 and AQP8) were analyzed in rat hepatocytes. These cells were obtained as mentioned earlier, and total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was prepared from total RNA with Omniscript Reverse Transcription (RT) Kit (Invitrogen). PCR was performed with HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany) and specific 5'- and 3'-primers for AQP3 and AQP8 [19], as well as acidic ribosomal protein [20] under the following conditions: 95 °C 15 min, 35 cycles of 95 °C 30 s, 63 °C 30 s, and 72 °C 45 s, and 72 °C 5 min. The amplified products were analyzed on SYBER green I (Roche Applied Science, Mannheim, Germany)-stained agarose gels. Kidney and testis were used as positive controls of AQP3 and AQP8,

respectively. Reactions substituting cDNA with water were used as negative controls.

### Western blot

Hepatocyte plasma membranes were disrupted in lysis buffer (1% NP-40 and 1 mM EDTA in phosphate-buffered saline) containing a protease inhibitor mixture (MP Biomedicals), with shaking for 30 min at 4 °C. Solubilized proteins were centrifuged at 14 000 *g* at 4 °C for 10 min, and supernatant was used as the soluble extract. Proteins (60  $\mu$ g) resolved by SDS/PAGE were transferred onto a poly(vinylidene difluoride) membrane (Millipore, Bedford, MA, USA), at 100 V for 1 h. Membranes were blocked overnight at 4 °C with Tris-buffered saline containing 5% nonfat dry milk and 0.1% Tween 20. Blots were incubated with antibodies against AQP3 and AQP8 then incubated with a horseradish peroxidase-conjugated secondary antibody, and protein bands were visualized by chemiluminescence (ECL Plus; Amersham, UK). Soluble extracts of rat kidney and AQP8-transfected 293T cells were used as positive control of AQP3 and AQP8, respectively [21,22].

### Determination of free cytosolic Ca<sup>2+</sup> in isolated hepatocytes

Hepatocytes were loaded with Fura2-AM as described [23,24]. Briefly, isolated hepatocytes (40 mg wet wt per mL) from fed rats were incubated in Ringer–Krebs bicarbonate supplemented with CaCl<sub>2</sub> 1.2 mM, for 20 min at 37 °C in O<sub>2</sub>/CO<sub>2</sub> (19 : 1) atmosphere, in the presence of 5  $\mu$ M Fura 2-AM, and were rinsed twice by centrifugation at 2700 *g* for 3 min. Liver cells were divided into 200- $\mu$ L aliquots, immersed in ice, and used within the subsequent 5 min. [Ca<sup>2+</sup>]<sub>cyt</sub> was measured as described [25].

### Statistical analysis

The results are shown as the mean  $\pm$  SEM obtained from at least three or four different experiments, each performed in duplicate. Each independent experiment corresponds to assays performed in different days with a different rat. Duplicates correspond to two cell aliquots tested in parallel the same day. For statistical purposes, the mean of duplicated results was considered as a single result. For immunoblots, one representative gel of at least three independent experiments is shown. All statistical analyses were performed using SIGMAPLOT ver. 12 (Systat Software, Inc., San Jose, CA, USA, <http://www.sigmaplot.com/>). Statistical differences were determined employing one-way analysis of variance (ANOVA) followed by a Holm–Sidak multiple comparisons test. The minimum level of significance was set at  $P < 0.05$ .

## Results

### H<sub>2</sub>O<sub>2</sub> production by $\alpha_1$ -AR stimulation is due to NOX2 activation

Our previously reported negative crosstalk between  $\alpha_1$ / $\beta$ -ARs for H<sub>2</sub>O<sub>2</sub> synthesis using hepatic plasma membranes [5], convinced us to seek for an adequate experimental model for the present study. As reported earlier by our group, H<sub>2</sub>O<sub>2</sub> synthesis by AR activation with adrenaline was slightly less than that of the  $\alpha_1$ -AR activation with  $\alpha_1$ -adr-ckt, which was maximal, and it was minimal by activating  $\beta$ -AR [4]. The reported negative crosstalk was attained with dual,  $\alpha_1$  and  $\beta$  activation ([4]; data not shown). Thus, in the rest of this paper, only  $\alpha_1$ - or  $\beta$ -AR activation was done. Additionally, our previous conclusion that NOX2 is stimulated by  $\alpha_1$ -AR activation was obtained by inhibiting H<sub>2</sub>O<sub>2</sub> synthesis with specific antibodies against NOX1, NOX2, and NOX4, or using diphenyleneiodonium (a nonspecific flavoenzymes inhibitor). Therefore, to confirm the validity of our previous conclusions, we tested now gp91ds-tat, a specific cell-penetrating peptide inhibitor of NOX2 assembly [13] on H<sub>2</sub>O<sub>2</sub> production. Adrenaline-stimulated  $\alpha_1$ -AR increased H<sub>2</sub>O<sub>2</sub> production in HPM from highly enriched hepatocytes, yielding after 4-min incubation, a maximum of  $104 \pm 1 \text{ nmol} \times \text{min}^{-1} \times \text{mg protein}^{-1}$  (Fig. 1A). With this peptide inhibitor, H<sub>2</sub>O<sub>2</sub> production in HPM diminished almost to basal values, and as expected, no inhibition occurred with the scrambled-tat control peptide (Fig. 1A). Latter results agree with previously published data by our group [4,5].

On the other hand, it is relevant to observe that, under the experimental conditions included for the prepared HPM, and without promoting their antioxidant systems, the measured *in vitro* H<sub>2</sub>O<sub>2</sub> production activated by  $\alpha_1$ -AR can reach H<sub>2</sub>O<sub>2</sub> concentration in the order of  $\sim 7 \mu\text{M}$ .

