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ORIGINAL ARTICLE

NOX2 activated by α_1-adrenoceptors modulates hepatic metabolic routes stimulated by β-adrenoceptors

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Abstract

The NADPH oxidase (NOX) family of enzymes oxidase catalyzes the transport of electrons from NADPH to molecular oxygen and generates $O_2^{\bullet-}$, which is rapidly converted into H_2O_2. We aimed to identify in hepatocytes the protein NOX complex responsible for H_2O_2 synthesis after α_1-adrenoceptor (α_1-AR) stimulation, its activation mechanism, and to explore H_2O_2 as a potential modulator of hepatic metabolic routes, gluconeogenesis, and ureagenesis, stimulated by the ARs. The dormant NOX2 complex present in hepatocyte plasma membrane (HPM) contains $gp91^{phox}$, $p22^{phox}$, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and Rac 1 proteins. In HPM incubated with NADPH and guanosine triphosphate (GTP), α_1-AR-mediated H_2O_2 synthesis required all of these proteins except for $p40^{phox}$. A functional link between α_1-AR and NOX was identified as the $G\alpha_{13}$ protein. Alpha$_1$-AR stimulation in hepatocytes promotes Rac1-GTP generation, a necessary step for H_2O_2 synthesis. Negative cross talk between α_1-/β-ARs for H_2O_2 synthesis was observed in HPM. In addition, negative cross talk of α_1-AR via H_2O_2 to β-AR-mediated stimulation was recorded in hepatocyte gluconeogenesis and ureagenesis, probably involving aquaporine activity. Based on previous work we suggest that H_2O_2, generated after NOX2 activation by α_1-AR lightening in hepatocytes, reacts with cAMP-dependent protein kinase A (PKA) subunits to form an oxidized PKA, insensitive to cAMP activation that prevented any rise in the rate of gluconeogenesis and ureagenesis.

Keywords: *H_2O_2, adrenaline, gluconeogenesis, ureagenesis, NADPH oxidase*

Introduction

It is generally accepted that reactive oxygen species (ROS) are continuously formed during aerobic metabolism, and due to their reactivity and pro-oxidant properties, molecular damage is produced. Although a major source of intracellular ROS appears to be generated accidentally as by-products of mitochondrial respiration [1,2], a ROS generation controlled by cellular machinery devoted to support organism homeostasis is of paramount importance [3]. To date, H_2O_2, considered the main signal face of ROS, can be produced in highly regulated and limited amounts by at least two specialized systems in specific cell sites to control key events in cellular life. The system that governs mitochondrial production is that of the enzyme $p66^{Shc}$, which responds to specific signals [4,5], generates H_2O_2 to trigger mitochondrial swelling and apoptosis [6] and has been closely related with lifespan [3]. The other specialized system is represented by the NADPH oxidase (NOX) family of enzymes, oxidases, which catalyzes the transport of electrons from NADPH to molecular oxygen generating $O_2^{\bullet-}$ rapidly converted into H_2O_2 [7]. NOX family is integrated by seven members: NOX1; NOX2 (also known as $gp91^{phox}$), NOX3, NOX4, NOX5, DUOX1 and DUOX2 [7,8]. The first widely studied NOX enzyme was NOX2, originally characterized in phagocytes membranes, with whom newly studied NOX enzymes are compared [9]. The

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physiological role of NOX2 in phagocytes is related with the microbe-killing mechanisms typical of these cells: microbial molecules present in phagocytes stimulate the dormant state of the NOX2 enzyme to form the corresponding activated state with catalytic properties [7]. The dormant state is a heterodimer composed of the electron transport protein $gp91^{phox}$ and the protein $p22^{phox}$, both of these known as cytochrome b_{558}. Upon appropriate phagocyte stimulation, four cytosolic proteins—$p67^{phox}$, $p47^{phox}$, $p40^{phox}$ and Rac—are recruited to bind cytochrome b_{558} in order to integrate the active NOX2 enzyme [10].

The presence and function of NOX enzymes in other cells are under study. The majority of NOX family members are broadly distributed in many cell types, and within these cells, in various subcellular localizations [7,8]. Growth factors and hormones have been shown to alter the activity or expression of NOX proteins and subunits, and particular emphasis has been placed on their activation mechanisms and on their physiological roles [7,8]. The controlled and compartmentalized synthesis of H_2O_2 within the cell produces reversible protein modifications by oxidizing cysteinyl thiols [11], which form disulfide bonds that regulate catalytic functions [12]; H_2O_2 protein targets include kinases, phosphatases and numerous other proteins [3], thus being responsible for modulating main metabolic pathways [13], physiological and oncogenic growth signals [3], apoptosis [14] and senescence [15].

Knowledge of NOX enzyme expression, activation and physiological functions in liver is fragmentary [16], in part, due to the existence of many cell types in the whole gland. Demonstration of mRNA for NOX1, NOX2 and NOX4, and DUOX 1 and 2 isoforms, as well as expression of NOX2 and $p47^{phox}$, have been reported in cultured adult rat hepatocytes [17]. The physiological role of NOX enzyme activation and ROS overproduction in hepatic normal and tumour cells has been associated with apoptosis and cell survival by two research groups. The Häussinger group reported ceramide synthesis- and PKCζ-dependent $p47^{phox}$ phosphorylation, with the subsequent activation of a NOX isoform observed in primary cultured hepatocytes after treatment with the CD95 ligand, or hyperosmotic exposure, or hydrophobic bile salts [17–19]. CD95 ligand-induced apoptosis was impaired by blocking NOX activation [17,18]. The Fabregat group reported modulation of NOX enzymes in normal, fetal, and tumor hepatic cells after treatment with transforming growth factor (TGF)-α or -β, epidermal growth factor (EGF), and an EGF receptor (EGFR) antagonist. In adult and fetal cultured rat hepatocytes, apoptosis promoted by TGF-β required NOX4 increased expression, while EGF treatment impaired both of these actions of TGF-β [20,21]. In FaO hepatoma cells, TGF-β induced NOX1 expression, which would have a cell protective effect, but antagonism of EGFR in these cells with tyrphostin AG 147 enhanced TGF-β-induced cell death, coinciding with a great increase in NOX4 expression [22,23]. In their most recent study, these authors reported the role of NOX1 as controlling the autocrine cell growth of FaO hepatoma cells through regulation of the EGFR pathway [24]. More recently, the Li group reported a critical role of NOX3-derived ROS in palmitate-induced insulin resistance in hepatocytes [25,26]. In addition to these works and by use of a different experimental strategy, it was shown that adrenaline, via α_1-adrenoceptors (α_1-ARs), activated a NOX2 enzyme localized in isolated plasma membranes of highly enriched hepatocytes from rat [27]. This NOX2 enzyme, identified with anti NOX2 antibodies, has the following characteristics: (a) activated NOX2 enzyme in liver plasma membranes incubated with NADPH and O_2 forms H_2O_2, as the enzyme from phagocytes do; (b) addition of cytochrome *c*, which binds $O_2^{\bullet -}$, impaired H_2O_2 generation; (c) disappearance of NADPH was correlated with H_2O_2 synthesis; (d) NOX2 is in a dormant state and might be activated by high concentrations of Mn^{2+}, or with submicromolar concentrations of α_1-AR agonists; and (e) hormone-mediated activation requires GTPγS, but does not require adenosine triphosphate (ATP) [27]. The aim of this work was to identify the protein complex responsible for H_2O_2 synthesis in response to α_1-AR lightening, their corresponding activation mechanism, and to explore a potential role of H_2O_2 in the modulation of classic hepatic metabolic routes stimulated by ARs, i.e. gluconeogenesis and ureagenesis.

