Nitric Oxide Bioavailability and Not Production Is First Altered During the Onset of Insulin Resistance in Sucrose-Fed Rats

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Although the role of nitric oxide (NO) in peripheral glucose uptake has been thoroughly described, little is known regarding the alterations in NO metabolism during the early onset of insulin resistance. During this study we investigated the alterations in NO synthesis and bioavailability in a model for dietary modulations of insulin sensitivity. For 6 weeks, rats were fed a standard diet (C), a high-sucrose diet inducing insulin resistance (HS), or high-sucrose diets supplemented with cysteine, which endowed protection against the high-sucrose–induced insulin resistance (T_i). Several markers of NO synthesis and bioavailability were assessed and confronted with markers of insulin sensitivity. After 5 weeks, although urinary cGMP excretion did not differ between the groups, insulin resistance in HS rats was associated with both a significant increase in NO oxidation, as determined by plasma nitrotyrosine concentrations, and in the inducible NO synthase (iNOS)/endothelial NO synthase (iNOS/eNOS) mRNA ratio in skeletal muscle compared with C rats. These alterations were prevented in rats fed the cysteine-rich diets. NO production, as assessed by urinary $^{15}NO_3^-$ excretion following a [$^{15}N_2$-(guanido)]-arginine intravenous bolus, independently and significantly correlated with insulin sensitivity but did not significantly differ between C, HS, and T_i rats; neither did the aortic eNOS protein expression or skeletal muscle insulin-induced eNOS activation. Our results indicate that in this model of dietary modulations of insulin sensitivity (i) NO production accounts for part of total inter-individual variation in insulin sensitivity, but (ii) early diet-related changes in insulin sensitivity are accompanied by changes in NO bioavailability. Exp Biol Med 232:1458–1464, 2007

Key words: glucose homeostasis; nitric oxide; insulin resistance; oxidative stress; dietary cysteine

Introduction

An increasing amount of data suggest that some defects in nitric oxide (NO) signaling concur with the onset of insulin resistance. In healthy subjects, insulin resistance correlates with impaired endothelium-dependent vasodilation (1–3) and, from a metabolic point of view, has been closely associated with lower urinary cGMP excretion, the NO second messenger (4). In agreement with these clinical data, several experimental studies conducted in rodents reported a decrease in NO synthesis and bioavailability under conditions of insulin resistance as a consequence of the decreased ability of insulin to increase endothelial NO release (5, 6), endothelial NO synthase (eNOS) being a downstream protein of the insulin signaling pathway (7, 8). Conversely, targeted disruption of the eNOS gene or acute pharmacologic eNOS blockade cause insulin resistance, reversed by the administration of an NO donor, suggesting that defective NO synthesis may drive the initiation of insulin resistance (9, 10). However, both in humans and in animal models, the alterations in NO metabolism associated with the early stages of insulin resistance are poorly characterized.

Oxidative stress plays an important role in the pathophysiologic alterations of NO signaling, consisting of decreased NO production and/or bioavailability. Indeed, a reduction in NO production has mainly been documented as originating from either the impairment of NOS activity ("NOS uncoupling"; Ref. 11) or increased levels of asymmetric dimethylarginine (ADMA; Refs. 12, 13), a competitive inhibitor of NOS. Both alterations are directly or indirectly related to oxidative stress. A decrease in NO

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bioavailability is also directly related to oxidative stress because it mainly consists of NO oxidative quenching (14), which generates peroxynitrite, a highly reactive nitrogen species that nitrates protein tyrosine residues. Impairments in NO production and NO bioavailability are interconnected in a vicious cycle, so that in clinical pathologic conditions, many alterations of NO signaling cluster together. However, during the early onset of insulin resistance, it is unclear which component of NO signaling is altered first, although this order is essential if we are to understand the pathophysiologic construct and develop strategies for early prevention.

In a model of diet-induced oxidative stress and insulin resistance (the high-sucrose–fed rat), we previously showed that increasing the dietary cysteine intake, using either a cysteine-rich protein or a cysteine donor, alleviated diet-induced oxidative stress and insulin resistance (15). In the current study, we investigated the alterations of several markers of NO production/bioavailability in this model of early insulin resistance induction/prevention. To directly assess *in vivo* whole-body NO production, we used an isotopic method to analyze the alterations in NO production *per se* and distinguish them from those in NO bioavailability.

