

# Pioglitazone Treatment Improves Nitrosative Stress in Type 2 Diabetes

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**OBJECTIVE**— The purpose of this study was to determine the effect of 24 weeks of treatment with 45 mg/day pioglitazone on peripheral skin blood flow (SkBF) and skin nitric oxide (NO) production in vivo in patients with type 2 diabetes.

**RESEARCH DESIGN AND METHODS**— This was a randomized, parallel, cross-over, double-blind, within- and between-subject study designed to compare vascular responses before and after treatment. We studied 12 subjects with type 2 diabetes (average age  $58.6 \pm 30.8$  years,  $HbA_{1c}$   $7.9 \pm 0.4\%$ , BMI  $31.3 \pm 1.2$  kg/m<sup>2</sup>). SkBF was measured using laser Doppler techniques in response to ischemia reperfusion and local skin warming, and NO production was assessed in vivo using an amperometric NO meter inserted directly into the skin. These measurements were performed before treatment and at 6 and 24 weeks.

**RESULTS**— The SkBF response was not significantly improved after 24 weeks in either of the groups. NO production was significantly decreased in the pioglitazone-treated group in the basal condition (area under the curve  $6.4 \pm 1.0$  vs.  $2.8 \pm 0.8$ ,  $P < 0.01$ ), after local heat stimulation at 40°C ( $12.9 \pm 2.2$  vs.  $5.7 \pm 1.7$ ,  $P < 0.01$ ), and after nociceptor stimulated flow with local heating at 44°C ( $36.4 \pm 6.3$  vs.  $16.6 \pm 3.4$ ). Differences were not significant in the placebo-treated group.

**CONCLUSIONS**— Treatment of patients with type 2 diabetes with pioglitazone for 24 weeks reduced skin NO production, thus probably reducing nitrosative stress without a demonstrable effect on SkBF. Because nitrosative stress is considered to be a factor in the pathogenesis of neurovascular dysfunction, these findings warrant further investigation.

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We have previously described both impaired peripheral vasoconstriction and vasodilatation in cutaneous vessels of people with diabetes, which strongly resembles the normal aging effects seen in peripheral vasculature (1). We have suggested both an endothelial and a neuronal component to these defects (2). The neuronal component is only activated at noxious temperatures (>40°C) or after tissue damage. Endothelial integrity is a major component under normal tissue demands for oxygenation

after ischemia. Other investigators have also proposed the notion that there is specific damage to vascular endothelium and/or smooth muscle in diabetes, but localization of such a defect remains elusive.

Insulin is a vasodilator hormone and facilitates endothelial release of nitric oxide (NO) and prostacyclin as well as up-regulation of prostacyclin receptors on vascular smooth muscle (3). Resistance to the action of insulin could theoretically contribute to impaired neurovascular

function. Jaap et al. (4) showed an endothelial or smooth muscle defect associated with insulin resistance that may precede the development of diabetes. We also showed that defective neurovascular function correlates with features of the metabolic syndrome, including insulin resistance, hypertension, dyslipidemia, and obesity (2). Diabetes is now recognized to be part of the metabolic syndrome and is considered to be a major risk factor for neurovascular disease (5). Additional features of the syndrome include a proinflammatory state, oxidative stress, impaired neurovascular function (6), and reduction of intraepidermal nerve fiber density (7). This raises the possibility that treatment with insulin-sensitizing agents would attenuate these defects and provide a means for ameliorating microvascular insufficiency in both diabetes and the metabolic syndrome.

The beneficial effects of thiazolidinediones (TZDs) in the human insulin-resistance syndrome have been well documented through peroxisome proliferator-activated receptor (PPAR) agonism (8). PPARs act as central transcriptional mediators of several important metabolic processes that influence adipogenesis, insulin sensitivity, glucose and lipid homeostasis, and vascular endothelial function (9). It has been established that PPAR $\gamma$  compounds favorably alter lipid metabolism, as well as reduce inflammation (10), although their effects on neurovascular inflammation have only been modest (11). Furthermore, TZDs have now been shown to exercise nonhypoglycemic actions related to cardiovascular risk, including decrease in blood pressure (12), correction of dyslipidemia (13), and improvement in inflammation, with reductions in C-reactive protein, metalloproteinase 9, interleukin-6, tumor necrosis factor- $\alpha$ , white blood cell (WBC) count, and carotid artery intima thickness (14). The ability of TZDs to reduce circulating levels of inflammatory markers in type 2 diabetes (15) suggests that their actions may be in addition to correcting insulin resistance and that there may be differences among the different members of this class of compounds (16).

If insulin resistance is the determinant of aberrant blood flow, we hypothe-

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**Abbreviations:** DPN, diabetic polyneuropathy; eNOS, endothelial nitric oxide synthase; MCHC, mean corpuscular hemoglobin concentration; NOCI, nociceptor stimulated flow; ONOO<sup>-</sup>, peroxynitrite; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; SkBF, skin blood flow; TZD, thiazolidinedione; WBC, white blood cell.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

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<b>Baseline 3 minutes</b>	<b>Limb occlusion 5 minutes</b>	<b>Hyperemic response 12 minutes</b>	<b>32°C 5 minutes</b>	<b>40°C 5 minutes</b>	<b>44°C 10 minutes</b>
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**Figure 1**—SkBF and NO protocol.

sized that treatment with pioglitazone, an insulin-sensitizing agent, would attenuate the functional defect in diabetic endothelium and/or smooth muscle that is responsible for impaired endothelium-mediated vasodilatation, thus improving local skin and subcutaneous tissue perfusion. We further proposed that resistance to NO was a specific functional defect in these microvessels, which is directly related to inflammation. Thus, if treatment with pioglitazone mitigated the functional blood flow defects, the mechanism would be through attenuation of NO resistance and inflammation. The result would be reflected in lowered NO levels associated with a decrease in insulin resistance and markers of inflammation and improvement of skin vascular perfusion.

