

# 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

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Recent studies have revealed that vascular cells can produce reactive oxygen species (ROS) through NAD(P)H oxidase, which may be involved in vascular injury. However, the pathological role of vascular NAD(P)H oxidase in diabetes or in the insulin-resistant state remains unknown. In this study, we examined the effect of high glucose level and free fatty acid (FFA) (palmitate) on ROS production in cultured aortic smooth muscle cells (SMCs) and endothelial cells (ECs) using electron spin resonance spectroscopy. Exposure of cultured SMCs or ECs to a high glucose level (400 mg/dl) for 72 h significantly increased the free radical production compared with low glucose level exposure (100 mg/dl). Treatment of the cells for 3 h with phorbol myristic acid (PMA), a protein kinase C (PKC) activator, also increased free radical production. This increase was restored to the control value by diphenylene iodonium, a NAD(P)H oxidase inhibitor, suggesting ROS production through PKC-dependent activation of NAD(P)H oxidase. The increase in free radical production by high glucose level exposure was completely restored by both diphenylene iodonium and GF109203X, a PKC-specific inhibitor. Exposure to palmitate (200  $\mu$ mol/l) also increased free radical production, which was concomitant with increases in diacylglycerol level and PKC activity. Again, this increase was restored to the control value by both diphenylene iodonium and GF109203X. The present results suggest that both high glucose level and palmitate may stimulate ROS production through PKC-dependent activation of NAD(P)H oxidase in both vascular SMCs and ECs. This finding may be involved in the excessive acceleration of atherosclerosis in patients with diabetes and insulin resistance syndrome. *Diabetes* 49:1939–1945, 2000

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AGE, advanced glycation end product; CxT, 4-carboxy-2,2,6,6-tetramethyl-piperidine-1-oxyl; CxT-AM, acetoxymethyl-2,2,6,6-tetramethylpiperidine-1-oxyl-3-carboxylate; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; ESR, electron spin resonance; FFA, free fatty acid; PKC, protein kinase C; PMA, phorbol myristic acid; ROS, reactive oxygen species.

The production of reactive oxygen species (ROS) has been shown to be increased in patients with diabetes (1–6). The increased ROS production may be involved in the onset or development of diabetic vascular complications. It has been postulated that hyperglycemia, a key clinical manifestation of diabetes, may produce ROS through formation of advanced glycation end products (AGEs) (3,4) and altered polyol pathway activity (5). Recently, however, a growing body of experimental evidence has shown that vascular cells, such as smooth muscle cells and endothelial cells, can produce ROS through activation of NAD(P)H oxidase (7–12). Mohazzab et al. (7) and Rajagopalan et al. (9) have shown that this enzyme is the most important source of ROS in intact arteries rather than arachidonic acid-metabolizing enzymes, xanthine oxidase, or mitochondrial sources. This enzyme has also been implicated in the pathogenesis of angiotensin II-induced hypertension and vascular smooth muscle hypertrophy (8,10,12) and may also contribute to impaired endothelium-dependent vascular relaxation secondary to inactivation of nitric oxide by ROS (9). However, to our knowledge, there is no report regarding the effect of diabetes or high glucose level on ROS production through NAD(P)H oxidase. In phagocytic cells, one of the regulators of NADPH oxidase activity may be protein kinase C (PKC) (13–15). Numerous studies, including ours, have shown that high glucose levels or diabetes may activate PKC in various vascular cells (16–27). Therefore, high glucose or diabetes might stimulate ROS production through PKC-dependent activation of NAD(P)H oxidase in vascular cells. The present study was undertaken to examine the effect of high glucose level on ROS production through activation of NAD(P)H oxidase in cultured aortic smooth muscle cells and endothelial cells. In the present study, we measured the reduction rate of nitroxiradical probe (spin clearance rate), which represents the rate of free radical production in intact cells, using electron spin resonance (ESR) spectroscopy (28–30,6).

Both diabetes and the insulin-resistant state are linked with atherosclerosis. One of the metabolic features of insulin resistance is a defect in the insulin-mediated lowering action of fatty acids as well as a defect in glucose disposal. Several reports have shown that plasma free fatty acids (FFAs) may affect vascular functions. Raising plasma FFAs in minipigs with intralipid and heparin acutely elevates vascular resis-

tance and raises blood pressure (31). Raising FFAs systematically in healthy normotensive volunteers impairs the lower extremity vascular response to methacholine, an endothelium-dependent dilator (32). The elevated FFAs in obese hypertensive subjects are extremely resistant to suppression by insulin and correlate with blood pressure (33,34). In addition, we showed that FFAs—especially palmitate, which is a major saturated FFA in plasma—could stimulate diacylglycerol (DAG) synthesis through de novo pathway in cultured endothelial cells and probably activate PKC (35). From this evidence, we speculated that FFA as well as high glucose level might affect ROS production and subsequently alter various vascular functions. In the present study, therefore, we also examined the effect of palmitate on ROS production in cultured vascular cells.

