Evaluation of the antioxidant peptide SS31 for treatment of burn-induced insulin resistance

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Abstract. After severe burn injury and other major traumas, glucose tolerance tests demonstrate delayed glucose disposal. This ‘diabetes of injury’ could be explained by insulin deficiency, and several studies have shown that soon after trauma (ebb phase) insulin concentrations are reduced in the face of hyperglycemia. After resuscitation of trauma patients (flow phase), β-cell responsiveness normalizes and plasma insulin levels are appropriate or even higher than expected, however, glucose intolerance and hyperglycemia persist. In the acute care setting, several approaches have been used for treating insulin resistance, including insulin infusion, propranolol and glucagon-like-peptide-1 (GLP-1). Recently, it was demonstrated that a tetrapeptide with antioxidant properties D-Arg-Dmt-Lys-Phe-NH\textsubscript{2} (SS31), but not its inactive analogue Phe-D-Arg-Phe-Lys-NH\textsubscript{2} (SS20) attenuates insulin resistance in mice maintained on a high fat diet. In this report the effects of SS31 and SS20 on burn-induced insulin resistance was studied in mice. Oral glucose tolerance tests (OGTT) were performed in 4 groups of 6 mice with thermal injury with or without pre-treatment with SS31 or SS20 and sham controls. In addition, biodistribution of \textsuperscript{18}FDG was measured in burned mice with and without SS31 treatment and shams (subsets of these animals were also studied by \textit{µPET}). For comparison purposes, groups of 6 cold-stressed mice with and without SS31 treatment were also studied. The results of these studies demonstrate that SS31 but not SS20 ameliorated burn-induced insulin resistance. In addition, SS31 treatment resulted in marked reduction in the increased \textsuperscript{18}FDG uptake by brown adipose tissue (BAT) in burned but not cold-stressed animals; suggesting that the stressors act by different mechanisms. Overall, these studies confirmed that SS31 can be used to reverse burn-induced insulin resistance and provide a firm pre-clinical basis for future clinical trials of SS31 for the treatment of insulin resistance in patients with burn injury.

Introduction

Maintenance of blood glucose levels is one of the most tightly regulated systems in the body, and although all cells require glucose, it is only available from exogenous sources in addition to hepatic and to a lesser extent renal cortical gluconeogenesis (1,2). Since glucose cannot be stored in significant amounts except as glycogen in liver and muscle, transport into the cell by specific glucose transport proteins is essential for normal cellular function (3).

Insulin plays a critical role in maintaining normal levels of plasma glucose. Its secretion is stimulated when blood glucose levels rise and it stimulates uptake of glucose by skeletal muscle and other tissues via specific glucose transporter proteins and decreases glucose production by the liver (4). There are several key proteins in the insulin/glucose regulatory pathway cascade, including insulin receptor substrate 1 (IRS-1) and Akt1/Protein kinase Ba (Akt1/PKBo) (5). When levels of insulin and glucose are abnormally high in the fasting state, in conditions such as type 2 diabetes, severe trauma and burn injury, a condition known as insulin resistance exists (6).

In the acute care setting, several approaches have been used for treating insulin resistance, including: insulin infusion (7), propranolol (8) and more recently, glucagon-like-peptide-1 (GLP-1) (9). Very recently, we demonstrated that simvastatin can also attenuate burn-induced insulin resistance (10). The use of insulin infusion is the most straightforward treatment, however, it can produce severe and potentially life-threatening hypoglycemia. Propranolol acts by inhibiting the effect of epinephrine (11,12). The mechanism(s) by which GLP-1 reverses insulin resistance is more complex and involves both insulinotropic effects and direct inhibition of glucagon (13,14). The mechanism for statin effects on insulin resistance are poorly defined but could be related to their anti-inflammatory actions.

Oxidative injury has been implicated in a wide variety of clinical disorders including, diabetes and burn injury. However, currently available antioxidants have not proven to be particularly effective for treating these conditions; possibly due to their
inability to reach the relevant sites of free radical generation, especially if mitochondria are the primary source of reactive oxygen species (ROS). Oxidative damage to mitochondria has been shown to impair their function and lead to cell death via apoptosis and necrosis. Because dysfunctional mitochondria produce more ROS, a feed-forward loop is set up whereby ROS-mediated oxidative damage to mitochondria favors more ROS generation, resulting in a vicious cycle (15).

