Insulin Reduces Plasma Arginase Activity in Type 2 Diabetic Patients

SANGEETA R. KASHYAP, MD[1]
ABIGAIL LARA, MD[2]
RENLIANG ZHANG, MD[2]
YOUNG MI PARK, MD[2]
RALPH A. DEFRONZO, MD[3]

OBJECTIVE — We sought to determine whether dysregulation of arginine metabolism is related to insulin resistance and underlies impaired nitric oxide (NO) generation in type 2 diabetic patients.

RESEARCH DESIGN AND METHODS — We measured plasma arginase activity, arginine metabolites, and skeletal muscle NO synthase (NOS) activity in 12 type 2 diabetic and 10 age-/BMI-matched nondiabetic subjects before and following a 4-h euglycemic-hyperinsulinemic clamp with muscle biopsies. Arginine metabolites were determined by tandem mass spectroscopy. Arginase activity was determined by conversion of [^{14}C] guanidoinoarginine to [^{14}C] urea.

RESULTS — Glucose disposal (R_d) was reduced by 50% in diabetic versus control subjects. NOS activity was fourfold reduced in the diabetic group (107 ± 45 vs. 459 ± 100 pmol · min^{-1} · mg protein^{-1}; $P < 0.05$) and failed to increase with insulin. Plasma arginase activity was increased by 50% in the diabetic versus control group (0.48 ± 0.11 vs. 0.32 ± 0.12 μmol · ml^{-1} · h^{-1}; $P < 0.05$) and markedly declined in diabetic subjects with 4-h insulin infusion (to 0.13 ± 0.04 μmol · ml^{-1} · h^{-1} vs. basal; $P < 0.05$). In both groups collectively, plasma arginase activity correlated positively with fasting plasma glucose ($R = 0.46$, $P < 0.05$) and A1C levels ($R = 0.51$, $P < 0.02$) but not with R_d.

CONCLUSIONS — Plasma arginase activity is increased in type 2 diabetic subjects with impaired NOS activity, correlates with the degree of hyperglycemia, and is reduced by physiologic hyperinsulinemia. Elevated arginase activity may contribute to impaired NO generation in type 2 diabetes, and insulin may ameliorate this defect via reducing arginase activity.

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Type 2 diabetes is an insulin-resistant state characterized by inflammation, oxidative stress, and accelerated atherosclerosis (1–3). Nitric oxide (NO) bioavailability, critical for normal vasomotor tone and function, is reduced in states of insulin resistance, including type 2 diabetes (4,5). In addition to enhancing endothelial function, NO has been shown to modulate insulin sensitivity and glucose disposal (6,7). Activation of NO synthase (NOS) augments blood flow to insulin-sensitive tissues (i.e., skeletal muscle, liver, adipose tissue), and its activity has been shown to be impaired by hyperglycemia and insulin resistance (8,9). Under conditions of low arginine levels, NOS is uncoupled, producing reactive oxygen species and oxidative stress in lieu of NO (10). Increased oxidative stress and concentrations of asymmetric dimethylarginine (ADMA), in turn, further reduce NO bioavailability and are predictive of cardiovascular risk (11). Previous reports have related increased ADMA, oxidative stress, and endothelial dysfunction in type 2 diabetes and obesity to the severity of insulin resistance (11,12). Our lab (13) and others (14,15) have previously demonstrated reduced NOS activity in patients with type 2 diabetes and an impaired ability of insulin to increase NOS activity compared with healthy nondiabetic subjects. However, the mechanism(s) responsible for decreased NOS activity in diabetic patients has yet to be determined. This has important therapeutic implications for the development of interventions to restore NO bioavailability.

