The Effect of Nitric Oxide Synthase Inhibitor on Improved Insulin Action by Pioglitazone in High-Fructose–Fed Rats

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The present study was performed to investigate whether nitric oxide synthase (NOS) inhibition influences the increased whole-body insulin action by pioglitazone in high-fructose–fed rats. Male Wistar rats aged 6 weeks were randomly divided into 3 groups and each group was fed one of the following diets for 3 weeks: standard chow diet (control group), high-fructose diet (fructose-fed group), and high-fructose diet plus pioglitazone (pioglitazone-treated group). The control and pioglitazone-treated groups were further divided into 2 subgroups respectively, and some rats of each subgroup were infused the NOS inhibitor, N^G-monomethyl-L-arginine (L-NMMA), during the euglycemic clamp studies. In vivo insulin action was determined by the 2-step (3 and 30 mU/kg body weight [BW]/min low- and high-dose, respectively) hyperinsulinemic euglycemic clamp procedure in the awake condition. Glucose infusion rate (GIR) was considered as the index of insulin action. Endothelium-type NOS (eNOS) and inducible NOS (iNOS) in skeletal muscle were also measured. At the low-dose clamp, high-fructose feeding produced a marked decrease in GIR compared with the control group. Pioglitazone-treated animals showed a significant increase in GIR, reaching a similar level as the control group. However, the improved GIR was decreased to the level of the fructose-fed group by L-NMMA infusion. The GIR of the control group was not affected by L-NMMA infusion. The same tendency as the low-dose clamp was found at the high-dose clamp. In skeletal muscle, eNOS and iNOS protein content were not affected by high-fructose feeding and/or pioglitazone treatment. These results suggest that NOS inhibition can decrease the improved insulin resistance by pioglitazone in high-fructose–fed rats. Therefore, although NOS protein content is not changed by high-fructose feeding and/or pioglitazone treatment, it could be concluded that nitric oxide (NO) plays an important role in the improvement of insulin action by pioglitazone.

INSULIN RESISTANCE is a common metabolic abnormality associated with hypertension, obesity, and type II diabetes.[1] Thiazolidinedione (TZD), including pioglitazone, is known as an antidiabetic agent that enhances insulin-stimulated glucose uptake in vivo and lowers blood glucose, triglyceride, and blood pressure in insulin-resistant humans as well as animals.[2] These drugs are believed to act by stimulating the peroxisome proliferator-activated receptor gamma (PPARγ) in fat cells. Recently, it has been reported that the antidiabetic action of these drugs occurs independently of their effect on adipose tissue,[3] and that direct skeletal muscle incubation with TZD affects metabolism independently of PPARγ.[4] Although TZD therapy has beneficial effects for the treatment of insulin resistance, the precise mechanisms are not yet clear.

Nitric oxide (NO) is a messenger molecule that plays an important role in a wide variety of physiological functions, including hemodynamics, glucose uptake, and anti-inflammatory action. Dilation of isolated skeletal muscle arterioles by insulin is endothelium-dependent and NO-mediated,[5] and insulin-stimulated glucose uptake in vivo is NO-dependent.[6] NO synthase (NOS) is expressed in skeletal muscle,[7] and NO also stimulates in vitro glucose uptake in this tissue.[7,8] Several studies have shown that NO is associated with insulin resistance.[9-12] Defective insulin-stimulated and endothelium-dependent vasodilation and accompanied redistribution of blood flow and detrimental insulin-stimulated glucose uptake are observed in insulin-resistant mellitus such as obesity and type II diabetes. Endothelium-type NOS (eNOS) knockout mice exhibit not only hypertension, but also hyperinsulinemia, hyperlipidemia, and an impairment in the insulin-stimulated glucose uptake.[13]

It is well known that high-fructose feeding leads to peripheral insulin resistance,[14-22] and that TZD enhances insulin action and corrects metabolic disturbance in rats fed this diet.[16-18] TZD also restores blunted endothelium-dependent vasodilation in high-fructose–fed rats.[17] Our previous report has shown that NO donor administration improves insulin-stimulated whole-body glucose uptake in insulin-resistant rats induced by high-fructose diet.[22] These results suggest that NO might have an important role in the improvement of insulin action by TZD administration in high-fructose–fed rats. However, there are no available data that prove the hypothesis that NO contributes to the insulin-sensitizing action by pioglitazone in vivo.

To test this possibility, we examined the effect of NOS inhibition on the improved insulin action by pioglitazone in high-fructose–fed rats.

