Pancreatic β-Cells Express Phagocyte-Like NAD(P)H Oxidase

Henriette R. Oliveira, Rozangela Verlengia, Carla R.O. Carvalho, Luiz R.G. Britto, Rui Curi, and Angelo R. Carpinelli

The presence of a phagocyte-like NAD(P)H oxidase in pancreatic β-cells was investigated. Three NAD(P)H oxidase components were found in pancreatic islets by RT-PCR: gp91^{PHOX}, p22^{PHOX}, and p47^{PHOX}. The components p67^{PHOX} and p47^{PHOX} were also demonstrated by Western blotting. Through immunohistochemistry, p47^{PHOX} was mainly found in the central area of the islet, confirming the expression of this component by insulin-producing cells. Activation of NAD(P)H oxidase complex in the β-cells was also examined by immunohistochemistry. The pancreatic islets presented slower kinetics of superoxide production than HIT-T15 cells, neutrophils, and macrophages, but they reached 66% that of the neutrophil nitroblue tetrazolium (NBT) reduction after 2 h of incubation. Glucose (5.6 mmol/l) increased NBT reduction by 75% when compared with control. The involvement of protein kinase C (PKC) in the stimulatory effect of glucose was confirmed by incubation of islets with phorbol myristate acetate (a PKC activator) and bysindoylmaleimide (GF109203X) (a PKC-specific inhibitor). Diphenylene iodonium [an NAD(P)H oxidase inhibitor] abolished the increase of NBT reduction induced by glucose, confirming the NAD(P)H oxidase activity in pancreatic islets. Because reactive oxygen species are involved in intracellular signaling, the phagocyte-like NAD(P)H oxidase activation by glucose may play an important role for β-cell functioning. *Diabetes* 52:1457–1463, 2003

NAD(P)H oxidase is a multicomponent enzyme composed of membrane-bound components (gp91^{PHOX} and p22^{PHOX}) that form the low-potential flavocytochrome b558 and cytosolic components (p47^{PHOX}, p67^{PHOX}, and GTPases Rac1 or Rac2) that translocate to the membrane after activation. NAD(P)H oxidase is an electron-transfer complex that uses NAD(P)H as its substrate and catalyzes the one-electron reduction of molecular oxygen to superoxide (1). NAD(P)H oxidase activity has been widely recognized as an important source of superoxide in phagocytes (neutrophils and monocytes) (2). Recent studies have suggested, however, that other tissues and cells also present NAD(P)H oxidase activity, such as endothelial cells, vascular smooth muscle cells (VSMCs) (4), thyroid cells (5), fibroblasts (6), lymphocytes (7), spermatozoa (8), and osteoclasts (9). In phagocytic cells, NAD(P)H oxidase produces large amounts of superoxide, which leads to the generation of other reactive oxygen species (ROS) devoted to killing microorganisms (bacteria, viruses, and fungi). In nonphagocytic cells, on the other hand, the role of NAD(P)H oxidase still remains to be defined. Protein kinase C (PKC) is the main regulator of NAD(P)H oxidase in leukocytes, inducing the phosphorylation-dependent activation of the cytosolic components (10). Inoguchi et al. (11) have also shown a PKC-dependent pathway for NAD(P)H oxidase activation in nonphagocytic cells (cultured aortic smooth cells and endothelial cells), which is stimulated by high levels of glucose and free fatty acids (palmitate).

There are several indications for the occurrence of oxidative stress induced by glucose in pancreatic β-cells. Isolated rat pancreatic islets incubated in the presence of a high glucose concentration (16.7 mmol/l) for a short period of time (60 min) show increased superoxide dismutase and glutathione peroxidase activities. This may result from ROS generation through glucose metabolism (12). Chronic high glucose levels increase the production of advanced glycosylation end products and 8-hydroxy-2′-deoxyguanosine in β-cells (13). These metabolites are important markers of ROS generation and oxidative stress that leads to stimulation of antioxidant enzyme expression (such as heme oxidase-1 and glutathione peroxidase) (14). The decreased transcription of the insulin gene induced by chronic elevation of glucose is prevented by antioxidants such as aminoguanidine and *N*-acetyl-L-cysteine (15). These findings support the proposition that hyperglycemia is associated with ROS generation by β-cells. In leukocytes, a glucose challenge induces an increase in ROS production and p47^{PHOX} expression (16). Despite the evidence for ROS generation in pancreatic β-cells, there is no direct demonstration that these cells express NAD(P)H oxidase.

In this study, the presence of phagocyte-like NAD(P)H oxidase in pancreatic β-cells was investigated. The following measurements were carried out: *1*) determination of the NAD(P)H oxidase components expression by RT-PCR (gp91^{PHOX}, p22^{PHOX}, and p47^{PHOX}) and by Western blotting (p47^{PHOX} and p67^{PHOX}); *2*) comparison of ROS pro-

From the Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo, Brazil.

Address correspondence and reprint requests to Dr. Angelo R. Carpinelli, Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of Sao Paulo, Av. Prof. Lineu Prestes, 1524, CEP: 05508-900, Sao Paulo, Brazil. E-mail: angelo@fisio.icb.usp.br.

Received for publication 9 December 2002 and accepted in revised form 24 February 2003.

ABC, avidin-biotin complex; DPI, diphenylene iodonium; KH, Krebs-Henseleit; PB, phosphate buffer; PKC, protein kinase C; PMA, phorbol myristate acetate; NBT, nitroblue tetrazolium; ROS, reactive oxygen species; VSMC, vascular smooth muscle cell.

TABLE 1
Primers used and PCR conditions

Genes	Size (bp)	Primer Sequence (5′-3′)	Annealing temperature (°C)	$MgCl_2$ (mmol/l)
$p22^{PHOX}$	435	Forward-GCTCATCTGTCTGCTGGAGTA Reverse-ACGACCTCATCTGTAACTGGA	59	1.5
$p47^{PHOX}$	374	Forward-AGGTGGTATGATGGGCAGCGTG Reverse-CCTCGGTTTGGCTTCATCTGGC	59	1.5
$gp91^{PHOX}$	336	Forward-TGACTCGGTTGGCTGGCATC Reverse-CGCAAAGGTACAGGAACATGGG	55	1.5
β-actin	549	Forward-GTGGGGCGCCCCAGGCAGCA Reverse-CTCCTTAATGTCACGCACGATTTC	55	1.5

duction by pancreatic islets with neutrophils, macrophages, and the HIT-T15 cell line; *3*) effect of glucose (and activators and inhibitors of PKC) and the inhibitor of NAD(P)H oxidase on ROS production. The presence of the NAD(P)H oxidase complex in the β-cells was confirmed by immunostaining of $p47^{PHOX}$ and insulin in sections (16 μm) of the pancreas. Activation of the NAD(P)H oxidase complex in the β-cells was examined by comparing the intracellular distribution of $p47^{PHOX}$ in isolated islet cells before and after incubation in the presence of glucose (5.6 mmol/l).