The objective of this study was to determine the effect of 12–24 weeks of treatment with pioglitazone on peripheral skin blood flow (SkBF) and NO production in the dorsum of the foot in patients with type 2 diabetes.

## RESEARCH DESIGN AND METHODS

This was a randomized, double-blinded trial with a placebo run-in period and two treatment arms. The primary efficacy measures were the vascular and NO responses of the foot dorsum to ischemic block and local warming.

Patients were divided into two groups. Group 1 received placebo for 12 weeks followed by 12 weeks of treatment with pioglitazone at 45 mg/day; group 2 received pioglitazone at 45 mg/day for 24 weeks.

Inclusion of a placebo-to-treatment crossover group and a pure pioglitazone-treated group enabled us to perform within-subject and between-subject statistical comparisons. The latter half of the treatment period was conducted in a single-blind manner, and all patients received active treatment during this period.

Sample size estimations were made on the basis of preliminary studies of normal healthy control subjects and age- and BMI-matched patients with type 2 diabetes (17–20). In the reported studies, the

peak postischemic hyperemia measured from the fingertip was  $38.25 \pm 4.71$  (mean  $\pm$  SE) in control subjects and  $23.40 \pm 3.18$  in matched type 2 diabetic subjects. Based on these data, a pooled standard deviation of 24.01 was used to determine power, assuming a 50% attenuation of the blood flow defect in diabetes with pioglitazone treatment (7.5 blood flow units) at 24 weeks of treatment versus placebo. For a power of at least 0.80 at  $\alpha = 0.05$  to detect a treatment effect of 50% attenuation, 10 subjects (5 in each arm) were required to complete the study.

All research subjects enrolled in this study were asked to sign an informed consent document. The research study was reviewed and approved by the Eastern Virginia Medical School Institutional Review Board.

We included subjects with type 2 diabetes (defined by current World Health Organization criteria) between ages 18 and 80 years. Subjects with type 1 diabetes (defined as fasting serum C-peptide concentration  $<1$  ng/ml or diabetes onset at  $<35$  years of age in a nonobese patient), severe retinopathy, significant proteinuria, neuropathy of a nondiabetic origin, amputations or foot ulcers, a past history of stroke or myocardial infarction, heart failure, or the presence of pedal edema, elevated liver enzymes, or any liver disease and any other serious illness that would preclude participation, including being enrolled in any other clinical trial, were excluded. Concomitant use of ACE inhibitors, angiotensin receptor blockers, and insulin was not allowed.

Tests for vascular responses and laboratory parameters were performed at weeks 0, 6, 18, and 24. At baseline a complete medical history was taken, and physical and neurological examinations were performed on each patient. HbA<sub>1c</sub> (A1C), serum lipids, C-peptide, and liver function were also measured. These tests were performed at Eastern Virginia Medical School Laboratories, Norfolk, Virginia, or LabCorp, Burlington, North Carolina. Study medication was discontinued at weeks 0 and 12.

Blood flow and skin NO production were measured at baseline and after dif-

ferent stimuli were applied following the protocol shown in Fig. 1. SkBF was measured noninvasively on the dorsal aspect of the left foot by continuous laser Doppler using Periflux small angled thermostatic laser Doppler probes (Perimed, North Royalton, OH), with core diameters of 0.125 mm, laser wavelengths of 780 nm, and fiber separation lengths of 0.25 mm. This system measures skin perfusion as erythrocyte flux. The depths of the measurements, influenced by tissue properties, light source, and probe configuration, were estimated to be between 500 and 1,000  $\mu\text{m}$ . NO was measured adjacent to the laser Doppler probe using a microsensors adapted for the detection of NO via an ion-exchange mechanism in the circulation of humans, which had a detection limit of  $5 \times 10^{-9}$  mol/l. The sensor was read by the ISO-NO Mark II NO meter (World Precision Instruments, Sarasota, FL), attached to a personal computer, and was calibrated before each use using a three-concentration nitrite standard. These methods have been described in detail elsewhere (2,17,18,20). Limb occlusion was performed with a sphygmomanometer cuff for 5 min; after baseline and ischemia-reperfusion measurements, local warming was maintained by the laser Doppler thermostatic probe at 32°C for 5 min, 40°C for 5 min, and 44°C for 10 min. Clinical safety measures included careful review of each subject before entry into the study and determination of liver enzyme elevation during treatment.