Arginase, an enzyme found predominantly in liver, kidney, and erythrocytes, converts L-arginine to urea and ornithine and thus decreases substrate availability for NOS to produce NO (16). There are two isoforms of arginase; both are present in human plasma and both are increased in inflammatory conditions, including pulmonary hypertension and sickle cell disease (17,18). In addition to serving as a substrate for NOS, arginine has vasodilatory, anti-inflammatory, and antiatherosclerotic properties (19,20). Although a recent study (21) failed to demonstrate a beneficial effect of arginine to decrease atherothrombotic events in the setting of acute myocardial infarction, this may have been explained by accelerated arginine catabolism secondary to increased arginase activity. Our objective was to measure arginase activity in plasma of subjects with uncomplicated type 2 diabetes and age-/BMI-matched nondiabetic control subjects and to examine the effect of physiologic hyperinsulinemia on plasma arginase activity.

RESEARCH DESIGN AND METHODS

— Twelve type 2 diabetic subjects (aged 50 ± 4 years, five male and seven female, BMI 31.1 ± 1.0 kg/m^2, A1C 6.8 ± 0.9%, fasting plasma glucose 135 ± 14 mg/dl, systolic blood pressure 136 ± 3 mmHg, and diastolic blood pressure 78 ± 3 mmHg) and 10 healthy (aged 45 ± 4 years, six male and four female, BMI 29.5 ± 0.9 kg/m^2, A1C 4.9 ± 0.2%, fasting plasma glucose 92 ± 2 mg/dl, systolic blood pressure 121 ± 4 mmHg, and diastolic blood pressure 72 ± 2 mmHg)

From the [1]Department of Endocrinology, Diabetes and Metabolism, Cleveland Clinic, Cleveland, Ohio; the [2]Department of Cell Biology, Lerner Institute, Cleveland Clinic, Cleveland, Ohio; and the [3]Diabetes Division, University of Texas Health Science Center, San Antonio, Texas.

Address correspondence and reprint requests to Sangeeta R. Kashyap, MD, 9500 Euclid Ave., Cleveland, OH 44195. E-mail: kashyas@ccf.org.

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Abbreviations: ADMA, asymmetric dimethylarginine; NOS, nitric oxide synthase.

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

age- and weight-matched nondiabetic control subjects participated and were described in a previous report (13). Type 2 diabetic subjects had elevated total cholesterol (198 ± 11 vs. 173 ± 8 mg/dl; $P = 0.03$) and triglyceride levels (206 ± 30 vs. 97 ± 15 mg/dl; $P < 0.01$) compared with nondiabetic subjects. Normal glucose tolerance was confirmed in all control subjects by a 75-g oral glucose tolerance test using American Diabetes Association criteria. The diabetic group was in reasonably good glycemic control, as reflected by mean A1C 6.8%, and was treated with diet ($n = 9$) or sulfonylureas ($n = 3$). No diabetic subject had received treatment with metformin, thiazolidinediones, or insulin. The mean duration of diabetes was <2 years. Five diabetic subjects had normal fasting glucose and were diagnosed with an oral glucose tolerance test (2-h plasma glucose >200 mg/dl). Oral antidiabetes agents were discontinued 24 h before the study. Other than diabetes, none of the subjects had any medical problems and none were taking any medications (other than sulfonylureas in three diabetic subjects) known to affect glucose metabolism. None of the participants smoked or were on hormone replacement therapy. The protocol was approved by the institutional review board of the University of Texas Health Science Center at San Antonio.

