Molecular Cardiology

c-Jun N-Terminal Kinase 2 Deficiency Protects Against Hypercholesterolemia-Induced Endothelial Dysfunction and Oxidative Stress

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Background—Hypercholesterolemia-induced endothelial dysfunction due to excessive production of reactive oxygen species is a major trigger of atherogenesis. The c-Jun-N-terminal kinases (JNKs) are activated by oxidative stress and play a key role in atherogenesis and inflammation. We investigated whether JNK2 deletion protects from hypercholesterolemia-induced endothelial dysfunction and oxidative stress.

Methods and Results—Male JNK2 knockout (JNK2−/−) and wild-type (WT) mice (8 weeks old) were fed either a high-cholesterol diet (HCD; 1.25% total cholesterol) or a normal diet for 14 weeks. Aortic lysates of WT mice fed a HCD showed an increase in JNK phosphorylation compared with WT mice fed a normal diet (P<0.05). Endothelium-dependent relaxations to acetylcholine were impaired in WT HCD mice (P<0.05 versus WT normal diet). In contrast, JNK2−/− HCD mice did not exhibit endothelial dysfunction (96±5% maximal relaxation in response to acetylcholine; P<0.05 versus WT HCD). Endothelium-independent relaxations were identical in all groups. A hypercholesterolemia-induced decrease in nitric oxide (NO) release of endothelial cells was found in WT but not in JNK2−/− mice. In parallel, endothelial NO synthase expression was upregulated only in WT HCD mice compared with WT normal diet (P<0.05 versus WT normal diet). JNK2−/− HCD animals, whereas expression of antioxidant defense systems such as extracellular superoxide dismutase and manganese superoxide dismutase was decreased in WT but not in JNK2−/− HCD mice. In contrast to JNK2−/− mice, WT HCD displayed an increase in O2− and ONOO− concentrations as well as nitrotyrosine staining and peroxidation.

Conclusions—JNK2 plays a critical role as a mediator of hypercholesterolemia-induced endothelial dysfunction and oxidative stress. Thus, JNK2 may provide a novel target for prevention of vascular disease and atherosclerosis. (Circulation. 2008;118:2073-2080.)

Key Words: atherosclerosis ■ endothelium ■ nitric oxide ■ JNK kinase ■ reactive oxygen species

Atherosclerosis is a systemic immunoinflammatory disease that develops in response to endothelial injury. Indeed, the endothelium is a key determinant of vascular integrity. Hypercholesterolemia, a well-known risk factor for cardiovascular disease, leads to accumulation and oxidation of low-density lipoprotein cholesterol within the intima of the vessel wall, triggering endothelial dysfunction and proinflammatory milieu as crucial steps in the early phase of the atherosclerotic process. Oxidative stress, resulting from an imbalance between reactive oxygen species (ROS) and the antioxidant defense system, is a crucial mediator of hypercholesterolemia-induced endothelial dysfunction. Indeed, ROS interact and inactivate nitric oxide (NO) and lead to protein nitration and lipid peroxidation.

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The c-Jun N-terminal kinases (JNKs), also known as stress-activated protein kinases, are serine/threonine protein kinases belonging to the mitogen-activated protein kinase superfamily. JNKs play a fundamental role in stress responses, cell survival, and apoptosis. The JNK pathway is activated by stress factors such as ultraviolet radiation, reperfusion injury, ceramides, and inflammatory cytokines. The dimerization of JNK leads to activation of other kinases,
their nuclear translocation, and subsequent modulation of the activity of different transcription factors such as c-Jun, ATF-2, Elk-1, p-53, and c-myc.7

