Effect of inhibition of glutathione synthesis on insulin action: *in vivo* and *in vitro* studies using buthionine sulfoximine

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Decreased cellular GSH content is a common finding in experimental and human diabetes, in which increased oxidative stress appears to occur. Oxidative stress has been suggested to play a causative role in the development of impaired insulin action on adipose tissue and skeletal muscle. In this study we undertook to investigate the potential of GSH depletion to induce insulin resistance, by utilizing the GSH synthesis inhibitor, L-buthionine-[*S*,*R*]-sulfoximine (BSO). GSH depletion (20–80 % in various tissues), was achieved *in vivo* by treating rats for 20 days with BSO, and *in vitro* (80 %) by treating 3T3-L1 adipocytes with BSO for 18 h. No demonstrable change in the GSH/GSSG ratio was observed following BSO treatment. GSH depletion was progressively associated with abnormal glucose tolerance test, which could not be attributed to impaired insulin secretion. Skeletal muscle insulin responsiveness was unaffected by GSH depletion, based on normal glucose response to exogenous insulin, 2-deoxyglucose uptake measurements in isolated soleus muscle, and on normal skeletal muscle expression of GLUT4 protein. Adipocyte insulin responsiveness *in vitro* was assessed in 3T3-L1 adipocytes, which displayed decreased insulin-stimulated tyrosine phosphorylation of insulin-receptor-substrate proteins and of the insulin receptor, but exaggerated protein kinase B phosphorylation. However, insulin-stimulated glucose uptake was unaffected by GSH depletion. In accordance, normal adipose tissue insulin sensitivity was observed in BSO-treated rats *in vivo*, as demonstrated by normal inhibition of circulating non-esterified fatty acid levels by endogenous insulin secretion. In conclusion, GSH depletion by BSO results in impaired glucose tolerance, but preserved adipocyte and skeletal muscle insulin responsiveness. This suggests that alternative oxidation-borne factors mediate the induction of peripheral insulin resistance by oxidative stress.

Key words: 3T3-L1 adipocytes, adipose tissue, insulin resistance, oxidative stress, skeletal muscle.

INTRODUCTION

Experimental and human diabetes is increasingly recognized as being associated with markers for increased oxidative stress. Despite methodological and conceptual difficulties in assessing increased oxidative stress *in vivo* [1], diabetic animals and humans display increased levels of oxidative products of lipids, DNA and protein [2–4]. Decreased antioxidant defence systems have been more difficult to demonstrate in diabetes. Assessment of the level of antioxidant vitamins and the activity of antioxidant enzymes have largely yielded conflicting results, attributable to cell type, patient selection and analytical methodology. Nevertheless, most available human studies have suggested decreased plasma vitamin C levels [5–7] and reduced activity of superoxide dismutase in leucocytes [8,9]. Moreover, a vast majority of the studies addressing the level of glutathione (either total or reduced form) demonstrate that glutathione levels in whole blood [10,11], platelets [12] and polymorphonuclear cells [13] are decreased in diabetic patients. In the streptozotocin-induced diabetic rat, GSH levels are also decreased in liver, kidney, heart and nerves [14–16]. As the major redox buffer of the cell, this decrease in the level of GSH may be viewed to reflect and/or contribute to the oxidative stress and redox imbalance which accompanies the diabetic milieu.

Defining whether increased oxidative stress plays a role in the pathophysiology of diabetes and its complications may be important both for understanding the disease processes, as well as for developing new therapeutic modalities. Various studies suggest that increased oxidative stress may play a role in the development of accelerated atherosclerosis in diabetes [17], in embryonal maldevelopment in diabetic pregnancies [18], and possibly in the pathophysiology of diabetic neuropathy, retinopathy and cataract formation [2,19]. The pancreatic beta-cell was also shown to be target for oxidative stress [20]. Expression of antioxidant enzymes is very low in pancreatic islets [21], making them rather sensitive to glycation-mediated reduction of insulin gene transcription and beta-cell apoptosis [20,22,23]. In addition, several lines of evidence suggest that increased oxidative stress may play a role in peripheral insulin resistance. These mainly include clinical studies, which correlated oxidative stress parameters with the metabolic control of patients [4,24,25]. Other studies reported beneficial metabolic effects of pharmacological doses of vitamin E [26], vitamin C [27], GSH [28] or lipoic acid [29], when administered to healthy volunteers or to diabetic subjects. A direct impairment in insulin responsiveness by reactive oxygen species has been demonstrated in cell culture systems [30–33]. In 3T3-L1 adipocytes, we have shown that micromolar H_2O_2 treatment impaired various metabolic effects of insulin, including the stimulation of glucose uptake activity. The cellular mechanism involved alterations in the expression and translocation capacity of the insulin-sensitive glucose transporter GLUT4 [34], and was associated with defective activation

Abbreviations used: BSO, L-buthionine-[*S*,*R*]-sulfoximine; NEFA, non-esterified fatty acid; GTT, glucose tolerance test; ITT, insulin tolerance test; PKB, protein kinase B; LMRT, low-molecular-mass reduced thiol.

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of protein kinase B (PKB) [32]. Since these studies demonstrate the potential causative relationship between oxidative stress and impaired insulin response, a crucial issue is to define which redox parameter acts as a biological mediator.

