Age-Accelerated Atherosclerosis Correlates With Failure to Upregulate Antioxidant Genes

Alan R. Collins, Christopher J. Lyon, Xuefeng Xia, Joey Z. Liu, Rajendra K. Tangirala, Fen Yin, Rima Boyadjian, Aliyfa Bikineyeva, Domenico Praticò, David G. Harrison, Willa A. Hsueh

Abstract—Excess food intake leads to obesity and diabetes, both of which are well-known independent risk factors for atherosclerosis, and both of which are growing epidemics in an aging population. We hypothesized that aging enhances the metabolic and vascular effects of high fat diet (HFD) and therefore examined the effect of age on atherosclerosis and insulin resistance in lipoprotein receptor knockout (LDLR−/−) mice. We found that 12-month-old (middle-aged) LDLR−/− mice developed substantially worse metabolic syndrome, diabetes, and atherosclerosis than 3-month-old (young) LDLR−/− mice when both were fed HFD for 3 months, despite similar elevations in total cholesterol levels. Microarray analyses were performed to analyze the mechanism responsible for the marked acceleration of atherosclerosis in middle-aged mice. Chow-fed middle-aged mice had greater aortic expression of multiple antioxidant genes than chow-fed young mice, including glutathione peroxidase-1 and -4, catalase, superoxide dismutase-2, and uncoupling protein-2. Aortic expression of these enzymes markedly increased in young mice fed HFD but decreased or only modestly increased in middle-aged mice fed HFD, despite the fact that systemic oxidative stress and vascular reactive oxygen species generation, measured by plasma F2α isoprostane concentration (systemic) and dihydroethidium conversion and p47phox expression (vascular), were greater in middle-aged mice fed HFD. Thus, the mechanism for the accelerated vascular injury in older LDLR−/− mice was likely the profound inability to mount an antioxidant response. This effect was related to a decrease in vascular expression of 2 key transcriptional pathways regulating the antioxidant response, DJ-1 and forkhead box, subgroup O family (FOXOs). Treatment of middle-aged mice fed HFD with the antioxidant apocynin attenuated atherosclerosis, whereas treatment with the insulin sensitizer rosiglitazone attenuated both metabolic syndrome and atherosclerosis. Both treatments decreased oxidative stress. A novel effect of rosiglitazone was to increase expression of Nrf2 (nuclear factor [erythroid-derived 2]-like 2), a downstream target of DJ-1 contributing to enhanced expression of vascular antioxidant enzymes. This investigation underscores the role of oxidative stress when multiple atherosclerotic risk factors, particularly aging, converge on the vessel wall and emphasizes the need to develop effective strategies to inhibit oxidative stress to protect aging vasculature. (Circ Res. 2009;104:e42-e54.)

Key Words: aging ■ atherosclerosis ■ basic research ■ mouse ■ oxidative stress

Age is a nonmodifiable risk factor for atherosclerosis. Among primates and rodents, older animals develop more extensive atherosclerosis than younger animals when both groups are fed an atherogenic diet.1,2 Age-accelerated vascular injury is commonly considered to result from increased oxidative stress, leading to inflammation and endothelial dysfunction, but no definite mechanisms have, yet, been identified.3 Tissues from aged animals demonstrate increased generation of reactive oxygen species (ROS) that lead to altered mitochondrial function, damage to vascular cells with age-associated remodeling changes, and oxidation of lipids which makes them more atherogenic.3–6 Age and other atherosclerotic risk factors appear to upregulate pathways that increase ROS production, whereas antioxidant mechanisms have been reported to be both enhanced and decreased in aging.7–10 Caloric restriction attenuates the increases in inflammation, oxidative stress, and endothelial dysfunction that accompany aging and has been shown to extend median lifespan in several animal models.11–15 Conversely, caloric excess leading to obesity worsens these factors and promotes insulin resistance, metabolic syndrome, and diabetes, all of which are frequently associated with
aging and are known contributors to accelerated atherosclerosis.\textsuperscript{16–18} We, therefore, examined the effect of age on atherosclerosis and insulin resistance in lipoprotein receptor knockout (LDLR\textsuperscript{−/−}) mice fed a high fat diet (HFD) to elucidate mechanisms by which aging promotes vascular injury. We found that aging was associated with increased expression of vascular antioxidant genes; however, in the presence of obesity, aging was associated with more severe metabolic syndrome and markedly accelerated atherosclerosis accompanied by an unexpected decrease in aortic antioxidant gene expression. This decrease resulted from loss of 2 key antioxidant pathways. Activation of the nuclear receptor peroxisome proliferator-activated receptor (PPAR)\textsubscript{γ}, attenuated both metabolic syndrome and atherosclerosis in aging mice through a novel mechanism to enhance vascular antioxidant responses. To our knowledge, this is one of the first models to link aging with markedly accelerated atherosclerosis through loss of antioxidant defense mechanisms, which may explain how multiple risk factors act synergistically to promote vascular injury.

Materials and Methods

Animal Procedures

Male LDLR\textsuperscript{−/−} mice were purchased from The Jackson Laboratory at 4 weeks of age and maintained on a standard chow diet (8604, Harlan Teklad). They were group housed in microisolator cages with a 12-hour light/12-hour dark cycle. Male mice at 3 months of age (young), 12 months of age (middle-aged), or 21 months of age (elderly) were randomly assigned to 3 months of standard chow (control) or HFD (D12079B, Research Diets) containing 41 kcal/%. Male mice at 3 months of age (elderly) were randomly assigned to 3 months of standard chow (control) or HFD (D12079B, Research Diets) containing 41 kcal/%.