Three distinct JNK genes have been described, JNK1, JNK2, and JNK3, encoding for different isoforms. JNK3 expression is restricted to brain, heart, and testis, whereas JNK1 and JNK2 proteins are ubiquitously expressed.8 Both JNK1- and JNK2-deficient mice are viable, indicating that neither JNK1 nor JNK2 plays an essential role in development and normal cellular functions; however, genetic disruption of both JNK1 and JNK2 is lethal.9 Studies using gene targeting as well as JNK inhibitors demonstrated the involvement of JNK1 and JNK2 genes in several pathological conditions including cancer, immune diseases, and neurological diseases as well as metabolic disorders and inflammatory conditions such as arthritis and atherosclerosis.10

JNKs are expressed in vascular smooth muscle cells and endothelial cells and activated by a wide range of stimuli such as oxidative stress, mechanical stretch, hypertension,11–13 hyperglycemia, apoptosis,14 and inflammation.10 A recent study suggested a role of JNK in endothelial dysfunction: short-term exposure of coronary arterioles to tumor necrosis factor-α–induced endothelial dysfunction through activation of JNK signal transduction pathway and generation of superoxide anion.15 We recently reported a critical role of JNK2 in atherogenesis showing that JNK2 is required for foam cell formation within the atherosclerotic plaque by activating the scavenger receptor A.16

The role of JNK2 in the early stage of atherosclerosis related to endothelial dysfunction as it occurs under hypercholesterolemic conditions remains unknown. Thus, we compared JNK2-deficient with wild-type (WT) mice exposed to a high-cholesterol diet (HCD) or a normal diet (ND). We found that endogenous JNK2 is critically involved in hypercholesterolemia-induced endothelial dysfunction and oxidative stress.

Methods

Animals and Diets

JNK2−/− and WT mice (both in a C57BL/6J background) were obtained from Jackson Laboratory (Bar Harbor, Maine) and kept on a regular diet. Mice were housed in temperature-controlled cages (20°C to 22°C), fed ad libitum, and maintained on a 12/12-hour light/dark cycle. At the age of 8 weeks, mice were fed a ND or a HCD (D12108 containing 1.25% cholesterol; Research Diets, New Brunswick, NJ) for 14 weeks, respectively. All animal experiments were approved by the local institutional animal care committee.

Plasma Lipid Measurements

At the time of tissue harvesting, 0.5 to 1 mL of blood was drawn from the right ventricle with heparinized syringes and immediately centrifuged at 4°C, and the plasma was stored at −80°C. Total cholesterol, triglycerides, and free fatty acids were analyzed with the reagents TR13421, TR22421 (both Thermoelectron Clinical Chemistry and Automation Systems, Thermo Fisher Scientific, Waltham, Mass), and 994-75409 (Wako Chemicals GmbH, Neu, Germany), as recommended by the manufacturer. The lipid distribution in plasma lipoprotein fractions was assessed by fast-performance liquid chromatography gel filtration with a Superose 6 HR 10/30 column (Pharmacia, Basking Ridge, NJ).

Tissue Harvesting

Mice were euthanized by intraperitoneal administration of 50 mg/kg sodium pentobarbital. The entire aorta from the heart to the iliac bifurcation was excised and placed immediately in cold modified Krebs-Ringer bicarbonate solution (pH 7.4, 37°C, 95% O2, 5% CO2) of the following composition (mM/L): NaCl (118.6), KCl (4.7), CaCl2 (2.5), KH2PO4 (1.2), MgSO4 (1.2), NaHCO3 (25.1), glucose (11.1), and calcium EDTA (0.026). The aorta was cleaned from adhering connective tissue under a dissection microscope and then snap-frozen in liquid nitrogen and stored at −80°C or used immediately for organ chamber experiments.