In the present study, we undertook to dissect out a potential role for decreased GSH levels in the development of impaired glucose homoeostasis and decreased response of insulin target tissues to its metabolic actions (insulin resistance). For this purpose, the γ-glutamylcysteine synthetase inhibitor L-buthionine-[*S*,*R*]-sulfoximine (BSO) was used to decrease tissue levels of GSH in normal rats, or to inhibit GSH synthesis in an insulin-responsive adipocyte cell line. Thereafter, glucose homoeostasis was assessed *in vivo*, and the cellular response to insulin was investigated either *ex vivo* or in the cultured cells. Our results suggest that the marked decrease in GSH levels was associated with impaired glucose tolerance, but with preserved insulin-responsiveness of skeletal muscle and adipocytes.

EXPERIMENTAL

Animal treatment

All protocols were approved in advance by the Ben-Gurion University Animal Studies Committee. Five-week-old male Sprague–Dawley rats were purchased from Harlan Laboratories (Jerusalem, Israel). They were kept on a 12-h light/dark cycle at 23 °C, housed four in a cage. Standard rat chow and tap water were provided *ad libitum* during the entire experiment. BSO (Sigma) was dissolved in saline and 2 mmol/kg body weight BSO or vehicle ($n = 8$ in each group) administered by a daily intraperitoneal injection between 9–11 a.m. for 20 days, following previously described protocols [35,36]. Glucose homoeostasis was evaluated during the treatment period by a glucose tolerance test (GTT) performed on days 8 and 16, following 18 h of fast (intraperitoneal injection of 2 g/kg glucose), and by an insulin tolerance test (ITT) performed on day 18 of the study after 4 h fast (intravenous administration of 0.5 unit/kg recombinant human insulin). Blood glucose levels during both the GTT and ITT were expressed for each rat as a percentage of its glucose level at $t = 0$, and plotted as a function of time. The area under the curve for each rat was calculated using the KaleidaGraph version 3.04 (Synergy Software, Reading, PA, U.S.A.), and the mean value $\pm$ S.E.M. calculated for each group.

At 20 h after the final treatment (day 20), and following a 12-h fast, animals were weighed, anaesthetized with phenobarbital (80 mg/kg, intraperitoneal), and the soleus muscle dissected out for *ex vivo* 2-deoxyglucose uptake measurements. Blood was then drawn by cardiac puncture into EDTA-containing test tubes, and an aliquot was taken for determination of low-molecular-mass reduced thiols (LMRTs), as described below. The remaining blood was centrifuged at 4 °C, and the plasma stored frozen in aliquots at −70 °C. Gastrocnemius muscle and liver were rapidly dissected out and frozen immediately in liquid nitrogen for storage at −70 °C until used for further analyses. The pancreas was removed and cut into two parts, one was frozen at −70 °C for subsequent determination of immunoreactive insulin content, and the other fixed in 10 % phosphate-buffered formalin for immunohistochemical analysis.

LMRTs, GSH and GSSG determinations

Whole blood, liver and muscle LMRTs were measured spectrophotometrically according to Ellman's method [37], as previously described [38]. Liver GSH and GSSG were measured spectrophotometrically by the glutathione reductase recycling assay, following a method described by Anderson [39].

Pancreatic insulin content

Frozen pancreatic tissue was weighed and homogenized in 1 ml of ice-cold HCl/ethanol, as described in [40], followed by extraction for 24 h at 4 °C. The supernatant was removed following centrifugation and used for determination of insulin content after the appropriate dilution in RIA buffer (0.1 % BSA in PBS, pH 7.4).

Immunohistochemical studies

Pancreatic tissue was fixed in 10 % phosphate-buffered formalin and embedded in paraffin. Multiple sequential sections (5 μm) were mounted on slides for immunohistochemical analysis. Deparaffinized sections were rinsed with PBS, and endogenous peroxidase blocked by exposure to 3 % H_2O_2 for 15 min at room temperature. For detection of islet hormones, sections were incubated with either guinea pig anti-insulin antibody at 1:100 (Sigma), or with rabbit anti-glucagon serum (Immustain™; DPC, Los Angeles, CA, U.S.A.), at 37 °C for 1 h followed by detection using a strepavidin–biotin peroxidase complex developed with aminoethylcarbazole substrate (Zymed Laboratories, San Francisco, CA, U.S.A.). Two sections per animal, containing 30–40 islets each, were evaluated by light microscopy at $\times 200$ magnification.

2-Deoxyglucose uptake into isolated soleus muscle

Soleus muscles were dissected out immediately after the induction of anaesthesia, and incubated in the absence or presence of 2 m-units/ml human insulin, after which 2-deoxyglucose uptake was determined exactly as described in [41].

