Endothelial and vascular dysfunctions and insulin resistance in rats fed a high-fat, high-sucrose diet

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1Department of Medicine and Lipid Research Unit from Centre Hospitalier Université Laval (CHUL) Research Center, 2Research Center of L’Hôtel Dieu de Québec, 3Diabetes Research, Centre Hospitalier Universitaire de Québec (CHUQ), Laval University, Quebec, Canada

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Endothelial and vascular dysfunctions and insulin resistance in rats fed a high-fat, high-sucrose diet. Am J Physiol Heart Circ Physiol 295: H1044–H1055, 2008. First published July 3, 2008; doi:10.1152/ajpheart.00516.2008.—This study was designed to examine the effects of a high-fat, high-sucrose (HFHS) diet on vascular and metabolic actions of insulin. Male rats were randomized to receive an HFHS or regular chow diet for 4 wk. In a first series of experiments, the rats had pulse Doppler flow probes and intravascular catheters implanted to measure blood pressure, heart rate, and regional blood flows. Insulin sensitivity and vascular responses to insulin were assessed during a euglycemic hyperinsulinemic clamp performed in conscious rats. In a second series of experiments, new groups of rats were used to examine skeletal muscle glucose transport activity and to determine in vitro vascular reactivity, endothelial nitric oxide synthase (eNOS) protein expression in muscle and vascular tissues and endothelin content, nitrotyrosine formation, and NAD(P)H oxidase protein expression in vascular tissues. The HFHS-fed rats displayed insulin resistance, hyperinsulinemia, hyperglycemia, hyperlipidemia, elevated blood pressure, and impaired insulin-mediated renal and skeletal muscle vasodilator responses. A reduction in endothelium-dependent vasorelaxation, accompanied by a decreased eNOS protein expression in muscles and blood vessel endothelium, and increased vascular endothelin-1 protein content were also noted in HFHS-fed rats compared with control rats. Furthermore, the HFHS diet induced a reduced insulin-stimulated glucose transport activity in muscles and increased levels of NAD(P)H oxidase protein and nitrotyrosine formation in vascular tissues. These findings support the importance of eNOS protein in linking metabolic and vascular disease and indicate the ability of a Westernized diet to induce endothelial dysfunction and to alter metabolic and vascular homeostasis.

Address for reprint requests and other correspondence: H. Bachelard, Lipid Research Unit, CHUL Research Center, CHUQ, 2705 Blvd. Laurier, Ste-Foy, Quebec, Canada G1V 4G2 (e-mail: helene.bachelard@crchul.ulaval.ca).

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thelium function in relation with the glucoregulatory and hemodynamic actions of insulin. Insulin resistance was induced in rats by feeding them an HFHS diet, which mimics to some extent the high-energy diets frequently consumed by many individuals. This diet maintains a high lipid flux and leads to increased fat accretion and alterations in glucose and lipid metabolism (34) that are similar to those observed in the human metabolic syndrome (16). In the present study, the rats were randomized to either HFHS or a normal chow diet for 4–5 wk, as we were interested in evaluating the effect of early experimental obesity on endothelial function and the metabolic and vascular actions of insulin. Although some other groups have reported insulin resistance and endothelial dysfunction in similar dietary models (1, 36, 43), none of these has conducted experiments looking in parallel at metabolic and regional hemodynamic actions of insulin in conscious HFHS-fed rats.

MATERIALS AND METHODS

Animals and feeding protocol. This study was approved by the Animal Care and Handling Committee of Laval University; animals were cared and handled in accordance with the Canadian Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats (n = 103) aged 6 wk and initially weighing 175–230 g were purchased from Charles River Laboratories (St-Constant, QC, Canada). The rats were individually housed in a light-controlled (12-h light cycle starting at 6 AM and temperature-regulated (20–22°C) space. They were randomly divided into two dietary groups and for 4 wk had free access to water and one of two diets. The control group (n = 52) was fed standard rat chow (Teklad Global 18% Protein Rodent Diet no. 2018), while the test group (n = 51) was fed a purified HFHS diet. The diet components are listed in Table 1. The fat content of the HFHS diet was predominantly saturated (49%).