The recently developed Szeto-Schiller (SS) peptide antioxidants represent novel compounds for targeted delivery of antioxidants to the inner mitochondrial membrane (16). The most common feature of these peptides centers on alternating aromatic and basic amino acid residues (aromatic-cationic peptides). These peptides can scavenge hydrogen peroxide and peroxynitrite and inhibit lipid peroxidation. Their antioxidant action can be attributed to the presence of a dimethyltyrosine residue. D-Arg-Dmt-Lys-Phe-NH₂ (SS31) is among the most potent ROS scavengers in this family of peptides. Since cells are highly permeable to SS31 (with a 3+ charge) and mitochondria are the most electronegative cellular organelles, the peptide accumulates >1,000-fold at the inner mitochondrial membrane (17) where it scavenges ROS, and protects mitochondria from permeability transition, swelling, and cytochrome c release (17). It is believed that the ROS scavenging effect of SS31 is mediated via the dimethyl tyrosine residue in its sequence. Since the dimethyl tyrosine residue in SS31 is not present in Phe-D-Arg-Phe-Lys-NH₂ (SS20) this peptide does not display ROS scavenging properties.

Among the SS peptides, SS31 has been most extensively studied and appears to be highly potent. Studies with isolated mitochondrial preparations and cell cultures have shown that SS31 can scavenge ROS, reduce mitochondrial ROS production, and inhibit the mitochondrial permeability transition (18). Excellent efficacy has also been demonstrated with animal models of myocardial ischemia-reperfusion injury (19), ischemic brain injury (20), neurodegeneration (21), islet isolation (20), renal fibrosis (23) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxicity (24). It was recently demonstrated that SS31 but not its inactive analogue, SS20, can reverse insulin resistance that occurs in obesity (25). It was recently demonstrated that SS31 but not its inactive analogue, SS20, can reverse insulin resistance that occurs in obesity (25). SS31 has been shown to impair their function and lead to cell death via apoptosis and necrosis. Because dysfunctional mitochondria produce more ROS, a feed-forward loop is set up whereby ROS-mediated oxidative damage to mitochondria favors more ROS generation, resulting in a vicious cycle (15).

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Effect of SS31 and SS20 on oral glucose tolerance tests (OGTT) performed in burn-injured and sham-treated mice. Burned mice treated with SS31 or SS20 and sham controls were studied at 24 h after injury. The doses of SS31 and SS20 were 4.0 mg/kg, injected i.p. in the resuscitation fluid immediately after injury, 12 h and 24 h later by i.p. injection. After fasting overnight a 2 g/kg glucose solution was administered via gavage. Glucose levels were determined on serial whole blood samples with a glucometer (Ascensia® Contour™, Bayer Healthcare LLC, Mishawaka, IN). Areas under the plasma glucose curves (AUCs) were calculated using the trapezoidal rule. Initial glucose levels in the sham mice used in these studies never exceeded 100 mg/dl.

Effect of SS31 and SS20 treatment on regional glucose metabolism. The biodistribution of 18F-FDG was measured in groups of burned mice, burned mice treated with SS31 or SS20, and sham-treated controls. One day after burn injury as described above, SS31 or SS20 was injected i.p. (4.0 mg/kg) followed by fasting overnight with free access to water. On the following morning, the unanesthetized mice were injected with 18F-FDG (50 µCi) via the tail vein. Approximately 60 min after tracer injection, the animals were sacrificed by cervical dislocation and complete biodistribution was measured. The results were calculated as the percentage of the injected dose per gram wet weight of tissue (% ID/g). All results were expressed as mean ± SEM. A subset of animals was injected with a larger dose of 18F-FDG (~500 µCi) and the distribution of tracer was evaluated by µPET.

Cold-stress in mice. To produce cold stress, mice were placed in a cold room at 4°C for 24 h with overnight fasting and access to water ad libitum. The mice were housed three to a cage, the radiopharmaceutical (18F-FDG) was administered on the following morning and biodistribution was measured as described above.