RESEARCH DESIGN AND METHODS

Animals. Female albino rats weighing 150–200 g (45–60 days old) were obtained from the Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo, Brazil. The animals were kept in groups of five at 23°C in a room with a light-dark cycle of 12:12 h (lights on at 7:00 A.M.) and free access to food and water. Ethics approval was granted for this study by the Animal Experimental Committee of the Institute of Biomedical Sciences, University of Sao Paulo.

Rat pancreatic islet isolation and islet cell dispersion. Rat pancreatic islets were isolated by collagenase digestion as described by Lacy and Kostianovsky (17). The isolated islets were free from blood vessels. The method used to disperse the cells of the pancreatic islets has been described elsewhere (18). Fresh islet cells were also incubated in RPMI-1640 medium containing 10% FCS and 5.6 mmol/l glucose at 37°C/5% CO_2 for 2 h.

Cell culture. HIT-T15 cells (provided by Dr. Mari C. Sogayar, Institute of Chemistry, Sao Paulo, Brazil) were maintained in CMRL/F12 HAM (1:1) (Life Technologies, Grand Island, NY) medium containing FCS (10% vol/vol), penicillin (100 units/ml), and streptomycin (0.1 mg/ml) at 37°C in an atmosphere of humidified air (95%) and CO_2 (5%). Cells were used at the eleventh passage and harvested using trypsin-EDTA.

Peritoneal neutrophil preparation. Rat neutrophils were obtained by washing the intraperitoneal cavity with 40 ml PBS 4 h after the intraperitoneal injection of 20 ml 1% oyster glycogen solution (type II; Sigma, St. Louis, MO) in PBS. The cells were centrifuged (1,200 rpm for 10 min at 4°C) three times in PBS. The number of viable cells was counted in a Neubauer chamber using an optic microscope after staining with 1% trypan blue solution in PBS.

Resident macrophages preparation. Rat macrophages were obtained by washing the intraperitoneal cavity with 40 ml PBS. The cells were centrifuged (1,200 rpm for 10 min at 4°C) three times in PBS. The number of viable cells was performed as described above.

Isolation of total RNA. Total RNA from fresh islets and neutrophils (positive control) was extracted, using the Trizol reagent (Life Technologies) as described in the product protocol. RNA concentration was spectrophotometrically determined by measuring the absorbance at 260 nm. The integrity of the RNA samples was evaluated in a 2% (wt/vol) agarose gel containing ethidium bromide and visualized with ultraviolet transillumination.

RT-PCR. The RT-PCR was performed on 2 μg total RNA using random hexanucleotide primers. The samples were treated with DNAse (Life Technologies) to avoid contamination and parallel amplification. Products of PCR were separated in a 1.5% (wt/vol) agarose gel containing ethidium bromide and were visualized with ultraviolet transillumination. The PCR conditions, such as the magnesium chloride concentration, number of cycles, and annealing temperature, were carefully optimized. The PCR products were of the expected size and showed authenticity when compared with the sequences deposited in the GenBank database of the National Center for Biotechnology Information ($gp91^{PHOX}$, accession no. AF298656; $p47^{PHOX}$, accession no. AF260779; $p22^{PHOX}$, accession no. AJ295951). All primers used, annealing temperature, magnesium chloride concentration, and the PCR product size for target sequences are shown in Table 1. Primers to amplify the

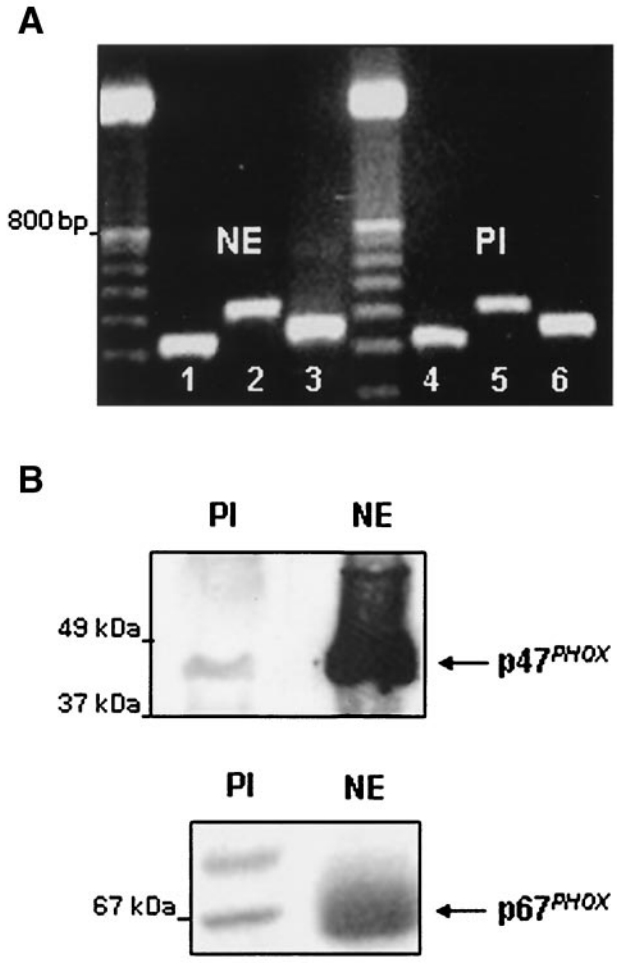

FIG. 1. Expression of NAD(P)H oxidase components. *A*: The enzyme components (neutrophils [NE]: 1, $gp91^{PHOX}$ [336 bp]; 2, $p22^{PHOX}$ [435 bp]; 3, $p47^{PHOX}$ [374 bp]; pancreatic islets [PI]: 4, $gp91^{PHOX}$; 5, $p22^{PHOX}$; 6, $p47^{PHOX}$) were analyzed by RT-PCR (40 cycles) using 2 μg RNA from neutrophils and pancreatic islet homogenates. The primer sequences and the PCR conditions are shown in Table 1. Products of RT-PCR amplification were separated in a 1.5% (wt/vol) agarose gel containing ethidium bromide and visualized under ultraviolet transillumination using 100-bp markers as molecular weight standards. *B*: Western blotting analysis of $p47^{PHOX}$ and $p67^{PHOX}$ of neutrophils (positive control) and pancreatic islets. The proteins of NE and PI were dissolved in a sample buffer and submitted to 8% SDS-PAGE. Western blotting was performed using rabbit anti-$p47^{PHOX}$ and goat anti-$p67^{PHOX}$ polyclonal antibodies. Similar results were obtained in three (*A*) and two (*B*) independent experiments.

housekeeping gene β-actin were used to set up general PCR conditions.

