Adipose Tissue Proadipogenic Redox Changes in Obesity*

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The role of inflammation and oxidative stress in the development of obesity and associated metabolic disorders is under debate. We investigated the redox metabolism in a non-diabetic obesity model, i.e. 11-week-old obese Zucker rats. Antioxidant enzyme activities, lipophilic antioxidant (α-tocopherol, coenzymes Q) and hydrophilic antioxidant (glutathione, vitamin C) contents and their redox state (% oxidized form), were studied in inguinal white fat and compared with blood and liver. The adipose tissues of obese animals showed a specific higher content of hydrophilic molecules in a lower redox state than those of lean animals, which were associated with lower lipophilic molecule content and lipid peroxidation. Conversely and as expected, glutathione content decreased and its redox state increased in adipose tissues of rats subjected to lipopolysaccharide-induced systemic oxidative stress. In these in vivo models, oxidative stress and obesity thus had opposite effects on adipose tissue redox state. Moreover, the increase in glutathione content and the decrease of its redox state by antioxidant treatment suggested that obesity is associated with an intracellular reduced redox state that promotes on its own the development of a deleterious proadipogenic process.

Redox metabolism corresponds to a complex interacting network involving the generation of reactive oxygen species and enzymatic and non-enzymatic cellular antioxidant defenses. Any small and transient disturbance of this balance induces redox signaling, which can act on several transduction pathways or enzyme and transcription factor activities. In contrast, when antioxidant defenses are chronically overwhelmed, the result is an oxidative stress in which free radicals may exert their deleterious effects (1). Oxidative stress has been implicated in the pathogenesis of several metabolic diseases as well as in the comorbidity of diabetes mellitus and atherosclerosis. In such studies, redox metabolism was evaluated by the pattern of various parameters such as manganese-dependent superoxide dismutase, glutathione peroxidase or catalase activities, and glutathione or α-tocopherol content in blood and liver (2–5). A similar pattern was observed in obesity and seemed to indicate that such pathology was related to pro-oxidative context. Indeed, obesity prevalence is correlated with decreased concentrations of plasma antioxidants (6). An increase in the markers of systemic oxidative stress has therefore been associated with obesity and metabolic syndrome (7, 8). Furthermore, it has been suggested that oxidative stress in accumulated fat, assessed by H₂O₂ generation and malondialdehyde (MDA), is an early instigator of metabolic syndrome (7). Such a conclusion is congruent with the emergent view characterizing obesity as a chronic inflammatory situation (9). It is noteworthy that most of these studies were conducted not in situations of strict obesity but with confused metabolic pathologies defined as metabolic syndrome. Taken together, all these reports argue in favor of an oxidative redox state in morbid obesity, although the occurrence of an oxidative stress in adipose tissue during the onset of obesity has never been investigated. Other studies also evidenced the importance of redox metabolism in obesity but in an opposite way. Indeed, we recently demonstrated that in vitro preadipocyte proliferation and differentiation can be controlled by redox metabolism (10, 11), while overexpression of cellular glutathione peroxidase has been reported to promote obesity in mice (12).

The aim of this work was to investigate changes in adipose tissue redox metabolism in an obese model without the confounding factors of diabetes and other metabolic dysregulations, i.e. the Zucker rat (13). The putative changes in redox defenses of the adipose tissues between obese and lean Zucker rats were compared with those occurring in blood and liver and also compared with those of rats subjected to an experimental known oxidative stress (14). Additionally, the importance of the redox state in the development of fat stores was assessed by studying the effect of antioxidants on lipid synthesis in cultured preadipocytes.

MATERIALS AND METHODS

Animals—Animals were acclimated for 7 days to 22 ± 1 °C in individual cages with a 12-h light-dark cycle and given commercial food and water ad libitum. There were 11-week-old obese and lean Zucker rats (Charles River Laboratories, L’Arbresle, France), 11-week-old Wistar rats (Janvier, Le Genest-St-Isle, France) intraperitoneally injected with 5 mg of LPS/kg (LPS dissolved in water) for 12 h, and C57 black 6N mice (Harlan, Gannat, France) for primary cultures of inguinal white adipose tissue (IW).