MATERIALS AND METHODS

Treatment of Animals

All experimental procedures were in accordance with the Guide for the Care and Use of Laboratory Animals of Nagoya University. Male Wistar rats (Chubu Kagakushizai, Nagoya, Japan) were obtained at 6 weeks of age and maintained in a 12:12-hour reversed light-dark environment at an ambient temperature of 23°C and with ad libitum access to food and water.

After a 1-week acclimation period, rats were divided into 3 groups,

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Table 1. Body Weights, Blood Glucose, and Plasma Insulin Concentrations Before and Immediately After the Euglycemic Clamp Procedure at Low-Dose (3 mU/kg BW/min) and the High-Dose (30 mU/kg BW/min) Insulin Infusions

	Body Weight (g)	Blood Glucose (mg/dL)			Plasma Insulin (μU/mL)		
		Basal	Low-Dose	High-Dose	Basal	Low-Dose	High-Dose
Control	262 ± 3	74 ± 2	81 ± 2	75 ± 2	6.8 ± 0.6	21.0 ± 1.5	630.4 ± 40.6
Control + L-NMMA	268 ± 4	72 ± 2	72 ± 4	83 ± 2	7.8 ± 1.1	31.1 ± 6.0	741.3 ± 110.9
Fructose	267 ± 2	72 ± 2	74 ± 2	70 ± 2	6.6 ± 1.2	26.4 ± 3.1	710.4 ± 70.3
Fructose + pioglitazone	270 ± 3	76 ± 1	82 ± 1	71 ± 1	7.2 ± 1.2	31.8 ± 3.5	681.5 ± 39.3
Fructose + pioglitazone + L-NMMA	272 ± 5	73 ± 3	76 ± 4	77 ± 5	9.4 ± 1.5	27.0 ± 4.1	808.4 ± 86.6

NOTE. Values are means ± SEM (n = 5-11).

and each group was fed one of the following diets for 3 weeks: (1) standard chow diet (MF, Oriental Yeast, Chiba, Japan), control group; (2) high-fructose diet (AIN-93M, Oriental Yeast), fructose-fed group; and (3) high-fructose diet plus pioglitazone (Takeda Chemical Industries, Tokyo, Japan), pioglitazone-treated group. The control and pioglitazone-treated groups were further divided into 2 subgroups respectively, and some rats of each subgroup were infused the NOS inhibitor, N^G-monomethyl-L-arginine (L-NMMA, Calbiochem, San Diego, CA) during euglycemic clamp studies. The caloric composition of MF is, as percent of total calories, 59% carbohydrate, 29% protein, and 12% fat. The high-fructose diet contained 60% carbohydrate, 28% protein, and 12% fat. Pioglitazone was given as a food mixture (0.015% wt/wt).

After 2 weeks on the respective diets, rats of each group were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight [BW]). The right jugular vein and the left carotid artery were cannulated with Silascon catheters (Kaneka Medix, Osaka, Japan). The catheters were tunneled subcutaneously to the back of the neck and were infused with 500 μL sodium penicillin G (10,000 U/mL). They were then filled with polyvinylpyrrolidone and sealed at one end until euglycemic clamp studies. After surgery, rats were kept at the same conditions as the preoperative states.

Euglycemic Clamp Study

Animals were allowed to recover from surgery for 6 days, and then a 2-step hyperinsulinemic euglycemic clamp procedure[23] was performed in the morning after a 16-hour overnight fast. Throughout the study, the rats were allowed to move freely within a large cage. Animals were allowed to rest 60 minutes before the initial blood sample was obtained. A continuous intravenous infusion of insulin was started at a rate of 3 mU/kg BW/min (low-dose clamp) for 90 minutes (0-90 minutes) and 30 mU/kg BW/min (high-dose clamp) for the subsequent following next 90 minutes (90-180 minutes). Assigned rats were infused with L-NMMA at a rate of 1 mg/kg BW/min during the euglycemic clamp study. This dosage was expected to be sufficient to inhibit NOS.[24] The other rats of the remaining groups were infused with saline. During the clamp study, blood glucose concentrations were measured every 10 minutes and maintained at the basal level with a variable infusion of glucose. Glucose infusion rate (GIR) for the low- and the high-dose clamps were calculated based on the glucose infusion during the last 30 minutes of each clamp and were regarded as indices of insulin action in peripheral tissues. After the completion of the clamp, animals were anesthetized intravenously with 20 mg/kg BW of sodium pentobarbital injection via vein. Plantaris muscles were dissected out and snap-frozen with liquid nitrogen. Frozen samples were kept at −70°C until analysis.