All studies were conducted in the General Clinical Research Center of the University of Texas Health Science Center at San Antonio and began at 0700 h after a 12-h overnight fast. Disposal of glucose, plasma arginase, and NOS activity was determined in response to physiologic infusion of insulin in both groups by a previously described euglycemic-hyperinsulinemic clamp testing protocol (13). A prime (25 μCi)-continuous infusion (0.25 μCi/min) of 3-[^{3}H]-glucose was started, and 2 h (3 h for diabetic subjects) was allowed for isotopic equilibration. Sixty minutes before the start of the insulin clamp (basal), a percutaneous biopsy of the vastus lateralis muscle was obtained for determination of NOS activity and protein content (13). Thirty minutes before the start of insulin, four baseline blood draws were performed 5–10 min apart for glucose, insulin, and free fatty acid levels. The last baseline blood (time 0) was determined for arginase activity, intercellular adhesion molecule and vascular cell adhesion molecule levels. At the end of the tracer equilibration period, a primed-continuous infusion of 80 mU/m^2 per minute of insulin was started along with a variable infusion of 20% glucose to maintain fasting glucose values (100 mg/dl for diabetic subjects). At 30 and 240 min after the start of the insulin infusion, repeat vastus lateralis muscle biopsies were obtained from a site 4 cm distal to the first. At time 0 and at 180 and 240 min after the start of insulin, plasma was obtained for intercellular adhesion molecule-1 and vascular cell adhesion molecule determination. The insulin infusion was continued for a total of 240 min.

Plasma glucose–specific activity was determined using barium hydroxide/zinc sulfate extracts of plasma. Plasma insulin concentration was determined by radioimmunoassay (Diagnostic Products, Los Angeles, CA). NOS activity produced by skeletal muscle was measured by the NOS Detect Assay Kit (Stratagene, La Jolla, CA) through the conversion of [^{14}C]L-arginine (Perkin Elmer Life Sciences) to [^{14}C]L-citrulline, according to the manufacturer's instructions (13). NOS activity data were normalized by the absolute amount of protein present. For measurement of arginine metabolites, including ADMA, plasma was subjected to cation exchange solid-phase extraction and analyzed by high-performance liquid chromatography–tandem mass spectroscopy (17). The coefficients of variation for intersample and intrasample variations tested with a pooled plasma sample were <3% for all analytes. The detection limit for dimethylarginines was 0.04 μmol/l. Nitrate, the stable metabolite for NO, was detected by the classical Greiss method (18).

Arginase activity assay

Plasma arginase activity was measured by the conversion of (^{14}C) guanidinoarginine to ^{14}C urea. Initiation of the arginase assay was performed as described by Russell and Ruegg (22). After a 30-min incubation at 37°C, the reaction was terminated by heating at 100°C for 3 min, and the reaction mixture was incubated for an additional 45 min at 37°C after addition of potassium phosphate buffer and urease, as described previously (22). Liberated $^{14}CO_2$, trapped as $Na_2{}^{14}CO_3$, was quantified by scintillation counting. One unit of arginase activity is defined as the amount of enzyme that produces 1 μmol of urea per minute at 37°C.

Arginase isoform protein concentration

The concentration of arginase isoform present in human plasma was determined by a direct enzyme-linked immunosorbent assay using an anti-arginase I and II polyclonal antibody raised in goat (Santa Cruz Biotechnology, Santa Cruz, CA) with appropriate secondary antibodies labeled to horseradish peroxidase.

Calculations

The rate of insulin-stimulated glucose disposal was calculated by adding the rate of residual hepatic glucose production to the cold glucose infusion rate (10,13).

Statistical methods

All data were normalized before analyzing. All data are presented as the mean ± SE, with some data demonstrating 95% CIs shown between brackets. Differences between control and diabetic groups were compared using the unpaired two-tailed *t* test, with statistical significance noted by $P < 0.05$. Differences between basal and insulin-stimulated values within groups were compared using the paired *t* test. Correlation analysis was performed by the Pearson product moment method using Stat View software (version 4.0; SAS, Cary, NC).

