Role of FoxO1 in FFA-induced oxidative stress in adipocytes

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OBESEITY IS ASSOCIATED WITH insulin resistance and an increased risk for diabetes and cardiovascular disease (16, 18). The strong association between obesity and insulin resistance implicates the adipocyte as an important link in the pathophysiology of these diseases. Adipose tissue from obese individuals show activation of inflammatory pathways, with the elaboration of cytokines such as monocyte chemotactic protein-1 (MCP-1), plasminogen activator inhibitor-1 (PAI-1), and IL-6 (20, 22) contributing to the systemic inflammation characteristic of obesity. Free fatty acids (FFA) are likely important mediators of this response, in part through the activation of PKCθ and IkB kinase β, each of which can activate inflammatory pathways and also inhibit insulin signaling (14, 33).

In addition to having an important role in the activation of inflammatory pathways, fatty acids are also implicated in the activation of oxidative stress, not only by uncoupling oxidative phosphorylation and increasing the generation of oxygen species but also by impairing endogenous antioxidant defenses (31). In diabetes, the increased flux of FFA increases mitochondrial reactive oxygen species (ROS) production, which in turn interferes with insulin signaling (15). If lipid oversupply is causally linked to the inflammatory response and to the generation of oxidative stress, it is reasonable to expect that approaches that have antioxidant and anti-inflammatory effects on adipocytes should have a beneficial effect on insulin sensitivity.

The FoxO (forkhead member of the class O) family of forkhead transcription factors comprises three functionally related proteins, FoxO1, FoxO3a, and FoxO4, which together with the mammalian Sirt1, an NAD-dependent deacetylase, have been found to be crucial in the suppression of processes associated with accelerated aging. One such process is decreasing the generation of ROS (23, 27). Environmental stresses, such as starvation, cause rapid nuclear localization of FoxO proteins, where it regulates genes associated with cell cycle and oxidative stress resistance (2). In conditions rich in nutrients, FoxO proteins are inhibited from entry into the nucleus. This response will allow organisms to grow and proliferate in the presence of nutrients, but this will result in a decreased ability to resist oxidative stress.

In the present study, we sought to determine the effect of fatty acid exposure on FoxO1, the main FoxO isoform present in adipocytes. We determined the relationship of FoxO1 and Sirt1 activation to cytokine production and oxidative stress in 3T3-L1 cells. The results suggest that fatty acids decrease FoxO1 levels in a dose-dependent manner. This effect is followed by the concordant increase in ROS generation and a dysregulated adipokine expression. Furthermore, treatment with the Sirt1 agonist resveratrol was able to increase FoxO1 levels and reverse the changes associated with fatty acid overloading.

MATERIALS AND METHODS

Animals. All animal experiments were conducted in accordance with the University of Michigan institutional guidelines for the care and use of laboratory animals and were approved by the University Animal Care and Use Committee. Male C57BL/6J and db/db mice at 16 wk of age were anesthetized using pentobarbital sodium, and intraperitoneal white adipose tissue was isolated.

Cell culture and induction of differentiation of preadipocytes. 3T3-L1 preadipocytes were propagated and maintained in DMEM containing 10% (vol/vol) FBS with antibiotic and antifungal drugs. To induce adipogenesis of 3T3-L1 cells, we incubated 2-day postconfluent preadipocytes (designated day 0) in adipogenic cocktail (MDI) containing 1 μg/ml insulin, 1 mM dexamethasone, and 0.5 mM isobutyl-1-methylxanthine for 2 days. Cells were switched to DMEM supplemented with 1 μg/ml insulin with 10% FBS for 2 days, followed by culture in 10% FBS alone. Adipocytes were used in the experiments 7–9 days after the initiation of differentiation.

