Pioglitazone prevents cardiac remodeling in high-fat, high-calorie-induced Type 2 diabetes mellitus

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METHODS

Diabetic mouse model. Three- to four-week-old male C57BL/6J (B6) mice were obtained from Jackson Laboratories (Bar Harbor, ME). The C57BL/6J inbred mouse model is highly susceptible to cardiac fibrosis. Although there is significant cardiac fibrosis in diabetic hearts, the role of MMP in the development of cardiac fibrosis in diabetes is unclear.

EVIDENCE suggests a higher risk of heart disease associated with 5 μmol/l (plasma) increases in homocysteine (Hcy) among diabetic groups compared with nondiabetic groups (10). In a diabetic rabbit model, concentrations of plasma Hcy in the physiological range dramatically inhibited arterial nitric oxide (NO) formation but had no effect in nondiabetic animals (27), suggesting a role of Hcy in diabetes. However, in an experimental study of insulin-resistant (obese) Type 2 diabetes, improvement of insulin resistance with intravenous insulin over a period of 17 days did not alter Hcy levels (24). This suggests that insulin reduces the circulating levels of other amino acids (3) and may promote uptake of Hcy into tissues, which results in lower plasma Hcy but increased tissue Hcy (2).

PEROXISOME proliferator-activated receptor-γ (PPARγ) influences insulin sensitivity and diabetic complications. Recent studies (18) suggest a role of PPARγ as an inhibitor of cardiac hypertrophy. In addition, PPARγ agonist improves left ventricular (LV) diastolic function, decreases collagen accumulation (fibrosis) in diabetic rats (28), and protects the myocardium from ischemic injury (1). Therefore, glitazones (PPARγ activator) may decrease cardiac fibrosis and MMP activation in diabetes. In addition, PPAR, upon induction, promotes the synthesis of superoxide dismutase and catalase. Meanwhile, PPAR decreases NADH oxidase. Therefore, high levels of Hcy are associated with increased reactive oxyradical species generation. There is evidence that in contrast to PPARα, which is abundant in vascular cells, PPARγ is abundant in cardiomyocytes (11), and thus treatment with glitazones may favorably alleviate the diabetes-mediated oxidative stress response in the heart, thereby protecting the cardiomyocytes. Hcy causes cardiovascular dysfunction by decreasing NO availability, resulting in MMP activation (12). Although several lines of evidence indicate robust MMP activation in diabetes, the role of tissue Hcy in decreased NO and increased MMP activation in E-M coupling is largely unknown. We hypothesize that increased LV tissue Hcy is associated with MMP-9 activation, E-M uncoupling, and remodeling, secondary to suppressing PPARγ responses in Type 2 diabetes mellitus.
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HOMOCYSTEINEMIC MECHANISM OF DIABETES

diet-induced Type 2 diabetes mellitus, central obesity, atherosclerosis, hyperglycemia, hyperinsulinemia, hyperhomocysteinemia, and hypertension (5, 23, 29, 30). The diet (45 kcal% fat) contains 45 kcal% fat, 35 kcal% carbohydrate, and 20 kcal% protein (Research Diets, New Brunswick, NJ). The normal diet has 10 kcal% fat, 70 kcal% carbohydrate, and 20 kcal% protein. These diets differ in terms of calories, i.e., fat diet contains 4.73 kcal/g and normal diet contains 3.85 kcal/g. The high-fat and high-calorie intake disturbs the glucose homeostasis and leads to diabetic complications. The mice were given the following diets for 6 wk according to the following groups: normal diet (N), normal diet with pioglitazone (N + Pi), diabetes fat diet (D), and fat diet with Pi (D + Pi).

Mice were fed rodent chow, and to induce PPARγ activation, Pi (50 µg/g food; CalBiochem) was administered. Based on the fact that mice eat ~4 g food/day, we estimated that each ingested ~200 µg/day Pi. The binding constant between Pi and PPARγ is in the micromolar range (20). Therefore, dietary consumption of Pi to produce a blood concentration of ~32 µmol/l was enough to saturate most binding sites on PPARγ. Others showed that 100 mg/day PPARγ agonist has a potent agonist effect (8). Because humans weigh ~75 kg and mice weigh ~25 g, we estimated that mice ingested approximately sixfold higher Pi than humans. To determine whether Pi treatment caused changes in food intake, food and water intake was measured every 2 days during the treatment period, and no changes in intake were found. The mice were euthanized at 6 wk after the start of the treatment. To determine whether there was peroxisome proliferation, the livers were weighed at the end of the protocol. Animal room temperature was maintained between 22°C and 24°C. A 12-h:12-h light-dark cycle was maintained by artificial illumination. In accordance with National Institutes of Health Guidelines for animal research, all animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University Of Louisville School Of Medicine.