Methods

Reagents and antibodies

Reagents were obtained from commercial sources and with the highest grade of purity available (Sigma Aldrich). Antibodies used in this work were anti-NOX1(sc-25545), anti-NOX2 (sc-5826), anti-NOX4 (sc-21860), anti-$p22^{phox}$ (sc-11712), anti-$p40^{phox}$ (sc-18253), anti-$p47^{phox}$ (sc-14015), anti-$p67^{phox}$ (sc-15342), anti-Rac1 (sc-217), anti-Rac 2 (sc-96), anti $G\alpha_{12}$ (sc-409), anti $G\alpha_{13}$ (sc-410), peroxidase-rabbit anti-goat IgG (sc-2004) (Santa-Cruz Biotechnology), monoclonal anti-$gp91^{phox}$ (611415, BD Transduction Laboratories), monoclonal anti-Rac 1 (MAB 3735, Chemicon), peroxidase-rabbit anti-mouse IgG (61-6020) and peroxidase-goat anti-rabbit IgG (656120) (Zymed Laboratories).

Cell isolation

All animal experiments were conducted according to the Federal Guidelines for the Care and Use of Animals (NOM 062-ZOO-1999, Ministry of Agriculture, Mexico), and the study protocol was approved

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by the Institutional Committee of the National Autonomous University of Mexico's (UNAM) Faculty of Medicine. Male Wistar rats weighing 200–250 g fed *ad libitum* and with free access to water were used. Hepatic cells were isolated by collagenase digestion with or without 10 mM glucose, according to the experiment, dissolved in Ca^{2+}-free Ringer–Krebs bicarbonate, pH 7.4 [28]. Viability was determined by trypan blue exclusion; experiments were conducted when >90% of cells excluded the dye. Hepatocytes were separated from total hepatic cells by elutriation (Centrifuge J2-21, Beckman, and rotor JE-6B, Beckman) [29]. Cells identified as hepatocytes, endothelial cells and Kupffer cells by immunohistochemistry as described [27] were collected by centrifugation in different batches. The hepatocyte batch obtained from one liver was suspended in 10 ml Ca^{2+}-free Ringer–Krebs bicarbonate, pH 7.4; enrichment of hepatic cells reached 99% [27]. Hepatocytes (5×10^5 cells) deprived of endothelial and Kupffer cells were incubated in a total volume of 1 ml Ringer–Krebs bicarbonate, pH 7.4 supplemented with 1.2 mM $CaCl_2$ at 37°C with continuous shaking to study metabolic pathways. Alternatively, larger batches of hepatocytes were employed to prepare cell plasma membranes.

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Hepatic cell plasma membrane preparation

Hepatocytes suspended as indicated previously were supplemented with 1 ml protease inhibitor cocktail (cat. no. 158837 from MP Biochemicals, Inc., Solon, OH, USA) and homogenized in a Teflon-glass Potter Elvejhem homogenizer during 2 min at 4°C, and then mixed with 100 ml of lysis buffer (containing 20 mM MES [2-(N-morpholino) ethanesulfonic acid], pH 5.8, 2 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM KCl) at 4°C. Cell lysis was completed by vigorous mechanical shaking during 2 min, and plasma membranes were prepared by differential centrifugations as detailed [27], in order to obtain a fraction identified as highly enriched hepatocyte plasma membranes (HPMs).

NOX activation in HPM

Activation of a dormant NOX enzyme in this preparation was conducted as follows: 20 μg protein of HPM, determined by Bradford method [30], were suspended in up to 1 ml of activation buffer maintained at 4°C and containing 120 mM NaCl, 10 mM $NaHCO_3$ and 1.2 mM $CaCl_2$ dissolved in 30 mM [4-morpholinepropanesulfonic acid] (MOPS) adjusted to pH 7.4. As rapidly as possible, the sample was incubated for 2 min at 37°C, and as indicated in figure legends, the following compounds were added: (a) commercial antibodies against NOX isoforms or NOX regulatory subunits; (b) an α_1-adrenaline cocktail (α_1-adr-ckt) containing in the final volume 1 μM adrenaline and selective adrenergic antagonists (propranolol a β-AR antagonist, yohimbine a α_{2A}-AR antagonist and rauwolscine, a α_{2B}-AR antagonist, all of these utilized at 0.1 μM concentration), with the purpose of stimulating only α_1-AR; (c) a β-adrenaline-cocktail (β-adr-ckt) to stimulate β-AR containing in the final volume 1 μM adrenaline and selective adrenergic antagonists (prazosin, a α_1-AR antagonist, yohimbine and rauwolscine, all of these employed at 0.1 μM concentration), in order to stimulate only β-AR, while the indicated receptors were antagonized; (d) 10 μM GTPγS, a non-hydrolizable analog of GTP and (e) different GTP concentrations as indicated in the figures. Final volume in the activation buffer was maintained constant. The activation step ended with centrifugation at 10 000 rpm during 5 min, the supernatant was discarded, and the pellet was suspended in the catalysis buffer to serve as the enzyme source for the NOX-isoform catalytic step. In some experiments, hormone activation with adrenaline cocktail was performed with undisrupted hepatocytes instead of HPM; in the latter cases, enriched hepatocytes after elutriation were maintained in Ca^{2+}-free Ringer–Krebs bicarbonate, pH 7.4 and incubated for 2 min at 37°C with α_1-adr-ckt (control hepatocytes without α_1-adr-ckt for basal values) in the absence of GTPγS, assuming that the intracellular pool of GTP should be sufficient to support eventual adrenaline stimulation. The step was ended by placing tubes at 0°C followed by immediate homogenization, and the addition of lysis buffer in a volume that was 10-fold higher than the original volume in which hepatocytes were incubated. Highly enriched HPM were prepared from these cells by the same procedure indicated previously, and the activation step for these membranes was conducted as usual during 2 min in the activation buffer, with or without adrenaline cocktail and GTPγS as indicated in the figures.

NADPH-dependent H_2O_2 generation

Immediately after activation, HPM were suspended in 540 μl of catalysis buffer (containing 120 mM NaCl, 1.2 mM KH_2PO_4, 1 mM NaN_3 and 100 μM flavin adenine dinucleotide (FAD) dissolved in 20 mM MES, pH 6.0) at 37°C. Enzymatic reactions were started by the addition of 60 μl of 0.25 mM NADPH dissolved in the catalysis buffer and were terminated after 4 min at 37°C by adding 1.5 M trichloroacetic acid. Samples were centrifuged at 10 000 rpm for 10 min, and the supernatant was utilized to quantify H_2O_2 [31].

