Proinflammatory Endothelial Activation Detected by Molecular Imaging in Obese Nonhuman Primates Coincides With Onset of Insulin Resistance and Progressively Increases With Duration of Insulin Resistance

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Background—Inflammation and insulin resistance (IR) are associated processes that potentiate risk for cardiovascular disease in obesity. The temporal relation between IR and inflammation is not completely characterized. We hypothesized that endothelial cell adhesion molecule expression in large arteries is an early event that coincides with diet-induced obesity and IR in primates.

Methods and Results—Ten adult male rhesus macaques were studied at baseline and every 4 to 6 months on a high-fat diet for 2 years. Truncal fat, carotid intima-media thickness, plasma inflammatory biomarkers, and carotid P-selectin and vascular cell adhesion molecule-1 expression by contrast-enhanced ultrasound molecular imaging were assessed. Intravenous glucose tolerance test was performed at baseline and at 4 and 18 months. A high-fat diet produced a rapid increase ($P<0.01$) in weight, truncal fat, and degree of IR indicated by the insulin area under the curve and glucose disappearance rate on intravenous glucose tolerance test, all of which worsened minimally thereafter. Molecular imaging detected a progressive increase in endothelial cell adhesion molecule expression over time (5- to 7-fold greater than control agent signal at 2 years; $P<0.01$). Changes in intima-media thickness were not detected until 2 years and, although there was a trend toward an increase in plasma markers of inflammation (monocyte chemotactic protein-1, C-reactive protein), the pattern of increase varied considerably over time.

Conclusions—In primates with diet-induced obesity, endothelial inflammatory activation is an early event that occurs coincident with the development of IR and long before any measurable change in carotid intima-media thickness. Endothelial activation is related more to the duration rather than to the severity of IR and is not mirrored by changes in plasma biomarkers. (Circulation. 2014;129:471-478.)

Key Words: endothelium $\bullet$ inflammation $\bullet$ insulin resistance $\bullet$ molecular imaging $\bullet$ obesity

Systemic inflammation and insulin resistance (IR) are mutually amplifying processes that together increase risk for cardiovascular disease and the development of type 2 diabetes mellitus in obese individuals. On one hand, proinflammatory cytokines produced by adipocytes and resident macrophages within inflamed adipose tissue contribute to IR in part by interfering with normal insulin receptor intracellular signaling. Conversely, obesity-related IR promotes inflammation through multiple pathways including an increase in oxidative stress and the production of proinflammatory adipokines, advanced glycation end products, and free fatty acids.

Activation of nuclear factor κB is one of the master pathways that link obesity, IR, and inflammation. Activation of nuclear factor κB leads to transcription and expression of endothelial cell adhesion molecules (ECAMs), which represents an important mechanism by which obesity increases risk for atherosclerotic disease. In mice fed a high-fat diet (HFD), nuclear factor κB activation, elevation of mRNA for ECAMs, and dysregulation of nitric oxide (NO) occur approximately at the same time that obesity develops but before any detectable increase in plasma biomarkers for inflammation. There is even evidence that vascular IR, measured by phospho-IκBα, precedes the development of IR in the liver and adipose tissue. However, the temporal onset of vascular inflammatory activation, endothelial dysfunction, and circulating biomarkers

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in humans or larger mammalian models of obesity and IR has not been characterized.

The aim of this study was to temporally evaluate pathologic changes in endothelial phenotype (ECAM expression, NO bioactivity) that contribute to atherosclerosis with the use of noninvasive molecular imaging and metabolic profiling in a primate model of diet-induced obesity. We hypothesized that endothelial inflammatory changes would occur coincident with the development of obesity and IR and before changes in circulating markers of inflammation and carotid intimal thickening. We also sought to determine whether the degree of ECAM expression correlated with either the degree or severity of IR.

Methods

Study Design

The study was approved by the Animal Care and Use Committee of the Oregon National Primate Research Center and conformed to US Department of Agriculture and Association for Assessment and Accreditation of Laboratory Animal Care guidelines for nonhuman primate care. Ten adult male rhesus macaques (Macaca mulatta) aged 9 to 11 years at baseline were studied. At baseline, animals were fed a standard primate chow diet consisting of 58% carbohydrates, 27% protein, and 15% fat by caloric content. Animals were studied at baseline and at 4, 8, 12, 18, and 24 months after they were started on a HFD (9 to 11 years at baseline were studied. At baseline, animals were fed a standard primate chow diet consisting of 58% carbohydrates, 27% protein, and 15% fat by caloric content. Animals were studied at baseline and at 4, 8, 12, 18, and 24 months after they were started on a HFD). The aim of this study was to temporally evaluate pathologic changes in endothelial phenotype (ECAM expression, NO bioactivity) that contribute to atherosclerosis with the use of noninvasive molecular imaging and metabolic profiling in a primate model of diet-induced obesity. We hypothesized that endothelial inflammatory changes would occur coincident with the development of obesity and IR and before changes in circulating markers of inflammation and carotid intimal thickening. We also sought to determine whether the degree of ECAM expression correlated with either the degree or severity of IR.

