Mitochondrial stress causes increased succination of proteins in adipocytes in response to gluco toxicity

Norma FRIZZELL*,†, Sonia A. THOMAS†, James A. CARSON† and John W. BAYNES*

*Department of Pharmacology, Physiology and Neuroscience, School of Medicine, University of South Carolina, Columbia, SC 29208, U.S.A., and †Department of Exercise Science, Arnold School of Public Health, University of South Carolina, Columbia, SC 29208, U.S.A.

INTRODUCTION

We have previously described 2SC [S-(2-succino)-cysteine], a chemical modification formed by a Michael addition reaction of fumarate with cysteine residues in proteins. Formation of 2SC, termed ‘succination’ of proteins, increases in adipocytes grown in high-glucose medium and in adipose tissues of Type 2 diabetic mice. However, the metabolic mechanisms leading to increased succination of protein in the adipocyte are unknown. Treatment of 3T3 cells with high glucose (30 mM compared with 5 mM) caused a significant increase in cellular ATP/ADP, NADH/NAD+ and Δψm (mitochondrial membrane potential). There was also a significant increase in the cellular fumarate concentration and succination of proteins, which may be attributed to the increase in NADH/NAD+ and subsequent inhibition of tricarboxylic acid cycle NAD+-dependent dehydrogenases.

Chemical uncouplers, which dissipated Δψm and reduced the NADH/NAD+ ratio, also decreased the fumarate concentration and protein succination. High glucose plus metformin, an inhibitor of complex 1 in the electron transport chain, caused an increase in fumarate and succination of protein. Thus excess fuel supply (gluco toxicity) appears to create a pseudohypoxic environment (high NADH/NAD+ without hypoxia), which drives the increase in succination of protein. We propose that increased succination of proteins is an early marker of gluco toxicity and mitochondrial stress in adipose tissue in diabetes.

Key words: adipocyte, cysteine, fumarate, mitochondrial hyperpolarization, pseudohypoxia, succination.
accumulation in other tissues, such as muscle and liver [17–19]. Therefore understanding the events that contribute to adipocyte metabolic stress are central to understanding the mechanisms underlying increased succination in diabetes.

Both glucotoxicity and lipotoxicity are associated with the development of insulin resistance and diabetes in several tissues, including the adipocyte [20–25]. However, fatty acid oxidation is a minor source of energy in the white adipocyte [26,27]. Accordingly, we hypothesized that glucotoxicity is the primary source of mitochondrial stress and increased fumarate concentration in the adipocyte during diabetes. In the present study, we examined the metabolic events which contribute to increased protein succination in adipocytes cultured in high glucose. We show that a high glucose concentration leads to an increase in total cellular ATP/ADP and NADH/NAD+ ratios, alongside the hyperpolarization of the IMM (inner mitochondrial membrane), and ultimately the accumulation of fumarate and succination of proteins. In support of this hypothesis we also show that chemical uncouplers, which dissipate ΔΨm (mitochondrial membrane potential), also lower the NADH/NAD+ ratio, decrease fumarate concentration and decrease succination of proteins. Consequently, we establish a mechanistic link between fuel excess, mitochondrial stress and succination of protein in adipocytes in Type 2 diabetes.

EXPERIMENTAL

Chemicals

Unless otherwise noted, all chemicals were purchased from Sigma–Aldrich. Criterion polyacrylamide gels and Precision Plus protein ladder were purchased from Bio-Rad Laboratories. PVDF membrane and ECL Plus chemiluminescent substrate were from GE Healthcare. The synthesis of 2-succinocysteamine and PVDF membrane and ECL Plus chemiluminescent substrate were from GE Healthcare. The synthesis of 2-succinocysteamine and preparation of a polyclonal anti-2SC antibody have been described previously [2].

