Increased glucose uptake promotes oxidative stress and PKC-δ activation in adipocytes of obese, insulin-resistant mice

Ilana Talior,1 Merav Yarkoni,1 Nava Bashan,2 and Hagit Eldar-Finkelman1

1Department of Human Genetics and Molecular Medicine, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978; and 2Department of Clinical Biochemistry, Faculty of Health, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel

Submitted 31 January 2003; accepted in final form 20 April 2003

Talior, Ilana, Merav Yarkoni, Nava Bashan, and Hagit Eldar-Finkelman. Increased glucose uptake promotes oxidative stress and PKC-δ activation in adipocytes of obese, insulin-resistant mice. Am J Physiol Endocrinol Metab 285: E295–E302, 2003; 10.1152/ajpendo.00044.2003.—Increased oxidative stress is believed to be one of the mechanisms responsible for hyperglycemia-induced tissue damage and diabetic complications. In these studies, we undertook to characterize glucose uptake and oxidative stress in adipocytes of type 2 diabetic animals and to determine whether these promote the activation of PKC-δ. The adipocytes used were isolated either from C57Bl/6J mice that were raised on a high-fat diet (HF) and developed obesity and insulin resistance or from control animals. Basal glucose uptake significantly increased (8-fold) in HF adipocytes, and this was accompanied with upregulation of GLUT1 expression levels. Insulin-induced glucose uptake was inhibited in HF adipocytes and GLUT4 content reduced by 20% in these adipocytes. Reactive oxygen species (ROS) increased twofold in HF adipocytes compared with control adipocytes and were largely reduced with decreased glucose concentrations. At zero glucose, ROS levels were reduced to the normal levels seen in control adipocytes. The activity of PKC-δ increased twofold in HF adipocytes compared with control adipocytes and was further activated by H2O2. Moreover, PKC-δ activity was inhibited in HF adipocytes either by glucose deprivation or by treatment with the antioxidant N-acetyl-D-cysteine. In summary, we propose that increased glucose intake in HF adipocytes increases oxidative stress, which in turn promotes the activation of PKC-δ. These consequential events may be responsible, at least in part, for development of HF diet-induced insulin resistance in the fat tissue.

oxidative stress; protein kinase C-δ; insulin resistance; glucose metabolism; C57Bl/6J mice

Address for reprint requests and other correspondence: H. Eldar-Finkelman, Dept. of Human Genetics and Molecular Medicine, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel (E-mail: heldar@post.tau.ac.il).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

http://www.ajpendo.org 0193-1849/03 $5.00 Copyright © 2003 the American Physiological Society E295
both to inhibit insulin sensitivity and to alter the expression levels of glucose transporters in 3T3-L1 adipocytes (52). To the best of our knowledge, however, increased ROS production in adipocytes isolated from diabetic animals has not been documented. In the present study, we undertook to characterize glucose metabolism in adipocytes isolated from obese, insulin-resistant C57BL/6J mice, an inbred mouse strain susceptible to diet-induced obesity and diabetes (60), and to examine whether this is linked to increased oxidative stress and changes in PKC-δ activity. The present studies indicate that glucose uptake is significantly increased in high-fat (HF) adipocytes and this, in turn, promotes oxidative stress and activation of PKC-δ.

MATERIALS AND METHODS

Materials. Insulin was a gift from Novo Nordisk (Bagvaerd, Denmark). The radioactive materials were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). GLUT1 and GLUT4 antibodies were from Chemicon International (Temecula, CA). PKC-δ and PKC-α antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), and the phospho-PKC-δ (Ser443) and PKB antibody were from Cell Signaling (Beverly, MA). Insulin receptor and phospho-PKC-α (Ser657) antibodies were from Upstate Biotechnology (Lake Placid, NY). All other reagents were purchased 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 6 h before experiments and then killed. Blood samples were taken immediately from the aorta. Plasma glucose was measured by the Sugar Accutrend Sensor (Roche, Mannheim, Germany), and plasma insulin was assayed with a radioimmunoassay kit (INSIK-5, DiaSorin, Saluggia, Italy). Animal care followed the institutional animal care and use committee guidelines.

