Dimethylarginine Dimethylaminohydrolase Overexpression Enhances Insulin Sensitivity

Karsten Sydow, Carl E. Mondon, Joerg Schrader, Hakuoh Konishi, John P. Cooke

Objective—Previous studies suggest that nitric oxide (NO) may modulate insulin-induced uptake of glucose in insulin-sensitive tissues. Asymmetrical dimethylarginine (ADMA) is an endogenous inhibitor of NO synthase (NOS). We hypothesized that a reduction in endogenous ADMA would increase NO synthesis and thereby enhance insulin sensitivity.

Methods and Results—To test this hypothesis we used a transgenic mouse in which we overexpressed human dimethylarginine dimethylaminohydrolase (DDAH-I). The DDAH-I mice had lower plasma ADMA at all ages (22 to 70 wk) by comparison to wild-type (WT) littermates. With a glucose challenge, WT mice showed a prompt increase in ADMA, whereas DDAH-I mice had a blunted response. Furthermore, DDAH-I mice had a blunted increase in plasma insulin and glucose levels after glucose challenge, with a 50% reduction in the insulin resistance index, consistent with enhanced sensitivity to insulin. In liver, we observed an increased Akt phosphorylation in the DDAH-I mice after i.p. glucose challenge. Incubation of skeletal muscle from WT mice ex vivo with ADMA (2 μmol/L) markedly suppressed insulin-induced glycogen synthesis in fast-twitch but not slow-twitch muscle.

Conclusions—These findings suggest that the endogenous NOS inhibitor ADMA reduces insulin sensitivity, consistent with previous observations that NO plays a role in insulin sensitivity. (Arterioscler Thromb Vasc Biol. 2008;28:692-697)

Key Words: arteriosclerosis ■ nitric oxide ■ asymmetrical dimethylarginine ■ dimethylarginine dimethylaminohydrolase ■ glucose

The insulin resistance syndrome (IRS) is associated with compensatory hyperinsulinemia, a constellation of metabolic perturbations, and increased risk for cardiovascular disease. The association of insulin resistance with cardiovascular disease may be mediated by its adverse effect on a variety of endothelial functions including those related to vascular reactivity, structure, inflammation, and thrombosis. A decreased bioavailability of nitric oxide (NO) contributes to these endothelial dysfunctions and to the development of arteriosclerosis. Intriguingly, a reduction in NO bioavailability may also play an important role in the development of insulin resistance. NO modulates insulin-mediated glucose disposal as well as reactivity of vessels in insulin-sensitive tissues (ie, skeletal muscle, liver, and adipose tissue). Furthermore, mice with gene disruption of either endothelial or neuronal NOS exhibit insulin resistance. Genetic disruption of NO synthesis may induce insulin resistance by reducing microvascular recruitment or muscle glucose uptake in response to insulin; indeed pharmacological inhibition of NOS by Nω-monomethyl-L-arginine (L-NMMA) has the same effect.

In this regard, it is intriguing that insulin resistance in humans is associated with increased levels of the endogenous NOS inhibitor asymmetrical dimethylarginine (ADMA). ADMA inhibits all 3 NOS isoforms, impairs endothelium-dependent vasodilation, increases vascular resistance, and is associated with hypertension. Plasma ADMA levels are elevated in patients with type 2 diabetes mellitus and insulin resistance. Metformin improves endothelial vasodilator function and decreases circulating ADMA in diabetic subjects and insulin-resistant rats. Interestingly, treatment with rosiglitazone improves insulin sensitivity and reduces plasma ADMA concentration in insulin-resistant subjects with hypertension, without having an effect on the mean blood pressure. In patients with type 2 diabetes, rosiglitazone ameliorates endothelial dysfunction independently of glucose control.

The elevation of plasma or tissue ADMA and inhibition of NOS may explain in part the coexistence of insulin resistance and endothelial dysfunction. We hypothesized that ADMA (by inhibiting NO synthesis) may contribute to insulin resistance. To test this hypothesis, we studied insulin sensitivity in wild-type mice and those with genetically reduced plasma and tissue ADMA levels.