Organ Chamber Experiments

For endothelial function experiments, aortas were cut into rings (2 to 3 mm long). Each ring was connected to an isometric force transducer (Multi-Myograph 610M, Danish Myo Technology A/S, Aarhus, Denmark), suspended in an organ chamber filled with 5 mL Krebs-Ringer bicarbonate solution (37°C, pH 7.4), and bubbled with 95% O2, 5% CO2. Isometric tension was recorded continuously. After a 30-minute equilibration period, rings were gradually stretched to the optimal point of their length-tension curve as determined by the contraction induced by potassium chloride (100 mM). Concentration-response curves were obtained in a cumulative fashion. Several rings cut from the same artery were studied in parallel. Responses to acetylcholine (10−9 to 10−6 mol/L; Sigma-Aldrich, St Louis, Mo) in the presence or absence of polyethylene glycol–superoxide dismutase (PEG-SOD, 150 U/mL, Sigma-Aldrich) were recorded during submaximal contraction to norepinephrine (10−8 mol/L). The NO donor sodium nitroprusside (10−10 to 10−3 mol/L; Sigma-Aldrich) was added to test endothelium-independent relaxation. Relaxations were expressed as a percentage of the precontracted tension.

Measurements of NO, O2•−, and ONOO−

Concurrent measurements of NO, O2•−, and ONOO− were performed with 3 electrochemical nanosensors combined into 1 working unit with a total diameter of 2.0 to 2.5 μm. Their design was based on previously developed and well-characterized chemically modified carbon-fiber technology.4,17,18 Amperometry was performed with a computer-based Gamry VFP600 multichannel potentiostat. A current at the peak potential characteristic for NO (0.65 V) oxidation and ONOO− (−0.40 V) or O2•− (−0.23 V) reduction was directly proportional to the local concentrations of these compounds in the immediate vicinity of the sensor. Linear calibration curves (current versus concentration) were constructed for each sensor from 10 mMol/L to 2 μMol/L before and after measurements with aliquots of NO, O2•−, and ONOO− standard solutions, respectively. At a constant distance of the sensors from the surface of the endothelial cell, the reproducibility of measurements is high (5% to 12%). The consumption of redox species by nanosensors depends on the area of the electrode (<0.12 μm2) and the duration time of electrolysis (≈5 to 10 seconds). For the amperometric measurements used, it varied between 0.04% and 0.1% of the NO, O2•−, and ONOO− peak concentration. This value is negligible compared with the experimental error. The position of nanosensors (x, y coordinates) versus the endothelial cell was established in two ways: (1) with the help of a computer-controlled micromanipulator. To establish a constant distance from cells, the module of sensors was lowered until it reached the surface of the cell membrane. After that, the sensors were slowly raised 4 to 1 μm (z coordinates) from the surface of cells. The sensors were then moved horizontally (x, y coordinates) and positioned above a surface of randomly chosen single endothelial cells in an aortic ring. Acetylcholine was then injected with a nanoinjector that was also positioned by a computer-controlled micromanipulator.

