PKC-δ-dependent activation of oxidative stress in adipocytes of obese and insulin-resistant mice: role for NADPH oxidase

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Oxidative stress is thought to play a causative role in the pathogenesis of type 2 diabetes and its complications and has been shown to increase insulin resistance both in animal models and in human patients with type 2 diabetes (3, 13, 14, 29). Once generated, reactive oxygen species (ROS) can act as second messengers and can activate a number of serine/threonine and tyrosine protein kinases (13, 29, 32). In addition, ROS have detrimental effects on proteins, lipids, and DNA, and their prolonged existence promotes severe tissue damage and cell death. It has been well demonstrated that hyperglycemia is a major factor responsible for the activation of oxidative stress (7, 9, 33, 36); however, little is known about the precise cellular mechanisms responsible for ROS generation in the diabetic tissues (7, 33). Previously, it was postulated that high glucose may activate ROS via multiple processes, such as enhanced formation of advanced glycation end products (AGE), dysfunction of the mitochondrial electron transport chain, and activation of the plasma membrane NADPH oxidase (3, 7). Among these possibilities, recent attention has been focused on NADPH oxidase as a potential source of ROS production in diabetic/hyperglycemic conditions (20, 29). This enzyme, which has been found primarily in phagocytic cells (1), was recently shown to exist in nonphagocytic cells, such as endothelial cells, vascular smooth muscle cells, fibroblasts, and adipocytes (21, 23, 28, 31). The phagocytic NADPH oxidase is a multicomponent enzyme, consisting of cytosolic components p47phox, p67phox, and the small G protein rac, as well as plasma membrane oxidase subunits (reviewed in Ref. 1). Assembly of the enzyme’s components in the membrane is required for its activation (1). Current evidence suggests that protein kinase C (PKC) may regulate the activation of NADPH oxidase (4, 18, 20, 29). PKCs are a family of serine/threonine protein kinases that are classified into conventional PKC (cPKC-α, -β, and -γ), which are calcium and diacylglycerol (DAG) dependent; novel PKC (nPKC-δ, -ε, -η, and -θ), which are DAG dependent but calcium independent; and atypical PKC (aPKC), which are insensitive to both calcium and DAG (34, 37). PKC has been implicated initially in NADPH oxidase-dependent activation of several responses in phagocytic cells, because PMA triggered the production of superoxides (1). Recent studies have implicated PKC in activation of NADPH oxidase and suggested that PKC may phosphorylate the cellular subunit p47phox that may induce its membranous translocation (4, 15, 38, 39, 46). PKC-δ was shown to increase NADPH oxidase activity in diabetic glomeruli (24), neutrophils (12), and HL60 cells (26). Other studies have implicated PKC-δ as another regulator of the oxidase (2) and reported PKC-δ’s requirement for complex assembly of the enzyme’s components (6). Still, the role of PKC in the activation of NADPH oxidase in nonphagocytic cells has not been well established.

In recent years, it became evident that fat tissue has important and integral roles in the development of insulin resistance and type 2 diabetes (22, 43). However, little is known about the mechanisms governing endogenous oxidative stress in the fat tissue, particularly those related to diabetes. In previous studies, we showed that oxidative stress is increased in adipocytes isolated from obese, insulin-resistant C57BL/6J mice (45) [an inbred mice strain susceptible to diet-induced obesity and diabetes (44)] and demonstrated that PKC-δ activity significantly increased in these cells compared with “normal” adipocytes.

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cytes (45). In the present study, we hypothesized that PKC-δ contributes to the rise in ROS production seen in high-fat diet-induced obese and insulin-resistant mouse (HF) adipocytes and examined their cellular source. Here, we show that PKC-δ is an important player affecting cellular ROS production in both normal and diabetic adipocytes and suggest that this process is mediated by high glucose and NADPH oxidase.