Methods

Animals

The enzyme dimethylarginine dimethylaminohydrolase (DDAH) degrades ADMA and is responsible for about 80% of the disposition...
of ADMA in healthy humans. Recently, DDAH-I transgenic mice have been generated in our laboratory. Male transgenic mice were mated with C57BL/6J (wild-type [WT]) female mice (Jackson Laboratory, Bar Harbor, Me). Offspring were screened for transgene expression by polymerase chain reaction (PCR) and Southern blot analysis using tail DNA as described earlier. Glucose tolerance tests (GTT) were performed on DDAH-I transgenic male mice, and age- and weight-matched WT littermates, at 22, 32, 44, and 70 wk of age. Assessment of insulin-induced 14C-glucose uptake into glycogen in isolated mouse skeletal muscle was performed on male WT (at 5 to 9 wk) and DDAH transgenic mice (at 10 to 11 months). Changes in liver insulin signaling were determined after i.p. administration of glucose in DDAH-I transgenic and WT littermates (12 to 16 wk of age). All mice were housed in the animal care facility at Stanford University Medical Center (Stanford, Calif), under standard temperature, humidity, and lighting conditions, and were provided mouse chow and water ad libitum. The investigation 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). The study protocol was approved by the Administrative Panel on Laboratory Animal Care, Stanford University.

**Assessment of Glucose Tolerance and Insulin Resistance**

The changes in plasma glucose and insulin were measured after i.p. administration of glucose (1 mg/10 μL/g body weight). Mice were fasted overnight (16 h) and weighed. Mice were wrapped in terry cloth to minimize the stress of handling, and the tip of the tail was clipped to collect blood into heparinized capillary tubes. After collecting blood in the fasting condition, the tail wound was sealed with a strip of adhesive tape for hemostasis. The animal received an i.p. injection of glucose and tail blood sampled after removing the adhesive tape at 30, 60, 44, and 150 min.

**Calculating the Insulin Resistance Index**

The insulin resistance (IR) index was calculated as previously described. The IR index is the product of the average elevation in plasma glucose concentration over the fasting value times the average plasma insulin concentration during the GTT. This product decreases with enhanced insulin sensitivity. At 22 wk, the data on DDAH and WT mice represent the average of 10 mice in each group. At 32 and 44 wk, exclusion of mice receiving an inadequate i.p. injection of glucose left 9 mice per group. At 70 wk of age, 2 mice in each group were excluded from testing because of spontaneous deaths or excessive weight loss.

**Glucose Uptake and Glycogen Synthesis Activity in Isolated Mouse Skeletal Muscles**

Soleus and extensor digitorum longus (EDL) muscle were isolated from overnight-fasted mice (5 to 9 wk of age), after an i.p. injection with 0.5 ketamine +0.1 mg xylazine/10g body weight. The muscles were mounted on a curved sharpened staple to their resting length. Each muscle was placed into a glass scintillation vial containing 1.5 mL of oxygenated Krebs Ringer bicarbonate (KR) buffer supplemented with 6 mM/L glucose and 0.05% bovine serum albumin in a shaking water bath (30°C). One half of the vials also contained ADMA (2 μmol/L). All vials (28) were gassed continuously with 95% O2 and 5% CO2 at a flow rate of 2L/min. A positive gas pressure was attained by sealing each vial with a rubber stopper containing a #18 gauge inflow needle and a #20 gauge outflow needle. After the initial incubations, timed additions of KR buffer or insulin were made to each flask to provide insulin concentrations of 0, 25, 50, and 200 μU/mL. Thirty min later, a 50 μL aliquot of U-14C-glucose (approximately 0.3 μCi/μL) was added to each vial. After another 30 min (60 min after the addition of insulin), the tissue was removed, blotted on absorbent paper, frozen in liquid nitrogen (LN2), and transferred to a tared test tube containing 30% KOH in saturated Na2SO4. After weighing, the muscle tissue (2.5 to 8.0 mg) was heated to 98°C, and after cooling, glycogen was precipitated with equal volumes of chilled 100% ethanol. After overnight refrigeration, free glucose was removed following serial (4×) centrifugation and washing with 50% ethanol. The recovered glycogen precipitate was dissolved in water and transferred to scintillation vials for determination of 14C radioactivity in a Beckman LS 6500 scintillation counter. Assessment of glucose concentration and 14C radioactivity in the incubation vial permitted assessment of nmol glucose conversion to glycogen per g muscle.

**Assessment of Hepatic Insulin Signaling**

Hepatic insulin signaling was assessed 30 and 60 min after i.p. administration of glucose (1 mg/10 μL/g body weight). Mice (12 to 16 wk of age) were fasted overnight (16 h) and weighed. The animals received an i.p. injection of glucose and were returned to their cage. The liver was isolated from mice, after an i.p. injection with 0.5 ketamine +0.1 mg xylazine/10g body weight.

**Measurement of Plasma ADMA, Insulin, Glucose, and Total Cholesterol**

Plasma ADMA concentrations were measured by ELISA (DLD Diagnostika GmbH) as previously described in duplicate using a microtiter plate reader (Tecan GENios). Tail blood samples were collected in 1.5 mL Eppendorf tubes, immediately centrifuged at 4°C (10 min, 4000 rpm), and plasma was stored at −20°C.