Intravenous Glucose Tolerance Test

After an overnight fast, animals received dextrose (600 mg/kg IV) over 1 minute. Venous blood samples were obtained at baseline and at 1, 3, 5, 10, 20, 40, and 60 minutes after dextrose injection for measurement of blood glucose and plasma insulin concentration by radioimmunoassay. Time-concentration curves were plotted to derive the insulin area under the curve (AUC). Glucose disappearance rate (Kg) was calculated as follows: Kg = Ln (20-minute glucose)–Ln (5-minute glucose)/15 minutes×100%, where Ln indicates natural log.11,12

Body Composition

Body composition was assessed by dual x-ray absorptiometry (Discovery A, Hologic Inc) to obtain muscle mass and fat mass. Percent truncal fat was calculated by dividing the truncal fat mass by the total truncal mass.

Carotid IMT and Compliance

Two-dimensional long-axis ultrasound imaging of the distal common carotid artery was performed with a linear array transducer (15L8, Sequoia, Siemens Medical Systems, Mountain View, CA) at 10 MHz with the use of a mechanical index of 1.3 and a dynamic range of 65 dB. The average far-wall IMT at end-diastole was measured at 9 separate sites within 1 cm proximal to the carotid bifurcation. M-mode ultrasound was used to measure the diastolic (Dd) and systolic diameter (Ds) and was averaged over 3 cardiac cycles. The following vascular mechanical properties were determined: vascular strain, calculated by (Ss–Dd)/Dd, and the elastic modulus, calculated by ((P2–P1)/(Ss–Dd))/Dd, where Ps and Pd are systolic and diastolic blood pressure, respectively.

Carotid Molecular Imaging

For CEU molecular imaging, microbubbles targeted to P-selectin (MB), vascular cell adhesion molecule-1 (VCAM-1) (MB), or control microbubbles (MB) were prepared by conjugating biotinylated anti-human P-selectin monoclonal antibody (mAb) (AK4, Biologend) with antimouse VCAM-1 (1.611B1, ABD Serotec), or IgG1 isotype control (BD Biosciences) to the surface of lipid-shelled defoamfluorobutane microbubbles containing a bifunctional molecular spacer with a membrane anchor and polyethylene glycol/streptavidin moiety (Micromarker 2, VisualSonics Inc, Toronto, Ontario, Canada). Longitudinal-axis imaging of the distal common carotid artery and bifurcation was performed with the use of multipulse amplitude-modulation imaging at 7 MHz, a mechanical index of 0.25, and a dynamic range of 55 dB. Imaging was performed 5 minutes after the intravenous injection of 1×108 MB, MB or MB, performed in a random order. Several frames were digitally averaged. Signal from any remaining freely circulating microbubbles was eliminated by digital subtraction of several averaged frames obtained after a high-power (mechanical index 1.9) pulse sequence.13 Regions of interest were placed on the near and far walls of the common carotid artery, with the former extending into the proximal portion of the internal carotid. Data were averaged for the right and left carotid arteries.

Flow-Mediated Vasodilation

Ultrasound imaging (10 MHz) of the brachial artery in its long axis was performed to measure diameter and centerline average peak velocity with the use of angle-corrected pulsed-wave Doppler. Data were acquired at baseline and then every 30 seconds for 3 minutes after release of a forearm cuff inflated to 60 mm Hg above systolic pressure for 5 minutes. Brachial artery diameter was measured by averaging 9 separate end-diastolic measurements over 3 cardiac cycles. Data were expressed as absolute change in diameter and percent change from baseline. Shear stress was calculated immediately after cuff deflation as μ×V/4D, where μ is the viscosity of blood and assumed to be 0.035 dynes/cm², V is Doppler peak average velocity, and D is vessel diameter at baseline.14

Serum Lipids and Biomarkers

Venous blood samples were used to measure plasma lipids, hemoglobin A1c and inflammatory biomarker profiles with the use of a multianalyte immunoassay (Human MAP V1.6, Rules Based Medicine, Inc).

