Key words: streptozotocin, glucose uptake, diabetes mellitus, glyceraldehyde-3-phosphate dehydrogenase

SUMMARY

The study investigated the effect of nitric oxide (NO) released from S-nitrosoglutathione (GSNO) and S-nitroso-N-acetylpenticillinamine (SNAP) on insulin-stimulated glucose uptake in adipocytes, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity in skeletal muscle strips of streptozotocin-induced type 2 diabetic and normoglycemic rats. GSNO at concentrations of 10 mM and 20 mM significantly decreased insulin-stimulated 2-deoxyglucose uptake by 60.9±5.6% and 62.5±4.6%, respectively (p<0.05), in adipocytes of diabetic rats. Similarly, SNAP at concentrations of 10 mM and 20 mM significantly decreased insulin-stimulated 2-deoxyglucose uptake by 65.6±6.1% and 67.2±7.2%, respectively (p<0.05). The GAPDH activity in skeletal muscle of diabetic rats (0.064±0.006 μmol/mg/min) was significantly lower than that in normoglycemic rats (0.110±0.010 μmol/mg/min; p<0.05). These results suggest that exogenous NO inhibits insulin stimulated glucose uptake in adipocytes of streptozotocin-induced type 2 diabetic rats, and this could lead to further pathologic sequelae in the diabetic state.

INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia, altered metabolism of lipids, carbohydrates and proteins, with an increased risk of vascular disease complications (1). Type 2 diabetes mellitus is characterized by derangement of insulin secretion and inability of peripheral tissues to respond to insulin (2). The study of the pathophysiology and treatment of diabetes mellitus requires well-characterized animal models that resemble the disease aspects in humans. Streptozotocin-induced type 2 diabetes rats have been reported to exhibit comparable features with human type 2 diabetes mellitus with regard to insulin responsiveness to glucose and sulfonylureas (3). Glucose intolerance in type 2 diabetes mellitus is manifested by defects in glucose transport into muscles (4) and adipose tissues (5). Decreased glucose uptake may be the consequence of impaired glucose transport activity and/or defective intracellular metabolism of the transported glucose (6). Once the glucose is transported into the cell, it is phosphorylated to glucose-6-phosphate and enters one of the two major pathways, glycogen synthesis or glycolysis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a very important enzyme, particularly in the glycolytic and gluconeogenic pathways (7). During glucose metabolism, the enzyme is responsible for reversibly catalyzing the oxidation and phosphorylation of...
glyceraldehyde-3-phosphate to produce glyceraldehyde-1,3-bisphosphoglycerate with the release of NADH (8). Glyceraldehyde-3-phosphate dehydrogenase has a highly reactive active-site thiol and hence is a potential in vivo biological target for reactive oxygen species (ROS). The enzyme, which is inactivated by superoxide (9) and nitric oxide (NO), can be covalently modified by NAD⁺ in the presence of NO (10).

In vivo, insulin-stimulated glucose uptake in rat white adipose tissue is dependent on intact NO synthesis as nitric oxide synthesis (NOS) blockade reduces insulin-mediated glucose uptake in both brown and white adipose tissues (11). In vitro, exogenous NO inhibits proliferation and stimulates the expression of two adipogenic marker genes, peroxisome proliferator-activated receptor γ and uncoupling protein 1, in rat brown preadipocytes (12). A previous study by Emani and Perry (13) found that sodium nitroprusside (SNP) had no stimulatory effect on glucose uptake in adipocytes. We designed experiments utilizing the NO donors, S-nitrosoglutathione (GSNO) and S-nitroso-N-acetylpenicillamine (SNAP) to test the hypothesis that exogenous NO is a potential modulator of insulin-stimulated glucose transport in adipocytes of normoglycemic and streptozotocin-induced type 2 diabetes rats. The study also examined basal and insulin stimulated glucose uptake, and the activity of GAPDH in skeletal tissue of type 2 diabetic rats.