MATERIALS AND METHODS

Materials. PKC-δ antibodies (Sc-213) were from Santa Cruz Biotechnology (Santa Cruz, CA). PKC inhibitors Rottlerin and GO®-6976 were purchased from Calbiochem (San Diego, CA), and LY-333531 was from Biomole (Plymouth Meeting, PA). Diphenyleneiodonium chloride (DPI), carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), rotenone, allopurinol, N³-nitro-l-arginine methyl ester hydrochloride (l-NAME), aminoguanidine, and 2',7'-dichlorodihydrofluorescein (DCHF) were from Sigma (Rehovot, Israel).

Animal and diets. Four-week-old mice were randomly assigned to receive either standard laboratory food or a high-fat diet containing 35% lard (BioServe, Frenchtown, NJ), in which 55% of the calories came from fat. The animals were housed in individual cages with free access to water in a temperature-controlled facility with a 12:12-h light-dark cycle; the animals were weighed periodically. Animals were used for testing after 16 wk on their designated diets. Animals were starved for 3 h before experiments and then killed. Animal care was followed according to the Tel Aviv University Institutional Animal Care and Use Committee.

Preparation of adipocytes. Adipocytes were isolated from the epididymal fat pad by digestion with 0.4 mg/ml collagenase (Worthington Biochemical, Freehold, NJ), as described previously (30, 45). Digested fat pads were passed through nylon mesh, and the cells were washed three times with Krebs-bicarbonate buffer (pH 7.4) containing 1% bovine serum albumin (BSA), fraction V; Boehringer Mannheim, Mannheim, Germany), 10 mM HEPES (pH 7.3), 5 mM glucose, and 200 mM adenosine. Cells were centrifuged and collected from the top layer. This process enables isolation of adipocytes from other cell types, such as macrophages, that were recently shown to accumulate in adipose tissue of HF mice (47). Aliquots of cells were used to determine the cell concentrations, as described by Talior et al. (45).

Measurement of intracellular ROS generation. The determination of ROS was based on the oxidation of the nonfluorescent DCHF into a fluorescent dye, 2',7'-dichlorofluorescein (DCF), by peroxide as previously described, with some modifications (45). In brief, adipocytes (10⁶) were incubated with indicated reagents for 1 h followed by the addition of DCFH (30 μM) for an additional 40 min. Cells were washed, and the fluorescence was measured in triplicate samples, using a multiplate fluorometer (FL-600, excitation at 488 nm and emission at 530 nm). Known concentrations of DCFH incubated with 20 mM NaOH were used as standards.

Tissue infections and tissue extraction. The recombinant adenovirus vectors were constructed and used as previously described (42). Epididymal fat tissues were removed from control or HF animals and infected with the PKC-δ recombinant adenoviruses or with a “control” virus encoding β-galactosidase (β-Gal) together with lipofectamine (10 μg/ml; Invitrogen Life Technologies Carlsbad, CA) in Dulbecco’s Modified Eagle’s Medium (DMEM), with low glucose (5 mM glucose), supplemented with 1% BSA for 18 h at 37°C. The tissues were then washed with Krebs-bicarbonate buffer, and adipocytes were isolated from the infected tissues as described in the previous section. Infection efficiency was monitored by X-Gal staining (of β-Gal-infected tissues), which showed an efficiency of >50% of cells. Tissues were homogenized with ice-cold buffer G (25 μg/ml each of 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerophosphate, 50 mM NaF, 5% glycerol, 1% Triton X-100, leupeptin, aprotinin, and pepstatin A). Homogenates were centrifuged at 10,000 g, and supernatants were collected. PKC-δ was immunoprecipitated from the supernatants with specific anti-PKC-δ antibody subjected to gel electrophoresis and immunoblot analysis with indicated antibodies. In other sets of experiments, adipocytes were isolated from infected tissues, as described in the previous section, and incubated with indicated concentrations of glucose. ROS were measured as described earlier.