µPET imaging. Groups of burn-injured mice, burn-injured mice treated with SS31 or SS20 (4.0 mg/kg) and sham-treated controls were studied by 18F-FDG-µPET. µPET imaging was performed with a P4 µPET camera (Concord Microsystems, Inc., Knoxville, TN). One hour after intravenous injection of 18F-FDG (~500 µCi)
via the tail vein without anesthesia, the mice were anesthetized,
positioned and stabilized in the gantry of the PET camera
and a 10 min image was acquired in list mode. The primary
imaging characteristics of the P4 camera are in-plane and axial
resolutions of ~2 mm FWHM, 63 contiguous slices of 1.21 mm
separation and a sensitivity of ~650 cps/µCi. In all animals, the
region from the head to the base of the tail was included in the
7.9 cm field of view of the camera. The PET images were recon-
structed using an iterative algorithm maximum a posteriori
(MAP) in a 256x256 matrix with zoom 4. Data for attenuation
correction was measured with a rotating point source containing
57Co. All projection data were corrected for non-uniformity of
detector response, dead time, random coincidences, and scat-
tered radiation. The PET camera was cross-calibrated to a well
scintillation counter by comparing the camera response from a
uniform distribution of an 18F solution in a 5.0 cm cylindrical
phantom with the response of a well counter to an aliquot of the
same solution.

Statistical analysis. Statistical analyses were performed using
1-or 2-way ANOVA (as appropriate) and individual means
were compared using Duncan’s New Multiple range test. All
results were expressed as mean ± SEM. P-values of <0.05 were
considered to be statistically significant.

Results

The glucose time-plasma concentration curves from OGTTs
performed on mice with burn injury, burned mice treated
with SS31 or SS20 and sham-treated controls are illustrated in
Fig. 1. Two-way ANOVA demonstrated highly significant main
effects of treatment, F3,44=58.06 P<0.00001 and time, F18,44=9.90; P<0.00001. Compared with sham-treated control animals,
burn injury produced marked elevation and delayed normaliza-
tion of blood glucose levels; baseline (P<0.0001), 5 (P<0.005),
10 (P<0.05), 15 (P<0.05), 30 (P<0.005), 60 (P<0.005), 120
(P<0.005) and 180 min (P<0.05). This elevation was signifi-
cantly (P<0.05) reduced at 30, 60 and 120 min by treatment
of the burned animals with SS31. In contrast, treatment with
SS20 did not reduce burn-induced elevations in glucose levels
at any time point. In fact, although not statistically significant,
at the early times (baseline to 15 min) glucose levels tended to
be higher in burned animals treated with SS20 compared with
untreated burned animals.

Fig. 2 shows the AUCs calculated from the blood glucose
curves in Fig. 1. One-way ANOVA demonstrated a highly
significant main effect of treatment on AUC, F3,29=20.15; P<0.00001. AUCs for burned animals and burned animals treated with SS20 were significantly greater than the values for
SS31-treated animals and sham-treated controls (P<0.0001). The AUC of the SS31-treated group was significantly (P<0.001) reduced compared to burned animals and burned animals
treated with SS20. Overall, the results of these studies clearly
demonstrate that burn injury markedly elevates plasma levels
of glucose (consistent with the results of previous studies) and
that this effect is reduced by treatment with SS31 but not SS20.

The effects of treatment with SS31 on the burn injury
and cold stress-induced changes in 18FDG uptake by brown
adipose tissue (BAT) in mice are shown in Fig. 3. ANOVA
demonstrated a highly significant main effect of treatment,
F4,45=30.65; P<0.0001. Compared with sham-treated mice,
animals with burn injury, cold stress and cold stress plus SS31
were reduced to highly significant increases in 18FDG uptake by
BAT (P<0.0001). Cold-stress and cold stress plus SS31 treat-
ment was associated with greater increases in 18FDG uptake by
BAT than burn injury alone or burn injury plus SS31 treatment
(P<0.0001). Burn plus SS31 treatment produced a significant
(P<0.01) increase in 18FDG uptake compared with shams.
Treatment with SS31 produced a significant (P<0.05) reduc-
tion in 18FDG uptake by BAT in burned animals. Surprisingly,
SS31 treatment had no effect on the increase in 18FDG uptake
by BAT that was produced by cold stress. There were no
significant effects of SS31 treatment on 18FDG uptake in any
of the other tissues that were sampled.

