Effects of Mitochondrial-Targeted Antioxidants on Real-Time Blood Nitric Oxide and Hydrogen Peroxide Release in Acute Hyperglycemic Rats

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Introduction

Diabetes and prediabetes are major public health concerns worldwide due to the high risk of developing micro- and macro-vascular complications. Hyperglycemia, the major criteria for diabetes diagnosis, is causally related to the pathogenesis of vascular complications in diabetic patients. An early event during hyperglycemia is vascular endothelial dysfunction. Normally, the vascular endothelium facilitates blood flow principally by releasing endothelial-derived nitric oxide (NO) via vascular endothelial NO synthase (eNOS) in the presence of an essential co-factor, tetrahydrobiopterin (BH₄). By contrast, acute and chronic hyperglycemia increase oxidative stress and reduce NO bioavailability [1,2]. The reduced endothelial-derived NO bioavailability promotes vasoconstrictive, pro-inflammatory, and pro-thrombotic events, initiating inflammation, thereby recruiting leukocytes, resulting in tissue/organ damage (Figure 1). Therefore, reduction of oxidative stress during hyperglycemia will mitigate vascular endothelial dysfunction and organ damage. Crabtree, et al. [1] found that mitochondria-derived superoxide (SO) contributes to hyperglycemia-induced oxidative stress in cultured vascular endothelial cells. Subsequently, the overproduction of SO promotes the oxidation of BH₄ to dihydrobiopterin (BH₂). The reduced BH₄/BH₂ ratio leads to BH₂, not BH₄, binding to oxygenase domain of eNOS, which causes eNOS to shift its product profile from NO to SO [1] (Figure 1). However, the role of mitochondria in acute hyperglycemia-induced oxidative stress and blood NO reduction has not been evaluated *in vivo*. Recently, our lab showed that mitoquinone (MitoQ) and SS31 (Szeto-Schiller, D-Arg-Dmt-Lys-Phe-Amide) peptide (Figure 2), mitochondria-targeted antioxidants, significantly reduced blood H₂O₂ (an index of oxidative stress) and increased blood NO levels in a hind limb ischemia/reperfusion (I/R) animal model [3]. Oxidative stress is also an important cause of reperfusion injury during I/R. Thus, we hypothesize that MitoQ and SS-31 will reduce blood oxidative stress and increase blood NO levels under acute hyperglycemic conditions.

Fig. 1. The putative role of mitochondrial-derived SO in hyperglycemia-induced oxidative stress, vascular endothelial dysfunction and tissue inflammation.

Fig. 2. Chemical structure of MitoQ and SS-31 [4,5].

Results and Discussion

Male Sprague-Dawley rats (275 to 325g, Charles River, Springfield, MA) were anesthetized with 60 mg/kg of pentobarbital sodium with 1000 units of heparin via intraperitoneal (i.p.) injections. The jugular vein was catheterized to allow for the infusion of saline, 20% D-glucose, or 20% D-glucose with 1.86 mg/kg MitoQ (MW=600 g/mol; complexed with cyclodextrin to improve water solubility, total MW=1714 g/mol) or with 2.7 mg/kg SS-31 (MW=640 g/mol, Genemed Synthesis, Inc., San Antonio, TX). The continuous infusion of 20% D-glucose solution was used to maintain a hyperglycemic state around 200 mg/dL for about 180 min. MitoQ or SS-31 was added to the 20% D-glucose infusate to achieve a blood concentration of approximately 13 μ M and 50 μ M, respectively. Both femoral veins were exposed and catheterized in order to randomly place calibrated NO or H₂O₂ microsensors (100 μ m diameter, WPI Inc., Sarasota, FL) into each femoral vein. These microsensors were connected to an Apollo 4000 free radical analyzer (WPI Inc., Sarasota, FL) to monitor blood NO and H₂O₂ levels in real-time. NO, H₂O₂ were monitored continuously while glucose levels were recorded at baseline and at 20 minute intervals throughout the 180 minute infusion period [2].

The changes of blood NO (nM) and H₂O₂ (μ M) levels were expressed as the relative change to the saline group. All the data were represented as a mean $\pm$ SEM, and then analyzed by ANOVA using post hoc analysis with the Student Newman Keuls test. $p < 0.05$ was considered significant.

The time course change in blood glucose in different experimental groups is illustrated in Figure 3. The blood glucose level in the saline group remained at baseline values of 80-100 mg/dl throughout the experiment. In contrast, after 20 min infusion of 20% D-glucose with or without the drug, the blood glucose levels rose to hyperglycemic levels at $\sim$ 200 mg/dl. All hyperglycemic rats urinated between 20-40 min after glucose infusion.

