Original Article

Effect of Enhanced Glycemic Control with Saxagliptin on Endothelial Nitric Oxide Release and CD40 Levels in Obese Rats

R. Preston Mason1, 2, Robert F. Jacob2, Ruslan Kubant3, Mary F. Walter4, Aouatef Bellamine5, Adam Jacoby3, Yoshiko Mizuno1, 2 and Tadeusz Malinski3

1Cardiovascular Division, Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts, USA
2Elucida Research LLC, Beverly, Massachusetts, USA
3Department of Biochemistry, Ohio University, Athens, Ohio, USA
4National Institutes of Health, Bethesda, Maryland, USA
5Bristol-Myers Squibb, Princeton, New Jersey, USA

Aim: Endothelial cell (EC) dysfunction contributes to insulin resistance in diabetes and is characterized by reduced nitric oxide (NO) release, increased nitrooxidative stress and enhanced inflammation. The purpose of this study was to test the effect of improved postprandial glucose control on EC function in insulin-resistant rats as compared to fasting glucose (FG) changes.

Methods: Obese Zucker rats were treated with 10 mg/kg/day saxagliptin, a dipeptidyl peptidase-4 (DPP4) inhibitor, for 4 or 8 weeks and compared to lean rats. NO and peroxynitrite (ONOO−) release from aortic and glomerular ECs was measured ex vivo using amperometric approaches and correlated with FG, postprandial glucose, insulin, soluble CD40 (sCD40) and L-citrulline levels.

Results: Saxagliptin treatment improved NO production and reduced ONOO− release prior to any observed changes in FG levels. In untreated obese animals, NO release from aortic and glomerular ECs decreased by 22% and 31%, respectively, while ONOO− release increased by 26% and 40%. Saxagliptin increased aortic and glomerular NO release by 18% and 31%, respectively, with comparable reductions in ONOO− levels; the NO/ONOO− ratio, an indicator of NO synthase coupling, increased by >40%. Improved glycemic control was further associated with a reduction in sCD40 levels by more than ten-fold (from 300 ± 206 to 22 ± 22 pg/mL, p < 0.001).

Conclusion: These findings indicate that enhanced glycemic control with DPP4 inhibition improved NO release and reduced inflammation in a manner not predicted by FG changes alone.


Key words: Saxagliptin, Endothelium, Nitric oxide synthase, Diabetes, CD40

Introduction

Endothelial dysfunction is causally associated with atherosclerosis and has been observed in patients with metabolic syndrome, diabetes and related disorders. A pathophysiologic relationship between reduced nitric oxide (NO) bioavailability and loss of insulin sensitivity has been established in experimental animal models of diabetes. Chronic metabolic diseases are also characterized by inflammation—a process that contributes to atherosclerotic plaque progression. The CD40/soluble CD40 ligand (sCD40L) axis represents a co-stimulatory pathway in immune response and inflammatory diseases1, 2). Attenuation of CD40 signaling pathways stabilizes plaque progression by reducing the accumulation of T lymphocytes and macrophages in atherosclerotic lesions3, 4). In addition, the activation of CD40 by its ligand is inhibited by NO and endothelial nitric oxide synthase (eNOS) activity—an effect that is reversed by inhibitors of NO
Saxagliptin is a dipeptidyl peptidase-4 (DPP4) inhibitor that causes significant reductions in postprandial plasma glucose and hemoglobin A1c levels. Inhibition of DPP4 increases the half-life and circulating levels of intact (active) glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide, which improve postprandial glucose clearance by stimulating pancreatic insulin release and inhibiting hepatic glucose release. Chronic inhibition of DPP4 leads to elevated GLP-1 levels and improved metabolic control, resulting in enhanced insulin sensitivity and protection against β cell apoptosis and hyperglycemia in experimental models. These agents reduce postprandial plasma glucose even in lean animals with normal insulin sensitivity.

In this study, we hypothesized that DPP4 treatment improves endothelial function in obese rats due to enhanced postprandial glucose clearance, prior to reductions in fasting glucose levels. This hypothesis was tested by measuring NO and ONOO⁻ release from aortic and glomerular endothelial cells and correlating these data with glucose, insulin, L-citrulline and sCD40 levels. The results of this study provide new insights into the contribution of enhanced glycemic control to endothelial nitric oxide bioavailability, oxidative stress, and mechanisms of inflammation associated with insulin resistance.