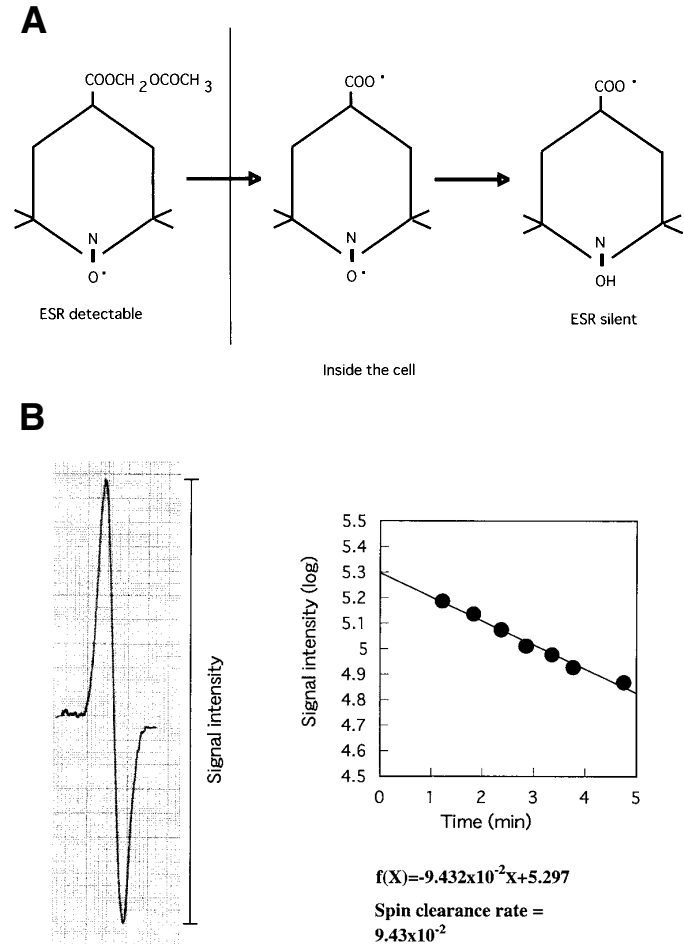
## RESEARCH DESIGN AND METHODS

**Cell culture.** Bovine aortic smooth muscle cells and endothelial cells were obtained from calf aorta as described (17,18). Both cell types were cultured with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 100 mU/ml penicillin, and 100 mg/ml streptomycin at 37°C in an atmosphere of 95% O<sub>2</sub>/5% CO<sub>2</sub>. Every 5–10 days, the cells were subcultured by 0.25% trypsin (Gibco) harvesting.

**Synthesis of acetoxymethyl-2,2,6,6-tetramethylpiperidine-1-oxyl-3-carboxylate.** We synthesized acetoxymethyl-2,2,6,6-tetramethylpiperidine-1-oxyl-3-carboxylate (CxT-AM), a nitroxiradical, as a spin probe for ESR measurement. 4-Carboxy-2,2,6,6-tetramethylpiperidine-1-oxyl (CxT) was purchased from Aldrich Chemical (Milwaukee, WI). Briefly, CxT-AM was synthesized by esterification of CxT with acetoxymethylbromide by slight modification of the synthesis of acetoxymethyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl-3-carboxylate, as described previously (36). Acetoxymethylbromide was prepared by stirring paraformaldehyde (2.44 g) in acetyl bromide (6.0 ml) for 30 min at 80°C. After the paraformaldehyde disappeared, the mixture was distilled at 130–138°C, and a yellow oil was obtained (4.54 g, yield 37%). The resulting acetoxymethylbromide (1.38 g) was added at 0°C to a mixture of CxT (1.0 g) and triethylamine (0.78 ml) in dimethylformamide (10 ml), and then stirred for 7 days at room temperature. After diluting with 40 ml of dichloromethane, washing once with water, and drying with manganese sulfate, the dichloromethane was evaporated. The residue was at first purified with silica gel column chromatography (Silicagel 60; Merck, Darmstadt, Germany), and then recrystallized from ether-hexane (bright red fine needle, yield 0.4 g [32%]). The purity was estimated with ESR, infrared spectroscopy, and fast atom bombardment mass spectrometry. Synthesized CxT-AM was used as a spin probe for ESR measurement.

**Measurement of ROS by ESR spectroscopy.** The procedures for ESR with spin probes and nitroxide radicals in cultured cells were described previously (37–40). Briefly, the cells were removed from the dishes by treatment with 0.25% trypsin, centrifuged at 800g for 5 min, and suspended in the medium without serum at a concentration of  $1 \times 10^7$  cells/ml. The nitroxide radical CxT-AM was added to the cell suspension at the final concentration of 20  $\mu$ mol/l, and the samples were mixed quickly but gently. Then, immediately, the samples were drawn into a gas-permeable Teflon tubes (Zeus Industries, Raritan, NJ) and inserted into a quartz ESR tube open at each end. Experiments were performed at 37°C. The typical time interval between addition of CxT-AM and the beginning of data collection was 2 min or less. ESR spectra were recorded with an ESR spectrometer (JES-RE-1X; JEOL) operating at X-band (9.45 GHz). The microwave power was 10 mW, the field modulation width was 0.2 mT, and the magnetic field range was swept at a scan rate of 5 mT/min. These procedures did not alter cell viability, as measured by exclusion of Trypan blue. As shown in Fig. 1, CxT-AM was hydrolyzed inside the cells, retained in the cytoplasm, and then reduced to the corresponding hydroxylamine, leading to the loss of paramagnetism (36). The ESR signal intensity decreased gradually after administration, and the signal decay curve of CxT-AM was obtained by semilogarithmically plotting the peak heights of the ESR signals. The curve was almost linear over the first 5 min of decay, allowing the initial velocity as the clearance constant (spin clearance rate) (Fig. 1). The spin clearance rate represents the rate of radical generation in intact cells.

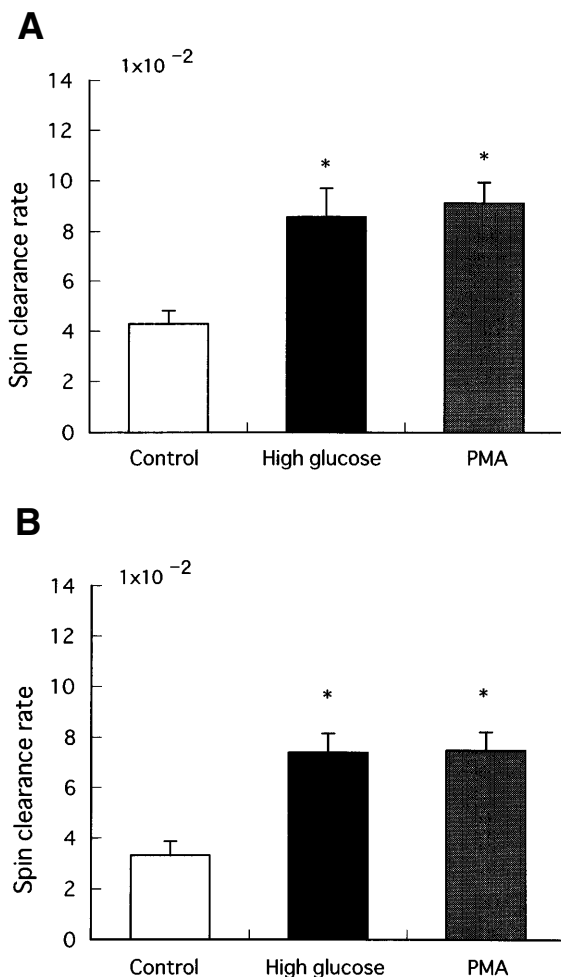
**Extraction and assay of DAG.** For the experiments, cells were allowed to reach confluence in 35-mm dishes, and then the medium was changed to DMEM supplemented with various concentrations of palmitate and 1% serum. At the end of the desired exposure time, the experiment was terminated by the addition of ice-cold methanol. Samples were harvested and transferred to chlo-



