

Addition of Pioglitazone and Ramipril to Intensive Insulin Therapy in Type 2 Diabetic Patients Improves Vascular Dysfunction by Different Mechanisms

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Running Title: “Mechanisms of Improved Vascular Dysfunction in Diabetes“

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Received for publication 11 April 2007 and accepted in revised form 27 September 2007.

Abstract

Objective: We examined the relationship between glycemic control, vascular reactivity and inflammation in T2DM subjects

Study Design: Thirty subjects with T2DM were initiated on intensive insulin therapy (continuous subcutaneous insulin infusion [n=12] or multiple daily injections [n=18]) and then randomized to either pioglitazone (PIO, 45 mg/d), ramipril (RAM, 10 mg/d), or placebo (PLC) for 36 wks. Euglycemic hyperinsulinemic clamp was used to quantify insulin resistance and plethysmography to assess changes in forearm blood flow (FBF) after: (i) 5 min of reactive hyperemia (RH); (ii) brachial artery infusion of acetylcholine (Ach: 7.5,15,30 μ g/min) and sodium nitroprusside (SNP: 3,10 μ g/min)

Results: The decrease in HbA_{1c} (~9.0 to 7.0%) and FPG (~190 to 128 mg/dl) were equal in all groups. In PIO, glucose disposal increased from 3.1 to 4.7 mg/kg•min, and there was a greater decrease in plasma triglycerides (~148 vs. 123 mg/dl) and FFA (~838 vs. 595 mEq/L) vs. RAM or PLC (p<0.05). Plasma adiponectin doubled with PIO (6.2±0.7 to 13.1±1.8 μ g/ml), while endothelin-1 decreased only with RAM (2.5±0.2 to 1.1±0.2 pg/ml, p<001). The increase in FBF during RH (215%) and Ach (from 132 to 205%, 216 to 262%, and 222 to 323%) was greater in PIO vs. RAM or PLC. In contrast, FBF during SNP was greater in RAM (141 to 221% and 218 to 336%) vs. PIO or PLC (all p<0.05).

Conclusion: Addition of PIO or RAM to intensive insulin therapy in T2DM further improves vascular dysfunction. PIO enhances endothelial-mediated, whereas ACE inhibition enhances endothelial-independent vasodilation. These different vascular effects, combined with the observation that PIO decreases FFA and triglycerides and increases adiponectin, while RAM reduces endothelin-1, suggest that different mechanisms underlie the vascular responses.

Introduction

Atherosclerotic cardiovascular disease affects more than 80% of type 2 diabetic patients and accounts for a substantial increase in the morbidity and mortality (1,2). The clustering of risk factors, including hyperglycemia, hypertension, dyslipidemia and visceral obesity, only can partially explain the 2 to 4-fold higher frequency of cardiovascular events reported in diabetic patients (3). Recent evidence suggests that insulin resistance, vascular endothelial dysfunction, and inflammation (4) play critical roles in the development of atherosclerosis. Thus, interventions aimed at improving insulin resistance and vascular dysfunction and inflammation may provide additional benefits to diabetic subjects at risk for cardiovascular disease over and beyond normalization of conventional risk factors (5).

Intensive insulin therapy (IT) to maintain near-normoglycemia has been associated with a decrease in long-term morbidity (6) and enhanced survival in selected diabetic (7) and non-diabetic (8) hospitalized patients. More recently, a reduction in cardiovascular events also has been demonstrated in type 1 diabetic patients treated aggressively with insulin for more than 10 years (9). In addition, the use of thiazolidinediones [TZD] (10) and agents that inhibit the angiotensin system (11,12) have been shown to result in improved cardiovascular outcomes in diabetic subjects; TZDs also improve dyslipidemia and enhance insulin sensitivity. Of note, both TZDs and ACE inhibitors enhance insulin signal transduction and increase phosphoinositol-3 kinase/Akt activity *in vitro*, which would be expected to augment nitric oxide synthetase activity (13), thereby improving endothelial

dysfunction. Previous studies have confirmed that vascular dysfunction and inflammation are attenuated with TZD therapy in diabetic subjects (14-16), though most studies could not discern whether these metabolic effects have a direct impact on the vascular structure and function, and whether these are mediated by the reduction in glycemia or some other action of the TZD. In some studies (16,17) amelioration of vascular dysfunction and reduction in inflammatory biomarkers was shown to occur independently of glycemic control. This finding, along with the observation that abnormal vascular and inflammatory responses are improved with angiotensin converting enzyme blocking agents (14-17), support the concept that vascular dysfunction cannot be attributed solely to elevated plasma glucose concentrations.

To gain insight into the pathophysiologic and molecular mechanisms responsible for endothelial dysfunction, we examined vascular reactivity and markers of inflammation in patients with type 2 diabetes who were intensively treated with insulin and subsequently randomized to receive either pioglitazone (45 mg/d), ramipril (10 mg/d), or placebo for 36 weeks, in a double-blind study.