Western blot analysis

HPM were disrupted by lysis buffer (NP-40 1%, EDTA 1 mM in phosphate-buffer saline) with a

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protease inhibitor mixture. Solubilized proteins were centrifuged at 12 000 rpm at 4°C for 10 min, and the supernatant protein was quantified [30]. Proteins (60 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinyldiene fluoride membrane (PVDF, Millipore), employing a Tank Transfer System (Bio-Rad) at 100 V for 1 h. Membranes were blocked overnight at 4°C with Tris-buffered saline containing 5% non-fat dry milk and 0.1% Tween 20. Blots were incubated with commercial antibodies as indicated in the figures. After incubation with the secondary antibody (horseradish peroxidase-conjugated antibody), the protein bands were visualized by chemiluminiscence (ECL plus, Amersham). Soluble extracts of THP-1 cells and rat brain were used as positive controls [32].

Rac activation assay

The HPM GTP-associated form of Rac 1 was detected using the Rac 1 activation assay kit (Upstate Biotechnology), following the manufacturer's protocol [33]. Hepatocytes and/or membranes were incubated with adrenaline and either GTPγS or GTP. Immediately after this treatment, HPM were obtained and disrupted in the Mg^{2+} lysis buffer supplied in the kit and were supplemented with protease inhibitor mixture. The lysate was centrifuged at 12 000 rpm for 5 min at 4°C, and 500 μg of the supernatant protein were then shaken with Rac/cdc42 assay reagent (PAK-1 PBD, agarose) at 4°C for 60 min. Agarose beads were collected by pulsing for 5 sec in the microfuge at 14 000 rpm and were washed three times with cold wash buffer (Mg^{2+} lysis buffer). The pellets were suspended in 40 μl of SDS-PAGE loading buffer and subjected to Western blotting. For control reactions, two equal aliquots of lysate of non-incubated cells with adrenaline were preloaded with either 0.1 mM GTPγS or 1 mM guanosine diphosphate (GDP), and processed as described previously. In all cases, HPM lysate was electrophoresed without further processing as controls (Total Rac 1). Rac 1 was visualized utilizing the anti-Rac 1 monoclonal antibody supplied with the kit.

Study of metabolic pathways in isolated hepatocytes

In order to measure the rate of gluconeogenesis, isolated hepatocytes from 24 h starved rats were incubated at 37°C during 60 min in Krebs–Ringer containing 1.2 mM $CaCl_2$, 10 mM lactate, and each of the chosen AR agonists and/or antagonists as indicated in the figures. Glucose release was measured in the extracellular fluid by the glucose oxidase method [34] after centrifugation. To determine the rate of ureagenesis, isolated hepatocytes from rats fed *ad libitum* were incubated at 37°C in Krebs–Ringer supplemented with 1.2 mM $CaCl_2$, 10 mM glucose, 5 mM $(NH_3)\ CO_3$, 3 mM ornithine, and each of the chosen AR agonists and/or antagonists as indicated in the figures. Synthesized urea was measured in the extracellular fluid after centrifugation as described [35].

Results

Identification of NOX isoform complex responding to α_1-AR stimulation

Hydrogen peroxide was formed during the catalytic step by the NOX present in HPM, only if dormant enzyme was incubated previously during the activation step in the presence of α_1-adr-ckt and GTPγS (Figure 1A), as described in the Methods section. The presence of antibodies during the activation step can prevent NOX lightening, as demonstrated experimentally in this work and can consequently affect the catalytic activity assayed immediately afterwards. Thus, a concentration as low as 0.25 μg/ml NOX2 antibodies completely avoided α_1-AR-mediated activation, maximal inhibition was 50% with 2 μg/ml anti NOX1 antibodies, and inhibition was absent with antibodies against NOX4 (Figure 1A). These data suggest that the NOX2 enzyme is the isoform mainly responsible for α_1-AR stimulation, and that NOX1 and NOX4 isoenzymes are expressed and catalytically active in rat liver cells. These findings were supported by identification of NOX1, NOX2 and NOX4 isoforms in HPM with Western blot (*Insert* in Figure 1A). Our results confirm previous findings [17,20,26] regarding the expression of these NOX enzymes in isolated hepatocytes, and indicate that some NOX isoforms are localized, at least, in HPMs.

It was important to know whether adrenaline-mediated NOX2 activation and NOX2 catalytic activity in hepatocyte membranes required all proteins integrating the active NOX2 complex, as in phagocytes, i.e. $p22^{phox}$, $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$. In HPM, $p22^{phox}$, $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ were identified (*Insert* in Figure 1B); accordingly, the reported expression of $p47^{phox}$ in liver cells [17] was confirmed, and its localization in cell plasma membrane was evident. When compared with similar subunits from THP 1 cells used as positive control, all of these proteins were found in liver membranes at lower levels. Once distinctive proteins from NOX2 complex were identified in HPM, the requirement of each one of these to integrate a functional system capable of generating H_2O_2 was explored. For this purpose, NOX2 catalytic activity promoted by α_1-adr-ckt was challenged by the use of the same strategy summarized previously, and employing specific antibodies directed against each of the identified subunits. Concentration-dependent inhibition in α_1-adr-ckt-mediated NOX2 activation

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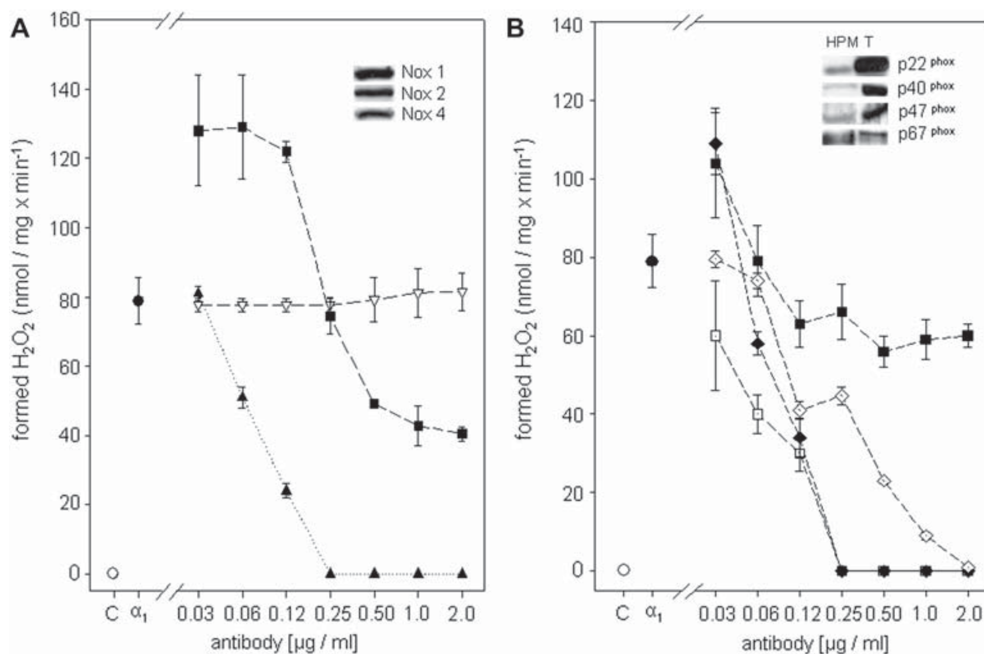