Surgical preparation. In vivo experiments were performed in HFHS- (n = 17) and Chow-fed rats (n = 19). Three weeks after the start of the diets, the rats were anesthetized with a mixture of ketamine-xylazine (100 and 10 mg/kg, respectively, iv) and had pulsed Doppler flow probes implanted to monitor changes in renal, mesenteric, and hindquarter blood flows, as previously described (20, 38). After surgery, the rats were given subcutaneous injections of buprenorphine (0.05 mg/kg) and returned to their cages and allowed to recover for at least 7 days. The Chow or HFHS diet continued during postsurgical recovery, and the latter was deemed satisfactory by the resumption of growth and normalization of 24-h food intake. After the recovery period, the rats were again anesthetized (as above), and two separate catheters were implanted in the right jugular vein (for iv infusions) and one catheter in the left carotid artery (for blood pressure and heart rate measurements) (35). The rats were given subcutaneous injections of buprenorphine (0.05 mg/kg) and returned to their cages. A euglycemic hyperinsulinemic clamp was then performed 72 h after this last surgical step. The rats were 10–11 wk old. Euglycemic hyperinsulinemic clamp studies. Before each experiment, blood glucose and plasma insulin were determined, and resting heart rate, blood pressure, and regional blood flows were recorded over 30 min in quiet, unrestrained, and overnight-fasted rats. Following this, a euglycemic hyperinsulinemic clamp was carried out over 2 h in the designated groups as previously described (38), while heart rate, blood pressure, and regional blood flows were continuously measured. Insulin (Humulin R; Eli Lilly, Toronto, ON, Canada) was infused at a rate of 4 mU•kg⁻¹•min⁻¹ in a first group of Chow-fed (n = 9) and HFHS-fed rats (n = 10). During this time, a dextrose solution (50% wt/vol) was infused at variable rates to maintain glycemia near the fasting level; the amount of dextrose infused to maintain euglycemia was plotted as whole body glucose infusion rate (GIR; expressed in mg•kg⁻¹•min⁻¹). In control experiments, a second group of Chow-fed (n = 10) and HFHS-fed rats (n = 7) was infused with saline-0.2% BSA instead of insulin and dextrose to match approximately the saline load delivered during the clamp studies. At the end of the clamp, the rats were killed by an overdose of anesthetic (pentobarbital sodium, 75 mg/kg, iv) followed by decapitation.

Table 2. Body, WAT, and muscle weights and average daily food and energy intakes of rats chronically fed either rodent chow or an HFHS diet for 4 wk

<table>
<thead>
<tr>
<th>Composition</th>
<th>Chow Diet (n = 34)</th>
<th>HFHS Diet (n = 35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body wt, g</td>
<td>189±5</td>
<td>197±5</td>
</tr>
<tr>
<td>Final body wt, g</td>
<td>373±5</td>
<td>419±7</td>
</tr>
<tr>
<td>Epididymal WAT, g</td>
<td>3.73±0.25</td>
<td>7.29±0.37*</td>
</tr>
<tr>
<td>Gastrocnemius muscle, g</td>
<td>2.27±0.05</td>
<td>2.44±0.04</td>
</tr>
<tr>
<td>EDL muscle, mg</td>
<td>181±4</td>
<td>179±4</td>
</tr>
<tr>
<td>Soleus muscle, mg</td>
<td>153±5</td>
<td>159±6</td>
</tr>
<tr>
<td>Liver, g</td>
<td>16.18±0.64</td>
<td>16.84±0.44</td>
</tr>
<tr>
<td>Food intake, g/day</td>
<td>23.9±0.3</td>
<td>20.1±0.5*</td>
</tr>
<tr>
<td>Energy intake, kcal/day</td>
<td>81±1</td>
<td>93±2*</td>
</tr>
</tbody>
</table>

Values are means ± 5E; n is the no. of rats. WAT, white adipose tissue; EDL, extensor digitorum longus. ∗Different from Chow-fed rats at P < 0.05.
tissue. The vessels were either snap frozen and stored at −80°C, immersed in Optimal Cutting Temperature (OCT) Embedding Medium (Tissue-Tek, Sakura Finetek, Torrance, CA), and quickly frozen for later immunofluorescence analysis or immediately processed for in vitro vascular reactivity studies. Epididymal white adipose tissues (WAT), gastrocnemius muscles, and liver were excised, weighed, snap frozen, and stored at −80°C.

Triacylglycerol, nonesterified fatty acids, glucose, and insulin measurements. Plasma glucose concentrations were measured by the glucose oxidase method (42), using a Beckman glucose analyzer (Beckman Instruments, Palo Alto, CA). Insulin levels were measured by radioimmunoassay (RIA), using porcine insulin standards and polyethylene glycol for separation (15). Plasma triglycerides were assayed by an enzymatic method (30), using a reagent kit from Roche Diagnostics (Basel, Switzerland) that allowed correction for free glycerol. Plasma nonesterified fatty acids (NEFA) were also determined enzymatically using commercially available kits (Wako Chemicals, Dallas, TX).