MATERIALS AND METHODS

Animals – experimental design

Sixteen Sprague-Dawley rats (8 male and 8 female) were obtained from the Preclinical Animal House of the Department of Basic Medical Sciences, University of the West Indies. The animals were housed in an animal room and fed standard laboratory diet and water ad libitum. All procedures were approved by and conducted in accordance with the guidelines of the University of the West Indies Animal Care and Use Committee. All experiments were conducted when the rats were 10-12 weeks of age after an overnight period of food restriction of 12 h.

The rats were divided into two groups, experimental (4 males and 4 females) and control (4 males and 4 females). Type 2 diabetes was induced in the experimental group of Sprague-Dawley rats as previously described (3). Briefly, nicotinamide (Sigma Chemical Co., St. Louis, MO, USA; 180 mg/kg body weight) dissolved in 0.2 mL of saline was administered intraperitoneally 15 min before an intravenous administration of streptozotocin (Sigma; 65 mg/kg body weight), also dissolved in 0.2 mL of saline. The normoglycemic (control) group received 0.2 mL of saline. Blood from the tail vein was tested for glucose concentration once per week for 4 weeks using a glucometer (Miles Inc. Diagnostics Division, IN, USA). At the end of 4 weeks, the rats were weighed and an oral glucose tolerance test administered using 1.75 mg/kg body weight of glucose. Diabetic [determined based on the World Health Organization (WHO)] values (14) and control rats were euthanized using diethyl ether in accordance with the UWI Ethics Committee standards.

Adipose cell incubation

Glucose transport in isolated adipocytes was measured by the use of radiolabeled 2-deoxy-D-glucose (2-deoxy-D-[1,2-3H(N)]glucose; 2-DG), as described previously (15). Isolated adipocytes were prepared from subcutaneous fat pads by collagenase digestion (1 mg/mL). Cells were incubated at 37 °C for 30 min with constant shaking (100 rpm) in a 2% suspension by volume in oxygenated Krebs-Ringer bicarbonate (KRB) buffer supplemented with 30 mM HEPES, 2.5% bovine serum albumin (BSA) [radioimmunoassay (RIA) grade], and 200 nM adenosine. After the incubation period, the adipocytes were separated from connective tissue by filtration through a nylon mesh.

2-Deoxyglucose uptake in adipocytes

GSNO was prepared as previously described (16). Briefly, reduced glutathione was reacted with sodium nitrite under acidic conditions at 0 °C, followed by the addition of cold acetone. The resulting precipitate was filtered off, washed, and dried under laboratory conditions to give the light-pink solid GSNO, with similar ultraviolet and visible absorption maxima and elemental composition as reported (16). SNAP was synthesized as previously described (17). The isolated adipocytes (300 μL/1.5 mL Eppendorf tube) were incubated with or without SNAP or GSNO (10 mM & 20 mM) for 15 min at 30 °C in oxygenated KRB buffer. After the initial period, adipocytes were incubated for 30 min in oxygenated KRB buffer in the presence or
absence of purified insulin (100 μM). Thereafter, cells were incubated for 30 min at 30 °C in KRB buffer containing 8 mM 2-DG (2.25 μCi/mL), 32 mM [14C]mannitol (0.3 μCi/mL) and 1.0% BSA. The reaction was terminated by spinning the suspension through dinonyl phthalate oil. The radioactivity was determined by liquid scintillation counting in a Beckman LS6000 scintillation counter programmed for dual-channel counting.

Muscle incubation

Glucose uptake in isolated skeletal muscle strips was measured by the use of radiolabeled 2-DG (Sigma Chemical Co., MO, USA), as described previously (18). Skeletal muscle strips were dissected out and rapidly cut into 20 to 30 mg strips. Muscle strips were then incubated initially in 25 ml Erlenmeyer flasks for 60 min. The flasks contained 3 mL of oxygenated KRB buffer supplemented with 8 mM glucose, 32 mM mannitol, and 0.1% BSA, RIA grade. Flasks were gassed continuously with 95% O2 : 5% CO2 throughout the experiment.