Materials and Methods

Animals and Materials

Zucker rats were obtained from Harlan Laboratories (Indianapolis, IN) at 4 weeks old. The animals were either homozygous (fa/fa, obese) or heterozygous (Fa/fa, lean) for the autosomal recessive mutation (fa) in the leptin receptor gene on chromosome 5. All animals were housed in a controlled environment that provided food and water ad libitum. Food was provided in the form of a standard, low-fat diet (19% protein, 12% fat, 69% carbohydrates, expressed as % of total energy content in units of kcal/g) or a high-fat diet (16% protein, 40% fat, 44% carbohydrates) obtained from Purina TestDiet (Richmond, IN) (AIN-76A Purified Diet and AIN-76A Western Diet, respectively). Polyethylene glycol-conjugated superoxide dismutase (PEG-SOD), N⁶-methyl-L-arginine (i-NMMA), and the calcium ionophore (Ca²⁺), A23187, were purchased from Sigma-Aldrich (St. Louis, MO). The synthetic polyphenol and NAD(P)H oxidase inhibitor, 6,8-diallyl 5,7-dihydroxy 2-(2-allyl 3-hydroxy 4-methoxyphenyl)1-H benzo(b)pyran-4-one (S17834), was provided by the Institut de Recherches Servier (Suresnes, France). Saxagliptin (in powder form) was provided by Bristol-Myers Squibb (Princeton, NJ). Plasma triglycerides and cholesterol levels were analyzed using a Cholestech LDX system (Alere, Inc., Waltham, MA).

Treatment Groups

Zucker lean (Fa/faith) and obese (fa/fa) rats at 12 weeks of age were assigned to one of six groups: (1) Zucker lean rats on a standard, low-fat diet; (2) Zucker lean rats on a high-fat diet; (3) Zucker obese rats on a high-fat diet and treated with vehicle for four weeks; (4) Zucker obese rats on a high-fat diet and treated with saxagliptin for eight weeks; (5) Zucker obese rats on a high-fat diet and treated with saxagliptin for four weeks; and (6) Zucker obese rats on a high-fat diet and treated with saxagliptin for eight weeks. For the drug-treated groups, saxagliptin (10 mg/kg/day) or vehicle alone (sterile saline solution) was administered orally. After completion of the various treatments, animals were sacrificed and tissue samples obtained immediately for further experimentation. All procedures were conducted in accordance with standard Institutional Animal Care and Use Committee guidelines and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Oral Glucose Tolerance Test

Glucose levels were measured using an Accu-Check Compact Plus glucometer (Sanmina-SCI, San Jose, CA). All glucose measurements were preceded by a 6-8 hr fasting period. Saxagliptin or vehicle was administered perorally 4 hr prior to starting the glucose tolerance test. Glucose was administered perorally at 1.5 g/kg/bw. Blood samples were collected from the tail vein at 5, 15, 30, 60, 90 and 120 minutes following glucose administration.

NO and ONOO⁻ Nanosensors

Concurrent measurements of NO and ONOO⁻ were performed with tandem electrochemical nanosensors combined into one working unit with a total diameter of 200-400 nm. Their design was based on previously developed and chemically modified carbon-fiber technology. Each of the nanosensors was made by depositing a sensing material on the tip of a carbon fiber (length 4-5 μm, diameter 100-200 nm). The fibers were sealed with nonconductive epoxy and electrically connected to copper wires with conductive silver epoxy. Conductive films of polymeric Ni(II) tetraakis (3-methoxy-4-hydroxy-phenyl) porphyrin and
Mn(III) [2,2] paracyclo-phenylporphyrin were used for the NO and ONOO- sensors, respectively.

The amperometric method (with a response time of 0.1 ms) provided a quantitative signal (current) that was directly proportional to changes (from basal levels) in NO or ONOO- concentration. Amperometric measurements were performed with a Gamry III double-channel potentiostat. Basal NO or ONOO- levels were measured by differential pulse voltammetry in separate experiments. The differential pulse voltammetry current at the peak potential for NO and ONOO- was directly proportional to the local concentrations of these compounds in the immediate vicinity of the sensor. For each set of analyses, linear calibration curves (current versus concentration) were constructed for each sensor from 1 nM to 2 µM before and after measurements with aliquots of NO and ONOO- standard solutions.