Animals were euthanized after anesthesia with 100 mg of ketamine and 10 mg of xylazine/kg body weight. All experiments were conducted in accordance with guidelines for animal care in the Institut Federatif de Recherche Louis Bugnard, Rangueil Hospital, Toulouse.

Tissues, Adipocytes, and Stromal Vascular Fraction (SVF) Isolation—Plasmas from abdominal aorta blood of anesthetized Zucker rats were collected into heparinized or EDTA tubes. Liver and epididymal and inguinal adipose tissues (EW and IW) that were considered as intra-abdominal and subcutane-

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2 The abbreviations used are: MDA, malondialdehyde; IW, inguinal white adipose tissue; EW, epididymal white adipose tissue; Gpx, glutathione peroxidase; SOD, superoxide dismutase; VitC, vitamin C; CoQ, coenzyme Q; α-Toc, α-tocopherol; LPS, lipopolysaccharide; SVF, stroma vascular fraction; BHA, butylhydroxyanisole.

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Circulating Glucose and Insulin—Plasma glucose and insulin were measured by glucose-dye-oxidoreductase with reactive strips (Accu-Chek Active, Roche Diagnostics) and radioimmunossay (RIA kit; Diasorin), respectively.

Adipocyte Glucose Uptake—Adipocyte suspensions were incubated with or without insulin for 45 min at 37°C in a final volume of 400 μl. Then, 2-deoxy-d-[3H]glucose (0.4 μCi) was added at a final concentration of 0.1 mM for 10 min. Assays were stopped with 100 μl of 100 mM cytochalasin B, and then aliquots were counted (16).

Cell Culture and Adipogenesis Assessment—3T3-F442A preadipocytes were routinely cultivated in a 21% O2, 74% N2, and 5% CO2 humidified atmosphere. At confluence, the medium was changed and antioxidant was added. Cell cultures were maintained for 7 days after confluence (the medium and antioxidant were changed every 2 days). For primary culture of mice, IW SVF cells were purified and cultured as previously described (17). At confluence, preadipocytes were induced to differentiate in the presence of 850 nM insulin, 2 nM triiodothyronine, 33 nM dexamethasone, and 1 μg/ml of transferrin. They were treated with antioxidant as described for 3T3-F442A preadipocytes. Cellular triglyceride content was determined using a commercially available test combination (Triglyceride enzymatique PAP150; bioMérieux). Triglycerides were also stained with Oil Red O (18) and visualized with phase contrast microscopy.

Real-time PCR—Total RNA from cells was extracted by the Extractall solution (Eurobio). Complementary DNA was synthesized with SuperScriptTM reverse transcriptase and poly-(dT) primers (Invitrogen). The real-time PCR measurement of individual cDNAs was performed using SYBR green dye to measure duplex DNA formation with the Gene Amp 5700 (Applied Biosystem) and normalized to the amount of 36B4 RNA. Increases of peroxisome proliferator-activated receptor γ2 and lipoprotein lipase and decrease of β actin mRNA expression were linked with adipocyte differentiation (19–20). Primers used were the following: 36B4 sense 5′-AGTCCGAGAATCAGATGAGGAT-3′, antisense 5′-GGCTGATTTGGTGTCTTGG-3′; PPARγ2 sense 5′-AGTGTGAATTACAGCAAATCTCTGTTT-3′, antisense 5′-AGTCGGAGGAATCAGATGAGGAT-3′. phosphorus uptake was reached at 100 nM in both lean and obese isolated mature adipocytes, this uptake was lower in obese (4.04 ± 0.82) than in lean (10.16 ± 0.94) (p <0.05). However, the fold increase (over basal) was equivalent to 7.20 ± 0.97 and 10.32 ± 1.68 in obese and lean, respectively (n = 10, not significant). This demonstrates that isolated mature adipocytes from obese rats were still responsive to maximal insulin stimulation but present signals of lower insulin sensitivity.

Changes in Liver and Blood Redox Metabolism of Obese Zucker Rats—Although the livers of obese Zucker rats were heavier and showed macroscopic steatosis (data not shown), no significant difference was observed for all redox parameters except a decrease of the CoQ9 content (Table 1). However, erythrocyte glutathione and plasma CoQ9 contents were significantly lower in obese than in lean rats. Moreover, erythrocyte glutathione redox state was significantly higher in obese rats.