Western Blotting

Frozen samples of aortas were pulverized and dissolved in lysis buffer (120 mMol/L sodium chloride, 50 mMol/L Tris, 20 mMol/L sodium fluoride, 1 mMol/L benzamidine, 1 mMol/L dithiothreitol, 1 mMol/L EDTA, 6 mMol/L EGTA, 15 mMol/L sodium pyrophos-
Chemiluminescence kit (Amersham Biosciences). Anti-
immunohistochemistry and Superoxide Detection
phate, 0.8 μg/mL leupeptin, 30 mmol/L p-nitrophenyl phosphate, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 1% NP-40) for
Immunoblotting. Cell debris was removed by centrifugation (12000 rpm) for 10 minutes at 4°C. The samples (20 μg) were treated with
5× Laemmli’s SDS-PAGE sample buffer (0.35 mol/L Tris-Cl, pH 6.8, 15% SDS, 56.5% glycerol, 0.0075% bromophenol blue), followed by heating at 99°C for 5 minutes and then subjected to
10% SDS-PAGE gel for electrophoresis. The proteins were then
transferred onto Immobilon-P filter papers (Millipore AG, Bedford, Mass) with a semidry transfer unit (Hoelter Scientific, San Francisco, Calif). The membranes were then blocked by use of 5% skim milk in
TBS-Tween buffer (0.1% Tween 20; pH 7.5) for 1 hour at room
temperature and incubated with anti-total JNK and p-JNK; (1:1000 dilution; Cell Signaling, Beverly, Mass) and anti-
Mn-SOD, sections were fixed in 4% PBS-buffered formalin for 5
minutes, blocked by heating at 95°C for 30 minutes with 1 μmol/L dithiothreitol for
superoxide detection. To stain for protein-bound nitrotyrosine or
free fatty acids was observed between the groups
Immunohistochemistry and Superoxide Detection
Freshly isolated aortic segments were immediately embedded in OCT medium and snap-frozen in pentane/liquid nitrogen. Cryosections of 6-μm thickness were mounted on SuperFrost glass slides and incubated at 37°C for 30 minutes with 1 μmol/L dithiothreitol for
superoxide detection. To stain for protein-bound nitrotyrosine or
Mn-SOD, sections were fixed in 4% PBS-buffered formalin for 5
minutes, blocked with 10% BSA in PBS, and incubated with polyclonal
anti-nitrotyrosine antibody (1:50; Upstate) and anti-Mn-SOD antibody (1:250; StressGen, Victoria, Canada) at 4°C overnight, respectively. For visualization, the secondary antibody (Alexa568 anti-rabbit IgG; 1:300; Molecular Probes, Carlsbad, Calif) was incubated for 1 hour at room temperature. Slides were then rinsed, embedded in glycerin-
PBS, and examined under a fluorescent microscope (DM-IRB; Leica, Heerbrugg, Switzerland) connected to a digital imaging
system (Spot RT; Diagnostic Instruments/Visitron Systems, Puch-
heim, Germany). Pictures were obtained with identical camera and
microscope settings. Dihydroethidium-stained specimens were back-
ground-corrected for autofluorescence of elastic fibers and the basal
lamina with the use of ImageJ/ National Institutes of Health
(rsb.info.nih.gov/ij/).

Table. Plasma Lipids From JNK2−/− and WT Littermates After
14 Weeks of ND or HCD

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<tr>
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<th>ND</th>
<th>HCD</th>
<th>JNK2−/−</th>
<th>ND</th>
<th>HCD</th>
<th>JNK2−/−</th>
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<tr>
<td>Total cholesterol,</td>
<td>3.3±0.2</td>
<td>7.1±2.4*</td>
<td>3.9±0.3†</td>
<td>9.6±1.2†</td>
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<tr>
<td>mmol/L</td>
<td></td>
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<tr>
<td>Triglycerides,</td>
<td>1.34±0.45</td>
<td>0.79±0.18</td>
<td>1.40±0.6</td>
<td>1.35±0.27</td>
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<td>mmol/L</td>
<td></td>
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<tr>
<td>Free fatty acid,</td>
<td>0.54±0.34</td>
<td>0.33±0.07</td>
<td>0.35±0.05</td>
<td>0.34±0.09</td>
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<tr>
<td>mmol/L</td>
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Values are mean±SEM. n=4 to 6 in each group. *P<0.05 vs ND WT and JNK2−/− mice, respectively; †P<0.05 vs diet-matched WT mice; ‡P<0.05 vs HCD WT mice.

Figure 1. Representative Western blots show phosphorylated JNK (p-JNK) protein expression in aortic lysates from WT mice after 14 weeks of ND or HCD. Expression of total JNK was used as a loading control.

Thiobarbituric Acid Reactive Substances Assay
In vitro assessment of aortic levels of lipid peroxidation was performed with the use of the thiobarbituric acid reactive substances (TBARS) assay kit (OxTert, ZeptoMetrix Corp, Buffalo, NY), according to the manufacturer’s instructions. Briefly, snap-frozen tissue was crushed in a prechilled mortar and pestle and resuspended at a concentration of 50 mg/mL in PBS. Then 100 μL of homogenate was added to SDS solution and mixed thoroughly. After TBA/buffer reagent addition, samples were incubated at 95°C for 60 minutes and centrifuged at 3000 rpm at 15 minutes. Absorbance was read at 532 nm.