RESULTS

Euglycemic insulin clamp

During insulin infusion, similar steady-state (time 210–240 min) plasma insulin concentrations were obtained in diabetic and control groups (133 ± 7 vs. 127 ± 8 μU/ml; P = NS). The rate of insulin-stimulated glucose disposal (R_d) was reduced in diabetic compared with control subjects (5.2 ± 0.4 mg · kg^{-1} · min^{-1} [95% CI 4.9–5.6] vs. 9.0 ± 0.9 mg · kg^{-1} · min^{-1} [7.2–11.2]; $P < 0.01$). Basal (time 0) plasma levels of vascular cell adhesion molecule and intercellular adhesion molecule were markedly higher in diabetic compared with control subjects (812 ± 33 ng/ml [692–926] vs. 672 ± 22 ng/ml [684–764]; 232 ± 26 ng/ml [190–279] vs. 155 ± 8 ng/ml [159–187]; $P < 0.05$), respectively (13).

NOS activity

Basal skeletal muscle NOS activity (time −60 min) was reduced in diabetic versus control subjects (107 ± 45 pmol · min^{-1} · mg protein^{-1} [95% CI 21.6–188] vs. 459 ± 100 pmol · min^{-1} · mg protein^{-1} [120–823]; $P < 0.05$). In response to hy-

perinsulinemia, NOS activity increased almost twofold in the control group after 4 h (757 ± 244 pmol · min^{-1} · mg protein^{-1} [305–1,564]; $P < 0.05$ vs. basal) but failed to increase in diabetic (105 ± 38 pmol · min^{-1} · mg protein^{-1} [12–165]; $P < 0.01$ vs. control) subjects (13). Basal NOS protein content in muscle was similar in nondiabetic and type 2 diabetic subjects and did not change significantly during the euglycemic insulin clamp (data not shown) (13).

Arginine metabolites

Plasma arginine levels (time 0) were similar in diabetic and control groups (22 ± 4 μmol/l [95% CI 23–36] vs. 29 ± 6 μmol/l [18–34]; $P = 0.32$), respectively. Plasma ornithine concentrations were higher in diabetic subjects (120 ± 6 μmol/l [115–135] vs. 95 ± 8 μmol/l [73–120]; $P < 0.05$). The arginine-to-ornithine ratio, a marker of arginase activity, tended to be lower in the diabetic group (0.18 [0.10–0.25] vs. 0.30 [0.2–0.34]; $P < 0.1$), suggesting increased arginase activity in this group. Only ADMA, compared with other methylated arginines (symmetric dimethylarginine and N^G-monomethylarginine), was higher in diabetic versus control subjects (0.48 ± 0.04 μmol/l [0.42–0.62] vs. 0.34 ± 0.04 μmol/l [0.28–0.52]; $P < 0.05$). Moreover, the ratio of arginine to ADMA, a nontraditional cardiovascular risk marker, was decreased in the diabetic versus control group (57 ± 6 μmol/l [35–60] vs. 78 ± 5 μmol/l [60–88]; $P < 0.05$). Plasma nitrate levels were significantly higher in control versus diabetic subjects (25 ± 4 vs. 14.6 ± 3 μmol/l; $P < 0.05$).

Plasma arginase isoform protein concentration

Plasma concentrations of arginase I (0.25 ± 0.08 vs. 0.31 ± 0.10 ng/ml; P = NS) and arginase II (0.16 ± 0.05 vs. 0.21 ± 0.09 ng/ml; P = NS) were similar in diabetic and control groups, respectively. No change in isoform concentrations was observed following 4 h of insulin infusion.

Plasma arginase activity (Fig. 1)

Basal plasma arginase activity (time 0) was significantly higher in diabetic versus control subjects (0.48 ± 0.11 μmol · ml^{-1} · h^{-1} [95% CI 0.37–0.59] vs. 0.32 ± 0.12 μmol · ml^{-1} · h^{-1} [0.25–0.40]; $P < 0.05$) and declined markedly to 0.13 ± 0.04 μmol · ml^{-1} · h^{-1} (0.1–0.18) during the 4-h insulin infusion (time 240). No decline in arginase activity was observed in the control group (0.24 ± 0.10 μmol · ml^{-1} · h^{-1} [0.14–0.35]; P = NS).