**Aortic NO and ONOO- Measurements**

Rats were euthanized with pentothal (150 mg/kg, IP) and the aortae removed and placed in modified Hanks' balanced salt solution (4°C, pH 7.4) containing 0.1 mM L-arginine. All measurements of NO and ONOO- were performed on intact endothelial cells. Aortic ring segments were isolated and immobilized on Sylgard film in an organ chamber containing fresh, oxygenated Hanks' balanced salt solution at 37°C. The NO/ONOO- nanosensor tandem module was positioned near the surface of individual endothelial cells using a computer-controlled M3301 micromanipulator (x-y-z resolution of 0.2 µm) and microscope (both from World Precision Instruments, Berlin, Germany) fitted with a CD camera.

Each analyte was measured with a maximum current from amperograms and standard calibration curves. The sensors had a high reproducibility of measurement (5% to 12%) at a constant distance from the surface of the endothelial cell. After establishing a background current, CaI (1 µM) was injected into the organ chamber using a nanoinjector (World Precision Instruments, Berlin Germany). Rapid changes in current (proportional to the molar concentrations of NO or ONOO- released) were observed after the addition of CaI and were monitored continuously. We have previously measured changes in NO and ONOO- release in isolated ECs and in animal models of cardiovascular risk using these approaches.

We also examined the direct effects of the eNOS inhibitor, L-NMMA, the superoxide dismutase mimetic, PEG-SOD, and the NAD(P)H oxidase inhibitor, S17834, on NO and ONOO- release from aortic endothelial cells isolated from obese Zucker rats maintained on a high-fat diet for eight weeks. Cells were incubated with these agents for 10 min at 0.1 mM, 100 U/mL and 40 µM (L-NMMA, PEG-SOD, and S17834, respectively) followed by CaI-stimulation and subsequent NO/ONOO- measurements.

**Glomerular NO and ONOO- Measurements**

Immediately after sacrificing animals as described above, the kidneys were removed, cut into 100 µm sections, and transferred to an organ chamber containing Dulbecco's phosphate-buffered saline and 5.6 M glucose at pH 7.4. The NO/ONOO- nanosensor module was positioned 5 ± 2 µm from the surface of a glomerular EC (cortical zone). All other aspects of NO and ONOO- measurement were performed as described for the aortic endothelial cells above.

**Measurement of Insulin and sCD40 Receptor Levels**

At various time points, blood was collected from the rat tail vein into MiniCollect K3 EDTA tubes (Greiner BioOne, Monroe, NC). Plasma was obtained by centrifugation (2700 g for 10 min at 4°C), transferred into cryogenic vials, and stored at −80°C for further analysis. Plasma insulin was quantified using a rat insulin ELISA (Alpco Diagnostic, Salem, NH). The ELISA method was also used to measure sCD40 levels (RayBiotech, Inc., Norcross, GA).

**Measurement of L-Citrulline Levels**

L-Citrulline was measured using HPLC equipped with an HP 1050 pump system with a ZORBAX Eclipse AAA column (4.6×150 mm, 5 µm column, PN 993400-902; Agilent Technologies, Inc., Santa Clara, CA) as previously described. Briefly, a 100 µL aliquot of each plasma sample was added to 10 µL of 30% sulfosalicylic acid. After 1 hr, the sample was centrifuged at 14,000 rpm for 12 minutes and the supernatant was collected. Pre-column derivatization was performed with a solution made up of 5 µL supernatant, 5 µL internal standard and 5 µL α-phthalaldehyde (OPA) reagent solution (containing 0.8 mg/mL OPA and mercaptoethanol in borate buffer, PN 26025; Thermo Scientific, Rockford, IL). The reaction was allowed to proceed for 1 min before stopping with 20 µL of 0.1 M sodium acetate. Samples were analyzed using a Shimadzu RF-551 fluorescence analyzer (Shimadzu Scientific Instruments, Kyoto, Japan). L-Citrulline was eluted at 9.5 ± 0.2 minutes. Sample L-citrulline concentrations were calculated using a calibration curve measured from standards of known concentrations.
Statistical Analyses

Data are presented as mean ± S.D. The significance of differences between results from independent experimental conditions was tested using either the two-tailed Student’s t-test (measurements of NO and \( \text{ONOO}^- \) release, including ratio calculations, from various treatments and diets) or one-way analysis of variance with Student-Newman-Keuls multiple comparisons post hoc analysis. Alpha error was set to 0.05 in this study.