In vitro kinase activity. PKC-δ activity was assayed as previously described (45). In brief, adipocytes were lysed with buffer H (25 μg/ml each of 20 mM Tris, pH 7.5, 50 mM β-glycerophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 10 mM NaF, 5% glycerol, 1% Triton X-100, leupeptin, aprotinin, and pepstatin A). The lysates were centrifuged, and equal protein aliquots were used for PKC-δ assays. The enzyme was immunoprecipitated for 2 h with anti-PKC-δ antibody bound to protein A-Sepharose. The immunocomplexes were washed extensively, and the kinase reaction was performed at 30°C in 20 mM Tris·HCl, pH 7.3, with 10 mM MgCl₂, 100 μM γ⁻³²P]ATP, 0.05 M), and FCCP (10 μM) for 1 h before addition of DCHF. Fluorescence of 2',7'-dichlorofluorescein (DCF) was measured in triplicate samples, as described in MATERIALS AND METHODS. Results represent means ± SE of 3 independent experiments, presented as fold inhibition of ROS levels determined in control adipocytes. B: HF adipocytes were treated with allopurinol (100 μM), N³-nitro-l-arginine methyl ester (l-NAME, 1 mM), aminoguanidine (AMG, 10 μM), and FCCP (10 μM) for 1 h before addition of DCHF. Fluorescence of 2',7'-dichlorofluorescein (DCF) was measured in a triplicate sample, as described in MATERIALS AND METHODS. Results represent means ± SE of 3 independent experiments, presented as fold inhibition of ROS levels determined in untreated HF adipocytes. *P < 0.05 treatment with DPI vs. no treatment.
phosphatidylserine (PS, 40 μM), and 3 μg of histone H1 (per reaction). Reactions were terminated after 20 min by the addition of SDS-PAGE load buffer and were analyzed in 15% SDS-PAGE. In some experiments, Rottlerin was added to the immunoprecipitates to measure Rottlerin’s inhibitory properties toward PKC-δ.

Graphics and statistical analyses were performed by Origin 6.0 Professional software. A difference was considered to be statistically significant when \( P < 0.05 \). Quantitation of gel bands was performed by densitometry analysis.

RESULTS

To determine the source of intracellular ROS production, HF and control adipocytes (i.e., isolated from diabetic or healthy animals) were incubated with increasing concentrations of the NADPH oxidase inhibitor DPI (31). The results presented in Fig. 1A indicated that DPI decreased ROS levels in both control and HF adipocytes (by \( \sim 50\% \)). Notably, ROS levels were higher in HF adipocytes compared with control adipocytes. Nevertheless, treatment with DPI reduced ROS levels to the “normal” levels seen in control adipocytes (Fig. 1A). In contrast, inhibition of other flavoproteins that may be sensitive to DPI, such as xanthine oxidase or nitric oxide synthase (NOS), with allpurinol (10–100 μM) or L-NAME (0.1–1 mM), respectively, had no effect on ROS levels (Fig. 1B).

Finally, inhibition of AGE formation by aminoguanidine [10 μM (35)] or treatment with the mitochondrial uncoupler FCCP had no effect on ROS levels (Fig. 1B). Similar results were obtained with control adipocytes treated with Rottlerin (17). In addition, the assays were performed in protein A beads to show that the phosphorylated signal is above background (Fig. 2B, bottom). These studies suggested that PKC-δ is involved in ROS production, and additional studies strengthen this point (Fig. 3).

We further examined whether other PKC isoforms affect ROS in HF adipocytes. Treatment with GO-6976, an effective inhibitor toward classical PKC isoforms [i.e., \( \alpha, \beta, \gamma \) (11)], or the selective PKC-β inhibitor LY-333531 slightly reduced ROS in HF adipocytes. Treatment with GO-6976, an effective inhibitor toward classical PKC isoforms [i.e., \( \alpha, \beta, \gamma \) (11)], or the selective PKC-β inhibitor LY-333531 slightly reduced ROS in HF adipocytes. We next examined whether PKC-δ might affect ROS production. HF adipocytes were incubated with the selective PKC-δ inhibitor Rottlerin (17) 1 h before incubation with DCFH. Indeed, increasing concentrations of Rottlerin decreased ROS levels by \( \sim 50\% \) (10 μM Rottlerin; Fig. 2A). Similar results were obtained with control adipocytes treated with Rottlerin. Interestingly, Rottlerin suppressed ROS to a greater extent in control cells than in HF adipocytes (68 ± 17 and 48 ± 6.5% control vs. HF at 10 μM Rottlerin; Fig. 2A).

