Tissue Distribution of S-(2-Succino)cysteine (2SC), a Biomarker of Mitochondrial Stress in Obesity and Diabetes

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S-(2-succino)cysteine (2SC) is a chemical modification of proteins produced by reaction of fumarate with thiol groups in protein, a process known as succination. We propose to use the name S-(2-succino)cysteine (instead of S-(2-succinyl) cysteine) from this point on. This is to distinguish protein succination (in which fumarate forms a thioether linkage with cysteine residues) from succinylation (in which an ester, thioester or amide bond would be formed). Succination of proteins is increased in muscle of type 1 diabetic rats and in adipose tissue in type 2 diabetic mice. The increase in 2SC is a direct result of tissue accumulation of fumarate in response to nutrient excess and resultant mitochondrial stress in diabetes. In this study, we examine the breadth of succination of tissue proteins in the *db/db* type 2 model of diabetes. We also determined the extent of succination in epididymal adipocytes of type 1 (Akita, streptozotocin (STZ)) and type 2 (*ob/ob, db/db*) diabetic mice, in diet-induced obese (DIO) mice, and in the adipose tissue of ground squirrels in various stages of hibernation. While succination was not increased in most tissues (brain, heart, kidney, liver, skeletal muscle) in the *db/db* model of diabetes, it was increased in all adipose beds of type 2 diabetic and DIO mice in comparison to their controls. Succination was not increased in adipocytes of type 1 diabetic mice. Adipose tissue from hibernating (HIB) 13-lined ground squirrels was also studied to determine if obesity in the absence of hyperglycemia affected succination of proteins. There were no differences in succination of proteins in brown or white adipose tissue over the torpor-arousal cycle. We conclude that 2SC is a biomarker of nutrient excess and mitochondrial stress in adipose tissue, increasing under the hyperglycemic and insulin resistant conditions associated with type 2 diabetes and obesity.

Obesity (2011) doi:10.1038/oby.2011.340

INTRODUCTION

S-(2-succino)cysteine (2SC) is a novel chemical modification of proteins, formed by reaction of the Krebs cycle intermediate, fumarate, with cysteine residues in proteins; this process is described as succination of protein (1). We propose to use the name S-(2-succino)cysteine (instead of S-(2-succinyl)cysteine) from this point on. This is to distinguish protein succination (in which fumarate forms a thioether linkage with cysteine residues) from succinylation (in which an ester, thioester or amide bond would be formed). Increased succination of tissue proteins is a direct result of increased fumarate concentration in the cells as a consequence of nutrient-derived mitochondrial stress. Succination is increased in adipose tissue of *db/db* (type 2) diabetic mice and in the skeletal muscle of streptozotocin (STZ)-induced (type 1) 6-month diabetic rats receiving insulin therapy (1–3). The increase in succination of adipose tissue protein in diabetes is mimicked by increased succination of proteins in adipocytes grown in high vs. low glucose medium (4). Succination inhibits the activity of the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (2). Brownlee has proposed that inhibition of GAPDH by oxidative stress leads to the buildup of glycolytic intermediates, which can activate several pathways (sorbitol, hexosamine, protein kinase C, and advanced glycation end-product formation) that are implicated in development of diabetic complications (5). Our results suggest, however, that succination may be the actual mechanism of inactivation of this enzyme during hyperglycemia in diabetes. Succination of adipose tissue adiponectin occurs at Cys-39, a cysteine residue that is essential for the polymerization and secretion of this protein (3). The extent of modification of adiponectin (~8%) in adipose tissue of *db/db* mice is consistent with the decrease in plasma adiponectin concentration in type 2 diabetes. Decreased secretion and polymerization of adiponectin is implicated in the development of insulin resistance in type 2 diabetes. In addition to GAPDH and adiponectin, we have identified a number of succinated proteins in adipose tissue of type 2 diabetic mice, including major cytoskeletal proteins and endoplasmic reticulum (ER)

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Received 27 April 2011; accepted 4 October 2011; advance online publication 1 December 2011. doi:10.1038/oby.2011.340

chaperone proteins (4). Considering the scope and quantitative significance of succination, we have proposed that succination plays a central role in the metabolic derangements characteristic of diabetes (6). Mechanistically, nutrient excess from hyperglycemia leads to feedback inhibition of oxidative phosphorylation and intracellular accumulation of mitochondrial intermediates, including fumarate, which then leads to increased succination of protein in diabetes (6).