Targeting Ligand Cross-Reactivity With Macaque Epitopes

The cross-reactivity of the anti-human P-selectin mAb used for microbubble targeting was tested by assessing immunofluorescence on activated platelets. Heparinized blood samples from normal rhesus macaques were obtained. Immunostaining of platelets, which were aggregated through vortex shear, was performed with the AK4 primary mAb and FITC-labeled polyclonal rat anti-mouse secondary antibody (BD Biosciences) (5 μg/mL). Control experiments were performed with secondary antibody alone. Cross-reactivity of the VCAM-1 mAb was performed by immunohistochemistry of tissue bank specimens of spleen and carotid artery from rhesus macaques fed a HFD for 2 years. Sections (5 μm) were fixed in formalin and paraffin embedded. After antigen retrieval, endogenous peroxidases were blocked for 15 minutes in 2% H2O2 in 0.05 mol/L potassium phosphate-buffered saline. Sections were then treated with avidin/biotin blocking reagents according to kit directions (Vector Labs catalog No. SP2001) before being blocked for 30 minutes in potassium.
phosphate-buffered saline containing 0.4% Triton-X and 2% normal donkey serum. Primary staining was performed with the biotinylated mouse anti-VCAM-1 mAb (1:20 dilution), rinsed, and stained with the Vectastain Elite ABC kit (Vector Labs catalog No. PK-6100) and a DAB peroxidase substrate kit (Vector Labs catalog No. SK-4100). Sections were counterstained with hematoxylin.

**Statistical Analysis**

Data were analyzed on Prism version 5.0 and are expressed as ±SD unless stated otherwise. The D’Agostino and Pearson omnibus tests were used to assess data normality. Changes from baseline through 18 months were analyzed with 1-way repeated-measures ANOVA with a Bonferroni posttest (parametric), a Friedman test with a Dunn posttest (nonparametric) for multiple comparisons, or a test for linear trend. Because of a loss in follow-up of 2 animals after the 18-month time point, 18- and 24-month differences were assessed with either a Student paired t test or a Wilcoxon matched-pairs signed rank test. IVGTT insulin AUC data were natural log transformed and fit to a quadratic model, and KG was fit to a cubic model to assess changes across follow-up. Correlations of parametric data were assessed by a Pearson product, and nonparametric data were assessed with a Spearman test. Best-fit linear regression analysis was performed on data when significant correlations were observed. Only correlations with a significant linear association are reported.

**Results**

**Diet-Induced Changes in Morphometry, Lipids, and Insulin Sensitivity**

All animals were studied at every predefined interval except for 2 animals that were not available for study at the final 24-month time point. The mean caloric intake increased by 68% from 711±167 kcal/d on the baseline chow diet to 1195±67 kcal/d when measured 4 months after the HFD was started.

Total body mass and truncal fat on dual x-ray absorptiometry both increased significantly over the first 4 months after animals were started on the HFD (Table 1). Over the remainder of the study period, there were only small additional increases in mass and truncal fat. After 4 months of HFD, there were significant increases in serum cholesterol that remained relatively stable thereafter, whereas a peak and fall were noted in serum triglycerides (Table 1). On IVGTT, there was evidence for the development of IR after 4 months of HFD. This early onset of IR was reflected by an increase in the insulin AUC and a decrease in the glucose disappearance rate (KG), with little change in these parameters at 18 months (Table 1). It was not until 18 months that the gradual rise in glycosylated hemoglobin was significantly elevated beyond baseline values (Table 1).

**Hemodynamics, Vascular Function, and Arterial Remodeling**

Systolic and diastolic arterial blood pressure increased significantly in the first 4 months and remained elevated over the remaining study period, whereas pulse pressure did not change (Table 2). There were also mild progressive increases in brachial and carotid artery dimensions over time (Table 2). Although there was no significant change in carotid arterial strain, there was a significant increase in the elastic modulus early after HFD was initiated, indicating an increase in vascular stiffness (Figure 1A). This increase in vascular stiffness demonstrated a linear relationship with the degree of hyperglycemia as measured by the glucose AUC during IVGTT (R²=0.39, P<0.001). There was a linear trend for a slight increase in carotid IMT over the 2-year study period (Figure 1B and 1C). However, IMT was not significantly different from baseline until 2 years after HFD was started, at which time it had increased by an average of 0.05±0.06 mm.

Bioactivity of NO from endothelial shear response was tested at each study interval by FMD. There was a significant trend for a progressive decrease in FMD beginning after the 4-month time interval (Figure 1D and 1E). However, FMD was not significantly different from baseline until 18 months to 2 years, indicating that the temporal decrease was gradual. Brachial artery hemodynamic shear stress measured immediately on cuff deflation did not change significantly over time (Figure 1F), confirming that changes in shear were not responsible for changes in FMD.