The time course change in blood NO levels relative to the saline infusion among the different experimental groups is shown in Figure 4. There was a significant decrease in blood NO levels in the

Fig. 3. Blood glucose level among saline and 20% glucose infusion groups.

20% glucose group (n=9) compared to the saline group (n=7) at 120 min (43.7 ± 11.3 nM lower), followed by a continued decrease throughout the infusion (all $p < 0.05$). At 180 min, blood NO level was 68 ± 13.5 nM lower relative to that in saline group ($p < 0.01$). However, the co-infusion of MitoQ (13 μ M, n=5) or SS-31 (50 μ M, n=6) with 20% glucose significantly reduced the fall in blood NO levels from 120 min or 140 min, respectively, which was sustained during the rest of the infusion time compared to those in the 20% glucose group. The NO levels in both MitoQ and SS-31

Fig. 4. The comparison of change in blood NO levels relative to saline group among 20% D-glucose, 20% D-glucose with MitoQ (13 μ M), and 20% D-glucose with SS-31 (50 μ M) (# p <0.05, ##<0.01 vs saline; * p <0.01 vs D-glucose).

blood H_2O_2 levels only at 160 min and 180 min by comparing to those in the 20% glucose group (both p <0.05). SS-31 treatment did show a trend of smaller increases in blood H_2O_2 levels compared to MitoQ treatment, but was not significant. Moreover, the H_2O_2 levels in both MitoQ and SS-31 treatment groups were not significantly different from H_2O_2 levels in the saline group throughout the experimental time course.

Researchers have shown that acute hyperglycemia can cause systemic oxidative stress and vascular dilatory dysfunction in non-diabetic human subjects [6,7]. By placing NO/ H_2O_2 sensors in rat femoral veins, we established the time course of blood NO and H_2O_2 levels during acute hyperglycemia. Blood H_2O_2 levels serve as an index of oxidative stress because H_2O_2 is principally derived from SO by SO dismutase. We found that acute hyperglycemia significantly increased blood H_2O_2 and reduced blood NO levels compared to the saline group. Moreover, increased blood H_2O_2 levels occurred earlier than reduction of blood NO levels during hyperglycemia. This result suggests that reduced blood NO levels may be due to direct quenching effect of free radicals on blood NO levels and/or eNOS product profile changing from NO to SO when BH_4 is oxidized to BH_2 (see Figure 1). Mitochondria may be a site of free radicals overproduction during hyperglycemia. Both MitoQ and

Fig. 5. The comparison of change in blood H_2O_2 levels relative to saline group among 20% D-glucose, 20% D-glucose with MitoQ (13 μ M), and 20% D-glucose with SS-31 (50 μ M) (# p <0.05, ## p <0.01 vs saline; * p <0.01 vs D-glucose).

treatment groups were similar to the NO levels in the saline group throughout the experimental time course.

The time course change in blood H_2O_2 levels relative to the saline infusion among the different experimental groups is shown in Figure 5. There was a significant increase in blood H_2O_2 levels in the 20% glucose group ($n=7$) compared to the saline group ($n=8$) at 60 min ($1.2 \pm 0.3 \mu$ M higher), followed by a continued increase throughout the infusion. At 180 min, blood H_2O_2 level was $3.0 \pm 0.5 \mu$ M higher relative to that in saline group. However, the addition of SS-31 (50 μ M, $n=5$) significantly reduced the increase in blood H_2O_2 levels from 60 min to 180 min compared to those in 20% glucose group (all p <0.05). Furthermore, the addition of MitoQ (13 μ M, $n=6$) significantly reduced

mitochondrial-targeted antioxidants by selectively diffusing into the mitochondria after administration [6,7]. We found that administration of MitoQ or SS-31 during hyperglycemia significantly reduced blood H_2O_2 levels and improved blood NO levels, similar to the saline group. Previous research has indicated that MitoQ possesses positive charges and lipophilic properties, and both facilitate its diffusion into the mitochondria. Meanwhile, MitoQ may collapse mitochondrial membrane potential at higher doses which slows its further concentration in the mitochondria [6]. By contrast, SS-31 has an alternating cationic-aromatic amino acid sequence which allows it to concentrate into the mitochondria without relying on mitochondrial membrane potential [4,5]. The different mechanism of action

between MitoQ and SS-31 may explain the observation in this study that SS-31 exerted earlier and better reduction in blood H₂O₂ levels compared to MitoQ. In summary, the data suggest that mitochondrial derived SO is a significant source of oxidative stress and vascular endothelial dysfunction under acute hyperglycemic conditions. Moreover, treatment with mitochondrial-targeted antioxidants, MitoQ or SS-31, may be beneficial to attenuate hyperglycemia-induced oxidative stress and vascular endothelial dysfunction.

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