The blood glucose concentration was determined with a YSI 2300 STAT glucose analyzer (Yellow Springs Instrument, Yellow Springs, OH). The plasma insulin level was assayed using a radioimmunological assay kit (Phadeseph Insulin RIA, Pharmacia, Stockholm, Sweden).

Nitric Oxide Synthase Protein Content

The plataris muscles were homogenized (1:10) in a 25 mmol/L HEPES buffer containing 2 mmol/L EDTA, 250 mmol/Lsucrose, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 7 μmol/L leupeptin, and trypsin inhibitor (0.1mg/mL), pH 7.4. After homogenization, samples were centrifuged at 175,000 × *g* for 60 minutes at 4°C to obtain supernatants and pellets (membrane fraction). Pellets were solubilized in homogenation buffer with 0.4 % sodium dodecyl sulfate (SDS) and without sucrose for 4 hours on ice. The aliquots of resulting suspensions were added to Laemmli sample buffer and were stored as samples for measuring eNOS and neural-type NOS (nNOS).

Supernatants containing 1 mg protein were incubated overnight with 1.25 μg of inducible NOS (iNOS) antibody (Transduction Laboratories, Lexington, KY) at 4°C. Fifty percent suspensions of protein A agarose beads (Upstate Biotecnology, Lake Placid, NY) in homogenate buffer (20 μL) were added to the samples and were incubated for an additional 4 hours at 4°C. Beads were washed 3 times with homogenation buffer. After the final wash and brief centrifugation, the precipitated beads were resuspended in Laemmli sample buffer and were stored as samples for measuring iNOS.

Protein concentrations were assayed by a dye-binding procedure (Bio-Rad, Hercules, CA) with fatty acid–free bovine serum albumin as standard. Samples were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) with a 7.5% polyacrylamide resolving gel and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes for 2 hours. Blots were then blocked with 3% (wt/vol) bovine serum albumin (BSA) for 2 hours at room temperature, and incubated overnight with polyclonal anti-eNOS (1:500, Calbiochem), anti-nNOS (1:500, Calbiochem), or anti-iNOS (1:500) antibodies at 4°C, followed by a 2-hour incubation with horseradish peroxidase-conjugated anti-rabbit IgG (Bio-Rad) at room temperature. Immunoreactive bands were detected by enhanced chemiluminescence reagent (Amersham, Chicago, USA) and subsequently analyzed by densitometry.

Statistical Analysis

Data are presented as means ± SEM. Significant differences among groups were evaluated using 1-way analysis of variance (ANOVA). When ANOVA showed significant differences, post-hoc analysis was performed by the Scheffe's method. Difference were considered statistically significant when $P < .05$.

RESULTS

Body Weight and Food Intake

The final body weights of the rats in the 5 groups were comparable throughout the study, as shown in Table 1. Although body weight decreased in all rats after the surgery for cannulation, it returned to preoperative levels within 5 days.

Pioglitazone administration produced no significant rise in body weight. Food intake throughout the study was comparable among the groups. The rats treated with pioglitazone consumed the medicine at about 12 mg/kg BW/d.

Whole-Body Insulin Action

As shown in Table 1, mean blood glucose and plasma insulin levels were not different among the groups before (fasting state) and during the euglycemic clamp study. Insulin levels reached physiological and supraphysiological levels at low- and high-dose clamp, respectively.

A plateau GIR was achieved in each animal for the last 30 minutes during both the low- and high-dose clamp studies (Fig 1). At the low-dose clamp (Fig 1A), high-fructose feeding produced a marked decrease in GIR compared with the control group (9.4 ± 0.2 *v* 5.5 ± 0.5 mg/kg BW/min; $P < .05$). Pioglitazone-treated animals showed a significant increase in GIR (9.1 ± 0.2 mg/kg BW/min; *v* fructose-fed group, $P < .05$) reaching a similar level as the control group. However, the improved GIR was decreased to the level of the fructose-fed group by L-NMMA infusion (4.7 ± 0.9 mg/kg BW/min; *v* pioglitazone-treated group, $P < .05$). The same tendency as the low-dose clamp was found at the high-dose clamp (Fig 1B). Compared with control group, high-fructose feeding reduced GIR (36.9 ± 0.8 *v* 31.5 ± 0.7 mg/kg BW/min; $P < .05$) and pioglitazone treatment improved GIR (39.6 ± 1.2 mg/kg BW/min; *v* fructose-fed group, $P < .05$). Increase in GIR by pioglitazone treatment was disappeared by L-NMMA infusion (29.5 ± 1.0 mg/kg BW/min; *v* pioglitazone-treated group, $P < .05$). The GIR of the control group was not affected by L-NMMA infusion during both the low- and the high-dose clamp studies (10.0 ± 1.2 and 36.6 ± 0.5 mg/kg, respectively).