## Statistics

All data, including those for skin perfusion, are presented as means  $\pm$  SE. The study design was parallel and crossover, with within- and between-subject analyses. The primary dependent variables were SkBF and NO responses to provocative stimuli. The independent variables were the treatment groups (two levels). For demographic data and laboratory results comparisons within and between groups, nonparametric tests (Wilcoxon's signed-rank test and the Mann-Whitney *U* test, respectively) were used. Nonparametric tests were also used to examine

**Table 1—Demographic characteristics of each group**

Characteristic	Group 1 (placebo)	Group 2 (pioglitazone)	P value*
n	5	6	
Age (years)	54 ± 4.5	62.2 ± 5.5	NS
Sex (female/male)	2/3	2/4	NS
Diabetes duration (years)	7.9 ± 2.5	7.1 ± 1.9	NS
SBP (mmHg)	135.5 ± 3.2	135.8 ± 4.9	NS
DBP (mmHg)	80.8 ± 2.1	80.8 ± 2.4	NS
BMI (kg/m <sup>2</sup> )	30.6 ± 1.1	32.3 ± 1.1	NS
FBG (mg/dl)	169 ± 22.4	166.2 ± 15.9	NS
A1C (%)	7.8 ± 0.3	7.6 ± 0.2	NS
C-peptide (ng/ml)	3.02 ± 0.3	3.4 ± 0.6	NS
Triglycerides (mg/dl)	127 ± 20.1	197.4 ± 38.6	NS
Total cholesterol (mg/dl)	179.7 ± 16.8	187.1 ± 10.2	NS
HDL (mg/dl)	56.5 ± 4.4	44.9 ± 2.5	NS
LDL (mg/dl)	97 ± 10.2	104 ± 8.2	NS
WBC (10 <sup>3</sup> /μl)	6.5 ± 0.4	6.5 ± 0.3	NS
Hemoglobin (g/dl)	13.7 ± 0.4	14.5 ± 0.3	NS
MCV (fl)	89.9 ± 0.9	92.6 ± 0.9	NS
MHC (pg)	30.2 ± 0.3	31.4 ± 0.2	NS
MCHC (%)	33.6 ± 0.2	33.9 ± 0.1	NS
RDW (%)	13.1 ± 0.2	13.1 ± 0.1	NS
Platelets (10 <sup>3</sup> /μl)	271.5 ± 8.8	288.3 ± 16.2	NS

Data are means ± SE. \*Comparisons between groups were made using Mann-Whitney *U* test. DBP, diastolic blood pressure; FBG, fasting blood glucose; MCV, mean corpuscular volume; MHC, mean hemoglobin concentration; RDW, red blood cell distribution width; SBP, systolic blood pressure.

treatment effects at 6 weeks (within and between groups) and at 24 weeks (within and between groups) on SkBF and skin NO production. Significance was accepted at the  $P < 0.05$  level.

**RESULTS**— Five patients in group 1 (one dropout) and six patients in group 2 completed the study. Demographic characteristics of and laboratory values for each group at baseline are given in Table 1. There were no differences in any of the variables between the groups.

Table 2 shows the blood pressure responses, BMI change, and laboratory parameter change in each group after 24 weeks of treatment. There were no significant changes. The fall in A1C in pioglitazone-treated patients showed a trend toward significance ( $P = 0.08$ ). No significant changes were seen at 12 weeks of treatment either (data not shown).

Figure 2A and B demonstrates the blood flow response to ischemia reperfusion (hyperemic response), local heating to 32 and 40°C, and nociceptor stimu-

lated flow (NOCI) with local heating to 44°C. There were no significant effects on ischemia reperfusion or increase in response to warming to 32 or 40°C or to NOCI-mediated vasodilatation at 44°C in the within-group analysis. A between-group analysis was also performed at baseline, 6 weeks, and 24 weeks and showed no differences between the groups (data not shown).

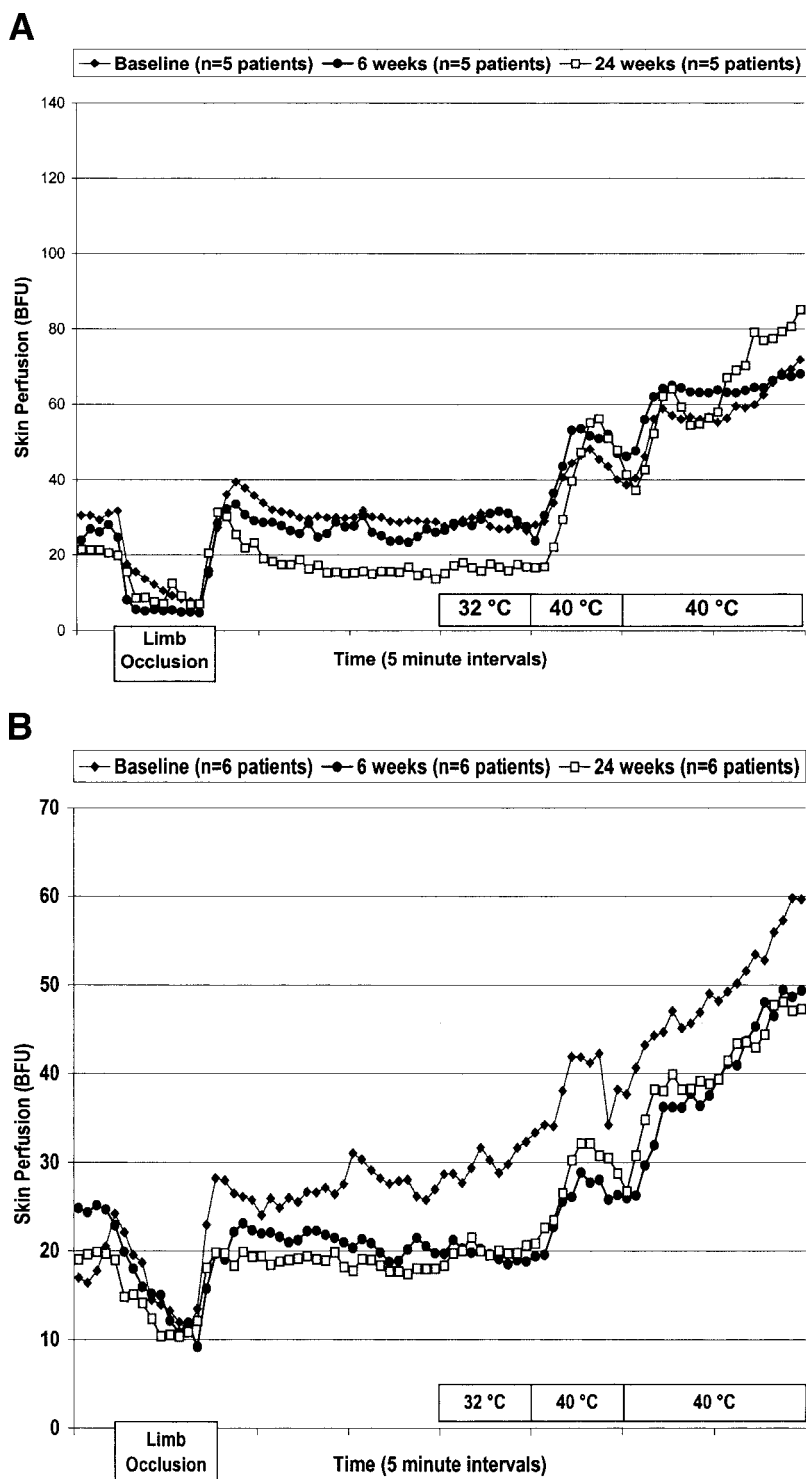
The basal and heat-stimulated NO responses are shown in Fig. 3A and B, comparing baseline NO levels to those after treatment in each group (after 6 and 24 weeks). After 6 weeks, no significant reduction in NO production was seen in any of the groups. In patients treated with placebo for 12 weeks and with pioglitazone for 12 weeks (group 1), there was a decrease in skin NO production after 24 weeks of treatment, but this did not reach statistical significance. In patients treated with pioglitazone for 24 weeks (group 2), the reduction in NO production was statistically significant at baseline and after ischemia reperfusion, local heating, and NOCI stimulation (Table 3). A between-group comparison was performed, but no differences were detected at baseline, 6 weeks, or 24 weeks (data not shown).