Western Blotting for p47^{PHOX} and p67^{PHOX}. Protein from islets and neutrophils (positive control) was diluted (1:5) in a 2-mercaptoethanol Laemmli buffer. An equal amount of proteins was subjected to SDS-PAGE in a Bio-Rad miniature slab gel apparatus and electrophoretically transferred onto a nitrocellulose sheet. The sheet was blocked in 5% nonfat dried milk solution and incubated overnight with partially purified rabbit anti-p47^{PHOX} (this antibody was raised in the laboratory of Professor Bernard M. Babior, Scripps Research Institute, La Jolla, CA, and was provided to us by Dr. Lucia R. Lopes) (19) and goat anti-p67^{PHOX} (Santa Cruz Biotechnology, Santa Cruz, CA) polyclonal antibodies. These antibodies were used at dilutions of 1:5,000 (p47^{PHOX}) and 1:500 (p67^{PHOX}). The blots were washed and probed with horseradish peroxidase–conjugated goat anti-rabbit IgG (Amersham-Pharmacia Biotech, Buckinghamshire, U.K.) and donkey anti-goat IgG (Amersham-Pharmacia Biotech), respectively. The proteins were finally visualized on autoradiography using the enhanced chemiluminescence Western blotting detection system (Amersham-Pharmacia Biotech).

Immunohistochemistry. Immunochemical detection of insulin and p47^{PHOX} protein was performed using the avidin-biotin complex (ABC) method. The animals were deeply anesthetized with ketamine (5 mg/100 g body wt i.m.) and xylazine (1 mg/100 g body wt i.m.) and perfused through the heart with 0.9% PBS and 4% paraformaldehyde in 0.1 mol/l phosphate buffer (PB) (pH 7.4). The pancreases were removed and postfixed in 4% paraformaldehyde for 4–6 h and transferred to a 30% sucrose solution in PB for cryoprotection. Frozen pancreas sections (16 μm) were cut on a cryostat and mounted on gelatin-coated slides. The pancreas sections were incubated with 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. After incubation, the sections were washed three times (5 min each) in 0.1 mol/l PB. The pancreas sections were incubated with rabbit anti-insulin (Santa Cruz Biotechnology) and rabbit anti-p47^{PHOX} polyclonal antibodies, and these were diluted 1:500 (insulin) and 1:400 (p47^{PHOX}) in 0.1 mol/l PB containing 0.3% Triton X-100 and 5% normal goat serum to block nonspecific binding of antibodies. The pancreas sections were incubated overnight at room temperature. After three washes in 0.1 mol/l PB (10 min each), the sections were incubated with biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA) diluted 1:200 in 0.1 mol/l PB containing 0.3% Triton X-100 for 2 h at room temperature. The sections were washed three times in 0.1 mol/l PB (10 min each) and then incubated for 2 h with the avidin-biotin peroxidase (ABC Elite Kit; Vector Laboratories). After incubation and three washes in 0.1 mol/l PB, the sections were incubated with 0.05% 3,3′-diaminobenzidinetetrahydrochloride and a 0.01% solution of hydrogen peroxide in 0.1 mol/l PB. The reaction was intensified with 0.05% osmium tetroxide. The sections were finally dehydrated, cleared, and cover slipped with Permount (Fisher, Pittsburgh, PA). Samples processed without primary antibodies served as negative controls. A similar procedure was used to show the glucose activation of the NAD(P)H oxidase complex in isolated islet cells. The cells were immunostained for p47^{PHOX} after being freshly obtained and at the end of a 2-h incubation in the presence of glucose 5.6 mmol/l. Neutrophils and macrophages were also stained with p47^{PHOX} antibodies and used as positive controls. For isolated islet cells, neutrophil and macrophage permeabilization was performed by treatment with 0.3% Triton X-100 for 10 min instead of overnight used for the pancreas section.

Superoxide generation determination: nitroblue tetrazolium assay.

Superoxide generation was detected by nitroblue tetrazolium (NBT) assay (20). NBT (yellow water soluble) was reduced by superoxide to formazan-NBT (dark-blue water insoluble). The assay was performed by incubating islets (100 islets/500 μl Krebs-Henseleit [KH] buffer), neutrophils (1×10^6 cells in 500 μl PBS), macrophages (1×10^6 cells in 500 μl PBS), and HIT-T15 cells (1×10^6 cells in 500 μl KH buffer) with 0.2% NBT (Sigma). The samples were incubated for 1 h (37°C/5% CO_2) in the presence or absence of glucose (0, 2.8, 5.6, 8.3, and 16.7 mmol/l), 20 nmol/l phorbol myristate acetate (PMA) (Sigma; a PKC activator) (21), 500 nmol/l bysindoylmaleimide (GF109203X; Sigma) (a PKC-specific inhibitor) (22), and 10 μmol/l diphenylene iodonium (DPI) (Sigma; a NAD(P)H oxidase inhibitor) (23,24). The cells were centrifuged (5,000 rpm for 2 min at 4°C), the supernatant was removed, and formazan (NBT reduced-insoluble) was dissolved in 100 μl 50% acetic acid by sonication (three pulses of 6 s; Vibra Cell; Sonics and Materials, Newtown, CT). The samples were briefly centrifuged (spin down), and the absorbance of the supernatant was determined at 560 nm in a microtiter plate reader (Spectramax Plus; Molecular Devices, Sunnyvale, CA).

Statistical analysis. Results are presented as means ± SE. ANOVA (Student-Newman-Keuls comparison test) was used to verify significance where appropriate, with confidence levels set at $P < 0.01$.