2-Deoxyglucose uptake in skeletal muscle strips

The skeletal muscle strips (300 μL/1.5 mL Eppendorf tube) were incubated with or without various concentrations of purified insulin (100 nM & 1000 nM) for 15 min at 30 °C in oxygenated KRB buffer and then washed for 10 minutes in KRB buffer containing 40 mM mannitol and 0.1% BSA. Subsequently, skeletal muscle strips were incubated for 30 minutes at 30 °C in KRB buffer containing 8 mM 2-DG (2.25 μCi/mL), 32 mM [14C]mannitol (0.3 μCi/mL) and 0.1% BSA. The reaction was stopped by centrifuging for 30 s and after rapidly aspirating the supernatant, the skeletal muscle strips were washed 3 times in ice-cold HEPES-buffered saline (140 mM NaCl, 5 mM KCl, 2.5 mM MgCl2, 1 mM CaCl2, 20 mM HEPES, pH 7.4), and solubilized in 1 mL of 1% Triton X-100 for 30 min at 37 °C. The Eppendorf tube with its contents was placed in a vial, and 10 mL of atomlight scintillation solution (Dupont Biotechnology Systems, Massachusetts, USA) were added. The associated radioactivity was determined by liquid scintillation counting in a Beckman LS6000 scintillation counter programmed for dual-channel counting. The specific uptake of 2-deoxyglucose by the skeletal muscle strips was calculated by subtracting the 3H activity in the extracellular space from the total 3H activity of each muscle strip. The protein concentration of the muscle cytosol fraction was determined by the Bradford method (19) using BSA as standard.

Measurement of GAPDH activity in skeletal muscle

Glyceraldehyde 3-phosphate dehydrogenase was purified from liver and skeletal muscle of streptozotocin-induced diabetic and normoglycemic (control) rats (20). Approximately 5 g of frozen tissue was minced and homogenized in 1 mL of buffer (30 mM M KOH, 1 mM EDTA, 3 mM mercaptoethanol, pH 7.4) per gram of tissue, in a chilled mortar. The homogenate was stirred for 15 min and centrifuged at 9000 g for 10 min. The supernatant was collected and the pH adjusted to 7.3-7.5 with 1 M ammonium hydroxide or 1 M acetic acid. An equal volume of saturated ammonium sulfate was added to the ice-cold supernatant, stirred for 15 min, and centrifuged for 15 min at 9000 g. For each 10 mL of supernatant obtained, 1.3 g of solid ammonium sulfate was added gradually. The extract was then centrifuged and the pH of the supernatant adjusted as before. The enzyme was stored at 0ºC until analysis.

In a 2-mL disposable cuvette, the following were added: 0.5 mL of 2X GAPDH assay buffer (100 mM Na2HPO4, 5 mM EDTA, pH 8.6) containing 1.6 mM glyceraldehyde 3-phosphate (Sigma Chemicals) and 10 μL of a 1:10 dilution of pure GAPDH or 100 μL of the enzyme preparation (20). The volume was made up to 0.98 mL with sterile distilled water and baseline levels of activity measured at 340 nm for 3 min. The reaction was started by adding 20 μL of 100 mM NAD+. The absorbance was measured at 340 nm every 5 s for 1 min and the rate of NADH formation calculated.

Statistical analysis

All results shown in figures are expressed as mean ± SEM. Data analysis was done using the Sigma Plot and Sigma Statistics software packages (Jandel Scientific). To evaluate the effects of SNAP and GSNO on insulin-stimulated glucose uptake in isolated adipocytes, or of insulin on glucose uptake in skeletal muscle of
normoglycemic and type 2 diabetic rats, values of each group were compared by either paired Student’s t-test or two-way analysis of variance (ANOVA) followed by Benferroni multiple comparison test. Statistical analysis was also done on the GAPDH activity in both groups. Values of p less than 0.05 were considered significant in all cases.