Western blot analysis. Immunoblotting was performed in thoracic aorta and gastrocnemius muscle of both diet groups to detect eNOS protein levels as previously described (35). Nitrotyrosine formation and NAD(P)H oxidase gp91phox protein levels were also determined in thoracic aorta. The aorta and gastrocnemius muscle of each rat were individually homogenized in 5 vol of homogenization buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1 mM DTT, 50 mM NaF, 5 mM Na pyrophosphate, 1% Triton X-100, 0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF), and protease inhibitors cocktail (P-8340; Sigma-Aldrich, Oakville, ON, Canada). Homogenates were centrifuged (10,000 g) and supernatant were determined by the glucose oxidase method (42), using a Beckman glucose analyzer (Beckman-Coulter, Inc., Brea, CA). Monoclonal -tubulin antibodies (1:5,000; Sigma-Aldrich, St. Louis, MO). While the rings were constricted, the endothelium-dependent dilator

Vascular reactivity. The effects of diets on vascular reactivity were evaluated in vitro using aortic rings prepared as previously described (9) which were allowed to equilibrate for 1 h in the incubation medium. The incubation medium was Krebs-Henseleit physiological solution, containing the following (in mM): 118 NaCl, 4.7 KCl, 25 NaHCO3, 1.18 MgSO4, 1.18 KH2PO4, 2.5 CaCl2, and 5.5 glucose, pH 7.4, maintained at 37°C and gassed with 95% O2:5% CO2. In some experiments, the NOS inhibitor L-NAME (100 μM) was added at the start of the incubation period and was present for the duration of the experiments. After equilibration, the arteries were constricted using a high-potassium Krebs solution (KCl, 18 mM) and were allowed to reequilibrate. The baths were then emptied and rinsed three times with Krebs solution and again allowed to equilibrate. The arteries were then constricted with 10–6 M phenylephrine (Sigma, St. Louis, MO). While the rings were constricted, the endothelium-dependent dilator responses to 10–5 M carbachol (Sigma) were obtained to ensure that the endothelium of each vascular ring was functioning. After washout of the phenylephrine and carbachol, a cumulative dose-response curve to phenylephrine (10–9–10–5M) was obtained. After a washout period, rings were constricted with phenylephrine (10–6 M), and cumulative relaxation curves to carbachol (10–8–10–4 M) and then to

Table 3. Fasting and unfasting plasma levels of glucose, insulin, triglycerides, and nonesterified fatty acids of chow-fed and HFHS-fed rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Chow Fed (n = 8)</th>
<th>Fasting</th>
<th></th>
<th>Nonesterified fatty acids, mmol/l</th>
<th>0.44 ± 0.03 *</th>
<th>0.53 ± 0.04 *</th>
<th>0.16 ± 0.02</th>
<th>0.37 ± 0.03 *</th>
</tr>
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</table>

Values are means ± SE; n is the no. of rats. Fasting values were measured in plasma obtained from overnight-fasted rats. *P < 0.05, HFHS-fed rats in fasted state vs. chow-fed rats in fasted state. †P < 0.05, HFHS-fed rats in unfasted state vs. chow-fed rats in unfasted state.

Immunofluorescence analysis. Three 7-μm transversal sections of each frozen thoracic aorta of chow- (n = 7) and HFHS-fed rats (n = 7) were mounted on glass slides, fixed in acetone at −20°C for 10 min, and washed in phosphate-buffered saline (PBS). They were incubated in the blocking solution containing 10% BSA diluted in PBS at room temperature for 45 min and then incubated overnight at 4°C with 1:200 diluted primary antibody for eNOS (Transduction Laboratories) and the Alexa Fluor-488 phallodin (Molecular Probes, Burlington, ON, Canada) diluted 1:150 in the blocking solution. Following that, the sections were washed three times with PBS for 10 min and incubated for 1 h with the Alexa Fluor-594 rabbit anti-mouse IgG, diluted 1:1,000 (Molecular Probes) in the blocking solution. After being rinsed in PBS, tissue slices were mounted on coverslips. Immunofluorescence was observed on a confocal microscope (MRC-1024 Confocal System, Bio-Rad) combined with the Laser Sharp image acquisition software v3.2. Comparison of fluorescence levels between groups was performed, under the same conditions of laser exposure, using the Metamorph software (Molecular Devices, Downingtown, PA).

Table 4. Baseline values of HR, MAP, and regional Doppler shift and vascular conductance in chow-fed and HFHS-fed rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Chow Fed (n = 8)</th>
<th>Fasting</th>
<th></th>
<th>Nonesterified fatty acids, mmol/l</th>
<th>0.44 ± 0.03 *</th>
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<th>0.16 ± 0.02</th>
<th>0.37 ± 0.03 *</th>
</tr>
</thead>
</table>

Values are means ± SE; n is the no. of rats. Groups represent those used to assess the hemodynamic effects of insulin intravenously infused during the euglycemic hyperinsulinemic clamp studies. HR, heart rate; MAP, mean arterial blood pressure; bpm, beats/min. *P < 0.05, HFHS-fed rats vs. chow-fed rats.
sodium nitroprusside (SNP; \(10^{-10}-10^{-7}\) M, Sigma) were quantified to assess the endothelium-dependent and endothelium-independent ability of the smooth muscle to relax. Each curve was separated by a 30-min washout period. The contracting curves to phenylephrine were expressed in gram of tension, and the relaxation curves were expressed as percent changes from the contraction induced by phenylephrine.