Reverse transcriptase-polymerase chain reaction analysis. Total cellular RNA was isolated with the RNeasy kit (Qiagen, Valencia, CA). Complementary DNA was synthesized using random hexamers and Moloney murine leukemia virus reverse transcriptase (Promega Biosciences) from 1 μg of RNA. Primer sequences were determined through established GenBank sequences (Table 1). Expression of the

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specific genes was assessed by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Two microliters of cDNA were used as template for RT-PCR, using the Quantitect SybrGreen kit (Qiagen) and 20 pM (each) primers in a final volume of 25 μl. Each measurement was performed in triplicate, and the threshold cycle number at which the amount of amplified target became detectable by fluorescence was determined. Serial dilutions of cDNA were run to ensure linearity and reliability of the assay. Analysis by agarose gel electrophoresis was performed to confirm product length and purity of the amplicon. Real-time qRT-PCR of 18S rRNA was also performed for every sample as an internal control. Relative levels of expression were determined by calculating differences in cycle threshold (Ct) normalized to 18s mRNA expression in all cases.

Fatty acid treatment. Lipid-containing media were prepared by conjugation of FFA with FFA-free BSA, using a method modified from that described by Chavez et al. (7). Briefly, FFA were dissolved in ethanol and diluted 1:100 in DMEM containing 2% (wt/vol) fatty acid-free BSA. Adipocytes were incubated for 24 h in serum-free DMEM containing 2% BSA with different concentrations of fatty acids. Ethanol (vehicle) was added accordingly to keep the concentrations equal between samples.

Western blotting. Cells were subcultured on six-well plates, washed once with ice-cold PBS, and lysed with PBS containing 50 mM HEPES (pH 7.5), 1% (vol/vol) Triton X-100, 150 mM sodium chloride, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 mM sodium pyrophosphate, 10 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotonin. Insoluble material was removed by centrifugation before SDS-polyacrylamide gel electrophoresis (PAGE).

For nuclear extracts, the NE-PER nuclear and cytoplasmic extraction reagents kit (Pierce, IL) was used following the manufacturer’s protocol. Cell lysates were normalized for total protein by using the bicinchoninic acid protein assay kit (Bio-Rad, Hercules, CA). For immunoblots, equal amounts of protein (25–50 μg) were separated by PAGE and transferred to nitrocellulose. The membranes were blocked with PBS, 0.1% Tween, and 5 or 10% (wt/vol) nonfat dry milk and incubated overnight at 4°C with primary antibody (FKHR antibody, 1:1,000; actin, 1:1,000; Cell Signaling). After washing, the bands were developed with horseradish peroxidase-conjugated secondary antibodies.
antibody and visualized with chemiluminescence reagent (Pierce, IL) according to the manufacturer’s directions.

**ROS production in 3T3-L1 adipocytes.** ROS production was detected by nitroblue tetrazolium (NBT) assay by incubating the cells for 90 min in PBS containing 0.2% NBT (29). NBT is reduced by ROS to a dark blue insoluble form of NBT called formazan, which was collected and dissolved in 50% acetic acid and its absorbance determined at 560 nm.

**Statistical analysis.** Data were analyzed using Student’s t-test or ANOVA as appropriate. P values <0.05 were considered statistically significant.

**RESULTS**

Fatty acid exposure increases oxidative stress and inflammatory cytokine production in adipocytes. We first investigated the effect of fatty acids on ROS generation and adipokine expression in adipocytes. To determine the effect of different fatty acids, we used both saturated fatty acids (stearic acid and palmitic acid) and unsaturated fatty acids (linoleic acid). Incubation of 3T3-L1 adipocytes with stearic acid or linoleic acid for 24 h increased NBT reductase activity in a dose-dependent manner, indicating an increase in oxygen radical production (Fig. 1A). In parallel, the expression levels of PAI-1, IL-6, and MCP-1 mRNA were increased (P < 0.05) following exposure to 600 μM stearic acid for 24 h. Similarly, 400 μM linoleic acid treatment also increased PAI-1 and IL-6 mRNA levels (P < 0.05); MCP-1 level did not reach statistical significance. In contrast, there was a significant downregulation of the mRNA for the purported insulin-sensitizing cytokine adiponectin for both stearic and linoleic acid (Fig. 1B). These results indicate that fatty acids, although increasing the generation of ROS in adipocytes, also alter adipokines, recapitulating characteristics of adipocytes seen in insulin-resistant states.