RESULTS

Table 1 gives a summary of body weight and plasma insulin concentration in study animals. There were no differences in body weight between streptozotocin-induced type 2 diabetic rats and normoglycemic (control) rats. In the streptozotocin-induced type 2 diabetic rats, fasting plasma insulin concentration was not significantly different from controls, however, postprandial plasma insulin concentration was approximately half that in control animals (p<0.05).

Table 1. Summary of the characteristics of the rats used in the study. Values are means ± SEM of 16 rats (8 control and 8 diabetic rats). Statistically significant differences between values indicated by *p<0.05 (vs control).

<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>DIABETIC</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td></td>
<td></td>
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<tr>
<td>Before treatment with</td>
<td>170.82 ± 15.83</td>
<td>238.79 ± 10.03</td>
</tr>
<tr>
<td>nicotinamide and streptozotocin in saline, or only saline</td>
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<td></td>
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<tr>
<td>After treatment with</td>
<td>251.51 ± 21.00</td>
<td>234.74 ± 7.34</td>
</tr>
<tr>
<td>nicotinamide and streptozotocin in saline, or only saline</td>
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<tr>
<td>Plasma insulin concentration (μIU/mL)</td>
<td></td>
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<tr>
<td>Postprandial</td>
<td>249.00 ± 7.56</td>
<td>119.83 ± 0.62*</td>
</tr>
<tr>
<td>Fasting</td>
<td>120.75 ± 7.07</td>
<td>112.25 ± 4.50</td>
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An abnormal glucose tolerance curve was obtained for the STZ-treated rats compared to controls (Fig. 1). Upon oral glucose load, the blood glucose concentration increased to a maximum of 19.00±0.90 mmol/L in diabetic rats compared to 6.74±0.50 mmol/L in controls at 1 h. After 2 h, the blood glucose approached fasting levels in controls but remained persistently high in diabetic rats (p<0.05).

GSNO and SNAP at 10 mM and 20 mM induced a concentration-dependent decrease in insulin-stimulated glucose uptake in adipocytes of normoglycemic rats (Fig. 2). GSNO at concentrations of 10 mM and 20 mM reduced insulin-stimulated 2-deoxyglucose uptake in adipocytes of normoglycemic
rats by 48.0±4.5% and 55.9±4.8%, respectively (p<0.05) compared to untreated adipocytes. Inhibition of insulin-stimulated glucose uptake by SNAP at both concentrations was more pronounced compared to GSNO. SNAP at 10 mM and 20 mM reduced insulin-stimulated 2-deoxyglucose uptake in adipocytes of normoglycemic rats by 60.4±6.7% and 61.2±5.6%, respectively (p<0.05).

GSNO and SNAP (10 mM and 20 mM) exhibited similar reduction in insulin-stimulated 2-deoxyglucose uptake in adipocytes of type 2 diabetic rats (Fig. 3). GSNO at 10 mM and 20 mM reduced insulin-stimulated 2-deoxyglucose uptake in adipocytes of type 2 diabetic rats by 60.9±5.6% and 62.5±4.6%, respectively, compared to untreated adipocytes (p<0.05). SNAP also had a significant effect on the reduction of insulin-stimulated 2-deoxyglucose uptake with values of 65.6±6.1% (10 mM) and 67.2±7.2% (20 mM) (p<0.05).

Figure 3. Graphic presentation of the concentration-dependent inhibitory effect of millimolar concentrations of GSNO and SNAP on insulin-stimulated glucose transport in adipocytes of diabetic rats. Statistically significant differences are indicated by *p<0.05 vs glucose uptake with insulin.