Preparation of adipocytes. To obtain an equal number of cells, the protocol required pooled cells harvested from three to four lean mice for each obese mouse. Adipocytes were isolated from the epididymal fat pad by digestion with 0.4 mg/ml collagenase (Worthington Biochemical, Lakewood, NJ), as described previously (33). Digested fat pads were passed through nylon mesh, and the cells were washed three times with Krebs-bicarbonate buffer (pH 7.4) that contained 1% bovine serum albumin (fraction V; Boehringer Mannheim, Mannheim, Germany), 10 mM HEPES (pH 7.3), 5 mM glucose, and 200 nM adenine. Aliquots of cells were used to determine the cell concentrations, as described (14), and to determine the amount of genomic DNA (Wizard Genomic; Promega, Madison, WI).

Histology and microscopy. Epididymal fat tissues were fixed in 4% buffered formalin and embedded in paraffin. The paraffin-embedded tissue was sectioned (4 μm) and stained with hematoxylin and eosin (Bio Optica, Milan, Italy). Micrographs were taken at ×100 magnification.

Glucose transport. Cells (1 × 10⁶) in 1- to 1.5-ml aliquots were divided in plastic vials and incubated with shaking at 37°C. For glucose transport measurements, cells were incubated with insulin at the indicated concentrations for 1 h, followed by the addition of 2-deoxy-[3H]glucose (0.5 μCi/vial) for 30 min. The assay was terminated by the centrifugation of cells through dinitolphalate (Irvine, CA), and 3H was quantitated with a liquid scintillation analyzer (Packard). Nonspecific uptake of 2-deoxy-[3H]glucose was determined by the addition of cytochalasin B (50 μM) 30 min before the addition of the radioactive material.

Total membrane and cell lysate preparations. Proteins were extracted from the adipocytes or from fat tissue with 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, and leupeptin, apro tinin, and pepstatin A). After centrifugation, supernatants were collected and subjected to gel electrophoresis followed by immunoblot analysis with the indicated antibodies according to the manufacturer's procedures. Total membranes were prepared from adipocytes of control and diabetic animals (5 × 10⁶ cells) as previously described (46), with some modifications. In brief, adipocytes were homogenized in buffer C (50 mM HEPES, pH 7.3, 10 mM NaF, 10 mM KCl, and 0.1 mM Na₃VO₄, 25 μg/ml each of protease inhibitors leu peptin, aprotinin, and pepstatin A) by 30 strokes and centrifuged at 1,000 g at 4°C to remove nuclear pellet. The resulting supernatants were centrifuged at high speed (100,000 g) at 4°C to precipitate membranes. Membranes were resuspended with buffer C (100 μl), and equal aliquots of membranes (30 μl) were subjected to gel electrophoresis followed by immunoblot analysis.

Measurement of intracellular ROS generation. The determination of ROS was based on the oxidation of 2′,7′-dichlorodihydrofluorescin (DCHF) by peroxide, as previously described, with some modifications (59). In brief, adipocytes were incubated either with various concentrations of glucose (0.5–5 mM), or with N-acetyl-L-cysteine (NAC; 0.1, 0.2, or 0.4 mM) for 1 h and then incubated with DCHF (30 μM) for an additional 40 min. Incubation with H₂O₂, a ROS donor, served as a positive control, and known concentrations of DCHF incubated with 20 mM NaOH were used as standards. Cells were washed, and the fluorescence of 2′,7′-dichlorofluorescein (DCF) was measured in triplicate samples in a multilple fluorometer (FL-600; excitation at 488 nm, emission at 530 nm). PKC-δ activity. PKC-δ activity was assayed as previously described (30). In brief, epididymal fat tissues were removed from both the control and the HF mice and immediately homogenized 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, and leupeptin, apro tinin, and pepstatin A). A similar procedure was performed in isolated adipocytes, except that cells were not homogenized. 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 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, 40 μM phosphatidylserine, and 3 μg histone H1 (per reaction). Reactions were terminated after 20 min by the addition of SDS-PAGE load buffer and were analyzed in 15% acrylamide gels by autoradiography. Autoradiography was quantitated by densitometry. Expression of results. Results were expressed as means ± SE for the number of cell preparations or animals, as indicated. Statistical analysis was performed by Origin Professional 6.0, using one-way ANOVA to compare the control and HF results. A difference was considered to be statistically significant when P < 0.05.