### Glycogenolysis and gluconeogenesis stimulated by $\alpha_1$ -AR need NOX2 activation

In order to analyze an eventual participation of H<sub>2</sub>O<sub>2</sub> on the metabolic routes activated by  $\alpha_1$ -AR stimulation, the experiments included in Fig. 1B,C were done. In absence of added H<sub>2</sub>O<sub>2</sub>, inclusion of NOX2-inhibitor ( $1.25 \mu\text{M}$  gp91ds-tat) completely impaired the  $\alpha_1$ -AR glycogenolytic stimulation in freshly isolated hepatocytes from fed rats (Fig. 1B, left panel). Likewise,  $\alpha_1$ -AR gluconeogenic stimulation in hepatocytes from fasted rats was impaired also (Fig. 1C, left panel). These last observed inhibitions recorded by the

peptide preventing NOX2 assembly were overcome by adding  $0.74 \mu\text{M}$  H<sub>2</sub>O<sub>2</sub> (Fig. 1B,C, right panels). These results indicate that H<sub>2</sub>O<sub>2</sub> is required to integrate a whole  $\alpha_1$ -AR-stimulated response dealing with glucose/glycogen regulation in fasted and in fed rats, respectively.

### Aquaporins (AQPs) are involved in NOX2-mediated actions stimulated by $\alpha_1$ -AR

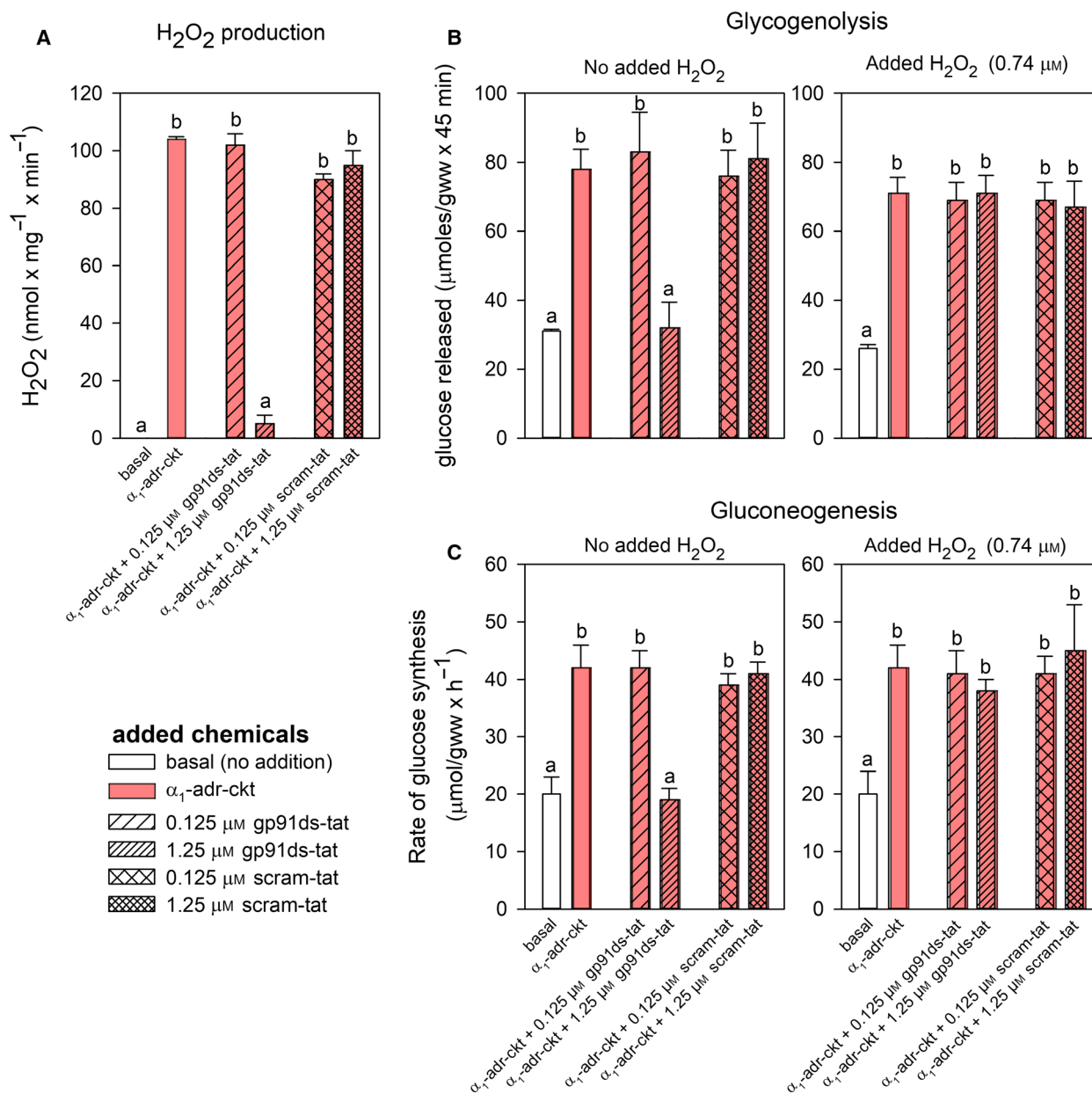
In isolated hepatocytes, activation of either  $\alpha_1$ -AR or  $\beta$ -AR reproduced the well-known stimulation of three metabolic pathways (gluconeogenesis, glycogenolysis, and ureagenesis; Table 1). Addition of exogenous H<sub>2</sub>O<sub>2</sub> ( $20 \mu\text{M}$ ) had no effect on  $\alpha_1$ -AR stimulation, but blocked  $\beta$ -AR stimulation (Table 1). In contrast, these classically activated metabolic pathways after adrenaline- $\alpha_1$ -AR stimulation were blocked by the nonspecific aquaporin inhibitor AgNO<sub>3</sub> (Table 1), suggesting that NOX2-derived H<sub>2</sub>O<sub>2</sub> must go through cell membrane AQPs before it acts. On the contrary, those metabolic pathways activated by adrenaline- $\beta$ -AR were not modified by AgNO<sub>3</sub> indicating that the signaling does not need H<sub>2</sub>O<sub>2</sub>, nor its inhibitory effect on  $\beta$ -AR stimulation can be observed if AQPs are not functional. As expected, if exogenous H<sub>2</sub>O<sub>2</sub> is added simultaneously with AgNO<sub>3</sub>, H<sub>2</sub>O<sub>2</sub> has no effect due to AQP blockade by AgNO<sub>3</sub> (Table 1).