Nitric Oxide Synthase Protein Content

High-fructose feeding and/or pioglitazone treatment did not affect eNOS protein expression in plantaris muscle (Fig 2A and B). The iNOS protein content was not expressed in skeletal muscle from all groups (Fig 3). The nNOS protein did not reach our detection level.

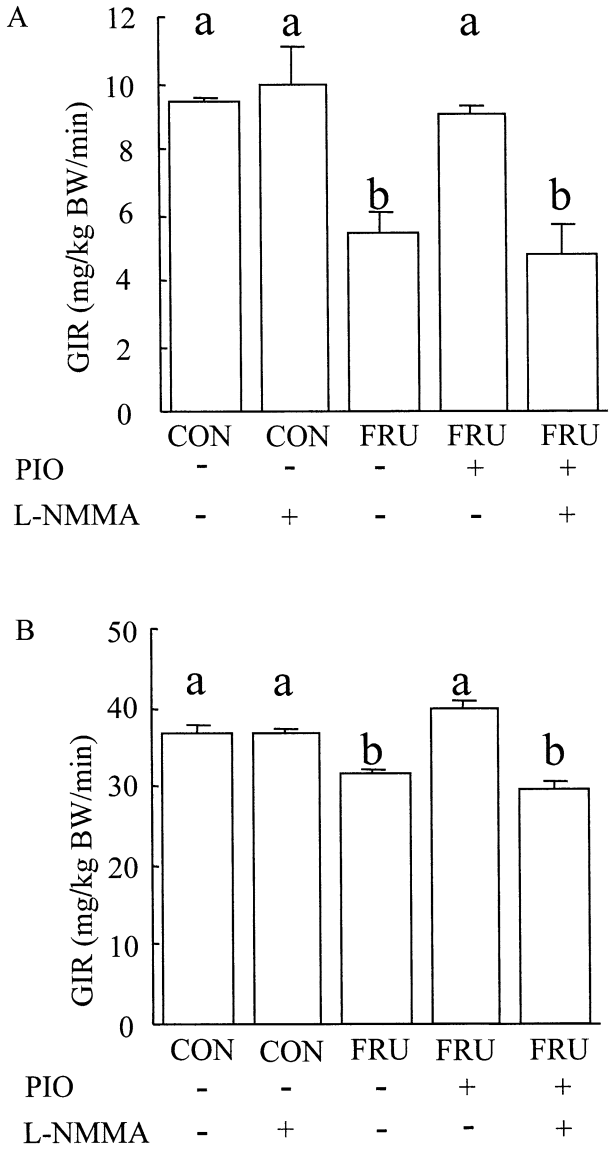

Fig 1. GIR during the euglycemic clamp procedure at (A) low-dose (3 mU/kg BW/min) and (B) high-dose (30 mU/kg/BW/min) insulin infusions. Rats were fed standard chow diet (CON), high-fructose diet (FRU), or FRU plus pioglitazone (PIO) for 3 weeks. L-NMMA was infused during the euglycemic clamp procedure. Data are presented as means ± SEM (n = 5-11). Values designated by different superscript letters are significantly different ($P < .05$).

DISCUSSION

The purpose of the present study was to examine the effect of L-NMMA, a NOS inhibitor, on insulin-sensitizing action by pioglitazone in high-fructose-fed rats. In vivo insulin action was measured by a 2-step hyperinsulinemic euglycemic clamp technique in chronically catheterized conscious rats. Our study showed that 3-week high-fructose feeding induced a marked insulin resistance and pioglitazone administration ameliorated the impaired insulin action. The major finding of this study was that the NOS inhibitor abolished improved insulin action by pioglitazone in the rats fed high-fructose.

Chronic high-fructose feeding results in impaired peripheral insulin resistance.[14-22] Tobey et al[14] reported that insulin resistance induced by high-fructose feeding was due to the diminished ability of insulin to suppress hepatic glucose output alone, and not to a decrease in insulin-stimulated glucose uptake by skeletal muscle. However, in their study the rats were fed high-fructose for only 7 days. High-fructose feeding for a longer period was shown decrease the action of insulin to stimulate tyrosine phosphorylation of IRS-1[21] and to enhance glucose uptake in skeletal muscle.[15,18] In our experiment, 3-week high-fructose feeding impaired insulin sensivity and responsiveness. Although physiological insulin level is not enough to suppress hepatic glucose output (HGO), we observed blunted GIR under supraphysiological insulin level, which sufficiently suppressed HGO, suggesting insulin resistance in skeletal muscle at least under maximal insulin stimulation.