To further elaborate on the relationship between glycemic control, insulin sensitivity, and inflammatory markers of diabetes with skin NO production, we analyzed the correlation between the changes of each variable (A1C, C-peptide, WBC count, and other parameters) and the change in NO in the basal state and after heating to 40 and 44°C, in each group after 24 weeks. The reduction in NO production correlated significantly with reductions in A1C, C-peptide, WBC count, and mean corpuscular hemoglo-

**Table 2—Change in blood pressure and laboratory parameters after 24 week of treatment in group 1 (12 weeks of placebo and 12 weeks of pioglitazone) and in group 2 (24 weeks of pioglitazone)**

Characteristic	Group 1 (placebo)		P value	Group 2 (pioglitazone)		P value
	Baseline	24 weeks		Baseline	24 weeks	
SBP (mmHg)	135.5 ± 3.2	130.1 ± 4.7	NS	135.8 ± 4.9	133 ± 9.9	NS
DBP (mmHg)	80.8 ± 2.1	76.6 ± 3.8	NS	80.8 ± 2.4	81 ± 5.1	NS
BMI	30.6 ± 1.1	30.1 ± 1.2	NS	32.5 ± 1.1	33.5 ± 1.0	NS
A1C (%)	7.8 ± 0.3	7.9 ± 0.7	NS	7.6 ± 0.2	7.2 ± 0.2	0.08
C-peptide (ng/ml)	3.02 ± 0.3	2.4 ± 0.3	NS	3.4 ± 0.6	2.7 ± 0.6	NS
Triglycerides (mg/dl)	127 ± 20.1	124 ± 34.3	NS	197.4 ± 38.6	139 ± 17.3	NS
Total cholesterol (mg/dl)	179.7 ± 16.8	174.5 ± 2.6	NS	187.1 ± 10.2	176.1 ± 11.9	NS
HDL (mg/dl)	56.5 ± 4.4	58.3 ± 6.8	NS	44.9 ± 2.5	44.6 ± 2.7	NS
LDL (mg/dl)	97 ± 10.2	91 ± 15.1	NS	104 ± 8.2	103.3 ± 12.3	NS

Data are means ± SE. \*Comparisons within groups were made using the Wilcoxon signed-rank test. DBP, diastolic blood pressure; SBP, systolic blood pressure.