**Table 1.** Body Morphometrics, Lipid Profiles, and Metabolic Changes in Response to High-Fat Diet

<table>
<thead>
<tr>
<th></th>
<th>Baseline (n=10)</th>
<th>4 mo (n=10)</th>
<th>8 mo (n=10)</th>
<th>12 mo (n=10)</th>
<th>18 mo (n=10)</th>
<th>24 mo (n=8)</th>
<th>ANOVA, Friedman, or t Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight, kg</td>
<td>11.0±1.9</td>
<td>13.2±2.6*</td>
<td>13.5±2.7*</td>
<td>13.7±2.9*</td>
<td>14.5±2.6†</td>
<td>14.1±2.3</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>Truncal fat, %</td>
<td>19.2±9.4</td>
<td>34.0±10.7*</td>
<td>34.5±10.8*</td>
<td>36.6±11.0*</td>
<td>40.4±9.8†</td>
<td>38.3±8.3</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>134±21</td>
<td>192±31*</td>
<td>195±39*</td>
<td>184±51*</td>
<td>185±46*</td>
<td>188±42</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>58±11</td>
<td>82±16†</td>
<td>87±20†</td>
<td>67±25</td>
<td>71±24</td>
<td>71±23</td>
<td>P=0.005</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>65±15</td>
<td>93±25*</td>
<td>91±31*</td>
<td>88±37*</td>
<td>91±24*</td>
<td>97±30</td>
<td>P=0.0005</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>42±22</td>
<td>70±56</td>
<td>53±41</td>
<td>121±97§</td>
<td>76±66</td>
<td>81±99</td>
<td>P=0.004</td>
</tr>
<tr>
<td>Ln insulin AUC, µU/mL×min</td>
<td>8.20±0.59</td>
<td>8.53±0.68#</td>
<td>…</td>
<td>…</td>
<td>8.67±0.67</td>
<td>…</td>
<td>P=0.04</td>
</tr>
<tr>
<td>Kg, %/min</td>
<td>3.16±1.24</td>
<td>2.40±0.81#</td>
<td>…</td>
<td>…</td>
<td>2.86±0.72</td>
<td>…</td>
<td>P=0.02</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>6.2±1.0</td>
<td>6.3±0.4</td>
<td>6.5±0.4</td>
<td>6.6±0.8</td>
<td>6.7±0.2‡</td>
<td>6.6±0.2</td>
<td>P=0.009</td>
</tr>
</tbody>
</table>

Data are mean±SD. AUC indicates area under the curve; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; Kg, glucose disappearance rate; LDL, low-density lipoprotein; and Ln, natural log.

*Repeated-measures 1-way ANOVA with Bonferroni posttest compared with baseline.
†Repeated-measures 1-way ANOVA with Bonferroni posttest compared with 4 mo.
‡Student paired t test compared with baseline.
§Repeated-measures 1-way ANOVA with Bonferroni posttest compared with 8 mo.
Table 2. Blood Pressure, Carotid Artery, and Brachial Artery Changes in Response to High-Fat Diet

<table>
<thead>
<tr>
<th></th>
<th>Baseline (n=10)</th>
<th>4 mo (n=10)</th>
<th>8 mo (n=10)</th>
<th>12 mo (n=10)</th>
<th>18 mo (n=10)</th>
<th>24 mo (n=8)</th>
<th>ANOVA or Freidman Test</th>
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</thead>
<tbody>
<tr>
<td>Systolic BP, mmHg</td>
<td>78±9</td>
<td>95±13*</td>
<td>94±13</td>
<td>90±12</td>
<td>95±15</td>
<td>90±8</td>
<td>P=0.02</td>
</tr>
<tr>
<td>Diastolic BP, mmHg</td>
<td>32±6</td>
<td>46±8*</td>
<td>42±14</td>
<td>42±9</td>
<td>45±12*</td>
<td>48±8</td>
<td>P=0.003</td>
</tr>
<tr>
<td>Pulse pressure, mmHg</td>
<td>46±8</td>
<td>49±9</td>
<td>51±8</td>
<td>48±7</td>
<td>49±6</td>
<td>42±9</td>
<td>P=0.56</td>
</tr>
<tr>
<td>Brachial artery diastolic diameter, mm</td>
<td>2.1±0.2</td>
<td>2.1±0.3</td>
<td>2.1±0.3</td>
<td>2.2±0.3</td>
<td>2.3±0.3†</td>
<td>2.3±0.2</td>
<td>P=0.01</td>
</tr>
<tr>
<td>Carotid artery diastolic diameter, mm</td>
<td>3.5±1.1</td>
<td>4.9±0.7†</td>
<td>5.5±1.1†</td>
<td>5.3±0.7†</td>
<td>5.5±0.6†</td>
<td>6.3±1.0†</td>
<td>P=0.0001</td>
</tr>
<tr>
<td>Carotid artery systolic diameter, mm</td>
<td>4.3±1.3</td>
<td>5.7±0.7†</td>
<td>6.4±1.3†</td>
<td>6.4±0.8†</td>
<td>6.5±0.7†</td>
<td>7.7±1.3</td>
<td>P=0.0001</td>
</tr>
<tr>
<td>Carotid artery strain, %</td>
<td>22±6</td>
<td>19±5</td>
<td>18±5</td>
<td>20±4</td>
<td>20±6</td>
<td>21±6</td>
<td>P=0.22</td>
</tr>
<tr>
<td>Carotid artery compliance, mm²/mmHg</td>
<td>0.10±0.28</td>
<td>0.17±0.47</td>
<td>0.29±0.85</td>
<td>0.17±0.49</td>
<td>0.20±0.58</td>
<td>0.40±1.0</td>
<td>P=0.17</td>
</tr>
</tbody>
</table>