Reverse Transcription and Quantitative Real-Time RT-PCR

RNA was isolated from whole aorta using an RNeasy kit (Qiagen), then reverse-transcribed into cDNA with TaqMan Reverse Transcription Reagent kit (Applied Biosystems). CD68, osteopontin (OPN), monocyte chemoattractant protein (MCP)-1, glutathione peroxidase (GPX)-1, GPX-4, catalase, superoxide dismutase (SOD)\textsubscript{2}, and uncoupling protein (UCP)\textsubscript{2} mRNA expression in the whole aorta was measured by quantitative RT-PCR of aorta cDNA using a TaqMan PCR Core Reagent Kit and specific primer/probe sets (Applied Biosystems). Samples were analyzed in triplicate and normalized to GAPDH mRNA expression.

Vascular Superoxide Generation

Briefly, vessels were removed and cut into 2-mm sections, which were stained with dihydroethidium at 37°C for 30 minutes in the dark. The segments were homogenized and the 2-hydroxyethidium determined by high-performance liquid chromatography.\textsuperscript{20}

Microarray Analyses

RNA was isolated from whole aorta with an RNeasy kit (Qiagen), reverse-transcribed with Superscript reverse transcriptase (Invitrogen), and converted to labeled cRNA with a High Yield RNA transcription labeling kit (Enzo Life Sciences). Labeled cRNA was hybridized to Amersham Codelink mouse whole genome microarrays (Amersham Biosciences), and data were analyzed using GeneSpring software (Silicon Genetics) and software developed at the BIOGEM microarray laboratory (University of California Los Angeles/University of California San Diego Diabetes Endocrinology Research Center).

Metabolic Analyses

Plasma lipids were assayed by the University of California at Los Angeles Lipid Core.\textsuperscript{21} Plasma glucose values were determined by the glucose oxidase method (Glucose Analyzer 2, Beckman). Plasma insulin, leptin, resistin, MCP-1, and total plasminogen activator inhibitor (PAI)-1 were measured with a multiplex adipokine assay (MADPK-71K, Millipore), and plasma adiponectin levels were determined by ELISA (K1002-1, B-Bridge). Plasma F2\textsubscript{α} isoprostane levels were measured using a highly specific gas chromatographic–mass spectrometric method.\textsuperscript{22}

Immunofluorescence and TUNEL Staining

Tissues for histology were fixed in 10% neutral buffered formalin, embedded in paraffin blocks, cut into 5-µm sections, and stained with hematoxylin/eosin. Slides were examined microscopically.

Experiments were carried out using appropriate positive and negative controls (including no primary and appropriate isotype antibody controls). F4/80 (eBioScience) was used as a mouse macrophage-specific rat monoclonal IgG2b. Cell apoptosis was assessed using a commercially available TUNEL kit (in Situ Cell Death Detection Kit-TMR red, Roche).

Sections were deparaffinized and pretreated with a microwave oven for antigen recovery. Sections were blocked of nonspecific binding sites with 2% normal serum from the host of the secondary antibody diluted in 2% BSA for 30 minutes. Sections were then incubated with primary antibodies to F4/80 (1:50) for overnight at 4°C. After washes in PBS, slides were then permeabilized in 0.1% Triton X-100 in PBS containing 0.1% sodium citrate for 2 minutes. Following washes in PBS, the fluorescent TUNEL mixture was added for 60 minutes at 37°C in the dark. Slides were incubated with Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes) as a second antibody after following washes. After further washes, the slides were mounted in Fluoromount-G mounting medium (SouthernBio- tech). Images were captured on a Nikon Eclipse 90i microscope using NIS-Elements software (Nikon).

Sample Collection

Blood samples were drawn from the abdominal vena cava into prechilled EDTA-coated tubes directly postmortem and centrifuged at 5000 g and 4°C for 5 minutes to isolate plasma samples that were stored at −80°C until use. Mice processed for gene expression studies were dissected for tissue collection immediately following blood draw, and their tissues were snap-frozen and stored at −80°C until analysis. Aortae were dissected in RNAlater to remove adventitial tissue before snap-freezing. Mice processed for atherosclerosis analyses were perfused for 15 minutes with 4% paraformaldehyde/7.5% sucrose in PBS, and after in situ dissection to remove adventitial tissue, aortae and fixed vessels were removed, split longitudinally, pinned to wax-filled dissection pans, and stained with Sudan IV to visualize en face atherosclerosis. Vessels images were captured using a stereomicroscope with an attached CCD camera and analyzed with ImagePro software. Atherosclerosis extent was expressed as the percentage of the aortic surface covered by Sudan IV–stained lesion. Following atherosclerosis quantification, Sudan IV–stained vessels were destained in 80% ethanol, embedded in paraffin, sectioned, and stained with Masson’s trichrome to determine lesion composition.}\textsuperscript{19}

Endothelial progenitor cells (EPCs) and monocytes were cultured to determine apoptosis and proliferation. EPCs were cultured in EGM-2 media and stimulated with PDGF-BB (10 ng/ml) for 24 hours. For proliferation, 4T1 cells were cultured in DMEM with 10% FBS and 1% Pen/Strep with and without 10 ng/ml FGF2 and 10 ng/ml PMA. For cell death, 4T1 cells were cultured in DMEM with 10% FBS and 1% Pen/Strep and treated with 10 ng/ml cisplatin for 24 hours.
Middle-aged mice demonstrate greater metabolic changes than young mice when both are fed HFD; middle-aged mice develop worse metabolic syndrome with increased fasting plasma glucose and triglycerides, as well as decreased HDL-cholesterol. Data are presented as means ± SEM. *P<0.05 vs the matching group of young LDLR⁺/⁻ mice (n=15 per group).