Materials and Methods

Animals and Diets. All experiments were carried out in accordance with the guidelines of the French Committee for Animal Care, using 6-week-old male Wistar rats (Harlan, Gannat, France). After a first week of adaptation to the laboratory conditions, the rats ($n = 40$) were randomly assigned to five groups (C, HS, T_1, T_2, and T_3) and fed the corresponding diets for 6 weeks, as previously described (15). Briefly, the C diet was a standard starch-based laboratory rodent diet, the HS diet was a high-sucrose diet (720 g sucrose per kg food), a model of diet-induced insulin resistance, and the T_i diets were similar high-sucrose diets but were enriched with cysteine—either with a cysteine donor, *N*-acetylcysteine (T_1, 5.8 and T_2, 20 g *N*-acetylcysteine/kg food), or with a cysteine-rich protein (whey protein enriched with α-lactalbumin: T_3). The Ti diets were previously shown to be protective against high-sucrose–induced oxidative stress and insulin resistance (15). During Week 4, rats were supplied with nitrate/nitrite-free water (Wattwiller, Wattwiller, France). At Week 5, *in vivo* NO production was measured. An oral glucose tolerance test was performed at Week 5, and the insulin sensitivity index (OGTT-ISI) was calculated as follows (16): 2 / [(Ins·Gly) +1], with Ins representing the area under the insulin curve above baseline during OGTT and Gly representing the area under the glycemic curve above baseline during OGTT. At Week 6, overnight fasted rats were anesthetized intraperitoneally (sodium pentobarbital, 30 mg/kg body wt), and blood, aorta, and gastrocnemius muscle samples were collected.

Biochemical Analysis. Unless specified otherwise, all chemicals were obtained from Sigma (La Verpilliere, France). Blood glucose concentrations were measured using a Glucometer (Roche Diagnostics). Commercial assay kits were used to measure insulin (Rat Insulin ELISA; Mercodia, Uppsala, Sweden), ADMA (ADMA ELISA; DLD Diagnostika GmbH, Hamburg, Germany), nitrotyrosine (NWK-NTR01 ELISA, Northwest Life Science, Vancouver, Canada), cGMP (cyclic GMP; Assay Designs, Ann Arbor, MI), nitrate (Nitrate/Nitrite Fluorometric Assay Kit; Cayman Chemical, Ann Arbor, MI) and creatinine (Creatinine; Biomérieux, Marcy l'Etoile, France) concentrations.

***In Vivo* NO Production Measurement.** NO production was measured as the relative recovery of ^{15}N as urinary nitrate following the intravenous administration of [$^{15}N_2$-(guanido)]-arginine, based on the method described by Forte and coworkers (17). Rats were injected with 6.03 mg [$^{15}N_2$-(guanido)]-arginine:HCl per kg body wt on a lateral tail vein and were placed in metabolism cages for the next 14 hrs for the collection of urine samples in glass tubes containing antibiotics, as described by Wu *et al.* (18). Enrichment of ^{15}N in urinary nitrate was determined by adapting previous methods (17, 19), as previously described in full (20). Briefly, urinary nitrate was extracted on a highly selective ion exchange resin (IMAC-HP-555; Rohm & Haas, Philadelphia, PA) by elution with NaCl (25%) before conversion into ammonia using the microdiffusion method and combustion in an elemental analyzer coupled with an isotope ratio mass spectrometer (MicroMass, Cary, NC). To determine whole-body NO production (17), the amount of $^{15}NO_3^-$ recovered from 14-hr urine was calculated, corrected by the excretion of creatinine, and expressed as ‰ of the amount of ^{15}N injected as $^{15}N_2$-arginine, as follows: (Q × E) / (d × C), where Q and C are the total amounts of nitrate and creatinine excreted in urine, E the $^{15}NO_3^-$ enrichment (as atom percent excess) in urine, and d the amount of ^{15}N injected as $^{15}N_2$-arginine.