Data are means±SD. BP indicates blood pressure.
*Freidman test with Dunn posttest compared with baseline.
†Repeated-measures 1-way ANOVA with Bonferroni posttest compared with baseline.
‡Difference between 18- and 24-mo values; P=0.03. Student paired t test.

Endothelial Activation and Serum Biomarker Inflammation

Cross-reactivity of the targeting ligands used for molecular imaging in rhesus macaques was confirmed with the use of immunohistochemistry (see figures in the online-only Data Supplement). CEU molecular imaging of the carotid arteries was well tolerated in all animals without hemodynamic change. Representative 2-dimensional and CEU-targeted images are shown in Figure 2A. At baseline, signal enhancement at the vascular wall for P-selectin– and VCAM-1–targeted microbubbles was low and similar to that for control microbubbles (Figure 2B).

Molecular imaging signal for both of these ECAMs increased progressively after HFD was initiated, whereas signal for control microbubbles did not change. A significant increase in signal was seen at 4 months for P-selectin and 8 months for VCAM-1. Molecular imaging signal for ECAMS was not elevated in control animals that were age matched to the final study period (Figure 2C). There was a modest but significant correlation between P-selectin signal enhancement and both plasma insulin (r=0.37, P<0.01) and truncal fat (r=0.39, P<0.01). There were no significant relations between ECAM expression on molecular imaging and the degree of IR indicated by fasting insulin, the IVGTT insulin AUC, or the glucose disappearance rate.

On biomarker profile, there were significant and sustained reductions in adiponectin and RANTES (regulated on activation, normal T cell expressed, and secreted) after 4 months of HFD (Table 3). There was a significant increase in monocye chemotactant protein-1 at 4 months, followed later by increases in C-reactive protein and interleukin-18. However, there was substantial variation in these markers over time. Soluble VCAM did not follow the same temporal pattern as that observed with endothelial VCAM-1 signal on molecular imaging, with levels declining over time. There were no significant associations observed between circulating plasma biomarkers and molecular imaging ECAM expression.

Discussion

Technologies for molecular imaging of inflammation in atherosclerosis are being developed to improve clinical care by early identification of high-risk patients. These same techniques are also able to provide insight into disease pathophysiology and treatment in preclinical models of disease. The basis of this study was to provide further information on the complex arrangement between IR and inflammation and to better understand the early events that may lead to atherosclerosis in an animal model designed to replicate patients who develop obesity and IR because of diet and inactivity. Our data indicate that endothelial activation, manifest by ECAM expression, (1) occurs in concert with the development of obesity and IR, (2) progresses rapidly with time, and (3) precedes changes in IMT by >1 year. The progressive pattern of endothelial activation on molecular imaging was not mirrored by the temporal changes seen in circulating markers of inflammation. Moreover, the progressive and steep increase in ECAM expression occurred without much further progression in the degree of IR.

In this study, we have defined the endothelial expression of P-selectin and VCAM-1 in a high-risk, proatherosclerotic phenotype that mimics the human conditions of obesity and IR. Both P-selectin and VCAM-1 were chosen as ECAM targets because they have been shown in some studies to be present in early atherosclerosis in animal models and humans with atherosclerotic disease.13,15–18 Although our histology results demonstrated VCAM-1 on the plaque endothelial surface, the bulk of data argues against significant macrovascular VCAM-1 expression in humans. However, our main objective was not to identify the specific ECAMs involved in atherogenesis; rather, it was to characterize the temporal relationship between diet-induced obesity and IR and inflammatory endothelial activation.