To gain broader understanding of the role and distribution of succination of proteins in diabetes and obesity, we report here on the analysis of succination of proteins in a range of tissues in the *db/db* model of type 2 diabetes and in the epididymal adipocytes of other models of type 1 and type 2 diabetes. The Akita and STZ-induced diabetic mouse were analyzed as models of type 1 diabetes, and *db/db* and *ob/ob* mice as models of type 2 diabetes. In addition, we studied the succination of protein in the diet-induced obese (DIO) mouse to determine if obesity and insulin resistance, independent of frank diabetes, affected succination. As a model for a natural condition characterized by a large increase in adiposity, we examined white and brown adipose tissue from hibernating (HIB) ground squirrels comparing animals sampled at different stages of the torpor-arousal cycle. Hibernators may double their body mass while accumulating sufficient adipose reserves to fuel their energy needs throughout the winter months (7). We show that succination increases early in type 2 diabetes, in both the *db/db* and *ob/ob* mice models, as well as in DIO mice before the development of frank diabetes, but is unchanged in adipocytes in type 1 diabetic mouse models and during hibernation in the squirrel model.

METHODS AND PROCEDURES

Materials

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich (St Louis, MO). Polyacrylamide gels, polyvinylidene difluoride membranes, ECL chemiluminescent substrate, and precast polyacrylamide gels were purchased from Bio-Rad (Hercules, CA). Rabbit anti-2SC polyclonal antibody was prepared as described previously (4).

Animal models of type 1 and type 2 diabetes and obesity

All mice were purchased from Jackson Laboratories (Bar Harbor, ME) including: (i) type 1, Akita (JAX 003548, $Ins2^{Akita}$) diabetic mice; (ii) type 1, STZ-induced diabetic mice (JAX 000664, C57BL/6J); (iii) DIO mice (JAX 000664) fed a high-fat diet and lean control C57BL/6 mice fed a normal diet; (iv) *db/db* obese type 2 diabetic mice (JAX000642, $Lepr^{db}$) and their lean littermates (*Db/+*, $Lepr^{db/+}$); and (v) *ob/ob* obese type 2 diabetic mice (JAX 000632, Lep^{ob}) and their lean littermates (*Ob/+*, $Lep^{ob/+}$). Akita mice were obtained at 14 weeks of age and housed for 2 weeks with *ad libitum* access to standard chow before sacrifice. STZ-diabetic mice were prepared at Jackson Labs, using a multiple low dose STZ protocol starting at 6 weeks of age. Mice were shipped at 9 weeks and blood glucose measurements were taken to confirm diabetes. The STZ mice were sacrificed at 16 weeks of age. The *db/db* and *ob/ob* mice were obtained at 10 weeks of age and housed for a further 5 weeks with full access to standard laboratory chow before sacrifice at 15 weeks. DIO mice were fed a 60% calorie fat diet starting at 6 weeks of age until 24 weeks of age, at which point they were sacrificed. All animal handling and experiments were conducted according to the guidelines of the University of South Carolina Institutional Animal Care and Use Committee. Animals were sacrificed by CO_2 asphyxiation and epididymal adipose tissue was removed immediately and placed in Dulbecco's modified Eagle's medium (DMEM) before isolation of adipocytes. Other tissues were snap frozen in liquid nitrogen, then stored at −70 °C until preparation for analysis.

Adipocyte extraction

Primary adipocytes were isolated from mouse epididymal adipose tissue. Briefly, the tissue was minced into 1 cm^2 pieces and placed in 10 ml of DMEM. Collagenase II (Sigma Aldrich; Product No. 6885) was added, and the samples were rocked gently at 37 °C for 30 min. Although bovine serum albumin is commonly added to the DMEM during collagenase digestion, it was omitted in these digestions to minimize albumin contamination of the adipocyte protein. The digest was filtered through a 100 µm strainer to remove tissue debris, and adipocytes were suspended in 10 ml of DMEM containing 1% penicillin–streptomycin. Following centrifugation for 10 min at 2,000*g* at 4 °C, the upper layer containing adipocytes was collected and washed twice by centrifugation in phosphate-buffered saline before lysis of the cells in radioimmunoprecipitation assay (RIPA) buffer (50 mm Tris-HCl, 150 mm NaCl, 1 mm EDTA, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, pH 7.4) with addition of 2 mmol/l diethylenetriaminepentaacetic acid, 1 mmol/l sodium orthovanadate, 50 mmol/l sodium fluoride, and 100 µl protease inhibitor cocktail (Sigma-Aldrich P8340).