### Specific AQP8 participation is needed to observe NOX2-mediated actions stimulated by $\alpha_1$ -AR in hepatocytes

Two identified AQPs that facilitate H<sub>2</sub>O<sub>2</sub> transport across the plasma membrane in mammalian cells are AQP3 [26] and AQP8 [27]. Thus, in order to gain information about the specific aquaporin(s) involved in the process, AQP3 and AQP8 antibodies were used. As observed, AQP8 antibody inhibited adrenaline- $\alpha_1$ -AR action on metabolic pathways in a concentration-related manner (Fig. 2A), but the AQP3 antibody had no effect (Fig. 2B), suggesting that AQP8 is present and is a new and necessary member of the adrenaline- $\alpha_1$ -AR transduction/signaling system, at least for hepatocytes. To corroborate this last finding, the absence of AQP3 mRNA and presence of AQP8 mRNA in rat isolated hepatocytes were confirmed by RT-PCR (Fig. 3A). Accordingly, AQP8 was identified by western blot in HPM, while AQP3 was not detected (Fig. 3B).

Interestingly and in contrast, adrenaline- $\beta$ -AR-stimulated metabolic pathways were inhibited by H<sub>2</sub>O<sub>2</sub>, and this effect was not modified by AQP3





**Fig. 1.** Gp91 ds-tat but not scram-tat inhibits: (A) H<sub>2</sub>O<sub>2</sub> generation, (B) glycogenolysis, and (C) gluconeogenesis triggered by activated  $\alpha_1$ -AR. (A) H<sub>2</sub>O<sub>2</sub> production in HPM freshly prepared from enriched hepatic cells [5] was measured in absence (basal) or in presence of GTP $\gamma$ S and  $\alpha_1$ -adr-ckt and two different concentrations of gp91 ds-tat or scram-tat (0.125 and 1.25  $\mu$ M). (B) Freshly isolated hepatocytes from fed rats were incubated in absence (left panel) or in presence (right panel) of 0.74  $\mu$ M H<sub>2</sub>O<sub>2</sub> as follows: in the absence of adrenaline cocktail (basal); with  $\alpha_1$ -adr-ckt, plus two concentrations of gp91 ds-tat or scram-tat (0.125 and 1.25  $\mu$ M). (C) Freshly isolated hepatocytes from starved rats were incubated in absence (left panel) or in presence (right panel) of 0.74  $\mu$ M H<sub>2</sub>O<sub>2</sub> as follows: in the absence of adrenaline cocktail (Basal); with  $\alpha_1$ -adr-ckt, plus two concentrations of gp91 ds-tat or scram-tat (0.125 and 1.25  $\mu$ M). Data are the mean  $\pm$  standard error of the mean (SEM) of three different experiments in duplicate. Significant differences ( $P < 0.05$ ) are denoted by different lowercase letters above each bar. Inside each metabolic pathway, equivalent data have the same letter; different letters indicate statistically significant differences.

antibody (Fig. 2B). Furthermore, AQP8 antibody blocked H<sub>2</sub>O<sub>2</sub>-induced inhibition of adrenaline- $\beta$ -AR stimulation of metabolic pathways (Fig. 2A),

indicating that H<sub>2</sub>O<sub>2</sub>, *via* AQP8, negatively regulates the metabolic pathways activated after adrenaline- $\beta$ -AR-stimulation.

**Table 1.** Effect of H<sub>2</sub>O<sub>2</sub> and AgNO<sub>3</sub> on gluconeogenesis, glycogenolysis, and ureagenesis stimulated by activation of  $\alpha_1$ - or  $\beta$ - ARs in rat hepatocytes. Hepatocytes from starved rats (for gluconeogenesis determination) or from fed rats (for glycogenolysis and urea synthesis determination) were incubated in absence (basal: 100% activity) or in presence  $\alpha_1$ - or  $\beta$ -adr-ckt. H<sub>2</sub>O<sub>2</sub> (20  $\mu$ M) and/or AgNO<sub>3</sub> (30  $\mu$ M) were added as indicated. The basal activities of nonstimulated hepatocytes were as follows: gluconeogenesis: 28.5  $\pm$  0.5  $\mu$ moles of glucose formed/ g wet weight  $\times$  h<sup>-1</sup>; glycogenolysis: 65.9  $\pm$  18.2  $\mu$ moles of glucose released per g wet weight  $\times$  45 min; ureagenesis: 18.3  $\pm$  0.9 nmoles of urea formed per g wet weight  $\times$  h<sup>-1</sup>. Each value represents the mean  $\pm$  SEM of three different experiments in duplicate. Significant differences ( $P < 0.05$ ) are denoted by different lowercase letters at the right of each figure. Inside each metabolic pathway, equivalent data have the same letter; different letters indicate statistically significant differences.

AR activated by	Stimulated metabolic pathway (% of basal)					
	Gluconeogenesis		Glycogenolysis		Ureagenesis	
	$\alpha_1$ -adr-ckt	$\beta$ -adr-ckt	$\alpha_1$ -adr-ckt	$\beta$ -adr-ckt	$\alpha_1$ -adr-ckt	$\beta$ -adr-ckt
Added chemicals						
Control (only adr-ckt)	147.5 $\pm$ 20.7a	150.0 $\pm$ 1.8a	451 $\pm$ 24c	412 $\pm$ 55c	200.0 $\pm$ 4.9e	200.0 $\pm$ 2.7e
H <sub>2</sub> O <sub>2</sub>	142.9 $\pm$ 3.9a	83.2 $\pm$ 5.0b	450 $\pm$ 39c	148 $\pm$ 46d	198.3 $\pm$ 6.6e	101.6 $\pm$ 4.9f
AgNO <sub>3</sub>	96.4 $\pm$ 1.8b	148.9 $\pm$ 3.2a	149 $\pm$ 47d	446 $\pm$ 38c	105.5 $\pm$ 6.6f	196.7 $\pm$ 6.0e
H <sub>2</sub> O <sub>2</sub> + AgNO <sub>3</sub>	90.4 $\pm$ 3.2b	141.4 $\pm$ 2.9a	127 $\pm$ 41d	428 $\pm$ 40c	96.2 $\pm$ 4.9f	191.3 $\pm$ 4.9e