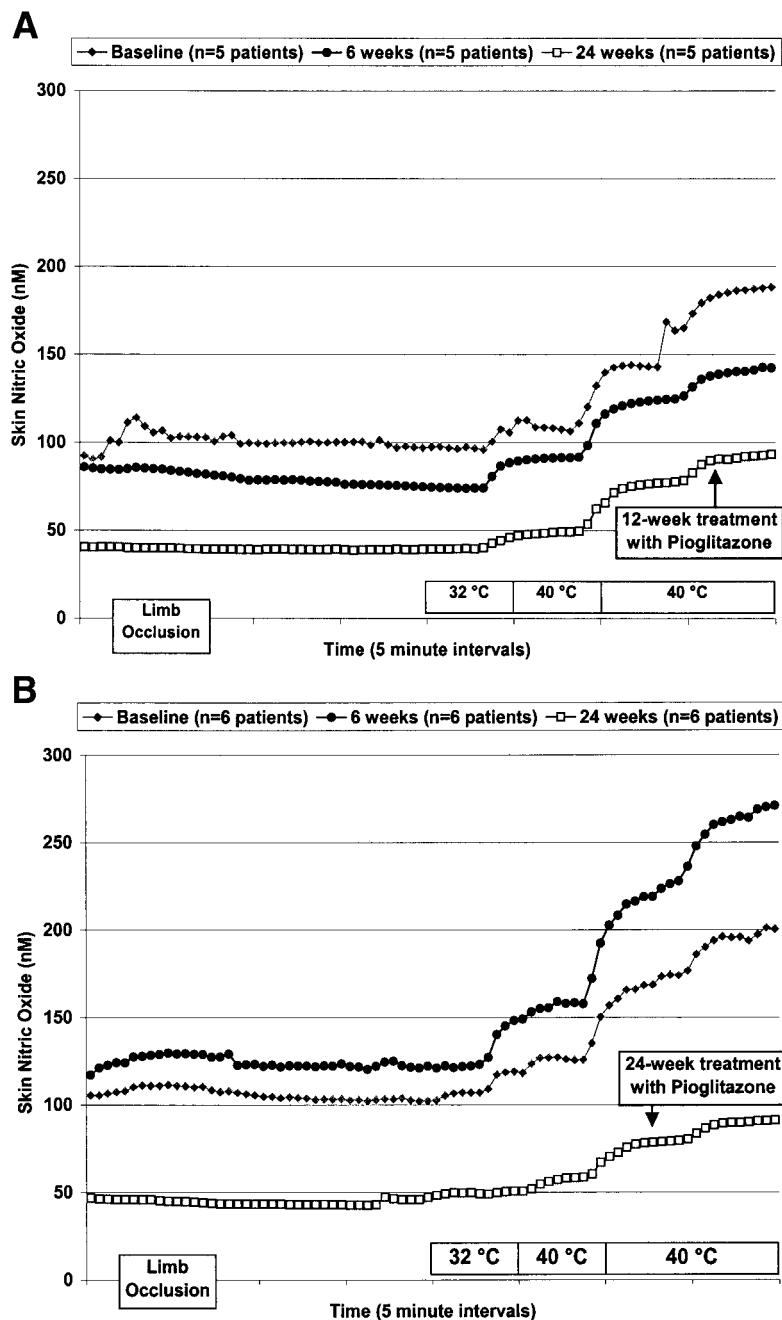
**Figure 2**—A: SkBF at baseline and after 6 weeks of treatment with placebo followed by 6 more weeks of placebo and then 12 weeks of treatment with pioglitazone (placebo group). B: SkBF at baseline and after 6 and 24 weeks of treatment with pioglitazone (pioglitazone group). For both groups, AUCs for each interval (baseline, hyperemic response, and 32, 40, and 44°C heating) were calculated and within-group comparisons were made using nonparametric tests (Wilcoxon signed-rank test). BFU, blood flow units.

bin concentration (MCHC) in the pioglitazone-treated group. This is shown in Table 4. We also evaluated the change in each variable in each group after 6 weeks, but no significant correlations were found at that time (data not shown).

**CONCLUSIONS**— In this study, we examined the effects of treatment with

pioglitazone on SkBF and NO production. The cardinal findings were that 24 weeks of treatment did not alter impaired blood flow of the skin of the dorsum of the foot but profoundly reduced the production of skin NO. Improvement in glycemic control and insulin sensitivity (shown by a reduction in A1C and C-peptide) correlated significantly with the

reduction in NO production after 24 weeks of treatment in the pioglitazone group. Furthermore, reductions in some parameters of inflammation and microvascular dysfunction in diabetes, such as WBC and MCHC, correlated significantly with the reduction in NO production as well. The lack of change in skin perfusion may suggest that regulation of SkBF is not



**Figure 3**—A: Skin NO levels at baseline and after 6 weeks of treatment with placebo followed by 6 more weeks of placebo and then 12 weeks of treatment with pioglitazone (placebo) group. B: Skin NO levels at baseline and after 6 and 24 weeks of treatment with pioglitazone (pioglitazone group). For both groups, AUCs for each interval (baseline, hyperemic response, and 32, 40, and 44°C heating) were calculated and within-group comparisons were made using nonparametric tests (Wilcoxon signed-rank test).

mediated by NO. Future studies with more patients treated for longer periods with pioglitazone may provide clarification on this relationship.

These findings, at first glance, are somewhat of an anathema, because NO is one of the primary agents eliciting a vasodilatory response in conduit and resistance vessels by relaxing smooth muscle and producing an increase in blood flow (21). Because there is a considerable body of evidence indicating that NO mediates vasodilatation of brachial and forearm vessels, which is compromised in diabetes (22), we sought to determine whether

there were abnormalities in NO production or utilization in the skin of people with diabetes. Using direct amperometric measurements of NO in the skin in response to heat and NOCI stimuli, we showed that there was no defect in interstitial NO production. It was produced in nanomolar amounts, as opposed to the picomolar amounts generated by vascular endothelial nitric oxide synthase (eNOS) and was only partially blockable by N<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME). There also was no defect in the vasodilatory response to the NO donor sodium nitroprusside (23). This finding sug-

gested that interstitial NO overproduction (presumably from macrophages, keratinocytes, and dendritic cells) might have been counterproductive and that the regulation of SkBF was not an interstitial NO-mediated phenomenon. There is an active vasodilative mechanism of skin blood vessels that is dependent on C-fiber nociceptors, which accounts for 75% of their dilative capacity (11), and there is an alternate pathway regulating blood flow that is associated with lipid levels, blood pressure, hematocrit, WBC count, and resistance to the action of insulin in diabetes and the metabolic syndrome. This in-