Ground squirrel adipose tissue

Adipose tissue was obtained as frozen biopsies from 13-lined ground squirrels (*Spermophilus tridecemlineatus*). Both brown and white adipose tissues were obtained from three conditions, as detailed in (8); (i) active in the cold room (ACR) for several days with a body temperature of ~37 °C, before entry into a bout of cold torpor, (ii) long-term hibernation, where the animals have been in constant torpor for 3–7 days with a core body temperature of 5–7 °C; or (iii) interbout arousal (recovery) from torpor (REC), in which animals have aroused from torpor and established a normal body temperature (37 °C) for about 18 h. The ACR squirrels maintain euthermic body temperatures and are metabolically active, continuing to storing body mass as needed. At this point they begin to become insulin resistant and have high circulating insulin levels. Upon entering torpor the HIB squirrels dramatically reduce their metabolic rates (typically becoming 1–5% of normal resting state). During HIB the squirrels switch to primarily utilize stored fatty acids for metabolic sustenance, as evidenced by respiratory quotients ~0.7. Upon recovery from torpor (REC) the squirrels significantly increase their metabolic rate as their body temperatures increases towards euthermia. They have a high ATP demand as arousal from torpor is a metabolically expensive shift. Adipose tissue samples were homogenized in RIPA buffer (as described below) before analysis by SDS-PAGE and western blotting.

Protein extraction from primary adipocytes and adipose tissue

Primary adipocytes extracted from epididymal adipose tissue were added to 300 µl of ice-cold RIPA buffer and sheared 10 times through a 21-gauge needle. The adipocytes were sonicated using a microprobe at 2 watts on a model 100 sonic dismembrator (Thermo Fisher Scientific, Waltham, MA) for 30 s and afterward allowed to rest on ice for 30 min. Insoluble debris was removed by centrifugation at 10,000*g* for 10 min in a Heraeus Biofuge 13 microcentrifuge (Thermo Fisher Scientific, Waltham, MA). A lipid layer was visible on the surface, and the infranatant containing soluble proteins was removed using a Hamilton syringe. The infranatant from squirrel adipose tissue remained opaque and was therefore treated for a second time by sonication and centrifugation. The infranatant was removed, then vortexed with 9 volumes of ice-cold acetone. The protein pellet was recovered by centrifugation at 2,000*g* for 10 min. The aqueous acetone, containing residual lipids and fumarate, was removed, and the protein pellet was dissolved in 500 µl of RIPA buffer. Removal of fumarate at this point is essential as excess fumarate may react with proteins during boiling in preparation for sodium dodecyl sulfate polyacrylamide gel