### H<sub>2</sub>O<sub>2</sub> and $\alpha_1$ -AR activation produces an increase in Ca<sup>2+</sup> mobilization from intracellular pools in hepatocytes

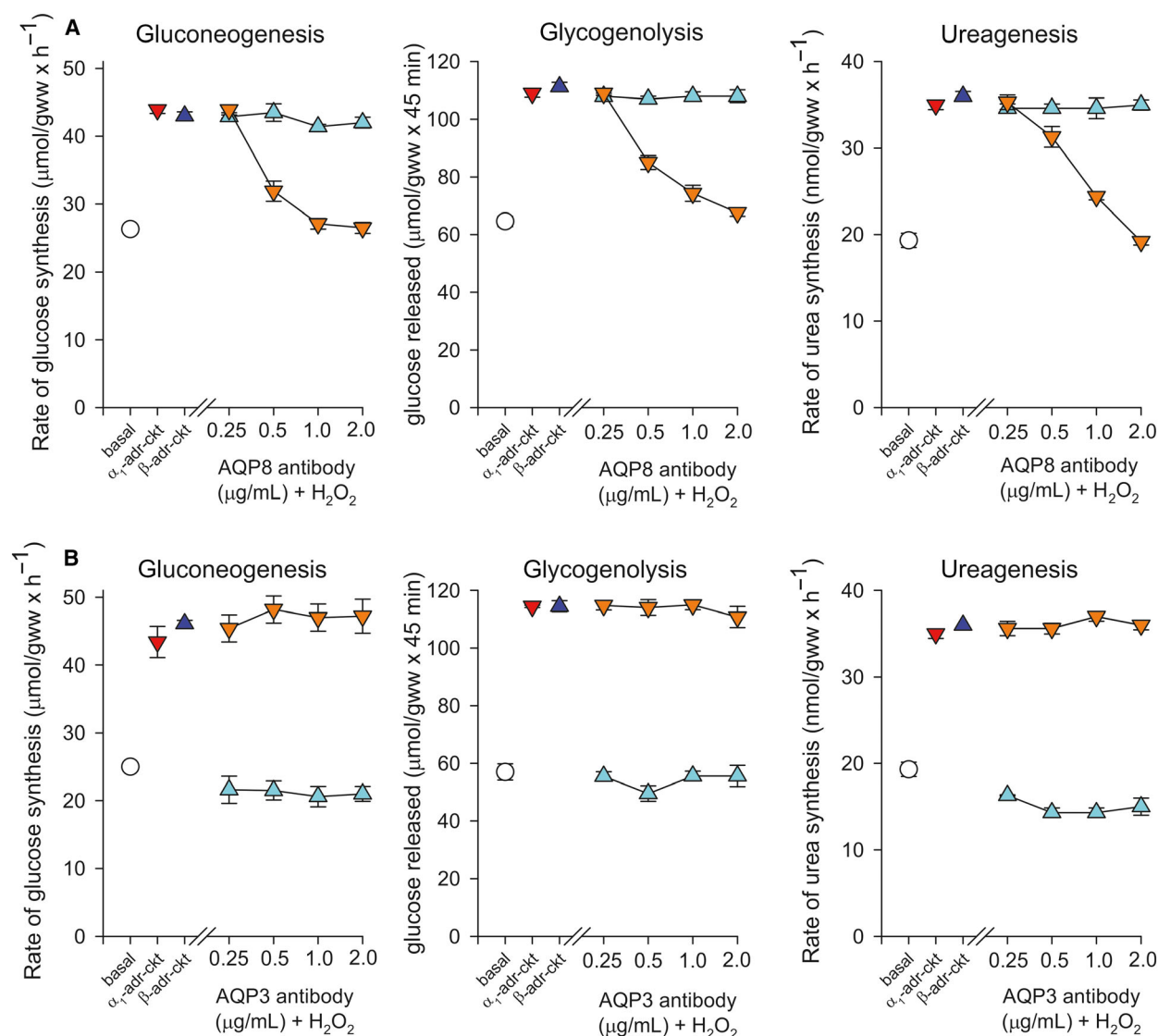
Figure 4 shows that adrenaline- $\alpha_1$ -AR stimulated cytosolic Ca<sup>2+</sup> mobilization in hepatocytes. H<sub>2</sub>O<sub>2</sub> also increased the ion mobilization *per se* but at a lower magnitude than the  $\alpha_1$ -AR agonist; interestingly, both stimuli were additive. In contrast, adrenaline- $\beta$ -AR stimulation did not modify basal cytosolic Ca<sup>2+</sup>, nor that induced by H<sub>2</sub>O<sub>2</sub> (Fig. 4). It is important to mention that BAPTA-AM chelated the H<sub>2</sub>O<sub>2</sub>-induced Ca<sup>2+</sup> augment, meaning that its origin is intracellular, probably from endoplasmic reticulum (ER), but not EGTA, an extracellular Ca<sup>2+</sup> chelator (Fig. 4). In concordance, AQP8 antibody diminished H<sub>2</sub>O<sub>2</sub>-induced Ca<sup>2+</sup> mobilization in a concentration-dependent manner (Fig. 5), indicating H<sub>2</sub>O<sub>2</sub> passage through the cell membrane to act, while addition of AQP3 antibody did not modify it. These results show that cytosolic Ca<sup>2+</sup> increase is involved in the metabolic actions of  $\alpha_1$ -AR and H<sub>2</sub>O<sub>2</sub> in hepatocytes, as well as H<sub>2</sub>O<sub>2</sub> inhibition of adrenaline- $\beta$ -AR-stimulated metabolic pathways.