Table 3—Skin NO area under the curve calculations: group 1 (placebo group) and group 2 (pioglitazone group)

	NO group 1 (nmol × 10 <sup>2</sup> )				NO group 2 (nmol × 10 <sup>2</sup> )			
	Baseline	6 weeks of placebo	12 weeks of placebo and 12 weeks of pioglitazone	P value*	Baseline	6 weeks of pioglitazone	24 weeks of pioglitazone	P value*
Baseline AUC	5.9 ± 1.8	5.1 ± 1.4	2.4 ± 0.7	NS	6.4 ± 1.0	7.4 ± 3.2	2.8 ± 0.8	0.01
Hyperemic response AUC	24.0 ± 0.7	18.6 ± 5.4	9.4 ± 3.8	NS	25.0 ± 4.7	29.5 ± 13.6	10.5 ± 3.3	0.008
40°C AUC	11.3 ± 3.1	9.4 ± 3.2	5.0 ± 1.9	NS	12.9 ± 2.2	16.1 ± 8.2	5.7 ± 1.7	0.01
44°C AUC	33.4 ± 8.9	26.2 ± 7.7	16.5 ± 4.8	NS	36.4 ± 6.3	48.2 ± 25.0	16.6 ± 3.4	0.03
Total AUC	95.0 ± 26.6	72.4 ± 2.0	41.5 ± 14.2	0.08	102.6 ± 16.9	126.9 ± 6.2	45.1 ± 12.0	0.04

Data are means ± SE. \*Within-group analysis was made using the Wilcoxon signed-rank test (baseline vs. 24 weeks). AUC, area under the curve.

crease in NO production has now been reported by other authors (24) and is thought possibly to be due to an excess of a circulating antagonist (asymmetric dimethylarginine). The suggestion has been made that this unbridled production results in NO reacting with reactive oxygen species (ROS) to form peroxynitrite (ONOO<sup>-</sup>), which is damaging to blood vessel structure and function (25).

**Treating insulin resistance to improve NO balance**

Hyperglycemia and oxidative stress deplete NO within the peripheral nerves and endothelium of the microvasculature by reducing eNOS and altering nerve perfusion (23). Depletion of endothelial NO has been observed to lead to low nerve blood flow, hypoxia, and mitochondrial dysfunction (26). Accumulation of ROS during hyperglycemia downregulates neuronal NOS. These observations suggest a role of NO repletion as a potential therapy in experimental diabetic polyneuropathy (DPN). There is, however, a paradoxical response of extravascular NO to hyperglycemia and inflammatory damage to peripheral nerves. Under inflammatory conditions, inducible NOS increases, which leads to nanomolar quantity production of NO as opposed to picomolar amounts production as with eNOS activation (27). High levels of NO causes nerve damage via mitochondrial dysfunction and formation of ONOO<sup>-</sup> by interaction with ROS. ONOO<sup>-</sup> nitrosylates proteins, thereby impairing their function. Antioxidants typically defend the cell against this damage. Some studies have shown that patients with diabetes have reduced levels of these antioxidants (28); however, there is conflicting evidence in the literature (29). Hyperglycemia and abnormalities in lipid metabolism generate the formation of ROS. This activates

β<sub>2</sub> protein kinase C-β, which increases the action of NAD(P)H oxidase, generating ROS (30). In addition, there is activation of the nuclear transcription factor κB with the induction of inducible NOS and reduction of eNOS (31). The increase in NO coupled with ROS to generate ONOO<sup>-</sup>, which nitrosylates proteins, damages the endothelia of blood vessels and damages DNA. Thus, if pioglitazone affects the NO balance in humans, we would expect to reverse the inability of subjects with diabetes to vasodilate in response to NO-mediated stimulation and reduce neurovascular damage.

Microvascular insufficiency, endoneurial blood flow, and hemodynamic factors lead to nerve damage in patients with DPN (32). Although the sequence of events is not well understood, investigators propose that microvascular vasoconstriction, edema, and ischemia play a role in DPN development (33). Endoneurial edema increases endoneurial pressure, thereby causing capillary closure and subsequent nerve ischemia and damage (34).

Diminished regulation of endoneurial blood flow and ischemia may result from decreased nerve density and innervation of vessels (35,36). Nerve ischemia stimulates vascular endothelial growth factor production, exacerbating DPN via overactivation of protein kinase C-β (37). As a result, ischemia and low blood flow reduce both endothelial- and NO-dependent vasorelaxation (38). Vascular defects also result in changes in endoneurial vessels. Changes in blood flow correlate with changes in oxygen saturation (39) and reduced sural nerve endoneurial oxygen tension. These changes are potentiated by increased expression or action of vasoconstrictors such as endothelin and angiotensin and decreased activity of vasodilators such as prostacyclin, substance P, calcitonin gene-related peptide, endothelial-derived hyperpolarizing factor, and bradykinin (40). In particular, in patients with DPN there is disruption in vasomotion, the rhythmic contraction exhibited by arterioles and small arteries (19). In type 2 diabetes, SkBF is abnor-

Table 4—Correlation between change in laboratory variables and blood pressure with NO response after 24-week treatment (pioglitazone group)

Variable	ΔNO AUC		
	32°C	40°C	44°C
ΔSBP	NS	NS	NS
ΔDBP	NS	NS	NS
ΔA1C	NS	NS	r = 0.8792, P = 0.0210
ΔC-peptide	NS	NS	r = 0.8049, P = 0.0534
ΔLDL	NS	NS	NS
ΔWBC	r = 0.8054, P = 0.0048	r = 0.8286, P = 0.0416	r = 0.8286, P = 0.0416
ΔMCH	NS	NS	NS
ΔMCHC	r = 0.7419, P = 0.0361	r = 0.9276, P = 0.0077	r = 0.9276, P = 0.0077