AJP-Endocrinol Metab • VOL 285 • AUGUST 2003 • www.ajpendo.org

E296 OXIDATIVE STRESS AND PKC-δ IN DIABETIC ADIPOCYTES
RESULTS

Characterization of HF mice and HF adipocytes. Table 1 characterizes C57BL/6J mice, which were fed the HF diet, and their respective controls, which were fed standard laboratory food. After 16 wk on their designated diets, the animals on the HF diet developed hyperglycemia and hyperinsulinemia. The HF mice increased their weight by 30–40% compared with control animals. Notably, there was about a fourfold increase in the number of adipocytes in each epididymal fat pad of HF mice compared with their respective controls. Determination of genomic DNA further confirmed this result and indicated that DNA content increased about fourfold in adipocytes isolated from HF fat pads compared with that of control (0.2 vs. 0.05 μg/fat pad of HF or control, respectively). Determination of the protein content of the fat tissues (Table 1) indicated that protein content also increased approximately fourfold in the HF tissue compared with control. Hence, protein content per cell did not significantly change in HF adipocytes compared with control adipocytes (calculated value 4 ng/cell), suggesting that the increase in the HF adipocytes size (~2.5-fold (cell diameter); Fig. 1) results mainly from accumulation of lipids.

Increased glucose uptake in HF adipocytes is accompanied by GLUT1 upregulation. Basal glucose uptake was strikingly higher in adipocytes from the HF mice compared with the controls (8.6-fold; Fig. 2A) and was blocked with cytochalasin B, indicating that glucose uptake is specific (data not shown). Insulin-stimulated glucose uptake is approximately threefold higher in control adipocytes, but insulin had no stimulatory effect in adipocytes from diabetic animals (Fig. 2A). This is consistent with studies performed in adipocytes derived from type 2 diabetic animals, showing a significant increase in basal glucose uptake and inhibition in insulin-stimulated glucose uptake (13, 50). Analysis of GLUT1 expression levels in total membranes prepared from an equal number of adipocytes indicated that GLUT1 is consistently higher in HF adipocytes (Fig. 2B). The elevation in GLUT1 in HF adipocytes seems to be specific, because the amount of another membrane protein, the insulin receptor, was not elevated but rather was reduced in HF adipocytes compared with controls (Fig. 2C). The expression level of GLUT4, which is known to be located in intracellular membranes, was reduced by 20 ± 4%, whereas no change in the cytosolic PKB was observed in either control or HF adipocytes (Fig. 2, D and E). Notably, alterations in GLUT1 and GLUT4 content were also observed in adipocytes of Zucker diabetic rats (46). All together, our results suggest that the increase in basal glucose uptake in HF adipocytes stems from specific upregulation of GLUT1 and is not due to a general phenomenon, namely increased protein expression levels, because other proteins were not elevated.

Intracellular ROS is increased in HF adipocytes. We next examined whether oxidative stress is increased in HF adipocytes and whether this may be related to the enhanced glucose uptake of these adipocytes. Intracellular ROS measurements presented in Fig. 3A indicate that ROS levels increased twofold in HF adipocytes compared with the control cells. Incubation of cells with a glucose-free medium resulted in a 50% decrease in ROS levels in the HF adipocytes (Fig. 3A). Figure 3B demonstrates the production of ROS in HF adipocytes as a function of medium glucose concentrations. As can be seen, elevation in ROS production correlates with elevation in medium glucose concentration, suggesting that elevation in glucose uptake in HF adipocytes is a major contributor to ROS production.

PKC-δ activity is increased in HF adipocytes. Previous studies demonstrated that PKC-δ is activated in various cell types by oxidative stress (30, 31). Thus we evaluated both PKC-δ activity (using histone H1 as substrate) and the abundance of PKC-δ and its phosphorylation state in adipocytes derived from both HF and control mice (Fig. 4). As can be evaluated (Fig. 4A),

Table 1. Characteristics of control and HF mice

<table>
<thead>
<tr>
<th>Animal</th>
<th>Body Weight, g</th>
<th>Plasma Glucose, mg/dl</th>
<th>Plasma Insulin, μU/ml</th>
<th>No. of Epididymal Adipocytes per Fat Pad ×10⁶</th>
<th>Protein Content, mg/fat pad</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30.3 ± 0.3</td>
<td>113.0 ± 4.47</td>
<td>15.7 ± 1.48</td>
<td>0.59 ± 0.04</td>
<td>2.54 ± 0.44</td>
</tr>
<tr>
<td>HF</td>
<td>40.35 ± 0.54*</td>
<td>287.8 ± 19.3*</td>
<td>53.6 ± 5.67*</td>
<td>2.34 ± 0.18*</td>
<td>10.6 ± 0.50*</td>
</tr>
</tbody>
</table>

