Free Radical Research, June 2007; 41(6): 663–672

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healthcare

Adrenaline stimulates H_2O_2 generation in liver via NADPH oxidase

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Accepted by Professor N. Taniguchi

(Received 24 November 2006; in revised form 15 January 2007)

Abstract
It is known that adrenaline promotes hydroxyl radical generation in isolated rat hepatocytes. The aim of this work was to investigate a potential role of NADPH oxidase (Nox) isoforms for an oxidative stress signal in response to adrenaline in hepatocytes. Enriched plasma membranes from isolated rat liver cells were prepared for this purpose. These membranes showed catalytic activity of Nox isoforms, probably Nox 2 based on its complete inhibition with specific antibodies. NADPH was oxidized to convert O_2 into superoxide radical, later transformed into H_2O_2. This enzymatic activity requires previous activation with either 3 mM Mn^{2+} or guanosine 5′-0-(3-thiotriphosphate) (GTPγS) plus adrenaline. Experimental conditions for activation and catalytic steps were set up: ATP was not required; $S_{0.5}$ for NADPH was 44 μM; $S_{0.5}$ for FAD was 8 μM; NADH up to 1 mM was not substrate, and diphenyleneiodonium was inhibitory. Activation with GTPγS plus adrenaline was dose- and Ca^{2+}-dependent and proceeded through α_1-adrenergic receptors (AR), whereas β-AR stimulation resulted in inhibition of Nox activity. These results lead us to propose H_2O_2 as additional transduction signal for adrenaline response in hepatic cells.

Keywords: *Adrenergic receptors, adrenaline, hydrogen peroxide, liver cell membranes, NADPH oxidase (Nox), ROS signaling*

Abbreviations: *AR, adrenergic receptors; DPI, diphenyleneiodonium chloride; EHCPM, enriched hepatic-cell plasma membranes; Glc 6-Pase, glucose 6-phosphatase; GPCR, G protein-coupled receptors; GTPγS, guanosine 5′-0-(3-thiotriphosphate); Nox, NADPH oxidase; ROS, reactive oxygen species; SuccDH, succinate dehydrogenase*

Introduction

Generation of reactive oxygen species (ROS) in phagocytes as its microbe-killing mechanism involved a catalytic subunit $gp91^{phox}$, a stabilizer subunit $p22^{phox}$, and some modular proteins including $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and the small GTPase Rac [1]. Catalytic subunit $gp91^{phox}$, also known as NADPH oxidase 2 (Nox 2), is embedded in phagosome membrane, FAD and NADPH binding sites are located in its cytoplasmic domain. In resting phagocytes, Nox 2 is dormant but becomes activated during phagocytosis to produce the superoxide radical, which by dismutation generates hydrogen peroxide [2,3]. Identification of ROS-generating oxidases in non-phagocytic cells, as well as increased information available in genome databases, led to the discovery of proteins with the homology with specific structural regions of $gp91^{phox}$, then grouped into Nox family [4–6]. For mammals, there are currently seven members of the family: Nox 1 through Nox 5, and

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2007 Informa UK Ltd.
DOI: 10.1080/10715760701268751

the dual oxidases Duox 1 and Duox 2 [3]. Function and expression regulation of these proteins in non-phagocytic cells are under study. Initial evidence of the presence of NADPH oxidase in liver was published in 2001 and deals with the presence of Nox 4 and 5 isoforms in human fetal liver [7]. A recent report showed that isolated rat hepatocytes cultured for 24 h expressed mRNAs of Nox 1, Nox 2 ($gp91^{phox}$), Nox 4, Duox 1, and Duox 2, as well as the regulatory subunit $p47^{phox}$; in addition, $gp91^{phox}$ and $p47^{phox}$ were also identified at the protein level [8]. In such experiments, activation of NADPH oxidase isoforms was associated with CD95 ligand-induced apoptosis in rat hepatocytes [8]. Once the presence of mRNA for several Nox and the existence of $gp91^{phox}$ in hepatic cells were demonstrated, it was decided in the present study to look for the identification of a catalytic activity for NADPH oxidase isoforms in these cells. In previous studies, it has been shown that adrenaline promotes, in a concentration-dependent manner via the activation of α_{-1B} adrenoceptors, hydroxyl radical production in rat hepatocytes [9]. Therefore, the initial goal of this study was to investigate the role of Nox isoforms from liver cells in generate ROS, and then to prove a potential activation of these isoforms through the stimulation of G protein-coupled receptor (GPCR) responding to adrenaline.

Materials and methods

Materials

MES [2-(*N*-morpholino)ethanesulfonic acid], $MgCl_2$, $MnCl_2$, $CaCl_2$, KCl, NaCl, $NaHCO_3$, MOPS [4-morpholinepropanesulfonic acid], GTPγS, KH_2PO_4, NaN_3, FAD, NADPH, trichloroacetic acid, collagenase, diphenyleneiodonium chloride (DPI), adrenaline, ATP, NADH, cytochrome *c*, prazosin, yohimbine, rauwolscine, propranolol and bovine serum albumin were obtained from Sigma-Aldrich Co. St Louis, MO, USA; protease inhibitor cocktail from MP Biochemicals, Inc. Solon, OH, USA; antibodies against Nox 1, Nox 2, and Nox 4 proteins {Nox 1 (H-75): sc-25545, gp91-phox, (K-15): sc-5826, and Nox-4 (N-15): sc 21860} from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA.