Nonparametric, Spearman rank correlation coefficients were calculated on the Δ (change of each variable) and the change in NO area under the curve (AUC) (in the basal state [32°C] and after heating at 40 and 44°C) after 24 weeks of treatment. DBP, diastolic blood pressure; MHC, mean hemoglobin concentration; SBP, systolic blood pressure.

mal, and the loss of neurogenic vasodilative mechanism in hairy skin may precede lower-limb microangiopathic processes and C-fiber dysfunction. Changes in endoneurial blood flow often are reflected by changes in nerve conduction (41). Therefore, both vascular or endoneural alterations may cause damage over time in the peripheral nerves of patients with diabetes. A multifactorial approach is needed to combat the many abnormalities associated with oxidative stress.

This study showed a reduction in skin NO production after treatment with pioglitazone but failed to demonstrate an improvement in SkBF. This may be due to the small amount of patients included and the short duration of the study. It remains to be tested whether the reduction of nitrosative stress by pioglitazone observed here translates to improved neurovascular function in the long term, thus preventing nerve damage and decreasing the predisposition to foot ulceration and amputation.

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## References

- Vinik A, Erbas T, Park T, Stansberry K, Scanelli J, Pittenger G: Dermal neurovascular dysfunction in type 2 diabetes. *Diabetes Care* 24:1468–1475, 2001
- Vinik AI, Erbas T, Park TS: Methods for evaluation of peripheral neurovascular dysfunction. *Diabetes Technol Ther* 3:29–50, 2001
- Baron AD: Insulin and the vasculature—old actors, new roles. *J Investig Med* 44:406–412, 1996
- Jaap AJ, Hammersley MS, Shore AC, Tooke JE: Reduced microvascular hyperaemia in subjects at risk of developing type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia* 37:214–216, 1994
- Resnick H, Jones K, Ruotolo G, Jain A, Henderson J, Lu W, Howard B: Insulin resistance, the metabolic syndrome, and risk of incident cardiovascular disease in nondiabetic American Indians: the Strong Heart Study. *Diabetes Care* 26:861–867, 2003
- LeRoith D, Fonseca V, Vinik A: Metabolic memory in diabetes—focus on insulin. *Diabetes Metab Res Rev* 21:85–90, 2005
- Pittenger G, Burcus N, McNulty P, Basta B, Vinik A: Intraepidermal nerve fibers are indicators of small fiber neuropathy in both diabetic and nondiabetic patients. *Diabetes Care* 27:1794–1779, 2004
- Nolan JJ, Ludvik B, Beerdsen P, Joyce M, Olefsky J: Improvement in glucose tolerance and insulin resistance in obese subjects treated with troglitazone. *N Engl J Med* 331:1188–1193, 1994
- Allan G, Brook CD, Cambridge D, Haldikiwsky J: Enhanced responsiveness of vascular smooth muscle to vasoconstrictor agents after removal of endothelial cells. *Br J Pharmacol* 79:334P, 1983
- Taberner A, Giraldo J, Vila E: Effect of NG-nitro-L-arginine methylester (L-NAME) on functional and biochemical  $\alpha$ 1-adrenoceptor-mediated responses in rat blood vessels. *Br J Pharmacol* 117:757–763, 1996
- Burnstock G, Ralevic V: New insights into the local regulation of blood flow by perivascular nerves and endothelium. *Br J Plast Surg* 47:527–543, 1994
- Ghazzi MN, Perez JE, Antonucci TK, Driscoll JH, Huang SM, Faja BW, Whitcomb RW: Cardiac and glycemic benefits of troglitazone treatment in NIDDM: the Troglitazone Study Group. *Diabetes* 46:433–439, 1997
- Freed MI, Ratner R, Marcovina SM, Kreider MM, Biswas N, Cohen BR, Brunzell JD: Effects of rosiglitazone alone and in combination with atorvastatin on the metabolic abnormalities in type 2 diabetes mellitus. *Am J Cardiol* 90:947–952, 2002
- Sidhu JS, Kaposzta Z, Markus HS, Kaski JC: Effect of rosiglitazone on common carotid intima-media thickness progression in coronary artery disease patients without diabetes mellitus. *Arterioscler Thromb Vasc Biol* 24:930–934, 2004
- Haffner SM, Greenberg AS, Weston WM, Chen H, Williams K, Freed MI: Effect of rosiglitazone treatment on nontraditional markers of cardiovascular disease in patients with type 2 diabetes mellitus. *Circulation* 106:679–684, 2002
- Beckley ET: Pioglitazone produces more lipid-lowering than rosiglitazone. *DOC News*, 1 Feb. 2005: no. 2, pg. 1
- Colberg S, Stansberry K, McNitt P, Vinik A: Chronic exercise is associated with enhanced cutaneous blood flow in type 2 diabetes. *J Diabetes Complications* 16:139–145, 2002
- Parson H, Colberg S, Nunnold T, Holton D, Swain D, Vinik A: Change in cutaneous blood flow and nitric oxide levels following ten weeks of aerobic training in type 2 diabetic individuals. *J Diabetes Complications* 19:276–283, 2005
- Stansberry KB, Shapiro SA, Hill MA, McNitt PM, Meyer MD, Vinik AI: Impaired peripheral vasomotion in diabetes. *Diabetes Care* 19:715–721, 1996
- Stansberry KB, Hill MA, Shapiro SA, McNitt PM, Bhatt BA, Vinik AI: Impairment of peripheral blood flow responses in diabetes resembles an enhanced aging effect. *Diabetes Care* 20:1711–1716, 1997
- Moncada S, Radomski MW, Palmer RM: Endothelium-derived relaxing factor: identification as nitric oxide and role in the control of vascular tone and platelet function. *Biochem Pharmacol* 37:2495–2501, 1988
- Morris SJ, Shore AC, Tooke JE: Responses of the skin microcirculation to acetylcholine and sodium nitroprusside in patients with NIDDM. *Diabetologia* 38:1337–1344, 1995
- Vinik A, Erbas T, Stansberry KB, Pittenger G: Small fiber neuropathy and neurovascular disturbances in diabetes mellitus. *Exp Clin Endocrinol Diabetes* 109 (Suppl. 2):S451–S473, 2001
- Hoeldtke RD, Bryner KD, McNeil DR, Hobbs GR, Riggs JE, Warehime SS, Christie I, Ganser G, Van Dyke K: Nitrosative stress, uric acid, and peripheral nerve function in early type 1 diabetes. *Diabetes* 51:2817–2825, 2002
- Ceriello A, Motz E: Is oxidative stress the pathogenic mechanism underlying insulin resistance, diabetes, and cardiovascular disease? The common soil hypothesis revisited. *Arterioscler Thromb Vasc Biol* 24:816–823, 2004
- Hoeldtke RD: Nitrosative stress in early type 1 diabetes: David H. P. Streeten Memorial Lecture. *Clin Auton Res* 13:406–421, 2003
- Stevens MJ: Nitric oxide as a potential bridge between the metabolic and vascular hypotheses of diabetic neuropathy. *Diabet Med* 12:292–295, 1995
- Jain SK, McVie R: Effect of glycemic control, race (white versus black), and duration of diabetes on reduced glutathione content in erythrocytes of diabetic patients. *Metabolism* 43:306–309, 1994
- Campoy C, Baena RM, Blanca E, Lopez-Sabater C, Fernandez-Garcia JM, Miranda MT, Molina-Font JA, Bayes R: Effects of metabolic control on vitamin E nutritional status in children with type 1 diabetes mellitus. *Clin Nutr* 22:81–86, 2003
- Jawa A, Kcomt J, Fonseca VA: Diabetic nephropathy and retinopathy. *Med Clin North Am* 88:1001–1036, xi, 2004
- Cooke CL, Davidge ST: Peroxynitrite increases iNOS through NF- $\kappa$ B and decreases prostacyclin synthase in endothelial cells. *Am J Physiol* 282:C395–C402, 2002
- Eaton S, Harris ND, Ibrahim S, Patel KA, Selmi F, Radatz M, Ward JD, Tesfaye S: Increased sural nerve epineurial blood flow in human subjects with painful diabetic neuropathy. *Diabetologia* 46:934–939, 2003
- Vinik AI: Advances in diabetes for the millennium: new treatments for diabetic neuropathies. *Med Gen Med* 6:13, 2004
- Myers RR, Powell HC: Galactose neuropathy: impact of chronic endoneurial

