S100B impairs glycolysis via enhanced poly(ADP-ribosyl)ation of glyceraldehyde 3-phosphate dehydrogenase in rodent muscle cells

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Abstract

S100 calcium-binding protein B (S100B), a multifunctional macromolecule mainly expressed in nerve tissues and adipocytes, has been suggested to contribute to the pathogenesis of obesity. To clarify the role of S100B in insulin action and glucose metabolism in peripheral tissues, we investigated the effect of S100B on glycolysis in myoblast and myotube cells. Rat myoblast L6 cells were treated with recombinant mouse S100B to examine glucose consumption, lactate production, glycogen accumulation, glycolytic metabolites and enzyme activity, insulin signaling, and poly(ADP-ribosyl)ation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Glycolytic metabolites were investigated by enzyme assays or metabolome analysis, and insulin signaling was assessed by western blot analysis. Enzyme activity and poly(ADP-ribosyl)ation of GAPDH was evaluated by an enzyme assay and immunoprecipitation followed by dot blot with an anti-poly(ADP-ribose) antibody, respectively. S100B significantly decreased glucose consumption, glucose analog uptake, and lactate production in L6 cells, in either the presence or absence of insulin. In contrast, S100B had no effect on glycogen accumulation and insulin signaling. Metabolome analysis revealed that S100B increased the concentration of glycolytic intermediates upstream of GAPDH. S100B impaired GAPDH activity and increased poly(ADP-ribosyl)ated GAPDH proteins. The effects of S100B on glucose metabolism were mostly canceled by a poly(ADP-ribose) polymerase (PARP) inhibitor. Similar results were obtained in C2C12 myotube cells. We conclude that S100B as a humoral factor may impair glycolysis in muscle cells independently of insulin action,

and the effect may be attributed to the inhibition of GAPDH activity from enhanced poly(ADP-ribosyl)ation of the enzyme.

Keywords: S100B, glucose, myoblast, poly(ADP-ribosyl)ation, glyceraldehyde-3-phosphate dehydrogenase

Introduction

Impaired glucose metabolism in the liver, muscles, and adipocytes causes insulin resistance and underlies the pathogenesis of type-2 diabetes. Multiple inflammatory cytokines have been reported to contribute to insulin resistance, and in obesity, chronic low-grade inflammation in the visceral fat may enhance the secretion of inflammatory cytokines such as monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6), and TNF-α (8, 18, 28, 40, 42). We recently identified S100 calcium-binding protein B (S100B) as a novel inflammatory adipokine that is likely involved in the interaction between adipocytes and macrophages (14).

S100B, a 21 kDa calcium-binding protein of the EF-hand type, has been investigated mainly in the central nervous system, and in cancer (2, 45). Adipocytes along with nerve tissues seem to be important sources of S100B (17). It should also be noted that serum S100B levels in obese mice and patients were reported to be higher than those in healthy controls (3, 36), and were closely correlated with the body mass index as well as with the serum levels of leptin and adipocyte fatty acid-binding protein (A-FABP) (35). These findings suggest that S100B may play some role in the pathogenesis of obesity and insulin resistance.

Intracellular S100B regulates protein phosphorylation, maintains calcium homeostasis, and acts as a stimulator of cell proliferation (2, 10). In contrast, extracellular S100B shows a variety of effects depending on its concentration and the cell types (10, 11, 29). Extracellular S100B is an intrinsic ligand for the receptor of advanced glycation end

products (RAGE) (38) and some effects of S100B, including the enhancement of insulin secretion from pancreatic β-cells, are believed to be mediated by this receptor (24, 27). Intriguingly, advanced glycation end products (AGEs) have been reported to induce insulin resistance via RAGE in skeletal muscles (4, 5), suggesting that extracellular S100B possibly affects insulin action through RAGE. Nevertheless, there has been no investigation, to the best of our knowledge, on the effect of S100B on glucose metabolism or insulin action in peripheral tissues.