Values are means ± SE. HF, high-fat diet. *P < 0.01.
PKC-δ activity was higher in HF adipocytes compared with controls. Immunoblot analysis with the anti-phospho-PKC-δ antibody, which recognizes the phosphorylated Ser643, a site known to be essential for the activation of PKC-δ when phosphorylated (35) and is phosphorylated in response to H2O2 (31), indicated that phosphorylation of Ser 643 increased in HF adipocytes compared with controls with no change in the expression of PKC-δ (Fig. 4, B and C). The phosphorylation of the conventional PKC-α isoform on Ser657, a site known to be crucial for the enzyme activity when phosphorylated (7), and its expression levels were similar in HF and control adipocytes (Fig. 4D).

PKC-δ activity is activated by H2O2 and inhibited by depletion of glucose and NAC. To further investigate the link between ROS and PKC-δ, the effect of H2O2 on PKC-δ in HF adipocytes was studied. As shown in Fig. 5A, exposure to H2O2 (200 μM or 2 mM) activated PKC-δ, suggesting that the increase in ROS production can contribute to the activation of PKC-δ in HF adipocytes. Furthermore, when cells were incubated with glucose-free medium (1 h), PKC-δ activity was greatly inhibited (Fig. 5B). To further assess the role of ROS in activating PKC-δ, we used NAC, a known free-radical scavenger previously shown to deplete intracellular ROS levels in adipocytes (37). Treatment of HF adipocytes with increasing NAC concentrations reduced ROS production (Fig. 5C) and basal PKC-δ activity (Fig. 5D). Taken together, these results suggest that increased oxidative stress in HF adipocytes is dependent on glucose efflux and promotes the activation of PKC-δ.

Fig. 3. Reactive oxygen species (ROS) levels in HF and control adipocytes. DCF, 2',7'-dichlorodihydrofluorescein. A: ROS levels were determined in control cells after 1-h incubation in glucose-containing medium and in HF adipocytes incubated for 1 h in presence or absence of glucose, as described in MATERIALS AND METHODS. *P < 0.05 C vs. HF or HF incubated in absence vs. HF presence of glucose. Results represent means ± SE of 5 independent experiments. B: same as A, except that HF adipocytes were incubated with various concentrations of glucose (1 h) as indicated. Results represent means ± SE of 3 independent experiments.
Oxidative stress and PKC-δ in diabetic adipocytes

DISCUSSION

Numerous reports have shown that oxidative stress is increased in animal models of diabetes and in patients with diabetes (5,18). In this study, we used an ex vivo system of isolated adipocytes from diabetic mice (HF adipocytes) to investigate whether oxidative stress is increased in these adipocytes and whether this may be related to hyperglycemia and/or activation of PKC. We showed that glucose uptake and ROS levels increased significantly in HF adipocytes compared with control adipocytes. Subsequently, ROS levels were reduced by decreased medium glucose concentrations in HF adipocytes, and in the absence of glucose its levels were reduced twofold, reaching the “normal” ROS levels observed in the control adipocytes. In addition, PKC-δ activity was higher in HF adipocytes compared with controls and could be further activated by H$_2$O$_2$. Moreover, the enzyme activity was inhibited by either the antioxidant NAC or the depletion of glucose. Taken together, these results enabled us to propose that the increase in glucose efflux into HF adipocytes may increase intracellular ROS production that, in turn, promoted the activation of PKC-δ. These intracellular events could represent, at least in part, one of the mechanisms responsible for induction of insulin resistance in the fat tissue. It should be noted, however, that our studies do not eliminate the role of additional in vivo factors such as hyperinsulinemia, hyperlipidemia, and adipocytokines that could contribute to the increased oxidative stress during the HF diet feeding period, as these factors were shown to elevate ROS levels in various cell systems (1,5,17,20,29,42,44,64).