**Measurement of endothelin level in mesenteric arterial bed.** Immunoreactive ET-1 concentrations were measured in the complete mesenteric arterial beds from HFHS- \(n = 8\) and chow-fed rats \(n = 8\) using a specific RIA in C18 Sep-Pak-extracted samples as described in detail elsewhere (35, 47). Briefly, the mesenteric vascular bed of each rat was individually homogenized twice with a Tissue-Tearor for 15 s in 2 ml of ice-cold extraction solution (1 M HCl, 1% acetic acid, 1% TFA, and 1% NaCl). The homogenate was centrifuged at 3,000 \(g\) for 30 min at 4°C. The supernatant was then collected, and 100 \(\mu l\) of \([^{125}I]\)ET-1 \((\sim 1,000 counts/min; \text{cpm})\) were added before extraction on a C18 Sep-Pak column. The Sep-Pak column was activated with 4 ml of 60% acetonitrile and 0.1% trifluoroacetic acid (TFA) and then rinsed twice with 10 ml of 0.1% TFA. After sample loading, the column was washed twice with 10 ml of 0.1% TFA, and the immunoreactive ET-1 (ir-ET-1) fraction was eluted with 3 ml of 60% acetonitrile and 0.1% TFA and then counted in a gamma counter (recovery is 90–95%). The sample extracts were dried overnight in a Speed-Vac and reconstituted in 500 \(\mu l\) of RIA buffer. Aliquots of 100 and 200 \(\mu l\) of extracted samples or 200 \(\mu l\) of standards (ET-1, Peninsula Laboratories) were added to 100 \(\mu l\) of anti-ET-1 antibody, and the final reaction volume was adjusted to 300 \(\mu l\) with RIA buffer. After a 24-h incubation period at 4°C, 100 \(\mu l\) of \([^{125}I]\)ET-1 \((15,000 \text{cpm})\) in RIA buffer were added, and the tubes were incubated for an additional 24 h at 4°C. Bound and free radioactivity was separated by the second antibody method. After a 2-h incubation period at room temperature, 0.5 ml of RIA buffer was added, and the tubes were centrifuged at 2,500 \(g\) for 20 min at 4°C. The supernatant was then discarded, and the pellet was counted in a gamma counter. ir-ET-1 concentrations were corrected for losses in extraction.

**Glucose transport activity in isolated rat skeletal muscles.** Basal and insulin-stimulated glucose utilization was examined in isolated soleus and EDL skeletal muscles from overnight-fasted chow- \(n = 9\) and HFHS-fed rats \(n = 11\). Glucose transport was measured by use of the glucose analog \([^{3}H]\)2-deoxy-D-glucose, according to the method developed by Hansen et al. (24) and as previously described (47). \([^{3}H]\)2-deoxy-D-glucose uptake rates were corrected for extracellular trapping using \([^{14}C]\)mannitol (24).

**Data analysis.** The results are expressed as means ± SE; \(n\) is the number of rats. Western blot and immunofluorescence analysis data are expressed relative to control, assigning a value of 1 to the chow-fed group baseline mean. Data describing the biological characteristics of the rats as well as those resulting from Western blot, RIA, and immunofluorescence analysis were evaluated using Student’s \(t\)-test for unpaired data. Vascular ring experiments and glucose transport activity measurements as well as results obtained over time, such as those from cardiovascular responses to insulin in HFHS- and chow-fed rats, were analyzed for statistical significance using a two-way repeated-measures ANOVA. Post hoc analyses were performed when significant differences were noted, using the Fisher’s least significance difference test. A \(P\) value < 0.05 was taken to indicate a significant difference.

**RESULTS**

**Body weight and metabolic changes.** Compared with chow, chronic ingestion for 4 wk of the HFHS diet resulted in larger increases in final body weight and epididymal adipose depot, whereas gastrocnemius muscle and liver weights were comparable (Table 2). These significant differences in final body weight and fat accretion were associated with a larger energy intake in the HFHS-fed group than in the chow-fed group. Furthermore, plasma insulin and NEFA levels were significantly higher in overnight-fasted HFHS-fed rats than in chow-fed rats, whereas no differences in plasma glucose or triglycerides levels were noted between the dietary cohorts (Table 3). The demonstration here of a higher plasma insulin level in HFHS-fed rats compared with their chow-fed counterparts is indicative of insulin resistance. In unfasted rats, we found...
significantly higher plasma insulin, glucose, triglycerides, and NEFA levels in HFHS-fed than in chow-fed rats.