The aim of this study is to clarify whether extracellular S100B has any effect on glucose metabolism or insulin action in skeletal muscle cells, whose disturbed glycolysis is associated with insulin resistance and glucose intolerance. Here we report the suppressive effects of S100B on glycolysis in myoblast and myotube cells via impaired glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity, due to enhanced poly(ADP-ribosyl)ation of the enzyme.

Materials and Methods

Cell culture

The rat myoblasts (L6) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were seeded in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS;Thermo Scientific Waltham, MA), penicillin, and streptomycin (Sigma-Aldrich) at 37°C in a humidified 5% CO2/ 95% air atmosphere. Cells were cultured for 1-2 days to reach 80-90% confluency and then used for experiments as myoblasts. Alternatively, confluent L6 myoblasts were cultured with DMEM containing 2% FBS for 5 additional days to induce differentiation into myotubes. The mouse myoblasts (C2C12) were also obtained from ATCC. The cells were cultured for 1-2 days in DMEM containing 10% FBS, penicillin, and streptomycin at 37°C in a humidified 5% CO2/ 95% air atmosphere and then differentiated into myotubes in DMEM without FBS as described previously (23). To examine the effect of S100B on glycolysis, cells were treated with recombinant mouse S100B (ATGen, Seongnam, South Korea) in serum-free DMEM containing 0.1 mM bovine serum albumin (BSA) for indicated time.

Measurement of glucose and lactate concentrations

The concentration of glucose and lactate in media was measured using Glucose Colorimetric™ assay kit (Cayman Chemical, Ann Arbor, MI) via oxidase-peroxidase reaction and Glycolysis Cell-based™ assay kit (Cayman) via lactate-dehydrogenase reaction, respectively, as per the manufacturer's instructions.

Measurement of glycogen content of the cells

Cells were collected with HPLC-grade water, washed 3-times with pre-cooled PBS, and lysed by two freeze-thaw cycles. The resultant cell lysates were centrifuged at 12,000xg for 5 min at 4°C, and the supernatant was collected and analyzed using Glycogen Assay™ kit (Cayman) by enzyme recycling reaction, as mentioned in the manufacturer's instructions.

Assays of glucose analog uptake

L6 cells were seeded in a black, 96-well tissue culture plate (BD Falcon, NJ, USA) and treated with 100 ng/mL recombinant-S100B protein for the indicated time. The cells were then exposed to PBS containing 400 μM 6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-6-deoxyglucose (6-NBDG, Setareh Biotech, OR, USA) or 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino-2-deoxyglucose (2-NBDG), (Wako, Osaka, Japan) for 30 min. After removing the media, the wells were washed three times with PBS and filled with 100 μl PBS. The fluorescence intensity in the cells was then analyzed in a multi-mode microplate reader (Bio-Tek, Winooski, VT, USA).

Enzyme activity assay for hexokinase and GAPDH.

The hexokinase (HK) activity was measured using Hexokinase Colorimetric Assay Kit (Abcam Inc., Cambridge, MA, USA) via glucose 6-phosphate dehydrogenase enzyme recycling reaction and GAPDH activity was measured using GAPDH Activity Kit™ (Bio Vision) as per the manufacturer's instructions.

Western blot analysis

Proteins were extracted from cells with RIPA buffer containing a cocktail of protease inhibitors and phosphatase inhibitors (Roche, Indianapolis, IN). Fifteen micrograms of proteins were loaded and fractionated on a SDS-PAGE gel, and transferred onto the PVDF membrane using iBlot system (Invitrogen, Carlsbad, CA, USA). The membrane was blocked with 5% milk powder in TBS-Tween 20, washed and then incubated with primary antibodies against phospho-Akt (phospho-protein kinase B; Ser 474, 1:1000; Cell Signaling), pan-Akt (pan-protein kinase B; 1:1000; Cell Signaling), phospho-GSK3β (phospho-glycogen synthase kinase 3 beta; Ser9; 1:1000; Cell Signaling), GSK3β (glycogen synthase kinase 3 beta; D5C5Z; 1:1000; Cell Signaling), GAPDH (1: 4000; GeneTex, San Antonio, TX, USA), HK (1:4000; GeneTex), or RAGE (1:1000; Cell Signaling). After washing, the membrane was incubated with HRP-conjugated secondary antibodies, and washed again. The protein bands were detected with an ECL Plus™ detection kit (GE Healthcare, Tokyo, Japan) on ChemiDoc XRS (BioRad, Richmond, CA, USA).