Cell culture and treatment

3T3-L1 pre-adipocytes (A.T.C.C.), were grown in six-well plates and differentiated to adipocytes, exactly as described in [30,34]. In all experiments, fully differentiated cells (10–12 days post-differentiation induction) were used. Cells were washed three times with PBS, and incubated for 18 h in serum-free medium, supplemented with BSA (0.5 % w/v, RIA grade), in the absence or presence of 0.5 mM BSO. Alternatively, cells were incubated after 16 h of serum deprivation for an additional 2 h with glucose oxidase (Sigma; 100 m-units/ml), which generated a constant medium H_2O_2 concentration of approx. 25 μM, as previously described [32]. Protein recovery and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) test confirmed cell viability. At the end of the incubation period, cells were washed three times with PBS, and incubated with or without 100 nM human insulin for either 7 min (for cell lysate preparation) or 20 min for glucose transport activity. Hexose transport activity was evaluated by the uptake of 2-deoxy-[^{3}H]glucose (50 μM, 1 μCi/ml), during 10 min incubation of the cells, as previously described [30]. Non-specific uptake was determined with 50 μM cytochalasin B, which was added to the reaction mixture.

Membrane preparations, total cell lysates and Western-blot analysis

Gastrocnemius muscle total membranes were prepared as previously described [42]. Total membranes of 3T3-L1 adipocytes (a single well of a six-well plate per condition) were prepared exactly as we previously described [32]. SDS/PAGE (7.5 % or 10 %) and Western-blot analysis, for detecting total membrane GLUT4 content, were carried out using anti-GLUT4 antibody (Chemicon International, Temecula, CA, U.S.A.). PKB phosphorylation was detected in total cell lysates using serine-473

PKB antibodies (New England Biolabs), and tyrosine phosphorylation state using anti-phosphotyrosine (4G-10) antibodies (Upstate Biotechnology, Lake Placid, NY, U.S.A.). Blots were then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Life Sciences), after which luminescence was detected by enhanced chemiluminescence (Pierce, Rockford, IL, U.S.A.). Density values of the different bands were obtained after scanning the blots with video densitometry using the UVP-GDF 5000 system (UVP Inc., San Gabriel, CA, U.S.A.), followed by quantification using Image Quant version 3.3 (Molecular Dynamics, Palo Alto, CA, U.S.A.).

Other assays

Blood glucose was measured using a glucometer (Glucometer Elite; Bayer Diagnostic, Tarrytown, NY, U.S.A.). Insulin RIA was performed using anti-insulin coated tubes (ICN Pharmaceuticals, Costa Mesa, CA, U.S.A.), and ^{125}I-insulin from Linco Research, St Charles, MO, U.S.A.). Rat insulin was used as a standard. Protein was measured using the Bio-Rad protein assay [43]. Plasma non-esterified fatty acid (NEFA) concentration was determined using a commercial kit (Roche Molecular Biochemicals, Mannheim, Germany).

Statistical analysis

The non-parametric Mann–Whitney test was used to determine the U value for differences between the control (vehicle treated) and BSO-treated animals. Student's t-test was used to determine significance of changes between treated and control cells. In both cases an α- or P-value of 0.05 was set as the threshold of statistical significance.

RESULTS

Five-week-old rats were treated for 20 days with the γ-glutamylcysteine synthetase inhibitor BSO, by a daily intraperitoneal injection (2 mmol/kg per day), following a previously described protocol [36]. BSO treatment was not associated with mortality, diarrhea, or any noticeable adverse effects. Consistent with previous reports [36], weight gain during the 20 days of treatment was decreased by approx. 8 % in the BSO-treated animals (Table 1). As expected, BSO treatment resulted in decreased plasma and tissue levels of LMRTs, of which GSH is the major contributor.

Table 1 Effect of 20 days BSO-treatment on weight gain, LMRT content and liver glutathione

Five-week-old rats were treated by a daily intraperitoneal injection of either vehicle or BSO for 20 consecutive days. At the end of the experiment, rats were fasted for 12 h then weighed and anaesthetized, after which tissues and blood were collected, as described in the Experimental section. Values are means ± S.E.M.

	Control (vehicle, $n = 8$)	BSO ($n = 8$)
Weight gain (g)	128.3 ± 4.4	118.0 ± 2.5*
LMRTs		
Whole blood (μmol/g Hb)	12.20 ± 1.05	7.63 ± 0.57*
Gastrocnemius muscle (μmol/g)	0.74 ± 0.06	0.11 ± 0.01*
Liver (μmol/g)	5.40 ± 0.60	4.23 ± 0.60*
Liver glutathione (μmol/g)		
GSH	3.25 ± 0.44	1.9 ± 0.33*
GSSG	0.082 ± 0.001	0.040 ± 0.001*
GSH/GSSG	40.0 ± 5.2	50.0 ± 7.43

* $P < 0.05$ compared with control animals.