Figure 1. NOX isoforms detected in HPM and their activation by α_1-AR agonists. (A) NOX enzymatic activity of HPM was measured in absence (○) or in presence of GTPγS and α_1-adr-ckt (●), with the indicated concentration of antibodies against NOX isoenzymes, NOX1 (■----■), NOX2 (▲----▲), and NOX4 (▽----▽). *Insert*. HPM were analyzed by Western blot using antibodies against NOX1, NOX2 (gp91^{phox}), and NOX4. (B) NOX enzymatic activity was measured after previous activation of HPM with α_1-adr-ckt and GTP was done, in presence of antibodies against p22^{phox} (◆----◆), p40^{phox} (■----■), p47^{phox} (□----□), and p67^{phox} (◇----◇). *Insert*. HPM were analyzed by Western blot using antibodies against p22^{phox}, p40^{phox}, p40^{phox} and p67^{phox}. THP-1 total extract (T) was used as positive control. Values represent means ± standard error (SE) of three independent experiments performed in duplicate. Western blot results are representative samples of three independent experiments.

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and H_2O_2 synthesis was detected with antibodies against p67^{phox}, p47^{phox} and p22^{phox}; the inhibition response with antibodies against p40^{phox} was ambiguous (Figure 1B).

Participation of G proteins in NOX2 response to α_1-AR stimulation

When the same experimental model was assayed with antibodies against Rac 1 and Rac 2, the so-called small G proteins, concentration-dependent inhibition of NOX activation and H_2O_2 synthesis by means of adrenaline-mediated α_1-AR stimulation was observed with antibodies against Rac 1 protein only (Figure 2A). Furthermore, Western blot in HPM identified Rac 1, but not Rac 2, proteins (*Insert* in Figure 2A). These data support previous reports dealing with the distribution of Rac 1 and Rac 2 proteins in different mammalian cell types. Whereas Rac 1 is localized in non-phagocytic cell types, Rac 2 is present exclusively in cells of hematopoietic origin to activate NOX2 in phagocytes [36]. The next step was to search for other G proteins that were eventually involved in α_1-adr-ckt-mediated H_2O_2 synthesis. G protein-related to α_1-AR comprise G_{q11}, $G\alpha_{i/o}$, $G\alpha_{12}$ and $G\alpha_{13}$ [37]. Our experimental model was utilized to analyze $G\alpha_{12}$ and $G\alpha_{13}$ proteins, which are known to modulate Rac 1 protein [38], here identified as part of the functional NOX2 system in liver cell membrane. Western blot clearly showed a $G\alpha_{13}$ protein presence in HPM, whereas $G\alpha_{12}$ was not detected (*Insert* in Figure 2B). Antibodies against the $G\alpha_{13}$ protein, inhibited, in a concentration-dependent manner, the H_2O_2 synthesis promoted by α_1-AR stimulation, probably due to impairment of NOX2 system activation, while anti $G\alpha_{12}$ antibodies slightly decreased α_1-adr-ckt-mediated H_2O_2 synthesis (Figure 2B). This result indicates that the α_1-AR and NOX2 systems in HPM are functionally linked through $G\alpha_{13}$ protein.

NOX2 activation with α_1-AR agonist does not involve recruitment of cytosolic proteins

Data from previously related experiments were useful in identifying proteins belonging to the NOX2 complex present in HPM, but its mechanism of enzyme activation after α_1-AR stimulation by adrenaline cocktail requires further experimental work. An initial approach was to explore, in the whole hepatocyte, a α_1-adr-ckt-mediated transfer of proteins from cytosol to plasma membrane. For this experiment, hepatocytes, rather than isolated cell membranes, were activated as detailed in the Methods section; subsequently, HPM were prepared as usual. These membranes were subjected to an equivalent activation step, but with an activation buffer free of both GTPγS and the α_1-adr-ckt. Control hepatocytes were included in which both activation steps—either with whole cells or with

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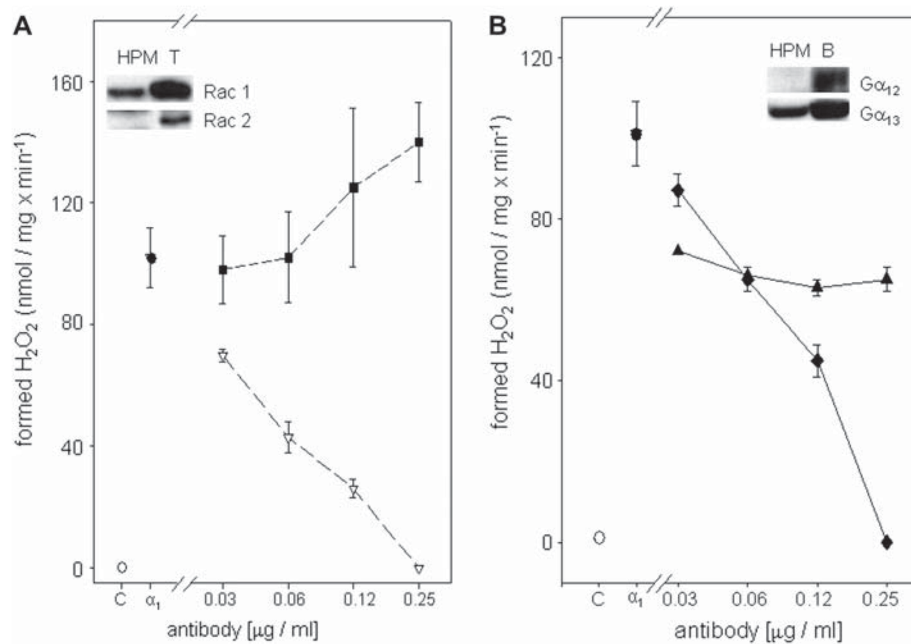

Figure 2. Involvement of Rac 1 and $G\alpha_{13}$ protein in NOX2 activation by α_1-AR lightening. (A) NOX enzymatic activity of HPM was measured after being incubated during the activation step in absence (○) or in presence of GTPγS and α_1-adr-ckt (●), and antibodies against Rac 1 (▽----▽) or Rac 2 (■----■) proteins. *Insert.* Western blot identification of Rac 1 and Rac 2 in HPM; (T) soluble extracts from THP-1 cells used as positive control. (B) NOX enzymatic activity of HPM measured after being incubated during the activation step in absence (○) or in presence of GTPγS and α_1-adr-ckt (●), and antibodies against $G\alpha_{12}$ (▲----▲) and $G\alpha_{13}$ (◆----◆). *Insert.* Western blot identification of $G\alpha_{12}$ and $G\alpha_{13}$ proteins in HPM; (br) brain-soluble extracts used as positive control. Values represent means ± SE of three independent experiments performed by duplicate. Western blot results are representative samples of three independent experiments.