### Discussion

NADPH oxidases can generate ROS as a response to activated G protein-coupled receptors (GPCRs) [28]. Actually, a variety of ligands after binding to their respective GPCRs promotes ROS production *via* NOX-dependent activation; among them, we can find AT1 receptor activated by angiotensin II [29,30], A<sub>2A</sub>-receptor activated by adenosine [31,32], P2Y<sub>1</sub> receptor activated by extracellular ATP [33], CXCR4 receptor activated by chemokine SDF-1 $\alpha$  [34],  $\beta_2$ -AR activated

by its agonist salmeterol [35] or isoproterenol [36,37], or  $\alpha_2$ -AR activated by noradrenaline [38]. Results of our work show the capacity of  $\alpha_1$ -ARs to promote ROS production *via* NOX2-activation, in both isolated rat liver cells [4], and prepared hepatic plasma membranes from isolated and highly enriched rat hepatocytes (Fig. 1A, and [5]). Even more, results in Fig. 1B, C showed that added 0.74  $\mu$ M H<sub>2</sub>O<sub>2</sub> alone cannot raise basal glycogenolysis or gluconeogenesis, suggesting that some unnoticed component, produced after  $\alpha_1$ -AR activation, is absent to activate both metabolic pathways under the used experimental conditions. Thus, after ROS production *via* NOX2 activation, an additional but unnoticed signaling component generated by  $\alpha_1$ -AR activation is absolutely required to fully obtain the classical metabolic response achieved on hepatic gluconeogenesis or glycogenolysis. In the same way, addition of exogenous H<sub>2</sub>O<sub>2</sub> does not produce a further increase in the metabolic responses produced by  $\alpha_1$ -AR activation, showing that the internal capacity of isolated hepatocytes to respond, probably reached its limit under the used experimental conditions; hence, H<sub>2</sub>O<sub>2</sub> produced *via* NOX2 activation is not a limiting factor in the metabolic responses induced by  $\alpha_1$ -AR activation.

Thereby, after  $\alpha_1$ -AR activation, two signaling cascades are coupled and activated simultaneously: its previously and properly established canonical signaling transduction route comprised of G<sub>q/11</sub>-PLC $\beta$ -IP<sub>3</sub>/DAG-Ca<sup>2+</sup> mobilization [39,40], as well as a second signaling transduction route integrated by Ga<sub>13</sub>-Rac1-GTP-NOX2-H<sub>2</sub>O<sub>2</sub> according to our previous reports [4,5], that here is enlarged with the participation of AQP8 and Ca<sup>2+</sup> mobilization, to become Ga<sub>13</sub>-Rac1-GTP-NOX2-H<sub>2</sub>O<sub>2</sub>(extracellular)-AQP8-H<sub>2</sub>O<sub>2</sub>(intracellular)-



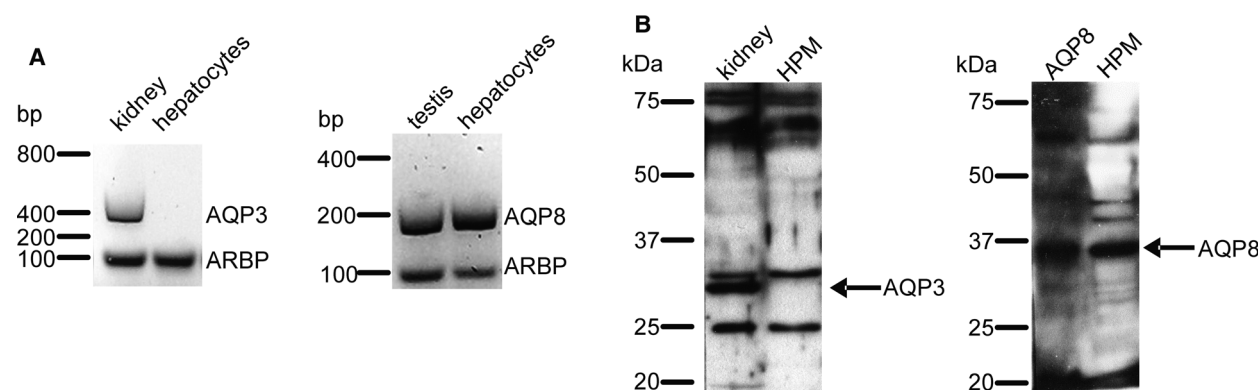
**Fig. 2.** Effect of H<sub>2</sub>O<sub>2</sub> and AQP8 (A) or AQP3 (B) antibodies on gluconeogenesis, glycogenolysis, and ureagenesis stimulated by activation of  $\alpha_1$ -AR or  $\beta$ -AR in rat hepatocytes. Freshly isolated hepatocytes from starved rats (to measure gluconeogenesis) or from fed rats (to measure glycogenolysis and urea synthesis) were incubated as follows: in the absence of adrenaline cocktail (basal, O); with  $\alpha_1$ -adr-ckt alone ( $\blacktriangledown$ ); with  $\beta$ -adr-ckt alone ( $\blacktriangle$ ); with  $\alpha_1$ -adr-ckt ( $\blacktriangledown$ ) or  $\beta$ -adr-ckt ( $\blacktriangle$ ) plus H<sub>2</sub>O<sub>2</sub> 20  $\mu$ M and the concentrations of antibody against AQP8 or AQP3 indicated in panels A and B, respectively. Significant differences ( $P < 0.05$ ) are denoted by different lowercase letters above each symbol in panels A and B. Inside each metabolic pathway, equivalent data have the same letter; different letters indicate statistically significant differences. Data are the mean  $\pm$  SEM of three different experiments in duplicate.

Ca<sup>2+</sup>-mobilization (Fig. 6). However, the subsequent steps in the signaling cascade are continuously being explored; for example, biased agonism [41], molecular dynamics through allosteric nanobodies [42], and possible short-lived intermediates [43] are among those steps.