Hemodynamic responses to insulin infusion during the euglycemic hyperinsulinemic clamp. Baseline values (before any iv infusion) for cardiovascular variables are listed in Table 4 for both groups of rats. Basal heart rate and mean blood pressure were higher in HFHS-fed rats than in the chow-fed group. This was accompanied by smaller basal superior mesenteric flow and vascular conductance in HFHS-fed rats than in chow-fed rats, while there was no significant difference in basal renal or hindquarter flows or vascular conductances between the two dietary groups.

Figure 1 shows that insulin infusion at a rate of 4 mU·kg⁻¹·min⁻¹ in chow-fed rats caused significant increases in renal and hindquarter blood flows, but it had no effect on heart rate, mean arterial blood pressure, or superior mesenteric flow compared with measurements following a control infusion of vehicle (saline-0.2% BSA). These responses were associated with significant increases in renal and hindquarter vascular conductances, whereas no change was noted in superior mesenteric vascular conductance (Fig. 2). In HFHS-fed rats, the same infusion of insulin had no effect on heart rate, mean arterial blood pressure, or renal or hindquarter flows, while a significant decrease in superior mesenteric flow was observed when compared with the effects of control infusion of vehicle (Fig. 1). The renal, superior mesenteric, and hindquarter blood flow responses to insulin differed significantly from those seen in chow-fed rats. Furthermore, in HFHS-fed rats the euglycemic infusion of insulin elicited a significant fall in superior mesenteric vascular conductance but had no consistent effect on renal or hindquarter vascular conductances when compared with the effects of control infusion of vehicle (Fig. 2). These responses differed significantly from those seen in chow-fed rats, in which insulin produced marked vasodilation in renal and hindquarter vascular beds but had no effect in the superior mesenteric vascular bed.

Responses during euglycemic hyperinsulinemic clamp. Figure 3 shows that, in the animals subjected to the euglycemic hyperinsulinemic clamp, the HFHS-fed rats had slightly but not significantly higher preinfusion insulin levels compared with their chow-fed counterparts (137 ± 14 vs. 96 ± 18 pmol/l). During the euglycemic infusion of insulin, we found that plasma insulin levels in both dietary cohorts rose acutely and achieved similar plateaus (770 ± 84 vs. 735 ± 75 pmol/l in...
HFHS- and chow-fed rats, respectively). Preinfusion glucose levels were similar in both dietary groups (4.3 ± 0.1 vs. 4.3 ± 0.1 mmol/l in HFHS- and chow-fed rats, respectively), and glycemia achieved during insulin infusion was comparable in the chow- and HFHS-fed rats (average levels between 60 and 120 min of infusion: 4.1 ± 0.1 mmol/l and 4.3 ± 0.1 mmol/l, respectively). The variation in glycemia during the infusions never exceeded 10%. The control infusion of saline-0.2% BSA did not alter plasma glycemia or insulin (data not shown). The steady-state glucose infusion rate needed to maintain euglycemia during the last hour of the clamp was found to be significantly lower in the HFHS-fed rats than in the chow-fed group, indicating frank whole body insulin resistance in the former.

Effect of HFHS diet on eNOS protein expression in thoracic aorta and skeletal muscle. The Western blot analysis revealed that eNOS proteins were detectable in vascular and muscle tissues from both dietary groups. The proteins migrated as a single band of ~140,000 M (Fig. 4A). Immunoreactivity of eNOS was quantified by scanning densitometry, and the mean data are presented. The results indicate that eNOS protein expression was significantly lower in thoracic aorta and gastrocnemius muscle from HFHS-fed rats than from chow-fed rats (Figs. 4B). Using confocal microscopy, we found a threefold reduction in eNOS immunofluorescence level in the endothelium of thoracic aorta from HFHS-fed rats compared with the chow-fed group (Fig. 5).

Vascular reactivity. Contractions induced by increasing concentrations of phenylephrine were not significantly altered by the HFHS diet compared with the chow diet (Fig. 6). The addition of L-NAME in the incubation medium was found to completely abrogate the vasorelaxing effects of carbachol in the two experimental groups. The evaluation of the endothelium-independent vasorelaxation by use of SNP in cumulative concentrations revealed that HFHS-fed rat vascular smooth muscles had a similar ability to relax as those from chow-fed rats (Fig. 7). The addition of L-NAME slightly but significantly reduced the vasorelaxations elicited by low doses of SNP (10^{-10}–10^{-9} M) in both groups. However, because we were using a consistent dose of phenylephrine to create the constriction, it is likely that the reduction observed here was due to changes in preconstriction, as L-NAME was found to enhance the contracting responses to phenylephrine.

