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Short-term exposure of high glucose concentration induces generation of reactive oxygen species in endothelial cells: implication for the oxidative stress associated with postprandial hyperglycemia

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Recent studies demonstrating a close relationship between postprandial hyperglycemia and the incidence of atherosclerotic cardiovascular disease prompted us to investigate the generation and source of reactive oxygen species (ROS) in endothelial cells stimulated by short-term exposure to a high glucose concentration. In addition, we investigated the effect of insulin on ROS production induced by high glucose concentration. Cultured bovine aortic endothelial cells demonstrated a significant increase in intracellular ROS generation after a 3-h exposure to 25 mM glucose (131.4% versus 5 mM glucose). This increased generation of ROS was suppressed by an inhibitor of NAD(P)H oxidase. Intracellular ROS production in cells exposed to 3 h of high glucose concentration was increased significantly by the presence of a physiological concentration of insulin. However, after a 1-h exposure to high glucose levels, ROS generation in cells incubated with insulin was only about 80% of that measured in cells incubated without insulin. The generation of intracellular nitric oxide (NO) resulting from an acute insulin effect may account for this difference. In conclusion, acute hyperglycemia itself may possibly cause endothelial oxidative stress in patients with postprandial hyperglycemia. Endothelial oxidative stress may be determined by the interaction between NO and superoxide generation.

Keywords: Postprandial hyperglycemia, oxidative stress, reactive oxygen species, short-term exposure, high glucose, endothelial cells

INTRODUCTION

Recently, epidemiological studies have shown there is a close relationship between postprandial hyperglycemia and the incidence of atherosclerotic cardiovascular disease.¹² A variety of factors, such as hyperglycemia itself and hyperlipidemia, have been proposed to contribute to this relationship.³ Oxidative stress has been implicated in the pathogenesis of atherosclerotic vascular disease,⁴⁵ and several studies have demonstrated that acute hyperglycemia after a meal and glucose loading induce oxidative stress in humans.⁶⁻⁸ Taken together, these findings indicate that oxidative stress may be an etiological factor for atherosclerosis in patients with postprandial hyperglycemia.

Convincing data have been reported to show that chronic exposure to high glucose concentration triggers the generation of reactive oxygen species (ROS) in endothelial cells.⁹¹⁰ Recent in vitro studies have suggested that protein kinase C dependent NAD(P)H oxidase activation and mitochondrial electron transport are involved in this mechanism.¹¹¹² Furthermore, saphenous veins and internal mammary arteries from diabetic patients have increased NAD(P)H oxidase activity and uncoupling of eNOS compared with those in matched...
non-diabetic subjects, resulting in increased vascular superoxide production in human diabetes mellitus. However, it remains unknown whether endothelial superoxide production is enhanced by the short-term exposure to high glucose concentration that occurs in postprandial hyperglycemia. In addition, little attention has been given to the interaction between high glucose concentration and insulin in the generation of intracellular ROS in the postprandial state.

In this study, we investigated the production and source of ROS in endothelial cells stimulated by short-term exposure to high glucose concentration. The effect of insulin on ROS generation induced by hyperglycemia also was investigated.

**MATERIALS AND METHODS**

**Cell culture**

Bovine aortic endothelial cells (BAECs) were obtained from Cell Systems (Kirkland, USA) and were maintained in Endo Media (IBL, Gunma, Japan) containing 10% fetal bovine serum (FBS), antibiotics, and 5 mM glucose. Confluent cells (passage less than 6) were placed in DMEM (Gibco, Grand Island, USA) containing 0.5% FBS, and 5 mM glucose for 24 h. The cells were then incubated for up to 24 h with DMEM containing 10% FBS and either 5 mM glucose, 25 mM glucose, or 25 mM glucose plus bovine insulin (Sigma, St Louis, USA). To determine which oxidative enzyme systems were contributing to intracellular ROS production, either diphenylene iodonium (an inhibitor of NAD(P)H oxidase), oxypurinol (an inhibitor of xanthine oxidase inhibitor), N-nitro-L-arginine methyl ester hydrochloride (L-NAME, an inhibitor of nitric oxide synthase), nordihydroguaiaretic acid (NDGA, an inhibitor of lipoygenase), rotenone (an inhibitor of mitochondrial respiration), or indomethacin (an inhibitor of cyclooxygenase) was added to the media containing 5 mM or 25 mM glucose.