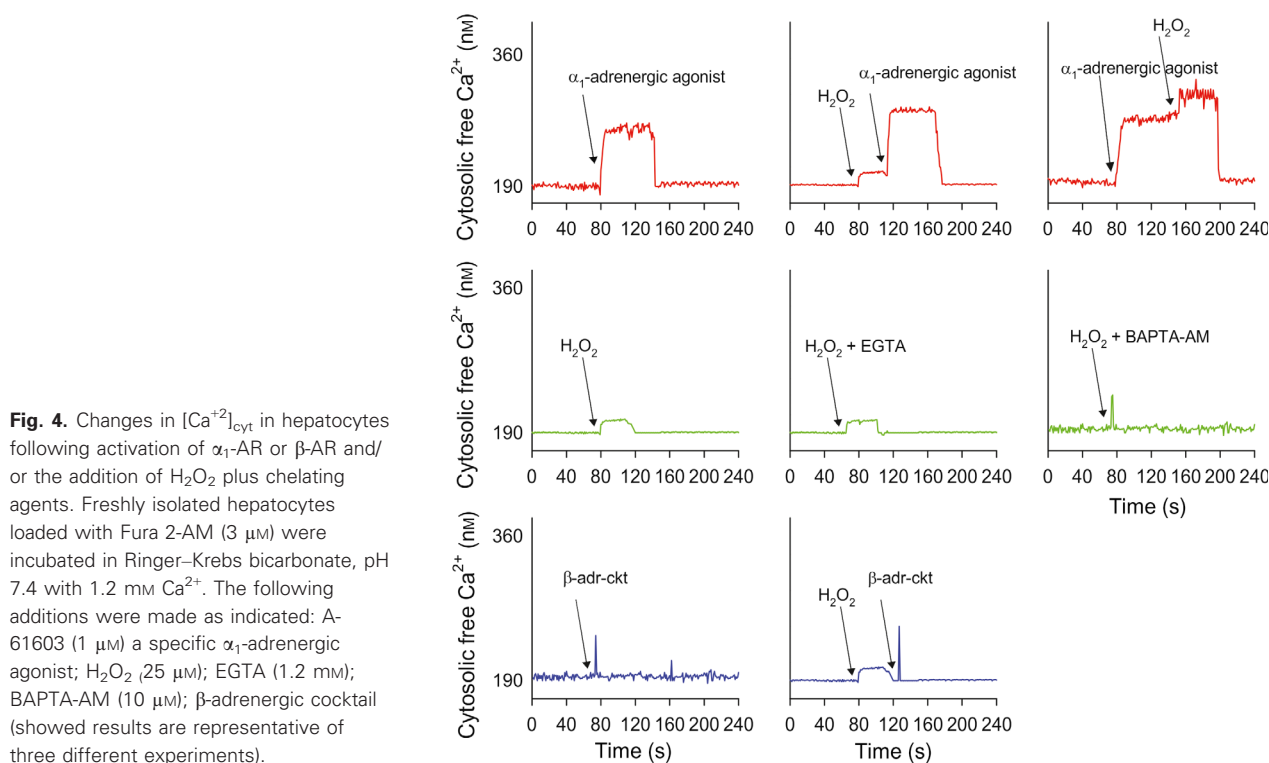
On the other hand,  $\beta$ -ARs are coupled to G<sub>s</sub>-AC-cAMP-PKA [40,43], where H<sub>2</sub>O<sub>2</sub> blocked the well-known stimulation of metabolic pathways (gluconeogenesis, glycogenolysis, and ureagenesis) produced by  $\beta$ -AR

activation (Table 1). This last reinforces our previously reported negative crosstalk between  $\alpha_1$ -ARs and  $\beta$ -ARs [5].

This inhibitory action of H<sub>2</sub>O<sub>2</sub> on  $\beta$ -AR-mediated metabolic action might be similar to the one experimentally analyzed with detail in adipose tissue, where type II $\beta$  cAMP-dependent protein kinase A (PKA) activation was impaired by H<sub>2</sub>O<sub>2</sub> at  $\mu$ Molar concentrations, either added directly or generated by insulin [9].



**Fig. 3.** Expression of AQP3 and AQP8 in hepatocytes. (A) expression of AQP3 and AQP8 mRNA was measured by RT-PCR in hepatocytes. (B) expression of AQP3 and AQP8 protein was assessed by western blot assay in HPM from highly enriched hepatocytes. Kidney was used as positive control for expression of AQP3, and testis or AQP8 transfected 293T cells (AQP8) were used as positive control of AQP8.

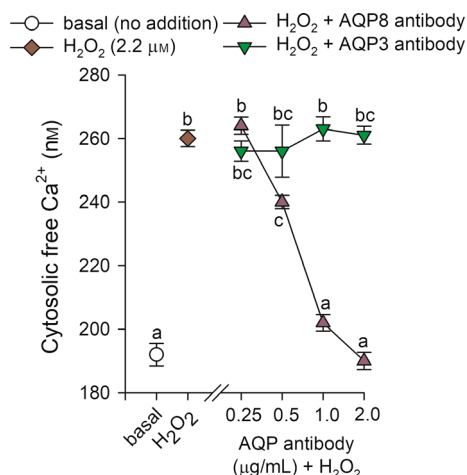


**Fig. 4.** Changes in [Ca<sup>2+</sup>]<sub>cyt</sub> in hepatocytes following activation of  $\alpha_1$ -AR or  $\beta$ -AR and/or the addition of H<sub>2</sub>O<sub>2</sub> plus chelating agents. Freshly isolated hepatocytes loaded with Fura 2-AM (3  $\mu$ M) were incubated in Ringer-Krebs bicarbonate, pH 7.4 with 1.2 mM Ca<sup>2+</sup>. The following additions were made as indicated: A-61603 (1  $\mu$ M) a specific  $\alpha_1$ -adrenergic agonist; H<sub>2</sub>O<sub>2</sub> (25  $\mu$ M); EGTA (1.2 mM); BAPTA-AM (10  $\mu$ M);  $\beta$ -adrenergic cocktail (showed results are representative of three different experiments).