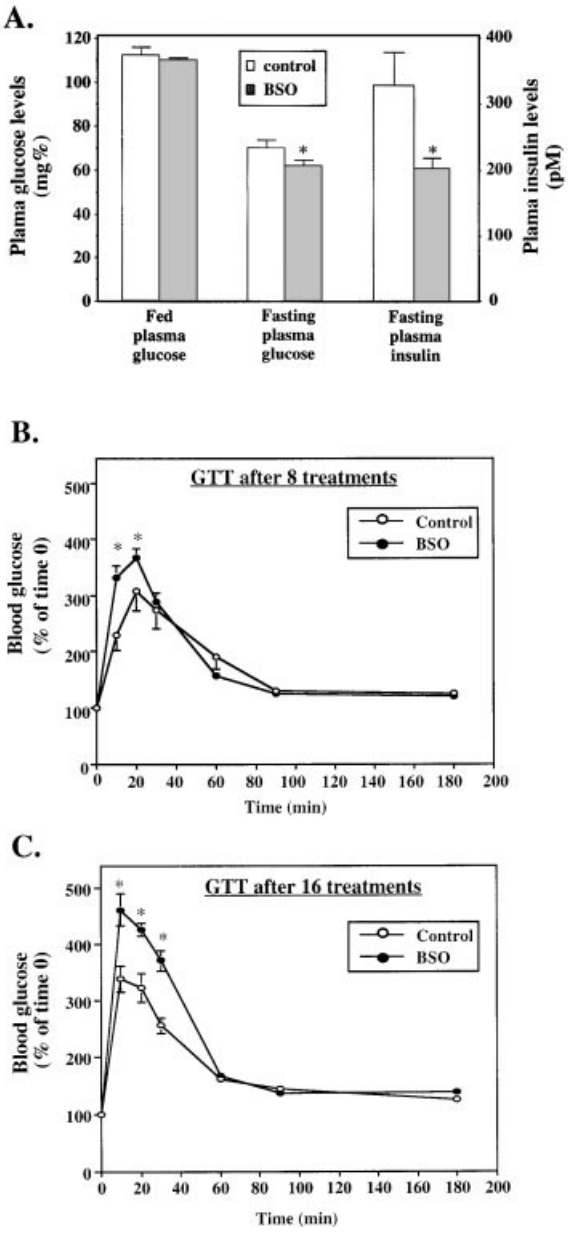

Figure 1 The effect of BSO treatment on steady-state glucose and insulin levels, and on the dynamic glucose levels during GTT

Five-week-old rats were treated with BSO or vehicle ($n = 8$ for each group) by a daily intraperitoneal injection. (**A**) At the end of the experiment (day 20), fed-state glucose levels were measured in tail blood, and again following 12 h of fasting. Fasting circulating insulin levels were also determined. After the 8th or the 16th treatment (**B,C** respectively), animals were fasted overnight and 2 g/kg glucose administered as described in the Experimental section. Glucose levels in tail blood was measured at the indicated times, and presented for each rat as % of the value at $t = 0$ (immediately before glucose administration). Each point is presented as means ± S.E.M. *$P < 0.05$ compared with vehicle-treated animals at the same time point.

Whole blood and skeletal muscle LMRT levels were reduced to 63 ± 5 % and 14 ± 1 % respectively of the levels measured in the control group (Table 1). In liver, the effect of BSO treatment on LMRTs and on the GSH/GSSG ratio was evaluated. Only a mild 20 % decrease in LMRTs was observed in BSO-treated rats, whereas the GSH/GSSG ratio was not decreased, consistent with a previous report using the same BSO dose [36].

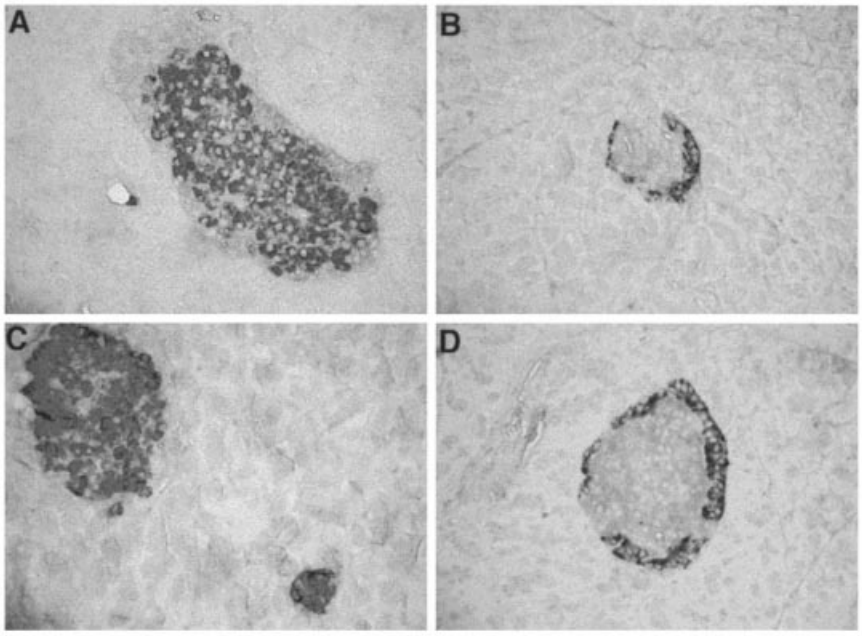

Figure 2 Photomicrographs of pancreatic sections from control and BSO-treated rats immunostained for insulin or glucagon

At the end of the experiment (see the Experimental section), control (**A**,**B**) and BSO-treated rats (**C**,**D**) were anaesthesized and the pancreas dissected out, followed by preparation of histological sections, and immunostaining with insulin (**A**,**C**) or glucagon (**B**,**D**) antibodies.