Experimental Design

Subjects

Thirty adult Mexican American patients with type 2 diabetes mellitus (T2DM) who required insulin therapy ($HbA_{1c} > 8.0$ despite optimized oral agent therapy) were recruited from the outpatient clinic at the Texas Diabetes Institute (TDI) in San Antonio, Texas. The protocol was approved by the Institutional Review Board of the University of Texas Health Science Center at San Antonio, and prior

to study all subjects provided informed voluntary written consent. Patients on insulin combination therapy with metformin, sulfonylurea and/or meglitinides were included, but those on thiazolidinediones were excluded. Patients taking angiotensin converting enzyme inhibitor (ACE) or angiotensin II-receptor blockade (ARB) agents were switched to alpha-methyl dopa and the dose was adjusted to re-establish blood pressure (<130/80 mmHg) control before they were enrolled in the study. The ACEI/ARB therapy was discontinued for at least 2 months prior to study, and other medications were allowed only if stable for at least 3 months.

Protocol

Subjects reported to the CRC after a 10 h overnight fast and blood samples for measurement of fasting plasma glucose, lipids, and hemoglobin A_{1c} and urine for the determination of microalbumin-to-creatinine ratio were obtained. Three consecutive sitting blood pressure measurements were recorded with an automated arm cuff. Patients then were enrolled in a 3-day comprehensive diabetes education and nutritional program conducted at the TDI. Patients were eligible for either therapy and allowed to select between IT using MDII or CSII. MDII consisted of a basal-bolus program with four daily insulin injections using a combination of insulin glargine (Sanofi-Aventis) at bedtime plus pre-meal insulin aspart (Novo Nordisk). CSII was implemented using the Medtronic/Minimed (n=6) or the Animas Corporation (n=6) pump using basal infusion and pre-meal boluses of insulin aspart (Novo Nordisk). Before randomization, patients received a euglycemic hyperinsulinemic clamp and vascular studies

Euglycemic HyperInsulinemic Clamp

Insulin sensitivity was quantitated with the euglycemic hyperinsulinemic (80 mU/m².min) clamp technique (18), which is sufficient to suppress endogenous glucose production by over 90% (19). The rate of exogenous glucose infusion was averaged during the last 60 minutes of the insulin clamp to provide a measure of insulin-stimulated total body glucose metabolism [M]. Prior to the start of the insulin clamp, baseline blood samples were obtained from the arterialized hand vein for the determination of plasma concentrations of: free of fatty acids, adiponectin, endothelin-1, adhesion molecules (VCAM, ICAM), high sensitivity C-reactive protein (hs-CRP); tumor necrosis factor alpha (TNF- α), interleukin-6 (IL-6), lipids (total/HDL/LDL cholesterol and triglycerides), and lipid particle size.

Vascular Studies

Endothelial function was evaluated in all patients using venous occlusion plethysmography [VOP] (Hokanson, Bellevue, WA). Changes in resting forearm blood flow (FBF) following (i) 5 minutes of transient ischemia (RH) and (ii) brachial artery infusion of acetylcholine [Ach] and sodium nitroprusside [SNP] were documented by VOP. In the morning, after an overnight fast and a rest of 30 minutes in the supine position, 5 measurements of FBF were performed to establish the baseline value. An arm cuff then was inflated above the patient's systolic BP for 5 minutes. Changes in FBF were documented at 5 seconds intervals after the arm cuff was released, and a mean value was calculated. Then, an 18-gauge catheter (Arrow International, Erding, Germany) was introduced into the brachial artery of the non-dominant arm under local

lidocaine anesthesia. After 60 minutes of rest, test substances were infused into the brachial artery according to previously published technique (14, 20). In brief, the initial 6 minute infusion of SNP (3 µg/min) was followed by an increase to 10 µg/min for an additional 6 minutes. After the line was cleared, Ach was infused at a constant rate of 7.5 µg/min for 8 minutes and then increased to 15 and 30 µg/min for 6 minutes in sequence. FBF was measured 5 times during the last 3 minutes of each intra-brachial infusion period and a mean value was calculated. Baseline FBF was expressed as ml/100 ml of forearm tissue per minute using a software program (Hokanson, Bellevue, WA) and expressed as a percent changes from basal. At the end of the experiment the arterial line was removed and compression pressure applied to the artery. All patients were contacted within 24 hrs and 1 week after the study to ascertain that there had been no complications.

Randomization

Upon successful completion of the baseline studies, patients were randomized either to pioglitazone (PIO, 45 mg/d), ramipril (RAM, 10 mg/d) or placebo (PLC), for 36 wks, in a double-blind fashion. Pioglitazone was started at the dose of 15 mg daily and then increased to 30 mg daily at week 2, and to 45 mg daily at week 4. Ramipril was started at the dose of 5 mg daily and increased to 10 mg daily as tolerated at 2 and 4 weeks. Placebo tablets were added to match the other treatment regimen. Participants were contacted by phone at least weekly during the first two months. Insulin dose was adjusted according to the UT Hospital protocol to achieve the following pre-established glycemic goals: fasting and pre-meal capillary blood glucose (BG)

values between 80-120 mg/dl; 2 hour post-meal <160 mg/dl; bedtime levels <140 mg/dl. If the pre-meal glycemic goal range was not attained, patients were instructed to supplement their usual insulin dose with an additional 1,2 or 3 units if the capillary BG measurement was >120 mg/dl, >150 mg/dl, or >180 mg/dl, respectively. If the capillary BG measurement was <80 mg/dl the calculated pre-meal insulin dose was reduced by 1-2 units. If the fasting BG concentration was >80<120 mg/dl for a minimum of three consecutive days, the insulin basal dose and the basal infusion rate were adjusted accordingly by ~10% daily.