3T3-L1 adipocyte experiments

Murine 3T3-L1 fibroblasts were obtained from the laboratory of Dr Howard Green (Harvard Medical School, Boston, MA, U.S.A.). The cells were maintained and differentiated into adipocytes as described previously [2]. After differentiation, the adipocytes were cultured in DMEM (Dulbecco’s modified Eagle’s medium) containing 5 μg/ml insulin and either 5 mM D-glucose, 30 mM D-glucose or 30 mM D-glucose containing drug treatments [CCCP (carboxyl cyanide m-chlorophenylhydrazone), DNP (2,4-dinitrophenol), SÂ (salicylic acid) or metformin (1,1-dimethylbiguanide hydrochloride)], for a further 2–8 days (maturation period); during this time lipid droplets accumulated in the cytoplasm. Preliminary experiments using L-glucose as an osmotic control indicated that it had no effect on protein succination (results not shown). Culture medium was changed every 48 h, but glucose was supplemented into the media at 24 h intervals to maintain glucose levels. Cells were harvested in 500 μl of RIPA lysis buffer [50 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS and 0.5% sodium deoxycholate, pH 7.4, with the addition of 2 mM DTPA (diethylenetriaminepenta-acetic acid) and a protease inhibitor cocktail (P8340, Sigma–Aldrich)]. The cell lysate was pulse-sonicated at 2 W RMS using a Model 100 sonic dismembrator (Fisher Scientific) for 1 min prior to resting on ice for 30 min in lysis buffer. The protein was precipitated with 9 vol. of ice-cold acetone for 10 min on ice. After centrifugation at 3000 g for 10 min and removal of the acetone, the protein pellet was resuspended in 500 μl of RIPA buffer. The protein content was determined by the Lowry assay [28].

Determination of the NADH/NAD+ and ATP/ADP ratio

Analysis of both NADH and NAD+ was performed in cell lysates after removal of protein and lipids by Folch extraction [29]. Briefly, 3-cm-diameter plates containing adipocytes were washed and scraped into ice-cold RIPA buffer, followed by the immediate addition of 20 vol. ice-cold chloroform/methanol (2:1, v/v). The samples were vortex-mixed and allowed to stand on ice for 10 min with intermittent vortex-mixing prior to the addition of 0.2 vol. H2O. The samples were vortex-mixed and allowed to stand on ice for an additional 2 min, followed by centrifugation at 2000 g for 10 min at 4°C. The supernatant was removed and rinsed in vacuo, then resuspended in H2O. Aliquots (100 μl) were removed for analysis of NADH and NAD+ (NAD+/NADH Quantification Kit, Biovision) according to the manufacturer’s instructions. ATP, ADP and AMP levels were measured immediately after nucleotide extraction using a bioluminescence assay (ApoSENSOR™, Biovision).

Measurement of ΔΨm

ΔΨm was measured using the fluorescent potentiometric dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3′,3′-tetrathylenemizidotolzylcarboyanine iodide) (Molecular Probes). Briefly, 3T3 adipocytes were cultured in 24-well plates and treatments in maturation medium were continued for 2 days, which was the optimal time point for measurement of ΔΨm, as increased lipid accumulation at later times interfered with fluorescence measurements. After 2 days in various treatments, the cells were incubated with 10 μg/ml JC-1 dye for 20 min at 37°C. The cells were then washed and resuspended in 200 μl of PBS, and JC-1 fluorescence was measured on a Tecan Safire® microplate reader (Tecan). The fluorescence of the JC-1 monomer was measured at λem = 485 nm/λex = 535 nm and the fluorescence of the JC-1 aggregate was measured at λex = 550 nm/λem = 600 nm. The aggregate/monomer ratio (600/535) was used to assess ΔΨm.

Cells treated with 100 μM CCCP for 20 min were used as a positive control to demonstrate complete depolarization of the mitochondrial membrane.

Cell viability assay

The CellTiter-Blue® Cell Viability Assay (Promega) was used to determine the effects of various compounds on 3T3-L1 adipocyte viability during prolonged incubations. Cells were also monitored daily using light microscopy to observe the effects on cell number and morphology, which were unaffected in any of the experiments. Prior to measurement of cell viability, the treatment medium was removed and replaced with 200 μl of fresh treatment medium and 40 μl of CellTiter-Blue Cell Viability reagent (Promega). The cells were then incubated in the dark for 1 h at 37°C and the fluorescent signal generated upon reduction of resazurin to resorufin was measured on a Tecan Safire® microplate reader at λex = 560 nm/λem = 590 nm, as described by the manufacturer.