Cell isolation

All animal experiments were conducted according to the Federal Guidelines for the Care and Use of Animals (NOM 062-Z00-1999, Ministry of Agriculture, Mexico) and the study protocol was approved by the Institutional Committee of the National Autonomous University of Mexico's (UNAM's) Faculty of Medicine. Male Wistar rats of 200–250 g body weight fed *ad libitum* and with free access to water were used. Hepatic cells were isolated by collagenase digestion in 10 mM glucose dissolved in Ca^{2+}-free Ringer Krebs bicarbonate, pH 7.4 [10], and viability was determined by trypan blue exclusion; experiments were performed when >90% of cells excluded the dye. *In vivo* treatment with $GdCl_3$ was followed with some animals to prepare isolated hepatocytes deprived of Kupffer cells, the resident hepatic macrophages [11]. In our research group's previous experiments, this was verified by commercial mouse monoclonal antibody (Serotec, Oxford, UK), which specifically recognizes a membrane glycoprotein in leukocytes [12].

Hepatic-cell plasma membrane preparation

Isolated hepatic cells from a liver maintained in 10 ml of Ca^{2+}-free Ringer Krebs bicarbonate, pH 7.4, were supplemented with 1 ml protease inhibitor cocktail and homogenized in a Teflon–glass Potter Elvehem 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 5 min. The lysate was subjected to the protocol summarized in Figure 1, and the corresponding results are reviewed in the Results section.

Activation of Nox from enriched hepatic-cell plasma membranes

Enriched hepatic-cell plasma membranes (EHCPM, precipitate 3 in Figure 1) showed scarce H_2O_2

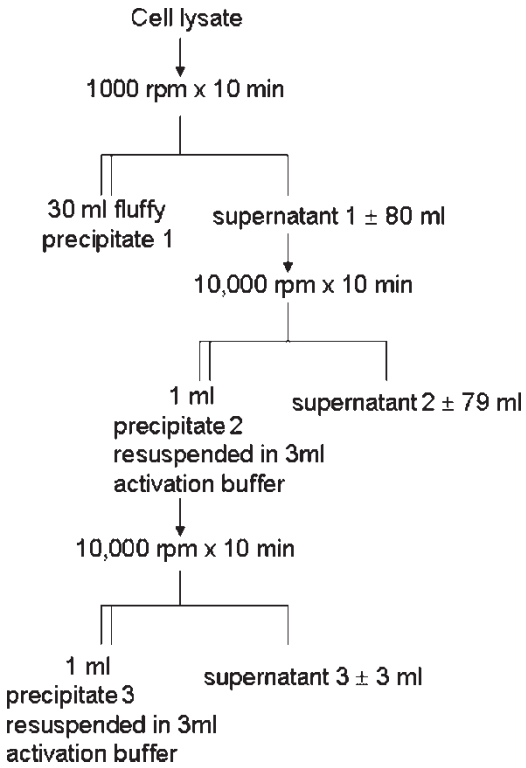

Figure 1. Summarized protocol to prepare enriched hepatic cell-plasma membranes from isolated rat liver cells.

generation in the presence of NADPH, in a similar manner to that of phagocytes in the absence of phagocytosis. Activation of a dormant Nox enzyme in this preparation appears desirable. After several assays, the following regular protocol to activate the enzyme was adopted: EHCPM (20 μg protein) were resuspended in up to 1 ml of activation buffer (containing 120 mM NaCl, 10 mM $NaHCO_3$, and 1.4 mM $CaCl_2$ dissolved in 30 mM [4-morpholine-propanesulfonic acid] (MOPS) adjusted to pH 7.4) and incubated for 2 min at 37°C with 3 mM $MnCl_2$. In order to study further important aspects of hepatic Nox activation, experiments were conducted using different concentrations of Mn^{2+} or Ca^{2+} in the activation buffer, to which one or more of the following reactants including GTPγS, ATP, commercial antibodies against Nox isoforms, diphenyleneiodonium chloride (DPI, a non-specific Nox inhibitor), adrenaline, and selective antagonists for particular AR, were added, maintaining the final volume constant. The activation step ended with centrifugation at 10,000 rpm during 5 min, the supernatant was discarded, and the pellet was the enzyme source for the Nox-isoform catalytic step.

Enzymatic assay

Immediately after activation, membranes were resuspended in 540 μl of catalysis buffer (containing 120 mM NaCl, 1.2 mM KH_2PO_4, 1 mM NaN_3, and 100 μM FAD dissolved in 20 mM MES, pH 6.0) at 37°C. Enzymatic reactions were started by addition of 60 μl of 0.25 mM NADPH dissolved in 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 used to quantitate H_2O_2 as described by Fioravanti [13]. In selected experiments addition of trichloroacetic acid was omitted and the enzymatic activity was determined by measuring the disappearance of NADPH at 340 nm. Furthermore, to prove that superoxide radical was formed prior to H_2O_2, ferricytochrome *c* was included in the incubation mixture before the addition of NADPH to trap the generated superoxide radical and to prevent its conversion into H_2O_2, as suggested by Allen [14].

Other assays

Succinate dehydrogenase (SuccDH) catalytic activity in liver fractions was measured as described by Scott et al. [15], and glucose-6-phosphatase and 5′ nucleotidase activity in the same liver fractions was measured as detailed by Aronson and Touster [16]. Protein concentration was determined with the Bradford method [17].