Follow-up Plan

All patients were asked to return for office visits at 2-4 week intervals during the first 3 months and every two months thereafter for the remainder of the 9-month study period. During each visit compliance with the assigned therapeutic regimen was ascertained and self-monitored glucose values were reviewed, and routine blood and urine samples were obtained. The hyperinsulinemic clamp, vascular studies, and measurements of markers of endothelial damage and inflammation were repeated at 36 weeks (study termination)

Laboratory Analyses

Plasma glucose concentration was measured using the glucose oxidase method with the Beckman Glucose Analyzer and tritiated glucose specific activity was determined in barium/zinc deproteinized plasma samples. HbA_{1c} was measured by affinity chromatography (Biochemical Methodology, Drower 4,350; Isolab, Akron, OH), plasma insulin (Diagnostic Products, Los Angeles, CA) and plasma free fatty acid (FFA)

concentration by enzymatic colorimetric quantification (Wako Chemicals, Neuss, Germany), adiponectin by commercially available radio-immunoassay (Linco, St. Charles, MO), and plasma CRP, VCAM, ICAM, TNF- α , IL-6, and endothelin-1 by ELISA assays (Linco, St. Charles, MO). Lipid profile and particle size were determined by nuclear magnetic resonance spectroscopy (Liposcience, Inc, Raleigh, NC).

Statistics

The sample size was estimated on the basis of our previous vascular data, and powered to demonstrate a difference between the mean basal value increase of 50% and the alternate mean increase of 75% between groups in forearm blood flow during reactive hyperemia, and following Ach and SNP brachial artery infusions before and after the interventions. A total number of 10 subjects per group was derived from a two-sided test with significance levels α ($=0.05$) and a power of $1-\beta$ ($=0.90$) using a mean standard deviation of $\pm 30\%$. Changes in metabolic (fasting plasma glucose, free fatty acids, hemoglobin A_{1c}, total and LDL, HDL cholesterol and triglyceride concentrations, and in insulin-mediated glucose disposal), in inflammatory biomarkers (hsCRP, adiponectin, endothelin-1, adhesion molecules VCAM, TNF- α and IL-6) and in vascular parameters (percent change in forearm blood flow above basal during reactive hyperemia, Ach-stimulated and SNP-stimulated vasodilation) were analyzed in the intensively treated diabetic patients (MDII and CSII together) assigned to each oral therapy and between groups using analysis of variance with repeated measures (ANOVA). Statistical significance ($p < 0.05$) was considered when the within group variability was

inferior to the inter-group variability. Univariate analyses was performed between vascular responses (RH, Ach- and SNP-vasodilation) and hemoglobin A_{1c} values, and multiple regression analyses (simultaneous regression) was conducted between the metabolic, the inflammatory biomarkers and the vascular parameters listed above, as continuous variables in a stepwise regression. Statistical significance was considered for p value < 0.05 . All statistical analyses were performed using a Sigma Stat software program.

Results

Baseline characteristics showed patients of comparable gender (~6F/4M), mean age (~46 yrs), duration of disease (6.2 to 8.4 yrs), BMI (~31-33 kg/m²), waist circumference (101-104 cm), blood pressure (~130/70 mm Hg), insulin dose (~1.2 U/kg.day), mean HbA_{1c} (~9.0%), and fasting plasma glucose (~190 mg/dl). The use of oral anti-diabetic medications (sulfonylureas, meglitinides, and metformin) was similar in all three groups. Nearly one-half of the patients was taking a statin, and one-third was on antihypertensive therapy, with alpha-methyl dopa substituted for ACE inhibitors ($n=15$) and ARB ($n=2$). After 36 weeks of IT, mean body weight increased in all patients, by 1.7 kg in the placebo group, 3.2 in the ramipril group and 4.4 kg in the pioglitazone group (all $p < 0.01$ vs. baseline). The average daily insulin requirement remained unchanged in the placebo group (~1.2 U/kg) and decreased similarly in the ramipril and pioglitazone groups to 1.0 ± 0.2 U/kg ($p < 0.05$). Except for the discontinuation of sulfonylureas and meglitinides (to avoid hypoglycemia), all other medications (including metformin) were

maintained stable in all patients during the entire study period.

IT using either MDII or CSII was well tolerated by patients, and there were no withdrawals. During the 36 weeks of observation, 14 patients (6 in the placebo, 4 in the pioglitazone and 4 in the ramipril group) had 33 hypoglycemic episodes (0.32 patients per year), defined as symptomatic hypoglycemia requiring glucose ingestion. Mechanical malfunction of the insulin pump was documented on four occasions, and patients had to temporarily (< 2 days) interrupt the insulin pump therapy and substitute multiple daily injections. One patient developed an abdominal skin infection, which resolved with oral antibiotic therapy and local treatment. Three patients in the pioglitazone and one in the ramipril group developed mild peripheral edema that did not require therapy.