The model of diet-induced obesity in macaques fed a HFD has been shown to produce a gradual worsening in neointimal formation that would be classified as mild to moderate on histology by 14 to 28 months.19 In our experience, there is substantial intersubject variability in the metabolic response to HFD in rhesus macaques, with some animals showing relative resistance to the effects of diet and other animals developing rather early and severe IR. In the present study, we found heterogeneity in the degree of IR on IVGTT at 4 and 18 months. However, in almost all study subjects, the relative worsening of the insulin AUCs and glucose disappearance rate on IVGTT was greatest in the first 4 months and progressed more gradually after that.

Inflammation and the production of proinflammatory cytokines play a role in the development or worsening of diet-induced...
IR. For example, cytokines such as tumor necrosis factor-α, interleukin-1, and interleukin-6 have been shown to increase the activity of serine kinases or suppressor of cytokine signaling pathways, which together inactivate and degrade insulin receptor substrates, which leads to IR. On the other hand, diet-induced obesity and IR can produce vascular inflammation by many different potential pathways. Direct glucotoxicity, toxic effects of fatty acids mediated by toll-like receptor-4, and increased production of oxygen free radicals in IR are all potential mediators of the vascular changes that predispose to atherosclerosis.

Figure 1. Ultrasound-based measurements of vascular morphology and function. Data represent mean±SEM values. A, Elastic modulus. *P=0.003 for repeated-measures Friedman test with Dunn posttest baseline compared with 8 months. B, Carotid intima-media thickness (CIMT). †P=0.01 for repeated-measures test for linear trend to 24 months; ‡P=0.05 for Wilcoxon matched-pairs signed rank test baseline to 24 months. D and E, Flow-mediated vasodilation quantified as percentage and absolute change in brachial artery diameter change. †P=0.04 (%) and ‡P<0.07 (absolute) for repeated-measures test for linear trend baseline to 18 months, respectively; †P=0.09 for Wilcoxon matched-pairs signed rank test; ‡P=0.08 for paired t test. F, Brachial artery shear stress during postischemic hyperemia. C provides representative image of CIMT measurement.

Figure 2. Ultrasound-based molecular imaging. A, Example of a carotid artery by 2-dimensional B-mode ultrasound imaging (left) and color-coded contrast-enhanced molecular imaging of P-selectin (right) demonstrating P-selectin signal at the endothelial surface in a subject after 1 year of high-fat diet (HFD). B, Mean±SEM background subtracted video intensity for P-selectin–targeted (MB,) vascular cell adhesion molecule-1–targeted (MB,), and control (MB,C) microbubbles. *P<0.01 for MB, vs MB,C at 4 to 24 months; †P<0.01 for MB,V vs MB,C at 8 to 24 months. C, Mean±SEM background subtracted video intensity for MB,, MB,V, and MB,C microbubbles in primates exposed to a HFD for 24 months (average age, 12 years) compared with age-matched controls on chow diet.
Vascular tissue IR is associated with upregulation of inflammatory genes independent of IR in other tissues such as adipose and skeletal muscle.¹⁰ Not all of these processes would necessarily be reflected by standard serological markers of total body inflammation or cytokines. For this reason, we directly assessed endothelial expression of P-selectin and VCAM-1 expression, which are critical participants in the vascular inflammatory cascade that initiates atherosclerotic disease.¹³,²²–²⁴

Our results in primates suggest that circulating biomarkers of inflammation may not necessarily correlate with the degree of IR. However, it should be cautioned that animals used in this study had been activity restricted for >1 year before starting HFD, which we have previously shown leads to a substantial decrease in FMD response compared with animals with normal activity patterns.²⁷

Our study tested the temporal relation between carotid artery molecular changes, IMT on ultrasound, and endothelial inflammatory activation in a primate model. Because endothelial activation is one of the very early events that lead to neointimal proliferation, it was not surprising that ECAM expression preceded any significant increases in IMT. Interestingly, however, we found an early increase in arterial stiffness that coincided with ECAM expression. Our histological data suggest that this dysfunction may be attributable to ultrastructural changes in the vessel wall content that can occur as a result of inflammation, hyperglycemia, or abnormal neurohormonal environment, although purely functional changes cannot be excluded either. The changes observed in arterial stiffness across follow-up displayed a strong linear relationship to the degree of hyperglycemia and are consistent with observational findings of a stepwise increase in arterial stiffness in humans with glucose intolerance and non–insulin-dependent diabetes mellitus.²⁸