**Measurement of intracellular ROS**

Intracellular ROS production was detected using the fluorescent probe 5-(and 6-) chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H$_2$DCFDA, Molecular Probes, Leiden, The Netherlands). Cells (1 x 10$^6$) were loaded with 10 µM CM-H$_2$DCFDA in HBSS (Ca$^{2+}$- and Mg$^{2+}$-free) for 45 min at 37°C. The fluorescence was then measured using a Fluorescent Microplate Reader (Bio-Rad) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm (1–5 x 10$^4$ cells/well). ROS production was evaluated as the percentage of the fluorescence of cells incubated in 5 mM glucose for each time period.

**Measurement of intracellular nitric oxide (NO)**

Intracellular NO levels were determined with the fluorescent probe 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM diacetate, Molecular Probes). Cells were loaded with 10 µM DAF-FM diacetate for 20 min at 37°C and the fluorescence was measured at an excitation wavelength of 485 nm and emission wavelength of 515 nm (1–5 x 10$^4$ cells/well).

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**Fig. 1** Time course of ROS generation in bovine aortic endothelial cells induced by high glucose concentration (25 mM glucose). Intracellular ROS production was detected using the fluorescent probe CM-H$_2$DCFDA with values at each time period expressed as a percentage relative to the fluorescence measured in cells incubated in 5 mM glucose. *P < 0.01 versus 5 mM glucose for each time period analyzed using Student’s t-test. Data are mean ± SEM of 7 different experiments.
**Statistical analysis**

Results are expressed as mean ± SEM. Student’s *t*-test was used for simple comparison between two values, while multiple comparisons were carried out using analysis of variance followed by Bonferroni’s post test. A *P* value < 0.05 was considered as statistically significant.

**RESULTS**

*Time course of intracellular ROS production by high glucose in BAECs*

The intracellular production of ROS did not increase until 24 h of incubation with 5 mM glucose (data not shown). A significant increase in intracellular ROS production was detected after a 3-h exposure to a high level of glucose (25 mM) compared with control (5 mM), with this production increasing in a time-dependent manner for up to 10 h of incubation (3 h, 131.4 ± 8.4%; 10 h, 158.9 ± 15.1%; Fig. 1).

*Source for intracellular ROS production by short-term exposure of high glucose*

Treatment of BAECs with 10 μmol/l diphenylene iodonium (an inhibitor of NAD(P)H oxidase) restored the increased intracellular ROS production induced by a 3-h exposure to 25 mM glucose. In contrast, treatment with 100 μmol/l oxypurinol, 10 μmol/l NDGA, 10 μmol/l L-NAME, 100 μmol/l rotenone or 10 μmol/l indomethacin had no detectable effect on increased intracellular ROS production (Fig. 2). These results suggest that short-term exposure to high glucose concentration stimulates ROS production mainly through NAD(P)H oxidase.

*Effect of insulin on intracellular ROS production by high glucose*

Intracellular ROS production in the cells exposed to high glucose concentration was increased slightly, but significantly, by the presence of a physiological concentration of insulin (100 μU/ml) after a 3-h incubation. This effect was not detected at 24 h of incubation. On the other hand, after 1 h of incubation with either 30 or 100 μU/ml insulin, ROS production in the cells was decreased to about 80% of that seen in cells incubated without insulin (Fig. 3).

To evaluate the role of NO production in the reduction of high glucose-induced intracellular ROS production by insulin, the effect of L-NAME, an inhibitor of NO synthase, was examined. Intracellular NO production in cells exposed to 25 mM glucose for 1 h was significantly lower than that exposed to 5 mM glucose (82.2 ± 1.2%; *P* < 0.01; *n* = 7), and that was increased after a 1-h incubation with...
insulin (Fig. 4A). On the other hand, the reduction of intracellular ROS production induced by insulin was abolished by the presence of 10 µmol/l L-NAME in the medium (Fig. 4B). These results suggest that short-term exposure to insulin stimulates NO production in the cells and results in scavenging of superoxide.

**DISCUSSION**

In this study, the fluorogenic probe 2',7'-dichlorodihydrofluorescein (DCFDA) was used to detect oxidative stress in endothelial cells. DCFDA diffuses across cell membranes and is hydrolyzed by non-specific cellular enzymes, producing a fluorescent product that can be measured. This probe is commonly used to detect intracellular ROS.

**Fig. 3.** Effect of insulin on ROS generation induced by high glucose concentration in bovine aortic endothelial cells. The intracellular ROS formation was evaluated after a 1-, 3- or 24-h exposure to high glucose concentration (25 mM glucose) in the presence or absence of insulin (30 and 100 µU/ml). At each time period, ROS production was expressed as a percentage relative to the fluorescence measured in cells incubated in 5 mM glucose. *P < 0.05, **P < 0.01 versus high glucose for each time period analyzed using ANOVA and Bonferroni’s comparison. Data are mean ± SEM of 7 different experiments.