**FIG. 1.** Measurement of ROS by ESR spectroscopy. **A:** The nitroxide radical CxT-AM was hydrolyzed inside the cells, retained in the cytoplasm, and then reduced to the corresponding hydroxylamine, leading to the loss of paramagnetism. **B:** The ESR signal intensity decreased gradually after the addition of CxT-AM to the cell suspension. The signal decay curve of CxT-AM was obtained by semilogarithmically plotting the peak heights of the ESR signals. The initial kinetic constant (spin clearance rate) was calculated from the slope of the signal decay curve.

roform-resistant tubes. After addition of 2 ml chloroform and 1 ml H<sub>2</sub>O to the samples, total lipids were extracted according to the methods of Bligh and Dyer (41). Total DAG was measured by an enzymatic assay kit using DAG kinase (Amersham, Arlington Heights, IL), as previously reported (35). Briefly, the resulting <sup>32</sup>P-phosphatidic acid that was converted from DAG by DAG kinase in vitro was separated on silica gel G thin layer plates and developed in chambers using a solvent of chloroform-acetone-methanol-acetic acid-water (10:4:3:1). The spots of phosphatidic acid visualized by autoradiography were scraped from the plates into vials, and radioactivity was determined by liquid scintillation counting.

**PKC assay.** PKC activity was measured by in situ PKC assay in digitonin-permeabilized cultured smooth muscle cells, as previously reported (42). For the assay, the cells were cultured on six-well flat-bottomed microtiter plates. After confluence, the medium was changed to DMEM containing various concentrations of palmitate and 1% serum. At the end of the desired exposure time, the medium was aspirated and replaced with a buffered salt solution containing 137 mmol/l NaCl, 5.4 mmol/l KCl, 10 mmol/l MgCl<sub>2</sub>, 0.3 mmol/l sodium phosphate, 0.4 mmol/l potassium phosphate, 25 mmol/l  $\alpha$ -glycerophosphate, 5.5 mmol/l D-glucose, 5 mmol/l EGTA, 1 mmol/l CaCl<sub>2</sub>, 100  $\mu$ mol/l <sup>32</sup>P ATP (Amersham), 50  $\mu$ g/ml digitonin, and 20 mmol/l HEPES (pH 7.2, 30°C). In addition, a 100  $\mu$ mol/l PKC-specific octapeptide substrate (VRKRLRRL) was added to the buffer. The kinase reaction proceeded for 10 min at 30°C before termination by the addition of 10  $\mu$ l of 25% (wt/vol) trichloroacetic acid.



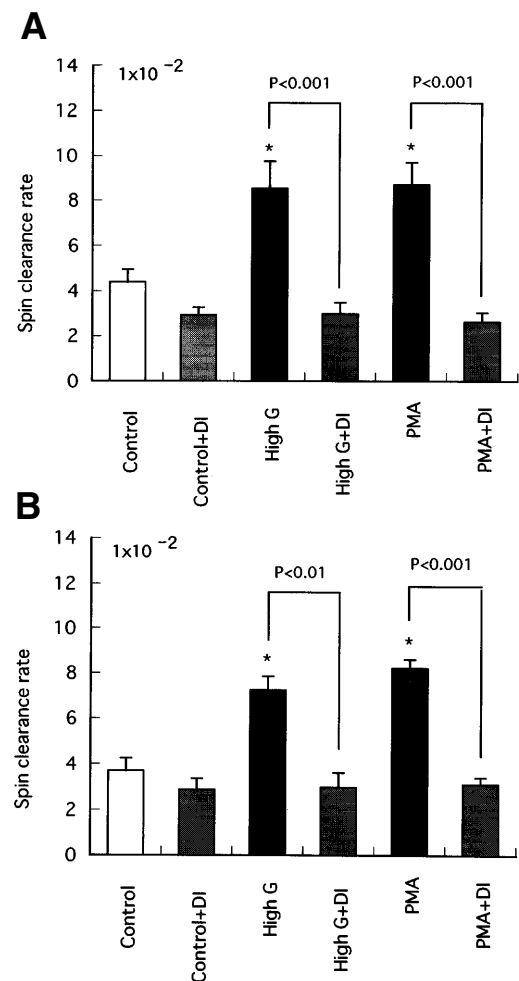
**FIG. 2.** Effect of high glucose level and PMA on ROS production in cultured aortic smooth muscle cells (A) and endothelial cells (B). The confluent cells were incubated with the test media containing 1% serum and 400 mg/dl glucose (High glucose) or 100 mg/dl glucose (Control) for 72 h. For the last 3 h of incubation, PMA ( $5 \times 10^{-7}$  mol/l) was added to the control media (PMA). ROS was measured by ESR spectroscopy as described in RESEARCH DESIGN AND METHODS. Results are expressed as means + SE from four independent experiments. \* $P < 0.01$  vs. control.

Aliquots (45  $\mu$ l) of the reaction mixture were spotted onto 2-cm phosphocelulose filter (Whatman P-81), and the filter was washed three times with 75 mmol/l phosphoric acid. The PKC-dependent phosphorylation of the peptide substrate bound to the filter was quantified by scintillation counting.

**Statistical analysis.** Statistical analysis was performed by analysis of variance followed by Fisher's comparison test.

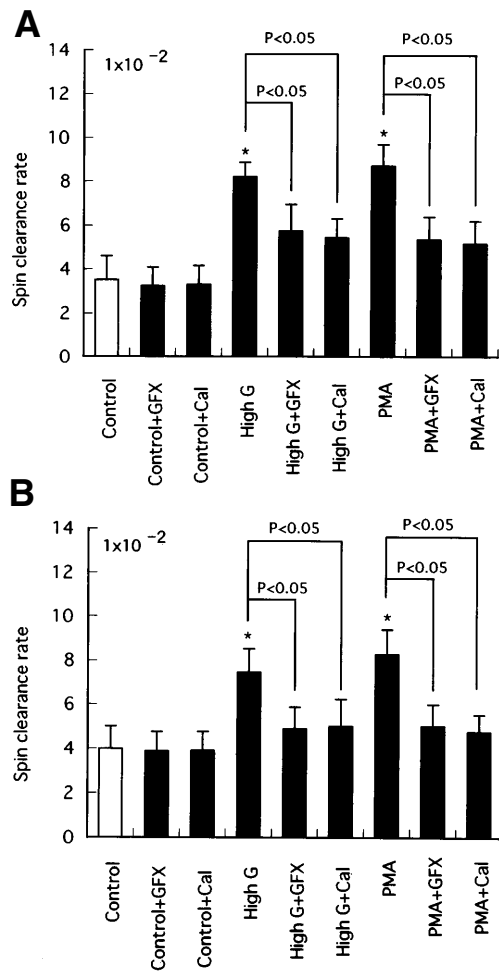
## RESULTS

For these experiments, aortic smooth muscle cells and endothelial cells were allowed to reach confluence in 100-mm dishes, and then the medium was changed to DMEM supplemented with 1% serum and high glucose level (400 mg/dl) or normal glucose level (100 mg/dl). Exposure of cultured smooth muscle cells to high glucose level for 72 h induced a significant ( $P < 0.01$ ) increase in the spin clearance rate compared with exposure to normal glucose level (Fig. 2A), suggesting a persistent stimulatory effect of high glucose level on free radical production. Treatment of the cells with phorbol myristic acid (PMA) ( $5 \times 10^{-7}$  mol/l) (Sigma, St. Louis, MO)