RESULTS

Antioxidant Systems in Inguinal White Adipose Tissue and Liver of Lean Zucker Rats—All enzymatic activities were significantly lower in IW than in liver (Fig. 1A). Hydrophilic molecules, glutathione, and VitC (Fig. 1B) in IW exhibited lower content and higher redox state (higher ratio of the oxidized forms) than liver (p <0.05). For lipophilic molecules (Fig. 1C), CoQ9 content was lower in IW, whereas αToc content was higher than in liver. MDA content (Fig. 1C) was lower in IW than in liver (p <0.05).

We then investigated the distribution of antioxidants between the mature adipocyte and stroma vascular fractions. As expected, the lipophilic molecules, CoQ9 and αToc, were mainly found in adipocytes (Fig. 2B). More surprisingly, the hydrophilic molecules, glutathione and VitC, were also mostly present in adipocytes (Fig. 2A).

Metabolic Status and Adipocyte Insulin Sensitivity of Obese Zucker Rats—Because the choice of animal model was crucial to investigate the interplay between obesity and diabetes, we carefully checked the insulin resistance status of adipose tissue of 11-week-old obese Zucker rats. These rats had glycemia (9.5 ± 0.5 mm) that did not significantly differ from lean rats (8.6 ± 0.5 mm) and higher insulinemia (422.5 ± 69.0 microunits/ml versus 63.6 ± 14.4 microunits/ml for lean rats, p <0.01), confirming that these animals were obese but not diabetic. The basal glucose uptake (nmol 2-deoxy-d-[3H]glucose/100 mg of lipids/10 min) was significantly lower in obese (0.67 ± 0.19) than in lean rats (1.18 ± 0.15) (p <0.01). Furthermore, even if maximal insulin stimulation of glucose uptake was reached at 100 nM in both lean and obese isolated mature adipocytes, this uptake was lower in obese (4.04 ± 0.82) than in lean (10.16 ± 0.94) (p <0.05). However, the fold increase (over basal) was equivalent to 7.20 ± 0.97 and 10.32 ± 1.68 in obese and lean, respectively (n = 10, not significant). This demonstrates that isolated mature adipocytes from obese rats were still responsive to maximal insulin stimulation but present signals of lower insulin sensitivity.

Adipocyte Peroxidation—Peroxidation of frozen tissues were homogenized in 3 ml EDTA, 154 mM KCl, pH 7.4 (1:10 w/v for liver, 1:2 w/v for IW or EW). The homogenates were sonicated (30 s), centrifuged at 8,000 × g for 10 min, defatted, and centrifuged at 105,000 × g for 45 min (all these steps were performed at 4°C). Antioxidant enzyme activities were assessed in erythrocytes and in tissular cytosol or homogenate (for catalase activity). Protein assays were performed in homogenate and cytosol (21). Superoxide dismutase (SOD) activities (Mn-SOD, Cu-Zn SOD) were assayed by inhibition of pyrogallol autoxidation (22). Catalase activity was determined by measuring decomposition of H2O2 at 240 nm (23). Glutathione peroxidase seleno-dependent (Gpx) activity was measured using t-butylhydroperoxide as substrate (24).

α-Tocopherol, Coenzyme Q9, Glutathione, and Vitamin C—Powdered frozen tissues, adipocytes, SVF, and plasma were used to measure α-tocopherol (αToc) and total coenzyme Q9 (CoQ9) contents. After solubilization and extraction in 2-propanol, αToc and CoQ9 were detected by reverse-phase high performance liquid chromatography with electrochemical detection on the same run (25). Glutathione and vitamin C (VitC) were also quantified from tissue homogenates, adipocytes, and SVF. Blood glutathione was evaluated in erythrocytes and VitC in plasma. After precipitation of proteins, reduced glutathione, oxidized glutathione, total VitC, and its oxidized form (DHAA) were detected by reverse-phase high performance liquid chromatography with electrochemical detection (26) and fluorimetric detection (27), respectively. For glutathione and VitC, we calculated the redox state as oxidized form/oxidized + reduced) × 100.