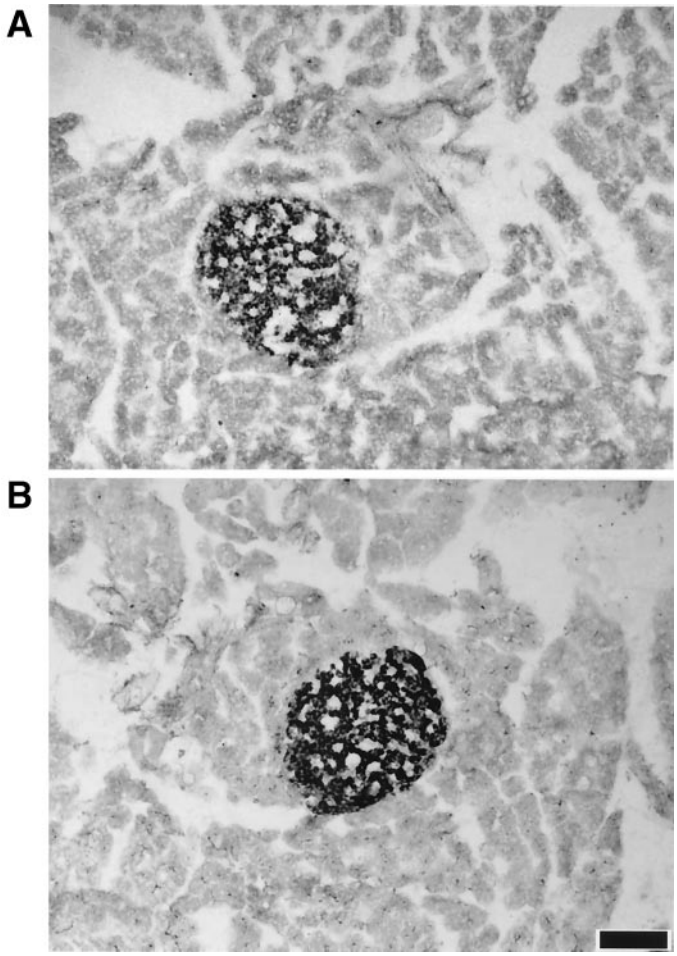

FIG. 2. Immunohistochemical localization of the NAD(P)H oxidase complex in rat pancreatic islets. Pancreas sections were labeled with rabbit anti-p47^{PHOX} (*A*) and anti-insulin (*B*) polyclonal antibodies. Scale bar represents 100 μm.

RESULTS

Expression of NAD(P)H oxidase components. Three NAD(P)H oxidase components were found in pancreatic islets by RT-PCR analysis: gp91^{PHOX} and p22^{PHOX}, both membrane-binding components, and p47^{PHOX}, a cytosolic component (Fig. 1*A*). The expression of these NAD(P)H oxidase components was also examined in neutrophils (positive control). The expression of the p47^{PHOX} and p67^{PHOX} components was also demonstrated by Western blotting (Fig. 1*B*). Interestingly, the p67^{PHOX} component was identified as a doubled band in the isolated islets instead of a single one detected in the positive control cells (neutrophils).

Immunohistochemistry. The expression of p47^{PHOX} was evaluated in pancreas sections and isolated cells (islet cells, neutrophils and macrophages) by immunohistochemistry (ABC method). The sections were probed with anti-p47^{PHOX} and anti-insulin antibodies (control to β-cell localization). The insulin and p47^{PHOX} distribution was compared. As well as insulin, p47^{PHOX} was mainly found in the central area of the islet, suggesting the expression of this NAD(P)H oxidase component by insulin-producing cells (Fig. 2*A* and *B*). Freshly obtained islet cells showed a wide distribution of p47^{PHOX} in the cytosol (Fig. 3*A*). Incubation in the presence of 5.6 mmol/l glucose promoted

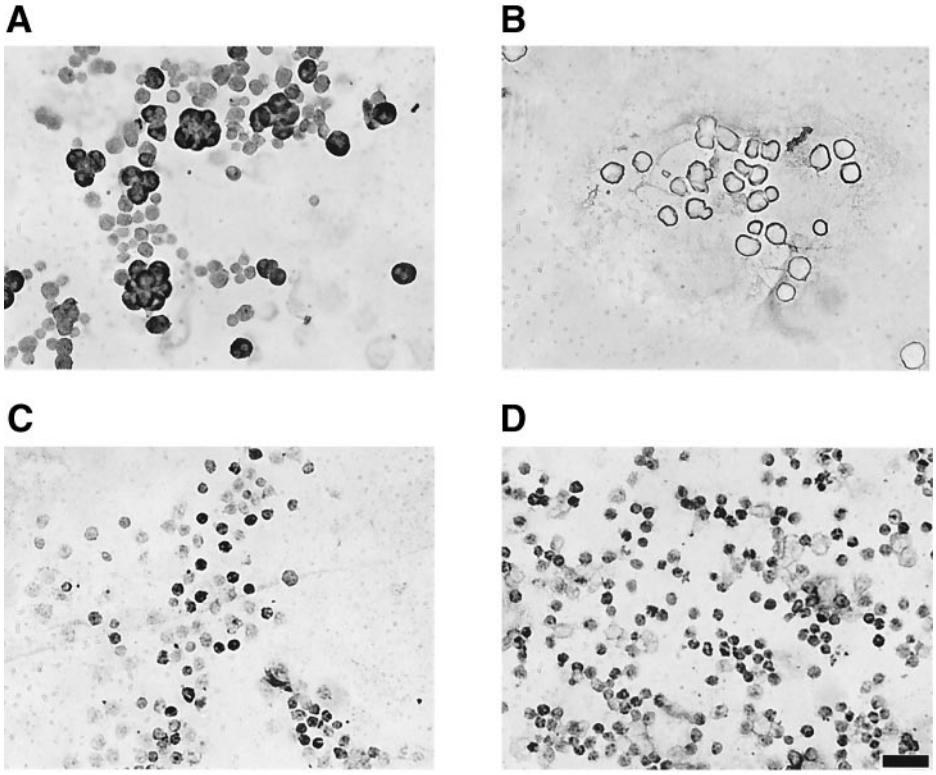

FIG. 3. Immunohistochemical localization of the NAD(P)H oxidase complex in rat pancreatic islet cells immediately after being isolated (*A*) and after incubation for 2 h in the presence of 5.6 mmol/l glucose (*B*). Isolated islet cells were labeled with rabbit anti-p47^{PHOX} polyclonal antibodies. Neutrophils (*C*) and macrophages (*D*) were also labeled with rabbit anti-p47^{PHOX} (positive controls). Scale bar represents 25 μm.

a translocation of the p47^{PHOX} from cytosol to plasma membrane (Fig. 3*B*). A strong staining for p47^{PHOX} was found in neutrophils and macrophages (positive controls) (Fig. 3*C* and *D*). These findings do not exclude the possibility that NAD(P)H oxidase is also expressed by non–insulin-secreting cells of the pancreatic islets.

Comparison of ROS production by pancreatic islets, neutrophils, and macrophages. The production of superoxide by rat pancreatic islets, as measured by NBT reduction, was compared with that of phagocytic cells (neutrophils and macrophages) and the HIT-T15 cell line (Fig. 4*A*). The pancreatic islets presented a slower kinetics of superoxide production than macrophages, neutrophils, and HIT-T15 cells, but islet superoxide production was linear up to 2 h (Fig. 4*A*). In pancreatic islets, the increase of superoxide production was closely correlated with the time (r = 0.98; Fig. 4*B*). The superoxide production by pancreatic islets reached 66% that of the neutrophils after a 2-h incubation (Table 2).

Effect of glucose on ROS production. The effect of glucose on NBT reduction was determined in 1-h–incubated rat pancreatic islets. In the presence of a basal glucose concentration (5.6 mmol/l), the NBT reduction increased by 75% when compared with control (absence of glucose). This effect remained unchanged in the presence of 8.3 and 16.7 mmol/l glucose (Fig. 5).