**FIG. 3.** Effect of diphenylene iodonium on high glucose- or PMA-induced increase in ROS production in cultured aortic smooth muscle cells (A) or endothelial cells (B). The confluent cells were incubated with the test media containing 1% serum and 400 mg/dl glucose (High G) or 100 mg/dl glucose (Control) for 72 h. For the last 3 h of incubation, PMA ( $5 \times 10^{-7}$  mol/l) was added to the control media (PMA). For the last 2 h of incubation, diphenylene iodonium ( $10^{-5}$  mol/l) was added to the control media (Control+DI), high glucose-containing media (High G+DI) or PMA-containing media (PMA+DI). ROS was measured by ESR spectroscopy as described in RESEARCH DESIGN AND METHODS. Results are expressed as means + SE from four independent experiments. \* $P < 0.01$  vs. control.

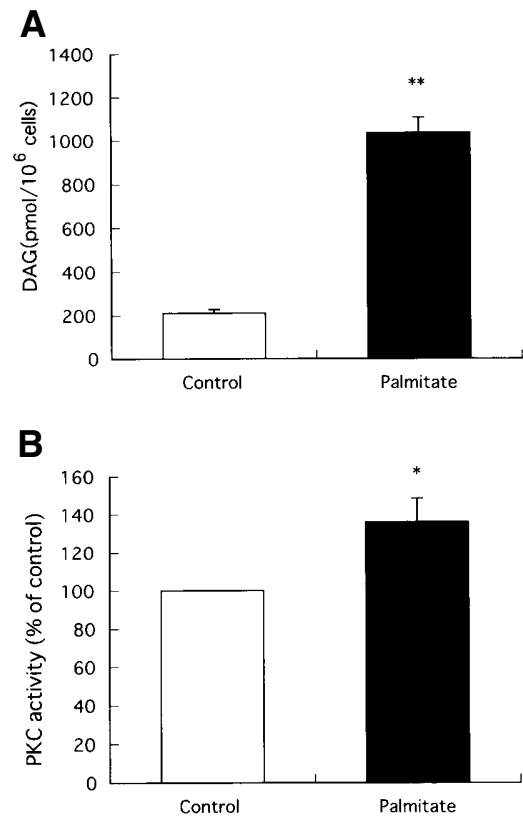
for 3 h also significantly ( $P < 0.01$ ) increased the spin clearance rate (Fig. 2A), suggesting a stimulatory effect of PMA on free radical production. In cultured endothelial cells, exposure to high glucose level and treatment with PMA also induced a significant ( $P < 0.01$ ,  $P < 0.01$ , respectively) increase in the spin clearance rate (Fig. 2B). To evaluate the role of NAD(P)H oxidase, the effect of diphenylene iodonium, a NADPH oxidase inhibitor, on the spin clearance rate was examined. Treatment of smooth muscle cells with diphenylene iodonium ( $10^{-5}$  mol/l) for 2 h completely restored the increased spin clearance rate induced by both high glucose level and PMA to the control value (Fig. 3A). In contrast, treatment for 2 h with inhibitors of other flavoproteins, such as xanthine oxidase (oxypurinol, 100  $\mu$ mol/l), nitric oxide synthase (1-N-monomethyl arginine, 10  $\mu$ mol/l), and mitochondrial electron transport chain (rotenone, 100  $\mu$ mol/l), did



**FIG. 4.** Effect of GF109203X or calphostin C on high glucose- or PMA-induced increase in ROS production in cultured aortic smooth muscle cells (A) or endothelial cells (B). The confluent cells were incubated with the test media containing 1% serum and 400 mg/dl glucose (High G) or 100 mg/dl glucose (Control) for 72 h. For the last 3 h of incubation, PMA ( $5 \times 10^{-7}$  mol/l) was added to the control media (PMA). For the last 2 h of incubation, GF109203X ( $5 \times 10^{-7}$  mol/l) or calphostin C ( $5 \times 10^{-7}$  mol/l) was added to control media (Control+GFX, Control+Cal), high glucose-containing media (High G+GFX, High G+Cal) or PMA-containing media (PMA+GFX, PMA+Cal). ROS was measured by ESR spectroscopy as described in RESEARCH DESIGN AND METHODS. Results are expressed as means + SE from four independent experiments. \* $P < 0.01$  vs. control.

not affect the increased spin clearance rate induced by high glucose level or PMA. Similar results were obtained in cultured aortic endothelial cells (Fig. 3B). These results suggest that both high glucose level and PMA may stimulate ROS production through activation of NAD(P)H oxidase. To confirm the role of PKC in the stimulatory effect of high glucose level, the effect of PKC inhibitor was also examined. Treatment with GF109203X ( $5 \times 10^{-7}$  mol/l) (Sigma) or calphostin C ( $5 \times 10^{-7}$  M) (Sigma) for 2 h significantly ( $P < 0.05$ ,  $P < 0.05$ , respectively) inhibited the increased spin clearance rate by high glucose level or PMA to the control value (Fig. 4A and B).