isolated membranes—were conducted with an activation buffer deprived of GTPγS and α_1-adr-ckt. The total amount of each of the visualized proteins from the NOX2 system remained constant in isolated HPM from hepatocytes, irrespective of whether the α_1-adr-ckt was present or not during the activation step with whole cells (Figure 3A). Figure 3B includes the NOX2 catalytic activity of HPM provided from hepatocytes non-preincubated with α_1-adr-ckt. The catalytic activity of these membranes incubated during 2 min in activation buffer alone or supplemented with GTPγS exhibited values near zero; addition of α_1-adr-ckt and GTPγS to these membranes increased NOX2 catalytic activity to values near 100 nmol of H_2O_2 formed per mg protein^{-1}min^{-1}. Figure 3C shows that membranes incubated during 2 min at 37°C in an activation buffer free of α_1-adr-ckt and GTPγS, provided from hepatocytes preincubated for 2 min at 37°C in an activation buffer without GTPγS, neither α_1-adr-ckt, were capable of activating NOX2 to values of 57.8 nmol of H_2O_2 formed per mg protein^{-1}min^{-1}, and to 157.9 nmol of H_2O_2 formed per mg protein^{-1}min^{-1}, when hepatocytes were preincubated during 2 min with α_1-adr-ckt (Figure 3C). Interestingly, subtracting this latter value from its basal value in the absence of the hormone yielded ≈100 nmol of H_2O_2 formed mg protein^{-1}min^{-1}. Thus, the magnitude of NOX2 activation via α_1-AR stimulation is the same, whether α_1-adrenaline-mediated activation is conducted in whole hepatocytes or in their plasma membranes. These results indicate that NOX2 activation after α_1-AR stimulation does not require membrane recruitment of proteins to manifest full catalytic activity. Furthermore, isolated hepatocytes apparently showed two mechanisms for activating NOX catalytic activity: one was adrenaline-dependent, while the other mechanism was adrenaline-independent, but temperature-dependent; this was observed in cells incubated for 2 min at 37°C and was not observed in cells incubated 2 min at 0°C (not shown). Both mechanisms of activation were additive.

Rac 1-GTP is required for α_1-AR agonist-mediated NOX2 activation

Once translocation of cytosolic factors was discarded as a mechanism for activating NOX2 in hepatocytes, as it occurs in phagocytes [39], phosphorylation of proteins integrating a NOX2 complex in hepatocytes was also considered an unlikely step for expediting such a translocation of factors. Moreover, ATP was ineffective for promoting NOX2 activation in HPM when tested under several experimental conditions [27]. The putative incorporation of guanine nucleotides into Rac 1 was an alternative to explore. This step was shown to take place in distinctive activation cases [40,41]. Consequently, Rac 1 activation assay

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was employed to detect binding of GTP to Rac 1 in a search for α_1-AR-adrenaline-mediated generation of Rac 1-GTP or Rac 1-GTPγS complex in membranes of hepatocytes. Rac 1-GTPγS was absent in non-activated membranes form non-preincubated hepatocytes (Lane 1, Figure 3B), but was observed after these membranes were incubated for 2 min at 37°C plus GTPγS, both with or without the α_1-adr-ckt (Lanes 2 and 3, Figure 3B). However, as depicted in Figure 3B, Rac 1-GTPγS generation in HPM with an excess of GTPγS alone cannot activate NOX2 unless adrenaline is also present in the activation

Figure 3. Requirement of non-catalytic proteins to activate NOX2 by α_1-AR agonist. (A) Western blot of HPM prepared from hepatocytes incubated in activation buffer 2 min at 37°C in the absence (NAdr) or presence of α_1-adr-ckt (Adr), using antibodies p22^{phox}, p40^{phox}, p47^{phox}, p67^{phox} and monoclonal antibodies against gp91^{phox} and Rac 1. (B) HPM were obtained from hepatocytes non-preincubated as usual and incubated in activation buffer supplemented or not with GTPγS and α_1-adr-ckt, as indicated in the figure and NOX catalytic activity was assayed. Rac1-GTP and total Rac1 were determined in these HPM as described in Methods. (C) Hepatocytes were incubated for 2 min at 37°C in activated buffer free of GTPγS and supplemented or not with α_1-adr-ckt as indicated in the figure. Immediately after this incubation, HPM were prepared, and NOX catalytic activity was assayed. Rac1-GTP and total Rac1 were determined in these HPM as described in Methods. (D) HPM obtained as usual were incubated in activation buffer supplemented or not with GTPγs, α_1-adr-ckt, and different GTP concentrations as indicated in the figure. After this treatment, NOX catalytic activity was measured. (E) HPM obtained as usual were incubated in activation buffer supplemented with different GTP concentration and, when indicated, with α_1-adr-ckt. Rac1-GTP and total Rac1 were determined as described in Methods. Panels A, B, C and E show Western blot representative results of three independent experiments. Panels B, C and D show means values ± SE of three independent experiments performed in duplicate.

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buffer. The following experiment was performed with hepatocytes preincubated during 2 min in activation buffer free of GTPγS, and with or without the hormone, to analyze Rac1-GTP generation from total Rac 1. In HPM from these cells incubated without the α_1-adr-ckt, a low ratio of Rac1-GTP/ total Rac was observed, while with the α_1-adr-ckt, a higher ratio of Rac1-GTP/total Rac was evident (Figure 3C). These data suggest than in whole hepatocytes, generation of Rac 1-GTP is more dependent on α_1-AR lightening than on isolated HPM, in which a high concentration of added GTPγS prevailed over α_1-AR action. To have more information on this point, HPM were incubated with GTP, the natural substrate for Rac 1 in the hepatocyte, and Rac 1-GTP formation along with NOX2 catalytic activity were evaluated. As previously shown with GTPγS (Figure 3B), α_1-AR stimulation was absolutely required to promote H_2O_2 synthesis as a function of added GTP (Figure 3D). Rac 1-GTP was evaluated from these HPM at all of the GTP concentrations employed, irrespective of α_1-AR presence and action. A low ratio of Rac1-GTP/total Rac was observed, except at 1 mM GTP, in which total Rac 1-GTP/ Rac was higher in the presence of the α_1-adr-ckt (Figure 3E). These data indicate that, within the cell, available endogenous GTP pool and α_1-AR lightening are required to generate Rac1-GTP and to activate the dormant NOX2 enzyme; while in HPM an artificial higher concentration of GTP is sufficient to form some Rac1-GTP but, in the absence of α_1-adr-ckt, NOX2 remains catalytically inactive. Furthermore, a low GTPγS concentration is better than high GTP concentration to activate the NOX2 complex; consistent with the expected role of a non-hydrolyzable analog.