Effect of activator and inhibitor of PKC and inhibitor of NAD(P)H oxidase. Isolated rat pancreatic islets were treated with PMA (20 nmol/l), an activator of PKC, for 1 h in the absence or presence of 5.6 mmol/l glucose. PMA caused marked NBT reduction by itself and potentiated the glucose effect (Fig. 6*A*). The effect of GF109203X (PKC inhibitor) was examined as to confirm the role of PKC in the stimulatory effect of glucose (Fig. 6*B*). GF109203X partially abolished the increase in NBT reduction (Fig. 6*B*). PKC is one of the main regulators of NAD(P)H oxidase activity in phagocytic cells (10). To confirm the role of NAD(P)H oxidase activity in the NBT reduction by isolated rat pancreatic islets, the effect of DPI, an inhibitor of NAD(P)H oxidase, was tested. The incubation for 1 h in medium containing DPI abolished the increase of NBT reduction induced by glucose, confirming the presence of NADPH oxidase activity in pancreatic islets (Fig. 6*C*).

DISCUSSION

We demonstrated herein the expression of a phagocyte-like NAD(P)H oxidase in β-cells of rat pancreatic islets. Expression of gp91^{PHOX}, p22^{PHOX}, and p47^{PHOX} mRNA by RT-PCR and p47^{PHOX} and p67^{PHOX} proteins by Western blotting in isolated pancreatic islets is shown. The NAD(P)H oxidase complex of pancreatic islets presents the same components as the one found in neutrophils. However, the anti-p67^{PHOX} blotting showed two bands (~76 and ~67 kDa), in contrast to the single band (~67 kDa) found in neutrophils. As compared with neutrophils, differences in oxidase component expression were also found in isolated nonphagocytic cells. In cultured VSMCs, for instance, the gp91^{PHOX} component is replaced by a homologue, termed "NOX1" or "Mox1" (for mitogenic oxidase), which presents 60% homology to the neutrophil component gp91^{PHOX} and conserved all the major functional domains, including pyrimidine binding sites, flavin nucleotide binding sites, and potential heme binding sites (25–27).

We have also examined the presence of p47^{PHOX} in pancreatic islets by immunohistochemistry. The pancreas sections staining showed that islet cells expressing p47^{PHOX} are mainly β-cells, judging from the central pattern of p47^{PHOX} distribution in the islets and from

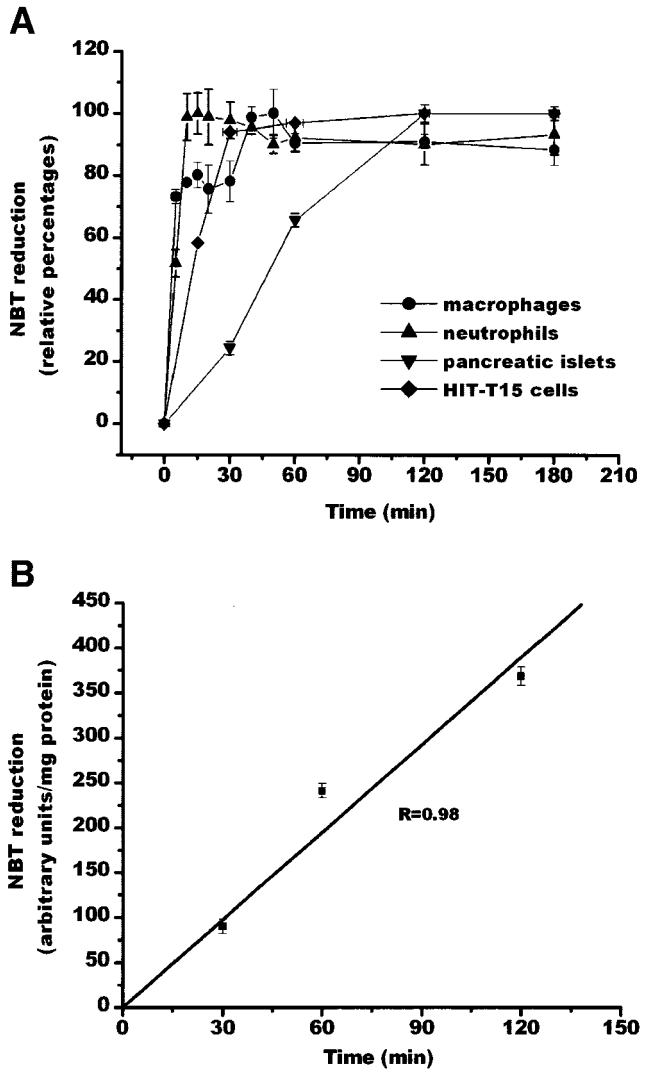

FIG. 4. *A*: Kinetics of NBT reduction in the presence of 5.6 mmol/l glucose by islets, leukocytes (neutrophils and macrophages), and HIT-T15 cells. Islets (100 in 500 μl KH buffer), neutrophils (1×10^6 cells in 500 μl PBS), macrophages (1×10^6 cells in 500 μl PBS) ($n = 6$), and HIT-T15 cells (1×10^6 cells in 500 μl KH buffer) ($n = 3$) were incubated for 3 h with 0.2% NBT and 5.6 mmol/l glucose. The results are expressed as relative percentages. *B*: Time course of NBT reduction by isolated pancreatic islets. The islets (100 in 500 μl KH buffer) were incubated for 2 h with 0.2% NBT and 5.6 mmol/l glucose ($n = 6$). The results are expressed as means ± SE from pools of 100 islets each.

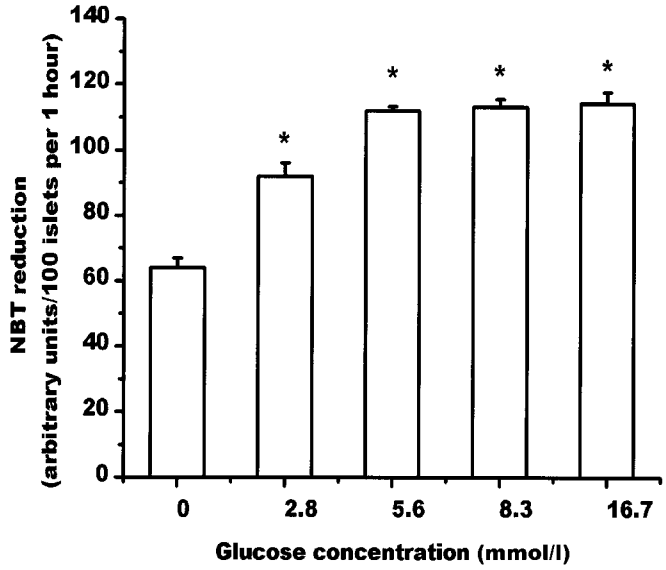

FIG. 5. Effect of glucose on NBT reduction. Pancreatic islets were incubated for 1 h in KH buffer containing 0.2% NBT in the absence (control) or presence of glucose 0, 2.8, 5.6, 8.3, and 16.7 mmol/l glucose ($n = 4$). The results are expressed as means ± SE from pools of 100 islets each. *$P < 0.01$, as compared with condition without glucose.

immunostaining with an anti-insulin antibody. In freshly isolated islet cells, $p47^{PHOX}$ was widely distributed in the cytosol.