MDA—Lipid peroxidation was estimated from the formation of MDA (28).

Statistical Analysis—Means ± S.E. were calculated, and statistically significant differences between two groups were determined by Student's t-test at p < 0.05.
Adipose Tissue Redox State and Obesity

FIGURE 1. Antioxidant systems and lipid peroxidation in liver (L) and inguinal white adipose tissue (IW) of lean Zucker rats. A, enzymatic activities. B, redox state ([oxidized form/(oxidized + reduced forms)] × 100) of the hydrophilic molecules glutathione and vitamin C (VitC) according to glutathione and VitC content. C, content of lipophilic molecules CoQ9 and α-tocopherol (αToc) and intensity of lipid peroxidation (MDA content). Mean ± S.E. (n = 9), **, p < 0.01; ***, p < 0.001.

and Cu-Zn SOD, but catalase activity was significantly higher in obese than in lean rats (Fig. 3A). In IW, total glutathione and VitC contents were significantly higher in obese rats and their redox states were lower than in lean rats (Fig. 3B). In contrast, obese rats had lower lipophilic molecule content as well as lower MDA values than lean rats (Fig. 3C).

Taken together, these results indicated that fat accumulation was associated with a greater abundance of hydrophilic antioxidants in their reduced state and lower lipid peroxidation. We then decided to use glutathione metabolism as a good index of redox changes.

The study of glutathione in IW and EW of obese rats showed that glutathione content is higher in the two fat pads when the lower redox state found in obese rats is specific to IW. Contrary to what occurred in obesity, IW and EW glutathione contents were significantly decreased in situations of systemic oxidative stress and inflammation induced by LPS treatment (Fig. 3D).

Reduced Redox State Parallels Triglyceride Content Increase and Adipocyte Differentiation—To evaluate the physiological consequences of the redox state evolution to a reduced set point, we treated cultured 3T3-F442A preadipocytes with an antioxidant, butylhydroxyanisole (BHA). Preadipocytes treated with increasing BHA concentrations showed a dose-dependent triglyceride increase (Fig. 4A). Similar results were obtained with another antioxidant, ascorbic acid, and confirmed with primary culture of mouse IW SVF cells (data not shown). Lipid staining by Oil Red O and adipocyte marker expression confirmed the adipocyte differentiation and lipid storage increase in BHA-treated cells (Fig. 4B and D). As expected, glutathione content was significantly increased while its redox state was significantly decreased in cells

TABLE 1
Enzymatic, non-enzymatic antioxidants, and lipid peroxidation in liver and blood of obese Zucker rats

In blood, enzymatic activities and glutathione content were measured in erythrocytes and the other parameters were measured in plasma. Results were expressed as % of the values obtained for lean (n = 6), *, p < 0.05, **, p < 0.01, and ***, p < 0.001.
treated with 10^{-4} \text{M} \text{BHA} \text{ (Fig. 4C). Increase in glutathione content, mainly in its reduced form, was concomitant with increase in triglyceride content and adipocyte differentiation.}