Statistical analysis

Values are expressed as means ± standard error (SE). Student *t*-test was applied to assess intergroup differences.

Results

Identification of NADPH oxidase activity

In preliminary experiments, a low but measurable activity consuming NADPH and forming H_2O_2 was detected in the supernatant of the cell lysate obtained after 10 min of centrifugation at 1000 rpm as outlined in Figure 1. The procedure presented in Figure 1 was followed to enrich the fraction containing plasmatic-cell membranes, and data on the presence of characteristic enzymes located in sub-cellular membranes [16] are included in the table. Comparing precipitate 3 with lysate data, it can be observed that 5′ nucleotidase activity, an index of cell-plasma membrane, increased its specific activity and there was a 25% recovery (Table I). Contrariwise, SuccDH from mitochondrial membrane and glucose 6 phosphatase from endoplasmic reticulum decreased their specific activity, and their recovery was 5% or even less in the same fraction identified as precipitate 3 (Table I). Therefore, despite a low yield of plasma membranes we decided to use precipitate 3 as a source of Nox

Table I. Specific and total activity of marker enzymes in sub-cellular membranes recovered from protocol in Figure 1.

	Specific activity⋆			Total activity†		
Fraction	5′ nucleotidase	Glc 6-Pase	SuccDH	5′ nucleotidase	Glc 6-Pase	SuccDH
Cell lysate	246.0 ± 6.0	0.119 ± 0.015	0.042 ± 0.009	11.7 ± 0.26 (100)‡	6.0 ± 0.06 (100)	2.20 ± 0.40 (100)
Precipate 1	165.0 ± 1.0	0.078 ± 0.008	0.008 ± 0.001	4.8 ± 0.08 (41)	6.3 ± 0.45 (105)	0.40 ± 0.08 (18)
Supernatant 1	62.0 ± 4.0	0.056 ± 0.009	0.008 ± 0.001	1.0 ± 0.10 (9)	2.1 ± 0.70 (35)	0.24 ± 0.07 (11)
Precipitate 2	196.0 ± 8.0	0.124 ± 0.019	0.015 ± 0.004	0.05 ± 0.02 (.5)	0.7 ± 0.20 (12)	0.03 ± 0.01 (1)
Supernatant 2	62.0 ± 4.0	0.0	0.017 ± 0.003	0.9 ± 0.06 (1)	0.0 (–)	0.10 ± 0.02 (5)
Precipitate 3	3454.0 ± 50.0	0.07 ± 0.009	0.006 ± 0.001	3.0 ± 0.08 (26)	0.3 ± 0.05 (5)	0.06 ± 0.03 (3)
Supernatant 3	65.0 ± 4.0	0.0	0.0	0.03 ± 0.01 (.3)	0.0 (–)	0.0 (–)

⋆ In μmoles/mg prot × min^{-1}; † in μmoles/min per total fraction; ‡ in parenthesis values in per cent, assuming 100 in cell lysate and the relative recovery of total activity in other fractions. Values are means ± standard error (SE) of three independent experiments.

isoforms. This protocol was simple, fast, and reproducible. The activation procedure as described in Materials and Methods was inspired by the activation of Nox enzyme from human adipocytes [18]; in particular, 3 mM $MnCl_2$ was included in the activation buffer, which potentiated the effects of GTP in some systems [19,20]. Thereafter, aliquots containing 20 μg of protein from plasma membranes were incubated for 2 min at 37°C in the activation buffer. Samples were spun at 10,000 rpm for 5 min, the supernatant was eliminated, and the precipitate was resuspended in catalysis buffer to measure enzymatic activity. Conditions for measuring enzymatic activity were established: pH for activation and catalysis steps, linear dependency for protein concentration, and times for measuring activation and catalysis within the linear section of the curve (Figure 2). ATP (1 mM) decreased the herein reported generation of H_2O_2 (Figure 2), probably by chellating Mn^{2+} ions required for the activation step (Figure 3).

Of paramount relevance is the fact that activation of the system and the enzymatic activity of Nox isoforms were observed in isolated hepatic cells that had been devoided of Kupffer cells by treatment with $GdCl_3$, i.e. the resident hepatic macrophages are not mainly involved in the measured enzymatic activity, as indicated in further experiments of this work in which 0.5 Vmax were quite similar in hepatic cell samples in the presence or absence of Kupffer cells.