Immunoprecipitation

GAPDH was separated from cell lysates by immunoprecipitation using magnetic beads. In brief, GAPDH antibody (Gene Tex) was buffer-exchanged by ultrafiltration to remove sodium azide and mixed with Protein G Mag Sepharose™ (GE Healthcare) pre-equilibrated with 10 mM Tris-buffered saline (TBS). The mixture was incubated on a rotator at room temperature for 30 min and washed. Cell supernatant pre-equilibrated with TBS was incubated with the prepared beads on a rotator for 60 min, and washed with TBS.

The beads were mixed with the loading buffer and heated at 95 °C for 5 min to liberate proteins. The resultant eluate was applied to dot blot analysis.

Dot blot analysis

One microliter of the immunoprecipitated sample was spotted onto the nitrocellulose membrane and dried. The membrane was blocked with 5% milk powder in TBS-Tween 20, washed, and then incubated with anti-poly(ADP-ribose) (10H) mouse IgG monoclonal Ab (1:100; Immuno-Biological Laboratories, Fujioka, Japan). After washing, the membrane was incubated with HRP-conjugated secondary antibodies, and washed again. The protein bands were detected with an ECL Plus™ detection kit (GE Healthcare) on ChemiDoc XRS (BioRad).

Quantitative real-time PCR

Total RNA was extracted from L6 cells using the High Pure™ RNA isolation kit (Roche) and reverse transcribed into cDNA using the ReverTraAce™ RT Kit (Toyobo, Osaka, Japan). The mRNA expression of HK and GAPDH was quantified by quantitative real-time PCR using specific primers and the qPCR MasterMix Plus for MESA Green Low ROX (Eurogentec, Seraing, Belgium) on MX 3000P qPCR system (Stratagene, La Jolla, CA). Levels of mRNA were normalized to those of GAPDH.

Inhibition of RAGE, Toll-like receptor (TLR) 4, and fibroblast growth factor receptors (FGFR)

L6 cells were pretreated with a RAGE-specific antagonist, FPS-ZM1 (EMD Millipore) for 0.5-48 h, with a TLR-4 inhibitor, TAK-242 (Merck Millipore, Guyancourt, France) for 0.5 h, or two kinds of FGFR inhibitors, PD173074 (Selleck, Houston, TX) and BGJ398

(Selleck, Houston, TX) for 2 h before the addition of S100B. After stimulation for 6 h with 100 ng/mL S100B, glucose concentrations in the media were measured using Glucose Colorimetric™ assay kit.

Inhibition of poly(ADP-ribose) polymerase (PARP)

L6 cells were pretreated with a PARP-inhibitor, 3-aminobenzamide (3ABA), for 1.5 h before addition of recombinant-S100B protein. After stimulation with 100 ng/mL S100B for 6 h, glucose concentration in the media was determined as describe above.

Metabolome analysis

Metabolome analysis was performed at Human Metabolome Technologies (HMT,Tsuruoka, Japan, http://humanmetabolome.com). In brief, L6 cells were incubated with or without 100 ng/mL S100B for 6 h and were washed with 5% mannitol solution. Cellular metabolites were then extracted using methanol containing HMT internal standard solution 1 at room temperature, as indicated by the manufacturer's instruction. Metabolome analysis was performed by CE-TOF MS/QqQ MS and the metabolite peaks were quantified with normalization by the cell number.