Cross talk: simultaneous activation of α_1- and β-ARs mutually impaired their physiological functions

In a recent report, it was shown that β-AR activation might decrease H_2O_2 synthesis in HPM [27], which prompted us to challenge the stimulatory action of α_1-AR generating H_2O_2, with the simultaneous activation of β-AR. A α_1-AR lightening-induced increase of H_2O_2 synthesis by NOX2 activation in isolated HPM, this was more noticeable with GTPγs than with GTP, and was completely impaired by simultaneous lightening of β-AR produced with an equimolecular concentration of adrenaline (Figure 4A). Similarly, the small increase in H_2O_2 synthesis mediated by β-AR activation was completely blocked by simultaneous α_1-AR activation (Figure 4A). The former effect is noticeable because more α_1- than β-ARs were found in liver of adult rats [42,43]. Then, cross talk phenomena that were recorded after hepatocyte activation of gluconeogenesis and ureagenesis many years ago [43] were brought up-to-date: stimulation of gluconeogenesis and ureagenesis rates by β-AR activation with isoproterenol (1 μM) was inhibited, in a concentration-dependent manner, by α_1-AR activation with phenylephrine (Figure 4B and C).

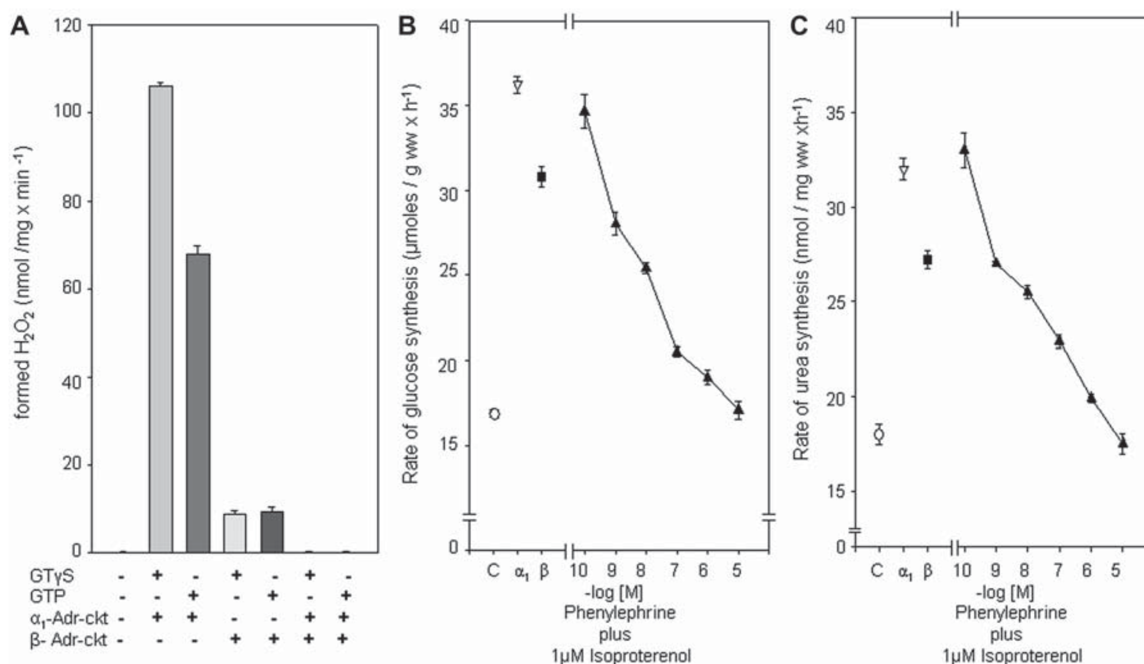

Figure 4. Cross-talk between α_1- and β-AR activating actions. (A) NOX enzymatic activity of HPM was measured in the presence of α_1-adr-ckt or β-adr-ckt, and supplemented with GTPγS or 10 μM GTP as indicated in the figure. (B, C) Hepatocytes from starved rats (glucose synthesis) and from fed rats (urea synthesis) were incubated as follows: in the absence of adr-ckt (○); with α_1-adr-ckt (▽); with β-adr-ckt (■), and with 1 μM isoproterenol plus different concentrations of phenylephrine as indicated in the figure (▲). Values represent means ± SE of three independent experiments performed in duplicate.

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Can H_2O_2 modulate adrenaline metabolic actions in hepatocytes?

Based on previous findings, we explored whether exogenous H_2O_2 might mimic H_2O_2 generated after α_1-AR stimulation and impinge upon the β-AR amplification cascade to impair their stimulatory effect on gluconeogenesis and ureagenesis. Indeed, the stimulatory effect by β-AR activation of gluconeogenesis and ureagenesis was inhibited by low concentrations of added H_2O_2. Hydrogen peroxide was active at submicromolar concentrations, whereas at micromolar concentrations, H_2O_2 effect led gluconeogenesis and ureagenesis rates to values even below the baseline (Figure 5A and B). Calculated K_i for this inhibitory action of H_2O_2 on β-AR-mediated activation of gluconeogenesis and ureagenesis was 0.1–0.2 μM (Figure 5A and B). Interestingly, H_2O_2 effect on α_1-AR activated gluconeogenesis and ureagenesis was smaller than effect on β-AR (Figure 5A and B), which coincides with the fact that α_1-AR lightening promotes much higher values of H_2O_2 synthesis than β-AR lightening (Figure 4A).

H_2O_2-described inhibitory actions on β-AR metabolic effects in hepatocytes can occur at the HPM external or internal face. A preliminary exploration on this point was made by assuming that $AgNO_3$, a described inhibitor of aquaporines [44], might inhibit the specific aquaporine identified to facilitate diffusion of H_2O_2 across membranes [45]. We found that $AgNO_3$ (30 μM) does not affect the whole signalling cascade of α-or β-adrenergic stimulation to raise glucose or urea rate synthesis, but it prevented completely the inhibitory action of H_2O_2 (20 μM) on β-AR-stimulated gluconeogenesis and ureagenesis (results not shown), suggesting the requirement of H_2O_2 transfer to act on the inner part of the hepatocyte.

Discussion

This discussion integrates the two complementary parts of this work. The first deals with the identification and activation of protein building blocks that integrate the NOX isoform to generate H_2O_2 in response to α_1-AR stimulation. The second part includes some metabolic consequences due to the action of this formed H_2O_2 acting upon the stimulated AR. The methodological strategy utilized in the first part of this work—gentle isolation of HPMs to be challenged with hormones, adrenergic antagonists, nucleotides and antibodies, alone or in combination—offered a reliable method, simpler than the use of complete cells, with the advantage of having compartmentalized the hormonal receptor, the transduction-amplifying ingredients, and the whole responding enzyme: i.e. ARs, $G\alpha_{13}$, Rac 1 and all NOX2 protein machinery to generate H_2O_2. Such a complex might be integrated into the reported adrenaline signalosome [46].