Further characterization of the system

Increasing Mn^{2+} concentrations for the activation step in Nox catalytic activity is shown in Figure 3A. Results suggest that Nox isoform activation is directly related with the logarithm of Mn^{2+} supraphysiologic concentrations employed, which is observed by an increase in catalytic activity measured as H_2O_2 generation. In this work, 3 mM of Mn^{2+} was used for to activate the system, as described in other cases [18–20]. The first product of NADPH oxidase is the superoxide radical instead of H_2O_2; to assess this in hepatic Nox isoforms, we conducted an experiment (Figure 3B), i.e. increasing ferricytochrome *c* concentrations were added to the complete incubation mixture prior to addition of the substrate, thus reacting with generated superoxide and avoiding H_2O_2 formation. At the end of the reaction (4 min incubation with NADPH), the H_2O_2 amount was compared with and without cytochrome *c*. As observed in Figure 3B, cytochrome *c* (Fe^{3+}) reacted with the superoxide radical, and this avoiding H_2O_2 synthesis. In another set of experiments, we conducted NADPH disappearance at 340 nm, finding that the oxidized NADPH was approximately equal to the H_2O_2 formed. The results obtained on modifying NADPH, NADH, and FAD concentrations in our experimental system are presented in Figure 4A and B. Mn^{2+}-activated Nox isoenzyme activity demonstrated a classic Michaelis kinetics on raising NADPH and FAD concentrations. NADPH is indispensable for registering enzymatic activity; the required concentration for obtaining 0.5 Vmax falls within the range of 44 μM this value changed to 69 μM in membranes prepared from rats previously treated with $GdCl_3$, (Figure 4A), thus emphasizing the idea of the impossibility of Kupffer cells as enzyme source for the herein reported experiments. In experiments reported in this work, we utilized a final concentration of 250 μM NADPH. In the absence of FAD, we obtained a basal activity of approximately 6% of the maximum activity recorded on modifying FAD concentration in the catalytic buffer. The concentration required for obtaining 0.5 Vmax was 8 μM, and we added a final concentration of 100 μM during the remainder of this work (Figure 4B). NADH assayed up to concentrations of 1 mM was not employed as the substrate of this H_2O_2-generating system (Figure 4A). It has been consistently reported that DPI is a Nox inhibitor [21]; when it was added during the activation stage to concentrations of 1×10^{-6} M, it inhibited H_2O_2 generation by 50%, the inhibition greater at greater DPI concentrations.

Adrenergic receptors and Nox activity

During the activation stage of the system with $MnCl_2$, we added adrenaline and additionally substituted the supraphysiologic concentration of Mn^{2+} with the GTP analog, GTPγS, which maintains protein G activation. Results demonstrated that activation of the system with Mn^{2+} elevated enzymatic activity, and that this catalytic activity rose even more on adding adrenaline, while activation of the system with GTPγS did not increase enzymatic activity but was elevated on additionally adding adrenaline (Figure 5). The experiment was repeated, eliminating Mn^{2+} from the activation buffer and adding GTPγS, supplemented or not, with increasing concentrations of adrenaline. Basal enzymatic activity rose proportionally to the adrenaline concentration employed in the experiment (Figure 6). Subsequently and by use of antagonists selected for each of the participating AR sub-types, we investigated the receptor involved in the studied response. We utilized prazosin to inhibit α_1-ARs, yohimbine, and rauwolscine to inhibit α_2-ARs, and propranolol to inhibit β-ARs [22]. The procedure we employed comprised stimulating the system by supplementing the activation buffer with GTPγS, adrenaline, and a cocktail with three selective AR antagonists to inhibit two adrenoreceptor sub-types; thus in each case, only the AR sub-type to which no antagonist was added could be activated with adrenaline. The receptor cited in Figure 7 is that which was not inhibited and that in case of being activated could