**Fig. 4.** (A) Effect of insulin on NO formation in bovine aortic endothelial cells incubated with high glucose concentration (25 mM glucose) for 1 h. Intracellular NO production was detected using the fluorescent probe DAF-FM diacetate and was expressed as the percentage of fluorescence relative to that measured in cells incubated with 5 mM glucose for 1 h. *P < 0.05, **P < 0.01 versus high glucose alone analyzed using ANOVA and Bonferroni’s comparison. Data are mean ± SEM of 7 different experiments. (B) Effect of L-NAME (10 µmol/l) on intracellular ROS generation induced by a 1-h exposure to high glucose concentration (25 mM glucose) in the presence of insulin. Results are expressed as the percentage of fluorescence relative to that measured in cells incubated with 5 mM glucose for 1 h. The reduction of intracellular ROS formation induced by insulin was abolished by the presence of 10 µmol/l L-NAME in the medium. *P < 0.05 versus HG + I(30); **P < 0.01 versus HG + I(100); #P < 0.05 versus HG + I(30); ##P < 0.01 versus HG + I(100) analyzed using ANOVA and Bonferroni’s comparison. Data are mean ± SEM of 7 different experiments. I(30), 30 µU/ml of insulin; I(100), 100 µU/ml of insulin.
esterases to the non-fluorescent compound dichlorofluorescein (DCFH). DCFH rapidly emits fluorescence upon reaction with hydroperoxide, peroxynitrate or their derivatives. Accordingly, in the series of experiments carried out in this study, it must be recognized that intracellular total ROS generation was measured as the result of co-production of NO and superoxide in the same cellular compartment.

We detected a significant increase in intracellular ROS generation in endothelial cells within 3 h of initial exposure to a high concentration of glucose. This result indicates that acute hyperglycemia in the postprandial state may itself produce oxidative stress in the endothelium. The present study also demonstrated the decreased NO generation in the cells exposed to high glucose for 1 h. Superoxide reacts rapidly with NO, reducing NO bioactivity. So this decrease may be associated with overproduction of superoxide, although a significant increase in net ROS generation was not detected. In the longer exposure to high glucose, it is assumed that an imbalance between NO and superoxide production may lead to intracellular oxidative stress. Under these conditions peroxynitrite, a powerful oxidant, is formed. Peroxynitrite has a variety of tissue damaging effects, such as lipid peroxidation, inactivation of enzymes and ion channels via protein oxidation, and nitration reactions that cause inhibition of mitochondrial respiration.

Although epidemiological studies indicate a close relationship between postprandial hyperglycemia and the incidence of atherosclerotic cardiovascular disease, a causal association between hyperglycemia and cardiovascular disease has not been clearly established. As postprandial hyperglycemia is associated with both insulin resistance and insufficient post-meal insulin secretion, and also hyperlipidemia, it has been argued that hyperglycemia may be a marker rather than a cause of atherosclerotic changes. However, the results of our study demonstrate that acute hyperglycemia itself may cause endothelial cell dysfunction by inducing oxidative stress and, therefore, contribute to atherosclerotic changes.

It has been shown that oxygen-derived free radicals produced during chronic hyperglycemia are generated by a variety of mechanisms. However, it is not clear which mechanisms are involved with acute hyperglycemia. Our results indicate that intracellular ROS production in endothelial cells is increased by acute hyperglycemia at least through the activation of NAD(P)H oxidase.

It is well known that insulin stimulates NO production in endothelial cells resulting in acute vasodilatation, thereby providing an anti-atherogenic effect. On the other hand, it has been demonstrated in an ex vivo system that insulin stimulates endothelial superoxide production through the activation of NAD(P)H oxidase. Our data show that total intracellular ROS generation is decreased 1 h after exposure to high glucose concentra-

**CONCLUSIONS**

The present study demonstrated that hyperglycemia itself could cause endothelial oxidative stress in patients with postprandial hyperglycemia. The activation of NAD(P)H oxidase appears to be the main pathway generating superoxide in endothelial cells exposed to acute hyperglycemia. Furthermore, endothelial oxidative stress may be determined by the interaction between superoxide and NO production induced by hyperglycemia and insulin action, respectively. Further analysis of the mechanism of ROS generation in endothelial cells associated with postprandial hyperglycemia may
provide a potential therapeutic intervention for reducing the risk of atherosclerotic cardiovascular disease.

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REFERENCES

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