Western Blot Analysis. Skeletal muscle eNOS activation (i.e., insulin-induced eNOS serine 1177 phosphorylation) and aorta eNOS protein expression were determined by Western blot. For the eNOS serine 1177 phosphorylation assay, anesthetized rats received a bolus of insulin (30 μg/kg body wt) into the vena cava. Skeletal muscle fragments were collected 5 mins later and homogenized in ice-cold buffer, and 2 mg total protein was purified using 2′,5′-ADP-sepharose (GE Healthcare, Orsay, France). After separation by sodium dodecyl sulfate–polyacrylamide gel eletrophoresis (SDS-PAGE), samples were transferred to nitrocellulose membranes and blotted with anti-phospho eNOS antibody (Ser 1177; Cell Signaling Technology, Danvers, MA). Membranes were stripped and reblotted with anti-eNOS antibodies (Becton Dickinson, Rungis, France). For aorta total eNOS protein concentration measurement, aorta fragments were homogenized in ice-cold buffer, and 100-μg total protein extracts were separated by SDS-PAGE and transferred to membranes. The upper part of the membrane was blotted with anti-eNOS antibody

Table 1. Glucose Control in Rats Fed the C, HS, and T_i Diets[a]

	C	HS	T_1	C versus HS	T_i versus HS
Blood glucose (m*M*)	5.2 ± 0.2	5.3 ± 0.3	5.6 ± 1.1	NS	NS
Plasma insulin (μg/l)	0.5 ± 0.1	1.0 ± 0.1	0.7 ± 0.1	<0.05	<0.05
OGTT ISI	33 ± 4	14 ± 2	34.1 ± 7.0	<0.05	<0.05

[a] Data are means ± SEM of $n = 8$ rats. NS indicates $P > 0.05$.

(Becton Dickinson), and the lower part with anti-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Detections were performed after incubation with horseradish peroxidase–linked secondary antibody, followed by chemiluminescence and quantification using densitometry.

Real Time RT-PCR. Skeletal muscle eNOS and iNOS gene expression analyses were performed with a Light-Cycler (7300; Applied Biosystem) using SYBR Green I DNA binding dye (Power SYBR Green PCR Master Mix; Applied Biosystems). The following primers were used: eNOS sense, TCCTCAGGAGGTCTTGCACATA; eNOS antisense, GCAGCGTGGAGTGTTTGGA; iNOS sense, GAGGAGAGAGATCCGGTTCACA; and iNOS antisense, GCGGGAAGCCATGACCTT. Samples contained 10 μl SYBR Green Master Mix, 600 pmol primers, and 50 ng cDNA in a 20-μl final volume. Ribosomal 18S RNA was used as the internal standard.

Statistical Analysis. Results are means ± SEM. The diet effect was analyzed with the ANOVA model. Because we focused on the effects of early induction/prevention of insulin resistance on NO metabolism, multiple comparisons were restricted to only two preplanned comparisons that were performed under the model using orthogonal contrasts: C compared to HS (induction effect), and HS compared to Ti groups taken together (prevention effect). For the same reason, comparisons between Ti groups were not performed, and Ti groups are presented as one single prevention group. Pearson correlations were calculated using the Corr procedure, and multiple linear regressions using the stepwise Reg procedure. The threshold of statistical significance was set at $P = 0.05$. All statistical analyses were performed using SAS software (SAS for Windows, Version 9.1; Cary, NC).

Results

Insulin Sensitivity. The consumption of the HS diet for 5 weeks induced insulin resistance, as shown by the significant differences in fasting plasma insulin concentrations and OGTT-ISI in HS rats compared with C rats (Table 1). T_i diets prevented the high-sucrose–induced insulin resistance.

NO Production and Related Parameters. NO production, as assessed by urinary $^{15}NO_3^-$ excretion after an intravenous administration of ^{15}N-labeled arginine, was not affected by the sucrose diet and remained unchanged in the T_i groups (Table 2). Plasma ADMA concentrations did not significantly differ between C and HS rats, and HS and T_i rats (Table 2). After 6 weeks, aortic eNOS protein concentrations did not vary as a function of the diet, nor did eNOS insulin-induced serine 1177 phosphorylation in the skeletal muscle (Fig. 1).

NO Bioavailability. Figure 2 shows the effects of the experimental diets on parameters related to NO bioavailability. Plasma nitrotyrosine concentrations were significantly lower in C rats than in HS rats, and lower in T_i than in HS rats (Fig. 2A). The skeletal muscle ratio of iNOS to eNOS mRNA concentrations, considered as an tentative explicatory marker of alterations in NO bioavailability (21, 22), was significantly higher in HS rats than in C rats, but it did not differ between T_i and C rats, and T_i and HS rats (Fig. 2B). However, urinary cGMP excretion did not differ significantly between groups (0.32 ± 0.06, 0.35 ± 0.04, and 0.36 ± 0.1 pmol/mmol creatinine in C, HS, and T_i rats, respectively).