**Statistical Analyses**
All data are presented as means±SE. Differences between groups were analyzed using either 1-way ANOVA or t test, as indicated in the figure legends. Differences between individual means were determined using Tukey’s least-significant difference. Differences in the slopes of the line were determined using linear regression analysis. Additionally, differences in the treatment groups were determined by comparing the treatment versus HFD using Dunnett’s post hoc test.

**Results**

Middle-aged Mice Develop Worse Metabolic Syndrome in Response to HFD Than Young Mice
Middle-aged mice had higher starting body weights than young mice (33 ± 0.05), but both groups demonstrated similar food intake during the 3 months of HFD (2.8 ± 0.4 g/d, P=NS) and gained similar amounts of body weight (18 ± 0.3 versus 18 ± 0.5 g, P=NS). However, as shown in Table 1, despite similar increases in total cholesterol in response to HFD, middle-aged mice had markedly greater triglyceride and glucose increases than young mice. Moreover, high-density lipoprotein (HDL)-cholesterol decreased in middle-aged but not young mice, indicating that the middle-aged mice developed worse metabolic syndrome in response to weight gain than the young mice. Middle-aged animals also had higher fasting insulin levels than young mice before HFD (1572 ± 251 versus 665 ± 113 pg/mL, respectively, P<0.01). Both young and middle-aged mice demonstrated significant increases in fasting insulin (young: 665 ± 113 to 1199 ± 150 pg/mL, P<0.01; middle-aged: 1572 ± 251 to 2609 ± 354 pg/mL, P<0.01) and glucose (Table 1) in response to a 3-month HFD. However, fasting insulin and glucose increases were markedly greater in middle-aged versus young mice, suggesting that the middle-aged mice were substantially more insulin resistant. Furthermore, oral glucose tolerance tests performed on middle-aged mice demonstrated that the glucose tolerance of these animals progressively decreased with time on HFD (data not shown). In contrast to metabolic parameters, blood pressure was not different between young and middle-aged mice, either at baseline or after HFD (data not shown).

Plasma adipokine concentrations, except leptin, were similar in chow-fed young and middle-aged mice (Figure 1). Elevated leptin concentrations in middle-aged mice may reflect absolute weight differences between the age groups that were maintained regardless of diet. HFD increased adipokine concentrations in both young and middle-aged mice, as indicated in Table 1. Fasting leptin levels were elevated in middle-aged mice compared to young mice (young: 113 ± 5 pg/mL, middle-aged: 90 ± 5 pg/mL, P<0.05). MCP-1, Resistin, and Total PAI-1 were increased in middle-aged mice compared to young mice, as indicated in Figure 1. Further studies are needed to determine the role of these adipokines in the development of metabolic syndrome in middle-aged mice.
animals. Both age groups demonstrated similar increases in leptin, MCP-1, PAI-1, and resistin in response to HFD, consistent with the observation that both age groups had identical weight gain. Plasma adiponectin was not significantly altered in response to HFD (data not shown), in agreement with previously reported results of diet-induced obesity in C57Bl/6J mice.

Middle-Aged Mice Demonstrate Accelerated Atherosclerosis in Response to HFD
Middle-aged mice had aortic atherosclerosis before HFD (Figure 2A and 2B; 5.8±1.0% aorta surface area), whereas young mice revealed only minimal lesion coverage (0.7±0.1%). Following 3 months of HFD, atherosclerosis increased from 5.8±1.0% to 16.9±0.9% (P<0.01) of the aorta surface area in middle-aged mice and from 0.7±0.1% to 4.2±0.4% (P<0.05) in young mice (Figure 2A and 2B). Middle-aged mice also demonstrated a greater rate of monthly atherosclerosis progression in response to HFD than young mice (Figure 2C), suggesting a predisposition to new lesion development or further expansion of existing lesions. Moreover, middle-aged mice developed markedly more complex lesions, primarily consisting of proteoglycan matrix covered lesions containing necrotic lipid cores and cholesterol clefts (Figure 2D), whereas lesions in young mice primarily consisted of fatty streaks, as previously reported in this model. Complex lesions were found in the aortic root of both young and middle-aged mice after 3 months of HFD, but only middle-aged mice had complex lesions throughout the length of the aorta (data not shown).