3-(4,5-Dimethylthiazol-2-yl)-5(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay

L6 cells were plated into a 96-well plate and cultured with 100 ng/mL recombinant S100B for 24 h. Twenty microliters of CellTiter 96 Aqueous One Solution Reagent™ (Promega, Madison, WI) was then added to each well and the plate was incubated for 1h, followed by the reading of absorbance at 490 nm.

Animals

S100B knockout mice (26) were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan and C57BL/6J mice were obtained from CLEA Japan (Osaka, Japan). All procedures were conducted according to the Nagoya University Medical School Guidelines for the Care and Use of Laboratory Animals. In brief, 12 month-old-mice were euthanized under anesthesia and quadriceps femoris muscles were resected. After homogenization and centrifugation, 100 μg of proteins in supernatant were applied to immunoprecipitation procedure.

Statistical analysis

All statistical analyses were performed using JMP 11.0 (Marlow, Buckinghamshire, UK). For comparison among groups, one-way analysis of variance (ANOVA) was applied. P-values <0.05 were considered significant.

Results

Effects of recombinant S100B protein on insulin activation of glycolysis in L6 cells

We first investigated whether extracellular S100B affect insulin action on glucose metabolism in skeletal muscle cells. The glucose level in culture media was significantly lowered by insulin when compared with that in non-treated cells at after 3 and 6 h of incubation, and the insulin effect was suppressed by 100 ng/mL S100B at 6 h (Fig. 1A). Changes in glucose levels from the baseline at 6 h showed that S100B affected glucose utilization not only under insulin stimulation but also under basal conditions (Fig. 1B). The suppressive effect of S100B on insulin-stimulated glucose consumption was dose-dependent (Fig. 1C).

Next, we measured the concentration of lactate, the end product of glycolysis and a biomarker for cellular glycolytic activity. Lactate concentration in the media after 6 h was significantly elevated by insulin, and the enhanced lactate production was attenuated by S100B. S100B also suppressed lactate production in the absence of insulin (Fig. 1D).

The intracellular glycogen content in L6 cells was significantly higher in the insulin group than the control group. Unexpectedly, S100B showed no effect on glycogen content, despite of lowered glucose disappearance and lactate production (Fig.1E).

MTS assay showed no effect of recombinant-S100B protein on cell viability of L6 cells at concentrations ranging from 10 to 1000 ng/mL for 24 h (Fig. 1F), indicating that the S100B used in our experiments at 100 ng/mL for 6 h was unlikely to be toxic to L6 cells.

The effect of S100B on glycolysis was independent of insulin

Western blot analysis of L6 cell lysate showed that insulin clearly enhanced the phosphorylation of Akt (Se474) and GSK3β. S100B was not able to suppress the insulin-induced phosphorylation of either protein (Fig. 2A and 2B), indicating that the S100B action is likely independent of the insulin signaling. Furthermore S100B alone has no effect on phosphorylation levels of either protein.

Because S100B showed no effect on insulin signaling, we next examined the independent effect of S100B protein on glucose metabolism. The recombinant-S100B protein reduced glucose consumption by L6 myoblasts at the concentration of 10-1000 ng/ml, though dose-dependency was not clear (Fig. 3A). The uptake of 6-NBDG, a fluorescent glucose analog that is not further metabolized to enter the glycolytic pathway (21, 43), was significantly reduced by S100B (Fig. 3B). In contrast, the fluorescence of 2-NBDG, a similar glucose analog but metabolized to non-fluorescent 2-NBDG-6-phosphate by HK activity (39, 43), was not diminished by S100B (Fig. 3C).