Triacylglycerol analysis

The triacylglycerol concentration was measured in 5 μl aliquots of cell lysates using the Thermo DMA enzymatic triacylglycerol assay kit and standard (Thermo Fisher Scientific), according to
Measurement of intracellular malate and fumarate
Malate and fumarate were measured in adipocyte lysates using an adaptation of the procedure described by Hatch [30]. Brieﬂy, adipocyte lysates harvested in RIPA buffer from confluent 100 mm × 20 mm plates were immediately acidiﬁed with 500 µl of 3 M hydrochloric acid, then extracted twice with 2 ml of ethyl acetate. The extracts were evaporated under nitrogen, redissolved in 1% TFA (trifluoroacetic acid) and applied to a 1 ml C-18 Sep Pak column (Waters) to remove endogenous ﬂuorophores, including NADH and NADPH. The columns were washed with 1% TFA, and the eluate was collected and dried in vacuo. The dried extracts were resuspended in assay buffer (25 mM Hepes/KOH, pH 7.5, 4 mM NADP+, 4 mM MgCl2; and 5 mM potassium phosphate) and aliquots of each sample corresponding to ∼30 µg of protein in the original cell extract were added to the wells of a 96-well plate. The formation of NADPH (λex = 340 nm/λem = 450 nm) from malate was measured at the 20 min endpoint after the addition of 1 unit of malic enzyme (Sigma–Aldrich; M1567). After this time, fumarate was measured by the addition of 1 unit of fumarase (Sigma–Aldrich; F1757) and NADPH ﬂuorescence was recorded after another 20 min. Background ﬂuorescence (samples without added enzymes) was subtracted from each measurement. A standard curve of NADPH ﬂuorescence with increasing amounts of fumarate was used to calculate the amount of malate and fumarate present in each sample.

2D (two-dimensional) gel electrophoresis and Western immunoblotting
Isoelectric focusing on pI 4–7 strips and 2D gel electrophoresis were performed as described previously [3]. The 2D gels were stained for protein using Sypro Ruby total protein stain (Invitrogen) [3] or transferred on to PVDF membrane to detect protein succination by Western blotting. Western blotting to probe for protein succination was performed as described previously [2]. ImageJ software (NIH; http://rsbweb.nih.gov/ij/) was used to quantify band intensity by densitometry.

Statistical analysis
Results are summarized throughout as means ± S.E.M. and are plotted using Sigma Plot 11 software (Systat Software). Statistical analyses were performed using SigmaPlot 11 and Prism 4 (GraphPad Software). Differences between groups were analysed using the unpaired two-tailed t test or one-way ANOVA with the Holm–Sidak post-test.

RESULTS
ATP/ADP and NADH/NAD+ ratios and protein succination are increased in adipocytes grown in high-glucose medium
To assess the effects of high-glucose medium on adipocyte metabolism, adipocytes were cultured in 5 mM compared with 30 mM glucose concentration. As shown in Figure 1, there was a ∼4.6-fold increase in the total cellular ATP/ADP ratio (12.26 compared with 2.69, P < 0.001, Figure 1A) and a ∼6-fold increase in the NADH/NAD+ ratio (2.69 ± 0.33 compared with 0.446 ± 0.076, P < 0.001, Figure 1B) in adipocytes cultured in 30 mM glucose compared with 5 mM glucose. Protein succination was also increased, even at an early stage (∼3 days) during adipocyte maturation in high glucose (Figure 1C). Although only one major protein (previously identiﬁed as tubulin; ∼55 kDa) was modiﬁed in the adipocytes cultured in 5 mM glucose (lane 1), an increase in succinated proteins was detected in adipocytes cultured in 30 mM glucose (lane 2), conﬁrming that succination increases in adipocytes in concert with the increase in the ATP/ADP and NADH/NAD+ ratios in response to high glucose. A duplicate 2D gel analysis of equal amounts of protein from 5 mM and 30 mM glucose-treated adipocytes (8 days maturation) was performed after isoelectric focusing across pI 4–7. As shown in Figures 1D (and 1E), there was no signiﬁcant difference in total proteins with the different glucose treatments. However, Figures 1F (and 1G) demonstrate that protein succination was increased only in 30 mM glucose (Figure 1G), conﬁrming that increased protein succination is not due to large changes in protein abundance, but is a result of increased post-translational modiﬁcation.

