Full Paper

Effects of Acarbose on the Acceleration of Postprandial Hyperglycemia–Induced Pathological Changes Induced by Intermittent Hypoxia in Lean Mice

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Abstract. Postprandial hyperglycemia (PPH) and intermittent hypoxia related to the sleep apnea syndrome are important predictors of cardiovascular disease. We investigated the effects of intermittent hypoxia on pathological changes in the left ventricular (LV) myocardium caused by PPH in lean mice and evaluated the influence of acarbose, an α -glucosidase inhibitor. Male C57BL/6J mice aged 8 weeks were exposed to intermittent hypoxia (8 h/day during the daytime) or kept under normoxia. PPH was induced by restriction of feeding to 1-h periods twice a day, with the restricted diet (RD) mice receiving either standard chow or chow containing 0.02% acarbose. Another group of mice were fed standard chow ad libitum (AL). Plasma glucose levels after food intake were significantly elevated in RD but not in AL mice, and glucose levels were suppressed by acarbose. Intermittent hypoxia exacerbated cardiomyocyte hypertrophy and interstitial fibrosis in the LV myocardium of RD mice. Superoxide production and expression of 4-hydroxy-2-nonenal in the LV myocardium with intermittent hypoxia were increased in RD mice, but not AL mice. In addition, expression of tumor necrosis factor α (TNF- α) mRNA was increased in hypoxic RD mice. Treatment with acarbose inhibited oxidative stress and TNF- α mRNA expression and preserved the histological architecture of the LV myocardium.

Keywords: α -glucosidase inhibitor, intermittent hypoxia, oxidative stress, postprandial hyperglycemia, sleep apnea

Introduction

Diabetes mellitus is a major risk factor for cardiovascular disease and is known to cause left ventricular (LV) remodeling including cardiomyocyte hypertrophy and interstitial fibrosis. A characteristic feature of type 2 diabetes is hyperglycemia, which promotes an imbalance between the generation and elimination of reactive oxygen species (ROS) (1). Oxidative stress induced by hy-

perglycemia may cause diabetes-related complications such as atherosclerosis, cardiomyopathy, and nephropathy (2, 3). Recently, Azuma et al. showed that postprandial hyperglycemia (PPH) exacerbates the adhesion of monocytes to endothelial cells compared with stable hyperglycemia in vivo (4). PPH has also been shown to play an important role in the occurrence of LV remodeling.

Acarbose (Glucobay[®]; Bayer Schering Pharma AG, Berlin, Germany) is known to suppress PPH by inhibiting α -glucosidase in the brush border of the small intestine (5). Rösen et al. reported that acarbose prevents increased oxidative stress and vascular dysfunction

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associated with hyperglycemia (6). In the STOP-NIDDM trial, treatment with acarbose was associated with a significant decrease in the incidence of cardiovascular disease and hypertension in patients with impaired glucose tolerance characterized by PPH (7).

Interestingly, sleep apnea syndrome (SAS), another risk factor for cardiovascular events (8), is also closely associated with diabetes mellitus as well as the metabolic syndrome (9, 10). Therefore, it is possible that intermittent hypoxia may accelerate cardiovascular remodeling in patients with diabetes. We have previously reported that intermittent hypoxia increases angiotensin II and oxidative stress, inducing LV remodeling in an animal model of SAS (11, 12). From this, we hypothesized that intermittent hypoxia in combination with PPH might act to further exacerbate the negative effects of oxidative stress on the LV myocardium.

In this study, we investigated the effects of intermittent hypoxia on pathological changes in the LV myocardium caused by PPH in lean mice and evaluated the preventive effect of acarbose on these changes.

Materials and Methods

Experimental protocol

We used 8-week-old male C57BL/6J mice (purchased from Clea Japan, Inc., Osaka) ($n = 50$). The mice were exposed to a 12-h light–dark cycle and were given free access to tap water. They were fed standard chow ad libitum (AL) or given restricted access to standard chow (with or without 0.02% acarbose) for 1 h twice daily (9:30 to 10:30 and 15:30 to 16:30). For the study, they were housed in a chamber and exposed to 30 s of 4.5%–5.5% oxygen followed by 30 s of 21% oxygen for 8 h per day during daytime for 10 consecutive days. A control group was maintained under normoxia. The dose of acarbose was determined from previous studies (13, 14), and plasma glucose levels were measured every hour from 9:30 to 19:30 in mice kept under normoxic conditions.

After the 10-day experimental period, we measured the systolic blood pressure in conscious mice using the tail-cuff method with a pneumatic pulse transducer (BP-98A; Softron, Tokyo). Animals were then anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg), and blood samples were collected for measurements of plasma glucose and lipid peroxide (LPO) levels. The heart was excised, and the upper half was used for light microscopic examination. The free wall of the left ventricle was excised for immunohistochemistry and reverse transcription polymerase chain reaction (RT-PCR). The experimental protocol and handling of animals during experiments were approved by the Experimental

Animal Research Committee of the Osaka University of Pharmaceutical Sciences.