To further reveal the mechanism by which S100B inhibited glycolysis, we carried out metabolome analyses of L6 cells in both the control and S100B-treated cells. S100B increased the concentration of the glycolytic intermediates upstream of GAPDH, such as glucose 6-phosphate (G6-P), fructose 6-phosphate, fructose 1,6-bisphosphate (F1,6-BP) and GAP. On the contrary, S100B showed no effect on the level of pyruvate that is located downstream of GAPDH (Fig. 4A). S100B also increased the concentration of some intermediates in glycogen metabolism, e.g., glucose-1-phosphate and UDP-glucose, and those in the pentose pathway such as 6-phosphogluconic acid and sedoheptulose 7-phosphate (Fig. 4B). Glycerol-3-phosphate, a downstream metabolite of

dihydroxyacetone phosphate, was also elevated by S100B treatment (Fig. 4B). In addition, fumaric acid and oxaloacetate-related amino acids were increased by S100B (Fig. 4C). The concentration of nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADP), nicotinamide adenine dinucleotide phosphate (NADPH), guanosine triphosphate (GTP), guanosine diphosphate (GDP), adenosine 5'-triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) showed no significant difference between the two groups, and the ratio of NAD/NADH, NADP/NADPH and ATP/ADP was not altered (Fig. 5).

Effects of S100B on the activity of HK and GAPDH

Expression levels of GAPDH mRNA and protein were similar between cells with and without S100B treatment (Fig. 6A and 6B), whereas GAPDH activity was significantly suppressed by S100B (Fig. 6C). Insulin stimulation resulted in an augmentation in HK protein expression, but S100B showed no effect on the expression of mRNA, protein, or activity of HK (Fig. 6D-F).

Effects of S100B on poly(ADP-ribosyl)ation of GAPDH

The activity of PARP, a nuclear enzyme that is involved in DNA repair and activated-DNA damage, is known to block glycolytic flux through the inactivation of GAPDH by its poly(ADP-ribosyl)ation (1, 9, 13,15). We therefore investigated the effect of S100B on poly(ADP-ribosyl)ation of GAPDH. Recombinant-S100B protein had no effect on the expression of PARP mRNA (Fig. 7A), whereas immunoprecipitation and immuno-dot blot analysis revealed that S100B enhanced poly(ADP-ribosyl)ation of GAPDH in L6 cells (Fig. 7B).

To ascertain that poly(ADP-ribosyl)ation is responsible for S100B inhibition of glycolysis, we pre-treated L6 cells with 3ABA, a PARP inhibitor, for 1.5 h followed by exposure to recombinant-S100B protein for 6 h. 3ABA canceled the decrease of glucose consumption by S100B in a dose-dependent fashion (Fig. 7C). The inhibitor also restored S100B-induced decrease in lactate production (Fig. 7D). It was assured that 3ABA was able to block S100B-induced poly(ADP-ribosyl)ation of GAPDH (Fig. 7E).

RAGE, TLR-4 and FGFR may not contribute to S100B effects on glycolysis

We also examined the effects of inhibitors of RAGE, TLR-4 and FGFR on glucose utilization in L6 cells, because S100B has been reported to interact with TLRs via binding to TLR ligands (32) and also to regulate myoblast proliferation and differentiation by activating FGFR1 (30, 32). None of the RAGE antagonist, FPS-ZM1, the TLR-4 antagonist, TAK242, and FGFR inhibitors, BGJ398 and PD173074 showed significant effects on the S100B suppression of glucose consumption (Fig. 8A, 8B and 8C). The phosphorylation of Erk, the GSH/GSSG ratio and RAGE mRNA and protein expression were not affected by S100B treatment (Fig. 8D-F).

Effects of S100B on glucose utilization and poly(ADP-ribosyl)ation of GAPDH in C2C12 myotubes and L6 myotubes, and in muscles from S100B knockout mice

To ascertain the observation in L6 myoblast cells, we examined the effects of S100B on glucose utilization and poly(ADP-ribosyl)ation of GAPDH in C2C12 myotubes and differentiated L6 myotubes. S100B protein reduced glucose consumption in C2C12 myotubes, and the effect was attenuated by a PARP inhibitor (Fig. 9A). S100B at the concentration of 100 ng/mL did not affect C2C12 cell proliferation at least for 6 h when

evaluated by MTS assay (Fig. 9B). S100B suppression of glucose utilization was observed also in L6 myotubes (Fig. 9C).