Statistical Analysis
Results are expressed as mean±SEM, and n indicates number of experiments. Statistical analysis was performed with Student t test for simple comparisons between 2 values. For multiple comparisons, results were analyzed by ANOVA followed by Bonferroni post hoc correction. A value of P<0.05 was considered statistically significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results
Lipid Profiles
We determined plasma cholesterol, triglycerides, and free fatty acids in JNK2−/− and WT mice fed either a HCD or a ND (Table). Both JNK2−/− and WT mice developed significant hypercholesterolemia after 14 weeks of HCD. Interestingly, JNK2−/− mice had slight but significantly increased levels of total plasma cholesterol compared with WT mice on either ND or HCD (Table). No difference in plasma triglycerides or free fatty acids was observed between the groups (Table).

Hypercholesterolemia Activates Aortic JNK
To determine the effect of hypercholesterolemia on JNK activation, we compared Western blot analyses of aortic lysates from normocholesterolemic and hypercholesterolemic WT mice using a phosphospecific JNK antibody. Aortas from WT mice on HCD showed increased JNK phosphorylation compared with vessels from WT on ND (Figure 1).
JNK2 Deletion Protects From Hypercholesterolemia-Induced Endothelial Dysfunction

Isometric tension studies demonstrated no difference in vascular contractions to norepinephrine between aortas obtained from WT or JNK2−/− mice fed either a HCD or ND (data not shown). Endothelium-dependent relaxations to acetylcholine were impaired in WT mice on HCD compared with WT mice on ND. Interestingly, endothelium-dependent relaxations remained normal in JNK2−/− mice, suggesting a preserved NO bioavailability favored by the lack of JNK2 (Figure 2A). Concurrent with this notion, addition of the free radical scavenger PEG-SOD (150 U/mL) significantly improved endothelium-dependent relaxations in HCD WT mice (Figure 2B). Endothelium-independent relaxations to sodium nitroprusside were similar in all groups (data not shown).

Preserved Endothelial NO Release in Hypercholesterolemic JNK2−/− Mice

For assessing NO bioavailability at the level of endothelial cells, we determined NO in single endothelial cells using nanosensors. After stimulation with acetylcholine (10−7 mol/L), maximal NO levels were 242±10 nmol/L in WT mice on ND and decreased ≈50% in WT mice on HCD (Figure 3A). In contrast, similar levels of NO release were found in JNK2−/− mice on ND or HCD (Figure 3A).

Decreased Oxidative Stress in Hypercholesterolemic JNK2−/− Mice

To determine the effect of hypercholesterolemia on oxidative stress in endothelial cells, we measured superoxide anion (O2−) and peroxynitrite (ONOO−) production in single aortic endothelial cells. A significant increase in O2− concentration was observed in WT mice exposed to HCD compared with WT mice on ND, whereas no significant hypercholesterolemia-induced changes were observed in JNK2−/− mice (Figure 3B and 3C). In agreement with preserved NO bioavailability and O2− findings, ONOO− concentrations were increased only in WT but not in JNK2−/− mice fed a HCD (Figure 4A). Because ONOO− leads to increased 3-nitrotyrosine–containing proteins, we performed in situ immunohistochemistry with a polyclonal antibody against 3-nitrotyrosine in aortic cross sections. JNK2−/− HCD mice showed a markedly reduced immunoreactivity both in the endothelium and in the media compared with diet-matched WT mice.
(Figure 4B). This favorable redox profile was confirmed by measuring aortic TBARS levels. After HCD exposure, JNK2−/− mice did not exhibit an increase in lipid peroxidation compared with WT mice (Figure 4C).