Previous data on adrenaline-mediated increase of H_2O_2 synthesis in isolated rat liver cells [47] and on NOX2 activation in HPM by α_1-AR agonists [27] were confirmed and better characterized in this work. Thus, the concentration-dependent inhibition

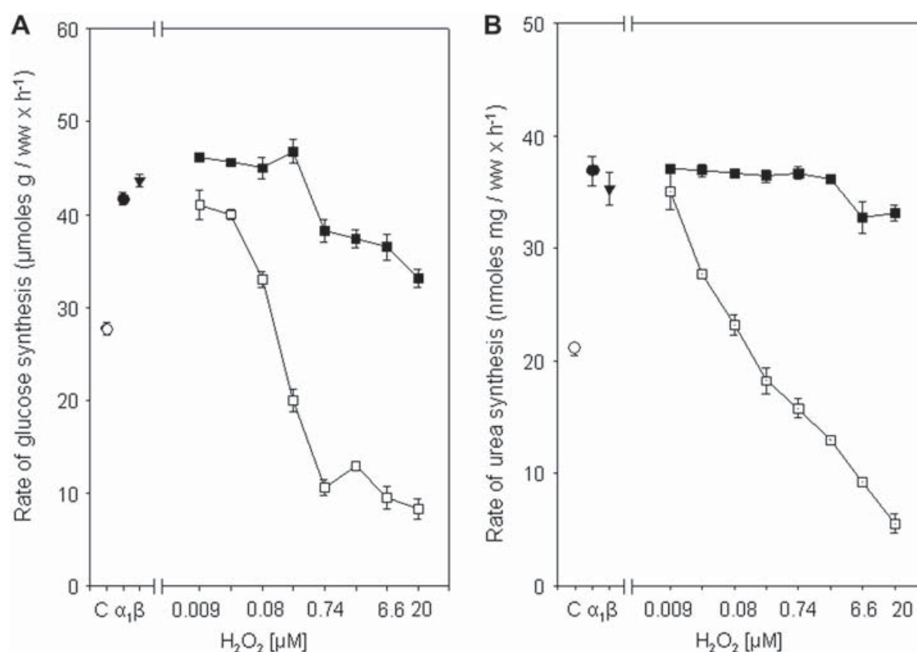

Figure 5. Inhibition by H_2O_2 of β-AR-mediated activated hepatocyte gluconeogenesis and ureagenesis. (A, B) Hepatocytes from starved rats (glucose synthesis) and from fed rats (urea synthesis) were incubated as follows: in the absence of adr-ckt (○); with α_1-adr-ckt (●); with β-adr-ckt (▼); with α_1-adr-cktal plus H_2O_2 at the concentrations of H_2O_2, indicated in the figures (■), and with β-adr-ckt plus H_2O_2 at the concentrations indicated in the figures (□). Values represent means ± SE of three independent experiments performed in duplicate.

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to α_1-AR agonist-mediated NOX catalytic activation with anti NOX2 antibodies, but not with antibodies against NOX1 and NOX4, led us to conclude that the isoform responding to α_1-AR stimulation is NOX2. Use of specific antibodies combined with Western blot analysis indicate that all proteins integrating the NOX2 complex are localized within isolated plasma membranes from hepatocytes, as it occurs in phagocytes, i.e. gp91^{phox}, p22^{phox}, p67^{phox}. p47^{phox}, p40^{phox} and Rac 1. The fact that antibodies against each of those proteins, except for p40^{phox} lowered, in a concentration-dependent form, NOX2 activation as the result of α_1-AR stimulation, indicated the requirement of all of these protein subunits to assemble NOX2 complex and to show full catalytic activity. The physiological role of p40^{phox} in modulating NOX2 activation has been questioned in phagocytes [48]; a similar question emerged in hepatocytes after analysis of results in which inhibition of NOX2 activation with anti p40^{phox} antibodies was small. Then, by use of the same experimental strategy, that is, concentration-dependent inhibition with antibodies of NOX2 activation after α_1-AR stimulation, the functional link between α_1-AR and NOX2 was identified as the Gα_{13} protein. Participation of Gα_{13} protein-mediating ROS production via NOX has been reported in rat neonatal cardiomyocytes using an equivalent methodology [49]. In these cells angiotensin receptor stimulation suggest that G$\alpha_{12/13}$-mediated ROS production through Rho, and Rac is essential for Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) activation [49].

NOX2 activation in phagocytes required the recruitment of cytosolic proteins to integrate the NOX2 complex in the phagosomal membrane [10], whereas NOX2 present in HPM freshly isolated from non-stressed rats is dormant, as takes place in non-activated phagocytes [10]. However, in HPM, NOX2 is pre-assembled with all of the protein subunits integrating the whole complex, while cells treated with the α_1-adr-ckt did not mobilize proteins from cytosol to the membrane; thus, the same amount of protein was detected both in the basal dormant state as well as in the activated complex. In this regard, the NOX2 complex in non-stimulated HPM seems to be similar to that localized in the cytoskeleton of non-stimulated cultured endothelial cells, in which all subunits integrating the NOX2 complex were already present in the dormant state [50]. With respect to the binding of GTPγS or GTP to Rac 1, our results are conclusive in α_1-AR stimulated hepatocytes when the cellular GTP pool is the only substrate available for coupling to Rac 1. However, in HPM, when an excess of added GTPγS or GTP is available, the nucleotide binds to Rac 1 even in the absence of adrenaline, although NOX2 activation is absent.

Addition of the α_1-adr-ckt to the GTP-enriched incubation mixture is absolutely required to observe NOX2 activation in HPM. These experiments indicate that switching on α_1-AR elicited the GTP binding to Rac 1, and fired an additional mechanism to activate dormant NOX2 in hepatocytes. Based on copious information on the modulation of signalling cascades by scaffold proteins [51–53] and in the eventual α_1-AR-conformational change upon receiving its agonist [53], an interaction between some scaffold proteins and the just now activated α_1-AR might be proposed as a candidate for this unidentified adjustment, which will ensure NOX2 activation.

A noticeable characteristic of the experimental model employed here is that the effect elicited in proteins from HPM, by compounds added to the acti-vation buffer (agonists, antagonists, antibodies), persisted even after these HPM were centrifuged and suspended in the catalysis buffer deprived of such compounds. Thus, measurements of NOX2 catalytic activity reflected the opposite interaction of α_1-AR agonists—activating—and that of antibodies—inhibiting—specific proteins from NOX2 system. In addition, NOX2 activation by α_1-AR turned on in isolated HPM was linear with time up to 4 min [27], at which time a plateau was reached. In this regard, such an immediate response was similar to the 1-min ceramide synthesis and phosphorylation of p47^{phox}, and required the later activation of a not fully identified NOX isoform (NOX1 or NOX2) in hepatocytes exposed to hyperosmolar conditions [18].