Middle-Aged Mice Demonstrate Altered Vascular Expression of Inflammatory Genes
We examined aortic expression of OPN, MCP-1, and cytokine receptor (CCR)2, because genetic knockout of each of these genes has been shown to attenuate atherosclerosis. Aortic expression of the macrophage marker CD68 and the macrophage chemoattractant OPN (Table 2) were markedly increased in chow-fed middle-aged versus young mice, whereas MCP-1 and CCR2 were not different. HFD has been shown to increase vascular expression of inflammatory genes in LDLR−/− mice and to potentiate leukocyte vascular adherence and invasion responses to inflammatory stimuli. We found that after 3 months of HFD, young mice displayed marked increases in vascular CD68 and MCP-1 mRNA expression, but not CCR2, and a dramatic 300-fold increase in OPN expression (Table 2). Middle-aged mice, however, demonstrated no apparent increases in aortic...
expression of any of these genes, so that after 3 months of HFD, young mice expressed nearly 2-fold more CD68 and MCP-1, and nearly 7-fold more OPN and than middle-aged mice. CD4, a marker of T cells in the lesions, also increased middle-aged mice on chow and decreased in the middle-aged mice fed HFD similar to CD68, suggesting that the lesions in the middle-aged mice on HFD were necrotic with decreased numbers of viable cells (Table 2). Middle-aged mice, thus, appeared to express much lower levels of important proinflammatory and proatherosclerotic genes in their vasculature after 3 months of HFD, despite developing more atherosclerosis with more complex lesions.

In an attempt to explain this seeming discrepancy, we measured vascular inflammatory gene expression in young and middle-aged mice after 0, 2, 4, 8, and 12 weeks of HFD or continued chow diet (Figure 3). Vascular CD68 expression was almost 5-fold greater in middle-aged versus young mice at baseline. However, CD68 expression progressively and markedly increased in young but not middle-aged mice fed HFD. Moreover, after 12 weeks of HFD, CD68 expression decreased to a level not different from baseline or middle-aged mice fed chow diet. Despite their different responses to HFD, vascular CD68 expression remained significantly greater in middle-aged versus young mice at all time points except for the dramatic drop between 8 and 12 weeks of HFD in the middle-aged mice. Changes in vascular OPN expression patterns were roughly similar to those of CD68 (Figure 3B). Vascular OPN expression was almost 50-fold higher in middle-aged versus young mice at baseline, progressively increased to 10-fold by 8 weeks, and then markedly increased by 12 weeks in young but not middle-aged mice fed HFD, and OPN expression (5-fold) peaked at 8 weeks in middle-aged mice fed HFD, but at 12 weeks of HFD in middle-aged mice. OPN expression was not significantly different from baseline. Similar to CD68 and OPN, vascular MCP-1 and CCR2 expression peaked at 12 weeks of HFD in young mice but changed relatively little during from 0 to 12 weeks of HFD in middle-aged mice (Figure 3C and 3D). Vascular MCP-1 and CCR2 expression were not different in HFD-fed young and middle-aged mice except at 8 and 12 weeks of HFD, because of their respective increases in expression in the young mice at these time points. Based on these data, young and middle-aged mice appear to respond differently to HFD; young mice demonstrate progressive and marked increases in proinflammatory genes during the 12 weeks of HFD, whereas middle-aged mice generally demonstrated increases from elevated baseline expression that subside to baseline expression by 12 weeks of HFD, or do not respond to HFD. In general, vascular expression of these proinflammatory genes did not change when young and middle-aged mice were fed chow for 12 weeks, suggesting that proinflammatory changes observed during HFD were not attributable to age-related effects acting during this time period.

Table 2. Inflammatory Gene Expression in the Vessels of Young or Middle-Aged Mice Fed Chow or HFD for Three Months

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chow 3 Months</th>
<th>Chow 12 Months</th>
<th>HFD 3 Months</th>
<th>HFD 12 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD68</td>
<td>3.8±0.4</td>
<td>14.9±2.6</td>
<td>17.4±3.8</td>
<td>911.8±193.6</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>3.0±0.5</td>
<td>97.5±18.0</td>
<td>140.8±57.9</td>
<td>0.5±4.0</td>
</tr>
<tr>
<td>MCP-1</td>
<td>1.6±0.1</td>
<td>1.9±0.5</td>
<td>3.8±0.5†</td>
<td>1.9±0.1</td>
</tr>
<tr>
<td>CCR2</td>
<td>4.0±0.6</td>
<td>5.5±1.3</td>
<td>7.8±2.4†</td>
<td>5.0±0.3</td>
</tr>
<tr>
<td>CD4</td>
<td>0.14±0.04</td>
<td>0.25±0.05</td>
<td>0.44±0.05†</td>
<td>0.16±0.06</td>
</tr>
</tbody>
</table>

Vascular expression of inflammatory genes substantially increases in young mice fed HFD but remains unchanged in middle-aged mice. Data are presented as ratios of gene expression vs GAPDH and expressed as means±SEM. *P<0.05 vs age-matched chow-fed mice; †P<0.05 vs matching diet by 1-way ANOVA with Tukey’s post test (n=4–5 per group).