In adipocytes, we showed previously (*op cite*) that H<sub>2</sub>O<sub>2</sub> reacts with type II holoenzyme and oxidizes the -SH from Cys-99 in the catalytic subunit and the -SH from Cys-97 in the type II regulatory subunit to constitute a disulfide bond between these two subunits that impair the cAMP-activating role in PKA holoenzyme. In these previous experiments, H<sub>2</sub>O<sub>2</sub> was unable to inhibit the enzymatic activity of the already cAMP-activated and therefore dissociated holoenzyme. The inhibitory effect of H<sub>2</sub>O<sub>2</sub> recorded for the RII $\beta$ S-

containing rat adipose tissue holoenzyme is shared by the type II PKA holoenzyme formed with RII $\alpha$ S present in bovine heart, but not by the type I regulatory subunits that possess a Ser instead of a Cys residue at position 97 [9]. Interestingly, rat liver possesses both type I and type II regulatory subunits of PKA, but type II regulatory subunit (with Cys-97) is the predominant isoform in liver [44]. All these previous data allowed us to suggest that type II PKA holoenzyme could play also a pivotal role in the H<sub>2</sub>O<sub>2</sub>-mediated





**Fig. 5.** Effect of AQP3 or AQP8 antibodies over cytosolic free Ca<sup>2+</sup> in rat hepatocytes. Freshly isolated hepatocytes loaded with Fura 2-AM (3 μM) were incubated in Ringer-Krebs bicarbonate, pH 7.4 with 1.2 mM Ca<sup>2+</sup>. Response to the addition of H<sub>2</sub>O<sub>2</sub> (2.2 μM) alone (control, ◆), or in addition to different concentrations of AQP8 antibody (▲) or AQP3 antibody (▼). Basal (○) indicates no addition. Significant differences ( $P < 0.05$ ) are denoted by different lowercase letters above of each symbol. Equivalent data have the same letter; different letters indicate statistically significant differences. Data are the mean  $\pm$  SEM of three different experiments in duplicate.

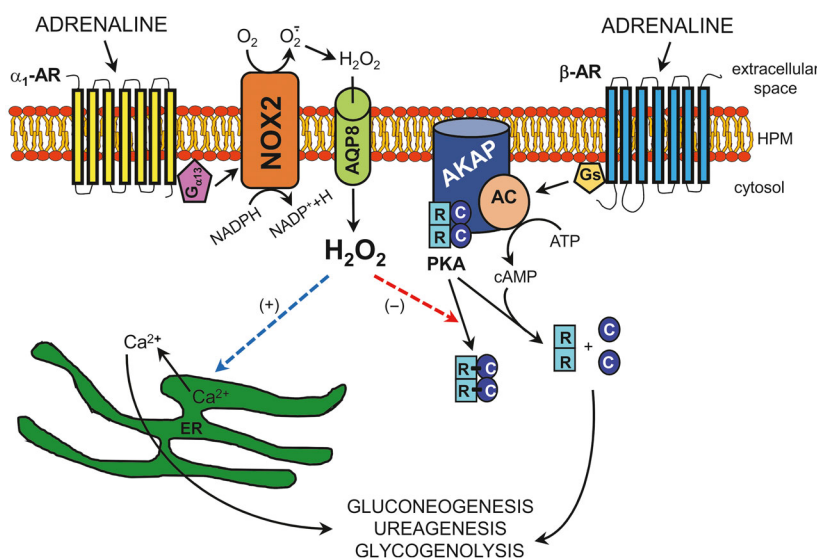
negative crosstalk obtained in isolated rat liver cells (Fig. 6). Above-mentioned actions explain the negative crosstalk between insulin and  $\beta$ -adrenergic agonists, since insulin-formed H<sub>2</sub>O<sub>2</sub> *via* NOX stimulation inhibits PKA activation in adipocytes [9]. Likewise, Valdés *et al.* [45] reported that NOX inhibition by apocynin and VAS2870 improves  $\beta$ -adrenergic stimulated contractility in cardiomyocytes. Furthermore, two papers from our group showed H<sub>2</sub>O<sub>2</sub>-mediated actions of completely different chemicals on adipocytes: Taurine *per se* does not modify baseline lipolysis in adipocytes, but taurine does prevent insulin-mediated H<sub>2</sub>O<sub>2</sub> generation, allowing a rise of isoproterenol and dibutyryl-cAMP-mediated lipolysis [46]. On other hand, four different nonsteroidal-anti-inflammatory-drugs (including aspirin) activate NOX in adipocytes, raising the H<sub>2</sub>O<sub>2</sub> pool that prevent cAMP-stimulated PKA activation and inhibit lipolysis [47]. In summary, PKA activation means physiological cAMP predominance, but inactive PKA means physiological H<sub>2</sub>O<sub>2</sub> predominance. Thus, the molecular point for crosstalk among heterologous signal transduction pathways (cAMP/H<sub>2</sub>O<sub>2</sub>), in adipocytes (and probably hepatocytes), is the well-identified PKA. We might speculate on the physiological meaning of the here described results. The metabolic route followed by hepatic cells after adrenaline stimulation

might be defined by its H<sub>2</sub>O<sub>2</sub> pool: With a high H<sub>2</sub>O<sub>2</sub> pool, the followed route will be through the  $\alpha_1$ -AR. With a low H<sub>2</sub>O<sub>2</sub> pool, the followed route will be through the  $\beta$ -one. Thus, cell response is obtained, and an over-response is avoided.

Besides, it is important to point out that in a variety of experimental models, the activation of different ARs has been associated with an increase of ROS production *via* NOX activation. For example, norepinephrine increases ROS produced by NOX2 in H9c2 cardiac myoblast [48], as well as in human peripheral blood mononuclear cells preferentially *via*  $\alpha_2$ -AR [38]. Otherwise, Qian *et al.* [35] reported in rat primary mesencephalic neuron-glia cell cultures, that salmeterol, a long-acting  $\beta_2$ -AR agonist, selectively increases the production of ROS by NADPH oxidase, through an ERK-dependent pathway, independent of PKA activation. Additionally, Moniri *et al.*, using human embryonic kidney (HEK293) cells transfected with  $\beta_2$ -AR, showed H<sub>2</sub>O<sub>2</sub> production when  $\beta_2$ -AR is stimulated by isoproterenol through activation of a putative NADPH oxidase isoform and that depletion of this H<sub>2</sub>O<sub>2</sub> production by receptors' antagonists, signaling inhibitors, or antioxidants impaired second messengers' formation [11,49]. This suggests that ROS generation, due to a specific AR activation, is cell specific since H<sub>2</sub>O<sub>2</sub> generation is coupled to  $\alpha_1$ -AR activation (this work) and not to  $\beta_2$ -AR activation (*op cit.*) in rat hepatocytes. Thus, the transduction pathway associated with a specific AR might be different depending on the cell/tissue/organ/species used to perform the experiments.