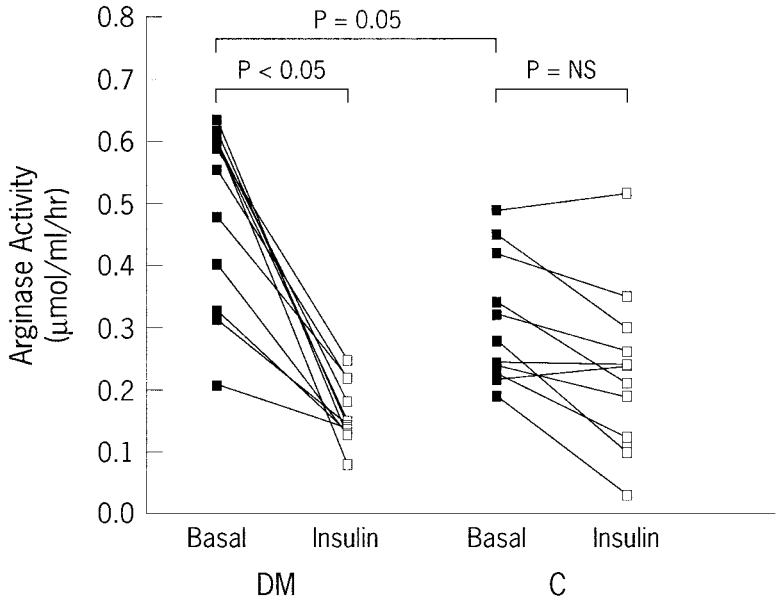

Figure 1— *Plasma arginase activity in diabetic (DM) and control (C) subjects during the basal (■) and insulin-stimulated (□) states. Arginase activity is expressed as μmol · ml^{-1} · h^{-1}.*

Relationship between metabolic parameters and plasma arginase activity (Fig. 2)

Basal plasma arginase activity in both groups combined correlated linearly with fasting plasma glucose concentration ($R = 0.46$, $P < 0.05$) and A1C levels ($R = 0.51$, $P < 0.05$) (Fig. 4 [available in an online appendix at http://dx.doi.org/10.2337/dc07-1198]). No correlation was observed with R_d, BMI, or fasting plasma insulin and free fatty acid levels.

CONCLUSIONS

Dysregulation of arginine metabolism may underlie reduced NO bioactivity in type 2 diabetes. The novel findings of the present study that deserve comment are *1*) plasma arginase activity is increased in insulin-resistant type 2 diabetic subjects with impaired muscle NOS activity compared with age-/weight-matched nondiabetic subjects, *2*) increased plasma arginase activity is correlated with the severity of hyperglycemia, and *3*) short-term (4-h) physiologic hyperinsulinemia in type 2 diabetic subjects markedly reduces the elevated plasma arginase activity, indicating an important regulatory effect of insulin on arginase enzymatic activity. Quantization of plasma metabolites involved in arginine metabolism demonstrated increased ornithine levels and a reduced arginine-to-ADMA ratio in type 2 diabetic versus nondiabetic control subjects, providing further support for increased arginase activity in type 2 diabetes.

Arginine is an exclusive substrate for NOS. Thus, understanding its metabolism in vascular health and disease is critical (23–25). Some studies have demonstrated a beneficial effect of arginine supplementation in patients with hypertension, angina, and erectile dysfunction (19), whereas a clinical trial with 6 months of arginine supplementation in patients with acute myocardial infarction (21) failed to demonstrate any vascular benefit. Increased arginine catabolism may provide an explanation for this. Although plasma arginine concentrations, determined by mass spectrometry, were relatively similar between diabetic and nondiabetic subjects in our study, conversion of arginine toward the NOS pathway was different. Elevated plasma ornithine levels (120 ± 6 vs. 95 ± 8 μmol; $P < 0.05$) and a reduced arginine-to-ornithine ratio in diabetic versus control patients (0.18 vs. 0.30; $P < 0.1$) suggests increased metabolism of arginine toward a pathway that counteracts the NOS pathway (15,23–25). Arginine and ornithine compete for the same transport system for cellular uptake (23–25). Thus, a decrease in intracellular arginine relative to ornithine concentrations would be expected, resulting from increased arginase activity as seen in our diabetic cohort, and