Figure 3. Time course of aortic gene expression data after beginning HFD (n=4 to 8 per group). A, CD68 expression progressively increases in young mice, whereas the middle-aged mice have increased basal CD68 expression that markedly decreases by 12 weeks of HFD, possibly because of macrophage necrosis within advanced atherosclerotic lesions. B, OPN expression in young middle-aged mice markedly increases at 3 months of HFD, whereas middle-aged mice exhibit higher basal levels that peak at 8 weeks and markedly decline by 12 weeks of HFD. Both MCP-1 (C) and CCR2 (D) expression increase with HFD in young mice, but not middle-aged mice. Expression of both genes is higher in middle-aged vs young mice at baseline but does not markedly change with HFD. Data for young and middle-aged mice are represented by black and gray bars, respectively, and are presented as means±SEM. *P<0.05 vs 12 week HFD; †P<0.05 vs 8 week HFD; ‡P<0.05 vs matching group of young mice by 1-way ANOVA (n=4 to 6 per group).
Histological analyses were performed to determine whether there was increased macrophage apoptosis after 3 months of HFD possibly accounting for decreased CD68 expression and release of free cholesterol. Macrophage apoptosis was uncommon in the rare fibrofatty lesion formed in the aorta of a young mouse on HFD (Figure 4, top images) but was present in a typical plaque from the aorta of a middle-aged mouse on HFD (Figure 4, bottom images).

HFD Inhibits the Expression of Antioxidant Pathways in the Vessels of Middle-Aged Mice

Vascular RNA was isolated from young and middle-aged mice fed chow or HFD for 3 months and analyzed using microarrays to identify gene changes that might explain the greater extent and complexity of atherosclerosis and decreased vascular proinflammatory gene expression in middle-aged versus young mice. Multiple genes involved in free radical detoxification were differentially expressed in young versus middle-aged mice, including GPX-1 and -4, SOD2, catalase, isocitrate dehydrogenase, and UCP2 (Table 3). Vascular antioxidant gene expression was increased in middle-aged versus young mice on chow diet. However, following 3 months of HFD, antioxidant gene expression increased in aortae of young mice but markedly decreased in the aortae of middle-aged mice. Plasma F2 isoprostane levels, measured as a surrogate marker of systemic oxidative stress, were 2-fold greater in middle-aged versus young mice on chow diet. HFD increased systemic oxidative stress in both young and middle-aged mice, but middle-aged mice demonstrated more oxidative stress than young mice when these mice were fed either chow or HFD (Figure 5A).

To determine differences in vascular superoxide production in middle-aged versus young mice, we quantified superoxide-selective oxidation of dihydroethidium to oxyethidium in mouse aortae using high-performance liquid chromatography, as previously described.20 Chow-fed young and middle-aged mice revealed similar vascular superoxide production, which increased in both groups in response to HFD (Figure 5B). Similarly, expression of p47phox, a key subunit of nicotinamide adenine dinucleotide phosphate oxide (NADPH oxidase) was also increased in response to the HFD. This increase could relate to changes in either vascular and/or phagocyte NADPH oxidase, but either source represents an overall increase in vascular oxidative stress. Levels
of vascular superoxide and p47phox mRNA were highest in the middle-aged mice on HFD. In general, increased reactive oxygen species production within a tissue should increase expression of antioxidant enzymes. However, in middle-aged mice, vascular expression of antioxidant enzymes were decreased at the same time as vascular superoxide production was increased, indicating that middle-aged mice were unable to mount a normal vascular and/or phagocyte response to oxidative stress.

The global loss of antioxidant enzyme gene expression prompted us to investigate upstream regulators of the antioxidant response. DJ-1 regulates nuclear transport of Nrf2, which enhances transcription of antioxidant enzymes. The FOXO family of transcription factors also regulate the expression of antioxidant enzyme genes but are decreased in hyperinsulinemia. Vascular DJ-1 expression was decreased with both HFD and age (Figure 6A). Two way ANOVA demonstrated that age and diet have separate effects on DJ-1 which do not interact. There were no differences in Nrf2 expression, but NADPH dehydrogenase quinone 1 (NQO1), an antioxidant enzyme highly regulated by Nrf2, decreased in middle-aged mice on HFD compared to middle-aged mice on chow (Figure 6B and 6C). FOXO1 and -4 expression were lowest in vessels of middle-aged mice on HFD with a trend for lower levels of FOXO3a (Figure 6D through 6F). Decreased activity of these 2 important antioxidant pathways likely contributes, at least in part, to the loss of the ability to mount an antioxidant response in middle-aged mice on HFD.

**Rosiglitazone and Apocynin Attenuate Atherosclerosis Progression in Middle-Aged Mice**

To determine the relative contributions of oxidative stress and insulin resistance on atherosclerosis progression, middle-aged LDLR/−/− mice were fed HFD for 3 months with or without supplemental treatment with the insulin-sensitizer rosiglitazone or the antioxidant apocynin. After 3 months of HFD, rosiglitazone-treated mice demonstrated atherosclerosis levels similar to those found in mice fed chow diet for 3 months (Figure 7A and 7B). Apocynin treatment also substantially decreased atherosclerosis, although to a lesser degree than rosiglitazone. However, although both treatments reduced the extent of atherosclerosis lesion coverage, complex lesions were still observed after either intervention (data not shown). Rosiglitazone treatment partially corrected the metabolic syndrome in these mice, decreasing fasting plasma triglycerides, glucose, and insulin (Table 4) without altering body...
weight. Rosiglitazone also decreased plasma leptin and resistin by 70% and 65%, respectively, but did not alter plasma MCP-1 or PAI-1 (data not shown).