- edema on nerve blood flow. *Ann Neurol* 16:587–594, 1984
35. Teunissen LL, Veldink J, Notermans NC, Bleyers RL: Quantitative assessment of the innervation of epineurial arteries in the peripheral nerve by immunofluorescence: differences between controls and patients with peripheral arterial disease. *Acta Neuropathol (Berl)* 103:475–480, 2002
36. Theriault M, Dort J, Sutherland G, Zochodne DW: Local human sural nerve blood flow in diabetic and other polyneuropathies. *Brain* 120 (Pt. 7):1131–1138, 1997
37. Oosthuysen B, Moons L, Storkebaum E, Beck H, Nuyens D, Brusselmans K, Van Dorpe J, Hellings P, Gorselink M, Heymans S, Theilmeier G, Dewerchin M, Laudenbach V, Vermynen P, Raat H, Acker T, Vleminckx V, Van Den BL, Cashman N, Fujisawa H, Drost MR, Sciort R, Bruyninckx F, Hicklin DJ, Ince C, Gressens P, Lupu F, Plate KH, Robberecht W, Herbert JM, Collen D, Carmeliet P: Deletion of the hypoxia-response element in the vascular endothelial growth factor promoter causes motor neuron degeneration. *Nat Genet* 28:131–138, 2001
38. Nangle MR, Cotter MA, Cameron NE: Protein kinase C  $\beta$  inhibition and aorta and corpus cavernosum function in streptozotocin-diabetic mice. *Eur J Pharmacol* 475:99–106, 2003
39. Ibrahim S, Harris ND, Radatz M, Selmi F, Rajbhandari S, Brady L, Jakubowski J, Ward JD: A new minimally invasive technique to show nerve ischaemia in diabetic neuropathy. *Diabetologia* 42:737–742, 1999
40. Schneider JG, Tilly N, Hierl T, Sommer U, Hamann A, Dugi K, Leidig-Bruckner G, Kasperk C: Elevated plasma endothelin-1 levels in diabetes mellitus. *Am J Hypertens* 15:967–972, 2002
41. Coppey LJ, Gellett JS, Davidson EP, Dunlap JA, Yorek MA: Changes in endoneurial blood flow, motor nerve conduction velocity and vascular relaxation of epineurial arterioles of the sciatic nerve in ZDF-obese diabetic rats. *Diabetes Metab Res Rev* 18:49–56, 2002