TZD treatment improves insulin resistance in human and various animal models including high-fructose–fed rats.[2] High-

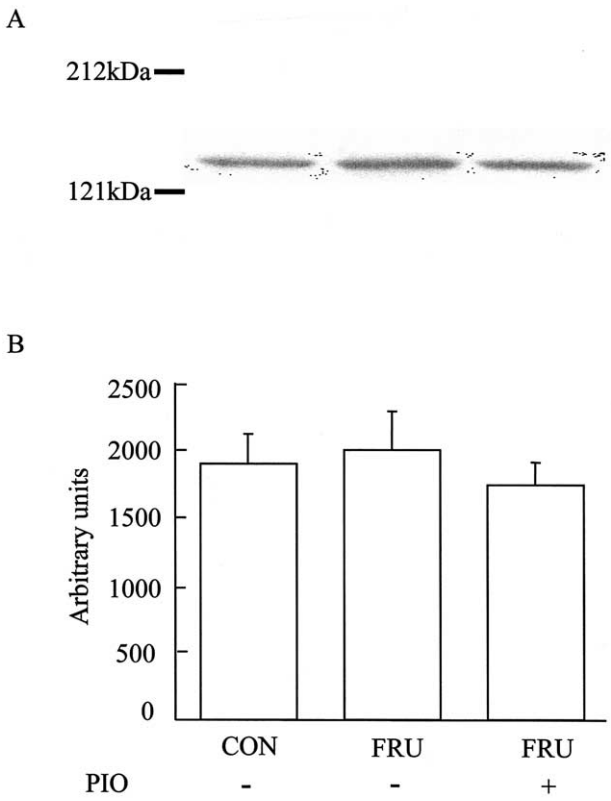

Fig 2. Analysis of the eNOS protein content in skeletal muscle. Representative blots are shown in A and respective densitometric quantifications are shown in B. Rats were fed standard chow diet (CON), high-fructose diet (FRU), or FRU plus pioglitazone (PIO) for 3 weeks. Data are presented as means ± SEM (n = 5-7).

fructose feeding results in impairment of endothelium-dependent vasodilation.[17,19,20] TZD treatment restores reduced endothelium-dependent vasodilation[17] and peripheral glucose uptake stimulated by insulin,[16,18] and corrects metabolic abnormality in high-fructose–fed rats.[16-18]

It has been reported that chronic NOS-inhibitor feeding induces insulin resistance with metabolic abnormality.[25] Furthermore, a previous report from our laboratory showed that sodium nitroprusside, a NO donor, infusion improves in vivo insulin action in rats fed high-fructose.[22] These results suggest the possibility that NO has an important role in pioglitazone-mediated insulin action. However, the effect of NOS inhibitor on enhanced insulin action by TZD is not clear. In accordance with this reasoning, L-NMMA administration abolished improved insulin action by pioglitazone in high-fructose–fed rats in the current study. It might be possible that L-NMMA administration induces hepatic insulin resistance,[26] which contributes to the reduced GIR by L-NMMA administration in pioglitazone-treated rats. In liver, the relation between NO and insulin resistance seems complicated since it has been reported that not only NOS inhibitor[26] but also NO donor[27] can enhance glucose output. Although we did not measure HGO, it is unlikely that NOS inhibition enhance HGO in our experimental condition because L-NMMA infusion in control rats had no effect on GIR (Fig 1). This result is consisted with our previous report.[23] Then, from the current data we might conclude that pioglitazone improves insulin-mediated glucose disposal in high-fructose–fed rats in a NO-dependent fashion.