µPET imaging. Representative 18FDG µPET images (sagittal
slices) of a sham control mouse (left panel), a mouse with
Once in the cytosol, H$_2$O$_2$ formation from the respiratory system and scavenging of H$_2$O$_2$ can alter the redox state by either reacting directly with thiol residues in redox-sensitive proteins or shifting the ratio of reduced to oxidized glutathione (GSH/GSSG); the main redox buffer of the cell. Thus, the rate at which H$_2$O$_2$ is emitted from mitochondria is considered an important index of mitochondrial function and modulator of the overall cellular redox environment (37). Two recent investigations have indicated that the rate of mitochondrial H$_2$O$_2$ emission is significantly greater when basal respiration is supported by fatty acid vs. carbohydrate-based substrates (38,39), raising the possibility that mitochondrial H$_2$O$_2$ emission may be a primary factor in the etiology of insulin resistance. Anderson et al demonstrated that hydrogen peroxide production by mitochondria is linked to a high-fat-diet induced insulin resistance in both rodents and humans (25).

Burn injury is associated with oxidative stress (40-42) as well as insulin resistance (8,9,28,29). Using the euglycemic insulin clamp technique it has been demonstrated that: i) maximal rate of glucose disposal is reduced in trauma patients, ii) the metabolic clearance rate of insulin is almost twice normal in these patients and iii) post-trauma insulin resistance appears to occur in peripheral tissues, probably skeletal muscle, and is consistent with a post-receptor effect (31,43). Ikezu et al (44) demonstrated that burn injury results in impaired insulin-stimulated transport of [H]-2-deoxyglucose into rat soleus muscle strips in vitro. These investigations, as well as other studies from our laboratory (45) have also demonstrated that insulin stimulated phosphoinositide 3-kinase (PI 3-kinase) activity, which is pivotal for glucose transport in muscle by glucose transporter 4 (GLUT-4), is decreased by burn injury to rats as measured by its IRS-1 associated activity. These data are consistent with alterations in post receptor signaling following burn injury, which results in burn-induced insulin resistance. The present study was designed to determine whether SS31, which has been shown to have protective effects at the mitochondrial level, effects glucose clearance from the blood after burn injury or uptake of the glucose analogue $^{18}$FDG. The mechanisms by which burn injury in this mouse model alters these two parameters of glucose metabolism is not understood at this point. However,
treatment with SS31 but not SS20 reduced the changes produced by burn injury. Whether this is related to changes in mitochondrial function in the burned mouse under these conditions is not clear. We have observed, however, that chronic treatment of burned rats with SS31 reduces the increased oxygen consumption produced by the burn injury (46,47). One possibility is that the increased oxygen consumption produced by burn injury to rats is related to altered mitochondrial metabolism, which might be affected by SS31.

The surprising observation that the increase in 18FDG uptake by BAT after burn injury is attenuated by treatment with SS31 whereas the peptide had no effect on the increase in 18FDG produced by cold-stress has important implications. Since pre-treatment with propranolol reduces the increased 18FDG uptake in BAT after cold stress in both animal models (48) and humans (49) and since that adrenalectomy reduces increased 18FDG uptake in BAT after both burn injury and cold stress (50), the differential effects of SS31 suggest that the two stressors produce increased 18FDG uptake by different mechanisms.

The observation that SS31-induced reductions in increased 18FDG uptake in BAT produced by burn injury closely parallel the results of OGTTs suggests that 18FDG-PET may be useful for evaluating new treatments for burn-induced insulin resistance and possibly for therapeutic monitoring of individual patients. This approach could have additional value for studying other types of insulin resistance such as in type 2 diabetes mellitus.

In conclusion, the results of these studies demonstrate that SS31, but not SS20, significantly attenuated burn-induced insulin resistance in mice. In addition, SS31 had major effects on the biodistribution of 18FDG in mice. In addition, SS31 had major effects on insulin sensitivity and possibly for therapeutic monitoring of individual patients. This approach could have additional value for studying other types of insulin resistance such as in type 2 diabetes mellitus.

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References