In order to determine the potential causative role of GSH depletion on the induction of peripheral insulin resistance and impaired glucose homoeostasis, the following experiments were performed: (1) steady-state levels of blood glucose and plasma insulin were evaluated in the fed and fasting states, and the dynamic changes in blood glucose concentrations determined following administration of a glucose (GTT) or insulin load (ITT); (2) skeletal muscle responsiveness to insulin was studied by an *ex vivo* measurement of glucose uptake into soleus muscle in the absence or presence of insulin, and by measuring GLUT4 protein content in gastrocnemius muscle; and (3) adipose insulin sensitivity was evaluated *in vivo* by measuring the dynamic changes in circulating NEFAs during GTT, and *in vitro* by the use of the 3T3-L1 adipocytes cell line model.

The effect of 20 days of BSO treatment on the steady-state levels of glucose in the fed and fasting states are presented in Figure 1(A). As can be noted, GSH depletion was not associated with elevated blood glucose levels, and in fasting conditions was found to be lower than in control animals. Fasting plasma insulin concentrations were also significantly lower in the BSO-treated animals. Figures 1(B) and 1(C) demonstrate glucose levels following an intraperitoneal injection of 2 g/kg glucose (GTT) after 8 and 16 treatments respectively. As can be noted, following 8 days of treatment, the increase in glucose levels 10 min and 20 min after the glucose load was greater in the BSO group relative to control. Following 16 days of BSO treatment, this impairment in glucose tolerance became more pronounced, with glucose levels at the 30-min time point also significantly increased. At day 8, the calculated area under the curve was 12258 ± 1560 and 13045 ± 642 (arbitrary units) for the control and BSO-treated groups respectively. Following 16 treatments, the respective area under the curve values were 12198 ± 1298 and 14990 ± 718, a difference which reached statistical significance ($P = 0.001$). To exclude impaired insulin secretion as the cause for the abnormal GTT in BSO-treated rats, circulating insulin levels were measured during the GTT, and were found to be identical between the two groups at time points 5, 15 and 60 min (results not shown). Moreover, there was no change in pancreatic immunoreactive insulin content (18.5 ± 4.4 and 19.8 ± 4.5 nmol/g tissue in control and BSO-treated rats respectively), and islet architecture was found to be normal (Figure 2). Collectively, the data presented in Figures 1 and 2 demonstrate that GSH depletion was associated with impaired glucose tolerance, but not with impaired beta-cell function.

We further assessed whether the abnormal GTT resulted from skeletal muscle and/or adipose tissue (peripheral) insulin resistance. Insulin administration (0.5 unit/kg) resulted in a rapid decrease in blood glucose levels, that were restored to the basal levels after 60 min. BSO treatment was not associated with blunting of the glucose curve during ITT (Figure 3A). Skeletal muscle responsiveness to insulin was also assessed *ex vivo* by measuring 2-deoxyglucose uptake into isolated soleus muscle in the absence and presence of insulin. In control animals, exposure of soleus muscle to insulin augmented hexose uptake from 66 ± 4 to 113 ± 11 pmol/mg muscle per 20 min. Similarly, in BSO-treated rats 2-deoxyglucose uptake increased from 68 ± 7 to 121 ± 13 pmol/mg muscle per 20 min. Both basal and insulin-stimulated glucose uptake values were not significantly different between the two groups. Accordingly, the protein content of the insulin-responsive glucose transporter GLUT4, previously shown to be reduced in gastrocnemius muscle membranes of diabetic rats [38,41], was not affected by BSO treatment (representative blot shown in Figure 3B).

The effect of GSH depletion by BSO on adipocyte insulin responsiveness was assessed both at the cellular level, by utilizing 3T3-L1 adipocytes, as well as *in vivo*. This cell line was selected since it is an acceptable model for adipose tissue, uniquely displaying both insulin signalling, as well as metabolic insulin

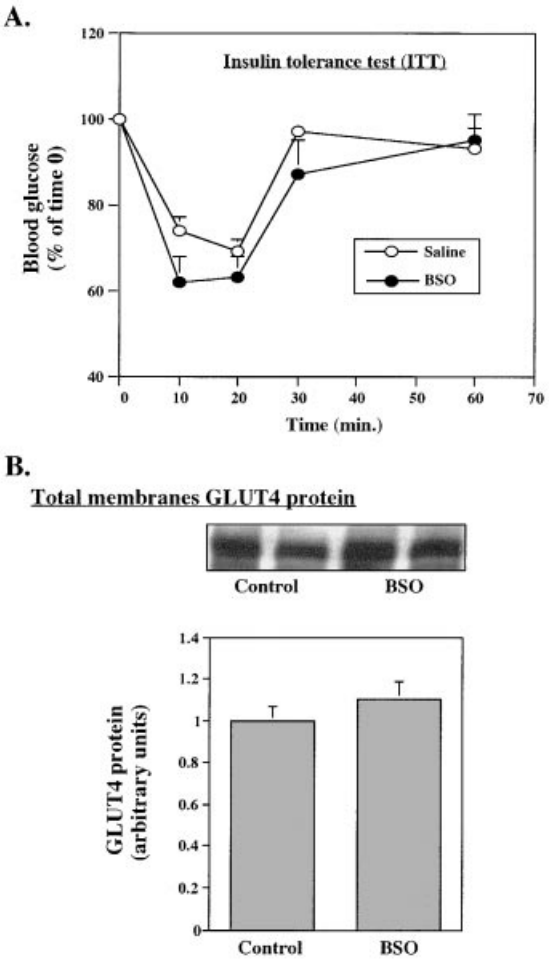

Figure 3 The effect of BSO treatment on dynamic glucose levels during ITT, and on gastrocnemius muscle total membrane GLUT4 protein content