Correlations Between Insulin Sensitivity and NO Production/Bioavailability. Figure 3 presents the most relevant and significant Pearson correlations obtained using the SAS Corr procedure. NO production was positively correlated with OGTT-ISI ($r = 0.52$, $P < 0.01$) and negatively correlated with fasting plasma insulin concentrations ($r = -0.36$, $P = 0.05$). When the variables were analyzed using stepwise multiple regressions, only urinary $^{15}NO_3^-$ excretion and iNOS/eNOS muscle mRNA ratio were independently and significantly correlated with OGTT-ISI ($P < 0.001$ and $P < 0.01$, respectively; Table 3).

Table 2. Effects of C, HS, and T_i Diets on NO Production and Linked Parameters[a]

	C	HS	T_i	C versus HS	HS versus T_i
NO production[b]	6.8 ± 0.5	6.6 ± 0.9	8.3 ± 0.7	NS	NS
Plasma ADMA (μ*M*)	0.8 ± 0.1	0.9 ± 0.1	1.7 ± 0.1	NS	NS

[a] Data are means ± SEM, $n = 6$ to 8. NS indicates $P > 0.05$.
[b] Urinary $^{15}NO_3^-$ excretion ‰^{15}N recovered after [$^{15}N_2$-(guanido)]-arginine:HCl intravenous bolus/mmol creatinine.

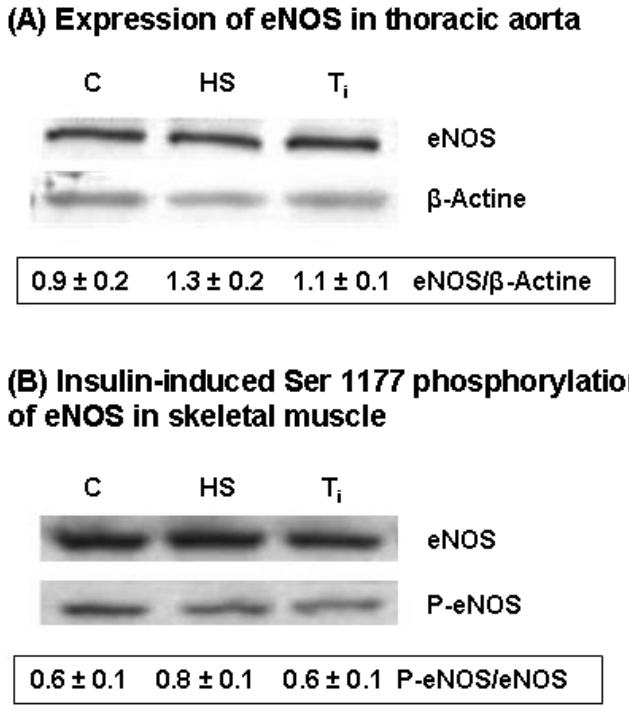

Figure 1. Endothelial NOS expression and phosphorylation in aorta and skeletal muscle of rats fed the C, HS, and T_i diets for 6 weeks. (A) Proteins from thoracic aorta homogenates were size fractionated by SDS-PAGE and immunoblotted for total eNOS and β-actin. (B) Rats were injected with insulin (30 μg/kg), and eNOS was affinity purified from gastrocnemius homogenates using 2′-5′-ADP-sepharose. Proteins were size fractionated by SDS-PAGE and immunoblotted with antibodies to eNOS or Ser1177-phospho-eNOS. Ratio of empirical densitometric units are shown on the bottom of each gel. Mean ± SEM of $n = 5$ rats (A) and $n = 3$ to 4 rats (B).