Figure 1 ATP/ADP, NADH/NAD+ and 2SC are increased during maturation of adipocytes in high glucose
(A) The ATP/ADP ratio in adipocytes grown in 5 mM compared with 30 mM glucose was measured after 2 days growth in maturation medium, as described in the Experimental section. Results are expressed as means ± S.E.M., n = 4, ***P < 0.001 for 30 mM compared with 5 mM. The ATP concentration in control cells was 0.8 mM. (B) The NADH/NAD+ ratio was measured in adipocyte lysates after 2 days growth in maturation medium, as described in the Experimental section. Results are expressed as means ± S.E.M., n = 4, ***P < 0.001 for 30 mM compared with 5 mM. The control (5 mM) level of NADH was ∼214 pmol/mg of protein and NADH was ∼69 pmol/mg of protein. (C) 2SC increases on adipocyte protein in association with an increased ratio of both ATP/ADP and NADH/NAD+. Total cell lysates (30 µg of protein) from adipocytes cultured in 5 or 30 mM glucose for 4 days were separated by SDS/PAGE. 2SC-modiﬁed proteins were detected using a polyclonal anti-2SC antibody, as described in the Experimental section. M indicates the marker lane and molecular masses of marker proteins are indicated on the right-hand side. (D–G) Protein (150 µg) from adipocytes grown in 5 mM of 30 mM glucose was analysed by 2D gel electrophoresis across a 4–7 pH range. Duplicate gels were either stained with Sypro Ruby to observe total protein (D and E) or transferred on to PVDF and immunoblotted with an anti-2SC antibody to detect succinated proteins (F and G). Protein succination was increased in adipocytes cultured in 30 mM glucose (G) compared with 5 mM glucose (F), although there were no major changes in the total protein proﬁle (D and E) respectively.
Figure 2 Chemical uncoupling lowers Δψ_m and NADH/NAD^+ ratio in adipocytes cultured in high glucose

(A) Adipocytes cultured in 5 mM glucose, 30 mM glucose or 30 mM glucose plus 10 μM CCCP, 10 μM DNP or 1 mM SA for 2 days were loaded with the dye JC-1 to measure the Δψ_m. After 20 min, the cells were washed and fluorescence was measured at λ_ex/λ_em 550/535 nm and λ_ex/λ_em 485/535 nm, as described in the Experimental section. The Δψ_m is a ratio of fluorescence at 600/535 nm and the results are expressed as a percentage of the control (5 mM). The results are representative of n = 4 measurements expressed as means ± S.E.M.; *P < 0.001 for 30 mM compared with 5 mM glucose, **P < 0.01 for 30 mM glucose compared with 30 mM glucose plus DNP, and ***P < 0.001 for 30 mM glucose compared with 30 mM glucose plus CCCP or SA. (B) NADH/NAD^+ levels were measured in adipocyte lysates after culture in 5 mM glucose, 30 mM glucose or 30 mM glucose plus 10 μM CCCP, 10 μM DNP or 1 mM SA for 2 days. Before expressing the data as a ratio, the control (5 mM glucose) levels of NAD^+ was measured as =182 pmol/mg of protein and NADH was measured as =78 pmol/mg of protein. The results are expressed as means ± S.E.M., n = 4; ***P < 0.001 for 30 mM glucose compared with 30 mM glucose plus CCCP, DNP or SA.