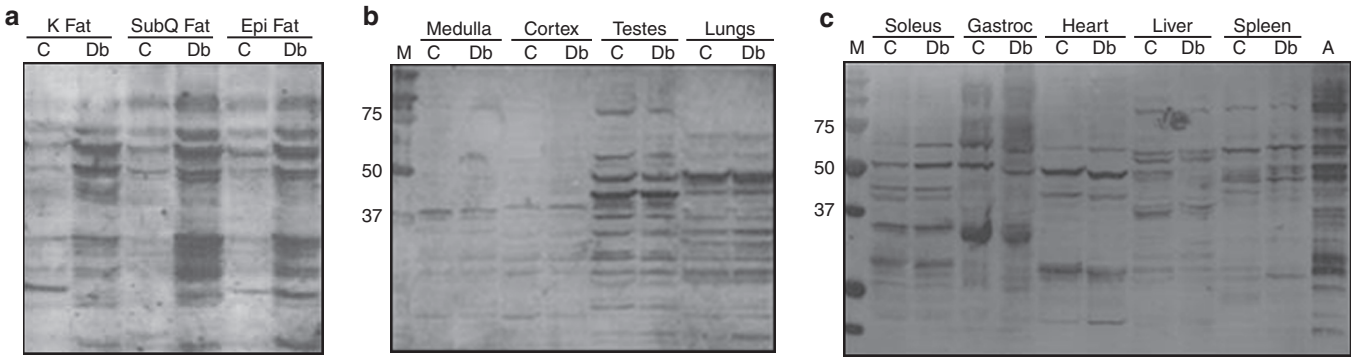

Figure 1 Detection of succinated proteins in tissues of *db/db* mice. (**a**) Representative western blotting analysis for suprarenal (K fat), subcutaneous, and epididymal adipose tissue of control C57BL/6 and diabetic mice. Densitometric analysis (not shown) indicated an approximately fivefold increase in succination of proteins in diabetic adipose tissue. (**b**) Analysis of renal medulla and cortex, testes, and lungs. (**c**) Analysis of various skeletal muscles, heart, liver, and spleen. For these analyses, protein was pooled in equal amounts from three mice. Separate prior analyses of tissues from each mouse were consistent with results observed for analysis of the pools. A, adipocytes grown in 30 mmol/l glucose for 8 days, used as a positive control; C, control; Db, diabetic; M, marker lane.

electrophoresis (SDS-PAGE) analysis, leading to an artifactual increase in succination of proteins. Protein concentration in cell and tissue extracts was measured by the Lowry assay (9).

Cell culture

3T3-L1 murine fibroblasts were obtained from the American Type Culture Collection (Manassas, VA) and cultured as described previously (4). Briefly, 3T3-L1 cells were cultured to full confluency before differentiation in DMEM containing 300 µmol/l dexamethasone, 500 µmol/l 3-isobutyl-1-methylxanthine, 10 µg/ml insulin, and 30 mmol/l glucose. After differentiation the cells were matured in 30 mmol/l glucose and 5 µg/ml insulin, with the medium replaced every 48 h, for 8 days before preparation of cell lysates for protein analysis. These cells were used on gels as reference standards to confirm sensitivity for detecting increased succination of proteins in tissues.

Western immunoblotting

Adipocyte protein lysate (70 µg protein) in RIPA buffer was diluted with deionized water, followed by addition of Laemmli (10) loading buffer containing 5% β-mercaptoethanol. Protein samples from other tissues (25–100 µg protein) were boiled for 15 min at 95 °C then loaded onto 12.5% Tris-HCl polyacrylamide gels and electrophoresed at 200 V for 60 min, as previously described (4). The separated proteins were wet blotted to a polyvinylidene difluoride membrane at 250 mA for 100 min, and the membranes were probed using polyclonal anti-2SC antibody (4). Blots were developed on a Konica Minolta Model SRX-101A film processor (Konica Minolta Medical & Graphic, Tokyo, Japan). Variations in the intensity of succination by western blotting was observed between different groups of animals; however, diabetic animals always demonstrated increased adipocyte succination vs. controls.

Glucose tolerance test

Glucose tolerance tests were performed on both the DIO mice and their controls. A 20% glucose solution (2 g glucose/kg body weight) was injected interperitoneally into overnight-fasted animals, and blood was sampled from the tail vein at 0, 15, 30, 60, 90, and 120 min postinjection. Blood glucose concentration was immediately measured using a Bayer (Mishawake, IN) Ascensia Contour glucometer.

100 µl of tail vein blood was collected for measurement of adiponectin levels in the serum of *db/db* mice. Adiponectin levels were measured by ELISA (R&D Systems, Minneapolis, MN).

Statistical analysis

Densitometry on western blotting images was evaluated using Image J software (National Institutes of Health, Bethesda, MD) by comparing the total density of all bands in the lane. Data are summarized throughout as means ± s.e. and are plotted using Sigma Plot 11 software (Systat Software, Point Richmond, CA). Statistical analyses were performed using InStat (GraphPad Software, San Juan, CA).

Table 1 Age, body weights, and blood glucose concentrations for various animal models

	Age (weeks)	Body weight (g)	Non-fasting glucose (mg/dl)
C57BL6/J	24	28.9 (±0.61)	139.7 (±15.8)
24-week DIO	24	35.3 (±0.95)***	223.3 (±15.01)**
Akita	15	19.6 (±1.4)***	542 (±87.2)***
STZ-diabetic	16	25.6 (±2.24)	378 (±104.9)*
db/+	15	30.2 (±1.35)	138.3 (±24.9)
db/db	15	48.7 (±1.81)***	507.3 (±67.8)***
ob/+	15	29.8 (±1.36)	139.7 (±14.4)
ob/ob	15	60.6 (±0.85)***	326.3 (±90.5)*