Plasma glucose was measured using a modification of the Trinder procedure, using reagents from Sigma-Aldrich. Plasma insulin concentrations were measured using the Mercodia 1-2-3 ultrasensitive mouse insulin ELISA (ALPCO Diagnostics), a solid phase 2-site enzyme immunoassay. Total cholesterol concentrations were determined using the Infinity cholesterol liquid stable reagent (ThermoDMA) as described by Allain et al with the modification of Roesch et al.

**Statistical Analysis**

The data were expressed as means±SEM. Differences between groups were assessed by Student paired t test. Differences in densitometric analysis in liver tissue was assessed by Mann–Whitney, nonparametric, unpaired test. For multiple comparisons, a 1 way analysis of variance was used (ie, to compare glucose, insulin, IR index, and glucose incorporation between DDAH and WT animals at several insulin concentrations). The level for significance was set at P<0.05.

**Results**

**Baseline Metabolic Values**

The metabolic characteristics of fasting male DDAH transgenic mice and their littermate WT controls are listed in the Table. Body weights were comparable between DDAH and WT mice with both groups showing a slight increase from 22 to 70 wk of age. Plasma glucose levels were also comparable between groups and were lower in younger mice than in older animals. Fasting plasma insulin levels tended to be lower in DDAH animals. Plasma cholesterol levels were comparable between groups from 22 to 70 wk of age. Plasma ADMA
Assessment of Insulin Sensitivity During a Glucose Tolerance Test

The plasma glucose and insulin responses of DDAH and WT mice (32 wk old) to an i.p. glucose challenge are illustrated in Figure 1. Plasma ADMA was significantly lower in DDAH animals (Figure 1A). Glucose and insulin levels in response to the glucose challenge were attenuated in DDAH animals (Figure 1B and 1C) and the IR index, representing the product of the average glucose elevation times the average insulin concentration, was significantly reduced (by 63%; \(P<0.005\)) in DDAH animals (Figure 1D).

Similar findings were observed at 22 and at 44 wk. At 22 wk, the IR index was significantly reduced by 43%. At 44 wk, both basal plasma glucose (124±6.2 versus 133±8.3 mg/dL) and insulin (0.19±0.04 versus 0.32±0.10 ng/mL) tended to be reduced in the DDAH transgenic mice. Two hours after the glucose challenge, plasma glucose levels (143±6.7 versus 155±16.1 mg/dL) tended to be less and plasma insulin levels (0.71±0.11 versus 1.34±0.21 ng/mL; \(P<0.05\)) were significantly less. To summarize, at all ages (22, 32, and 44 wk) the same metabolic pattern was observed in response to a glucose challenge, with attenuated increases in plasma glucose and insulin, and a reduced IR index, observed in the DDAH transgenic animals.

**Glucose Challenge Increases Plasma ADMA Concentrations**

In 1 group of animals (44 wk old DDAH and WT mice), plasma ADMA levels were measured in serial fashion after a glucose challenge. Intriguingly, plasma ADMA increased by 0.44 \(\mu\)mol/L (165±18%) in the WT mice 2 hours after the glucose challenge (Figure 2). Notably, in DDAH transgenic mice the absolute (0.18 \(\mu\)mol/L) or relative (124±7%) increase in plasma ADMA levels was significantly less (both \(P<0.05\)) than observed in the WT mice at the same timepoint.

To confirm these results, a modified GTT was performed in 70-wk-old mice (n=8 in each group). Under fasting conditions, plasma glucose (124±6.2 versus 133±8.3 mg/dL) and plasma insulin levels (0.19±0.04 versus 0.32±0.10 ng/mL) tended to be less in the DDAH transgenic mice. Two hours after the glucose challenge, plasma glucose levels (143±6.7 versus 155±16.1 mg/dL) tended to be less in the