Confirming the data from array analyses, vascular GPX-1, GPX-4, and SOD2 mRNA expression decreased with HFD in middle-aged mice, whereas catalase and UCP2 expression showed a trend toward decrease. Rosiglitazone added to HFD increased or revealed a trend toward increasing expression of all of these enzymes (Figure 7C through 7G). Apocynin treatment did not alter body weight, fasting plasma triglycerides, glucose, or insulin (Table 4) and did not change plasma adipokine levels versus HFD controls. Apocynin also did not increase the low levels of GPX-1, GPX-4, catalase, or SOD2 in the aortae of mice fed HFD but tended to increase UCP2 (Figure 7C through 7G). Both rosiglitazone and apocynin decreased urinary F2α isoprostane levels, although apocynin was more effective than rosiglitazone (Figure 7H). There was a modest, nonsignificant decrease in p47phox.

Table 4. Plasma Metabolic Markers of Middle-Aged Male LDLR<sup>−/−</sup> Mice Fed Chow or HFD With or Without Rosiglitazone or Apocynin for Three Months

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cholesterol</th>
<th>Triglycerides</th>
<th>HDL</th>
<th>Glucose</th>
<th>Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow</td>
<td>309±20</td>
<td>112±9</td>
<td>93±5</td>
<td>177±16</td>
<td>822±247</td>
</tr>
<tr>
<td>HFD</td>
<td>1781±111*</td>
<td>388±47*</td>
<td>85±5</td>
<td>358±20*</td>
<td>2583±352*</td>
</tr>
<tr>
<td>HFD+RSG</td>
<td>951±128†</td>
<td>102±21†</td>
<td>74±4†</td>
<td>210±26†</td>
<td>855±134†</td>
</tr>
<tr>
<td>HFD+ACN</td>
<td>1530±160*</td>
<td>301±56</td>
<td>97±4</td>
<td>286±15*</td>
<td>2351±495</td>
</tr>
</tbody>
</table>

Unlike humans, rosiglitazone (RSG) decreases HDL levels in mice. Data are presented as means±SEM. *P<0.05 vs chow; †P<0.05 vs HFD; ††P<0.05 vs HFD+apocynin (ACN) by 1-way ANOVA (n=10–14 per group).
expression with both rosiglitazone and apocynin (data not shown). In addition, neither rosiglitazone nor apocynin changed vascular expression of CD68, OPN, MCP-1, or CCR2 (data not shown), which were only modestly different in middle-aged mice on chow versus HFD (Table 2).

Neither rosiglitazone nor apocynin altered vascular DJ-1 expression. However, rosiglitazone, but not apocynin treatment, increased Nrf2 expression, as well as that of NQO1 (Figure 8A through 8C). In addition, rosiglitazone administration tended to increase expression of FOXOs (Figure 8D through 8F).

**Metabolic Syndrome and Atherosclerosis in Elderly Mice**

We also examined the effect of advanced age on atherosclerosis administering HFD for 3 months to 21-month-old (elderly) LDLR/−/− mice. Elderly mice developed aortic atherosclerosis similar to middle-aged mice in lesion coverage and complexity (Figure 9) and demonstrated a metabolic phenotype consistent with a model of the metabolic syndrome (Table 5). After 3 months of HFD, vascular expression of proinflammatory genes was similar in elderly and middle-aged mice, but vascular expression of antioxidant genes tended to be lower in elderly versus middle-aged mice (data not shown).

**Discussion**

In the present investigation, HFD clearly induced a worse metabolic phenotype and substantial acceleration of atherosclerosis extent and complexity in middle-aged and elderly versus young LDLR/−/− mice, despite similar food intake, weight gain, and total serum cholesterol levels. The middle-aged and elderly mice developed 4 of 5 human metabolic syndrome criteria, which progressively worsened during the 3 months of HFD: hyperglycemia, hypertriglyceridemia, low HDL-cholesterol, and visceral adiposity. Vascular expression of antioxidant enzymes important for conferring protection against oxidative stress, such as GPX-1 and -4, catalase, SOD2, UCP2, and isocitrate dehydrogenase, were increased in chow-fed middle-aged versus young LDLR/−/− mice. However, vascular expression of these antioxidant enzymes increased only in young HFD- versus chow-fed LDLR/−/− mice and typically decreased in HFD- versus chow-fed middle-aged LDLR/−/− mice, despite substantial increases in systemic oxidative stress (F2 isoprostane) and more modest increases in vascular ROS production (dihydroethidium oxidation) and a 2.5-fold increase in vasculature p47phox expression. Thus, middle-aged mice, unlike young mice, were unable to mount a response to increased vascular ROS production and oxidative stress. This precipitous decline in vascular antioxidant gene expression in the face of aging, obesity, and hypercholesterolemia likely contributed to the marked acceleration of atherosclerosis associated with these risk factors. It is possible that the accelerated atherosclerosis may represent repeated cycles of inflammation and lipid accumulation potentially inherent in the LDLR/−/− mice. Although the middle-aged mice had 5% of their aortic surface covered by lesions at the start of HFD, after 3 months of continued chow, the lesions only progressed to 7%, compared to the 20% on HFD; the lesions were complex on HFD- but
not on chow-fed mice. Thus, the interaction of age, HFD and metabolic syndrome likely contributed to accelerated lesion extent and complexity.