Blood glucose was measured in the morning under non-fasting conditions. Data are shown as mean ± s.d. vs. respective controls.
DIO, diet-induced obese; STZ, streptozotocin.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

RESULTS

Distribution of succinated proteins in tissues of *db*/*db* vs. control mice

In previous studies (3), we have shown that succination of protein is increased in adipose tissues of *db/db* mice, compared to lean littermates (3); this is illustrated in **Figure 1a** for comparative purposes, showing the increase in succination in renal, subcutaneous, and epididymal adipose tissue of *db/db* mice. **Figure 1b,c** present the results of analysis for succinated proteins in other tissues of the *db/db* mouse. These results were obtained from animals at 15 weeks of age, when non-fasting blood glucose was ~500 mg/dl (**Table 1**) and fasting blood glucose was ~230 mg/dl (data not shown). The serum adiponectin levels in the *db/db* mice were ~9% lower than control mice (4.42 µg/ml vs. 4.86 µg/ml, respectively), consistent with previous observations that ~8% increases in adipocyte succination

correlate with decreased serum adiponectin levels (3). All animals were sacrificed in the fed state as this corresponds with increased protein succination. Succinated proteins were detected in all tissues, and varied significantly among organs and tissues of *db/db* mice, Figure 1b,c. However, except for adipose tissue, there was no evidence of increased succination of proteins in tissues of the *db/db* mouse. Testes, lung, and skeletal muscle showed prominent staining for succinated proteins, even in control animals. Whether specific cell types in these tissues or cells at various stages of the cell cycle are targets of succination is unknown at this time.

Figure 2 shows results of representative analysis of isolated epididymal adipocytes from both *db/db* and *ob/ob* mouse models of type 2 diabetes. Succination of proteins was increased in the adipocytes from both animal models, compared to the lean heterozygote controls. Densitometric analysis indicated an approximately threefold increase in succination of adipocyte protein in the diabetic mice. It is possible that the differences in staining of proteins from whole adipocytes in Figure 1a, of adipocytes grown in high glucose *in vitro* (Figure 1a, lane A) and of isolated adipocytes (Figure 2) may be explained by the differential content of preadipocytes in these samples.

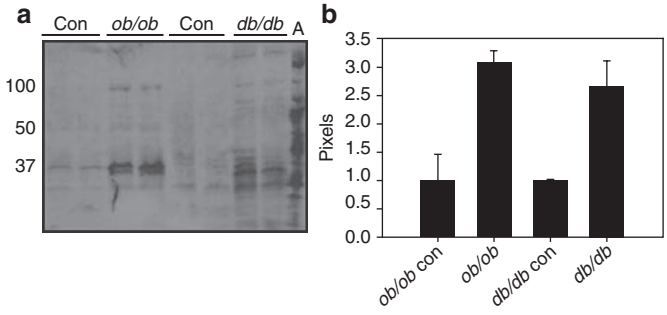

Figure 2 Analysis of succinated proteins in epididymal adipocytes of *ob/ob* and *db/db* diabetic mice. (**a**) Representative analysis of epididymal adipocytes of *db/db* and *ob/ob* mice ($n = 2$) and their controls, *ob/ob* ($Lep^{ob/+}$) and *db/db* ($Lepr^{db/+}$), respectively. (**b**) The blot in Figure 2a was replicated $n = 3$ and densitometric analysis was performed, normalized to intensity for control animals. The data shows increased succination (approximately threefold) of proteins in adipocytes of type 2 diabetic mice at 15 weeks of age; in both genetic models, $^*P < 0.05$. A, adipocytes grown in 30 mmol/l glucose for 8 days, used as a positive control; Con, heterozygote control for each homozygote diabetic animal.