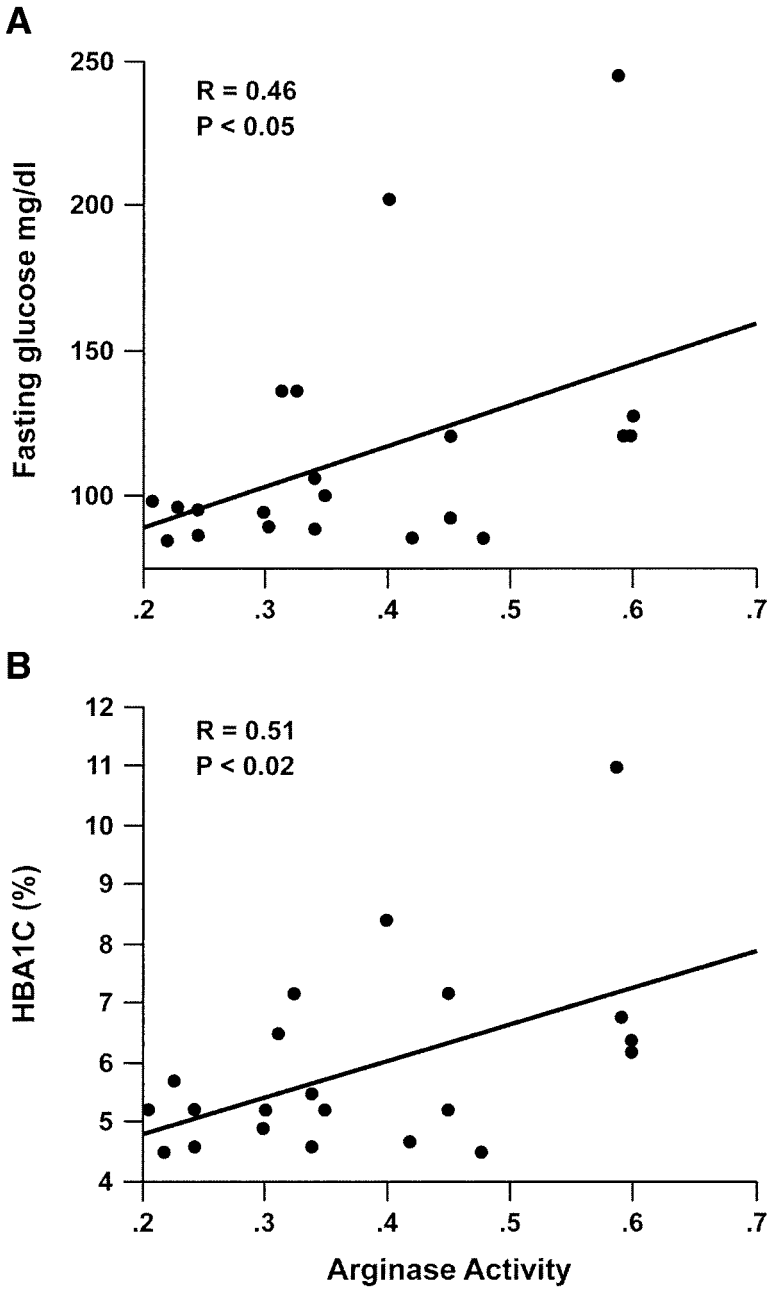

Figure 2—*Correlation analysis between plasma arginase activity (x-axis) and fasting plasma glucose (A) and A1C (B) levels for control and diabetic groups combined.*

could limit NO generation via impaired NOS activity. Other factors recognized in diabetes that could play a role in reducing intracellular arginine levels include increased inducible NOS activity and peroxynitrite generation (26,27). Both may lead to accelerate NO consumption and worsening endothelial dysfunction (23–25,28).