There is a debate about the ability of Rottlerin to inhibit PKC-δ (10, 16, 17); therefore, we examined its in vitro effect on adipocyte PKC-δ. The enzyme was immunoprecipitated from lysates prepared from HF adipocytes, and PKC-δ activity was assayed in the immunoprecipitate complex with increasing concentrations of Rottlerin. The ability of PKC-δ to phosphorylate the substrate histone H1 was significantly reduced by 5 and 10 μM Rottlerin (Fig. 2B, top), indicating that Rottlerin can inhibit PKC-δ in vitro. To further confirm the specificity of the assay, we show that the kinase activity was dependent on the PKC lipid activator PS (Fig. 2B, middle). In addition, the assays were performed in protein A beads to show that the phosphorylated signal is above background (Fig. 2B, bottom). These studies suggested that PKC-δ is involved in ROS production, and additional studies strengthen this point (Fig. 3).

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ROS levels (10 ± 1.5 and 22 ± 1.2%, respectively; Fig. 2C). These results suggested that conventional PKC isoforms are probably not involved in ROS production. They do not exclude, however, the possibility that novel or atypical PKC isoforms are involved. We next examined the effect of a combination treatment of Rottlerin with DPI on ROS levels. Apparently, administration of both Rottlerin and DPI into HF adipocytes or control adipocytes did not further enhance the reduction in ROS levels achieved by each reagent added separately (Fig. 2D). These results suggested that Rottlerin and DPI affect ROS via a common intracellular target, most likely NADPH oxidase.

To further assess the role of PKC-δ in ROS production, PKC-δ proteins were overexpressed in control and HF adipocytes by use of adenovirus-mediated gene delivery. An adenoviral vector encoding β-Gal was used as a control. Infection protocols were developed and optimized. Tissues infected with β-Gal construct were processed for X-Gal staining, and blue staining of the tissue shown in Fig. 3A confirmed the expression of β-Gal. In addition, adipocytes were isolated from β-Gal-infected tissues and processed for X-Gal staining as well. Blue staining of adipocytes confirmed the expression of β-Gal in adipocytes (~30% efficiency; Fig. 3B). Fat tissues were infected with either the recombinant adenovirus encoding wild-type (WT) PKC-δ (WT-PKC-δ) or its kinase dead (DN) mutant, DN-PKC-δ. The degree of expression of PKC-δ protein above endogenous levels was determined by Western blot analysis (Fig. 3C). Densitometry analysis of PKC-δ bands indicated that overexpression of WT-PKC-δ or DN-PKC-δ was 5 ± 1.6-fold and 3 ± 1-fold, respectively, above endogenous PKC-δ of β-Gal-infected adipocytes. ROS levels were determined in control or HF adipocytes infected with PKC-δ- or β-Gal-infected tissues. Shown is fluorescence of DCF measured in triplicate and presented as fold activation/inhibition of ROS levels determined in β-Gal-infected cells. Results represent means ± SE of 3 independent experiments, presented as arbitrary units. *P < 0.05, PKC-δ-infected adipocytes vs. β-Gal-infected adipocytes.