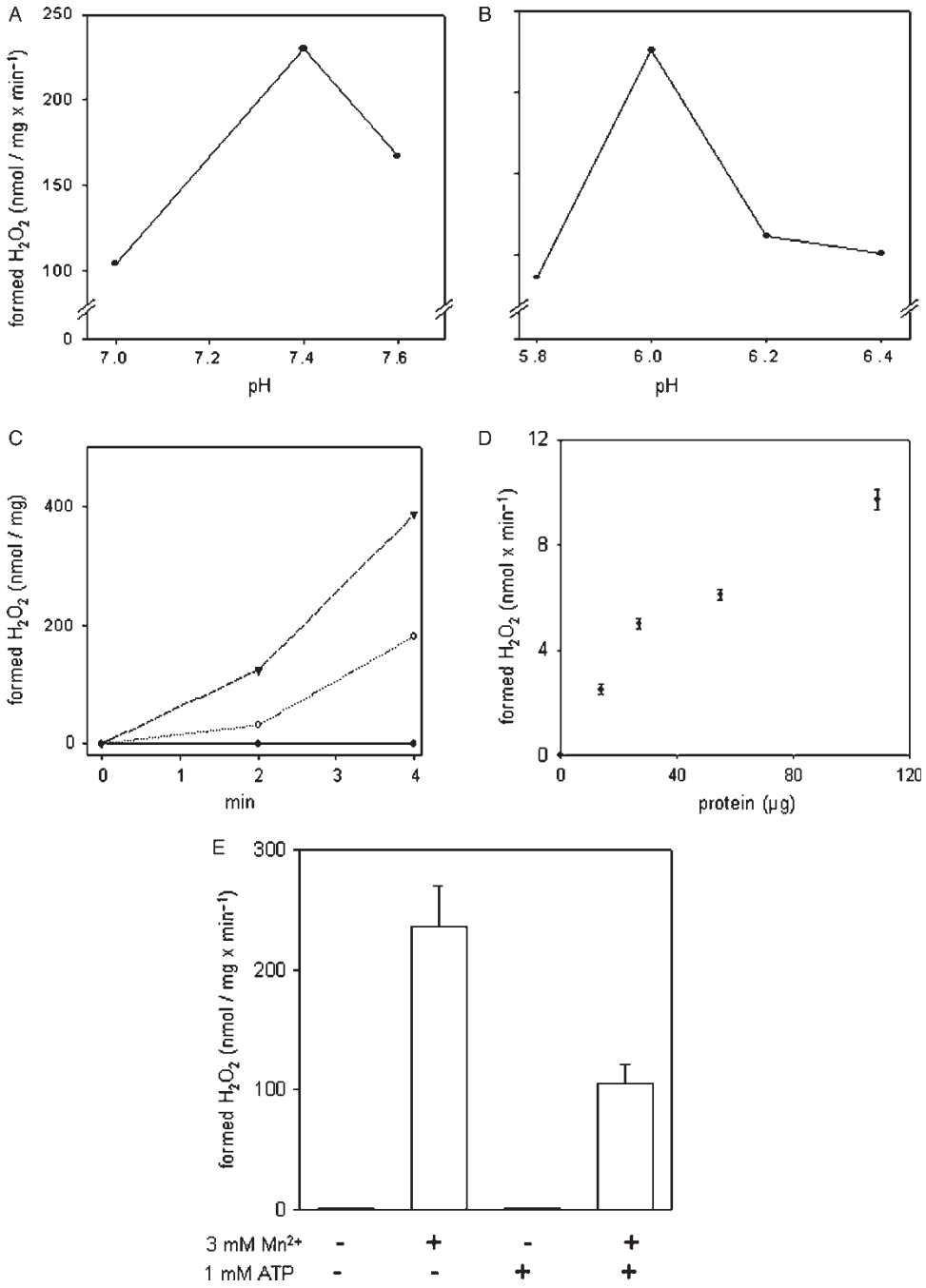

Figure 2. Basic experimental conditions to generate H_2O_2 by an NADPH oxidase present in plasma membranes (fraction 3, Figure 1), preparated from isolate rat hepatocytes. In all cases, generation H_2O_2 was used to detect the enzymatic activity, maintaining identical experimental conditions for the experiments in each panel in which one single variable was tested. A. pH activation: activation time 25 min, catalysis time 10 min, protein concentration 100 µg, catalysis buffer pH 6.0. B. pH catalysis: identical conditions as in A but activation buffer pH 7.4. C. Enzymatic reaction as a function of time: activation buffer pH 7.4, catalysis buffer pH 6.0, protein concentration per sample 10 µg, formation of H_2O_2 as a function of time after 0 min (•), 1 min (○), or 2 min (▼) of activation. D. protein concentration dependency of the reaction: activation time 2 min, catalysis time 4 min, activation buffer pH 7.4, catalysis buffer pH 6.0. E. Effect of ATP: identical conditions as in D but using 20 µg of membrane protein per experimental tube, Mn^{2+} and ATP were included to the activation step. Typical experiments performed in duplicate are present in panels A, B, and C. Average of duplicate experiments repeated three times are presented in panels D and E.

respond to the presence of adrenaline. With the procedure employed, maximum response to adrenaline was observed on stimulating the α_1-ARs. On the other hand, it is interesting that ARs α_1, α_{2A}, and α_{2B}, on remaining inhibited, afforded an observation of inhibition in H_2O_2 synthesis by NADPH (Figure 7); this inhibition disappeared with the addition of propranolol. Both data suggest that activation of AR β produces Nox isoform activity inhibition and a lower generation of H_2O_2, nearly 60%, in relation to the

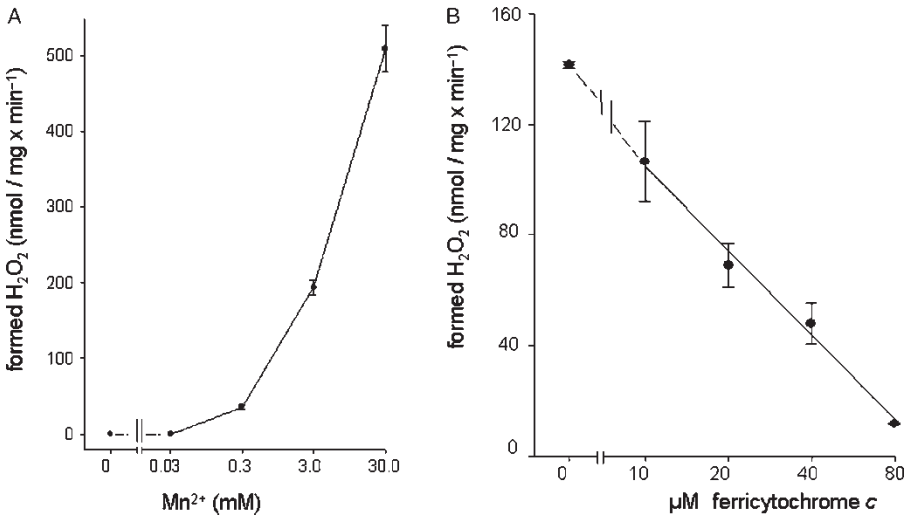

Figure 3. A: NADPH oxidase (Nox) isoform enzymatic activity of enriched hepatic-cell plasma membrane (EHCPM) previously activated with increasing concentrations of Mn^{2+} as indicated in the figure, added to the activation buffer. B: Inhibition of H_2O_2 generation by EHCPM previously activated with 3 mM Mn^{2+} in the presence of increasing concentration of ferricytochrome *c* as indicated in the figure, and added to the catalysis buffer. Values are means ± standard error (SE) of three independent experiments.