**Free Radical Scavenger Expression and Activity**

Protein expression of 3 pivotal free radical scavengers was assessed to determine whether an upregulation of antioxidant defense mechanisms might explain the preserved NO bioavailability in hypercholesterolemic JNK2−/− mice. Cu/Zn-SOD was similar in all experimental groups (data not shown), whereas aortic expression of Mn-SOD and EC-SOD was significantly decreased in WT mice fed a HCD compared with WT mice on a ND (Figure 5A and 5B). By contrast, levels of Mn-SOD and EC-SOD in JNK2−/− HCD were similar to WT ND mice (Figure 5A and 5B). Unexpectedly, JNK2−/− ND mice exhibited a reduced expression of both SOD isoforms compared with diet-matched WT mice (Figure 5A and 5B). Accordingly, immunofluorescent stainings for Mn-SOD showed similar results in aortic cross sections (Figure 5C).

**Increased eNOS Expression in JNK2−/− Mice**

To obtain more insight into the mechanisms of preserved NO bioavailability in JNK2−/− mice with hypercholesterolemia, we quantified eNOS expression in aortic lysates. eNOS expression was not affected by HCD in WT mice, whereas JNK2−/− HCD mice showed a significantly increased expression of eNOS (Figure 6A). Furthermore, to determine whether NO production was also regulated by eNOS activity, we performed additional blotting of phosphorylated Ser1177-eNOS in pooled samples. pS1177-eNOS protein levels were
similar in aortic lysates from JNK2−/− on either diet and WT mice on ND. In contrast, we found reduced eNOS phosphorylation in WT mice on HCD (Figure 6B).

Discussion

The present study demonstrates for the first time that genetic deletion of JNK2 protects against hypercholesterolemia-induced and ROS-mediated endothelial dysfunction. The following findings support our conclusion: (1) Long-term exposure of WT mice to a HCD induces aortic JNK phosphorylation. (2) In contrast to WT mice, long-term exposure of JNK2−/− to HCD did not impair endothelium-dependent relaxation to acetylcholine. (3) Lower ONOO− levels in hypercholesterolemic mice were associated with decreased protein nitration and lipid peroxidation. Accordingly, (4) expression of the antioxidant enzymes Mn-SOD and EC-SOD was increased in JNK2−/− mice. In contrast, we observed a downregulation of these enzymes after 14 weeks of HCD in WT mice.

Genetic deletion of JNK2 did not affect severity of hypercholesterolemia, although JNK2−/− mice had slightly but significantly increased levels of total cholesterol compared with WT mice fed with either ND or HCD. Thus, we could rule out that differences observed among the 2 groups were caused by different experimental conditions. In contrast to our findings, a recent study showed similar total plasma cholesterol levels in JNK2−/− and WT controls.19

Preserved bioavailability of NO is a key marker of vascular integrity. In vivo, activity of the l-arginine/NO pathway is determined by a balance between synthesis and breakdown of NO for its reaction with O2•−. This balance is impaired in hypercholesterolemia and atherosclerosis.20,21 Endothelial dysfunction, reflected by impaired endothelium-dependent relaxation, occurs in experimental models of hypercholesterolemia, as was confirmed in WT mice of this study.22,23 Similarly, many clinical studies reported abnormal endothelium-dependent vasodilation in hypercholesterolemic patients.24 Hypercholesterolemia induces a series of molecular events that increase the production of ROS and inactivate NO to form ONOO−.25 In this study, acetylcholine-induced relaxation did not differ between JNK2−/− and WT mice in control conditions of normocholesterolemia following ND. However, on chronic hypercholesterolemia induced by 14 weeks of HCD, WT mice but not JNK2−/− mice developed endothelial dysfunction.