Histological examination

Isolated ventricular tissues were fixed in 10% formaldehyde, embedded in paraffin, and cut into 4- μ m sections. To evaluate mean cardiomyocyte diameter, we measured the shortest diameter of each nucleated cardiomyocyte in transverse sections stained with hematoxylin–eosin under a light microscope at a $\times 400$ magnification. After staining with Sirius Red, color images of each section were taken with a digital camera (Fujix Digital Camera HC-300Z; Fujifilm, Tokyo) mounted on a Nikon Microphot-FXA (Nikon, Tokyo). Images were obtained of five randomly selected low-power fields ($\times 200$) in five sections per mouse. The percentage of interstitial fibrosis was then calculated by a previously described method (15).

Assay of NADPH-dependent superoxide production

NADPH-dependent superoxide production was measured using a lucigenin-enhanced chemiluminescence assay method (15, 16). The lucigenin concentration in the final reaction mixture was 5 mM. NADPH-dependent superoxide production was expressed as relative light units per min per mg of protein.

Immunohistochemistry for 4-hydroxy-2-nonenal (4-HNE) and angiotensin II

Immunohistochemical staining was performed by using a previously described method (12, 15). Briefly, sections cut from the paraffin blocks were incubated with a monoclonal antibody directed against 4-HNE (No. MHN-20; Japan Institute for the Control of Aging, Shizuoka) or with a polyclonal antibody targeting angiotensin II (IgG Corporation, Nashville, TN, USA) (12). The percentage areas of 4-HNE and angiotensin II staining were determined by quantitative analysis (17).

Quantitative real-time RT-PCR

Total RNA was extracted from myocardial tissues using an RNeasy mini kit (Qiagen, Valencia, CA, USA). Reverse transcription was performed with random hexamers and superscript reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Real-time quantitative PCR was performed by using an ABI Step One sequence detector (PE Applied Biosystems, Foster City, CA, USA), with Taqman Probe and primers for the target mRNA [Ma00443258 for tumor necrosis factor α (TNF- α)] purchased from Applied Biosystems. The reaction conditions were as follows: 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 60°C for 1 min. The level of target mRNA was normalized to that of 18s

mRNA.

Statistical analyses

Data were expressed as the mean $\pm$ S.E.M. Variables between groups were compared using one-way analysis of variance followed by the Tukey-Kramer multiple comparison test. Plasma glucose levels were compared by the unpaired Student's *t*-test. We considered $P < 0.05$ to denote significance.

Results

Plasma glucose, plasma LPO, heart rate, and blood pressure

Plasma glucose levels of mice fed AL were constantly high and ranged from 116 to 169 mg/dl. On the other hand, plasma glucose levels of mice fed a restricted diet (RD) were significantly elevated after feeding ($P < 0.05$), indicating that restriction of food intake induced PPH (Fig. 1). Although hypoxic stress significantly lowered plasma glucose levels measured pre-sacrifice in mice fed AL, no change was seen in glucose levels in mice fed RD; furthermore, the difference in the AL group was augmented by hypoxic stress (Fig. 2). Acarbose completely inhibited the postprandial increase in plasma glucose levels (Figs. 1 and 2). Plasma LPO levels were similarly elevated in both normoxic and hypoxic RD mice compared with normoxic AL mice, and LPO levels were significantly reduced by acarbose (Fig. 2). In contrast, no significant differences were seen in heart rate or

systolic blood pressure between groups (Fig. 3).

Histological findings

Intermittent hypoxic stress increased mean cardiomyocyte diameter in mice fed a RD. In contrast, no significant changes were observed in mice housed under normoxic conditions (Fig. 4). Acarbose attenuated cardiomyocyte hypertrophy in mice fed a RD and exposed to intermittent hypoxia (Fig. 5A). The percentage area of interstitial fibrosis in the LV myocardium was significantly increased by PPH and was higher in RD mice exposed to intermittent hypoxia than in RD mice housed under normoxic conditions ($P < 0.01$). In contrast, treatment with acarbose suppressed interstitial fibrosis (Figs. 4 and 5B).

NADPH-dependent superoxide production

NADPH-dependent superoxide production in the LV myocardium was significantly increased in hypoxic mice compared with normoxic RD mice (Fig. 6A). This increase was suppressed by acarbose, although the difference did not reach statistical significance.

Myocardial 4-HNE and angiotensin II expression

Expression of 4-HNE and angiotensin II in the LV myocardium was significantly increased in hypoxic mice compared with normoxic RD and hypoxic AL mice. Treatment with acarbose tended to inhibit the increase of 4-HNE ($P = 0.09$) and significantly suppressed the expression of angiotensin II (Figs. 6B and 7).