Table. Baseline Metabolic Values in DDAH Transgenic and Wild-Type Mice

<table>
<thead>
<tr>
<th>Age Group</th>
<th>22 Weeks</th>
<th>32 Weeks</th>
<th>44 Weeks</th>
<th>70 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DDAH</td>
<td>WT</td>
<td>DDAH</td>
<td>WT</td>
</tr>
<tr>
<td>n</td>
<td>10</td>
<td>10</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Weight, g</td>
<td>26.4±0.4</td>
<td>26.6±0.6</td>
<td>27.5±0.4</td>
<td>26.4±0.8</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>96±4.1</td>
<td>92±4.4</td>
<td>87±6.2*</td>
<td>104±5.0</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>0.53±0.17</td>
<td>0.87±0.19</td>
<td>0.22±0.04</td>
<td>0.28±0.04</td>
</tr>
<tr>
<td>Cholesterol, mg/dl</td>
<td>104±4.5</td>
<td>107±2.1</td>
<td>108±6.3</td>
<td>115±9.0</td>
</tr>
<tr>
<td>ADMA, (\mu)mol/L</td>
<td>0.63±0.03†</td>
<td>1.00±0.04</td>
<td>0.65±0.03†</td>
<td>1.05±0.05</td>
</tr>
</tbody>
</table>

Each value is the mean±SEM for 10 animals from 22 weeks of age, 9 animals from 32 and 44 weeks of age, and 8 animals per group at 70 weeks of age. DDAH indicates DDAH-I transgenic mice; WT, wild-type littermates. *\(P<0.05\) DDAH vs WT, †\(P<0.01\) DDAH vs WT.
DDAH transgenic, and plasma insulin levels (0.71±0.11 versus 1.34±0.21 ng/mL; P<0.05) were significantly less. The increase in plasma insulin levels after glucose challenge in the DDAH transgenic mice was only 51% of that observed in WT. Under fasting conditions, plasma ADMA levels were less in the DDAH transgenic mice (0.63±0.03 versus 0.85±0.03μmol/L, P<0.01). After the glucose challenge at 2 hours plasma ADMA levels increased by 59% in WT mice. By contrast, in the DDAH transgenic mice, ADMA levels only increased by 22% and remained significantly different from WT (0.77±0.02 versus 1.35±0.07μmol/L; P<0.01).

**Effect of ADMA on Glycogen Synthesis in Incubated Muscle**

To understand the mechanisms by which ADMA may affect insulin sensitivity in adult mice, we assessed the effect of exogenous ADMA on the insulin sensitivity of isolated skeletal muscle from C57BL/6J mice (5 to 9 wk of age). Specifically, we measured skeletal muscle uptake of glucose and conversion to glycogen in response to clinically relevant concentrations of insulin, ranging from 25 to 200 μU/mL (167 to 1333 pmol/L) ex vivo using isolated EDL and soleus muscles. In the presence of vehicle alone, glucose incorporation into glycogen was increased in a dose-dependent fashion by insulin (Figure 3A). By contrast, in the presence of ADMA (2 μmol/L), glucose uptake and incorporation into glycogen was shifted rightward, indicating that ADMA significantly increased resistance to insulin. In contrast to EDL muscle, soleus muscle manifested a higher basal incorporation of glucose into glycogen and a greater sensitivity to insulin (Figure 3B). By contrast to EDL, incubation with ADMA did not affect insulin sensitivity.

We performed additional studies in 10- to 11-month-old DDAH transgenic and WT mice using EDL muscle. Tissue was incubated with 2 μmol/L ADMA before adding insulin (50 μU/mL) and radioactively labeled glucose. There was a trend for increased glucose uptake in EDL muscle from DDAH transgenic mice compared to WT littermates at baseline (12.6±1.9 versus 8.7±0.8 nmol/min/g muscle), consistent with increased insulin sensitivity in the DDAH transgenic mice. However, after adding exogenous insulin no differences in glucose uptake were observed between the groups (11.0±1.2 versus 14.2±1.4 nmol/min/g muscle).

**Insulin Signaling Cascade in Liver Tissue After an i.p. Glucose Challenge**

To evaluate the contribution of another insulin-sensitive tissue to the accelerated glucose disposal in DDAH transgenic mice, we assessed glucose-stimulated insulin signaling in the liver. Despite lower blood insulin levels (0.42±0.12 versus 1.03±0.38 ng/mL) 30 min after glucose challenge, we observed an increased Akt phosphorylation in the DDAH transgenic mice compared to WT littermates indicative of a more effective insulin signaling in the liver (276±15 versus 100±13 p-Akt/total Akt in %, P<0.03; Figure 4). This increase in insulin signaling was not attributable to differ-
Plasma ADMA levels are increased in type 2 diabetic subjects, and is reduced by metformin treatment in humans and insulin-resistant subjects. Treatment with exogenous ADMA, glycogen synthesis in slow-twitch soleus muscle was not. These discordant responses may be a result of insulin resistance has been further clarified using mice genetically deficient in NOS. Within skeletal muscle, NOS is present in myocytes (µ-NOS, a variant of nNOS) as well as the endothelium (eNOS) of the skeletal muscle microvasculature. Mice lacking the endothelial (eNOS) or neuronal (nNOS) isoform of NOS each develop insulin resistance. However, there are subtle phenotypic differences in the insulin resistance associated with these knockouts. In hyperinsulinemic-euglycemic clamp studies, the glucose infusion rate is reduced to a greater extent in the eNOS knockout mice compared with the nNOS knockout mice. The endogenous glucose output is completely suppressed in the nNOS knockout and WT mice during insulin infusion, but not in the eNOS knockout mice. In addition, the eNOS knockout mice display significantly reduced whole-body glucose disposal rates compared with WT mice. These data suggest that different NOS isoforms may modulate insulin sensitivity with different tissue preferences. Duplain et al. made similar observations in eNOS-deficient mice. They observed that eNOS−/− mice were hypertensive and had fasting hyperinsulinemia, hyperlipidemia, and a 40% lower insulin-stimulated glucose uptake than control mice. The development of insulin resistance in the eNOS−/− mice was related to impaired NO synthesis rather than hypertension per se, because equally hypertensive 1-kidney/1-clip mice manifested normal insulin-stimulated glucose uptake.