Effect of HFHS diet on nitrotyrosine expression in vascular tissue. Western blot analysis of the thoracic aortas isolated from both experimental groups revealed higher levels of nitrotylated proteins in vascular tissues isolated from HFHS-fed rats than from chow-fed rats (Fig. 8). This was associated with higher NAD(P)H oxidase protein expression in vascular tissues isolated from HFHS-fed rats than from chow-fed rats (Fig. 9).

ET-1 protein content in vascular tissues. Higher concentrations of ir-ET-1 were noted in the mesenteric arterial beds isolated from the HFHS-fed rats than from the chow-fed rats (Fig. 10).

Effect of HFHS diet on [3H]-2-deoxy-D-glucose uptake in isolated skeletal muscles. The effect of the diets on basal and insulin-stimulated glucose transport activities in isolated soleus and EDL muscles is shown in Fig. 11. In both skeletal muscles, we found that HFHS feeding induced a significant decrease in basal glucose transport activity compared with chow feeding. Furthermore, in the presence of insulin, we found a significantly smaller glucose transport activity in soleus (at doses of 0.002, 0.02, and 0.2 μM/ml insulin) and EDL muscles (at doses of 0.02, 0.2 and 2 μM/ml insulin) isolated from HFHS-fed rats than in those isolated from chow-fed rats.

Fig. 4. Effects of HFHS diet on endothelial nitric oxide synthase (eNOS) protein expression in thoracic aorta and skeletal muscle. eNOS protein content was measured in thoracic aortas and gastrocnemius muscles isolated from overnight-fasted HFHS-fed (n = 8) and chow-fed rats (n = 8). A: representative immunoblots showing eNOS immunoreactivity in aorta and gastrocnemius muscle of one animal in each dietary group are shown. B: densitometric band intensities of eNOS are summarized. The mean level of eNOS in chow-fed rats was assigned a value of 1, and the relative levels of eNOS in each group were adjusted with α-tubulin. Data are expressed as means ± SE and were analyzed using unpaired Student’s t-tests. *P < 0.05, significant difference between HFHS-fed rats and chow-fed rats.
DISCUSSION

The rats fed an HFHS diet for 4 wk were found to be significantly heavier than the chow-fed rats. They had increased fat accretion and developed insulin resistance that was associated with vascular endothelium dysfunction and significant increases in blood pressure and unfasting plasma levels of insulin, glucose, triglyceride, and free fatty acid. Increased plasma levels of insulin and free fatty acid were also noted in overnight-fasted HFHS-fed rats. Therefore, the HFHS-fed rat represents a good model of diet-induced metabolic perturbations that are quite similar to those that define the metabolic syndrome in obese subjects (40). They also represent a suitable model to further explore vascular endothelium function in relation to the glucoregulatory and hemodynamic actions of insulin. Although some others have shown insulin resistance, hyperinsulinemia, hyperlipidemia, hypertension, and endothelial dysfunction in similar dietary models (1, 36, 37, 43, 45, 58), none of them has conducted experiments looking in parallel at vascular and metabolic actions of insulin in conscious, unrestrained HFHS-fed rats. An obvious strength of this study is the present status of the preparation in which the metabolic, vascular, and NOS responses can be evaluated in the same animal. These in vivo experiments were completed with in vitro studies to assess skeletal muscle glucose transport activity, vascular reactivity, eNOS and ET-1 protein expression and localization, and parameters of oxidative stress and metabolic homeostasis.

The major findings that emerged from this study are that a 4-wk HFHS diet in rats markedly impaired the vascular responses to insulin and induced insulin resistance, as concomitantly determined during a euglycemic hyperinsulinemic clamp. Thus, in chow-fed rats, the euglycemic infusion of insulin was found to induce vasodilations in renal and hindquarter vascular beds, whereas in HFHS-fed rats, the same euglycemic infusion of insulin had no vasodilatory effects but caused a vasoconstriction in the superior mesenteric vascular bed. The mechanism underlying the impaired vascular responses to insulin in the HFHS-fed group remains unclear. However, since a role for endothelium-derived NO has been shown in the mediation of the insulin vasodilator effect (48, 49), it is likely that a reduced production of NO, consecutive to a downregulation of eNOS protein in the vasculature of HFHS-fed rats, could have contributed, at least in part, to the impaired renal and hindquarter vasodilator responses. Indeed, our results clearly indicate a decreased abundance of eNOS protein expression in skeletal muscles and thoracic aortas from HFHS-fed rats compared with their chow-fed counterparts. This contention is further supported by the demonstration here of a threefold