Increased succination of protein in isolated adipocytes from epididymal adipocytes of DIO mice

DIO mice develop insulin resistance, hyperlipidemia, and hyperglycemia in response to a high-fat diet (11). After 16 weeks on a high-fat diet (24 weeks age), there was a significant increase in the mean body weight and non-fasting blood glucose of DIO vs. control mice (Table 1). Although fasting blood glucose levels were similar in DIO mice and lean controls (95 ± 15 vs. 105 ± 10 mg/dl), DIO mice had abnormal glucose tolerance tests (Figure 3a) characterized by an approximately twofold increase in area under the curve for blood glucose measurements (Figure 3a). Figure 3b shows that there was a significant increase in succination of protein in epididymal adipocytes from DIO vs. lean controls after 16 weeks of feeding a high-fat diet. Thus, an increase in succination of protein was detectable at an early stage in the development of diabetes where insulin resistance, but not frank diabetes, was present. Both soleus and gastrocnemius muscles were analyzed to determine if high-fat feeding affected protein succination in the muscle of DIO mice. However, as in *db/db* mice (Figure 1c), there was no increase in succination of proteins in the muscles of DIO vs. lean control mice (data not shown).

Succination is not increased in epididymal adipocytes of type 1 diabetic mice

Analysis of succinated proteins in isolated epididymal adipocytes of Akita and STZ models of type 1 diabetes is shown in Figure 4. Although succination was detectable in proteins from all of these mice, there was no evidence for an increase in succination in diabetic compared to control mice, whereas significant increases in succination were detected in *db/db* mice.

Succination of protein is not altered in brown or white adipose tissue of ground squirrels during hibernation

Figure 5 presents the results of analysis of succinated proteins in brown and white adipose tissue from HIB 13-lined ground squirrels at three stages during the torpor-arousal cycle. Only

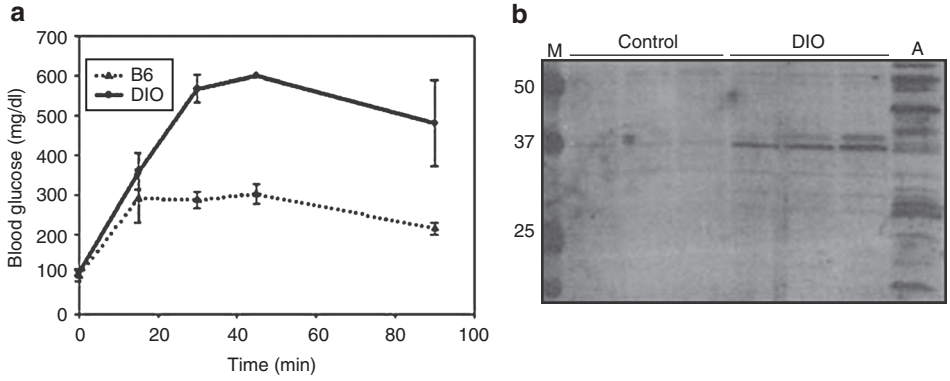

Figure 3 Glucose tolerance testing and analysis of succinated proteins in epididymal adipocytes of C57BL/6 and diet-induced obese (DIO) mice. (**a**) Blood glucose concentrations during an IGTT; and (**b**) western blotting for succinated proteins in adipose tissue from control vs. DIO mice. In all cases, means ± s.e. for $n = 3$ animals. IGTT, intravenous glucose tolerance test.

one succinated protein, tubulin (~55 kDa), was detected at similar levels in both brown and white adipose tissues; however, no consistent changes in succination were detected in these tissues over different stages of the hibernation cycle.

DISCUSSION

Succination of protein in animal models of diabetes and DIO

Diabetes and obesity are characterized by increased mitochondrial and oxidative stress in various tissues including skeletal muscle, kidney, retina, peripheral nervous system, cardiovascular system, β-cells, and adipose tissue (12–18). This stress occurs, in part, as a consequence of nutrient excess, characterized by hyperglycemia and hyperlipidemia (glucotoxicity and lipotoxicity), which induces mitochondrial dysfunction, generation of reactive oxygen species, and ER stress (12,17,19,20). One index of mitochondrial stress in response to nutrient excess in diabetes is the accumulation of fumarate (4), which leads to an increase in succination of proteins in adipose tissue of *db/db* mice (3). Since mitochondrial number and function vary in different tissues, we analyzed various tissues in order to assess the breadth of changes in succination of protein in diabetes and obesity.