The mechanism behind the global drop in antioxidant gene expression in this model appears to be related to the decline in DJ-1 expression and FOXOs, both important regulators of pathways mediating the antioxidant response. Treatment of HFD-fed middle-aged LDLR⁻/⁻ mice with the antioxidant apocynin reduced plasma F₂α isoprostane levels, decreased aortic atherosclerosis lesion area by 50%, and modestly increased vascular anti-oxidant enzyme gene expression when rosiglitazone was administered with HFD. These novel observations demonstrate age-dependent metabolic and vascular responses to HFD and underscore a role for oxidative stress and its regulation in both of these processes. Maintaining vascular antioxidant enzyme expression may, thus, be an essential mechanism for attenuating the increased age-associated risk for development of type 2 diabetes and cardiovascular disease.

Antioxidant enzymes are crucial for tissues to detoxify free radical species and protect organisms against oxidative stress. SOD2 converts oxygen free radicals to hydrogen peroxide, which is then neutralized by GPXs and catalase; UCP2 deficiency enhances ROS generation; and isocitrate dehydrogenase generates α-ketoglutarate, a powerful antioxidant. The activity of these well-coordinated systems normally maintains redox balance, because redox imbalance can damage multiple organs and tissues. Several studies have reported that older animals, particularly those approaching senescence, have reduced antioxidant enzyme gene expression and activity compared to their younger counterparts.

These changes are likely environment-, species-, and tissue-dependent. In rabbit cornea: antioxidant enzyme activity peaked in the young adult animals; SOD fell in middle-aged animals; and GPX, catalase, and SOD were dramatically reduced in elderly animals. We also found decreased liver antioxidant gene expression in middle-aged versus young mice, similar to what we found in the aortae (A.R. Collins, et al, unpublished observations, 2008). In vasculature, aging is associated with loss of nitric oxide bioavailability, because of increased superoxide production primarily from increased NAD(P)H oxidase and other prooxidant systems involving lipoxygenase, xanthine oxidase, cyclooxygenase, etc. The imbalance between prooxidant and antioxidant activity leads to endothelial dysfunction and vascular damage.

Hypercholesterolemia, hyperglycemia, and excess adiposity from HFD all likely contribute to oxidative stress in our model, similar to humans with obesity and multiple cardiovascular disease risk factors, which can synergistically increase myocardial ischemia. HFD was associated with increased plasma levels of adipokines such as leptin and resistin, which are predominantly produced by adipose, and MCP-1 and PAI-1, which are secreted by adipose and other tissues. Both resistin and leptin were somewhat increased in the plasma of middle-aged versus young mice following HFD; resistin has been implicated in insulin resistance and may have contributed to a worse metabolic syndrome in middle-aged mice. ROS production is reportedly increased in the adipose tissue of obese versus lean mice. Measurement of ROS production by white adipose tissue (WAT) in the obese KKAY mice and their C57BL/6 controls revealed that a ~12-g body weight difference resulted in a 2-fold increase in ROS production, which was associated with decreased expression of SOD, GPX, and catalase in WAT but not liver.

Table 5. Plasma Levels of Lipids, Glucose, and Insulin in Fasting Elderly (Twenty-Four-Month) Mice

<table>
<thead>
<tr>
<th>Diet</th>
<th>Cholesterol (mg/dL)</th>
<th>Triglycerides (mg/dL)</th>
<th>HDL (mg/dL)</th>
<th>Glucose (mg/dL)</th>
<th>Insulin (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow</td>
<td>267±6</td>
<td>63±3</td>
<td>94±2</td>
<td>287±26</td>
<td>700±126</td>
</tr>
<tr>
<td>HFD</td>
<td>1143±155†</td>
<td>164±40†</td>
<td>88±6</td>
<td>396±32*</td>
<td>1297±94</td>
</tr>
</tbody>
</table>

Data is presented as means±SEM. *P<0.05, †P<0.001 vs old chow fed mice by t test (n=15 per group).
or skeletal muscle. Apocynin treatment decreased WAT superoxide production, as well as plasma glucose, insulin, triglycerides, and tumor necrosis factor α, leading Furukawa et al to conclude that oxidative stress generated in adipose tissue contributed to metabolic syndrome. However, the authors did not describe the mechanism responsible for decreased antioxidant gene expression in WAT.

Both metabolic and age-related factors can regulate antioxidant gene expression. For example, activation of tyrosine kinase activity by insulin and insulin-like growth factor-1 decreases FOXO forkhead transcription factor activity, which leads to decreased expression of SOD2, catalase and other antioxidant enzymes. Conversely, KLOTHO, an important antiaging hormone, increases FOXO-mediated transcription of SOD2, which is implicated as an important KLOTHO-regulated antiaging mechanism. Circulating KLOTHO decreases with age (we found decreased renal expression of KLOTHO in middle-aged versus young mice; A.R. Collins, et al, unpublished observations, 2008), and this decrease, coupled with the marked increase in plasma insulin in our HFD-fed middle-aged mice, could potentially explain their decrease in antioxidant enzyme gene expression. Rosiglitazone markedly suppressed insulin levels, tended to increase vascular FOXOs, and increased vascular expression of GPX-4, catalase, and SOD2 in these mice.