As for palmitate, we first examined the effect of palmitate on the DAG-PKC pathway in cultured smooth muscle cells. A stock solution of palmitate was prepared by dissolving it in

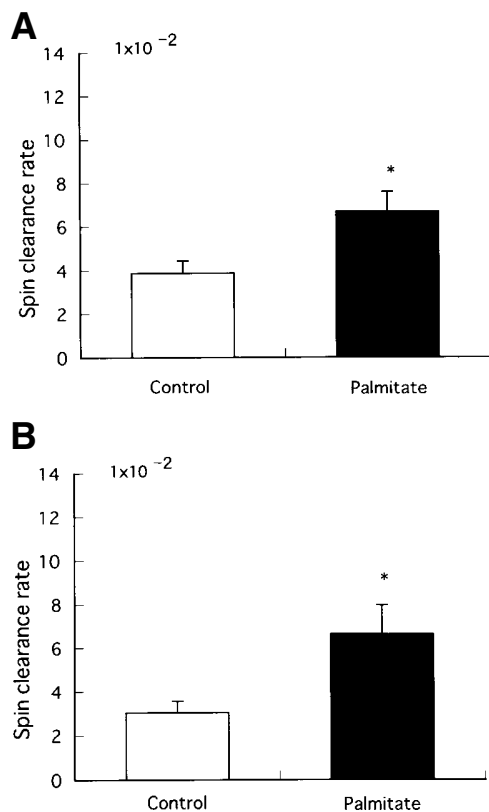


**FIG. 5.** Effect of palmitate on DAG level (A) and PKC activity (B) in cultured aortic smooth muscle cells. The confluent cells were incubated with test media containing 1% serum with palmitate (Palmitate) or without palmitate (Control) for 72 h. DAG was measured by the DAG kinase method, and PKC activity was measured by in situ PKC assay, both described in RESEARCH DESIGN AND METHODS. Results are expressed as means + SE from four independent experiments. \* $P < 0.05$ ; \*\* $P < 0.01$  vs. control.

0.1 N NaOH solution in a bath of boiling water. Appropriate volumes of these stock solutions, freshly prepared before each experiment, were then added slowly and during continuous agitation to the test medium with 1% serum. Palmitate at a concentration of 200  $\mu$ mol/l, which is a physiological concentration in plasma, increased DAG level in a time-dependent manner (0–24 h), and the maximal DAG level lasted up to 72 h in cultured smooth muscle cells. As shown in Fig. 5, exposure of the cells to palmitate (200  $\mu$ mol/l) for 72 h significantly increased both DAG level and PKC activity ( $P < 0.01$ ,  $P < 0.05$ , respectively). Next, exposure of both smooth muscle cells and endothelial cells to palmitate (200  $\mu$ mol/l) for 72 h induced a significant increase in the spin clearance rate ( $P < 0.01$ ,  $P < 0.01$ , respectively) (Fig. 6). Again, diphenylene iodonium ( $10^{-5}$  mol/l) completely restored the increased spin clearance rate induced by palmitate to the control value in cultured smooth muscle cells (Fig. 7A). PKC inhibitors, such as GF109203X or calphostin C, also significantly ( $P < 0.05$ ,  $P < 0.05$ , respectively) inhibited the increased spin clearance rate (Fig. 7A). Similar results were obtained in cultured endothelial cells (Fig. 7B).

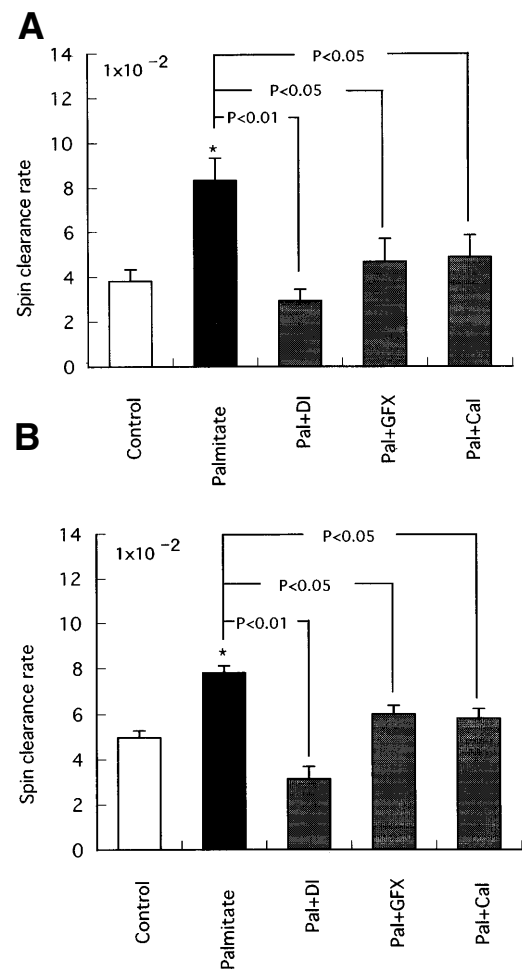
## DISCUSSION

Numerous reports have shown that oxidative stress is increased in animal models of diabetes and in patients with dia-



**FIG. 6.** Effect of palmitate on ROS production in cultured aortic smooth muscle cells (A) and endothelial cells (B). The confluent cells were incubated with test media containing 1% serum with palmitate (Palmitate) or without palmitate (Control) for 72 h. ROS was measured by ESR spectroscopy as described in RESEARCH DESIGN AND METHODS. Results are expressed as means + SE from four independent experiments. \* $P < 0.01$  vs. control.

betes (1–5). Recently, we also confirmed the increased oxidative stress in streptozotocin-induced diabetic rats using non-invasive in vivo ESR measurement (6). It has been postulated that hyperglycemia may produce ROS through AGE formation (3,4) and altered polyol pathway activity (5). However, recent studies have revealed that vascular cells, such as smooth muscle cells and endothelial cells, can produce ROS through activation of NAD(P)H oxidase (7–12). In addition, Mohazzab et al. (7) and Rajagopalan et al. (9) have shown that this enzyme is the most important source of ROS in intact arteries rather than arachidonic acid-metabolizing enzymes, xanthine oxidase or mitochondrial sources. In the present study, we showed that chronic exposure of aortic smooth muscle cells and endothelial cells to a high glucose level induced a significant increase in free radical production, as evaluated by ESR spectroscopy. Furthermore, this increase in free radical production induced by high glucose was completely restored to the control value by diphenylene iodonium, a NAD(P)H oxidase inhibitor. Although diphenylene iodonium can inhibit several other flavoproteins, such as xanthine oxidase, nitric oxide synthase, and mitochondrial electron transport chain, the present results showed that specific inhibitors of these enzymes were ineffective for restoring the increase in free radical production induced by high glucose. These results suggest that high glucose level may stimulate ROS production through activation of NAD(P)H oxidase in aortic smooth mus-