Glucose, Hcy, and insulin. At the end of the protocol, plasma glucose and Hcy levels were measured by collecting 1 ml blood in heparinized tubes from anesthetized mice via a PE-10 catheter in the right common carotid artery. Glucose was measured using a Bio-Rad glucose measurement kit. Hcy was separated by HPLC and detected by a spectrophotometer (33). Plasma insulin was measured with the use of a K-Assay kit, Mouse Insulin ELISA Kit, Cat. No. KG-011 (Kamiya Biomedical, Seattle, WA).

Tissue NO and Hcy. Fresh LV tissue was homogenized in 50 mM Tris·Cl (pH 7.4) buffer. Colorimetric-based measurements of nitrite and nitrate, the stable end products of NO metabolism, were used for assessment of NO production. Total nitrite production was measured with a commercial colorimetric assay (2.5 µM, detection limit, Cayman Chemical) by the Greiss method. Total Hcy, protein bound and free, was measured as previously described (33).

Urinary protein. Mouse urine has large quantities of proteins [primary mouse major urinary protein (MUP)], and males have much higher MUP than females. Only male mice were used. To determine multorgan damage in diabetes, MUP was measured. To collect urine, the mice were caged in 24-h metabolic cages for several days of acclimatization to reduce separation effects, before hemodynamic measurements. MUP was measured by using the Bio-Rad dye binding assay.

Zymographic analysis of MMP activity. To determine MMP-2, -7, and -9 activities, gelatin substrate gel zymography, containing 1% gelatin in 8% SDS-PAGE, was performed (33). The LV tissue homogenates were loaded onto the gel with identical total protein concentration. The bands were scanned with a Bio-Rad GS-700 densitometer with band intensity normalized to β-actin.

Echocardiography and hemodynamic parameters. Mice were anesthetized with tribromoethanol (100 mg/kg ip), which has minimal effects on cardiovascular function in mice (21). To determine in vivo cardiac diastolic relaxation, M-mode echocardiography (Philips SONO-5500) was performed. To determine cardiac relaxation, a pressure-tipped Millar catheter was inserted into the LV through the right common carotid artery. The aortic blood pressure, heart rate, and blood pressure were measured. After arterial pressure measurements were taken, the catheter was advanced to the LV, and LV systolic pressure, end-diastolic pressure, and the first derivative of the rise or fall in LV pressure (±dP/dt) were measured. The pulsatile arterial pressure signal at the level of heart was analyzed by using a computer with customized software (Micro-Med).

Preparation of ACh and nitroprusside solutions. The concentrations of Ach (10−10 to 10−4 M) and nitroprusside (10−9 to 10−4 M) were based on weight measurements. All dilutions from stock solutions in PBS were made before the experiment. PBS was used as a vehicle control.

Endocardial E-M function. The doughnut-shaped LV rings were prepared by sectioning the heart transmurally (1–2 mm thick). To prepare LV rings, the right ventricular (RV) wall was removed. To prepare RV rings, the LV wall was removed. The LV rings were mounted on a polygraph in a tissue myobath. One of the two mounted wires was connected to a force transducer. The ring was stretched and brought to resting tension at which the 20 mM of CaCl2 were added. At the maximum CaCl2 contraction, ACh (endothelial-dependent) or nitroprusside (endothelial-independent) was added. To minimize the differences due to the orientation of cardiac muscle, the rings were rotated 90°, and contraction was measured again. The average of these two contractions was recorded. The percent relaxation was calculated based on 100% contraction to 20 mM CaCl2, and concentration-response curves were generated. To avoid ischemia, 95% O2–5% CO2 was continuously bubbled through the myobath. This was sufficient for penetrating the cardiotoxic agent into the tissue (32). The experiments were completed within 40 min to minimize injury due to ischemia (32).

Statistical analysis. Values are given as means ± SE from n = 6 mice in each group. Differences between groups were tested using a two-way ANOVA, followed by the Bonferroni post hoc test (31), focusing on the respective effects of diabetes, comparing D with N and D + Pi with D. P < 0.05 was considered significant.

RESULTS

Hemodynamic and gravimetric parameters. Although there was no change in body weight with or without the Pi treatment in N mice, the caloric intakes and growth rate (body weight) were higher in high-fat diet compared with control diet. This was associated with an increase in blood pressure. The MUP was significantly increased in the D group compared with the N group. The treatment with Pi normalized the blood pressure and MUP in the D group with no effect on body weight (Table 1). To confirm the Type 2 diabetes nomenclature, we looked at insulin sensitivity in this model. The levels of insulin were increased 2.5-fold in D groups (Table 1).