basal activity (Figure 7). Figure 6 also depicts the dose-response curve of the α_1 receptor to adrenaline in the presence of selected antagonists for ARs α_2 and β. The response is very similar to that obtained in the absence of the selected antagonists, indicating an abundance of AR α_1 in the generation of H_2O_2 from NADPH in rat-hepatocyte plasmatic membranes, and that AR β-mediated inhibition in Nox isoform activity was solely observed on maintaining AR α_1 inactive. An initial attempt to identify a Nox isoform responsible for the described enzymatic reaction was conducted using commercial antibodies against Nox 1, Nox 2, and Nox 4 proteins. Results in Figure 8 suggest that Nox 2 is the main catalytic isoform present in liver exhibiting NADPH oxidase activity under the experimental conditions detailed in this work. Additional experiments are required to interpret the results obtained for Nox 1 and Nox 4 (Figure 8), as well as to identify in liver samples the presence of other Nox 2 activity-associated proteins in phagocytes, such as $p22^{phox}$, Rac, $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$. Finally, we found that the effect of 1 µM adrenaline on α_1-ARs promoting H_2O_2 synthesis is partially dependent on Ca^{2+}: on eliminating $CaCl_2$ from the activation buffer, the response was 60% less than in the presence of the cation. In addition, the effect of 1 µM of adrenaline was completely blocked by the addition of 10 µM DPI during the activation stage.

Discussion

In this work, we reported on the enzymatic system present in the EHCPM whose functional capacity based on information available for phagocytes [3] corresponds to that of Nox isoforms. These findings increase information on the presence of mRNAs for

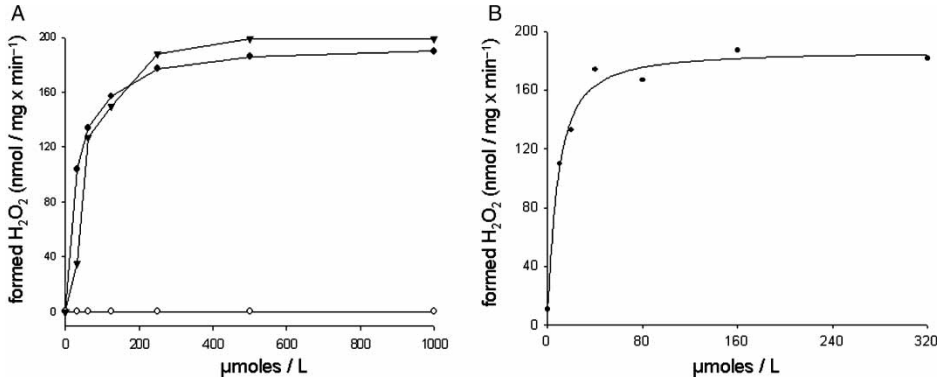

Figure 4. Nox isoform enzymatic activity of EHCPM previously activated with 3 mM Mn^{2+} as a function of increasing concentrations of NADPH in fraction prepared from control (•) or GdCl (▼) treated rats, and NADH (○) in panel A, and FAD in B. Data in the figures are representative of three independent experiments with very similar results.

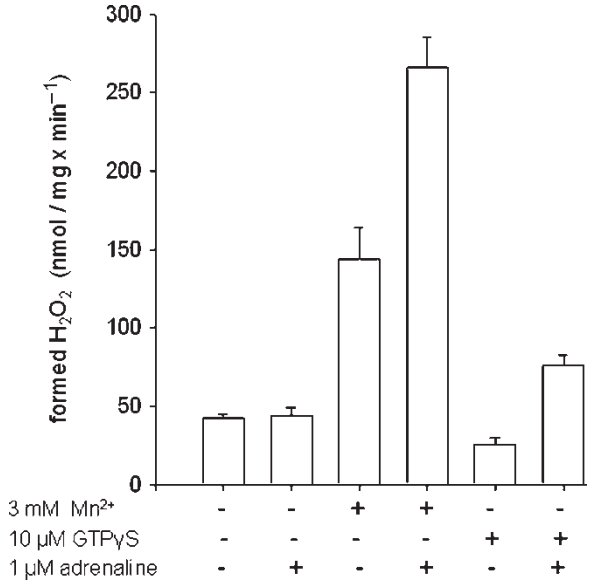

Figure 5. Effect of 1 µM adrenaline on Nox isoform enzymatic activity from EHCPM previously activated with either Mn^{2+} or GTPγS as indicated in the figure. Values are means ± standard error (SE) of three independent experiments.

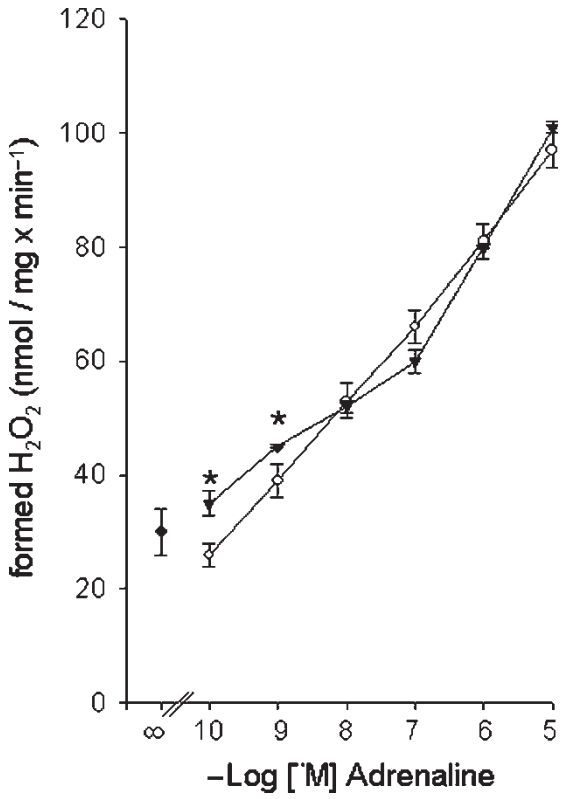

Figure 6. Dose response of increasing concentrations of adrenaline on Nox isoform enzymatic activity from EHCPM previously activated with 10 µM guanosine 5′-0-(3-thiotriphosphate) (GTPγS). Adrenaline alone (○), and adrenaline plus 0.1 µM of the following adrenergic receptor (AR) antagonists: yohimbine; rauwolscine, and propranolol (▼). Values are means ± standard error (SE) of three independent experiments. Statistically different values comparing results of adrenaline alone vs. adrenaline plus AR antagonists: $\star P < 0.05$.