We observed along this work increases in NOX(s) activity independent of the adrenaline route when isolated hepatic plasma membranes were: (a) not used immediately but maintained on ice for 45–60 min; (b) used after freeze-thawing and (c) used immediately after preparation as usual, but provided by stress-subjected rats. Neither the stimulatory agent, i.e. the activated NOX isoform, nor the mechanism(s) of activation were studied here in order to clarify the observed increase, which was insensitive to GTPγS and/or adrenaline activation.

The main results from the experiments in Figure 4 demonstrate reciprocal cross talk in AR responses: the ability of α_1-AR to generate H_2O_2 was abolished by turning on β-AR, and the increased rates of gluconeogenesis and ureagenesis observed after β-AR stimulation were inhibited by lightening α_1-AR. It is noteworthy that exogenously added H_2O_2 prevented the raised rates of gluconeogenesis and ureagenesis promoted by β-AR, in a similar fashion to the way α_1-AR activation accomplished it. The effect was concentration-dependent, and the approximate K_i was below micromolar range.

The scheme in Figure 6 included a proposal to integrate the results presented in this work in a hypothetical adrenaline signalosome, and to advance in a probable pathway for the negative cross talk observed in α_1-AR-generating H_2O_2 to inhibit β-AR-mediated activation of gluconeogenesis and

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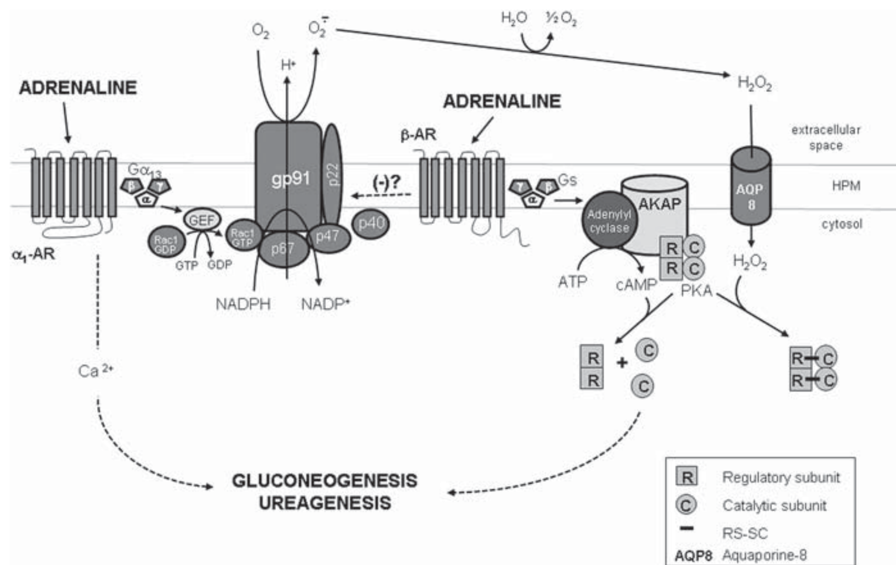

Figure 6. Hypothetical signalosome showing molecular steps underlying the negative cross talk between α_1-AR lightening, via H_2O_2, on β-AR-mediated stimulation of hepatic gluoneogenesis and ureagenesis. Findings of present work were included to the scheme along the discussion. Two details were not mentioned previously and merit a comment: between $G\alpha_{13}$ and Rac 1-GTP, a RhoGTPase nucleotide exchange factor (GEF) was included based on information from Kreutz et al. [54], and AKAP-adenylyl cyclase were drawn into contact after identification of these types of complexes according to Dessauer [55].

ureagenesis. Lightening α_1-AR activates both a Ca^{2+}-and a protein-kinase C (PKC)-amplification cascade to accelerate gluconeogenesis and ureagenesis [46] and NOX2 to generate extracellular H_2O_2 (Figure 5). Lightening β-AR includes a cAMP-mediated amplification chain to activate protein kinase A (PKA) and to accelerate gluconeogenesis and ureagenesis in the end [43], and an unknown pathway to inhibit H_2O_2 synthesis (Figure 4A). We reported previously that H_2O_2 reacts with type II PKA holoenzyme and oxidizes a –SH from Cys-199 in a catalytic subunit and –SH from Cys-97 in the regulatory subunit to constitute an –S–S– bond that impairs the cAMP-activating role in PKA holoenzyme [13]. Phylogenetic analysis of catalytic and regulatory PKA subunits showed that Cys-199 in catalytic subunits is strictly conserved in all reported cAMP-dependent PKA. In contrast, Cys-97 in regulatory subunits is conserved only in RIIα and in β ortholog proteins, but not in RIα and β ortholog proteins, which possess Ser at this position [13]. In consequence, H_2O_2 inhibitory action was observed with the type II PKA holoenzyme, either the α-subtype from bovine heart (containing the regulatory II α-ortholog protein) or the β-subtype from rat adipocytes (containing the regulatory IIβ-ortholog protein [13]). H_2O_2-mediated inhibitory action was not observed with type I PKA from muscle formed with regulatory subunit type I [21]. Here, we suggest that the inhibitory action of H_2O_2 could be extended to PKA type II from rat liver [56] (Figure 6). Such inhibition will prevent target proteins from being phosphorylated by active catalytic PKA subunits, and gluconeogenesis and ureagenesis will be lowered, as may be observed in Figures 4 and 5. Results with $AgNO_3$ impairing the inhibitory effect of added H_2O_2 on β-AR-activated gluconeogenesis and ureagenesis (Figure 5) led us to include, in the hypothetical scheme (Figure 6), the presence of an aquaporine-8 to incorporate H_2O_2 generated by NOX2 in the extracellular space after its stimulation by α_1-AR agonists [57]. In this way, H_2O_2 might be channelled towards PKA type II complexed with some protein kinase-anchoring protein (AKAP) targeted to plasma membrane [58]. The spatial interaction of proteins comprised in the scheme of Figure 6 appears to be a requirement to insure proper protein function in a larger adrenaline signalosome [46].

In conclusion, lightening α_1-AR promotes H_2O_2 synthesis in HPM by activating a dormant NOX2. All protein subunits from this enzyme system are already integrated into the membrane, and its activation required the presence of α_1-AR agonist and Rac 1-GTP formation. Thus formed, or added, H_2O_2 impaired a rise in the rate of β-AR stimulation-mediated gluconeogenesis and ureagenesis. Based on previous work, we propose that H_2O_2 generated by α_1-AR activation oxidizes highlighted cysteines from catalytic and regulatory PKA subunits to form a disulfide bridge between them in order to hinder PKA activation by cAMP, which is generated in response to β-AR stimulation. A non-activated PKA cannot increase the gluconeogenesis and ureagenesis rate. One step in understanding a negative cross talk is proposed; nevertheless, a fundamental question remains: How can the hepatocyte integrate a single metabolic response after adrenaline stimulation of all of the AR present in this cell?

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Declaration of interest

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