We next examined whether ROS and PKC-δ are regulated by glucose. This question was of particular importance because glucose uptake is dramatically increased in HF adipocytes (45). Incubation of HF adipocytes with decreased glucose concentrations gradually decreased ROS levels (Fig. 4A). Similarly, increased PKC-δ activity correlated with increased glucose concentrations (Fig. 4B). Moreover, suppression of ROS in reduced glucose concentrations was stronger in DN-PKC-δ-overexpressing cells than in control β-Gal-expressing cells (Fig. 4C). These results suggested a functional link between glucose, PKC-δ activity and ROS production.
DISCUSSION

The present study demonstrates the novel findings that PKC-δ/H9254 enhances oxidative stress in insulin-resistant adipocytes and provides evidence that this process is most likely mediated by NADPH oxidase. This oxidase, which has been described mainly in phagocytic cells (1), was recently shown to exist in nonphagocytic cells (1, 31). However, the mode of activation of this oxidase in these cells is not known well. In 3T3-L1 adipocytes, it has been shown that ROS production was largely sensitive to the flavoprotein inhibitor DPI, a potential NADPH oxidase inhibitor (31). We report that DPI significantly decreased ROS in HF adipocytes. On the other hand, inhibitors for xanthine oxidase and NOS, and for AGE formation, or the mitochondrial uncoupler FCCP, had no effect on ROS, further suggesting that the major source for enhanced ROS production is NADPH oxidase. Our studies also suggest that PKC-δ is an important regulator of ROS in HF adipocytes. Rottlerin, a selective PKC-δ inhibitor, suppressed ROS levels, and adenoviral-mediated expression of PKC-δ protein affected ROS: expression of the dominant negative mutant PKC-δ reduced ROS levels by 30%, and overexpression of the wild-type PKC-δ increased ROS levels twofold (Fig. 3D). It is noteworthy that PKC-δ has been previously implicated in NADPH oxidase activation in various cells (2) and has been shown to be recruited to the complex assembly formation of the enzyme’s components (6). The mechanisms by which PKC-δ activates NADPH oxidase are not fully clear. It may be that PKC-δ phosphorylates the cytoplasmic subunits of the oxidase, such as p47phox, and initiates its translocation of the membrane (15, 38). Future studies should further address this problem.

PKC-δ regulates intracellular ROS production in normal adipocytes, which indicates that its role is not unique to HF adipocytes. Rather, abnormal regulation of PKC-δ in HF adipocytes leads to sustained/enhanced activation of ROS production. These differences are crucial for the well-being of the cell. Although PKC-δ and ROS are tightly regulated in normal adipocytes, their activity levels are increased in HF adipocytes, provoking/enhancing intracellular pathways, which leads to the detrimental effects associated with oxidative stress (3, 13, 14, 29).

How is PKC-δ activated in HF adipocytes? Our studies suggest two alternative routes that can activate PKC-δ, glucose, and oxidative stress. Hyperglycemia is a known factor...
activating PKCs, and it has been shown by numerous studies that, in cells chronically exposed to high glucose or in hyper-glycemic animals, PKC is activated (20, 27, 29, 48). The fact that glucose suppressed PKC-δ (Fig. 4B) suggested that high glucose influx into HF adipocytes and PKC-δ. This, in turn, promotes the activation of NADPH oxidase. It is noteworthy that similar observations were recently reported in vascular cells, showing that high glucose activated NADPH oxidase in a PKC-dependent fashion, although the specific PKC isofrom has not been determined (19, 20). An additional route for activation of PKC-δ is oxidative stress. This has been initially shown in H2O2-treated COS-7 cells (25) and vascular muscle cells (41). In HF adipocytes, we previously showed that oxidative stress activates PKC-δ (45). Thus it appears that a vicious cycle operates between ROS and PKC-δ. Once activated by glucose, PKC-δ enhances ROS production, which, in turn, feedback loops into the activation of PKC-δ (Fig. 5). These consequential events amplify oxidative stress and may be attenuated by glucose depletion or inhibition of PKC-δ.

In summary, we propose a new role for PKC-δ in the activation of oxidative stress in HF adipocytes and suggest that its inhibition may be a way to prevent the deleterious effects of oxidative stress that develop in the diabetic fat tissue.

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