S100B showed similar, though statistically not significant, effects on poly(ADP-ribosyl)ation of GAPDH in C2C12 myotubes as seen in L6 myoblasts (Fig. 9D). We also analyzed poly(ADP-ribosyl)ation of GAPDH in quadriceps femoris muscles from S100B knockout mice and control C57B6 mice to clarify the role of S100B in vivo. Interestingly, poly(ADP-ribosyl)ated GAPDH was obviously reduced in muscles from S100B knockout mice as compared to wild control mice (Fig. 9E).

Discussion

In this study, we found that extracellular S100B disturbed glucose utilization in L6 myoblast cells, and also in C2C12 or L6 myotubes. We hypothesized that S100B might interact with the insulin pathway because insulin-stimulated glucose utilization was impaired by S100B. Unexpectedly, insulin-induced phosphorylation of Akt and GSK3β were not suppressed by S100B, suggesting that S100B inhibited glycolysis in L6 cells independent of insulin signaling. In fact, S100B showed suppressive effects on glucose utilization even in the absence of insulin.

It was curious that glycogen accumulation was unchanged by S100B treatment in spite of reduced glucose utilization and lowered lactate production. The results seemed to indicate that S100B suppressed glycolysis but not glycogenesis from G6-P. A metabolome analysis revealed that S100B increased G6-P, F1, 6-BP and other glycolytic intermediates upstream of GAPDH, whereas pyruvate was not increased. In addition, some metabolites in the pentose pathway were increased. These data may be considered with the observation that the fluorescence of 2-NBDG, a glucose analog metabolized to a non-fluorescent molecule by HK activity (41, 44), was not reduced by S100B in contrast to the decrease of another non-metabolized glucose analog 6-NBDG (21, 43), because HK activity is known to be suppressed by G6-P by allosteric regulation (25). Although our HK assay did not show decrease in activity, the procedure for separated enzymes does not detect allosteric regulation. Furthermore, it should also be noted that fumaric acid and total

oxaloacetate-related amino acids were increased, possibly resulting from the reduction in energy generation due to inhibited glycolysis by S100B.

From these findings, we speculated that S100B might disturb GAPDH-activity followed by reduced glucose utilization and increased levels of intermediates in the early steps of glycolysis and its by-pass pathways. In fact, our data showed that GAPDH activity was significantly decreased by S100B treatment in myoblast cells, without changes in its mRNA or protein expression levels.

GAPDH can be modified with polymers of ADP-ribose by the activity of PARP (1, 9, 13, 15). The modified GAPDH loses its activity irrespective of expression levels of mRNA and protein (13). We therefore further examined poly(ADP-ribosyl)ation of the enzyme. The immunoprecipitation results clearly demonstrated that S100B treatment enhanced the poly(ADP-ribosyl)ation of GAPDH. The overt reduction of poly(ADP-ribosyl)ated GAPDH in muscles from S100B knockout mice may support the in vitro finding, although the interpretation should be circumspect because the knockout mice lack not only extracellular S100B but also intracellular S100B. It is puzzling, however, why NAD content or the NAD/NADH ratio was not decreased despite the enhanced poly(ADP-ribosyl)ation.

Because the specific PARP inhibitor—3-aminobenzamide—restored S100B-induced reduction in glucose utilization and lactate production, the S100B-PARP interaction may be a central mechanism by which extracellular S100B impairs glucose metabolism, independent of insulin signaling, in muscle cells, as tentatively depicted in Fig. 10.