Insulin enhances glucose transport and stimulates glycogen synthase in skeletal muscle fibers. We observed that a physiologically relevant concentration of ADMA (2 µmol/L) markedly inhibited insulin-induced glycogen synthesis in isolated EDL skeletal muscle of young wild-type mice. In aged DDAH transgenic mice, there was an enhanced basal glycogen synthase in skeletal muscle fibers. Insulin enhances glucose transport and stimulates glycogen synthase in skeletal muscle fibers. We observed that a physiologically relevant concentration of ADMA (2 µmol/L) markedly inhibited insulin-induced glycogen synthesis in isolated EDL skeletal muscle of young wild-type mice. In aged DDAH transgenic mice, there was an enhanced basal glycogen synthase in skeletal muscle fibers. Insulin enhances glucose transport and stimulates glycogen synthase in skeletal muscle fibers. We observed that a physiologically relevant concentration of ADMA (2 µmol/L) markedly inhibited insulin-induced glycogen synthesis in isolated EDL skeletal muscle of young wild-type mice. In aged DDAH transgenic mice, there was an enhanced basal glycogen synthase in skeletal muscle fibers.
was observed in liver tissue from the DDAH transgenic mice. This effect occurred in spite of lower blood insulin levels in the DDAH transgenic mice after the glucose challenge. This insulin sensitivity was not associated with alterations in the JNK pathway or inhibitory phosphorylation sites of IRS-1—each of which may also be involved in insulin sensitivity.29

We suggest that the relationship between endogenous ADMA and insulin resistance may be reciprocal. Specifically, the metabolic perturbations associated with insulin resistance can increase plasma ADMA levels, but it also seems true that ADMA may increase insulin resistance. With respect to this reciprocal relationship, we observed that the glucose challenge acutely increased plasma ADMA levels in wild-type animals (an effect that was attenuated in the DDAH transgenic mice). We have previously documented that exposure of cultured endothelial cells to high glucose reduces the activity of DDAH and increases the accumulation of ADMA in the conditioned medium. This effect is reversed by antioxidants.30 The sensitivity of DDAH to oxidative stress is likely conferred by a critical sulfhydryl moiety in its catalytic site.31

In conclusion, we demonstrate that the DDAH transgenic mouse is insulin-sensitive. This observation is consistent with previous studies suggesting a link between NOS activity and insulin sensitivity. We suggest that the enhanced sensitivity to insulin in DDAH mice is attributable to reduced plasma or tissue levels of ADMA. ADMA may affect skeletal muscle glycogen synthesis in an age- and fiber type–dependent manner. Furthermore, DDAH overexpression is associated with enhanced insulin signaling in the liver. Accordingly, modification of DDAH expression or activity may provide a new therapeutic avenue for treatment of insulin resistance, diabetes mellitus, and cardiovascular disease.

Sources of Funding
Dr Sydow has been a recipient of a research grant provided by the Deutsche Forschungsgemeinschaft (DFG; Sy 41/1-1). This work was supported by grants to Dr Cooke from the National Institutes of Health (R01 HL-63685; R01 HL-75774; R01 CA098303 and P01 AG18784; and P01A150153) and the Tobacco Related Disease Research Program (11RT-0147).

Disclosures
Dr Cooke is an inventor and receives royalties from patents owned by Stanford University relating to diagnostic and therapeutic applications of the NOS pathway.

References
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Arterioscler Thromb Vasc Biol. 2008;28:692-697; originally published online January 31, 2008;
doi: 10.1161/ATVBAHA.108.162073

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