Reduced NO bioavailability, endothelial dysfunction, and increased oxidative stress have been demonstrated in individuals with type 2 diabetes (4, 5,16,17,29). Insulin resistance, a prominent feature of our type 2 diabetic cohort, is thought to parallel the development of endothelial dysfunction, suggesting that the two may share a common pathogenic mechanism (29). At the cellular level, insulin resistance is characterized by impaired insulin stimulation of phosphoinositol-3-kinase activity and subsequent downstream signaling of Akt (30). In metabolically active tissues, Akt phosphorlyation is required for GLUT4 translocation. In vascular endothelial cells, Akt has been shown to modulate generation of NO (31). Inhibition of insulin signaling by Wortmanin (32) and hyperglycemia (33) reduces NO generation. Altered Akt pathway in humans has been linked to inactivation of dihydropterine reductase and GTP-cyclohydrolase that results in reduced BH4 (cofactor for NOS) availability, while promoting eNOS decoupling and peroxynitrite generation (29). Impaired insulin signaling and chronic hyperglycemia provide a common mechanism for reduced NO generation and decreased glucose disposal in insulin-resistant states. This could explain our previous observation of severely reduced NOS activity during both basal and insulin-stimulated conditions in this cohort of type 2 diabetic subjects.

Another important mechanism that could explain reduced NO generation is reduced substrate concentration brought about by accelerated enzymatic conversion of arginine to ornithine by arginase. Consistent with this, arginase has been shown to reduce the vasodilatory properties of NOS (11,12). Arginase is an intracellular enzyme that appears in plasma only after cell damage or death. The type 1 isoform of arginase predominantly is located in liver and kidney, while the type 2 isoform predominantly is found in endothelial cells and can be induced in many cell types by a variety of inflammatory cytokine factors (34). Thus, chronic, low-grade inflammation and liver disease are potential sources of elevated arginase activity in type 2 diabetes. In the present study, we found a similar and approximately equal distribution of the type 1 and 2 isoforms in diabetic and control subjects. However, in diabetic erectile dysfunction, increased expression and activity of arginase II was found, suggesting a potential role of arginase II in negative regulation of NO production (28). Subjects with diabetes in this study had no evidence of microvascular or macrovascular complications, were treated with diet alone or sulfonylurea therapy, and demonstrated reasonably good glycemic control (A1C = 6.8%). Not unexpectedly, these diabetic individuals demonstrated significant insulin resistance and had many features of metabolic syndrome including increased adiposity, dyslipidemia, hypertension, and pro-inflammatory mileau including hyperglycemia and increased circulating soluble cellular adhesion molecules. Diminished arginase activity was not associated with reduced insulin-stimulated whole-body glucose disposal or increased fasting plasma insulin concentration. However, reduced arginase activity was correlated with the level of glycemic control (fasting plasma