GLUT1 levels were significantly increased in HF adipocytes (Fig. 2B). Previous studies demonstrated that GLUT1 expression levels might be affected by stress conditions such as hyperglycemia, hyperinsulinemia, or TNF-α (24,34,40). Although we do not know the exact mechanisms leading to increased GLUT1 expression in HF adipocytes, we suggest that the elevation of GLUT1 was responsible for the increased glucose uptake of HF adipocytes, which in turn led to the elevation of ROS. On the other hand, ROS production could further lead to increased GLUT1 transcription rates. The latter supposition is based on previous studies that indicated that induction of oxidative stress in 3T3-L1 adipocytes or L6 muscle cells resulted in elevation of GLUT1 (32,52). Thus it is suggested that a vicious cycle may be operating between GLUT1 content and increased oxidative stress in HF adipocytes.

The generation of ROS as a byproduct of the mitochondrial electron transport chain has long been attributed to the high rates of glucose metabolism (4,16,54,63). Nevertheless, additional mechanisms may be operating to produce ROS under hyperglycemic conditions, including the formation of advanced glycation end products (5,42) or activation of oxidases such as NADPH oxidase (26,41,62). We do not know at this point the precise source of the production of ROS in HF adipocytes. Further studies should elucidate this problem.

Our studies indicated that insulin receptor levels significantly decreased in HF adipocytes (Fig. 2C). This phenomenon was reported in various tissues and cells in conditions associated with insulin resistance (21,38,65). Hyperinsulinemia has been suggested to be a prominent factor responsible for this phenomenon (27,48), thereby indicating that downregulation of insulin receptors is a secondary effect in insulin resistance.
Nevertheless, it is still possible that reduced levels of insulin receptors in HF adipocytes could limit the sensitivity of the cells to insulin. On the other hand, it should be noted that the remaining insulin receptors (≈50%) could still transmit intracellular insulin signaling, as the receptors are most likely not defected (38).

The novel PKC isoform PKC-δ was shown to be activated by oxidative stress in various cell systems. Initially, it was shown that H$_2$O$_2$ led to activation in COS7 cells of PKC-δ, which was constitutively active and independent of the lipid coactivator, diacylglycerol (30, 31). Additionally, oxidative stress was shown to stimulate PKC-δ and c-Abl tyrosine kinase association, facilitating the activation of c-Abl by PKC-δ (58). In vascular smooth muscle cells, H$_2$O$_2$ activation of PKC-δ was required for the activation of platelet-derived growth factor receptor (53). PKC-δ was also shown to mediate oxidative stress-induced activation of several signaling components, including the nonreceptor tyrosine kinases JAK2 and PYK2 (19), as well as the serine/threonine protein kinase MAPK (12). Thus PKC-δ may play a central role in oxidative stress-induced cellular processes important in the development of insulin resistance in HF adipocytes. Further studies are needed to examine the downstream targets activated by PKC-δ in these cells.

PKC was initially shown to play a role in glucose transport in primary rat adipocytes (43, 57). Overexpression of PKC-δ in 3T3-L1 adipocytes enhanced both basal and insulin-induced glucose transport (61). Another study reported that PKC-α, PKC-β, PKC-δ, and PKC-ε were not involved in insulin-induced GLUT4 translocation (3). Thus it may be concluded that elevated PKC-δ activity could contribute to increased basal glucose uptake in HF adipocytes; on the other hand, and in agreement with previous findings (3), it has no effect on insulin-induced GLUT4 translocation in these adipocytes.
In summary, we suggest that the consequential events of glucose uptake, ROS production, and activation of PKC-δ may represent one of the mechanisms responsible for the development of insulin resistance in HF adipocytes in response to HF diet feeding.

We thank Prof. Zvi Nevo from the Department of Clinical Biochemistry for conducting the histological studies.

DISCLOSURE

This work was supported by the Israeli Diabetes Foundation and by the Annual Award of the Hendrik and Irene Gutwirth Research Prize in Diabetes Mellitus, which was awarded to H. Eldar-Finkelstein.

REFERENCES

34. Laybutt DR, Thompson AL, Cooney GJ, and Kraegen EW. Selective chronic regulation of GLUT1 and GLUT4 content by

E301OXIDATIVE STRESS AND PKC-δ IN DIABETIC ADIPOCYTES