Pancreatic islets present low activity of antioxidant enzymes (28), being highly susceptible to cellular injury. High glucose, the main stimulator of insulin release, stimulates the activity and expression of antioxidant enzymes (12,14). Activity and expression of antioxidant enzymes can also be induced by ROS (12,29,30). In this study, glucose stimulated superoxide production in pancreatic islets incubated in the presence of 2.8 and 5.6 mmol/l glucose. However, the increase of glucose concentration did not change ROS production. This stabilization of superoxide production possibly occurred because of the acute increase in the activity of CuZnSOD induced by high glucose levels (12). In leukocytes and endothelial cells, glucose also stimulates the production of ROS and the expression of antioxidant enzymes (16,30). In these cells, the main source of ROS is NAD(P)H oxidase. Similar oxidases have been identified in several nonphagocytic cells, but the role and the significance of this enzyme expression is unknown.

To confirm the role of NAD(P)H oxidase activity in pancreatic islets, glucose-induced ROS production was measured in the absence and presence of DPI, a NAD(P)H inhibitor. The glucose-induced (5.6 mmol/l) increase of ROS production was restored to control values by DPI. Incubation of islet cells in the presence of 5.6 mmol/l glucose promoted translocation of $p47^{PHOX}$ from cytosol to plasma membrane. This is the feature expected for activation of the NAD(P)H oxidase complex, as reported for phagocytes (1). These results indicate that glucose

TABLE 2
NBT reduction by islets, leukocytes, and HIT-T15 cells after incubation in the presence of 5.6 mmol/l glucose

	NBT reduction					
	0 min	15 min	30 min	60 min	120 min	180 min
Pancreatic islets	—	—	90 ± 8	241 ± 8	369 ± 10	374 ± 8
Neutrophils	—	623 ± 41	609 ± 36	573 ± 27	559 ± 41	577 ± 41
Macrophages	—	827 ± 42	804 ± 69	931 ± 31	935 ± 23	911 ± 50
HIT-T15	—	694 ± 6	1118 ± 39	1151 ± 45	1187 ± 27	1187 ± 24

Data are means ± SE and are given in arbitrary units per milligram protein.

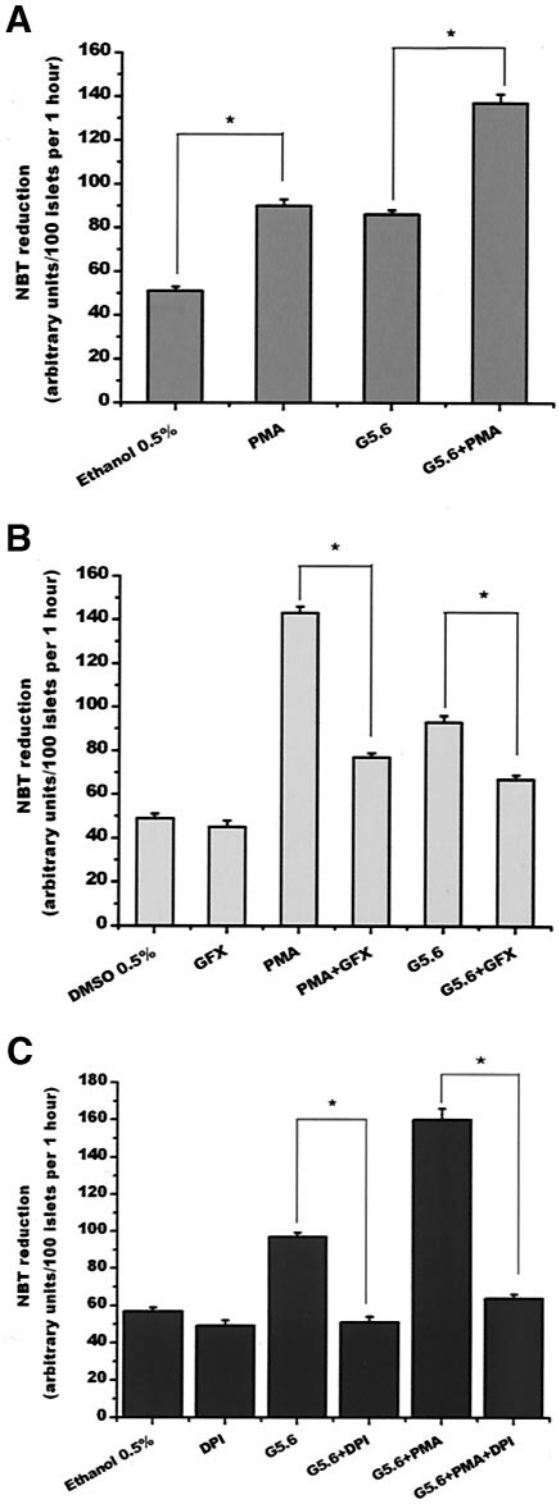

FIG. 6. *A*: Effect of PMA (PKC activator) on NBT reduction. The islets were incubated for 1 h in KH buffer containing 0.2% NBT in the absence (ethanol 0.5%) or presence of 20 nmol/l PMA and 5.6 mmol/l glucose (G5.6) (n = 3). *B*: The effect of GF109203X (PKC inhibitor) on NBT reduction. The islets were incubated for 1 h in KH buffer containing 0.2% NBT in the absence (DMSO 0.5%) or presence of 500 nmol/l GF109203X (GFX), 5.6 mmol/l glucose (G5.6), and 20 nmol/l PMA (n = 4). *C*: Effect of DPI (NADPH oxidase inhibitor) on NBT reduction. The islets were incubated for 1 h in KH buffer containing 0.2% NBT in the absence (ethanol 0.5%) or presence of 10 μmol/l DPI, 5.6 mmol/l glucose (G5.6), and 20 nmol/l PMA (n = 4). The results are expressed as means ± SE from pools of 100 islets each. *P < 0.01.

stimulates ROS production in pancreatic islets through activation of a phagocyte-like NAD(P)H oxidase. We compared ROS production induced by glucose in pancreatic islets, phagocytic cells (neutrophils and macrophages), and HIT-T15 cells. Pancreatic islets presented slower kinetics of production. However, the amount of ROS produced after 2 h was only 34% lower than that in neutrophils. This result demonstrated a significant production of superoxide during glucose metabolism in pancreatic islets. The kinetics of production presented by the islet oxidase mimics that of vascular oxidase, which is low-output, in contrast to neutrophil oxidase (2). Superoxide is then produced by islets and vascular cells (endothelial cells and VSMCs) in minutes to hours. On the other hand, superoxide production by neutrophils is quickly triggered, in general, in seconds. The NAD(P)H oxidase complex of HIT-T15 cells is more likely to function as that of neutrophils and macrophages.