(**A**) ITT was performed on BSO- or vehicle-treated animals ($n = 4$ in each group) after the 17th treatment, as described in the legend for Figure 1. At each time point, 250 μl of tail blood was collected, assayed for glucose and the data analysed (see the Experimental section). Results are means $\pm$ S.E.M. (**B**) Total membranes were prepared from gastrocnemius muscle, and GLUT4 protein content was assessed using Western-blot analysis. Shown is a representative blot, as well as densitometry analysis (means $\pm$ S.E.M., in arbitrary units) of GLUT4 content as measured in muscles of eight rats in each group.

Table 2 Cellular glutathione content in 3T3-L1 adipocytes treated with BSO or H_2O_2

Fully differentiated 3T3-L1 adipocytes were incubated for 18 h in serum-deprived medium with or without 0.5 mM BSO, or exposed for the last 2 h of incubation to approx. 25 μM H_2O_2 produced by adding glucose oxidase to the culture medium. Cells were then washed three times with PBS, and assessed for GSH and GSSG content, as described in the Experimental section. Results are means $\pm$ S.E.M. of three experiments, each performed in duplicates. UD, undetectable.

	Control	BSO (0.5 mM, 18 h)	H2O2 (25 μM, 2 h)
GSH (nmoles/mg of protein)	32.07 $\pm$ 2.28	5.40 $\pm$ 0.80†	18.65 $\pm$ 2.62*
GSSG (nmoles/mg of protein)	0.26 $\pm$ 0.06	UD	0.27 $\pm$ 0.04
GSH/GSSG	123.5 $\pm$ 18.5	–	69.3 $\pm$ 2.5*

* $P < 0.05$ compared with control cells.
† $P < 0.001$ compared with control cells.

responsiveness. In addition, we have previously shown in this cell line that oxidative stress, characterized by a significant decrease in cellular GSH, results in impaired insulin action [30,34]. As shown in Table 2, 18 h incubation with 0.5 mM BSO resulted in an 80 % reduction in cellular GSH content, with GSSG levels below the detection limits of the glutathione reductase recycling assay. As expected, exposure of the cells for 2 h to approx. 25 μM H_2O_2 resulted in a significant decrease in GSH, but also in decreased GSH/GSSG ratio. The insulin-signalling cascade was analysed under these conditions. In control cells, insulin stimulated the tyrosine phosphorylation of the β-subunit of the insulin receptor (95 kDa band) and of insulin-receptor-substrate proteins (approx. 180 kDa). In BSO-treated cells, insulin-stimulated phosphorylation of these two major phosphotyrosine bands tended to decrease (Figure 4A). The effect of H_2O_2 treatment of the cells on insulin-stimulated tyrosine phosphorylation is shown as comparison. Incubation with H_2O_2 was associated with several phosphotyrosine bands, one of which is a high-molecular-mass (approx. 200 kDa) protein, as previously described [44]. Following insulin stimulation, an exaggerated, rather than decreased, tyrosine phosphorylation of insulin-receptor-substrate and the β-subunit of the insulin receptor could be observed. The effect of BSO on more distal events in the insulin-signalling cascade was addressed by measuring insulin-induced serine-473 phosphorylation of PKB, a key enzyme in the metabolic signalling pathway of insulin. As shown in Figure 4(B), BSO treatment resulted in a mild increase ($P > 0.05$) of insulin-induced phosphorylation, without affecting total PKB-α content (results not shown). In contrast, cells exposed to 25 μM H_2O_2 displayed increased basal PKB phosphorylation, as well as a significant reduction in the ability of insulin to stimulate PKB phosphorylation. Consistent with this observation were the findings on the effects of BSO and H_2O_2 on insulin-stimulated hexose transport activity. While GSH depletion by BSO did not significantly affect basal or insulin-stimulated 2-deoxyglucose uptake activity, H_2O_2 exposure significantly impaired the capacity of insulin to stimulate 2-deoxyglucose uptake (Figure 4C). Moreover, consistent with the data obtained in skeletal muscle, total membrane GLUT4 protein content in BSO-treated 3T3-L1 adipocytes was comparable with control cells (results not shown).

The *in vivo* relevance of these findings to adipose tissue insulin responsiveness was addressed by measuring plasma NEFA levels during GTT, since adipose tissue acutely responds to insulin by inhibition of lipolysis. As shown in Figure 4(D), fasting NEFA levels rapidly decreased following glucose administration and endogenous insulin secretion. In BSO-treated rats, this response was comparable with that observed in control rats, suggesting normal adipose tissue responsiveness *in vivo*. Collectively, these data indicate that skeletal muscle and adipocyte insulin-responsiveness remained intact, despite GSH synthesis inhibition and a significant depletion of tissue and cellular GSH.