Results

Effects of Saxagliptin on Fasting Glucose, Glucose Intolerance, Weight Gain, and Plasma Cholesterol and Triglyceride Levels

Fasting glucose and insulin levels were significantly elevated in obese \((fa/fa)\) rats as compared to lean \((Fa/fa)\) animals on matching diets. Saxagliptin treatment did not reduce fasting glucose levels until 8 weeks as compared to lean animals (Table 1).

Obese Zucker rats also showed evidence of glucose intolerance in response to a glucose challenge test as compared to lean controls (Fig. 1). Treatment with saxagliptin (10 mg/kg/day) for 8 weeks (black squares) reproduced the normal response. Saxagliptin or vehicle was administered perorally 4 hr prior to starting the glucose tolerance test. Glucose was administered at 1.5 g/kg/bw.

![Image](image.png)

**Fig. 1.** Saxagliptin treatment normalized the response of obese, diabetic animals to a glucose challenge test. Obese Zucker \((fa/fa)\) rats (black triangles) showed evidence of insulin resistance in response to a glucose challenge test as compared to lean \((Fa/fa)\) animals (white circles). Treatment with saxagliptin (10 mg/kg/day) for 8 weeks (black squares) reproduced the normal response. Saxagliptin or vehicle was administered perorally 4 hr prior to starting the glucose tolerance test. Glucose was administered at 1.5 g/kg/bw.

Table 1. Biometric data collected from animals examined in this study

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Body Weight (g)</th>
<th>Glucose (mg/dL)</th>
<th>Insulin (ng/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean ((Fa/fa)), low-fat diet, vehicle treatment 8 wk</td>
<td>429 ± 22</td>
<td>104 ± 4</td>
<td>0.54 ± 0.20</td>
</tr>
<tr>
<td>Lean ((Fa/fa)), high-fat diet, vehicle treatment 8 wk</td>
<td>478 ± 35</td>
<td>116 ± 4</td>
<td>1.13 ± 0.40</td>
</tr>
<tr>
<td>Obese ((fa/fa)), high-fat diet, vehicle treatment 4 wk</td>
<td>646 ± 65*</td>
<td>129 ± 7*</td>
<td>4.15 ± 1.03*</td>
</tr>
<tr>
<td>Obese ((fa/fa)), high-fat diet, saxagliptin treatment 4 wk</td>
<td>648 ± 55*</td>
<td>124 ± 7*</td>
<td>5.39 ± 1.77*</td>
</tr>
<tr>
<td>Obese ((fa/fa)), high-fat diet, vehicle treatment 8 wk</td>
<td>709 ± 66*</td>
<td>136 ± 9*</td>
<td>4.01 ± 1.26*</td>
</tr>
<tr>
<td>Obese ((fa/fa)), high-fat diet, saxagliptin treatment 8 wk</td>
<td>717 ± 87*</td>
<td>115 ± 6*</td>
<td>5.76 ± 1.69*</td>
</tr>
</tbody>
</table>

Values are reported as the mean ± S.D. \((n=6-8)\). *p < 0.05 versus either lean group; †p < 0.05 versus other obese groups; ‡p < 0.05 versus obese, high-fat animals treated with vehicle for 4 weeks (Student-Newman-Keuls multiple comparisons post hoc test; overall ANOVA-body weight data: p < 0.0001, \(F = 28.894\); glucose data: p < 0.0001, \(F = 21.336\); insulin data: p < 0.0001, \(F = 21.971\)).

Effects of Obesity and Saxagliptin Treatment on NO and \( \text{ONOO}^- \) Release from Aortic Endothelial Cells

We measured CaI-stimulated (maximal) NO and \( \text{ONOO}^- \) release from aortic endothelial cells isolated from untreated and saxagliptin-treated Zucker rats increased to 520 ± 45 mg/dL and 430 ± 40 mg/dL (triglyceride and cholesterol concentrations, respectively) in Zucker obese rats maintained on a high-fat diet for 8 weeks. No statistically significant changes were observed in these levels with saxagliptin treatment (data not shown).
In the absence of an exogenous eNOS agonist, basal concentrations of NO (30 ± 2 nM) and ONOO⁻ (10 ± 3 nM) were detected near the endothelial surface; these levels increased significantly following stimulation with CaI.