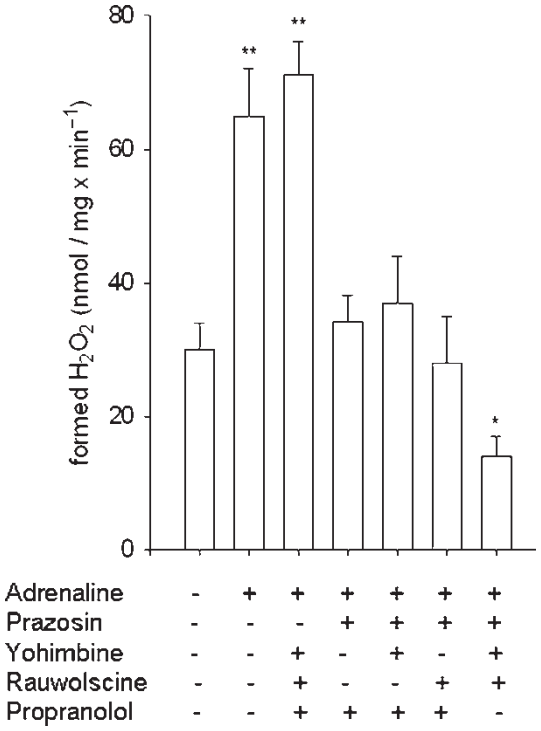

Figure 7. Effect of four adrenergic receptor (AR) selective antagonists on Nox isoform enzymatic activity from EHCPM previously activated with 10 µM GTPγS. Adrenaline was used at a final concentration of 1 µM. Antagonists indicated in the figure were used at a final concentration of 0.1 µM. Values are means ± standard error (SE) of three independent experiments. Statistically different values comparing results with basal values: $\star P < 0.05$; $\star\star P < 0.001$.

Nox 4 and 5 isoforms in human fetal liver [7] and for Nox 1, Nox 2, and Nox 4 in cultured rat hepatocytes [8]; in these cell cultures, Nox 2 and $p47^{phox}$ were also identified by Western blots assays [8]. According to our findings, the liver NADPH oxidase system is dormant and requires an activation stage, and the greatest specific activity was obtained in enriched plasmatic membranes (Figure 1). Enzymatic activity increases with exogenous FAD (Figure 4B) and is inhibited by DPI (see Results), which suggests the presence of a flavoprotein in the exchange of electrons within NADPH—that disappears on following the spectrophotometric reaction—and the molecular oxygen from which the superoxide radical is formed that can be bound by ferricytochrome *c* (Figure 3B), or that can even react with H_2O to generate H_2O_2. NADPH was not substituted by NADH (Figure 4A). The reaction disappears on heating the protein; this possesses an optimal pH of 7.4 for the activation stage and of 6.0 for the catalytic stage; it is time-linear (Figure 2) and persists on eliminating *in vivo* rat liver Kupffer cells (Figure 4A).

Basal Nox activity in the absence of activation can be the response to the moderate stress state in which rats are found and that perhaps corresponds to a part of *in vivo* Nox activity regulation. On taking

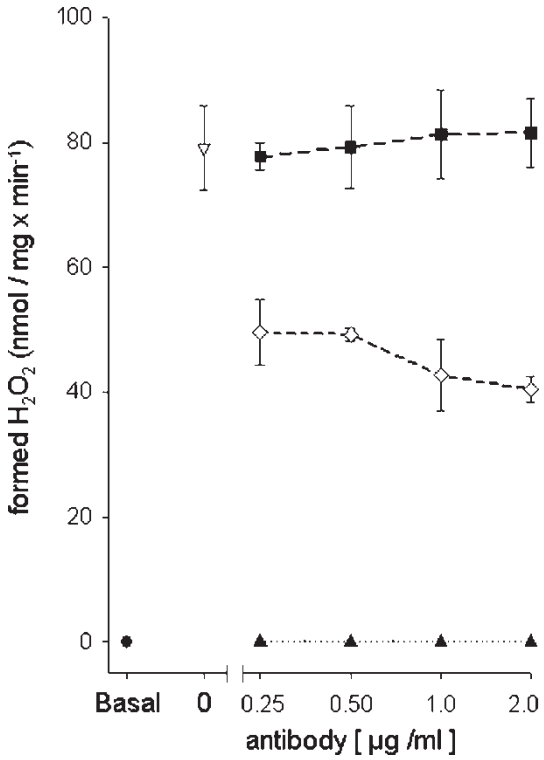

Figure 8. Effect of commercial antibodies prepared against particular NADPH oxidase (Nox) isoforms (Nox 1◇----◇, Nox 2▲----▲ and Nox 4 ■----■) on NADPH oxidase enzymatic activity present in EHCPM activated with 10 µM (GTPγS) and a cocktail of 1 µM adrenaline plus 0.1 µM of the following antagonist: yohimbine; rauwolscine, and propranolol. Values are means ± standard error (SE) of three independent experiments.