**High fatty acid medium decreases total and nuclear FoxO1.** FoxO transcription factors play a role in the response to oxidative stress (11, 23) and are known to respond to nutritional states with increasing transcriptional activity under conditions of starvation (13, 26). Therefore, we examined the effect of fatty acids on FoxO1 expression in adipocytes. Western blot analysis of nuclear and cytoplasmic extracts of 3T3-L1 adipocytes treated with increasing concentrations of linoleic acid (A) or palmitic acid (C) for 24 h. D: relative FoxO1 mRNA expression in cells treated with 600 μM stearic acid for 24 h. Results are means (SD) of 2 experiments performed in triplicate.

**Fig. 2.** Effect of fatty acids on FoxO1 expression in 3T3-L1 adipocytes. A: nuclear and cytoplasmic FoxO1 levels in adipocytes were treated with increasing concentrations of linoleic acid for 24 h. B and C: whole cell FoxO1 protein levels in 3T3-L1 adipocytes treated with increasing concentrations of stearic acid (B) or palmitic acid (C) for 24 h. D: relative FoxO1 mRNA expression in cells treated with 600 μM stearic acid for 24 h. Results are means (SD) of 2 experiments performed in triplicate.

**Fig. 3.** FoxO1 expression in adipose tissue of control and db/db mice. A: weight and blood glucose of wild-type (WT) and db/db mice. B: FoxO1 protein expression in nuclear and cytoplasmic extracts of intraperitoneal adipose tissue from WT and db/db mice. Each bar represents means (SD) of results (WT, n = 3; db/db, n = 4). *P < 0.05, db/db vs. WT.

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**EFFECT OF FFA AND RESVERATROL ON ADIPOCYTES**
levels (Fig. 2, B and C). The downregulation of FoxO1 appears primarily due to posttranscriptional events, since a 24-h exposure to 600 μM stearic acid had no significant effect on FoxO1 mRNA levels (Fig. 2D). These results show that in adipocytes, FoxO1 levels decrease in the presence of FFA concordantly with the generation of reactive oxygen and the induction of inflammatory cytokines.

FoxO1 in db/db mice. To determine whether a similar alteration in FoxO1 protein levels occurs in vivo, we examined FoxO1 levels from white adipose tissue from 16-wk-old db/db mice and age-matched control C57BL/6 mice. Expectedly, db/db mice were significantly heavier and were hyperglycemic (Fig. 3A). db/db mice have a higher concentration of plasma fatty acids compared with controls (1, 3). Adipose tissue showed an ~50% decrease in FoxO1 protein compared with wild-type controls for the nuclear compartment (Fig. 3B). A less pronounced effect was seen on the cytoplasmic compartment, demonstrating an in vivo parallel to the in vitro observations.

Resveratrol ameliorates the effect of fatty acids on adipocytes. A number of studies have demonstrated that Sirt1-dependent FoxO1 deacetylation increases FoxO1-depandant transcription (5, 8, 34). Furthermore, the Sirt1 activator resveratrol is known to promote the nuclear retention of FoxO1 with subsequent stabilization (10). Therefore, we next determined the effect of resveratrol on adipocytes treated with a high concentration of fatty acids. As before, exposure of 3T3-L1 adipocytes to stearic acid for 24 h increased the generation of ROS (Fig. 4A). The addition of resveratrol suppressed fatty acid-induced ROS production by stearic acid (Fig. 4, A and B). Addition of resveratrol to cells treated with stearic acid restored the expression of FoxO1 protein in both the cytoplasmic and nuclear fractions (Fig. 4C). We were also able to correlate these reductions in NBT reductase activity...
with an increase in the mRNA expression of the antioxidant enzymes MnSOD and glutathione peroxidase (Fig. 4D). The expression of these enzymes has been previously described as diminished in adipose tissue of obese individuals (12, 31). Thus the effect of resveratrol to decrease oxidative stress may be the ability to modulate the expression of antioxidant enzymes.