Maximal NO release decreased by 16% and 22% in obese rats after 4 and 8 weeks, respectively, on a high-fat diet. Over the same time course, ONOO⁻ levels increased by 18% and 26% in obese animals. The overall NO/ONOO⁻ ratio, which reflects the balance between endothelial NO and ONOO⁻ (nitroxidative stress) levels, decreased by 29% and 38% in obese animals at 4 and 8 weeks, respectively, as compared to lean animals on the high-fat diet (Fig. 2).

Saxagliptin significantly increased the capacity of aortic endothelial cells to produce NO while simultaneously reducing ONOO⁻ production, consistent with increased eNOS coupling efficiency (Fig. 2). Following treatment with saxagliptin for 4 and 8 weeks at 10 mg/kg/day, maximal NO release increased by 5% (n.s.) and 17% (p < 0.001), while ONOO⁻ levels decreased by 9% (p < 0.01) and 16% (p < 0.001), respectively, as compared to vehicle-treated controls. The overall NO/ONOO⁻ ratio increased significantly by 16% and 40% in obese animals after 4 and 8 weeks on saxagliptin treatment, respectively, as compared to vehicle-treated obese animals (Fig. 2). These changes were in contrast to little or no change in blood glucose levels over the same time course (Fig. 3).

Effects of Obesity and Saxagliptin Treatment on NO and ONOO⁻ Release from Glomerular Endothelial Cells

We also measured NO and ONOO⁻ release from the glomerular endothelium after stimulation with CaI. As shown in Fig. 4, maximal NO release decreased by 24% and 31%, while ONOO⁻ levels increased by 27% and 40%, in obese rats maintained on a high-fat diet for 4 and 8 weeks, respectively. The overall NO/ONOO⁻ ratio, over the same time course, decreased by 40% and 51% in obese animals as compared to lean animals on the high-fat diet. This large ratio change indicates a marked loss of eNOS coupling and the balance between bioavailable NO and ONOO⁻ produced after maximum stimulation.

As observed in aortic ECs, saxagliptin significantly increased the capacity of glomerular ECs to release NO while simultaneously reducing ONOO⁻ production (Fig. 4). Following treatment with saxagliptin for 4 and 8 weeks at 10 mg/kg/day, maximal NO release increased by 15% (p < 0.01) and 31% (p < 0.001), while ONOO⁻ levels decreased by 16% (p < 0.001) and 23% (p < 0.001), respectively, as compared to vehicle-treated controls. The overall NO/ONOO⁻ ratio increased significantly by 16% and 40% in obese animals after 4 and 8 weeks on saxagliptin treatment, respectively, as compared to vehicle-treated obese animals (Fig. 2). These changes were in contrast to little or no change in blood glucose levels over the same time course (Fig. 3).

Fig. 2. Effects of obesity and saxagliptin treatment on nitric oxide (A) and peroxynitrite (B) release from aortic endothelial cells isolated from study animals. NO and ONOO⁻ were measured from single endothelial cells following stimulation with calcium ionophore. The NO/ONOO⁻ release ratio (C) was calculated as the arithmetic quotient of separate NO and ONOO⁻ measurements. Values are mean ± S.D. (n = 6-8). *p < 0.01 and **p < 0.001 versus lean, high-fat, vehicle-treated controls; †p < 0.01 and ‡p < 0.001 versus cognate obese, vehicle-treated control (Student-Newman-Keuls multiple comparisons post-hoc test; overall ANOVA-aortic NO data: p < 0.0001, F = 19.696; aortic ONOO⁻ data: p < 0.0001, F = 20.597; aortic NO/ONOO⁻ ratio data: p < 0.0001, F = 38.363).
Fig. 3. Comparative effects of saxagliptin on blood glucose levels and the ratio of NO/ONOO$^-$ release from aortic endothelial cells. (A) In obese animals maintained on a high-fat diet for 4 weeks, glucose levels were unaffected by saxagliptin treatment (black bars) as compared to vehicle-treated controls (white bars); however, the NO/ONOO$^-$ ratio increased by 16% with saxagliptin treatment over this same time period. (B) Following treatment for 8 weeks, glucose levels were significantly reduced with saxagliptin treatment as compared to controls. The NO/ONOO$^-$ ratio increased by 40% with saxagliptin treatment over this treatment period. Values are mean ± S.D. (n = 5–6). *$p<0.0001$ versus vehicle-treated animals (Student’s t-test).