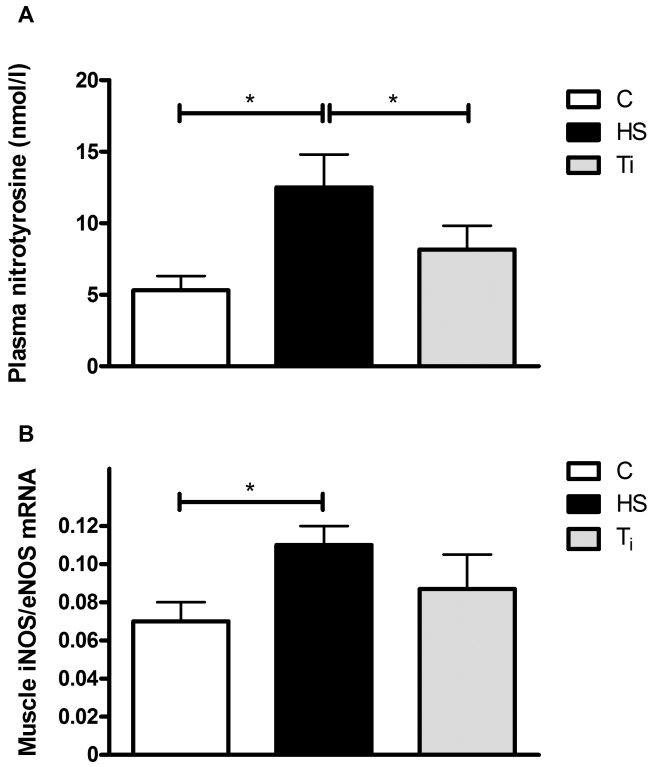

Figure 2. Plasma nitrotyrosine concentrations (A) and skeletal muscle iNOS/eNOS mRNA ratio (B) in rats fed the C, HS, and T_i diets for 6 weeks. Data are means ± SEM, $n = 7$ to 8 or $n = 5$ for mRNA data. $^*P < 0.05$.

Discussion

Using rats fed a high-sucrose diet for 6 weeks as a model for the early induction of insulin resistance and cysteine donors as effective preventive agents, we showed that (i) NO production could explain part of the total variation of insulin sensitivity in rats, but (ii) decreased NO bioavailability was the main impairment in NO metabolism that came along with the initiation of insulin resistance.

Consumption of the HS diet for 5 weeks significantly increased plasma nitrotyrosine concentrations, a stable footprint of NO oxidation. This result indicated a decrease in NO bioavailability in HS-fed rats, even though urinary cGMP excretion did not vary as a function of the diet (this invariance did not preclude local differences in cGMP levels that we could not assess from measurements of whole-body cGMP production). Interestingly, this decreased NO bioavailabitity was associated with an increase in the skeletal muscle iNOS/eNOS mRNA ratio. Although this result was not confirmed by measuring the iNOS/eNOS protein expression ratio, an increased iNOS/eNOS mRNA ratio indicates the initiation of an imbalance in the type of NO production in HS rats that might contribute to a reduction in NO bioavailability. Indeed, an imbalance in the fine tuning between eNOS and iNOS pathways in favor of the latter has been incriminated in the initiation of numerous pathologic conditions, including insulin resistance (21, 22), partly by favoring the oxidative degradation of NO to peroxynitrite (23). In the context of the high-sucrose diet, higher production of superoxide anion is very likely to account for the increase in NO scavenging and the resulting production of peroxynitrite. Possible underlying mechanisms include upregulation of NAD(P)H oxidase and/or downregulation of superoxide dismutase, as already reported in similar models of diet-induced oxidative stress (24–27).

The HS diet did not affect aortic eNOS protein levels and insulin-induced eNOS activation, which is in line with the findings of a study that measured no change in eNOS expression and phosphorylation in rats fed a high-fructose diet for 8 weeks (28). In addition, the HS diet did not affect NO production and plasma ADMA concentrations, the values measured in HS rats being remarkably similar to those measured in rats receiving the C diet. This result apparently contrasts with what had previously been reported under conditions of oxidative stress and with other models of diet-induced oxidative stress in rodents. Indeed, oxidative stress has been reported to be associated with tetrahydrobiopterin deficiency and/or to elevated plasma ADMA, both leading to a reduction in NO production (11, 29). In rats fed a high-refined sugar and/or high-fat diet, an impairment of endothelial-dependant vasodilation was associated with a