Several aspects of this study may affect clinical practice. We found that systemic IR and the timing and extent of ECAM expression are not necessarily reflected by often-used blood markers of systemic inflammation. C-reactive protein is the most often-used marker of vascular inflammation in atherosclerosis and has been validated in rhesus macaque cohorts as a reasonable pentraxin to follow the progression of inflammation.²⁹–³¹ Interestingly, in our cohort of primates, C-reactive protein, interleukin-18, and monocyte chemotactic protein-1 all peaked by 1 year and did not follow the same progressive pattern found for endothelial ECAM expression. Our results in primates suggest that circulating biomarkers of inflammation may not necessarily correlate with the degree of endothelial activation. It should be noted, however, that large population studies have demonstrated that elevations of C-reactive protein have been associated with an increased relative risk for developing complications of atherosclerotic disease.³² Our results also suggest that once IR develops,

### Table 3. Inflammatory and Thrombotic Biomarker Changes in Response to High-Fat Diet

<table>
<thead>
<tr>
<th></th>
<th>Baseline (n=10)</th>
<th>4 mo (n=10)</th>
<th>8 mo (n=10)</th>
<th>12 mo (n=10)</th>
<th>18 mo (n=10)</th>
<th>24 mo (n=8)</th>
<th>ANOVA or Freidman Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adiponectin, μg/mL</td>
<td>11.7±2.4</td>
<td>8.4±1.7*</td>
<td>8.4±1.9*</td>
<td>8.5±1.8*</td>
<td>6.6±1.3*</td>
<td>6.7±1.7</td>
<td>P=0.003</td>
</tr>
<tr>
<td>RANTES, ng/mL</td>
<td>10.7±4.3</td>
<td>3.1±1.3</td>
<td>2.5±0.4</td>
<td>2.3±0.6</td>
<td>2.3±0.5†</td>
<td>1.9±0.7</td>
<td>P=0.01</td>
</tr>
<tr>
<td>MCP-1, pg/mL</td>
<td>246±41</td>
<td>430±74*</td>
<td>386±67</td>
<td>476±72*</td>
<td>344±48</td>
<td>367±27</td>
<td>P=0.006</td>
</tr>
<tr>
<td>CRP, μg/mL</td>
<td>0.17±0.04</td>
<td>0.40±0.17</td>
<td>0.49±0.16</td>
<td>1.39±0.83†</td>
<td>0.10±0.03</td>
<td>0.09±0.03</td>
<td>P=0.002</td>
</tr>
<tr>
<td>Interleukin-18, pg/mL</td>
<td>116±12</td>
<td>144±17</td>
<td>303±144</td>
<td>307±110†</td>
<td>173±26</td>
<td>159±14</td>
<td>P=0.01</td>
</tr>
<tr>
<td>sVCAM, μg/mL</td>
<td>212±18</td>
<td>206±12</td>
<td>212±17</td>
<td>213±14</td>
<td>155±10*</td>
<td>167±9</td>
<td>P=0.0001</td>
</tr>
<tr>
<td>Interleukin-8, pg/mL</td>
<td>1704±509</td>
<td>1189±324</td>
<td>941±128</td>
<td>986±206</td>
<td>788±93</td>
<td>761±204‡</td>
<td>P=0.02</td>
</tr>
<tr>
<td>vWF, μg/mL</td>
<td>54±4</td>
<td>59±5</td>
<td>56±6</td>
<td>64±7</td>
<td>79±10*</td>
<td>83±9</td>
<td>P=0.0001</td>
</tr>
</tbody>
</table>

Data are mean±SE. CRP indicates C-reactive protein, MCP; monocyte chemotactic protein; RANTES, regulated on activation, normal T cell expressed, and secreted; sVCAM, soluble vascular cell adhesion molecule; and vWF, von Willebrand factor.

*Repeated-measures 1-way ANOVA with Bonferroni posttest.
†Freidman test with Dunn posttest from baseline to 18 mo. Each time point is compared with baseline.
‡ANOVA posttest for linear trend.
ECAM expression progressively increases even if the degree of IR does not rapidly progress. Studies examining the effect of dietary intervention or exercise training will be needed to determine whether ECAM expression can be reduced or reversed by eliminating the causal factors for IR. Studies have demonstrated that absolute carotid IMT, as well as the rate of carotid IMT progression over time, is associated with an increased risk of coronary revascularization, nonfatal myocardial infarction, and cardiovascular death.\textsuperscript{33,34} Identification of the endothelial molecular signal that precedes these morphometric changes has the potential to identify those at highest risk for the development of atherosclerotic disease or can be utilized in a temporal manner to evaluate therapeutic response to lifestyle, diet, and pharmacological interventions.