Effect of glucose concentration and uncouplers on Δψ_m and the NADH/NAD^+ ratio

The overall increase in the NADH/NAD^+ ratio is consistent with feedback inhibition of the electron transport chain by respiratory control at a high ATP/ADP ratio. To test this hypothesis, we examined the effects of a high glucose concentration on Δψ_m and also the effects of uncouplers of oxidative phosphorylation. As shown in Figure 2(A), there was a significant increase in the Δψ_m in adipocytes cultured in 30 mM compared with 5 mM glucose (fluorescence ratio = 0.757 ± 0.44 compared with 0.369 ± 0.03, P < 0.001). The uncouplers CCCP and DNP at 10 μM concentration reversed the increase in Δψ_m in adipocytes grown in 30 mM glucose (P < 0.001 and P < 0.01 for CCCP and DNP respectively). SA, at a concentration of 1 mM, was also an effective uncoupler in cells grown in 30 mM glucose (P < 0.001). Treatment with CCCP, DNP and SA also caused a 46%, 58% and 82% decrease in the NADH/NAD^+ ratio in adipocytes grown in high-glucose medium (P < 0.001, Figure 2B), consistent with uncoupling of oxidative phosphorylation and activation of the electron transport chain. The toxicity of the uncouplers was assessed by a cell viability assay. The adipocytes were cultured for 8 days (to determine viability at the time when scission was measured) in concentrations ranging from 1 to 100 μM CCCP and DNP and 1 to 10 mM SA. After 8 days’ exposure, cells cultured in 10 μM CCCP or DNP showed no significant cell death compared with untreated control cells (Supplementary Figures S1A and S1B at http://www.BiochemJ.org/bj/445/bj4450247add.htm), indicating that these low concentrations were well tolerated and did not contribute to cell death. However, there was a significant decrease in cell viability in both 25 and 50 μM concentrations of CCCP and DNP against untreated cells; and at 100 μM concentrations, no cells survived. SA appeared to be well tolerated at all concentrations used; however, a significant ~33% reduction in cell viability was observed at 10 mM SA compared with untreated cells in 30 mM glucose (P = 0.015, Supplementary Figure S1C). CCCP and DNP, used at 10 μM concentrations, had a minimal effect on triacylglycerol biosynthesis in cells grown in 30 mM glucose, whereas SA reduced lipogenesis by approximately 50%, to levels observed in cells grown in 5 mM glucose (P = 0.012, Supplementary Figure S1D). Consequently, CCCP and DNP were used in all experiments at 10 μM and SA was used at 1 mM.

Figure 3 Malate and fumarate are reduced after treatment with chemical uncouplers

Adipocytes were matured in 5 mM or 30 mM glucose or 30 mM glucose plus 10 μM CCCP, 10 μM DNP or 1 mM SA for 2 days. Malate and fumarate levels were measured in an enzymatic assay as described in the Experimental section. The results are expressed as nmol/mg of protein calculated from a standard curve of NADPH production. The results are expressed as means ± S.E.M.; n = 3; ***P < 0.001, **P < 0.01 or *P < 0.05 for 30 mM glucose compared with 30 mM glucose plus CCCP, DNP or SA or #P < 0.001 for 5 mM compared with 30 mM glucose.

Figure 3 Malate and fumarate concentrations

To validate the role of NADH as the inhibitor of tricarboxylic acid cycle activity in adipocytes grown in high-glucose medium, we examined the effects of uncouplers on fumarate concentration. Since the enzymatic assay also measures malate concentration (see the Experimental section), both results are reported. Both the malate and fumarate concentration were significantly elevated in adipocytes grown in high- compared with low-glucose medium (Figures 3A and 3B, 30 mM compared with 5 mM: 28.3 ± 3.24 compared with 6.7 ± 0.47 nmol/mg of protein, P < 0.01, for malate; and 19.9 ± 5.03 compared with 2.4 ± 0.91 nmol/mg of protein, P = 0.001 for fumarate). Although the malate concentration in high glucose was slightly greater than the fumarate concentration, the fold increase between 30 mM and 5 mM glucose was greater for fumarate than for malate (~7.6-fold compared with ~4-fold). Malate levels were significantly decreased by chemical uncoupling (68% and 84% for DNP and SA respectively, P < 0.01; and 43% for CCCP, P < 0.05;
Protein succination is reduced by uncouplers of oxidative phosphorylation

To determine whether the effects of uncouplers on the NADH/NAD⁺ ratio and fumarate concentration ultimately affected protein succination, we examined succination of proteins by Western blotting with an anti-2SC antibody. As can be observed in Figure 4(A), there were multiple succinated proteins in the adipocytes cultured in 30 mM compared with 5 mM glucose. However, when the adipocytes were treated with the chemical uncouplers, a significant reduction in succination of a range of proteins was detected (Figure 4A), consistent with the decrease in Δψm and NADH/NAD⁺ ratio (Figure 2). The gel was stripped and reprobed with an anti-β-tubulin antibody to assess protein loading (Figure 4B), and the densitometry obtained for the 2SC staining was normalized to the tubulin content of each lane. There was a statistically significant reduction in protein succination with all uncouplers (Figure 4C; P < 0.001). Under normal conditions, protein succination is directly related to the high glucose environment. When adipocytes have been matured in 30 mM glucose for 4 days, and are then switched to 5 mM glucose for a further 4 days, succinated proteins revert to levels seen in cells grown in 5 mM glucose for 8 days (Figure 4D).