The finding that addition of the free radical scavenger PEG-SOD restored endothelium-dependent relaxation in WT mice on HCD suggests an important role of ROS in this context. Hypercholesterolemia-induced oxidative stress has been attributed to activation of oxidases in the vasculature and in infiltrating leukocytes.26 Moreover, hypercholesterolemia has been shown to impair antioxidant defense mechanisms against ONOO− formation.25

To investigate whether preserved endothelial function in hypercholesterolemic JNK2−/− mice was associated with increased bioavailability of NO, we assessed NO release from single endothelial cells after stimulation with acetylcholine. In hypercholesterolemic control mice, NO levels decreased by ~50%, whereas they remained unchanged in JNK2−/− mice. Because O2•− is the main inactivator of NO, we tested whether decreased endothelial production of O2•− contributes to increased NO bioavailability in JNK2−/− mice. We found enhanced O2•− production in hypercholesterolemic WT mice compared with mice on ND, whereas no significant diet-induced changes were detected in JNK2−/− mice. Dihydroethidium stainings of aortic segments confirmed these findings.

In aortas exposed to chronic hypercholesterolemia, the reaction of NO and O2•− leads to enhanced ONOO− formation and, in turn, increased nitrotyrosine residues, which are typical end products of the reaction of ONOO− with biological compounds.26 Tyrosine nitration is responsible for inactivation of several enzymes.27 Our group has shown that nitration of Mn-SOD and prostacyclin synthase occurs in aged and diabetic mice, respectively.28,29 In agreement with the notion that JNK2 deficiency induces preserved NO bioavailability but reduced O2•− production, we found that ONOO− concentrations were increased in WT but not in JNK2−/− mice fed a HCD. In parallel, nitrotyrosine immunoreactivity was detected in both endothelium and smooth muscle cells of hypercholesterolemic mice, as shown previously by our group.30 However, aortas from hypercholesterolemic WT mice exhibited enhanced immunostaining compared with diet-matched JNK2−/− mice. ONOO− contributes to atherogenesis by promoting lipid peroxidation.31 In contrast to hypercholesterolemic WT mice, JNK2−/− mice were protected against lipid peroxidation, as determined by TBARS in aortic lysates.

To investigate whether antioxidant defense mechanisms contribute to the preserved endothelial function in hypercholesterolemic JNK2−/− mice, we assessed protein expression of 3 pivotal O2•− scavengers. Aortic expression of Mn-SOD and EC-SOD was decreased in hypercholesterolemic WT mice
compared with normocholesterolemic controls. By contrast, both Mn-SOD and EC-SOD were induced after 14 weeks of hypercholesterolemia in JNK2+/− mice. Furthermore, in situ immunohistochemistry showed that changes in Mn-SOD expression occur throughout the aortic vascular wall. These findings suggest that the ability of the SOD scavenging system to respond to oxidative stress remains intact in JNK2−/− mice. Unexpectedly, normocholesterolemic JNK2−/− mice exhibited a reduced expression of Mn-SOD and EC-SOD compared with diet-matched WT mice. However, these changes did not translate into differences in endothelium-dependent, NO-mediated responses, as shown by organ chamber experiments and in situ measurements of NO release. At low concentrations, O$_2^-$ diffusion is slow, and O$_2^-$ is scavenged by highly diffusible NO. Therefore, at low O$_2^-$ in JNK2−/− mice on ND, SOD may be less competitive for O$_2^-$ than NO. This process may change under high O$_2^-$ and low NO levels, as found in the context of hypercholesterolemia, in which the role of SOD becomes more substantial. Accordingly, the reduced expression of Mn-SOD and EC-SOD in JNK2−/− mice on ND did not translate into changes of NO, O$_2^-$, and ONOO$^-$ production. To obtain more insight into the mechanisms of preserved NO bioavailability in hypercholesterolemic JNK2−/− mice, we assessed eNOS expression in aortic lysates. Western blot analysis revealed higher eNOS expression in hypercholesterolemic JNK2−/− compared with WT mice. Conflicting data have been reported related to the regulation of eNOS during hypercholesterolemia. Evidence exists of reduced transcription and enhanced breakdown of eNOS transcripts on increasing concentrations of oxidized low-density lipoprotein. Long-term stimulation with oxidized low-density lipoprotein may also lead to a decrease in the amount of NOS protein through induction of cytokines. Experimental atherosclerosis is associated with an increased eNOS expression and NO production, whereas decreased eNOS expression and NO release are found in advanced human atherosclerosis. To determine whether NO production was also regulated by eNOS activity, we determined eNOS phosphorylation. Interestingly, the observed upregulation of eNOS protein in JNK2−/− on HCD did not translate into increased eNOS phosphorylation, justifying unchanged NO concentrations and endothelium-dependent relaxations in JNK2−/− mice. On the other hand, decreased eNOS phosphorylation in WT HCD mice matched reduced NO levels found in this group.