One of the main regulators of phagocytic NAD(P)H oxidase, PKC, activates the enzyme by phosphorylation of cytosolic components such as $p47^{PHOX}$, which is phosphorylated on several serine residues (11,31) and translocated to the membrane. Neutrophils contain 5 of the 11 known isoforms of PKC (α, βI, βII, δ, and atypical isoform [ζ]) (32–36), and PKC-β seems to play a major role in neutrophil oxidase activation (37,38). PMA, an activator of PKC, stimulated ROS production in isolated pancreatic islets and potentiated the glucose effect. The role of PKC in the activation of nonphagocytic NAD(P)H oxidase is unclear. However, similar to the situation in the islet, PMA also increases NAD(P)H oxidase activity in VSMCs (11). Pancreatic islets were incubated in the presence of glucose plus GF109203X, a PKC-specific inhibitor. The ROS production induced by glucose was partially abolished by GF109203X, suggesting that glucose stimulates ROS production mainly through a PKC-dependent mechanism. Interestingly, glucose also stimulates ROS production in cultured vascular cells, and an increase of ROS production occurred through PKC-dependent activation of NAD(P)H oxidase (11).

The activation mechanism and physiological role of NAD(P)H oxidase in pancreatic islets still remain to be fully established. In several cell types, ROS produced through NAD(P)H oxidase may have a signaling role (29,39). In endothelial cells, for instance, NAD(P)H oxidase-derived H_2O_2 increases the release of intracellular Ca^{2+} (40). In pancreatic islets, this effect on Ca^{2+} mobilization would be of great importance for regulation of insulin secretion. In general, ROS act as second messengers on cell signaling cascades and on regulation of several biological processes, such as cell growth (41), gene expression (42), kinase activation (29,43), and modulation of ionic channels (44,45). Therefore, activation of NAD(P)H oxidase may play an important role for β-cell functioning.

In summary, we found that pancreatic islet cells express phagocyte-like NAD(P)H oxidase components. The cytosolic component $p47^{PHOX}$ is mainly expressed in β-cells, as shown by immunostaining of pancreas sections and isolated islet cells with an anti-insulin antibody. Glucose stimulates ROS production in islets through a PKC-dependent activation of NAD(P)H oxidase.

ACKNOWLEDGMENTS

This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq), and Programa de Apoio a Núcleos de Excelência (PRONEX).

We are grateful to Marlene Santos Rocha and Adilson da Silva Alves for excellent technical assistance. We also are indebted to Dr. Lucia R. Lopes (Department of Pharmacology, Institute of Biomedical Sciences, University of Sao Paulo) and Anna Karenina Azevedo Martins for help with Western blotting analysis and HIT-T15 cells cultures.