Fig. 4. Effects of obesity and saxagliptin treatment on nitric oxide (A), peroxynitrite (B), and the ratio of NO/ONOO$^-$ (C) release from glomerular endothelial cells isolated from study animals. Values are mean ± S.D. (n = 6–8). *$p<0.05$ and **$p<0.001$ versus lean, high-fat, vehicle-treated controls; †$p<0.01$ and ‡$p<0.001$ versus cognate obese, vehicle-treated control (Student-Newman-Keuls multiple comparisons post-hoc test; overall ANOVA-aortic NO data: $p<0.0001$, $F=33.377$; aortic ONOO$^-$ data: $p<0.0001$, $F=67.529$; aortic NO/ONOO$^-$ ratio data: $p<0.0001$, $F=71.789$).
Enzymatic Sources of NO and ONOO\(^{-}\) in the Endothelium

To determine the potential contribution of oxidative stress and the underlying enzymatic mechanisms associated with observed changes in NO and ONOO\(^{-}\) levels, we measured the effects of L-NMMA, PEG-SOD, and S17834 on the release of these molecules from aortic endothelial cells isolated from obese Zucker animals maintained on a high-fat diet for 8 weeks (Fig. 6). The eNOS inhibitor, L-NMMA, reduced both NO and ONOO\(^{-}\) levels by >90%. Superoxide dismutation with PEG-SOD resulted in a 38% increase in NO release concomitant with a 52% decrease in ONOO\(^{-}\) levels. The NAD(P)H oxidase inhibitor, S17834, increased NO release by 19% and reduced ONOO\(^{-}\) production by 24%. These data indicate that the majority of bioavailable ONOO\(^{-}\) is derived from eNOS.

Effects of Saxagliptin on sCD40 Receptor and L-Citrulline

In addition to metabolic parameters and EC
function, we also evaluated the effect of treatment on plasma levels of an inflammatory biomarker (sCD40) and a product of eNOS function, L-citrulline. After 8 weeks on the high-fat diet, the obese Zucker rats showed high levels of sCD40 (300 ± 206 pg/mL). With treatment, these levels were reduced to 22 ± 22 pg/mL (p < 0.001), similar to the levels observed in lean animals (Fig. 7). After 8 weeks of treatment, L-citrulline levels increased from 65 ± 14 to 83 ± 17 µM, although this increase was not statistically significant based on analysis of variance assessment of all treatment groups (data not shown). Comparison to vehicle treatment alone, using Student’s t-test, yielded a p value of 0.05.

Discussion

The key finding from this study is that enhanced postprandial glycemic control with a DPP4 inhibitor improved NO release from aortic and glomerular endothelium prior to reductions in fasting glucose levels. The increase in NO release with treatment was associated with a pronounced decrease in nitroxidative stress in these vascular tissues. The NO/ONOO⁻ ratio, an indicator of eNOS coupling efficiency, increased in aortic and glomerular endothelial cells by 40% and 64%, respectively, with saxagliptin treatment. Enhanced eNOS function was further evidenced by an increase in L-citrulline levels by 30% with treatment. L-Citrulline is the enzymatic metabolite of L-arginine and serves as an independent biomarker of eNOS activity and endothelial cell function. Improved glycemic control was also associated with reduced sCD40 levels. These findings provide new insights into the effects of a DPP4 inhibitor on endothelial function in an established animal model of insulin resistance.

After 4 weeks of treatment, improvements in NO release were observed in the absence of changes in mean glucose (c.f., Table 1, Fig. 3, 5). Inhibitors of DPP4, such as saxagliptin, improve postprandial glucose changes even in lean animals [10]. Saxagliptin may also enhance eNOS function by reducing postprandial glucose and increasing levels of GLP-1, an incretin hormone that increases endothelial-dependent NO release, independent of changes in glucose levels [17, 18]. Saxagliptin treatment has also been shown to increase levels of GLP-1 in obese Zucker rats [19]. Other important substrates for DPP4 may be involved in this process, including brain natriuretic peptide (BNP), a 32-amino acid hormone that has vasodilating and natriuretic properties [20]. Pharmacologic inhibition of the DPP4 enzyme prevents the metabolic degradation of BNP, thereby potentiating its vascular benefits [21]. The production of stromal derived factor-1 (SDF-1) is also enhanced with DPP4 inhibition, leading to increased production of endothelial progenitor cells essential for normal vascular function and repair [22].