In contrast with the mitochondrial uncouplers, the anti-diabetic drug metformin, which at suprapharmacological concentrations is an inhibitor of mitochondrial complex I [31], caused an increase in both fumarate and malate concentrations (P < 0.001, Figures 5A and 5B), as well as an increase in protein succination after 4 days treatment in maturation medium (P < 0.01, Figures 5C and 5D). The increase in protein succination was most evident for tubulin (55 kDa) at the 4 day time point when cells were harvested. Longer incubations at these concentrations of metformin (1 mM and 2 mM) resulted in toxicity.

**DISCUSSION**

The results from the present study outline our current understanding of the mechanisms effecting the increase in succination of proteins in adipocytes in high-glucose medium and in diabetes. Elevated carbohydrate fuel supply exceeds the...
metabolic needs of the cell and drives mitochondrial stress due to the accumulation of ATP in adipocytes, which in turn leads to feedback inhibition of oxidative phosphorylation. The corresponding decrease in ADP concentration inhibits the F_{0}F_{1} ATP synthase (respiratory control) and produces a high Δψ_m. Accordingly, electron transport is inhibited and the cellular redox status shifts towards an increase in NADH concentration. The increase in the NADH/NAD⁺ ratio in diabetes, also known as reductive stress, was first described by Williamson and co-workers as ‘pseudohypoxia’ [32,33], i.e. an increase in the NADH/NAD⁺ ratio in the absence of hypoxia. The increase in NADH causes an increase in fumarate and malate, consistent with feedback inhibition of the NAD⁺–dependent dehydrogenases of the tricarboxylic acid cycle. Fumarate then reacts with cysteine residues on proteins within the mitochondrion, or it may be transported into the cytosol and other organelles via dicarboxylate carriers [34], leading to succination of a wide range of intracellular proteins. In collaboration with Dr Thomas Metz at the Pacific Northwest National Laboratories, we have identified ∼70 succinated proteins in adipocytes grown in high-glucose medium (N. Frizzell, T. O. Metz and J. W. Baynes, unpublished work). This sequence of events leading to increased succination of proteins requires the synergistic effects of both high glucose and high insulin concentrations: succination is not significantly increased when the adipocytes are matured in fasting insulin (⩽0.05 nM) concentrations (A. Manuel, S. A. Thomas, J. W. Baynes and N. Frizzell, unpublished work). Thus the combined effects of elevated insulin and high glucose concentrations are necessary to induce mitochondrial stress and protein succination in the 3T3 adipocyte [20,21], providing an excellent model to understand how succination is also increased in the db/db mouse adipose tissue [3].

**Protein succination can be reduced by chemical uncouplers**

The use of chemical uncouplers in the present study has demonstrated the mechanistic link between high glucose concentration and elevated Δψ_m, the increase in the NADH/NAD⁺ ratio, the accumulation of fumarate and increase in succination of protein – all of these events may be viewed as a consequence of glucoxicity. Uncouplers, such as CCCP and DNP, decrease Δψ_m and stimulate mitochondrial fuel metabolism. At high concentrations, they completely uncouple mitochondria and induce cell death; however, at a 10 μM concentration, adipocytes could be cultured for 8 days without evidence of toxicity (Supplementary Figures S1A and S1B). Under these conditions, the high glucose-induced increase in Δψ_m was normalized (Figure 2A) and the accumulation of tricarboxylic acid cycle intermediates and protein succination was significantly reduced (Figures 3 and 4). We also observed that sodium SA, a much weaker uncoupler [35], typically used at 1000-fold higher concentrations than DNP or CCCP, significantly lowered the Δψ_m, the NADH/NAD⁺ ratio and protein succination without evidence of toxicity. The recently published results of the TINSAL-T2D [Targeting Inflammation using Salsalate (salicylsalicylate) in Type 2 Diabetes] study suggests that the treatment of Type 2 diabetes with salsalate, which is hydrolysed to SA (t½ ∼60 min) offers improved therapeutic potential with significant reductions in HbA₁c (glycated haemoglobin) levels [36]. The beneficial effect of salsalate therapy is generally attributed to its anti-inflammatory activity; however, the results of the present study suggest that mild mitochondrial uncoupling by SA, with the consequent increase in fuel (glucose) utilization, may assist in the reduction of the metabolic stresses induced by hyperglycaemia in diabetes. Although reductions in triacylglycerol content in 3T3 adipocytes have been reported both with chemical uncoupling and UCP (uncoupling protein) overexpression [37], we did not observe any significant decrease in triacylglycerol content in the uncoupled adipocytes compared with high glucose controls, with the exception of SA treatment (Supplementary Figure S1D). Whether SA affects adiposity in Type 2 diabetic patients remains to be explored.