REFERENCES

1. Babior BM, Lambeth JD, Nuseef W: The neutrophil NADPH oxidase. *Arch Biochem Biophys* 397:342–344, 2002
2. Pithon-Curi TC, Levada AC, Lopes LR, Doi SQ, Curi R: Glutamine plays a role in superoxide production and the expression of p47^{PHOX} in rat neutrophils. *Clin Sci* 103:403–408, 2002
3. Li JM, Shah AM: Intracelullar localization and preassembly of the NADPH oxidase complex in cultured endothelial cells. *J Biol Chem* 277:19952–19960, 2002
4. Griendling KK, Minieri CA, Ollerenshaw JD, Alexander RW: Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ Res* 74:1141–1148, 1994
5. Dupuy C, Virion A, Ohayon R, Kaniewski J, Deme D, Pommier J: Mechanism of hydrogen peroxide formation catalyzed by NADPH oxidase in thyroid plasma membrane. *J Biol Chem* 266:3739–3743, 1991
6. Jones SA, Wood JD, Coffey MJ, Jones TG: The functional expression of p47-*PHOX* and p67-*PHOX* may contribute to the expression of superoxide by an NADPH oxidase-like system in human fibroblast. *FEBS Lett* 335: 178–182, 1994
7. Pani G, Colavitti R, Borrello S, Galeotti T: Endogenous oxygen radicals modulate protein tyrosine phosphorylation and JNK-1 activation in lectin-stimulated thymocytes. *Biochem J* 347:173–181, 2000
8. Aitken J, Fisher H: Reactive oxygen species generation and human spermatozoa: the balance of benefit and risk. *Bioessays* 16:259–267, 1994
9. Steinbeck MJ, Appel WH, Verhoeven AJ, Karnovsky MJ: NADPH oxidase expression and in situ production of superoxide by osteoclasts actively resorbing bone. *J Cell Biol* 126:765–772, 1994
10. El Benna J, Faust LP, Babior BM: The phosphorylation of the respiratory burst oxidase component p47PHOX during neutrophil activation: phosphorylation of sites recognized by protein kinase C and by proline-directed kinases. *J Biol Chem* 269:23431–23436, 1994
11. Inoguchi T, Li P, Umeda F, Yu HY, Kakimoto M, Imamura M, Aoki T, Etoh T, Hashimoto T, Naruse M, Sano H, Utsumi H, Nawata H: High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C-dependent activation of NAD(P)H oxidase in cultured vascular cells. *Diabetes* 49:1939–1945, 2000
12. Oliveira HR, Curi R, Carpinelli AR: Glucose induces an acute increase of superoxide dismutase activity in incubated rat pancreatic islets. *Am J Physiol* 276:C507–C510, 1999
13. Ihara Y, Toyokuni S, Uchida K, Odaka H, Tanaka T, Ikeda H, Hiai H, Seino Y, Yamada Y: Hyperglycemia causes oxidative stress in pancreatic beta-cells of GK rats, a model of type 2 diabetes. *Diabetes* 48:927–932, 1999
14. Laybutt DR, Kaneto H, Hasenkamp W, Grey S, Jonas JC, Sgroi DC, Groff A, Ferran C, Bonner-Weir S, Sharma A, Weir GC: Increased expression of antioxidant and antiapoptotic genes in islets that may contribute to beta-cell survival during chronic hyperglycemia. *Diabetes* 51:413–423, 2002
15. Tanaka Y, Gleason CE, Tran PO, Harmon JS, Robertson RP: Prevention of glucose toxicity in HIT-T15 cells and Zucker diabetic fatty rats by antioxidants. *Proc Natl Acad Sci U S A* 14:10857–10862, 1999
16. Mohanty P, Hamouda W, Garg R, Aljada A, Ghanim H, Dandona P: Glucose challenge stimulates reactive oxygen species (ROS) generation by leucocytes. *J Clin Endocrinol Metab* 85:2970–2973, 2000
17. Lacy PE, Kostianovsky M: Method for the isolation of intact islets of Langerhans from the rat pancreas. *Diabetes* 6:35–39, 1967
18. Gobbe P, Herchuelz A: Effects of verapamil and nifedipine on gliclazide-induced increase in cytosolic free Ca2+ in pancreatic islet cells. *J Endocrinol Invest* 12:469–474, 1989
19. Lopes LR, Hoyal CR, Knaus UG, Babior BM: Activation of the leukocyte NADPH oxidase by protein kinase C in a partially recombinant cell-free system. *J Biol Chem* 274:15533–15537, 1999
20. Schrenzel J, Serrander L, Banfi B, Nusse O, Fouyouzi R, Lew DP, Demaurex N, Krause KH: Electron currents generated by the human phagocyte NADPH oxidase. *Nature* 392:734–737, 1998
21. Babior BM: NADPH oxidase: an update. *Blood* 93:1464–1476, 1999
22. El-Benna J, Park JW, Ruedi JM, Babior BM: Cell-free activation of the respiratory burst oxidase by protein kinase C. *Blood Cells Mol Dis* 21:201–206, 1995
23. Cross AR, Jones OT: The effect of the inhibitor diphenylene iodonium on the superoxide-generating system of neutrophils: specific labeling of a component polypeptide of the oxidase. *Biochem J* 237:111–116, 1986
24. Suzuki Y, Ono Y, Hirabayashi Y: Rapid and specific reactive oxygen species generation via NADPH oxidase activation during Fas-mediated apoptosis. *FEBS Lett* 425:209–212, 1998
25. Suh YA, Arnold RS, Lassegue B, Shi J, Xu X, Sorescu D, Chung AB, Griendling KK, Lambeth JD: Cell transformation by the superoxide-generating oxidase Mox1. *Nature* 401:79–82, 1999
26. Griendling KK, Sorescu D, Ushio-Fukai M: NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res* 86:494–501, 2000
27. Lambeth JD, Cheng G, Arnold RS, Edens WA: Novel homolog of gp91PHOX. *Trends Biochem Sci* 25:459–461, 2000
28. Lenzen S, Drinkgern J, Tiedge M: Low antioxidant enzyme gene expression in pancreatic islets compared with various other mouse tissues. *Free Radic Biol Med* 20:463–466, 1996
29. Lu D, Maulik N, Moraru II, Kreutzer DL, Das DK: Molecular adaptation of vascular endothelial cells to oxidative stress. *Am J Physiol* 264:C715–C722, 1993
30. Ceriello A, dello Russo P, Amstad P, Cerutti P: High glucose induces antioxidant enzymes in human endothelial cells in culture: evidence linking hyperglycemia and oxidative stress. *Diabetes* 45:471–477, 1996
31. Babior BM: The activity of leukocyte NADPH oxidase: regulation by p47^{PHOX} cysteine and serine residues. *Antioxid Redox Signal* 4:35–38, 2002
32. Majumdar S, Rossi MW, Fujiki T, Phillips WA, Disa S, Queen CF, Johnston RB Jr, Rosen OM, Corkey BE, Korchak HM: Protein kinase C isotypes and signaling in neutrophils: differential substrate specificities of a translocatable calcium- and phospholipid-dependent beta-protein kinase C and a phospholipid-dependent protein kinase which is inhibited by long chain fatty acyl coenzyme A. *J Biol Chem* 266:9285–9294, 1991
33. Dang PM, Hakim J, Perianin A: Immunochemical identification and translocation of protein kinase C zeta in human neutrophils. *FEBS Lett* 349:338–342, 1994
34. Kent JD, Sergeant S, Burns DJ, McPhail LC: Identification and regulation of protein kinase C-delta in human neutrophils. *J Immunol* 157:4641–4647, 1996
35. Nixon JB, McPhail LC: Protein kinase C (PKC) isoforms translocate to Triton-insoluble fractions in stimulated human neutrophils: correlation of conventional PKC with activation of NADPH oxidase. *J Immunol* 163: 4574–4582, 1999
36. Fontayne A, Dang PM, Gougerot-Pocidalo MA, El-Benna J: Phosphorylation of p47PHOX sites by PKC alpha, beta II, delta, and zeta: effect on binding to p22PHOX and on NADPH oxidase activation. *Biochemistry* 41:7743–7750, 2002
37. Korchak HM, Rossi MW, Kilpatrick LE: Selective role for beta-protein kinase C in signaling for O $^{-2}$ generation but not degranulation or adherence in differentiated HL60 cells. *J Biol Chem* 273:27292–27299, 1998
38. Dekker LV, Leitges M, Altschuler G, Mistry N, McDermott A, Roes J, Segal AW: Protein kinase C-beta contributes to NADPH oxidase activation in neutrophils. *Biochem J* 347:285–289, 2000
39. Sanders KA, Hueckstead T, Xu P, Sturrock AB, Hoidal JR: Regulation of oxidant production in acute lung injury. *Chest* 116:56S–61S, 1999
40. Hu Q, Zheng G, Zweier JL, Deshpande S, Irani K, Ziegelstein RC: NADPH oxidase activation increases the sensitivity of intracellular Ca^{2+} stores to inositol 1,4,5-trisphosphate in human endothelial cells. *J Biol Chem* 275:15749–15757, 2000
41. Burdon RH: Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. *Free Radic Biol Med* 18:775–794, 1995
42. Schoonbroodt S, Piette J: Oxidative stress interference with the nuclear factor-kappa B activation pathways. *Biochem Pharmacol* 60:1075–1083, 2000
43. Lo YY, Wong JM, Cruz TF: Reactive oxygen species mediate cytokine activation of c-Jun NH2-terminal kinases. *J Biol Chem* 271:15703–15707, 1996
44. Vega-Saenz de Miera E, Rudy B: Modulation of K+ channels by hydrogen peroxide. *Biochem Biophys Res Commun* 186:1681–1687, 1992
45. Wang D, Youngson C, Wong V, Yeger H, Dinauer MC, Vega-Saenz Miera E, Rudy B, Cutz E: NADPH-oxidase and a hydrogen peroxide-sensitive K+ channel may function as an oxygen sensor complex in airway chemoreceptors and small cell lung carcinoma cell lines. *Proc Natl Acad Sci U S A* 93:13182–13187, 1996