Metabolic and Inflammatory Parameters (Table 1)

Patients in all three groups showed a substantial improvement in their metabolic control over the 9 month period, with reduction in fasting plasma glucose concentration to 123-128 mg/dl and decrease in mean HbA_{1c} to ~7.0% (p<0.01). Plasma free fatty acid (838±84 to 595±65 mEq/L) and triglyceride (148±17 to 123±11 mg/dL) concentrations decreased significantly (p<0.01 vs. baseline and vs. placebo and ramipril groups) after 9 months of pioglitazone therapy. In the pioglitazone group, although plasma LDL concentration did not change (107±7 vs. 105±12 mg/dl), both HDL concentration (45±3 to 51±3 mg/dl) and LDL particle size (20.2±0.5 nm to 21.2±0.8 nm) increased significantly (p<0.05 vs. placebo and

ramipril). Plasma adiponectin levels more than doubled (6.2±0.7 to 13.1±1.8 µg/ml) with pioglitazone therapy (p<0.001 vs. baseline, and vs. placebo and ramipril), and remained unchanged in the placebo and ramipril groups. Plasma levels of endothelin-1 (from 2.5±0.2 to 1.1±0.2 pg/ml) and IL-6 (from 3.6±0.3 to 2.4±0.2) decreased significantly with the addition of ramipril to intensive insulin therapy (p<0.05 vs. baseline and vs. placebo and pioglitazone). After 36 weeks, M was significantly enhanced by ~50% in the IT group that received pioglitazone, (3.1 to 4.7 mg/kg.min; p<0.01 vs. baseline and vs. placebo and ramipril groups). The increments in M in the placebo (4.5 vs. 4.9 mg/kg.min) and ramipril (3.2 vs. 3.6 mg/kg.min) groups were not statistically significant.

Vascular Reactivity (Figure 1)

Vascular reactivity was assessed by the changes in FBF during RH, and during intra-brachial infusion of Ach and SNP. Basal FBF was comparable in all groups: 2.4-2.6 ml/100ml.min. The percent rise in FBF following 5 minutes of brachial artery occlusion was similar (~30-40% above baseline) in all groups prior to the start of insulin/drug therapy (**Figure 1A**). After 36 weeks of therapy, the percent increase in FBF during RH was significantly (p<0.05) greater in PIO (215%) compared to PLC (170%) and RAM (181%) groups. Pioglitazone-treated patients achieved the highest absolute increase in FBF during RH (5.2 ml/100ml•min) compared to PLC (4.3) and RAM (4.2) groups (both p<0.05). The percent rise in FBF in the ramipril group during Ach infusion was: 139 to 181% [7.5 µg/min], 147 to 196% [15 µg/min], and 193 to 230% [30 µg/min] and did not differ from placebo (**Figure 1B**). The percent increase in FBF with

Ach infusion was greater with PIO vs. both PLC & RAM (132 to 205% [7.5 µg/min], 216 to 262% [15 µg/min], and 222 to 323% [30 µg/min]) (all $p < 0.05-0.01$). In contrast, endothelial-independent SNP-stimulated vasodilation (**Figure 1C**) was significantly greater in the ramipril group ($p < 0.05$) with percent increments of 141 to 221% [3 µg/min] and 218 to 336% [10 µg/min]. Percent change in FBF in the PLC (151 to 182% at 3 µg/min and 237 to 265% at 10 µg/min) and PIO (138 to 184% at 3 µg/min and 230 to 257% at 10 µg/min) groups were small and did not reach statistical significance.

Multivariate analyses were performed between the metabolic, inflammatory biomarkers and vascular continuous variables. Positive correlations between the change in HbA_{1c} and reactive hyperemia ($p = 0.04$), and between fasting plasma glucose ($p < 0.01$) and insulin-mediated glucose disposal ($p < 0.05$) and Ach-stimulated vasodilation [30 µg/min] were obtained, indicating that glycemic control and insulin sensitivity were closely associated with vascular reactivity. In multiple regression analyses, there were also strong negative correlations between changes in plasma triglyceride concentration ($p = 0.04$) and in plasma levels of hsCRP ($p < 0.02$) with SNP-stimulated [10 µg/min] vasodilation, and positive correlations between plasma HDL cholesterol and both low [3 µg/min] and high [10 µg/min] SNP-stimulated vasodilation in all subjects, which imply also that there was a role for plasma lipid concentrations in vascular responses. Amongst the inflammatory parameters, plasma adiponectin concentration showed an independent strong positive correlation with Ach-stimulated vasodilation [30 µg/min] ($p < 0.001$), whereas plasma

endothelin-1 levels showed a negative correlation with SNP-induced vasodilation [3 µg/min] ($p = 0.04$).

Discussion

In this study we have demonstrated that the addition of either the insulin sensitizer pioglitazone or the angiotensin-converting enzyme blocking agent ramipril further improves vascular dysfunction and markers of inflammation independent of glycemic control. Our data indicate that pioglitazone primarily enhances endothelial-mediated, whereas ramipril augments endothelial-independent vasodilation. These different vascular effects, combined with the observation that pioglitazone increases the vasodilator adiponectin concentration, while ramipril reduces the vasoconstrictor endothelin-1 levels suggest that different and complementary mechanisms underlie the observed improvements in vascular reactivity. In agreement with previous studies, pioglitazone therapy also was accompanied by a reduction in plasma fatty acids and triglyceride concentrations, both of which may play a role in the correction of vascular dysfunction (14-16).