The basal glucose uptake in skeletal muscle strips of streptozotocin-induced type 2 diabetic rats (0.58±0.10 pmol/mL/min) was significantly lower than in normoglycemic rats (2.93±0.88 pmol/mL/min) (Fig. 4). A significant difference was also observed for insulin-stimulated glucose uptake (100 nM of insulin) with values of 1.88±0.03 pmol/mL/min in type 2 diabetic rats and 4.12±1.40 pmol/mL/min in normoglycemic rats. However, no significant difference was observed with increased concentration of insulin (1000 nM). The specific activity of GAPDH in the skeletal muscle of diabetic rats was significantly lower than that in the normoglycemic rats (Fig. 5). GAPDH activity in the skeletal muscle of diabetic rats was 0.064±0.006 μmol/mg/min vs. 0.110±0.010 μmol/mg/min in normoglycemic rats (p<0.05).

Figure 4. Graphic presentation of insulin-stimulated glucose uptake in skeletal muscle of streptozotocin-induced diabetic and normoglycemic rats.

Figure 5. GAPDH activity in skeletal muscle of streptozotocin-induced diabetic and normoglycemic rats.

**Figure 4. Graphic presentation of insulin-stimulated glucose uptake in skeletal muscle of streptozotocin-induced diabetic and normoglycemic rats**

**Figure 5. GAPDH activity in skeletal muscle of streptozotocin-induced diabetic and normoglycemic rats**
DISCUSSION

The major finding of this study is the observation that nitric oxide released from GSNO and SNAP inhibits insulin-stimulated glucose uptake in adipocytes of normoglycemic and streptozotocin-induced diabetic rats. However, the degree of inhibition of insulin-stimulated glucose uptake was greater in adipocytes of type 2 diabetic rats compared with those from normoglycemic rats. The present work also demonstrated a decreased basal and insulin-stimulated glucose uptake, and a reduced GAPDH activity in skeletal muscle of streptozotocin-induced type 2 diabetic rats.

Streptozotocin is a deoxy-s [(methyl-nitrosoamino), carbonyl-aminol]-D glucopyranose molecule that produces a selective toxic effect on β-cells and induces diabetes mellitus in most laboratory animals (21). The 2-deoxyglucose moiety which acts as a carrier for N-methyl-N-nitrosourea decomposes to form NO (22). The overproduction of NO during the development of streptozotocin-induced diabetes is probably an important part of the complex autoimmune reaction that leads to destruction of the pancreatic β-cells (23). The NO produced from streptozotocin has been implicated in damage to the β-cell membrane and alkylation of DNA bases. There is breakage of the DNA strand resulting in the activation of poly (ADP-ribose) synthetase and NAD depletion, ultimately leading to β-cell death (24). Hyperglycemia in type 2 diabetes mellitus is the result of both insulin resistance in the muscles and other key insulin target tissues, and decreased β-cell insulin secretion (2). In this study, insulin deficiency was observed as the postprandial plasma insulin concentration in type 2 diabetic rats that was significantly lower than that in normoglycemic rats. The reduced plasma insulin concentration may be due to a reduction in the pancreatic β-cell function due to the destruction of β-cell, as nicotinamide only partially protects the β-cells from the toxic effect of streptozotocin (25).

The decreased glucose uptake in response to insulin in skeletal muscle of type 2 diabetic rats is in accordance with similar results of other investigations where the ability of insulin to stimulate glucose uptake in skeletal muscle was impaired in animal models of diabetes (26). Studies in incubated or perfused skeletal muscle of diabetic rodents showed a decreased maximal insulin-stimulated glucose uptake, which may be due to reduced recruitment of GLUT-4 to the plasma membrane, reduced recruitment of GLUT-4 vesicle fusion at the plasma membrane, and/or defective intracellular metabolism of the transported glucose (27).