Liver proliferation. Although we did not measure blood concentration of Pi, we measured liver proliferation as an index of peroxisome proliferation. The ratio between liver weight and body weight was lower in the D group compared with the N group. However, the ratio increased in the D + Pi group compared with the D group (Table 1). The results suggested liver proliferation after Pi treatment.

Plasma levels of glucose and Hcy. Plasma glucose levels increased in the high-calorie D group (126 ± 4 in D group vs. 84 ± 1 mg/dl in N group, P < 0.01). On the other hand, the group that was fed the high-fat calorie diet plus Pi did not experience increased plasma glucose levels. Plasma Hcy levels were increased in the D group (18 ± 5 in D and 2.5 ± 2.0...
Values are means ± SE. Pi, pioglitazone; BW, body weight; HW, heart weight; KW, kidney weight; L/BW, liver-to-BW ratio; MAP, mean arterial pressure; SBP and DBP, systolic and diastolic blood pressure, respectively; HR, heart rate; EDP, end-diastolic pressure; LVP, left ventricular (LV) systolic pressure; dP/dt, first derivative of rise (↑) or fall (↓) in LV pressure; FS, LV fractional shortening, measured by echocardiography; MUP, major urinary protein. *P values compare diabetic + Pi group with diabetic group.

μmol/l in N groups, P < 0.01), and Pi had no effect on the plasma Hcy levels (Fig. 1, A and B).

LV tissue levels of Hcy and NO. LV tissue Hcy levels were increased in D mice (12 ± 1 of protein in D group vs. 3.0 ± 0.5 ng/mg of protein in N group, P < 0.01). Treatment with Pi ameliorated the increase in LV tissue Hcy levels. LV tissue NO levels were decreased in the D group compared with the N group, whereas the LV tissue NO levels in the group treated with Pi were normal (Fig. 1, C and D).

MMP activity. Plasma and LV tissue levels of MMP-2 and MMP-9 activities were higher in the D group than in the N group and were normalized after treatment with Pi. The levels of MMP-7 were decreased in the D group compared with the N group (Fig. 2). We measured MMP activity with or without the effects of Pi in numerous rounds of separate gel experiments. The results showed a consistent decrease in MMP activity in the D + Pi group. Figure 2B shows accumulative data from zymographic gels of LV tissue homogenates. The data represent total MMP activity (pro- and cleaved forms). The changes in zymographic activity resulted from an increase in expression as well as an increase in activation.

E-M coupling. The basal tissue levels of NO may not reflect stimulated levels in response to ACh. Therefore, the LV relaxation to ACh was dose dependent (Fig. 3). Treatment with Pi, and D.

![Plasma](A)

![LV](B)

![Hcy](C)

![NO](D)

**Fig. 1.** Plasma levels of glucose and homocysteine (Hcy). A: mice were made diabetic (D) by feeding 45 kcal% fat diet. Control (N) mice received 10 kcal% fat diets. Peroxisome proliferator-activated receptor-γ was induced by pioglitazone (Pi) in diet for 6 wk. Mice were grouped into N, D, N + Pi, and D + Pi. Blood was taken from tail vein. Fasting levels of plasma glucose were measured by standard kit (test strips). B: levels of Hcy were measured by HPLC and spectrophotometry. A and B: *P < 0.01 compared with N; **P < 0.05 compared with D. C: left ventricular (LV) homogenates were prepared, and total Hcy was extracted, separated by HPLC, and quantitated by spectrophotometry. Hcy (in ng/mg of protein) was expressed. *P < 0.01 compared with N; **P < 0.02 compared with D. D: total nitrate/nitrite was measured by Greiss method. Nitric oxide levels (in μmol/l of LV homogenate) were expressed. Identical amounts of total protein were used for each sample. *P < 0.02 compared with N; **P < 0.01 compared with D; n = 6 mice in each group. Note that tissue levels of Hcy were normalized by Pi treatment.
Pi ameliorated the diabetes-mediated impairment in relaxation to ACH. Relaxation to sodium nitroprusside (SNP) was also attenuated in D versus N mice (Fig. 3). The results suggested that Pi ameliorated the E-M uncoupling associated with diabetes mellitus.

**Cardiac muscle function.** Both the in vivo rate of cardiac diastolic relaxation and the rate of cardiac systolic function were decreased in D mice compared with N mice. Treatment with Pi restored this impairment (Fig. 4), suggesting that in diabetes there is impairment of the endothelial-dependent cardiac relaxation and that treatment with the PPARγ agonist ameliorates this impairment.