There are several important limitations of this study, the most notable of which is the number of primates studied across the follow-up. Our initial cohort of 10 primates was reduced to 8 animals after 18 months. The 2 animals lost to follow-up had the largest gains in both weight and truncal fat by 18 months, and each demonstrated severe IR. This loss limited the use of paired statistical testing over time and likely contributed to the decline in markers of IR and adiposity between 18 and 24 months. Although the biomarkers reported in our study have been shown to have cross-reactivity to human markers, the mean ratios of human to primate levels vary from 0.4 to 3.3, and thus the absolute values reported should not be compared directly with human values. In addition, the cross-reactivity studies were performed in Macaca fuscata\textsuperscript{r} (cynomolgus monkeys), whereas our study was performed in a cohort of rhesus macaques (Macaca mulatta). Although these are different species, they are both of the same genus and the same old-world primate lineage. It is estimated that there is no more than 1\% difference in their genetic sequence.\textsuperscript{35} Additionally, although dietary caloric intake was markedly increased after 4 months of a HFD and remained elevated and unchanged over 12 months, we do not have data on caloric intake after 1 year. Finally, we do not have confirmatory histology data in this study because these animals are part of a shared animal resource and because of an effort to avoid animal euthanasia for ethical reasons. We are, however, reassured by the fact that CEU molecular imaging signal for both P-selectin and VCAM-1 has been shown to quantify the degree of vascular inflammation in murine models of progressive atherosclerosis.\textsuperscript{13} In addition, VCAM-1 has been shown to quantify the degree of vascular inflammation in murine models of progressive atherosclerosis.\textsuperscript{13} In the initial inflammatory response in atherosclerosis: implications for early detection of disease. Arterioscler Thromb Vasc Biol. 2009;30:54–59.

In summary, endothelial inflammatory activation is an early event in the development of diet-induced obesity and IR without diabetes mellitus in primates and occurs before substantial increases in circulating inflammatory biomarkers or carotid IMT. ECAM expression progressively increases over time despite a relatively constant degree of IR. From a methodological standpoint, we have also shown that CEU molecular imaging of large-vessel endothelial phenotype in primates can provide an early indication of proatherosclerotic phenotype long before changes in morphology can be detected.

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Disclosures
None.

References


**CLINICAL PERSPECTIVE**

Because inflammation and diabetes mellitus are interwoven processes that potentiate each other, there is interest in temporally characterizing insulin resistance and the endothelial inflammatory processes that lead to accelerated atherosclerosis. To closely mimic the human condition, we studied adult nonhuman primates (rhesus macaques) at baseline and at regular intervals for 2 years after they were started on a high-fat diet. There was rapid onset of diet-induced obesity and insulin resistance on intravenous glucose tolerance testing in the first 4 months, which then progressed very gradually over the rest of the study period. Carotid artery endothelial activation assessed by contrast ultrasound molecular imaging of P-selectin and vascular cell adhesion molecule-1 was increased over baseline by 4 to 8 months and, in contrast to insulin resistance, progressively increased over the 2-year period (5- to 7-fold greater than control at 2 years). Endothelial expression of adhesion molecule-1 occurred before any detectable changes in carotid intima-media thickness and did not directly correlate with systemic markers of inflammation such as C-reactive protein. These data indicate that endothelial expression of adhesion molecules involved in atherogenesis coincides with the development of obesity and insulin resistance and progressively increases according to the time, but not necessarily the severity, of insulin resistance. From a clinical perspective, the progressive nature of the vascular inflammatory response may explain in part the increased risk for atherosclerotic disease in obese patients and patients with insulin resistance who do not necessarily progress to overt diabetes mellitus.
Proinflammatory Endothelial Activation Detected by Molecular Imaging in Obese Nonhuman Primates Coincides With Onset of Insulin Resistance and Progressively Increases With Duration of Insulin Resistance
Scott M. Chadderdon, J. Todd Belcik, Lindsay Bader, Melissa A. Kirigiti, Dawn M. Peters, Paul Kievit, Kevin L. Grove and Jonathan R. Lindner

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Supplemental Figure 1.

Supplemental Figure 1. Anti-human P-selectin Antibody Cross-Reactivity with Rhesus Macaque Platelet P-selectin.

Fluorescent microscopy of whole blood showing platelet aggregates identified by immunofluorescence with primary and FITC-labeled secondary antibody. No staining was seen with secondary antibody alone (not shown).
Supplemental Figure 2. Anti-human VCAM-1 Antibody Cross-Reactivity with Rhesus Macaque Perivascular and Endothelial VCAM-1.

Immunohistochemistry of spleen and the carotid artery with DAB staining demonstrating VCAM-1 expression detected by the primary antibody used for molecular imaging. Control experiments were performed with blocking conditions alone. Splenic VCAM-1 expression is seen in red pulp macrophages, particularly in the periarteriolar region (A). Carotid artery VCAM-1 staining was found primarily on the vascular endothelium (C).