**FIG. 7.** Effect of diphenylene iodonium or PKC inhibitor on palmitate-induced ROS production in cultured smooth muscle cells (A) and aortic endothelium cells (B). The confluent cells were incubated with test media containing 1% serum with palmitate (Palmitate) or without palmitate (Control) for 72 h. For the last 2 h of incubation, diphenylene iodonium ( $10^{-5}$  mol/l), GF109203X ( $5 \times 10^{-7}$  mol/l), or calphostin C was added to palmitate-containing media (Pal+DI, Pal+GFX, or Pal+Cal, respectively). ROS was measured by ESR spectroscopy as described in RESEARCH DESIGN AND METHODS. Results are expressed as means + SE from four independent experiments. \* $P < 0.01$  vs. control.

cle cells and endothelial cells. The NADPH oxidase system has been well characterized in phagocytic cells, where it consists of cytosolic components, p47<sup>phox</sup> and p67<sup>phox</sup> (43,44), a low molecular weight G-protein, Rac 1 or Rac 2 (45,46), and a membrane-associated cytochrome b558. In phagocytes, cytochrome b558 consists of a 22-kDa  $\alpha$ -subunit (p22<sup>phox</sup>) and a glycosylated 91-kDa  $\beta$ -subunit (gp91<sup>phox</sup>) (47). In a variety of nonphagocytic cells, including vascular smooth muscle cells and endothelial cells, these components of phagocyte-type oxidase (8,11,48) or Mox-1 (49), a variant form of gp91<sup>phox</sup>, have been detected, confirming the role of NAD(P)H oxidase in ROS production in vascular cells. In phagocytic cells, it is well recognized that PKC can activate NADPH oxidase by phosphorylation-dependent activation of p47<sup>phox</sup> (13), p67<sup>phox</sup> (14), and/or Rac (15). The present study shows that PMA, an activator of PKC, stimulated ROS production in both cultured aortic smooth muscle cells and endothelial cells. Furthermore, the increased ROS production induced by high glucose level in cul-

tured vascular cells was significantly inhibited by both GF109203X and calphostin C, PKC-specific inhibitors, suggesting the role of PKC. It is well established that high glucose activates PKC through an increase in de novo DAG synthesis in vascular cells (17–27). Taken together, the present results strongly suggest that high glucose level stimulated ROS production through PKC-dependent activation of NAD(P)H oxidase in cultured vascular cells. Although PKC inhibitor normalized the increased ROS production induced by high glucose level statistically completely, it should be stated that phosphatidic acid also can stimulate NADPH oxidase (50,51). Because phosphatidic acid is an intermediate of de novo DAG synthesis, its level should be increased by high glucose level. Increased phosphatidic acid level might in part contribute to the increased ROS production induced by high glucose level. In addition, this oxidase preferentially utilizes NADH rather than NADPH as a substrate in vascular cells (8), which is in contrast to the phagocytic enzyme. High glucose level was reported to induce an increase in the ratio of cytosolic NADH:NAD<sup>+</sup> through an increased polyol pathway (5). This might also be in favor of the high glucose-induced increase in ROS production through NAD(P)H oxidase in vascular cells.

Migration and proliferation of vascular smooth muscle cells are contributory events in vascular lesions associated with atherosclerosis and restenosis after vascular injury. ROS are reported to elicit specific growth and induce the expression of growth-related genes, including c-fos, c-myc, and c-jun (52–54). Furthermore, this ROS production through NAD(P)H oxidase has been implicated in the pathogenesis of angiotensin II-induced hypertension and vascular smooth muscle hypertrophy (8,10,12). In endothelial cells, cytokine-induced expression of vascular cell adhesion molecule-1 has been reported to involve ROS-mediated mobilization of nuclear factor- $\kappa$ B and can be blocked by antioxidant (55–56). The expression of vascular cell adhesion molecule-1 promotes monocyte adhesion to endothelial cells and may be an important event in the development of atherosclerosis (57). Because the ROS generated by vascular NAD(P)H oxidase appears to be released outside the cell as well as inside the cell (49), the ROS released outside the cell may contribute to impaired endothelium-dependent vascular relaxation by inactivation of nitric oxide (9). Thus, taken together with these findings, the present results suggest that the increased ROS production through PKC-dependent activation of NAD(P)H oxidase in vascular cells induced by high glucose level may contribute to excessive acceleration of atherosclerosis in patients with diabetes.

Both diabetes and the insulin-resistant state are linked with atherosclerosis. One of the links between insulin resistance and atherosclerosis may be elevated plasma FFAs. This notion is supported by several reports showing that FFA may affect vascular functions. Raising plasma FFAs in minipigs with intralipid and heparin acutely elevates vascular resistance and raises blood pressure (31). Raising FFAs systematically in healthy normotensive volunteers impairs the lower extremity vascular response to methacholine, an endothelium-dependent dilator (32). The elevated FFAs in obese hypertensive subjects are extremely resistant to suppression by insulin and correlate with blood pressure (33,34). Previously, we showed that palmitate, which is a major saturated FFA in plasma, increased DAG level in cultured aortic endothelial cells (35). Because excessive influx of palmitate into vascular

cells could lead to an increase in acyl-CoA levels, the mechanism for the stimulatory effect of palmitate on DAG levels was supposed to be an increase in the de novo DAG synthesis by step-wise acylation. In the present study, we confirm that palmitate increased DAG level and PKC activity in cultured aortic smooth muscle cells. The detailed mechanism for palmitate-induced activation of the DAG-PKC pathway should be determined in future study. The present results showed that palmitate also stimulated ROS production in cultured aortic smooth muscle cells and endothelial cells. Again, this increased production of ROS was completely restored to control value by diphenylene iodonium and significantly inhibited by the PKC inhibitors GF109203X and calphostin C. These results suggest that palmitate as well as high glucose level may stimulate ROS production through PKC-dependent activation of NAD(P)H oxidase in cultured vascular cells. If increased DAG levels by palmitate may be due to the increased de novo synthesis, an increased phosphatidic acid level might in part contribute to the increased ROS production induced by palmitate. The increased ROS production induced by FFAs, such as palmitate, in vascular cells may lead to the altered vascular functions and may in part account for excessive acceleration of atherosclerosis in patients with insulin resistance syndrome.

In conclusion, the present study provides the first evidence that high glucose level and FFA (palmitate) stimulate ROS production through PKC-dependent activation of NAD(P)H oxidase in cultured aortic smooth muscle cells and endothelial cells. This may in part account for the excessive acceleration of atherosclerosis in patients with diabetes and patients with insulin resistance syndrome.