Fig. 1. Plasma glucose profile in AL (black diamonds), RD (black squares) mice, and RD mice treated with acarbose (black circles) under normoxic conditions. PPH was observed in RD mice, and acarbose significantly suppressed the plasma glucose profile after feeding in RD mice. Columns and bars represent the mean $\pm$ S.E.M. * $P < 0.05$ and ** $P < 0.01$ vs. normoxic mice fed AL. # $P < 0.05$ and ## $P < 0.01$ vs. RD mice. AL: ad libitum, RD: restricted diet, PPH: postprandial hyperglycemia, α -GI: alpha-glucosidase inhibitor.

Fig. 2. Plasma glucose (A) and LPO levels (B) examined under anesthesia before sacrifice. Both plasma glucose and LPO levels in mice fed RD were significantly higher than those in mice fed AL kept under normoxic and hypoxic conditions, which were significantly suppressed by acarbose. Columns and bars represent the mean $\pm$ S.E.M. * $P < 0.05$ and ** $P < 0.01$ vs. normoxic mice fed AL. # $P < 0.05$ and ## $P < 0.01$ vs. RD mice. †† $P < 0.01$ vs. normoxic RD mice treated with acarbose (α -GI). LPO: lipid peroxide, RD: restricted diet, AL: ad libitum, α -GI: alpha-glucosidase inhibitor.

Fig. 3. Effect of intermittent hypoxic stress on heart rate (A) and systolic blood pressure (B). Columns and bars represent the mean $\pm$ S.E.M. AL: ad libitum, RD: restricted diet, α -GI: alpha-glucosidase inhibitor.

TNF- α mRNA expression

In RD mice, intermittent hypoxia increased the expression of TNF- α mRNA. This increase was significantly reduced by treatment with acarbose (Fig. 8).

Discussion

In this study, we found that intermittent hypoxic stress accelerated pathological changes caused by PPH in lean mice. Acarbose attenuated these pathological changes in the LV myocardium by suppressing PPH and oxidative stress. These findings support the use of treatment strategies including acarbose aimed at preventing cardiovascu-

lar complications in diabetes.

The prevalence of SAS is increased in patients with type 2 diabetes (18), and intermittent hypoxia due to SAS may play a crucial role in the progression of diabetes mellitus (19–21). PPH is a primary characteristic of type 2 diabetes and is an independent risk factor for cardiovascular disease (22, 23). We have shown that continuous exposure to hypoxia for 2 weeks accelerated myocardial degeneration in a rat model of diabetes, in spite of weight loss due to a reduced food intake (24). In this study, restriction of food intake induced PPH, and intermittent hypoxia accelerated cardiomyocyte hypertrophy and interstitial fibrosis in lean mice. Ceriello et al.

Fig. 4. Representative light micrographs of LV myocardium from AL mice (a – d), RD mice (e – h), and RD mice treated with acarbose (α -GI) (i – l). Hematoxylin–eosin, original magnification $\times 200$; Sirius Red, original magnification $\times 100$. LV: left ventricular, AL: ad libitum, RD: restricted diet, α -GI: alpha-glucosidase inhibitor.

Fig. 5. Mean cardiomyocyte diameter (A) and percentage of interstitial fibrosis (B) in the LV myocardium. Columns and bars represent the mean $\pm$ S.E.M. * $P < 0.05$ and ** $P < 0.01$ vs. normoxic AL mice, ## $P < 0.01$ vs. normoxic RD mice, †† $P < 0.01$ vs. normoxic RD mice treated with acarbose (α -GI). LV: left ventricular, AL: ad libitum, RD: restricted diet, α -GI: alpha-glucosidase inhibitor.

suggested that oscillating levels of plasma glucose may impact on endothelial function and oxidative stress more than stable high glucose, and our results support theirs obtained with diabetic patients (25). Treatment with acarbose prevented these pathological changes in the LV

myocardium through the suppression of PPH and oxidative stress.

Several studies have found that intermittent hypoxic stress increases blood pressure in rats (26 – 29). In contrast, we previously reported that mean blood pressure

Fig. 6. NADPH-dependent superoxide production (A) and 4-HNE expression (B) in the LV myocardium. Columns and bars represent the mean $\pm$ S.E.M. $**P < 0.01$ vs. normoxic mice fed AL, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. normoxic RD mice, $^{\dagger\dagger}P < 0.01$ vs. normoxic RD mice treated with acarbose (α -GI). NADPH: reduced form of nicotinamide adenine dinucleotide phosphate, 4-HNE: 4-hydroxy-2-nonenal, LV: left ventricular, AL: ad libitum, RD: restricted diet, α -GI: alpha-glucosidase inhibitor.