Finally, we determined whether resveratrol treatment reversed the effect of fatty acids on the expression of adipokines in 3T3-L1 adipocytes. There was a partial reversal of the stearic acid-induced reduction of adiponectin in cells treated with resveratrol and a concomitant decrease in the mRNA levels of IL-6, MCP-1, and PAI-1 (Fig. 5; \( P < 0.05 \)).

**DISCUSSION**

Starvation has been shown to increase both FoxO and Sirt1 levels in multiple cell types, and this is associated with increased longevity and defense against oxidative stress. In these studies, we have demonstrated the converse with nutrition, in the form of FFA exposure, resulting in decreases in FoxO1 levels in adipocytes. This effect occurs in vitro and in vivo with a similar decrease in FoxO1 levels in the adipose tissue of an animal model of obesity.

These findings may have important implications for the mechanism by which FFA have adverse effects on metabolism. The increased flux of FFA increases free radical production in mitochondria. The reduction in the levels of FoxO1 following FFA exposure magnifies this effect because of decreased transcription of oxygen radical scavengers, such as MnSOD and glutathione peroxidase. This effect seems independent of fatty acid species, since both saturated and unsaturated fatty acids produce similar changes in inflammatory marker production and changes in FoxO1 levels. Increases in oxidative stress induced by fatty acids can activate multiple cellular pathways that lead to insulin resistance, such as JNK, \( \text{I\kappaB} \), PKC, and mTOR (mammalian target of rapamycin), each of which can enhance serine phosphorylation of IRS (insulin receptor substrate) proteins and inhibition of insulin signaling cascade (9). The cellular oxidative stress is directly linked to increases in inflammatory cytokine production and decreases in adiponectin levels. Indeed, inhibition of NADPH oxidase, which generates ROS in the mitochondria, prevents proinflammatory cytokine production by fatty acids (12). Similarly, a recent study (21) demonstrated that exposure of 3T3-L1 adipocytes to \( \text{H}_2\text{O}_2 \) results in decreased adiponectin expression and an increase in the inflammatory marker PAI-1, suggesting that direct exposure to oxidative stress (\( \text{H}_2\text{O}_2 \)) and indirectly increasing oxidative stress by FFA exposure result in similar alterations of adipokine production. Thus the alteration in adipokine expression following FFA exposure appears to be dependent on the generation of oxidative stress.

Our data suggest that resveratrol protects cells from fatty acid induced increases in inflammatory adipokine production by increasing FoxO1/Sirt1-dependent antioxidant defenses, such as MnSOD and glutathione peroxidase. Sirt1-dependent deacetylation sequesters FoxO1 in the nucleus (10), which may be the reason why resveratrol treatment prevents the decrease in FoxO1 levels. Several studies have shown that FoxO1 degradation is dependent on the insulin signaling pathway through Akt phosphorylation and subsequent Skp2-mediated degradation (17, 24, 30). \( \text{I\kappaB} \) kinase, which is known to be activated after FFA exposure (4, 19, 32) and oxidative stress (28), has been shown to phosphorylate and inhibit another member of the FoxO family, FoxO3a, independently of Akt. One of the limitations of these studies is that although the effects of resveratrol on FoxO1 have previously been shown to be Sirt1 dependent (25), resveratrol is not a specific Sirt1 agonist (6).

In conclusion, in vitro exposure to high fatty acids recapitulates in large part the dysmetabolic adipocyte that is found in insulin-resistant individuals. We have shown that an excess flux of fatty acids causes an altered adipokine production and an increase in oxidative stress in mature adipocytes. These changes are associated with a decrease in total FoxO1 protein in vitro that is also seen in vivo in \( \text{db/db} \) mice. This study also provides evidence that alterations of the Sirt1/FoxO1 pathway have an important role in the generation of a dysmetabolic adipocyte. This pathway can be considered a potential therapeutic target for obesity and the metabolic syndrome through its effects on adipose tissue.

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**REFERENCES**