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**Fig. 5.** Effects of HFHS diet on eNOS immunofluorescence level in the endothelium of thoracic aortas from chow- and HFHS-fed rats. A: confocal microscopy photographs of the thoracic aortas from a chow-fed rat (left) and an HFHS-fed rat (right). Endothelium-specific eNOS protein expression was revealed using an Alexa Fluor-594-conjugated rabbit anti-green fluorescent protein (GFP) (red). The aortas were counterstained with Alexa Fluor-488-conjugated phalloidin (green), which has a high affinity for F-actin. B: densitometric band intensities of eNOS are summarized. The mean level of eNOS in chow-fed rats \( (n = 7) \) was assigned a value of 1, and the relative level of eNOS in HFHS-fed rats \( (n = 7) \) was calculated. Data are means ± SE and were analyzed using unpaired Student’s \( t \)-tests. \( *P < 0.05 \), significant difference between HFHS-fed rats and chow-fed rats.
reduction in eNOS immunofluorescence level in the endothelium of thoracic aorta, using confocal microscopy, and by the depressed endothelium-dependent vasorelaxation response to carbachol noted in aortas from the HFHS-fed rats. The latter finding cannot be explained by smooth muscle dysfunction, because the vascular smooth muscle contracting response to phenylephrine, and the response to the endothelium-independent vasodilator SNP, did not differ between control and insulin-resistant rats. These findings indicate that the ability of vascular smooth muscle to contract or to relax in response to exogenous NO was not impaired in the HFHS-fed rats, and that diet selectively impaired endothelium-dependent vasodilation. These findings agree with previous data from this laboratory and others showing impaired endothelium-mediated relaxation and depressed eNOS protein expression in obese subjects, and in animal models with insulin resistance (36, 37, 43, 45, 47, 50).

On the other hand, enhanced ROS-mediated inactivation and sequestration of NO, leading to depressed NO bioavailability (43, 45) and then limiting the NO-dependent vasodilator capacity at the level of the vascular smooth muscle (36, 37, 43), might have contributed to the altered vascular responses noted in our group of HFHS-fed rats. In agreement with that, we found an increased level of NAD(P)H oxidase protein expression, a major vascular source of ROS, as well as an increased formation of nitrotyrosine, which is the stable “footprint” of NO oxidation by ROS, in vascular tissues of rats fed an HFHS diet. Therefore, these findings indicate that increased vascular oxidative stress induced by the HFHS diet, leading to NO breakdown and endothelial dysfunction, might be responsible, at least in part, for the impaired insulin-mediated vasodilator responses in renal and skeletal muscle vascular beds. This is supported by two recent studies carried out in female Fischer rats and demonstrating that long-term consumption of an HFHS diet (for 2 mo or longer) induces endothelial dysfunction and oxidant/antioxidant imbalance, resulting in upregulation of the ROS-generating enzyme NAD(P)H oxidase and downregulation of several key antioxidant enzymes in the kidney and vascular tissues (43, 44). Added to that, accelerated ROS production and diminished bioavailable NO caused by eNOS uncoupling in vascular tissues of our rat model might be an interesting hypothesis that remains to be verified.
Another potential mechanism that could contribute to impaired vascular responses to insulin in HFHS-fed rats is an exaggerated production of endothelium-derived contracting factors such as ET-1, which is a powerful vasoconstrictor that has been previously reported to modulate endothelium-dependent vasoconstriction in rats and mice fed a high-fat diet (12, 54). Consistent with that is the demonstration here that the mesenteric arteries from the HFHS-fed rats contained a greater amount of ET-1 protein than those from the chow-fed rats. This could have contributed to the insulin-mediated vasoconstrictor effect noted in this vascular bed. Insulin is known to stimulate gene expression of vascular ET-1 in vitro and to modulate ET-1 receptor mRNA and increased plasma levels of ET-1 have been shown in fructose-fed rats (27, 28), and we recently reported increased ET-1 concentration in mesenteric arteries isolated from sucrose-fed rats (47). Therefore, diet-induced insulin resistance/hyperinsulinemia may have created an environment resulting in activation of the ET system, thus causing impaired endothelium-dependent vasodilation.