Because deletion of JNK2 in hypercholesterolemic mice was associated with upregulation of SOD, it is likely that this antioxidant defense system contributes to protect against hypercholesterolemia-mediated oxidative stress in JNK2−/− mice. Thus, our findings suggest that JNK2 is involved in the pathways regulating vascular endothelial ROS production and antioxidant defense systems under hypercholesterolemic conditions. Our results are in accordance with previous studies that associate JNK activation with increased levels of oxidative stress and ROS-mediated cell death. In particular, JNK is known to play a major role in cardiovascular disease and is activated on mechanical stress, hypertension, and ischemia/reperfusion. JNK has also been reported to be activated in advanced atherosclerotic plaques in rabbits as well as in humans and in disease progression of abdominal aortic aneurysm in mice and humans. Along this line, JNK2 is necessary for scavenger receptor A− or CD36-mediated foam cell formation in atherogenesis. JNK2−/− mice have also been demonstrated to be involved in insulin resistance and type I diabetes mellitus. Pharmacological JNK inhibition is a promising strategy given its beneficial effects in mouse models of atherogenesis, abdominal atherosclerosis, and cerebral ischemia. JNK inhibition may even be more rewarding considering its critical role in obesity and insulin resistance. Thus, JNK inhibition could represent a therapeutically attractive target to prevent progression of atherosclerosis and metabolic disease. Our findings suggest that JNK may also be a promising target for preventing atherosclerosis at its early stage of endothelial dysfunction.

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Disclosures

None.

References


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**CLINICAL PERSPECTIVE**

Atherosclerosis is a systemic immunoinflammatory disease that develops in response to endothelial injury. Hypercholesterolemia, a crucial risk factor for cardiovascular disease, leads to accumulation and oxidation of low-density lipoprotein cholesterol within the intima of the arterial wall. Thereby, hypercholesterolemia triggers endothelial dysfunction and creates a proinflammatory milieu, both critical initial steps of atherogenesis. The c-Jun N-terminal kinases (JNKs) play a fundamental role in inflammation, stress responses, cell survival, and apoptosis. Three distinct JNK genes have been described, JNK1, JNK2, and JNK3, encoding for different isoforms. We recently reported that genetic JNK2 deletion decreases progression of atherosclerosis by inhibiting foam cell formation. However, the role of JNK2 in early atherogenesis related to hypercholesterolemia-induced endothelial dysfunction remains unknown. In the present study, we compared JNK2-deficient mice with wild-type mice exposed long term to a high-cholesterol or a normal diet. Our results show that genetic JNK2 deletion inhibits hypercholesterolemia-induced and oxidative stress–mediated endothelial dysfunction, suggesting a critical role of endogenous JNK2 in this context. Thus, the concept of JNK inhibition with proven protective effects in atherogenesis, abdominal aeurysm formation, myocardial infarction, and cerebral ischemia may be extended to endothelial dysfunction as the initial step of vascular disease. Given the additional beneficial effects of pharmacological JNK inhibition in mouse models of obesity and diabetes, JNK blockade may represent an attractive therapeutic target for both cardiovascular and metabolic disease.
c-Jun N-Terminal Kinase 2 Deficiency Protects Against Hypercholesterolemia-Induced Endothelial Dysfunction and Oxidative Stress

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