Fig. 7. Immunohistochemistry for angiotensin II (A) and quantitative analysis (B) in the LV myocardium. Columns and bars represent the mean $\pm$ S.E.M. $**P < 0.01$ vs. normoxic AL mice, $^{\#}P < 0.05$ vs. normoxic RD mice, $^{\dagger\dagger}P < 0.05$ and $^{\dagger\dagger\dagger}P < 0.01$ vs. normoxic RD mice treated with acarbose (α -GI). LV: left ventricular, AL: ad libitum, RD: restricted diet, α -GI: alpha-glucosidase inhibitor.

was unchanged in mice exposed to intermittent hypoxia for 10 days (11). This apparent discrepancy may be due to differences in the duration of exposure to intermittent hypoxia and the species studied.

Consistent with our previous study, no differences were seen in heart rate or systolic blood pressure between

groups in this study, suggesting that pressure overload is not an important factor in the pathological changes detected in this model. Sympathetic nervous activity is also reported to increase under hypoxic conditions and is associated with increases in blood pressure (27, 29). No elevation was seen in heart rate or systolic blood pressure

Fig. 8. Expression of TNF- α mRNA (shown as TNF- α /18s ratio) in the LV myocardium by RT-PCR. Columns and bars represent the mean $\pm$ S.E.M. ** P < 0.01 vs. normoxic mice fed AL. †† P < 0.01 vs. normoxic RD mice treated with acarbose (α -GI). TNF: tumor necrosis factor, mRNA: messenger ribonucleic acid, LV: left ventricular, RT-PCR: reverse transcription polymerase chain reaction, AL: ad libitum, RD: restricted diet, α -GI: alpha-glucosidase inhibitor.

in this study, however, so further investigation is needed to determine the role of sympathetic activity in mice exposed to intermittent hypoxia.

Oxidative stress arises from an imbalance between the generation and elimination of ROS and is involved in the progression of cardiovascular diseases such as heart failure and atherosclerosis (30, 31). In this study, we detected elevation of plasma LPO levels and increased myocardial 4-HNE expression in RD mice exposed to intermittent hypoxia. Hyperglycemia enhances ROS production through several mechanisms, including defective mitochondrial metabolism (32) and the formation of AGE products (33). Activation of NADPH oxidase through the protein kinase C pathway has been shown to be a major source of ROS production in response to high glucose levels (34, 35). Guzik et al. reported significantly increased NADPH oxidase activity and protein levels in the venous and arterial walls of patients with diabetes (36).

On the other hand, Chen et al. reported increased oxidative stress in the heart tissue of rats exposed to intermittent hypoxia (37). We previously reported that hypoxic stress enhances NADPH-dependent superoxide production in the aorta (16) and LV myocardium (15). In this study, intermittent hypoxia produced a significant

increase in NADPH-dependent superoxide production in RD mice, suggesting that intermittent hypoxia further exacerbates oxidative stress due to PPH via the activation of NADPH oxidase, at least in part. In addition, we found that acarbose decreased NADPH-dependent superoxide production in RD mice exposed to intermittent hypoxia. Liao et al. previously reported that control of PPH by α -glucosidase inhibitor therapy suppressed cardiac remodeling in mice with LV pressure overload (38). Accordingly, the beneficial effects of acarbose in RD mice exposed to intermittent hypoxia may be due to its suppression of NADPH oxidase expression.

The proinflammatory cytokines TNF- α and angiotensin II have been implicated in the pathogenesis of cardiovascular disease and diabetes-related complications. They have also been reported to induce the generation of ROS through activation of NADPH oxidase, leading to oxidative stress in type 2 diabetes (39–41). A recent study found that serum levels of TNF- α are elevated in patients with SAS (42). In this study, intermittent hypoxia increased the expression of TNF- α in RD mice, an effect that was significantly attenuated by treatment with acarbose. Accordingly, TNF- α may enhance oxidative stress mediated by activation of NADPH oxidase in the presence of PPH and intermittent hypoxia.

Activation of the renin–angiotensin–aldosterone system has also been reported in patients with SAS (43), and plasma angiotensin II levels are increased under hypoxic conditions (44). In addition, increased angiotensin II type 1 (AT₁) receptor expression has been demonstrated in hypoxic heart tissue (45). In this study, angiotensin II expression in the LV myocardium was increased by PPH, an effect enhanced by exposure to intermittent hypoxia. This suggests that AT₁ receptor-dependent angiotensin II signaling contributes to PPH-induced pathological changes in lean mice exposed to intermittent hypoxia. Quantitative evaluation of the renin–angiotensin–aldosterone system, including AT₁ receptor expression, should be performed in this model.

In conclusion, intermittent hypoxic stress accelerated pathological changes caused by PPH in lean mice, and acarbose attenuated these pathological changes in the LV myocardium by suppression of PPH and oxidative stress.

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