It should be emphasized that intensive insulin therapy alone, in the placebo group, was effective in decreasing the circulating inflammatory biomarker hsCRP, and that this decrease was equivalent to that seen with the addition of pioglitazone or ramipril. Moreover, endothelial-mediated vasodilation, measured by: (i) the percent increase in FBF during RH and during the low Ach [7.5] infusion and (ii) endothelial-independent vasodilation following both low [3.0] and high [10.0] dose SNP infusion were improved in the placebo group, and there was a significant

correlation between glycemic control and vascular reactivity in all groups. These findings indicate that, in addition to attenuating inflammation, intensive insulin therapy improves vascular endothelial dysfunction. The independent association between the decrease in plasma hsCRP levels and the improvement in vasodilation support the concept that intensive insulin therapy improves vascular endothelial dysfunction, in part, via attenuation of the inflammatory response. The possibility that acute glycemic fluctuations, induced by insulin therapy interfere with vascular reactivity is not ruled out, however, since these may not be adequately reflected in the HbA_{1c} values (21). The relationship between hyperglycemia and vascular reactivity previously has been examined in both diabetic (22) and non-diabetic subjects (8) and, in the presence of hyperglycemia, endothelial-mediated vascular dilation is impaired (23, 24). Correction of hyperglycemia, regardless of the therapy, has been shown to enhance endothelial dependent vasodilation (24-26), and intensive insulin therapy is believed to reduce the formation of reactive oxygen and nitrogen species in tissue (22,24,26) and macrophages (27), thus minimizing the inflammatory response.

Our findings indicate that additional mechanisms, beyond glycemic control, and more directly related to changes in insulin resistance and vascular reactivity must play an important role in the improvement in endothelial dysfunction observed with these agents. There is increasing evidence that, in order to prevent/retard atherosclerotic cardiovascular complications in type 2 diabetic patients, the underlying defects of both insulin resistance and endothelial

dysfunction need to be addressed (5). Enhanced insulin sensitivity consistently has been demonstrated with pioglitazone (14,19), although the beneficial effect of ramipril on insulin sensitivity is more controversial (30), and in a recent trial the diabetes conversion rate was unaffected (30). Both pioglitazone and ramipril have been shown to improve vascular reactivity (16,30,31). In the present study, a 50% increment in insulin-mediated glucose disposal was demonstrated during the hyperinsulinemic clamp in the pioglitazone-treated group. The modest non-significant increase in insulin sensitivity in the ramipril-treated group is consistent with previously reported results (28). We observed a highly significant correlation between the improvements in peripheral insulin resistance and in endothelial-mediated vasodilation, suggesting that enhanced insulin sensitivity, induced by pioglitazone, contributes to the improvement in endothelial-mediated vasodilation. However, previous studies by us (14) and others (15,16) also have shown that pioglitazone can improve endothelial dysfunction independent of changes in blood glucose levels. Thus, it is likely that the beneficial effects of TZDs on vascular function are mediated by: (i) direct effects on the vascular endothelium, (ii) improved glycemic control, and (iii) enhanced insulin-mediated glucose metabolism.

Pioglitazone did not improve endothelial-independent vasodilation beyond that observed with intensive insulin therapy alone. In contrast, ramipril significantly increased endothelial-independent vasodilation above that observed with insulin alone. The improvement in SNP-stimulated vasodilation (an index of endothelial-independent nitric oxide-

mediated vaso-relaxation) was closely correlated with the reduction in plasma endothelin-1 levels. Following pioglitazone treatment for 36 weeks, plasma adiponectin levels doubled, and the increase correlated closely with improved endothelial-dependent vasodilation. Based on these findings, we speculate that increased levels of the vasodilator adiponectin (36) plays a role in mediating the improvement in vascular reactivity following pioglitazone, while a decrease in the vasoconstrictor endothelin-1 (33) levels contributes to the enhanced vascular function observed with ramipril. These complementary mechanisms suggest that combined TZD-ACEI therapy may be especially beneficial in improving vascular health in T2DM patients.

Our experiments are limited by the inherent difficulty of clinical observations performed in diabetic patients who are being treated with multiple drugs. The effects of prior treatment with agents that interfere with the angiotensin system were minimized by switching all ACEI/ARB-treated patients to alpha-methyldopa, as is done routinely in pregnant women. The number of diabetic patients receiving metformin therapy was similar in all three groups (n=3-4) and we did not observe any differences in any parameters in patients treated with and without metformin. Statins were used in 4-6 patients in each group but the dose of statin therapy was stable for at least 6 months prior to the study commencement and the statin dose was not changed during the study. Although no clear

difference has been reported regarding the effect of intensive therapy provided with an insulin pump versus multiple insulin injections, it must be recognized that our groups were heterogeneous with respect to this modality of therapy. Nevertheless, each of the three treatment groups contained equal number of individuals treated with CSII and MDII. Other limitations of our study are the absence of a group receiving pioglitazone combined with ramipril and the period of observation (36 weeks) in the present study that is relatively short, considering that these patients have had diabetes for 6-8 years. Whether our observations would continue to be observed over longer periods of therapy and translate into significant clinical benefits remain to be determined.

In conclusion, the addition of PIO or RAM to intensive insulin therapy in T2DM further improves vascular dysfunction. PIO enhances endothelial-mediated, whereas ACE inhibition enhances endothelial-independent vasodilation. These different vascular effects, combined with the observation that PIO decreases FFA and triglycerides and increases adiponectin, while RAM reduces endothelin-1, suggest that different mechanisms underlie the vascular responses.

Acknowledgments

This work was supported by the American Diabetes Association and in part by Takeda Pharmaceuticals of North America.