Although protein succination can be lowered by chemical uncouplers, Figure 4(D) demonstrates that a reduction in glucose to 5 mM after 4 days exposure to 30 mM glucose limits the accumulation of succinated proteins over the next 4 days and permits the turnover of existing succinated proteins. The factors that determine the stability and turnover of succinated proteins are currently under further investigation in our laboratory. However, these results suggest that chronic high-glucose exposure is analogous to a sedentary individual who maintains hyperglycaemia through sustained feeding. Healthy individuals may also have a transient increase in protein succination with a large meal; however, glucose utilization due to physical activity and/or caloric restriction should lower succination due to protein turnover. The results suggest that the succinated protein load may be highest and most pathogenic in the adipocytes of early-stage diabetics who are not receiving hyperglycaemia-lowering treatments or reducing their calorie intake.

If the sequence of events we have proposed is correct, electron transport chain inhibitors should also increase both fumarate levels and protein succination by reducing electron flow. We evaluated the effects of rotenone, sodium amobarbital and oligomycin; however, all of these compounds caused significant cytotoxicity during the long-term incubations, which are necessary to detect protein succination, even at submicromolar concentrations (results not shown). In contrast, the biguanide drug metformin has been documented to act as a weak inhibitor of complex I at millimolar concentrations [31,38,39]. Although the doses used in the present study (1–2 mM) are relatively high compared with peak plasma concentrations in metformin-treated diabetics (∼10 μM), this drug is known to bioaccumulate in mitochondria to ∼100–500 μM [40,41]. More specifically, Wang et al. [41] have demonstrated that the EC₅₀ for blood lactate accumulation in rats is 734 μM, and this is sufficient to lower oxygen consumption and promote lactic acidosis in rat hepatocytes. This indicates that, at concentrations >734 μM, metformin is acting as an inhibitor of oxidative phosphorylation, and the concentrations used in the present study (1–2 mM) are a model of acute electron transport chain inhibition, as demonstrated previously [42]. At physiological pH, metformin is positively charged and accumulates slowly at a membrane potential-driven rate that requires prolonged exposure, such as the 4 day treatment in the present study. Metformin treatment also increases glucose uptake via GLUT4 (glucose transporter 4) in white subcutaneous adipocytes [43], which in combination with complex I inhibition might be expected to lead to an increased fumarate concentration.

The present study demonstrates that exposure to elevated concentrations of metformin increases the concentration of the tricarboxylic acid cycle intermediates malate and fumarate, and increases protein succination at high glucose concentration (Figures 5A–5C), consistent with its action as a complex I inhibitor, and indirectly as an inhibitor of tricarboxylic acid cycle activity. Although micromolar concentrations of metformin in clinical use are clearly beneficial in Type 2 diabetes treatment, elevated concentrations of metformin are known to be deleterious, given the clinical association with lactic acidosis, particularly during renal injury or metformin intoxication suicide attempts [44–51]. The results from the present study suggest that
metformin-associated lactic acidosis and hepatorenal damage in a clinical setting may be associated with increased protein succination.