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#### REFERENCES

1. Wolf SP, Jiang ZT, Hunt JV: Protein glycation and oxidative stress in diabetes mellitus and aging. *Free Radic Biol Med* 10:339–352, 1991
2. Oberley LW: Free radicals and diabetes. *Free Radic Biol Med* 5:113–124, 1988
3. Mullarkey CJ, Edelstein D, Brownlee M: Free radical generation by early glycation products: a mechanism for accelerated atherogenesis in diabetes. *Biochem Biophys Res Commun* 173:932–939, 1990
4. Baynes JW: Role of oxidative stress in development of complications in diabetes. *Diabetes* 40:405–412, 1991
5. Williamson JR, Chang K, Fragos M, Hasan KS, Ido Y, Kawamura T, Nyengaard JR, van den Enden M, Kilo C, Tilton RG: Hyperglycemic pseudohypoxia and diabetic complications. *Diabetes* 42:801–813, 1993
6. Sano T, Umeda F, Hashimoto T, Nawata H, Utsumi H: Oxidative stress measurement by in vivo electron spin resonance spectroscopy in rats with streptozotocin-induced diabetes. *Diabetologia* 41:1355–1360, 1998
7. Mohazzab KM, Kaminski PM, Wolin MS: NADH oxidoreductase is a major source of superoxide anion in bovine coronary endothelium. *Am J Physiol* 266:H2568–H2572, 1994
8. 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
9. Rajagopalan S, Kurz S, Munzel T, Tarpey M, Freeman BA, Griendling KK, Harrison DG: Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. *J Clin Invest* 97:1916–1923, 1996
10. Ushio-Fukai M, Zafari AM, Fukui T, Ishizuka N, Griendling KK: p22phox is a critical component of the superoxide-generating NADH/NADPH oxidase sys-