On a quantitative basis, skeletal muscle is the predominant site of insulin-stimulated glucose disposal and the major tissue responsible for postprandial hyperglycemia in insulin-resistant states (4, 14). Therefore, we believe that the impaired hindquarter vasodilator response to insulin could have contributed to the insulin resistance noted in our HFHS-fed rats by reducing delivery of glucose and insulin to muscle beds, as previously suggested in humans and rats.
However, to distinguish the direct effects of insulin on glucose uptake from its effects on blood flow, we carried out experiments in isolated skeletal muscles to examine the effect of HFHS feeding on glucose transport activity, thus in the absence of blood flow influence. We found that, compared with the chow diet, the HFHS diet caused resistance of skeletal muscle glucose transport to stimulation by insulin, suggesting that there is an intrinsic defect in the tissue itself. Thus impairment of insulin’s hemodynamic actions in vivo would contribute to insulin resistance and endothelial dysfunction and hypertension in comparable animal models (1, 29). Another factor that could potentially contribute to the rise in blood pressure is the presence of increased sympathetic drive and hyperlipidemia in the HFHS-fed rats. Fat feeding in rats was shown to stimulate sympathetic nervous system activity (59), and increased sympathetic activity is known to stimulate free fatty acid release from fat depots (46). Previous studies have indicated an association between increased circulating levels of triglycerides or free fatty acids and endothelial dysfunction and hypertension (22, 32, 51, 52). The sucrose component of the diet could have enhanced the deleterious effect of the saturated fats on blood pressure and endothelial function, as we previously reported that feeding a high-sucrose diet (for 4 wk) to rats leads to insulin resistance, impaired insulin-mediated skeletal muscle vasodilation, and reduced eNOS protein content in muscle, but it has no effect on resting blood pressure (47). In the latter study, neither effect of the sucrose diet was observed on body weight or plasma levels of triglycerides or free fatty acids. Therefore, the hypertensive effect of the HFHS diet could be a facet of fat deposition and/or the fat content of the diet, as saturated fat feeding without sucrose was shown to cause endothelial dysfunction and hypertension (58, 59). Further studies are required to clarify this point.

In conclusion, metabolic insulin resistance is a problem of utmost clinical importance and a major risk factor for cardiovascular morbidity and mortality. Loss of the modulatory role of the endothelium may be a critical and initiating factor in the development of diabetic vascular disease. Our findings in HFHS-fed rats provide further evidence of the importance of eNOS protein in linking metabolic and vascular disease and indicate the ability of a relatively short-term HFHS diet to induce insulin resistance and endothelial dysfunction and to affect regulation of vascular NO synthesis and glucose and vascular homeostasis.

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Fig. 11. Insulin dose-response curve for stimulation of glucose uptake in soleus and extensor digitorum longus (EDL) muscles. Muscles were dissected out from chow-fed rats (n = 9) and HFHS-fed rats (n = 11). Values are means ± SE. Comparisons were made between insulin-evoked responses in chow-fed rats and those in HFHS-fed rats, and data were analyzed using a 2-way repeated-measures ANOVA followed by Fishers test. *P < 0.05, HFHS-fed rats vs. chow-fed rats.

(5, 10, 11). However, to distinguish the direct effects of insulin on glucose uptake from its effects on blood flow, we carried out experiments in isolated skeletal muscles to examine the effect of HFHS feeding on glucose transport activity, thus in the absence of blood flow influence. We found that, compared with the chow diet, the HFHS diet caused resistance of skeletal muscle glucose transport to stimulation by insulin, suggesting that there is an intrinsic defect in the tissue itself. Thus impairment of insulin’s hemodynamic actions in vivo would contribute to insulin resistance by reducing access for hormone and nutrient but would not be the sole cause of insulin resistance in these animals. These findings are consistent with our previous data and with those of other laboratories indicating an impaired ability of insulin to stimulate glucose uptake in muscles isolated from rats fed a high-fat and/or high-sucrose diet (1, 25, 47). The extent to which a reduction in the total cellular content of GLUT4 proteins or an impairment in the translocation process of the GLUT4 protein to the cell surface and/or a change in its intrinsic activity contributes to the reduced insulin-stimulated glucose transport activity remains to be verified.

In the present study, the HFHS diet-induced insulin resistance was accompanied with a significant rise in blood pressure. The reduction in baseline value of the superior mesenteric vascular conductance noted in the HFHS-fed group, together with the altered endothelial function and the insulin resistance/hyperinsulinemia induced by the diet, could be the trigger for the development of high blood pressure, as previous data indicated that insulin resistance and endothelial dysfunction precede hypertension in comparable animal models (1, 29). Another factor that could potentially contribute to the rise in blood pressure is the presence of increased sympathetic drive and hyperlipidemia in the HFHS-fed rats. Fat feeding in rats was shown to stimulate sympathetic nervous system activity (59), and increased sympathetic activity is known to stimulate free fatty acid release from fat depots (46). Previous studies have indicated an association between increased circulating levels of triglycerides or free fatty acids and endothelial dysfunction and hypertension (22, 32, 51, 52). The sucrose component of the diet could have enhanced the deleterious effect of the saturated fats on blood pressure and endothelial function, as we previously reported that feeding a high-sucrose diet (for 4 wk) to rats leads to insulin resistance, impaired insulin-mediated skeletal muscle vasodilation, and reduced eNOS protein content in muscle, but it has no effect on resting blood pressure (47). In the latter study, neither effect of the sucrose diet was observed on body weight or plasma levels of triglycerides or free fatty acids. Therefore, the hypertensive effect of the HFHS diet could be a facet of fat deposition and/or the fat content of the diet, as saturated fat feeding without sucrose was shown to cause endothelial dysfunction and hypertension (58, 59). Further studies are required to clarify this point.

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