References

1. Isomaa B, Almgren P, Tuomi T, Forsen B, Lathi K, Nissen M, Taskinen M, Groop L. Cardiovascular morbidity and mortality associated with the metabolic syndrome. *Diabetes Care* 2001; 24:683-689
2. Haffner SM, Letho S, Rönnemaa T, Pyörälä K, Laako M. Mortality from coronary heart disease in subjects with type 2 diabetes and in non-diabetic subjects with and without prior myocardial infarction. *N Engl J Med*. 1998; 339:229-234
3. Stamler J, Vaccaro O, Neaton JD, Wentworth D. Diabetes, other risk factors, and 12-yr cardiovascular mortality for men screened in the multiple risk factor intervention trial. *Diabetes Care* 1993; 16:434-444
4. Ross R. 1993. The pathogenesis of atherosclerosis: a perspective for the 1990's. *Nature* 362:801-809
5. Cersosimo E., DeFronzo RA. Insulin Resistance and Endothelial Dysfunction: The Road Map for Cardiovascular Diseases. *Diabetes Metab Res Rev* 2006; 22:423-436
6. The DCCT Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med* 1993; 329:977-986
7. Malberg K, Norhammar A, Wedel H et al. Glycometabolic state at admission: important risk marker of mortality in conventionally treated patients with diabetes mellitus and acute myocardial infarction. Long-term results from the diabetes and insulin-glucose in acute myocardial infarction (DIGAMI) study. *Circulation* 1999; 99:2626-2632
8. Van Der Berghe G, Wouters P, Weekers F et al. Intensive insulin therapy in critically ill patients. *N Engl J Med* 2001; 345:1359-1367
9. The Diabetes Control and Complications Trial/ Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) Study Research Group. Intensive diabetes treatment and cardiovascular disease in patients with type 1 diabetes. *N Engl J Med* 2005; 353 (25): 2643- 53
10. Dormandy JA, Charbonnel B, Eckland DJA, Erdmann E, Massi-Benedetti M, Moules IK, Skene AM, Tan MH, Lefebvre PJ, Murray GD. Secondary prevention of macrovascular events in patients with type 2 diabetes in Proactive study (Prospective pioglitazone clinical trial in macrovascular events): a randomized controlled trial. *The Lancet* 2005; 366:1279-1289
11. HOPE (The Heart Outcomes Prevention Evaluation Study Investigators). 2000. Effects of an angiotensin-converting enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. *N Engl J Med* 2000; 342:145-153
12. Julius S, Kjeldsen SE, Weber M et al. Outcomes in hypertensive patients at high cardiovascular risk treated with regimens based on valsartan or amlodipine; the VALUE randomized trial. *Lancet* 2004; 363: 2022-31
13. Quyyumi AA, Dakak N, Andrews NP, et al. Nitric oxide activity in the human coronary circulation: impact or risk factors for coronary atherosclerosis. *J Clin Invest* 1995; 95:1747-1755

14. Wajcberg E, Sriwijilkamol A, Musi N, DeFronzo R, Cersosimo E. Relationship between vascular reactivity and lipids in Mexican American with T2DM treated with pioglitazone. *J Clin Endo and Metabol* 2007;92: 1256-62
15. Natali A, Baldeweg S, Toschi E, Capaldo B, Barbaro D, Gastadelli A, Yudkin J, Ferrannini E. Vascular effects of improving metabolic control with metformin/rosiglitazone in type 2 diabetes. *Diabetes Care* 2004; 27:1349- 1357
16. Pfutzner A, Marx N, Lubben G, Langenfeld M, Walcher D, Konrad T, Forst T. Improvement of cardiovascular risk markers by pioglitazone is independent from glycemic control. Results from the Pioneer study. *J Am Coll Cardiol* 2005; 45:1925-1931
17. Powell J.S., Clozel J.P., Muller R.K.M., Kuhn H. Hefite F, Hosang M, Baumgartner HR. Inhibitors of angiotensin-converting enzyme prevent myointimal proliferation after vascular injury. *Science* 1898; 245:186-188
18. DeFronzo RA, Tobin R, Andres R. The glucose clamp technique. A method for quantifying insulin secretion and resistance. *Am J Physiol* 1979;:E214-223
19. Miyazaki Y, Mahankali A, Matsuda M, Glass L, Mahankali S, Ferrannini E, Cusi K, Mandarino LJ, DeFronzo RA. Improved glycemic control and enhanced insulin sensitivity in type 2 diabetic subjects treated with pioglitazone. *Diabetes Care* 2001; 24: 710-719
20. Bergholm, R.S., S. Makimatilla, M. Valkonen, M.L. Liu, S. Lahdenpara, M.R. Taskinene, A. Sovijarvi, P. Malmberg, and H. Yki-Jarvinen. Intense physical training decreases circulation antioxidants and endothelium-dependent vasodilatation in vivo. *Atherosclerosis* 1999; 145: 341-349
21. Monnier L, Mas E, Ginet C, Michel F, Vilon L, Cristol JP, Colette C. Activation of oxidative stress by acute glucose fluctuations compared with sustained chronic hyperglycemia in patients with type 2 diabetes. *JAMA* 2006; 295(14):1681-87
22. Evans JL, Goldfine IA, Maddux BA, Grodsky GM. Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes. *Endocrine Reviews* 2002; 23(5):599-622
23. Nieuwdorp M, van Haeften WT, Gouverneur MCLG, et al. Loss of endothelial glycocalyx during acute hyperglycemia coincides with endothelial dysfunction and coagulation activation *in vivo*. *Diabetes* 2006; 55: 480-86
24. Van den Berghe G. How does blood glucose control with insulin saves lives in intensive care? *J Clin Invest* 2004. 114(9): 1187-95.
25. Williams SB, Goldfine AB, Timimi FK, Ting HH, Roddy MA, Simonsen DC, Creager MA. Acute hyperglycemia attenuates endothelium-dependent vasodilation in humans *in vivo* *Circulation* 1998; 97: 1695-1701
26. Rask-Madsen C, Ihlemann N, Krarup T et al. Insulin therapy improves insulin-stimulated endothelial function in patients with type 2 diabetes and ischemic heart disease. *Diabetes* 2001; 50:2611-2618
27. Dandona P, Ajada A, Mohanty P, Ghanin H, Hamouda W, Assian E, Ahmad S. Insulin inhibits intra-nuclear factor κ B and stimulates I κ B in mononuclear cells in obese subjects: evidence for an anti-inflammatory effect? *J Clin Endocrinol Metabol* 2001; 86:3257-65