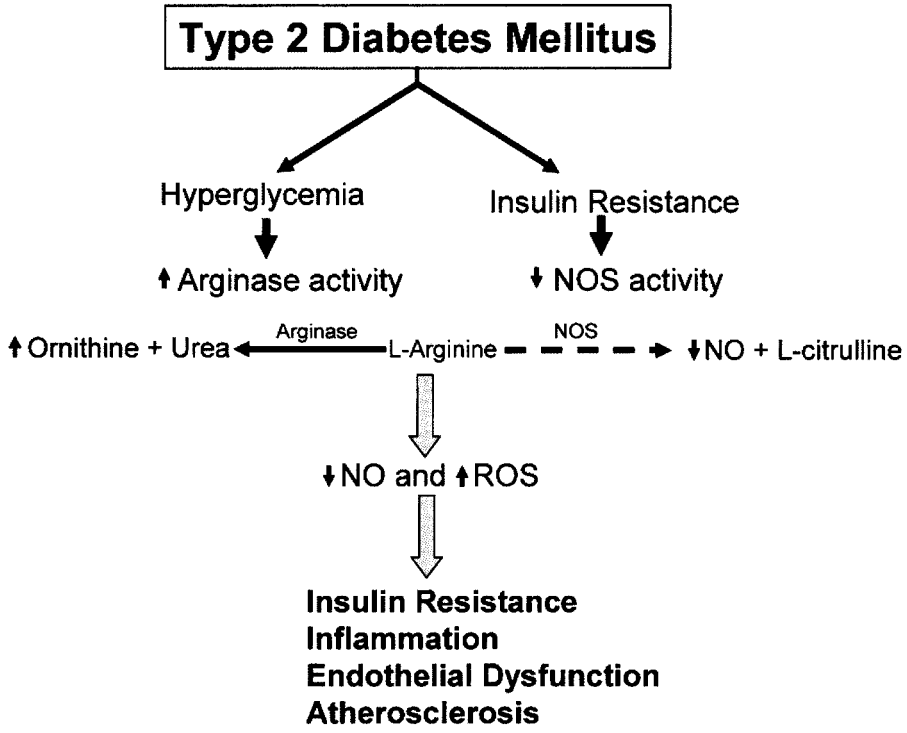

Figure 3—*Schematic representation of the effects of hyperglycemia and insulin resistance on NO generation. We propose that hyperglycemia is associated with increased arginase activity, leading to decreased arginine availability. Insulin resistance in type 2 diabetic patients is associated with decreased NOS activity. Impaired activity of NOS and increased arginase activity contribute to decreased NO generation and result in endothelial dysfunction, inflammation, insulin resistance, and accelerated atherosclerosis.*

glucose concentration and A1C levels). Consistent with this, studies have shown that hyperglycemia increases arginase activity in renal cortex (35). Recent evidence in human endothelial cells has identified a regulatory effect of the RhoA/ROCK pathway on increasing arginase activity (36). In apolipoprotein E–null mice, higher arginase (isoform II) activity in the atherosclerotic aortas was associated with higher RhoA protein levels, suggesting a role of RhoA in upregulation of arginase activity (36). It is tempting to speculate that hyperglycemia directly or indirectly stimulates this pathway to enhance arginase activity and that insulin may ameliorate this effect.

Insulin has been shown to have vasodilatory effects, as well as anti-inflammatory properties (1,4). The insulin signaling cascade mediates insulin action in insulin responsive tissues and has been shown to stimulate NO generation in vascular smooth muscle and skeletal muscle (6,7,13,29,32,33). In our study, physiologic hyperinsulinemia, while maintaining euglycemia, markedly decreased the elevated plasma arginase activity in diabetic but not in control subjects. Several possible explanations could explain these results: *1*) insulin inhibits arginase activity only when the activity of the enzyme is increased, as in our type 2 diabetic subjects; *2*) during the insulin clamp, the plasma glucose concentration is allowed to decline to baseline, thereby removing the stimulatory effect of hyperglycemia on plasma arginase activity; and *3*) during the insulin clamp, restoration to normal of some other metabolic factor(s) (i.e., elevated plasma free fatty acid levels) results in reduced plasma arginase activity.

In summary (Fig. 3), insulin resistance in type 2 diabetes may contribute to reduced NOS activity by generation of methylated arginine, while hyperglycemia in type 2 diabetic individuals contributes to increased arginase activity and reactive oxygen species, which further inhibit NOS activity. Based upon this scenario, inhibition of pathways that reduce NO bioavailability could prove to be an important target to enhance endothelial function in type 2 diabetes. Other therapeutic modalities, including intensive control of hyperglycemia with insulin, should be assessed for their ability to increase arginine bioavailability and block arginase activity.

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