The increase in succination of protein in adipose tissue of *db/db* mice was mirrored by similar changes in the *ob/ob* mouse; both of these mice are obese, insulin resistant, hyperglycemic, and hyperlipidemic models of type 2 diabetes (**Table 1**, **Figure 2**). Similar, but less pronounced, changes in succination were observed in adipose tissue of the DIO mouse after 16 weeks on a high-fat diet (**Figure 3b**). These mice also had an increased body weight compared to their respective controls (**Table 1**), but had a normal fasting blood glucose concentration (~105 mg/dl). However, their abnormal glucose tolerance test, indicative of tissue insulin resistance, is characteristic of the early stages of type 2 diabetes. The disease-dependent increase in succination in the DIO mouse is consistent with the glucose concentration-dependent increase in succination of proteins in 3T3-L1 adipocytes in cell culture (4). In this case, the DIO model is similar to a "prediabetic" state where hyperinsulinemia is evident but hyperglycemia is mild, in contrast, the *db/db* and *ob/ob* models correspond to "frank" diabetes. The combined data from the type 2 mouse models and the DIO mice suggest that greater increases in succination are dependent on the presence of both hyperglycemia and insulin resistance.

Although succinated proteins were detected at various concentrations in all tissues examined in type 2 diabetic and DIO mice and their respective controls, there were no changes in succination of proteins in nonadipose tissues of diabetic compared to control animals (**Figure 1**). Thus, the increase in succination of proteins in diabetes is restricted to adipose tissue, indicating unique metabolic stresses on this tissue in type 2 diabetes. In other work (N. Frizzell, S.A. Thomas, J.W. Baynes, unpublished data), we have demonstrated that in adipocytes cultured in high glucose for only 2 days there is a significant increase in ATP:ADP ratio, suggesting energy intake greatly exceeds the requirements of the adipocyte. This results in pseudohypoxia and leads to inhibition of the Krebs cycle, and may be a major contributor to the increase in mitochondrial stress and accumulation of fumarate in adipose tissue. Alterations in adipose tissue biochemistry and adipokine secretion are increasingly recognized as important mediators of systemic inflammation and development of pathology in other tissues. Succination of proteins may therefore be useful as a biomarker of adipose tissue dysfunction in diabetes.

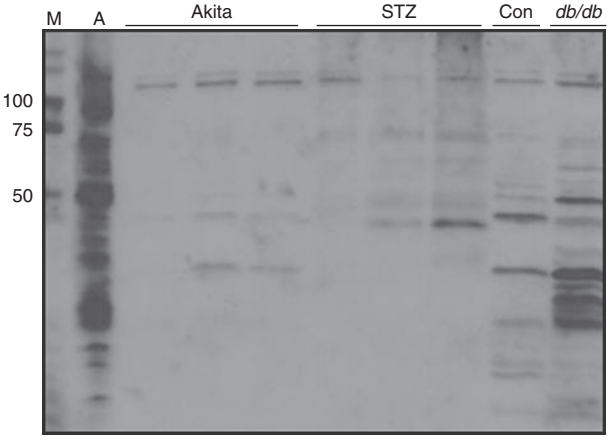

Figure 4 Analysis of succinated proteins in epididymal adipocytes of Akita and streptozotocin (STZ)-induced type 1 diabetic mice. Lane 1: M = protein molecular weight markers. Lane 2: A = adipocytes grown in 30 mmol/l glucose. Lanes 3–5 and 6–8: epididymal adipose tissue from Akita and STZ-diabetic mice ($n = 3$), respectively. Lanes 9 and 10: epididymal adipose from nondiabetic control ($Lepr^{db/+}$) and *db/db* diabetic mice, respectively.

Succination of proteins in animal models of type 1 diabetes

There was no increase in succination of proteins, even in adipose tissue, in type 1 models of diabetes. In both the STZ-induced diabetic mouse and the Akita genetic-model, diabetes results from dysfunction of pancreatic β-cells. In contrast to type 2 diabetes, which is characterized by obesity, type 1 diabetes is a wasting model, characterized by reduced

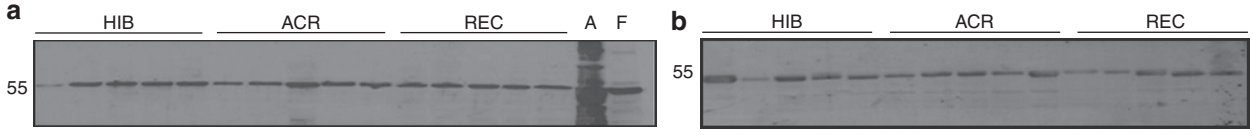

Figure 5 Analysis of succinated protein in brown and white adipose tissue from 13-lined ground squirrels before (active in cold room; ACR), during (hibernating; HIB), and after (interbout arousal recovery; REC) a bout of cold torpor. (**a**) Brown adipose tissue of HIB, ACR, and REC animals. (**b**) White adipose tissue of HIB, ACR, and REC squirrels. Tissues were analyzed from five different animals for each group. A, adipocytes grown in 30 mmol/l glucose for 8 days, F, undifferentiated 3T3-L1 fibroblast control.