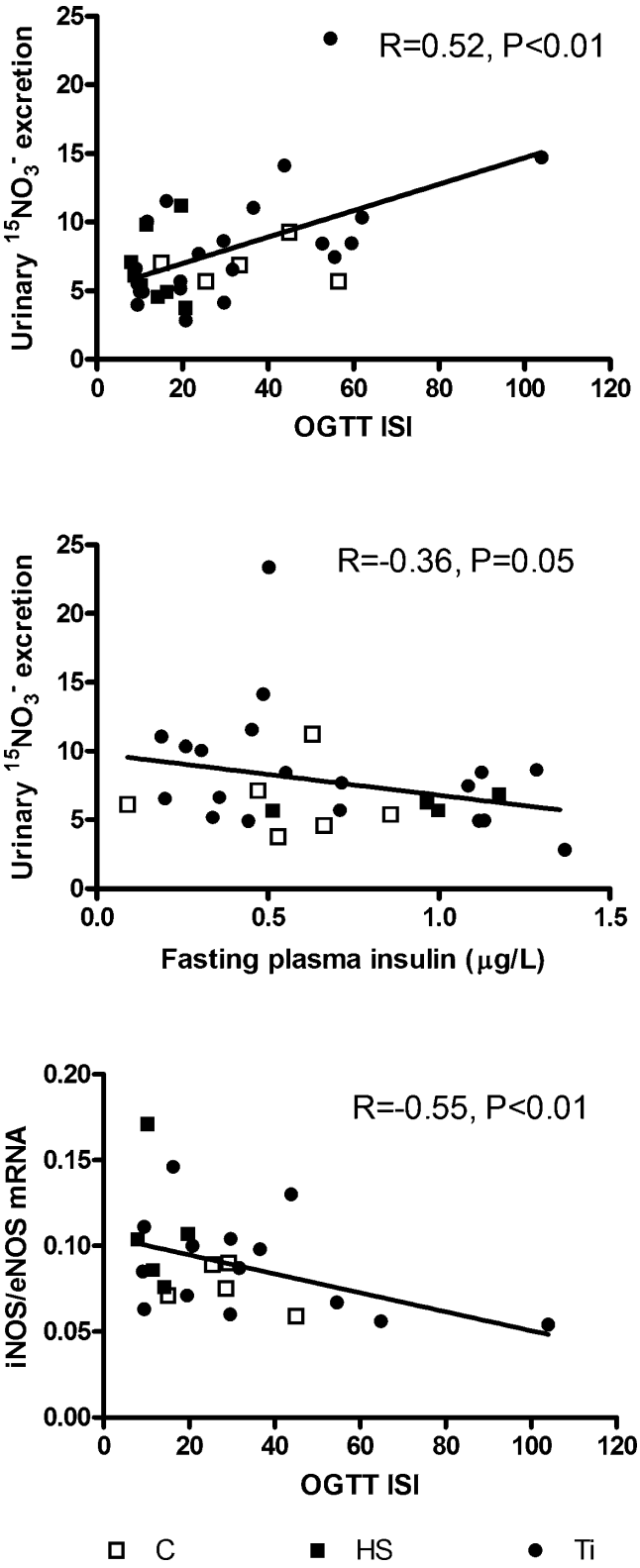

Figure 3. Pearson correlations between NO production (‰^{15}N recovered as urinary nitrate after [$^{15}N_2$-(guanido)]-arginine intravenous bolus/mmol creatinine; $n = 7$ to 8), skeletal muscle iNOS/eNOS mRNA ratio ($n = 6$), OGTT-ISI ($n = 7$ to 8), and fasting plasma insulin ($n = 7$ to 8).

Table 3. Stepwise Multiple Regression for Insulin Sensitivity Index, As Determined During the Oral Glucose Tolerance Test[a]

	Step	Estimated parameter	Model R^2	*P*
Intercept		−25.9		0.075
NO production[b]	1	2.25	0.38	0.0062
Muscle iNOS/eNOS mRNA	2	−3.04	0.58	0.017

[a] Variables entered in the model: plasma nitrotyrosine concentrations, plasma ADMA concentrations, muscle iNOS/eNOS mRNA, NO production, and urinary cGMP excretion.
[b] Urinary $^{15}NO_3^-$ excretion ‰^{15}N recovered after [$^{15}N_2$-(guanido)]-arginine:HCl intravenous bolus/mmol creatinine.

decrease in eNOS expression, NO production and bioavailability, and reduced insulin-induced eNOS activation (30). However, in the latter studies, these observations were made after more than 4 months of treatment. Similarly, an increase in plasma ADMA and an apparent decrease in NO production have mostly been demonstrated in a setting of pathologic conditions or in at-risk subjects who already displayed a cluster of risk factors, such as those associated with the metabolic syndrome (13). Therefore, based on the present pathophysiologic model (rats fed an HS diet for 6 weeks), we propose that a decrease in NO bioavailability is the first impairment that affects NO metabolism in the course of insulin resistance, and that subsequent impairment in NO metabolism lags behind.