Along this line of thinking, our study is controversial since we found that stimulation of  $\alpha_1$ -AR with adrenaline induces H<sub>2</sub>O<sub>2</sub> formation, which is necessary for metabolic pathways to proceed, after its entry *via* AQP8, reaching probably a high concentration ( $\sim 7$  μM); that is, it could be an oxidative stress [1]. In contrast, adrenaline-stimulated  $\beta$ -AR increases metabolic pathways, which were inhibited by H<sub>2</sub>O<sub>2</sub> either by  $\alpha_1$ -AR activation [5] or by addition of the ROS, that is, blockade of AQP8 by antibodies avoided H<sub>2</sub>O<sub>2</sub>-induced inhibition of  $\beta$ -AR effects and confirmed AQP8's role in the signaling (this work). In addition, it has been reported that H<sub>2</sub>O<sub>2</sub> (100 μM) induces Ca<sup>2+</sup> increase in endothelial cells due to 5-HT<sub>1</sub> receptor activation, increase that is similar to the one we found here [50].

Finally, it is quite interesting that H<sub>2</sub>O<sub>2</sub>, a main redox metabolite, has emerged as a signaling molecule for several hormones [1], such as insulin [8,9], growth factors, cytokines, and several agonists of GPCRs (for a review, see Ref. [44,51]). On the other hand,



**Fig. 6.** Scheme summarizing molecular steps underlying the negative crosstalk between  $\alpha_1$ -AR activation, *via* H<sub>2</sub>O<sub>2</sub> generation by NOX2 and calcium mobilization, on  $\beta$ -AR-mediated stimulation of hepatic metabolic pathways. Under starvation or stress conditions, adrenaline is released and activates the NOX2 located in the hepatocyte plasma membrane through the  $\alpha_1$ -adrenoreceptors ( $\alpha_1$ -AR) [5]. NOX2 generates superoxide anion toward the extracellular space; this anion is transformed into H<sub>2</sub>O<sub>2</sub> presumably by superoxide dismutase 3 [53] and enters the hepatocyte through AQP8. Thus, H<sub>2</sub>O<sub>2</sub> promotes the release of calcium from the ER. On the other hand, adrenaline activates the adenylyl cyclase (AC) through  $\beta$ -adrenoreceptors ( $\beta$ -AR) and increases the cAMP pool. This cyclic nucleotide activates the PKA holoenzyme by separating the two catalytic (C) subunits from the two regulatory subunits (R). The H<sub>2</sub>O<sub>2</sub> generated in the extracellular space, with the assistance of the AQP8 gets to the type II PKA that is anchored to a PKA-anchoring protein (AKAP) [54]. Following this previously proposed route [9], PKA holoenzyme is oxidized by H<sub>2</sub>O<sub>2</sub> producing a disulfide bond (—) between regulatory and catalytic subunits that impairs the cAMP-activating role on the PKA holoenzyme. Thus, the blank proteins are not phosphorylated by the PKA active catalytic subunit and therefore downstream biochemical cascade is inhibited.

H<sub>2</sub>O<sub>2</sub> could be present in physiological oxidative eustress concentrations (1–10 nM) [1], which is derived mainly from mitochondrial activity and other sources even though H<sub>2</sub>O<sub>2</sub> seems to be kept compartmentalized, or in oxidative stress concentrations (> 100 nM) [1]. We have to mention that the H<sub>2</sub>O<sub>2</sub> concentration we found after  $\alpha_1$ -AR stimulation is around  $\sim 7 \mu\text{M}$ , while in other experiments, we used 0.74–25  $\mu\text{M}$  as pharmacological ones, and it is clear that these H<sub>2</sub>O<sub>2</sub> concentrations show a singular action in our study: the spatiotemporal coordination of the signaling cascades of  $\alpha_1$ -AR and  $\beta$ -AR, that is,  $\alpha_1$ -AR negatively modulates  $\beta$ -AR actions. To do so, H<sub>2</sub>O<sub>2</sub> has to enter the cell *via* an aquaporin and we show that AQP8 is involved in H<sub>2</sub>O<sub>2</sub> crossing plasma membrane, as well as in ER membrane [52], suggesting that this messenger could promote cytosolic Ca<sup>2+</sup> increase released from ER. Here, it is interesting to point out that metabolic effects stimulated by  $\alpha_1$ -AR, like glycogenolysis and gluconeogenesis, need the simultaneous action of both: the canonical signaling transduction route and the signaling transduction route mediated by NOX2 and H<sub>2</sub>O<sub>2</sub> described in this

work. Otherwise, the inhibition produced by the NOX2-specific inhibitor gp91ds-tat over these metabolic pathways (Fig. 1B,C) cannot be explained, opening novel avenues to study the interactions between different signaling transduction routes. Obvious questions following the results showed herein are as follows: (a) Does H<sub>2</sub>O<sub>2</sub> have similar roles in other cell types and (b) does H<sub>2</sub>O<sub>2</sub> generated by other hormones resemble the role reported for it here? Definitive answers to these questions cannot be reached here, but cumulative available evidence suggests that an affirmative answer is probable, though further experiments are needed for confirmation.

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## Author contributions

EP conceived, designed and coordinated the study, MVL and RG performed the experiments. RVM write the original draft. EP, HRR, RVM, MVL analyzed data. HRR, RG and MVL made Figures. EP, HRR and RVM contributed to the general discussion and wrote final version of manuscript. All authors read and approved the final manuscript.

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