adipose tissue mass. Thus, tissue from the type 1 models had to be analyzed at an early stage in their diabetes, before severe loss of adipose tissue mass. The differences in succination of adipose tissue protein in type 1 vs. type 2 diabetes can be attributed to differences in metabolism. Adipose tissue in type 1 diabetes is lipolytic and, in the absence of insulin, glucose uptake and metabolism is limited. In contrast, in type 2 diabetes, adipose tissue is lipogenic; adipose tissue anabolism is supported by increased glucose uptake and metabolism for lipogenesis. Limitations in the capacity for lipogenesis may lead to inhibition of mitochondrial metabolism and accumulation of fumarate, setting the stage for succination of proteins. In recent studies (S.A. Thomas, J.W. Baynes, and N Frizzell, unpublished data) on 3T3-L1 adipocytes in cell culture, we have observed that both high glucose and high insulin concentrations characteristic of insulin resistance are required to increase succination of protein. These conditions exist in the models of type 2 diabetes studied, and to a much lesser extent in the DIO model. However, as insulin was absent in type 1 diabetic mice despite hyperglycemia, we observed low levels of succinated proteins in these type 1 models. It is important to note that earlier studies in our laboratory demonstrated increased succination of GAPDH in skeletal muscle of type 1 diabetic rats (2), however, these animals were diabetic for ~6 months (vs. 10 weeks in the mice used in current study) and were receiving regular insulin therapy to ameliorate their chronic hyperglycemia. In the older rats the insulin therapy may have facilitated glucose uptake resulting in increased fumarate and protein succination in the muscle (2).

Succination in adipose tissues of ground squirrels during the hibernation cycle

Many small mammals undergo circannual periods of obesity in preparation for winter hibernation (7). Huge reserves of fat are laid down to fuel metabolism over the nonfeeding winter months when animals undergo prolonged periods of cold torpor. Lipids fuel both basal energy needs for many months and power high rates of thermogenesis by brown adipose tissue during periodic arousals from torpor back to euthermic body temperature. We expected that the limited mitochondrial metabolism, effected by respiratory control (high ATP; low ADP) during hibernation, might produce increased concentrations of Krebs cycle metabolites and increased succination of proteins in adipose tissue during deep cold torpor in HIB animals. By contrast, we predicted that succination might be minimal both before (ACR) and after (REC) excursions into deep torpor. However, the results show that, succination was limited under all conditions and only succinated tubulin was detectable, and was unchanged, in either brown or white adipose tissue during the three phases of the hibernation cycle. Ground squirrel adipose tissue had levels of succinated tubulin comparable to that in adipocytes grown in 30 mmol/l glucose (**Figure 5a**, lane A). Although HIB animals become hyperinsulinemic and insulin resistant in preparation for hibernation, their blood glucose concentration appears to remain in the normal range (7,21,22). The lack of hyperglycemia is consistent with the failure to detect increased succination of adipose tissue proteins in ground squirrels.

Conclusion

Overall this work shows that changes in succination of proteins in obesity and diabetes are clearly demonstrable in adipose tissue under conditions of hyperglycemia and insulin resistance. In other work, discussed in ref. (6), we have detected a link between mitochondrial stress and ER stress in diabetes, possibly attributable in part to succination of ER chaperone proteins in the adipocyte (4). ER stress is also increased in retina, kidney, and β-cells in diabetes (23–26), so that changes in succination of protein may also be observable in these tissues at a later stage in the disease. Future studies are directed at analysis of these tissues in addition to the continued investigations in adipose tissue on the relationships between mitochondrial stress, protein succination, and development of ER stress in diabetes.

ACKNOWLEDGMENTS

This work was supported by a research grant from the National Institute of Diabetes, Digestive and Kidney Diseases (DK-19971) to J.W.B. and NSERC (Canada) to K.B.S. We thank James Carson for helpful discussion and suggestions.

DISCLOSURE

The authors declared no conflict of interest.

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