The increase in the Nrf2 antioxidant pathway could also explain the action of rosiglitazone: DJ-1 stabilizes Nrf2 protein by impairing its proteasomal degradation. DJ-1 protein is modified with age in fruit flies, mice, and humans and by H2O2. DJ-1 message levels have not previously been reported to decrease with aging as seen in our model. The decrease in NQO1 in the vasculature of middle-aged mice on HFD suggests altered Nrf2 nuclear translocation, even though message levels of Nrf2 did not change. Nrf2 when translocated to the nucleus, binds to the antioxidant response element to promote transcription of catalase, GPX, and other antioxidant enzymes. Genetic ablation of Nrf2 enhances mortality to septic shock, as well as cigarette smoke–induced emphysema in mice. We found decreased vascular expression of DJ-1 in the middle-aged mice that was related to both age and HFD but no changes in Nrf2 message levels compared to young mice on chow. Neither rosiglitazone nor apocynin altered DJ-1 levels, but rosiglitazone enhanced expression of Nrf2, as well as NQO1 and other enzymes regulated by Nrf2. Previous studies suggested that PPARγ activation in cells could increase catalase and SOD2 expression, possibly through a putative PPRE. Our study suggests a novel upstream antioxidant effect of PPARγ activation through increasing Nrf2 expression. We found similar changes in the Nrf2 pathway in the livers of these mice (A.R. Collins, et al, unpublished observations, 2008). Further investigation is necessary to determine whether these observations result from a direct or indirect effect of rosiglitazone.

Surprisingly, despite the enhanced oxidative stress and accelerated atherosclerosis, monocyte accumulation and vascular inflammation were not increased in middle-aged versus young LDLR−/− mice at 3 months of HFD. Young LDLR−/− mice fed HFD for 3 months demonstrated a 5-fold increase in vascular expression of CD68, a marker of macrophage accumulation, consistent with 2- to 5-fold enhanced mRNA levels of MCP-1, CCR2, and OPN, all of which promote monocyte tissue entry. Knockout of any one of these chemotactants rescues young LDLR−/− or ApoE−/− mice from atherosclerosis, providing evidence for a critical role of these inflammatory proteins in the atherosclerotic process. Whereas vessels from middle-aged animals on chow diet had increased expression of these genes compared to vasculature of young animals, when challenged with HFD, middle-aged animals had an earlier increase in expression of CD68 and OPN than young mice but no increase in MCP-1 or CCR2. CD4 expression, a marker of T-cell accumulation, paralleled that of CD68. Although we saw only a 46% increase in vascular ROS generation in vessels of middle-aged mice on HFD versus chow, substantial oxidative stress, including both extent and length of time of exposure, likely contributed to necrosis of foam cells, as suggested by the extensive necrotic lipid cores and cholesterol clefts seen in lesions of middle-aged animals and to the marked drop in CD68 and OPN expression seen in these animals between 8 and 12 weeks of HFD associated with apoptosis of macrophages demonstrated by tunnel staining of the lesions. Although treatment with rosiglitazone and apocynin attenuated atherosclerosis, there was little impact on expression of CD68 or other markers of vascular inflammation, but both decreased oxidative stress, which could decrease phagocytic cell death to allow cholesterol transport out of vessels. Thus, our data suggest oxidative stress, rather than inflammation, is a primary driver of the accelerated vascular damage in middle-aged and elderly LDLR−/− mice. These observations have implications regarding the potential effect of an antiinflammatory-based strategy for atherosclerosis in young animals versus an antioxidant approach in older models.

The primary mechanism through which apocynin reduced atherosclerosis appeared to be through its antioxidant activity, likely through actions at the vessel wall. Unlike its effect in KKAy mice, we found that apocynin did not decrease fasting glucose, insulin, or triglycerides (Table 4) or hepatic steatosis (data not shown) in LDLR−/− mice. Apocynin inhibits NADPH oxidase to reduce ROS production and has other antioxidant properties. Other approaches to decrease oxidative stress have also improved endothelial function, decreased blood pressure, and attenuated atherosclerosis in mouse models of vascular injury. Although the vascular protective effects of decreasing oxidative stress in mouse models appear consistent, approaches in human investigations have been less successful. It is possible that more powerful, more specific antioxidant strategies may be necessary in humans. Importantly, our data demonstrate that multiple stresses to the vessel wall, such as obesity and hypercholesterolemia, impair antioxidant defenses even in middle-aged animals and even in the face of an apparently decreased inflammatory response. These results emphasize the need for early, aggressive prevention and treatment of cardiovascular risk factors, all of which increase oxidative stress, and for the development of approaches that enhance production or activity of antioxidant enzymes, whose expression appears to be impaired in aging vasculature exposed to...
metabolic abnormalities. Targeting of FOXOs and Nrf2 may be important strategies.

Sources of Funding
This work was supported in part by NIH grants U01 HL70526 ("Novel Models of Cardiovascular Complications of Diabetes") (to W.A.H.) and R01 HL75171-2 (to W.A.H.).

Disclosures
W.A.H. is a scientific advisor to GlaxoSmithKline, Merck, Novartis, and Daiichi Sankyo.

References


Age-Accelerated Atherosclerosis Correlates With Failure to Upregulate Antioxidant Genes
Alan R. Collins, Christopher J. Lyon, Xuefeng Xia, Joey Z. Liu, Rajendra K. Tangirala, Fen Yin, Rima Boyadjian, Alfiya Bikineyeva, Domenico Praticò, David G. Harrison and Willa A. Hsueh

Circ Res. 2009;104:e42-e54; originally published online March 5, 2009; doi: 10.1161/CIRCRESAHA.108.188771

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/104/6/e42

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/