The important role played by the impairment of NO bioavailability in this pathophysiologic process is further demonstrated by the observation that the prevention of insulin resistance observed with cysteine-rich diets was associated with a significant reduction in plasma nitrotyrosine levels, resulting in partial normalization of this marker. Although the skeletal muscle iNOS/eNOS mRNA ratio in rats fed cysteine-rich diets did not differ from that of HS rats, an observation that could be related to a lack of statistical power, this variable was significantly correlated with plasma nitrotyrosine values ($r = 0.46$, $P < 0.05$), suggesting that the imbalance in the type of NO production was partly prevented by cysteine supplementation.

As far as the preventive effect of dietary cysteine is concerned, our results may also be helpful in inferring any underlying mechanism. Because we have previously shown that providing the cysteine-rich diets prevented the oxidation of tissue GSH/GSSG redox potential resulting from high-sucrose feeding (15), we propose that the positive impact of the cysteine-rich diets on NO bioavailability is mediated by the maintenance of the body redox status. Increased glutathione synthesis, as reported after a similar increase in dietary sulfur amino acid intake (31), could favor the formation of *S*-nitrosothiol, a stable adduct of nitric oxide and glutathione that protects nitric oxide from oxidative destruction (32, 33). Previous studies already

reported the beneficial effects of *N*-acetylcysteine supplementation on oxidative stress, glycemic control, and NO production/bioavailability over the short term in streptozotocin-induced diabetic rats (34, 35). However, in the present study, these effects were investigated at the very beginning of the onset of insulin resistance, and the effects of *N*-acetylcysteine supplementation were duplicated by the consumption of a cysteine-rich protein, an original finding. It is not excluded that the response to the three different Ti treatments could display some heterogeneity, suggesting possible different underlying mechanisms. Indeed, according to the multiple regression analysis, changes in NO bioavailability account only for 25% of changes in insulin sensitivity. However, we found no significant effect of the type of cysteine supplementation within the Ti group regarding insulin sensitivity and NO-related parameters.

Lastly, in order to gain further insight into the relative importance of NO production versus NO bioavailability in explaining the total variation of insulin sensitivity in our experimental setting, we analyzed the correlations between NO-related markers and insulin sensitivity. The results confirmed the importance of NO bioavailability, the muscle iNOS/eNOS mRNA ratio being negatively correlated with OGTT-ISI. However, we also found that NO production significantly explained part of the total variance in both fasting plasma insulin and OGTT-ISI (i.e., both the basal and dynamic indicators of insulin sensitivity). A multiple regression analysis revealed that NO production was the first predictor of OGTT-ISI. Therefore, our data show that NO production was the most important contributor to insulin sensitivity within the groups, despite the lack of difference in NO production between groups. Recently, polymorphisms in the promoter and coding regions of the eNOS gene have been reported to affect NO production and insulin sensitivity in humans (36–38). Whether such polymorphisms exist in outbred rats and could account for the differences in NO production and insulin sensitivity remains to be demonstrated.

Taken together, our data strongly suggest that in our animal model, (i) NO production is a major inherited factor of insulin sensitivity, whereas (ii) a decrease in NO bioavailability by NO oxidative scavenging is the first hit in the induction of insulin resistance. Other impairments to NO metabolism, which cluster together in a vicious cycle, may occur later during the progression of insulin resistance. Further studies are required to gain further insights on the importance of early defects in NO bioavailability to the initiation of insulin resistance.

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ERRATUM

In the article entitled, ''Nitric Oxide Bioavailability and Not Production Is First Altered During the Onset of Insulin Resistance in Sucrose-Fed Rats'' by C. Blouet, F. Mariotti, V. Mathe, D. Tome, and J.-F. Huneau, which was published in the December 2007 issue of *Experimental Biology and Medicine* (Vol. 232:1458–1464, 2007), please note the following correction. In Table 2, the plasma ADMA of the T_i group is 0.8 ± 0.1 μM. The authors apologize for this error.