Insulin stimulation increased glucose uptake in adipocytes of both normoglycemic and diabetic rats, however, the elevation of glucose uptake was greater in adipocytes of normoglycemic rats. Streptozotocin-induced diabetes is associated with a dramatic decrease in GLUT-4 mRNA and protein in rat adipose tissue (28). The reduction in adipocyte GLUT-4 expression is due to a decrease in the rate of transcription of the GLUT-4 gene (29). Investigators have found that in diseases characterized by insulin resistance such as obesity and non-type 2 diabetes mellitus, cellular depletion of GLUT-4 is a major mechanism in adipocytes (30). The decreased response to insulin may have resulted from defect in the trafficking and translocation of GLUT-4 to the plasma membrane (31) and cellular abnormality in adipocytes which lies intrinsically to the glucose transport effector system. Insulin receptor kinase activity and the expression and insulin-stimulated phosphorylation of IRS-1 are reduced in adipose tissues of diabetic subjects. Events downstream of IRS-1 are also impacted, as insulin stimulation of phosphoinositol-3-kinase (PI3K) activity and phosphorylation of protein kinase B/Akt are impaired (32).

The well-known diversified and multifunctional role of NO in biological systems depends on its level, type of tissue, location, redox status, and whether the study is carried out in vivo or in vitro (33). In the present study, millimolar concentrations of GSNO and SNAP significantly reduced insulin-stimulated glucose uptake in diabetic and normoglycemic rats. The detailed mechanism of NO-induced inhibition of insulin-stimulated glucose uptake is poorly understood. In this study we suggest that reduction of glucose uptake by NO released from these donors in both type 2 diabetic and normoglycemic rats could be due to its direct inhibition of the intrinsic or functional activity of GLUT-4 (34), which negatively alters the recruitment of GLUT-4 from an intracellular pool to plasma membrane (35). Another viable explanation could be the possible cellular toxicity of NO at higher concentrations and its effect on insulin action (36). Increasing NO released from GSNO and SNAP can
become problematic in the diabetic state as it potentiates the formation of peroxynitrite, which has enormous potential to increase the oxidative modification of proteins by oxidizing different amino acids such as cysteine, tryptophan, methionine and tyrosine (37). This could result in further pathologic sequel in the diabetic state.

There is ample clinical and experimental evidence showing diabetes per se as a state of oxidative stress (38). The overproduction of superoxide in mitochondria of skeletal muscle and adipose tissues exposed to high glucose concentration has been suggested as a common mechanism for all diabetic complications (39). A possible consequence of excess ROS generation in the mitochondria would be ROS leakage and inhibition of GAPDH. In this study, we found that the specific activity of GAPDH in skeletal muscle of type 2 diabetic rats was significantly lower than in normoglycemic rats accounting for one of the defects in the glycolytic pathway. This enzyme has displayed sensitivity to ROS in several different conditions of oxidative stress (40), and this sensitivity resides in the thiol group of cysteine residue 149 in the active site of the enzyme (41). Oxidation of the thiol group by NO or ROS leads to decreased enzyme activity (42), and blocking of this process by antioxidants protects the activity of the enzyme (43). The major metabolic effect of inhibited GAPDH would be the accumulation of glycolytic intermediates upstream of this enzyme, which could lead to enhanced activity in the polyl and hexosamine pathways, and to increased glycation (44). Another possible effect of inhibited GAPDH activity is the accumulation of diacylglycerol (DAG) and, consequently, increased protein kinase C (PKC) activity (45).

In summary, we provide experimental evidence that NO released from GSNO and SNAP inhibits insulin-stimulated glucose uptake in adipocytes of normoglycemic and diabetic rats. Additionally, these data also show decreased basal and insulin-stimulated glucose uptake, and reduced GAPDH activity in skeletal muscle of streptozotocin- and nicotinamide-induced type 2 diabetic rats, which accounts for the possible defect in the glycolytic pathway. This suggests that exogenous NO modulates insulin stimulated glucose uptake in adipocytes of streptozotocin-induced type 2 diabetic rats, and this could lead to further deterioration of the diabetic state. Future investigations should focus on the interactions of NO with early insulin signaling factors in adipocytes of streptozotocin-induced type 2 diabetic rats, and should also address the important issue of whether the inhibition of insulin-stimulated glucose uptake is caused by decreased glucose transporter translocation and/or activity.

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