**DISCUSSION**

In this work we have demonstrated that, in obesity, adipose tissue is not associated with an oxidized redox state as observed in other tissues during inflammatory situations. In contrast, obesity is associated with a reduced redox state that promotes in vitro accumulation of triglycerides. Obesity is now considered a chronic inflammatory disease often associated with a decrease in antioxidant defenses and the emergence of markers of oxidative stress in blood and liver (2–5, 29). This view is not consistent with the recently observed induction of obesity in transgenic mice overexpressing one of the most important antioxidant enzymes, glutathione peroxidase (12). Moreover, reactive oxygen species have been demonstrated to inhibit adipogenesis in vitro (11). In addition, no change in antioxidant defenses or markers of lipid peroxidation was evidenced in the blood of young obese men when compared with their age-matched controls (30). A major difficulty in studying the link between obesity and oxidative stress is that the animal models used exhibit the coexistence of congruent metabolic dysfunctions such as insulin resistance. The genetically obese Zucker rats gave us the opportunity to partly overcome this problem because it is well known that, depending on their age, they can be considered as being obese without being diabetic. Indeed, such obese animals display hyperinsulinemia but without hyperglycemia, although lower insulin sensitivity was indeed present in isolated adipocytes of obese animals. Under these conditions, enzymatic activities, hydrophilic antioxidant molecule content, and lipophilic CoQ9 content were first determined in adipose tissue of lean Zucker rats and were shown to be lower than in liver. Results for CoQ9 could be related to the low mitochondrial activity described for white adipose tissues (31). The lower level of lipid peroxidation in adipose tissues when compared with liver could be attributed to a lower polyunsaturated fatty acid content (32) and to a higher \(	ext{aToc} \) content. Despite low antioxidant defenses (except \( \alpha \text{Toc} \) ), adipose tissue seemed to display little sensitivity to lipid peroxidation. Our study then showed that most of the hydrophilic and lipophilic components of the antioxidant defenses were strongly modified in the adipose tissue of obese rats and consisted of an increase in glutathione and vitamin C content in their reduced form. The differences observed between obese and lean adipose tissue can be attributed essentially to mature adipocytes because of their predominant proportion in the contents of antioxidant molecules.

The most surprising finding of this study was that the adipose tissue of obese rats showed fewer signs of lipid peroxidation than that of lean rats, as well as a higher content of hydrophilic molecules in a more reduced state despite a lower content of lipophilic molecules. The particularly marked reduced state of glutathione and VitC could be due to their synergic functioning. Indeed, the reduced form of VitC was regenerated by reduced glutathione, itself regenerated by glutathione reductase in the presence of NADPH, a reduced substrate generated by the potent pentose phosphate pathway in adipocytes (33). Conversely, antioxidant defenses in the lipid phase of adipose tissue were lower in obese
than in lean rats. As polyunsaturated fatty acid was markedly lower in adipose tissues of obese than of lean Zucker rats (34), these differences could explain in part the results obtained for lipid peroxidation. The low endogenous lipid peroxidation level in obese adipose tissue was probably also related to the high content of the reduced forms of synergic glutathione and VitC, the latter being able to regenerate $\alpha$-tocopherol radical.

These changes are contrary to those observed under systemic oxidative stress induced by LPS injection. Indeed, LPS-induced oxidative stress (14) was, as expected, associated with an oxidized state, whereas obese Zucker rats were surprisingly in a reduced state. These results are consistent with our in vitro observation. Thus, the addition of antioxidants increased glutathione content in its reduced form and, in parallel, promoted triglyceride synthesis. Similarly, a decrease in reactive oxygen species or a decrease in antioxidant defenses impaired preadipocyte differentiation. First, intracellular reactive oxygen species inhibited in vitro preadipocyte differentiation by specifically controlling expression of the adipogenic repressor CHOP10/GADD153 (11). Second, in vivo treatment of rats with buthionine sulfoximine, an inhibitor of glutathione synthase, decreased glutathione level and resulted in smaller adipocytes and lower body weight (35). Finally, our conclusion is supported by studies aimed at genetic invalidation or overexpression of Gpx in mice. Genetic inactivation of Gpx1 resulted in increased oxidative stress and 20% decreased body weight (36). In contrast, overexpression of Gpx1 resulted in an obese phenotype (12). It is noteworthy that in this study the authors focused their investigations on liver and muscle and demonstrated that Gpx1 overexpression dramatically reduced intracellular reactive oxygen species. However, our conclusion seems to be contradictory to the previous one (7). This apparent discrepancy could be explained by the different obese model (KKAy mice). We carefully checked that the obese rats used in our study are lower insulin sensible but not hyperglycemic, whereas KKAy mice, whatever their age, are obese and hyperglycemic (37). We also demonstrated that adipose tissue of obese organisms shows an active and still adaptable redox metabolism.

Thus, taking into consideration our results and other reports, we can assume that obesity per se is characterized by a reduced redox state in adipose tissues. This reduced redox state could in turn encourage cell proliferation and differentiation by modulation of redox-sensitive transcription factors (11). A deleterious cycle thus takes place that promotes and amplifies the onset of obesity (Fig. 5).

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