It remains to be seen how extracellular S100B enhances the poly(ADP-ribosyl)ation. RAGE has been reported to be the receptor via which S100B acts as an agonist to promote multiple signals such as NFκB and Erk (12). It should be noted that AGEs possibly reduce insulin sensitivity (4, 5). However, we failed to detect the antagonizing effects of a RAGE inhibitor on S100B suppression of glucose utilization. Some effects of S100B are known to be RAGE-independent (6, 19, 20), and our results indicate that RAGE is unlikely involved in the S100B effects in L6 cells, although the results may not be applicable to other types of muscle cells because of low expression of RAGE in L6 myoblasts and myotubes (data are not shown). S100B may also affect TLR signals (33) and TLR-4 has been reported to be upregulated by S100B in chondrocytes (34). Nevertheless, our data with a TLR-4 antagonist TAK242 indicate that this receptor also seems unlikely to contribute to the S100B effects.

S100B has been also reported to stimulate ERK1/2 phosphorylation in myoblasts via activation of the bFGF-FGFR1 complex (31, 32). In present study, ERK1/2 phosphorylation was not changed by S100B and FGFR inhibitors did not affect the S100B suppression of glucose utilization, indicating that ERK 1/2 and FGFR1 may not play a substantial role in the observed S100B effect on glycolysis. The discrepancy between previous and present results is possibly attributable to different experimental conditions. Previous study used medium containing FBS, whereas we incubated cells without FBS in order to avoid interference of hormones. Our data, due to the lack of bFGF in medium, do not exclude the role of bFGF-FGFR1 in vivo but suggest that there is an FGF-independent mechanism(s) by which S100B affects glucose metabolism.

Oxidative stress is a well-known enhancer of PARP activity (16, 39) and H2O2 has been reported to block glycolysis via enhanced ADP-ribosylation (7). However, the reduced (GSH)/ oxidized glutathione (GSSG) ratio, a surrogate marker of oxidative stress, was unchanged by S100B treatment in the present metabolome analysis. We also failed to observe S100B-induced impairment of insulin signaling that is usually suppressed by oxidative stress. Other markers such as 4-hydroxynonenal and oxygen radical antioxidative capacity were also not altered by S100B (data are not shown). From these results, oxidative stress does not appear to account for the effects of S100B on glycolysis.

Considering that known receptors for S100B were unlikely involved in S100B effects on glucose metabolism in present experiments, the possible influence of extracellular S100B on its intracellular concentration may warrant discussion. Interestingly, the latest study in astrocytes has reported that extracellular S100B is captured by endocytotic vesicles and transferred into cells within 3 h (22). Although we have not verified the phenomenon in muscle cells, the cellular uptake of S100B could be a candidate mechanism that underlies the observed S100B effects. This hypothesis seems relevant to decreased poly(ADP-ribosyl)ation in muscles from S100B knockout mice that lack both intracellular and extracellular S100B.

The limitations of this study are the sole use of cell lines and the lack of in vivo data on glucose metabolism. The detailed mechanism by which extracellular S100B activates poly(ADP ribosyl)ation has yet to be clarified. The inference from these data is hence limited.

In conclusions, our results demonstrate that S100B as a humoral factor may inhibit glycolysis in muscle cells by suppressing GAPDH activity via enhanced poly(ADP ribosyl)ation, independent of insulin action. Given that serum S100B levels are increased in obese subjects, our data may suggest the possible involvement of this protein in impaired glucose tolerance in obesity, and thus warrants further investigation.

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Disclosures

The authors declare no conflict of interest.

Author contributions

KH conceived and carried out the experiments. ST, YS and HA contributed to data interpretation. MM, RM, YN, and TI contributed to some of the experiments and data analyses. YH contributed to the study design, data interpretation, and manuscript writing. All authors gave final approval to the submitted and published versions.