DISCUSSION

This study addresses the hypothesis that decreased cellular or tissue levels of GSH could mediate the effects of oxidative stress on glucose homoeostasis and insulin responsiveness. Recent studies from several groups, including our own, have demonstrated that oxidative stress impairs the normal cellular response to insulin [30,31,33]. Decreased GSH levels were found in both 3T3-L1 adipocytes exposed to a H_2O_2-generating system [44], and also in blood and tissues of streptozotocin-induced diabetic rats [38], in which impaired peripheral insulin sensitivity was observed. These data established an association between decreased GSH content and impaired insulin response, raising the

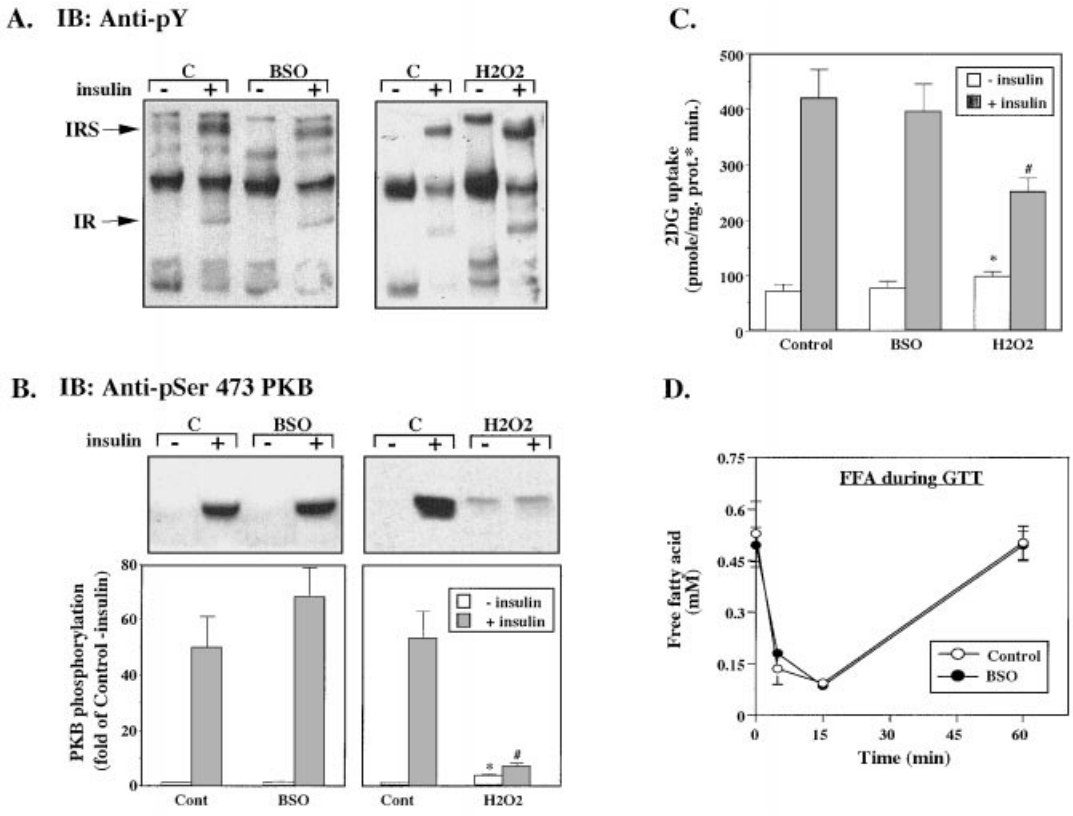

Figure 4 The effect of BSO treatment on adipocyte insulin sensitivity

(**A**,**B**) Differentiated 3T3-L1 adipocytes were either treated with 0.5 mM BSO in serum-free medium for 18 h or exposed for the last 2 h to glucose oxidase in the culture medium, generating H_2O_2 at a concentration of approx. 25 μM. Cells were then rinsed three times with PBS and incubated for an additional 7 min with or without 100 nM insulin, after which cell lysates and Western-blot analyses were performed as described in the Experimental section. Blots shown are representative of at least five different experiments yielding similar results. $^*P < 0.05$ compared with control cells (C) without insulin. $\#P < 0.001$ compared with insulin-stimulated control cells. Abbreviations: Anti-pY, anti-phosphotyrosine; IR, insulin receptor; IRS, insulin-receptor substrate. (**C**) At the end of the BSO or H_2O_2 treatments, cells were rinsed and incubated for an additional 20 min with or without 100 nM insulin, after which 2-deoxyglucose uptake was assessed. Results are means $\pm$ S.E.M. of six independent experiments performed at least in duplicate. $^*P < 0.05$ compared with control cells without insulin. $\#P < 0.001$ compared with insulin-stimulated control cells. (**D**) During a GTT performed after the 17th day of treatment, plasma NEFA (FFA) concentrations were measured at the indicated time points. Results are means $\pm$ S.E.M. of four animals in each group.

possibility that decreased GSH is causative in impairing insulin action.

Several potential cellular mechanisms, to explain how decreased GSH content would potentially affect peripheral insulin action, could be raised. Insulin stimulates glucose transport and metabolism in adipocytes and in skeletal muscle by inducing the translocation of glucose transporters (GLUT4) from internal membrane pools to the plasma membrane [45,46]. This complex process may potentially be affected by GSH depletion, modulating either the metabolic insulin signalling or GLUT4 protein expression.