- tem and regulates angiotensin II-induced hypertrophy in vascular smooth muscle cells. *J Biol Chem* 271:23317–23321, 1996
11. Bayraktutan U, Draper N, Lang D, Shah AM: Expression of a functional neutrophil-type NADPH oxidase in cultured rat coronary microvascular endothelial cells. *Cardiovasc Res* 38:256–262, 1998
  12. Warnholtz A, Nickenig G, Schulz E, Machazina R, Brazen JH, Skatchkov M, Heitzer T, Stasch JP, Griendling KK, Harrison DG, Bohm MD, Meinertz T, Munzel T: Increased NADH-oxidase mediated superoxide production in the early stages of atherosclerosis. *Circulation* 99:2027–2033, 1999
  13. Swain SD, Helgeson SL, Davis AR, Nelson LK, Quinn MT: Analysis of activation-induced conformational changes in p47phox using tryptophan fluorescence spectroscopy. *J Biol Chem* 272:29502–29510, 1997
  14. Benna JE, Dang PM, Gaudry M, Fay M, Morel F, Hakim J, Gougerot-Pocidal MA: Phosphorylation of the respiratory burst oxidase subunit p67(phox) during human neutrophil activation: regulation by protein kinase C-dependent and independent pathway. *J Biol Chem* 272:17204–17208, 1997
  15. Akasaki T, Koga H, Sumimoto H: Phosphoinositide 3-kinase-dependent and independent activation of the small GTPase Rac2 in human neutrophil. *J Biol Chem* 274:18055–18059, 1999
  16. King GL, Johnson S, Wu G: Possible growth modulators involved in the pathogenesis of diabetic proliferative retinopathy. In *Growth Factors in Health and Disease*. Westermark B, Betscholtz C, Hokfelt B, Eds. Amsterdam, Elsevier Science, 1990, p. 303–317
  17. Inoguchi T, Battan R, Handler E, Sportsman JR, Heath W, King GL: Preferential elevation of protein kinase C isoform  $\beta$  II and diacylglycerol levels in the aorta and heart of diabetic rats: differential reversibility to glycemic control by islet cell transplantation. *Proc Natl Acad Sci U S A* 89:11059–11063, 1992
  18. Inoguchi T, Xia P, Kunisaki M, Higashi S, Feener EP, King GL: Insulin's effect on protein kinase C and diacylglycerol induced by diabetes and glucose in vascular tissues. *Am J Physiol* 267:E369–E379, 1994
  19. Xia P, Inoguchi T, Kem TS, Engerman RL, Oates PJ, King GL: Characterization of the mechanism for the chronic activation of diacylglycerol-protein kinase C pathway in diabetes and hypergalactosemia. *Diabetes* 43:1122–1129, 1994
  20. Craven PA, DeRubertis FR: Protein kinase C is activated in glomeruli from streptozotocin diabetic rats: possible mediation by glucose. *J Clin Invest* 83:1667–1675, 1989
  21. Wolf BA, Williamson JR, Easom RA, Chang K, Sherman WR, Turk J: Diacylglycerol accumulation and microvascular abnormalities induced by elevated glucose levels. *J Clin Invest* 87:31–38, 1991
  22. Shiba T, Inoguchi T, Sportsman JR, Heat WF, Bursell S, King GL: Correlation of diacylglycerol level and protein kinase C activity in rat retina to retinal circulation. *Am J Physiol* 265:E783–E793, 1993
  23. Tesfamariam B, Brown ML, Cohen RA: Elevated glucose impairs endothelium-dependent relaxation by activating protein kinase C. *J Clin Invest* 87:1643–1648, 1991
  24. Studor PK, Craven PA, DeRubertis FR: Role of protein kinase C in the mediation of increased fibronectin accumulation by mesangial cells grown in high-glucose medium. *Diabetes* 42:118–126, 1993
  25. Ishii H, Jirousek MR, Koya D, Takagi C, Xia P, Clermont A, Bursell S-E, Kern TS, Ballas LM, Heath WF, Stramm LE, Feener EP, King GL: Amelioration of vascular dysfunctions in diabetic rats by an oral PKC  $\beta$  inhibitor. *Science* 272:728–731, 1996
  26. Kuroki T, Inoguchi T, Umeda F, Ueda F, Nawata H: High glucose induces alteration of gap junction permeability and phosphorylation of connexin-43 in cultured aortic smooth muscle cells. *Diabetes* 47:931–936, 1998
  27. Koya D, King GL: Protein kinase C activation and the development of diabetic complications. *Diabetes* 47:859–866, 1998
  28. Utsumi H, Muto E, Masuda S, Hamada A: In vivo ESR measurement of free radicals in whole mice. *Biochem Biophys Res Commun* 172:1342–1348, 1990
  29. Takeshita K, Utsumi H, Hamada A: Whole mouse measurement of paramagnetism-loss of nitroxide free radical in lung with a L-band ESR spectrometer. *Biochem Mol Biol Int* 29:17–24, 1993
  30. Ide T, Tsutsui H, Kinugawa S, Suematsu N, Hayashidani S, Ichikawa K, Utsumi H, Machida Y, Egashira K, Takeshita A: Direct evidence for increased hydroxyl radicals originating from superoxide in the failing myocardium. *Circ Res* 86:152–157, 2000
  31. Bulow J, Madsen J, Hojgaard L: Reversibility of the effect on local circulation of high lipid concentrations in blood. *Scand J Clin Lab Invest* 50:291–296, 1990
  32. Steinberg HO, Tarshoby M, Monestel R, Hook G, Cronin J, Johnson A, Bayazeed B, Baron AD: Elevated circulating free fatty acid levels impair endothelium-dependent vasodilation. *J Clin Invest* 100:1230–1239, 1997
  33. Hennes MM, O'Shaughnessy IM, Kelly TM, Labelle P, Egan BM, Kissebah AH: Insulin resistant lipolysis in abdominally-obese hypertensives: role of the renin-angiotensin system. *Hypertension* 28:120–126, 1996
  34. Egan B, Hennes MMI, O'Shaughnessy IM, Stepniakowski KT, Kissebah AH, Goodfriend TL: Obesity hypertension is more closely related to impairment of insulin's fatty acid than glucose lowering action. *Hypertension* 27 (pt 2):723–728, 1996
  35. Kuroki T, Inoguchi T, Umeda F, Nawata H: Effect of eicosapentaenoic acid on glucose-induced diacylglycerol synthesis in cultured bovine aortic endothelial cells. *Biochem Biophys Res Commun* 247:473–477, 1998
  36. Sano H, Naruse M, Matsumoto K, Oi T, Utsumi H: A new nitroxyl-probe with high retentivity in brain and its application to brain imaging. *Free Radic Biol Med* 28:959–969, 2000
  37. Kaplan J, Canonico PG, Caspary WJ: Electron spin resonance studies of spin-labeled mammalian cells by detection of surface-membrane signals. *Proc Natl Acad Sci U S A* 70:66–70, 1973
  38. Chen K-Y, McLaughlin MG: Differences in the reduction kinetics of incorporated spin labels in undifferentiated and differentiated mouse neuroblastoma cells. *Biochim Biophys Acta* 845:189–195, 1985
  39. Swartz HM, Sentjurs M, Morse PD II: Cellular metabolism of water-soluble nitroxides: effect on rate of reduction of cell/nitroxide ratio, oxygen concentrations and permeability of nitroxides. *Biochim Biophys Acta* 888:82–90, 1986
  40. Chen K, Morse PD II, Swartz HM: Kinetics of enzyme-mediated reduction of lipid soluble nitroxide spin labels by living cells. *Biochim Biophys Acta* 943:477–484, 1988
  41. Bligh EG, Dyer WJ: A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 37:911–917, 1959
  42. Williams B, Schrier R: Characterization of glucose-induced in situ protein kinase C activity in cultured vascular smooth muscle cells. *Diabetes* 41:1464–1472, 1992
  43. Nunoi H, Rotrosen D, Gallin JI, Malech HL: Two forms of autosomal chronic granulomatous disease lack distinct neutrophil cytosol factors. *Science* 242:1298–1301, 1988
  44. Volpp BD, Nauseef WM, Clark RA: Two cytosolic neutrophil oxidase components absent in autosomal chronic granulomatous disease. *Science* 242:1295–1297, 1988
  45. Abo A, Pick E, Hall A, Totty N, Teahan CG, Segal AW: Activation of the NADPH oxidase involves the small GTP-binding protein p21rac1. *Nature* 353:668–670, 1991
  46. Knaus UG, Heyworth PG, Kinsella BT, Curnutte JT, Bokoch GM: Purification and characterization of Rac2: a cytosolic GTP-binding protein that regulates human neutrophil NADPH oxidase. *J Biol Chem* 267:23575–23582, 1992
  47. Parkos CA, Allen RA, Cochrane CG, Jesaitis AJ: Purified cytochrome b from human granulocyte plasma membrane is comprised of two polypeptides with relative molecular weights of 91,000 and 22,000. *J Clin Invest* 80:732–742, 1987
  48. Fukui T, Lassegue B, Kai H, Alexander RW, Griendling KK: Cytochrome b-558  $\alpha$ -subunit cloning and expression in rat aortic smooth muscle cells. *Biochim Biophys Acta* 1231:215–219, 1995
  49. Suh Y-A, 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
  50. Bellavite P, Corso F, Dusi S, Grzeskowiak M, Della-Bianca V, Rossi F: Activation of NADPH-dependent superoxide production in plasma membrane extracts of pig neutrophils by phosphatidic acid. *J Biol Chem* 263:8210–8214, 1988
  51. Agwu DE, McPhail LC, Sozzani S, Bass DA, McCall CE: Phosphatidic acid as a second messenger in human polymorphonuclear leucocytes: effects on activation of NADPH oxidase. *J Clin Invest* 88:531–539, 1991
  52. Rao GN, Berk BC: Active oxygen species stimulate vascular smooth muscle cell growth and proto-oncogene expression. *Circ Res* 70:593–599, 1992
  53. Rao GN, Lassegue B, Griendling KK, Alexander RW: Hydrogen peroxide stimulates transcription of c-jun in vascular smooth muscle cells: role of arachidonic acid. *Oncogene* 8:2759–2764, 1993
  54. Rao GN, Lassegue B, Griendling KK, Alexander RW, Berk BC: Hydrogen peroxide-induced c-fos expression is mediated by arachidonic release: role of protein kinase C. *Nucleic Acids Res* 21:1259–1263, 1993
  55. Tsai J-C, Jain M, Hsieh C-M, Lee W-S, Yoshizumi M, Patterson C, Perrella MA, Cooke C, Wang H, Haber E, Schlegel R, Lee M-E: Induction of apoptosis by pyrrolidinedithiocarbamate and N-acetylcysteine in vascular smooth muscle cells. *J Biol Chem* 271:3667–3670, 1996
  56. Marui N, Offermann MK, Swerlick R, Kunsch C, Rosen CA, Ahmad M, Alexander RW, Medford RM: Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through an antioxidant-sensitive mechanism in human vascular endothelial cells. *J Clin Invest* 92:1866–1874, 1993
  57. Weber C, Erl W, Pietsch A, Strobel M, Ziegler-Heitbrock HWL, Weber PC: Antioxidants inhibit monocyte adhesion by suppressing nuclear factor- $\kappa$ B mobilization and induction of vascular cell adhesion molecule-1 in endothelial cells stimulated to generate radicals. *Arterioscler Thromb* 14:1665–1673, 1994