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Figure Captions

Figure 1. Effects of recombinant-S100B protein on insulin-activated glycolysis in L6 myoblast cells. (A) Time course of glucose concentration in the media. (B) The effects of insulin and S100B on glucose consumption 6 h after insulin stimulation. (C) The dose dependency of S100B effect on glucose consumption after 6 h of incubation with 100 nM insulin.(D) The effects of insulin (100 nM) and S100B (100 ng/mL) on lactate concentration in the media after incubation for 6 h. (E) Glycogen accumulation in L6 cells after 6 h of incubation with or without insulin (100 nM) and S100B (100 ng/ml). (F) MTS assay after 24 h-culture with S100B. Data are mean±S.D. values. (n=8). *P<0.05, **P<0.01, and ***P<0.001.

Figure 2. The effects of recombinant S100B protein on insulin signaling. (A) Western blot and (B) densitometric analysis of Akt and GSK3β in the lysates of L6 myoblast cells treated or untreated with insulin and/or S100B for 6 h. Data are mean±S.D. values (n=3). *P<0.05

Figure 3. Insulin-independent effects of recombinant-S100B protein on glucose utilization and uptake in L6 myoblast cells. (A) The dose dependency of S100B on glucose consumption for 6 h. (B) Uptake of 6-NBDG and (C) 2-NBDG by cells pre-treated or untreated with S100B for 2 h prior to 6-NBDG and 2-NBDG addition. Data are mean±S.D. values (n=8). *P<0.05, **P<0.01, ***P<0.001.

Figure 4. Effects of recombinant-S100B protein on concentrations of metabolites

involved in the glycolytic pathway (A), the pentose and polyol pathways (B) and the tricarboxylic acid cycle (C). Data are mean±S.D. values (n=3-5). *P<0.05

Figure 5. Effects of S100B on concentrations and ratio of coenzymes. Data are mean±S.D. values (n=3-5).

Figure 6. Messenger RNA expression, protein expression and enzyme activity of GAPDH (A-C) and of hexokinase (D-F) in L6 myoblast cells treated or untreated with recombinant S100B for 6 h. Data are mean±S.D. values (n=4-6). * P<0.05

Figure 7. Effects of S100B and a PARP inhibitor (3ABA) on poly(ADP-ribosyl)ation and glucose utilization in L6 myoblast cells. (A) Gene expression of PARP1 and PARP2 in cells treated or untreated with 100 ng/mL recombinant-S100B for 6 h. (B) Dot blot analysis using anti-poly(ADP-ribose) antibody following immunoprecipitation by anti-GAPDH antibody in cells after treatment with or without S100B for 6 h. (C-E) Effects of 3ABA, a PARP inhibitor, on S100B-induced changes in glucose utilization, lactate production and poly(ADP-ribosyl)ation. Data are mean±S.D. values (n=3-6). *P<0.05

Figure 8. Effects of antagonists for RAGE (FPS-ZM), TLR-4 (TAK-242) and FGFR1 (PD and BGJ) on the S100B-induced alterations in glucose metabolism in L6 cells treated for 6 h. (A) Effects of FPS-ZM, (B) TAK-242 and (C) PD and BGJ on the S100B inhibition of glucose consumption. (D-F) No effects of S100B on Erk phosphorylation, GSSG/GSH ratio and RAGE mRNA and protein expression. Data are mean±S.D. values (n=7-8). *P<0.05

Figure 9. Effects of S100B on glucose utilization or poly(ADP-ribosyl)ation of GAPDH in C2C12 myotube cells, L6 myotubes and in muscles from S100B knockout mice. (A) Effects of S100B and 3ABA on glucose utilization in C2C12 myotubes. (B) MTS assay in

C2C12 myotubes. (C) Effects of S100B in glucose utilization in L6 myotubes. (D) Poly(ADP-ribosyl)ation of GAPDH in C2C12 myotubes and (E) in quadriceps femoris muscles from mice. Data are mean±S.D. values (n=3). *P<0.05 and **p<0.01.

Figure 10. A tentative scheme for the mechanisms by which S100B affects glucose metabolism in muscle cells.