3T3-L1 adipocytes exposed to oxidative stress characterized by decreased GSH and GSH/GSSG ratio ([44] and Table 2), demonstrated impaired insulin-stimulated glucose transport and GLUT4 translocation [34]. These could be attributed to impaired capacity of insulin to stimulate the phosphorylation and activity of PKB, a key enzyme in the metabolic signalling of insulin [32]. When cellular GSH content was increased by pretreating the cells with lipoic acid, these effects of oxidative stress were significantly prevented [44]. In addition, GLUT4 translocation has been demonstrated to depend on *N*-ethylmaleimide-sensitive proteins (reviewed in [45,46]), and SH reagents have been shown to affect the insulin signalling apparatus [47,48], providing additional potential targets for GSH depletion. In the present study, however, cellular depletion of GSH which was not associated with a decreased GSH/GSSG ratio (Tables 1 and 2), did not cause impaired capacity of insulin to trigger the serine phosphorylation of PKB (Figure 4B). Consistently, insulin-stimulated glucose transport activity, severely impaired by exposure to H_2O_2, was not significantly affected by BSO treatment (Figure 4C).

The importance of normal GLUT4 levels for maintaining glucose homoeostasis has been conclusively demonstrated using heterozygous GLUT4-knockout mice, which develop insulin resistance and a Type II diabetes-like syndrome [49]. 3T3-L1 adipocytes exposed for 18 h to micromolar H_2O_2 concentrations exhibit decreased GLUT4 protein and mRNA expression [30]. In skeletal muscle of streptozotocin-induced diabetic rats, GLUT4 protein levels were decreased, which was associated with decreased GSH content [38,41]. Moreover, multiple regression analysis demonstrated skeletal muscle GSH content as an independent predictor for GLUT4 protein levels [38]. Furthermore, when skeletal muscle GSH content was increased by treating diabetic rats with lipoic acid, GLUT4 protein content

was increased [38,41]. Yet, in the present study, decreased GSH levels induced by inhibiting its synthesis by BSO (Table 1), were not associated with decreased GLUT4 protein content, neither in the cell line, nor in rat skeletal muscle (Figure 3B). This was despite the fact that the degree of GSH depletion achieved was at least comparable, if not greater than, GSH depletion observed following H_2O_2 treatment of the cells (Table 2), or in diabetes [38]. Thus the present study does not support the notion that GSH levels in themselves regulate GLUT4 protein levels.

Several interpretations of the data, demonstrating lack of effect of GSH depletion on peripheral insulin action, can be generated. Firstly, the degree or duration of GSH depletion achieved *in vivo* may not have been sufficient to produce an effect on insulin signalling. However, GSH depletion in BSO-treated cells and animals was greater than that observed with H_2O_2-treated cells (Table 2) or in diabetic rats [38], both of which displayed impairment in insulin action. Thus it is possible that other antioxidant defence mechanisms compensated for the decrease in GSH content under our experimental conditions. At the cellular level, it could be speculated that GSH depletion by BSO did not occur at a cellular compartment critical for normal insulin responsiveness.

An alternative line of reasoning may be, that GSH depletion in itself is not the mediator of oxidative stress-induced insulin resistance, at least not as an independent factor. It is noteworthy, that BSO treatment at higher doses is associated with both vitamin E and vitamin C depletion and elevated lipid peroxidation products [50]. The dose used in the present study, however, allowed us to assess the isolated effect of GSH depletion without simultaneous generation of lipid peroxidation products [36]. Inhibiting GSH synthesis resulted in decreased GSH content, but also in decreased GSSG (Table 1), whereas oxidative stress is associated with increased formation of oxidized moieties, such as GSSG (manifested by a decreased GSH/GSSG ratio) and protein-SG. Oxidative- and lipid peroxidation-induced modification of proteins may alter their function, as well as their degradation through the proteasome protein-degradation system [51,52]. It is therefore suggested that isolated inhibition of GSH synthesis does not mimic the complex reactions associated with oxidative stress, in which oxidative modification of proteins and additional cell components occurs, each acting alone or in concert as the biologically significant mediator in the induction of insulin resistance.

Although GSH depletion did not cause measurable insulin resistance at the skeletal muscle and adipocyte level, progressive impairment of glucose tolerance did occur with BSO treatment (Figures 1B and 1C). Since no evidence for beta-cell dysfunction could be demonstrated, the reason for glucose intolerance induced by BSO treatment remains elusive. It could either reflect alterations in glucose utilization by tissues other than skeletal muscle and adipocytes addressed in this study (e.g. liver), or by tissues in which glucose utilization is not insulin dependent. In that sense, GSH depletion in itself may contribute to the overall impairment in glucose homoeostasis in diabetes.

In conclusion, this study demonstrates that decreasing cellular or tissue levels of GSH by inhibiting its synthesis, cannot reproduce the impaired insulin responsiveness of skeletal muscle and adipocytes that occurs in response to oxidative stress or to chronic hyperglycaemia. These data suggest that alternative or additional oxidation-borne factors function as biologically relevant mediators of oxidative stress-induced insulin resistance.

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