conditions in rat management to the extreme, basal activity reached nearly zero. With these basal values, the strength of this work was oriented toward reviewing the NADPH oxidase *in vitro* activation present in EHCPM. Use of a supraphysiologic concentration of Mn^{2+} or the addition of GTPγ S allows us to suggest that the initial activity of some G protein occasions the later activation of the catalytic subunit of some Nox isoform. This is supported by previous work in phagocyte cells: Rac-2 can directly bind with cytochrome b_{558}, comprising Nox 2 and $p22^{phox}$ in a GTP-dependent manner [23], and recent work has shown that cytosol-recruited Rac GTPases play a central role in regulating ROS production and other neutrophyl functions [23–25]. In liver cells, it might be argued that G proteins are already present in the plasma membranes and are coupled with hormonal receptors, among others, with adrenergic receptors. In the case of Mn^{2+}, G protein activation is sufficient to demonstrate catalytic activity and can be equivalent to the activation of other Nox with phorbol esters [26] or with arachidonic acid [3]. It is noteworthy that Nox activation with 3 mM $MgCl_2$ is sensitive to the presence of 10^{-6} M adrenaline (Figure 5), which permits us to suppose that the activated protein G is interacting on the one hand with the hormone-receiving AR, and on the other with the cytochrome b_{558} that responds by increasing its activity.

GTPγS reminds the G protein physiological activator, but on impeding its hydrolysis protein G remains switched on. Treatment of EHCPM with GTPγS is insufficient for increasing the Nox isoform's catalytic activity unless adrenaline is added (Figure 5); for its part, adrenaline alone does not activate Nox. It is important to underscore that the activation response is proportional to the adrenaline dose utilized (Figure 6). With the use of inhibitors selected for each AR, we were able to identify the receptors involved in the described response. The response that is most important quantitatively was that of the activation of a Nox isoform by means of α_1 receptors (Figure 7). Note that this is the most abundant of rat hepatocytes [27] and the one that is bound to the $G_{q/11}$ protein [28]. Coincidentally, the response to the α_1 receptor stimulus elevates the cytosolic Ca^{2+} concentration [28], and adrenaline-mediated activation of the hepatic Nox isoform is sensitive to changes in Ca^{2+} concentration (see Results). In experiments employing adrenaline doses within the range of 10^{-10}–10^{-9} M and in which adrenergic antagonists were added, a greater statistically significant response was observed, in comparison with experiments in which these agonists are absent (Figure 6). This greater response in the presence of adrenergic antagonists can be due to propanolol's inhibitory action on adrenaline-stimulated β receptors, thus avoiding the reported inhibitory effect of activated β-ARs on Nox catalytic activity (Figure 7). Based on initial experimental evidence, complete NADPH oxidase enzymatic activity inhibition in the presence of an antibody prepared against $gp91^{phox}$ (Figure 8) lead us to suggest that the isoform mainly involved in the adrenergic agonist response is the Nox 2 isoform.

$Alpha_1$-mediated adrenaline receptor action on hepatic Nox isoform activation and ROS generation can be of physiologic importance for one or more of the following complementary reasons. According to data produced in this work and at least for liver, it is proposed to consider the superoxide radical and H_2O_2 as other physiological transduction signals for adrenaline that can be added to those existing at present, such as cAMP and Ca^{2+}. Although to date the target on which the previously mentioned adrenaline intermediation-formed ROS can act is unknown, evidence is growing that in general these ROS function as signal transducers. Otherwise, adrenaline-mediated ROS production might cast some light on the striking potentiation in hepatotoxicity when co-treatment of chemicals (CCl_4, acetaminophen, and methylphenidate) with certain adrenergic agonist drugs is performed [9,29]. A greater pool of adrenaline

action-associated ROS might underlie the hepatotoxic potentiation in cells in which homeostasis is already compromised by the independent effect of the toxic compound itself. In addition, the reported Kupffer-cell activation by environmental stress, which may subsequently sensitize hepatocytes to xenobiotics [30], should contribute to enhance hepatotoxicity. The fact of finding opposing actions to a same stimulus, such as adrenaline, by means of the activation of different ARs, stimulation mediated by α_1, and inhibition by β (Figure 7), is a common observation on studying physiological responses to receptor activations. While we must delve more deeply into the finding, the results illustrate the fact that AR α must be inactive in order to manifest AR β inhibitory action (Figure 6). The generation of ROS reported herein by the activation of a Nox isoform in an adrenaline-mediated process appears to be one additional case of the most generalized biological response schema that includes different tissues and involves three constituents that interact successively: a hormone or cytokine receptor susceptible to stimulation; a member of the G protein family that can be activated, and a Nox isoform that responds to the generation of ROS.

Acknowledgements

We are grateful Mrs Alejandra Palomares for her secretarial contribution, to Rafael Villalobos-Molina for stimulating discussion and for careful reading of the manuscript, and to Maggie Brunner, MA, for her advice on improving the manuscript. We gratefully acknowledge financial support from CONACyT, México, grant 45003-M and IN202106-2 from DGAPA, UNAM.

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