Enhanced glycemic control in obese Zucker rats was associated with improved NO release and diminished production of superoxide (O₂⁻), as evidenced by marked reductions in ONOO⁻ production [23]. Membrane-bound eNOS, as well as NAD(P)H oxidase, may contribute to the overall generation of ONOO⁻. At low to moderate activity of eNOS, the main source of O₂⁻ in endothelium is NAD(P)H oxidase, which can be stimulated by angiotensin II and other proatherogenic factors [24]; however, as shown in this study, under conditions of high eNOS activity (such as occurs with calcium ionophore stimulation), eNOS itself serves as the primary source of O₂⁻ and ONOO⁻ in the endothelium (Fig. 3). These pro-oxidant pathways may be inhibited through increased insulin-coupling efficiency, improved glycemic control, and enhanced incretin activity.

Treatment of obese Zucker rats with saxagliptin improved eNOS coupling, increased bioavailable NO levels, and reduced nitroxidative stress in the endothelium. The favorable increase in eNOS coupling efficiency observed with saxagliptin treatment may be due to several factors, such as increased levels of enzyme substrate (L-arginine) or cofactors (especially
tetrahydrobiopterin). Saxagliptin may also cause a decrease in the amounts of asymmetrical dimethylarginine (ADMA), an endogenous eNOS inhibitor. Finally, saxagliptin treatment may enhance NO bioavailability by decreasing levels of superoxide, which rapidly oxidizes tetrahydrobiopterin.

Insulin resistance is considered a core metabolic dysfunction of type 2 diabetes and is associated with abnormalities in glucose and lipid levels. The insulin-mediated delivery of glucose to skeletal muscle can be reversibly blocked by specific eNOS inhibitors. Transgenic mice that are eNOS-deficient have vascular abnormalities typically associated with insulin resistance, including hyperinsulinemia. Elevations in L-citrulline and ADMA levels have been shown to reduce insulin sensitivity in a transgenic animal model. In a recent report, plasma ADMA levels were shown to correlate significantly with reduced glomerular filtration rate and coronary flow velocity reserve, thus linking renal and cardiac microvascular disturbances to NO bioavailability. This is consistent with the observation that patients with type 2 diabetes have elevated plasma ADMA levels linked to reduced vascular compliance and glucose uptake. Restoring eNOS activity with DPP4 inhibition may thus represent a novel mechanism for improving insulin sensitivity, vascular vasodilation, and glucose control.

Another important finding from this study was the pronounced reduction in levels of the inflammatory marker sCD40 with saxagliptin treatment. Patients with diabetes are at higher risk for atherosclerosis, a chronic disease of the arterial wall causally associated with endothelial dysfunction, inflammation, and the development of acute or chronic tissue ischemia. CD40/sCD40 ligand coupling has a co-stimulatory role in immune response as well as in inflammatory diseases. CD40 expression on inflammatory cells is essential to plaque initiation, progression and stability. Activation of CD40 is regulated by eNOS through direct nitrosylation and changes in cell membrane distribution. This is the first study to show a reduction in CD40 levels with a DPP4 inhibitor and is consistent with the observed reversal in endothelial dysfunction. This anti-inflammatory effect with saxagliptin may slow disease progress, as observed with other inhibitors of the CD40 pathway.

The results of this study provide further insight into the relationship between NO release, metabolic changes associated with insulin resistance, and inflammatory processes. Obese Zucker rats demonstrated significant reductions in NO release and pronounced increases in oxidative stress as compared to normal (lean) animals. DPP4 inhibition improved NO release in both aortic and glomerular endothelial cells. This effect may be attributed to improved eNOS coupling efficiency through enhanced glycemic control, although other potential mechanisms cannot be excluded at this time. Additional studies into the effects of postprandial glucose control on vascular and eNOS function may be useful to extend these findings, especially in combination with other anti-diabetic agents.

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**References**