28. Furuhashi M, Ura N, Takizawa H *et al.* Blockade of the renin-angiotensin system decreases adipocyte size with improvement in insulin sensitivity. *J Hypertens* 2004; 22:1977-82
29. Tomiyama H, Motobe K, Zaydun G *et al.* Insulin sensitivity and endothelial function in hypertension. A comparison of temocapril and candesartan *Am J Hypertens* 2005; 18: 278-82
30. The DREAM Trial Investigators. The effect of ramipril on the incidence of diabetes. *N Engl J Med* 2006; 355-355
31. Hornig B, Landmesser U, Kohler C *et al.* Comparative effect of ACE inhibition and angiotensin II type 1 receptor antagonism on bioavailability of nitric oxide in patients with coronary artery disease: role of superoxide dismutase. *Circulation* 2001; 103:799-805
32. Bajaj M, Suraamornkul S, Piper P, *et al.* Decreased plasma adiponectin concentrations are closely related to hepatic fat content and hepatic insulin resistance in pioglitazone-treated T2DM. *J Clin Endo Metab* 2004;89: 200-206
33. Takahashi K, Ghatei M, Lam H, O'Halloran D, Bloom S. Elevated plasma endothelin in patients with diabetes mellitus. *Diabetologia* 1990; 33:306-310

Table 1 – Changes in metabolic parameters, markers of endothelial dysfunction, and inflammation in patients with type 2 diabetes after 36 weeks of intensive insulin therapy in combination with placebo, pioglitazone, or ramipril.

Parameters	Placebo		Pioglitazone		Ramipril	
	Pre	Post	Pre	Post	Pre	Post
FPG (mg/dl)	196±19	128±19	190±14	123±13	189±11	126±6
FPI (µU/ml)[@]	16±5	28±9	14±4	24±7	11±4	16±10
HbA_{1c} (%)	9.2±0.4	7.2±0.1	9.0±0.7	6.9±0.3	9.1±0.5	7.0±0.2
FFA (mEq/l)	778±54	695±88	838±84	595±65*	753±64	705±73
Total Chol (mg/dl)	195±9	180±8	176±9	175±16	190±12	199±13
LDL Chol (mg/dl)	121±8	115±7	107±7	105±12	117±8	122±10
HDL Chol (mg/dl)	49±4	46±3	45±3	51±3*	50±4	51±7
VLDL Chol (mg/dl)	113±24	93±19	109±16	88±15	120±28	92±10
Trig (mg/dl)	146±15	132±18	148±17	123±11*	145±12	142±17
Adiponectin (µg/ml)	8.9±1.3	9.1±1.1	6.2±0.7	13.1±1.8*	7.1±0.2	8.6±0.3
hs-CRP (mg/l)[@]	2.5±0.3	2.1±0.2	1.7±0.2	1.1±0.2	1.5±0.2	1.1±0.1
TNF-α (mg/l)	1.8±0.2	1.3±0.1	1.6±0.1	1.3±0.2	1.9±0.4	1.6±0.3
IL-6 (pg/ml)	2.4±0.2	2.1±0.3	3.7±0.7	2.9±0.6	3.6±0.3	2.4±0.2**
VCAM (ng/ml)	574±33	522±38	556±38	487±32	590±40	529±32
ICAM (ng/ml)	278±9	269±8	247±11	235±10	257±25	242±29
Endothelin-1 (pg/ml)	1.8±0.2	2.0±0.3	2.3±0.3	2.1±0.4	2.5±0.2	1.1±0.2**

*p<0.05-0.01 pioglitazone vs. baseline; **p<0.05 ramipril vs. baseline; [@]p<0.05 vs. baseline for all groups

FPG = fasting plasma glucose; FPI = fasting plasma insulin; FFA = free fatty acids; Chol = cholesterol; Trig = triglycerides; hs-CRP = high sensitive C-reactive protein; TNF-α = tumor necrosis factor alpha; IL-6 = interleukin-6; VCAM = vascular cell adhesion molecules; ICAM = inter-cellular adhesion molecules