Skeletal muscle is a major site of glucose disposal during the hyperinsulinemic euglycemic clamp,[28] and plays a predominant role in TZD-induced improvement of glucose homeostasis.[29] Dilation of skeletal muscle arterioles by insulin is endothelium-dependent and NO-mediated,[5] and insulin-stimulated skeletal muscle glucose uptake in vivo is NO-dependent.[6] Skeletal muscle expresses eNOS,[7] and eNOS knockout mice exhibit insulin resistance in this tissue.[13] These findings indicate that eNOS might be considered a principal mediator of insulin-stimulated glucose uptake. However, the contribution of eNOS protein content in skeletal muscle to metabolic changes by high-fructose feeding and/or TZD is not known. In this study we tried to clarify the effect of high-fructose feeding and/or pioglitazone on the eNOS protein content in skeletal muscle. We found that the eNOS protein content in skeletal muscle do not change in those rats. This result is consistent with previous reports in which eNOS protein[30] and mRNA[20] were not reduced but NOS activity was reduced[20] in rat aorta endothelium of high-fructose–fed rats. It is known that skeletal muscle can express nNOS and iNOS, as well as eNOS. Shankar et al[31] reported that mice with knockout of nNOS exhibited peripheral insulin resistance. The nNOS is expressed in skeletal muscle, which is composed of predominantly glycolytic fivers.[32] The present study could not reveal alterations in nNOS protein because it was not detected. The reason may be the existence of much oxidative fibers in plantaris muscle. Recent reports have shown that iNOS is associated with insulin resistance.[7,32,33] Some cytokines are increased in skeletal muscle under insulin-resistant condition,[35] and cytokine-induced iNOS expression is enhanced by TZD.[34] The iNOS might become a NO source arranged by high-fructose feeding and/or pioglitazone. However, it is unlikely because we found no effect of these treatments on iNOS protein content in skeletal muscle. This result suggests that iNOS does not necessarily contribute to high-fructose–induced insulin resistance and/or to insulin-sensitizing action by pioglitazone, at least in skeletal muscle.

Although we have not measured NOS activity in skeletal muscle, NO valance, which is arranged by NO production and

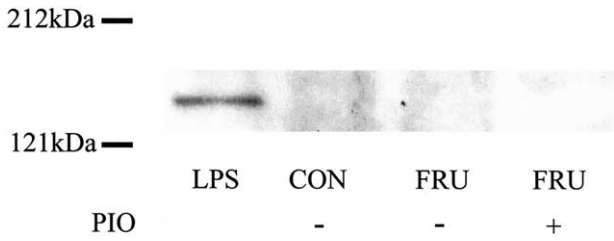

Fig 3. Analysis of the iNOS protein content in skeletal muscle. The panel shows representative blots for iNOS. Rats were fed standard chow diet (CON), high-fructose diet (FRU), or FRU plus pioglitazone (PIO) for 3 weeks. Muscle from a lipopolysaccharide (7 mg/kg BW)-injected rat (after 5 hours) was used as positive control for iNOS.

NO inactivation, might be disturbed by high-fructose feeding and is corrected by pioglitazone.

Mechanisms of increased GIR by pioglitazone may be attributed to an improved insulin action in skeletal muscle cells and/or blood flow in skeletal muscle. In skeletal muscle cells, however, the relation among NO, insulin, and pioglitazone is complicated. TZD incubation[4,37,38] and acute TZD perfusion[39] increase skeletal muscle glucose uptake independent of insulin.[4,38] Fürnsinn et al[37] reported that TZD- and contraction/hypoxia-induced glucose uptake is similar. If glucose uptake is measured in in vitro, in vitro contraction[40,41] or TZD treatment[42] cannot enhance insulin-stimulated glucose uptake, otherwise in vivo these stimulations increase insulin action. Additionally, NOS inhibitor does not inhibit contraction-[8] or insulin-stimulated[7] glucose uptake in vitro, but contraction-induced glucose uptake *in vivo* is reduced by NOS inhibitor independent of blood flow.[43] It is not clear, in the current study, why insulin-sensitizing action of TZD is observed only in vivo treatment, and how NO contributes to it.

Improved insulin-stimulated blood flow in skeletal muscle is also a plausible candidate of enhanced GIR by pioglitazone. It is known that L-NMMA can affect blood flow, but our control rats did not reduce GIR under L-NMMA treatment. A lesser contribution of blood flow to GIR, however, is thought in control conditions. If pioglitazone can improve endotherium-dependent vasodilation, it is possible that improved GIR by pioglitazone is achieved by the increased contribution of insulin and glucose delivery, then the inhibitory effect of L-NMMA on GIR appears.

In conclusion, our study suggests that in vivo insulin-sensitizing action by pioglitazone is NO-dependent and this effect is not related with skeletal muscle eNOS and iNOS protein content in high-fructose–fed rats. L-NMMA might have a direct effect on pioglitazone-induced changes in GIR and we cannot exclude this hypothesis. Further studies with other NOS inhibitors and tracer techniques are needed to understand the in vivo effect of pioglitazone.

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