**Echocardiography of mice.** The M-mode echocardiographic data indicated an increase in cardiac diameter and decrease in LV wall thickness in diabetes mice. Pi normalized the LV wall thickness and diameter in diabetes (Fig. 5). Although we observed temporal incremental changes in metabolic syndrome, such as increases in body weight and changes in LV parameters, here, we reported the changes only at 6 wk. To correlate well with the contractility measurements in LV rings, we estimated the fractional shortening (FS) from echocardiodynamics. Even though this parameter is a load-dependent variable, the data suggest decreased FS in diabetes compared with controls (Table 1). Treatment with Pi mitigates this decrease in FS.

**DISCUSSION**

In this study, we found that treatment with Pi, a PPARγ activator, ameliorated diabetes-associated increases in blood pressure and decreased hyperglycemia yet had no effect on
plasma Hcy in high-fat calorie, diet-induced diabetes mellitus. In addition, our findings suggest that in diabetes there are increases in plasma levels of Hcy as well as tissue Hcy levels that are associated with decreased tissue NO levels. This suggests that high-fat calorie diet impairs LV Hcy metabolism in diabetes mellitus and causes an increase of Hcy levels in cardiac tissue. Interestingly, treatment for 6 wk with a PPARγ agonist Pi normalized these conditions in tissue but not in plasma. This suggests that normally there is basal constitutive activity of PPARγ activity and that Pi does not cross this normal threshold of PPARγ activity. Conflicting studies (24) show increases as well as decreases in plasma Hcy in diabetes. However, we observed increased plasma and LV tissue Hcy in high-fat calorie-induced Type 2 diabetes. Pi decreased LV Hcy levels and had no effect on plasma Hcy. Because plasma Hcy reflects a contribution from multiorgans, it cannot predict specific changes in tissues. On the other hand, Pi has a specific role in cardiac tissue Hcy metabolism.

Fig. 4. Cardiac muscle function. Typical LV pressure wave in N mice (A) and D mice (B). C: diastolic relaxation. D: systolic function. 

Fig. 5. A: M-mode echocardiogram of N, D, and D + Pi mice. B: LV wall-to-LV diameter ratio for all 4 groups. *P < 0.02 compared with N; **P < 0.05 compared with D; n = 6 mice in each group.
There is an association between Hcy and NO levels. The causal associations are assumed but not proven. However, Hcy levels were inversely related to NO levels. Therefore, the LV relaxation to ACh is attenuated in the myocardium in diabetes. These results suggest that in high-fat calorie-induced diabetes, there is an Hcy-mediated endothelial-dependent myocyte uncoupling. Treatment with a PPARγ agonist ameliorates the diabetes-induced cardiac dysfunction (such as the E-M uncoupling), in part by decreasing Hcy and MMP-9, which increases cardiac NO availability.

PPARα and -γ are downregulated in streptozotocin-induced Type 1 diabetes in rats and are associated with decreased endothelial function (13). Pi can reverse the decrease in aortic endothelial NO synthase that occurs in fructose-fed, salt-sensitive hypertensive rats (19). We suggest that decreased NO in diabetes is associated in part with increases in LV Hcy levels. Previously, Tyagi and colleagues (12, 16) showed that increased MMP activity was associated with decreased NO and increased Hcy levels. The literature (4) presents evidence that increased levels of MMP-9 activity are associated with endocardial endothelial apoptosis and therefore contribute to cardiac dysfunction in Type 2 diabetes. Cardiac remodeling during a hyperglycemic state is likely associated with glucotoxicity, endothelial dysfunction, and collagen turnover. MMP-9 can specifically induce apoptosis of endothelial cells in diabetes (4). In patients with diabetes, it has been shown that high glucose alters MMP expression in vascular cells (7), as well as in smooth muscle cells (9). Another study with patients with Type 2 diabetes revealed increased levels of MMP-9 that were significantly reduced after treatment with PPARγ agonist (14). We found that plasma levels of MMP-2 and MMP-9 activities were increased in D compared with N groups. These activities were normalized after Pi treatment. Our findings using Pi may offer a mechanism to reduce or eliminate the detrimental effects produced via elevated levels of MMP-9. Importantly, PPARγ signaling could attenuate cardiac remodeling in diabetes via pathways such as Hcy-MMP activation.