FIGURE 1 LEGEND

Figure 1A – Brachial arterial vasodilation expressed as forearm blood flow [FBF, in ml per 100 ml.min] in response to reactive hyperemia (5 minutes of cuff inflation) at baseline and following 36 weeks (Post-Tx) of combined intensive insulin therapy with placebo, pioglitazone or ramipril. There was a significant increase in the vascular response to reactive hyperemia after therapy in all three groups (** $p < 0.05$ vs. baseline), and the magnitude of vasodilation was significantly greater in the group treated with pioglitazone versus the placebo and ramipril groups (* $p < 0.05$)

Figure 1B – Brachial arterial vasodilation expressed as forearm blood flow [FBF, in ml per 100 ml.min] during a dose-response stimulation test with intra-brachial arterial acetylcholine (Ach) infusion at doses of 7.5, 15, and 30 μg per minute at baseline (\square) and after 36 wks of treatment with intensive insulin therapy plus placebo (Δ), pioglitazone (\bullet) or ramipril (\blacksquare). There was a significant increase in the vascular response at low dose (7.5) Ach in all three groups (** $p < 0.05$ vs. baseline). The degree of vasodilation achieved at the intermediate (15) and high (30) dose Ach infusion was significantly greater in diabetic subjects treated with pioglitazone vs. both ramipril and placebo (* $p < 0.05$)

Figure 1C – Brachial arterial vasodilation expressed as forearm blood flow [FBF, in ml per 100 ml.min] during a dose-response stimulation test with intra-brachial arterial sodium nitroprusside (SNP) infusion at 3.0 and 10 μg per minute at baseline (\square) and after 36 wks of treatment with intensive insulin therapy plus placebo (Δ), pioglitazone (\bullet) or ramipril (\blacksquare). There was a significant increase in the vascular response at the low (3) and high (10) SNP dose in all three groups (* $p < 0.005$ vs. baseline). The degree of vasodilation achieved at the high SNP dose was significantly greater in diabetic subjects treated with ramipril vs both placebo and pioglitazone (** $p < 0.05$)

Figure 1A: Reactive Hyperemia

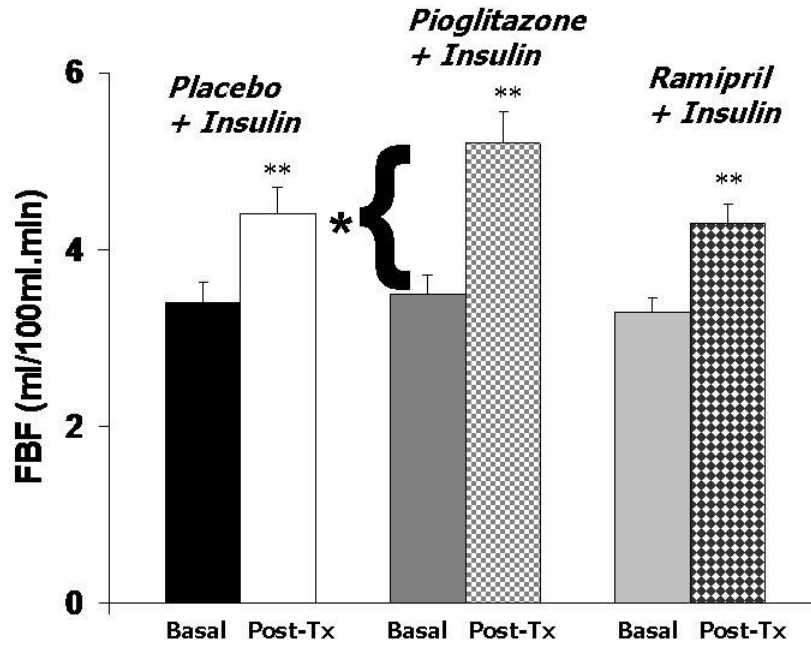


Figure 1B: Ach-Stimulated Vasodilation

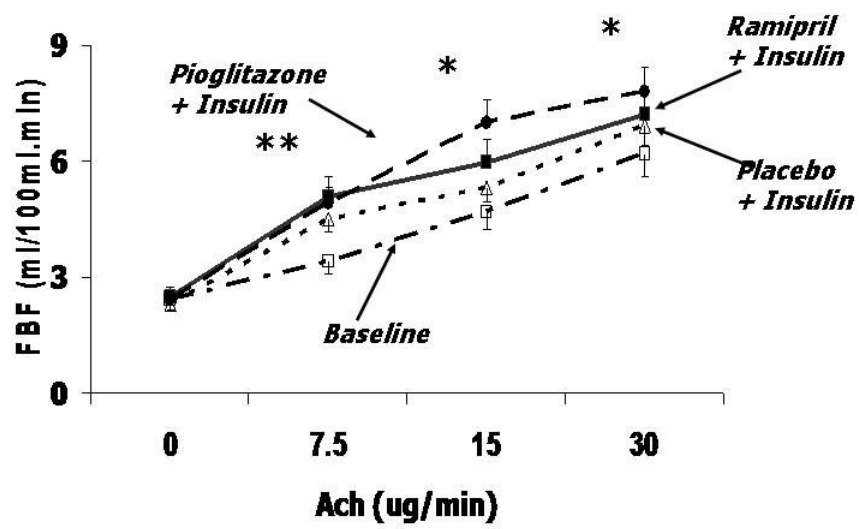


Figure 1C: SNP-Stimulated Vasodilation