Role of pseudohypoxia in succination of proteins

The increase in NADH/NAD\(^+\) in adipocytes grown in high-glucose medium implies a critical role for hypoxia or pseudohypoxia (increased NADH/NAD\(^+\) in the absence of hypoxia) in the increased succination of proteins. However, there is no evidence that oxygen supply is limiting to adipocytes in vitro, since cellular maturation and lipogenesis proceed efficiently. When the oxygen tension is altered either by growing the adipocytes in variable depths of medium (from 8 to 12 ml of medium on a 100-mm-diameter plate), or in 2% oxygen, we observe no change in succination in vitro (S. A. Thomas, J. W. Baynes and N. Frizzell, unpublished work). Thus, when the energy supply exceeds the requirements of cellular metabolism, the overfed adipocyte appears to employ the pseudohypoxic response (accumulation of NADH) for feedback regulation of the tricarboxylic acid cycle, which sets the stage for increased succination of proteins. At the same time, there is strong evidence for true hypoxia in adipose tissue in obesity and diabetes [52–55], so that both true hypoxia and pseudohypoxia may be relevant in understanding changes in adipose tissue metabolism in vivo. It remains to be determined whether the (pseudo)hypoxic response and increased succination of proteins is a common response to glucotoxicity in other cell types, such as the β-cell, or specific retinal, renal and vascular cells, which are subject to glucotoxicity in Type 2 diabetes.

Conclusions

Although the sequence of events documented in the present study illustrates how protein succination occurs as a result of pseudohypoxia, alternative or complementary mechanisms may also be involved. Increased oxidative stress in the diabetic adipocyte has been documented extensively [56–60], and recent studies by Wang et al. [60] suggest that elevated ROS (reactive oxygen species) production in adipocytes can also inhibit respiration. Lin et al. [61] have shown that ROS production in high-glucose treated adipocytes is related to decreases in insulin sensitivity; interestingly, the ‘rescue’ strategies employed by Lin et al. [61] included overexpression of UCP2 and treatment with CCCP; both of which should simultaneously lower protein succination. Although these studies [56–61] demonstrate that ROS and markers of oxidative damage are produced, the adipocyte is well equipped with antioxidant defence systems. The most abundant of these, glutathione, rapidly reverses the oxidation of cysteine residues to sulfenic acid, regenerating the protein thiol. In contrast, the succination of cysteine residues cannot be reversed by known cellular antioxidant systems and appears to be a cumulative irreversible biomarker of protein damage until the protein is turned over intracellularly. The interplay between oxidative stress and protein succination remains to be elucidated. Further investigations on specific succinated proteins should provide a novel insight into how succination contributes to adipose tissue damage during the development of diabetes.

AUTHOR CONTRIBUTION

Norma Frizzell designed and performed the experiments, contributed to the discussion and wrote the paper. Sonia Thomas performed the experiments and contributed to the discussion. James Carson reviewed/edited the paper prior to submission. John Baynes designed the experiments, contributed to the discussion and reviewed/edited the paper prior to submission.

FUNDING

This work was supported by the National Institutes of Diabetes and Digestive and Kidney Diseases [grant number DK-19971 (to J.W.B. and N.F.)] and the American Diabetes Association [grant number 1-11-JF-13 (to N.F.)].

REFERENCES


© The Authors Journal compilation © 2012 Biochemical Society
SUPPLEMENTARY ONLINE DATA

Mitochondrial stress causes increased succination of proteins in adipocytes in response to glucotoxicity

Norma FRIZZELL*†, Sonia A. THOMAS†, James A. CARSON† and John W. BAYNES*

*Department of Pharmacology, Physiology and Neuroscience, School of Medicine, University of South Carolina, Columbia, SC 29208, U.S.A., and †Department of Exercise Science, Arnold School of Public Health, University of South Carolina, Columbia, SC 29208, U.S.A.

Figure S1  Cell viability and triacylglycerol accumulation

(A–C) Cell viability was measured as described in the Experimental section of the main text in adipocytes cultured with the following drug concentrations; 1–100 μM CCCP (A), 1–100 μM DNP (B) or 1–10 mM SA (C). ***P < 0.001 and **P < 0.01 for CCCP- or DNP-treated cells compared with untreated controls (0). (D) Triacylglycerols were measured in an aliquot of cell lysate as described in the Experimental section of the main text. The measurements were normalized to the protein content of the cells, and the results are expressed as means ± S.E.M., n = 3, *P = 0.012 for 30 mM glucose compared with SA.

Received 12 December 2011/3 April 2012; accepted 24 April 2012
Published as BJ Immediate Publication 24 April 2012, doi:10.1042/BJ20112142

1 To whom correspondence should be addressed (email norma.frizzell@uscmed.sc.edu).