Insulin resistance has been implicated in the development of myocardial remodeling. Pi, acting through increasing PPARγ activity, has insulin-sensitizing properties and may exert an antifibrotic effect in cardiac remodeling by sensitizing insulin receptors as an upstream target. Experimental studies of the effects of PPARγ activators on the heart have demonstrated an inhibition of cardiac hypertrophy (18, 34), improved LV diastolic function, decreased collagen accumulation (fibrosis) in diabetic rats (28), and protection of the myocardium from ischemic injury (1). Our study strengthens these reports by showing that cardiac diastolic and systolic function (i.e., E-M coupling) decreased in diabetic compared with the nondiabetic mice. The treatment with Pi augmented this impairment, in part, by decreasing fibrosis and MMP activation.

The determination of endothelial function in isolated papillary muscle preparation does not demonstrate what happens in the entire transmural myocardial wall. To determine cardiac function, the Langendorff preparation has been used. However, this does not differentiate the specific contribution due to regional ischemia, hypertrophy, stunning, and/or hibernation of myocytes in the myocardial wall. Rather, it indicates the global contractile response to cardiotonic agents. Furthermore, it does not separate the effects of LV from RV. We compared data obtained from cardiac rings prepared from hypertensive rats (17) and found similar pressure-volume curves as obtained by the Langendorff preparation. In addition, the cardiac ring preparation separated the effect of LV from RV. To determine specific regional differences in contractile function, rings can be prepared to include or to exclude the homogeneous or nonhomogeneous regions of the transmural myocardial wall (6, 15, 32).

This study presents four novel findings. First, tissue levels of Hcy are highly accurate in predicting endothelial dysfunction in diabetic hearts (Fig. 1). Second, although numerous studies have suggested impaired diastolic and systolic function in Type 2 diabetes, there is lack of information regarding the diabetic response to stress in the heart. Our data suggest that the cardiac rings from control mice responded significantly to stress by relaxing to basal levels. Conversely, rings from diabetic hearts had a diminished overall relaxation. Although contractile responses were similar in control and diabetic hearts, ACh-induced dilation was attenuated in diabetic hearts. These results suggest a diminished NO-mediated relaxation in the hearts from high-fat calorie-induced Type 2 diabetes (Fig. 3). The treatment with PPARγ augmented the impaired cardiac relaxation in diabetic hearts. Third, the data with nitroprusside also suggest myocytic cell dysfunction in diabetes. The treatment with PPARγ agonist ameliorated the Hcy metabolic derangement and E-M uncoupling in diabetes (Fig. 3D). Finally, PPARγ agonists ameliorated the Hcy metabolic derangement and E-M uncoupling in diabetes (Fig. 4).

Our observations of attenuated relaxation in cardiac rings from diabetic mice were ameliorated by Pi treatment and support a significant role for PPARγ in reducing metabolic dysfunction associated with Type 2 diabetes. This study suggests that a chronic high-fat calorie diet leads to metabolic derangement in the LV that increases Hcy, causing decreased NO in LV and E-M coupling. The treatment with Pi ameliorates the E-M uncoupling and diastolic impairment in high-fat calorie-induced Type 2 diabetes mellitus.

There was a significant increase in blood pressure in diabetes. It is known that increased blood pressure causes diastolic pressure overload heart failure and instigates cardiac remodeling. The treatment with Pi ameliorated both the increase in blood pressure and cardiac remodeling.

**Limitations.** The inner lining of the endocardium is the endothelium. Moreover, toward the end of a capillary, the endothelium is embedded into the muscle. Therefore, most of the cardiac relaxation is normally endothelial dependent. However, this does not suggest that other dilatory mechanisms, such as prostaglandins or endothelial-derived hyperpolarizing factor, are not involved. The ACh dose-response curve suggests that at a lower dose of ACh, other mechanisms may be important.

We may not truly demonstrate E-M uncoupling. However, because every cardiomyocyte is surrounded and embedded with four to five capillary endothelium, endothelium may therefore control the cardiac relaxation by coupling with the muscle during systolic/diastolic cycle. We measured endothelium-dependent cardiac relaxation in a cardiac ring preparation.

Paradoxically, the SNP data seem a little counterintuitive, because one might expect that in the face of reduced tissue levels of NO, the LV would be more sensitive to exogenously administered NO in the form of SNP. However, this may only be true if the number of myocytes increased. In contrast,
myocyte response to SNP is decreased in diabetes. This may suggest a decreased number of myocytes. The treatment with Pi mitigates the decrease in SNP response (Fig. 3D).

The decrease in diabetes-induced systolic and diastolic function may be just as likely due to changes in LV wall thickness and chamber diameter as well as changes in Ca2+ homeostasis than just due to impairment of endothelial-dependent cardiac diastolic relaxation.

To determine the receptor-mediated responses in cardiac rings, future studies in LV rings should be repeated in rings precontracted by a receptor-mediated agonist, such as isoproterenol or ANG II.

GRANTS

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