Oxidative Stress in Human Abdominal Aortic Aneurysms
A Potential Mediator of Aneurysmal Remodeling

Francis J. Miller, Jr, William J. Sharp, Xiang Fang, Larry W. Oberley, Terry D. Oberley, Neal L. Weintraub

Abstract—Abdominal aortic aneurysm (AAA) is an inflammatory disorder characterized by localized connective tissue degradation and smooth muscle cell (SMC) apoptosis, leading to aortic dilatation and rupture. Reactive oxygen species are abundantly produced during inflammatory processes and can stimulate connective tissue–degrading proteases and apoptosis of SMCs. We hypothesized that reactive oxygen species are locally increased in AAA and lead to enhanced oxidative stress. In aortas from patients undergoing surgical repair, superoxide levels (measured by lucigenin-enhanced chemiluminescence) were 2.5-fold higher in the AAA segments compared with the adjacent nonaneurysmal aortic (NA) segments (6638±2164 versus 2675±1027 relative light units for 5 minutes per millimeter squared, respectively; n=7). Formation of thiobarbituric acid–reactive substances and conjugated dienes, 2 indices of lipid peroxidation, were increased 3-fold in AAA compared with NA segments. Immunostaining for nitrotyrosine was significantly greater in AAA tissue. Dihydroethidium staining indicated that increased superoxide in AAA segments was localized to infiltrating inflammatory cells and to SMCs. Expression of the NADPH oxidase subunits p47phox and p22phox and NAD(P)H oxidase activity were increased in AAA segments compared with NA segments. Thus, oxidative stress is markedly increased in AAA, in part through the activation of NAD(P)H oxidase, and may contribute to the disease pathogenesis. (Arterioscler Thromb Vasc Biol. 2002;22:560-565.)

Key Words: aneurysm • aortas • free radicals • antioxidants • NADPH oxidase

A bdominal aortic aneurysms (AAAs) occur in ≈3% of humans aged >65 years and are characterized by localized structural deterioration of the aortic wall, leading to progressive aortic dilation.1 The morbidity and mortality associated with AAAs are considerable. Surprisingly, however, little is known about the mechanisms responsible for aneurysmal formation and progression.

Infiltration of inflammatory leukocytes is a prominent pathological feature of AAA.2–4 The inflammatory response is believed to play a key role in the degeneration of the elastic media, which, in turn, promotes aneurysmal remodeling of the aortic wall.4 Degeneration of the elastic media has been attributed to proteolytic degradation of structural proteins, such as elastin, consequent to the activation of matrix metalloproteinases (MMPs) and other proteases.5–7 Recently, apoptosis of smooth muscle cells (SMCs) has also been detected in AAAs and may contribute to the disease process.8–10 SMCs are the most abundant cellular component of the arterial media and promote structural integrity by synthesizing matrix proteins, such as elastin. The emerging concept is that the increased destruction and decreased production of matrix proteins, which are due to the activation of proteases and apoptosis of SMCs, respectively, act in concert to promote the aneurysmal remodeling process.

One potential mechanism by which inflammatory responses could promote aneurysm formation is through enhanced production of reactive oxygen species (ROS), such as superoxide (O2·−) and hydrogen peroxide (H2O2). These toxic substances are produced by inflammatory leukocytes and contribute to the tissue destruction observed in a variety of immunologic and infectious disorders. In addition, O2·− is produced by the 3 major types of cells resident in the aortic wall: SMCs, fibroblasts, and endothelial cells. Importantly, recent studies indicate that ROS activate MMPs and induce apoptosis of vascular SMCs.11,12

In the present study, we measured levels of O2·− and lipid peroxidation products in segments of AAA and in adjacent nonaneurysmal aortic (NA) tissue removed from patients undergoing elective AAA repair. We examined the cellular production of O2·− in situ and the expression and activity of NAD(P)H oxidase. Our results indicate that levels of ROS are increased locally in AAAs, partially because of NAD(P)H oxidase activity, and lead to marked increases in oxidative stress.

Methods

Segments of infrarenal AAA and adjacent NA tissues were obtained from 12 patients undergoing elective aneurysm repair at the University of Iowa Hospitals and Clinics and at the Department of Veterans Affairs Medical Center in Iowa City, Iowa. The patients ranged in age from 60 to 85 years (mean, 71±12 years). All patients were men, and none had a history of smoking or diabetes. The patients underwent resection of an AAA with endovascular graft placement. Control segments of infrarenal NA aorta were obtained from patients undergoing elective abdominal aortic aneurysm (AAA) repair. The patients ranged in age from 65 to 85 years (mean, 74±9 years). None of the patients had a history of smoking or diabetes.

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age from 60 to 90 years, and all but 3 were male. Removal of these tissues was accomplished as part of the normal operative procedure, in which AAA and adjacent NA segments were trimmed in preparation for graft anastomosis. Care was taken to ensure that none of the tissues was subjected to surgical manipulation or cross-clamping. Aortic tissues were obtained from 7 additional patients without AAA (aged 16 to 70 years). These included organ donors (n=4), individuals with aortic dissection (n=2), and individuals with Marfan’s syndrome (n=1). Immediately after procurement, segments were placed in 0.9% saline (4°C) and transported to the laboratory. The protocol was approved by the Human Subjects Office at the University of Iowa.

**Histological Analyses**

Segments were snap-frozen in Tissue-Tek OCT mounting media for histological analysis. Cryosections (8 μm thick) were stained with hematoxylin and eosin or Verhoeff–van Gieson stain.

Immunohistochemical staining for nitrotyrosine was performed by using Vectastain ABC-AP kit (Vector Laboratories). Anti-nitrotyrosine (Upstate Biotechnology Inc) was used at a dilution of 1:100. After they were washed, the slides were incubated with biotinylated anti-mouse antibody and then washed and incubated with Vectastain ABC-AP reagent. Visualization was accomplished after treating the slides with alkaline phosphatase substrate solution and counterstaining with nuclear fast red.

The presence of macrophages was assessed by immunohistochemistry with the mouse monoclonal anti-human macrophage antibody NCL-MACRO (1:80 dilution). Immunostaining for the NADPH oxidase subunits p47phox (1:20, Santa Cruz Biochemicals) and 22phox (1:100, Santa Cruz Biochemicals) and 22phox (1:100, monoclonal antibody 44.1, obtained by permission from Dr A.J. Jesaitis, Department of Microbiology, Montana State University, Bozeman) was also performed.

**Assessment of O$_2^-$ Levels**

Aortic segments were prepared for in situ imaging of O$_2^-$ generation with the fluorescent dye dihydroethidium (DHE, Molecular Probes, Inc), as previously described. Unfixed frozen aortic segments were cut into 30-μm-thick sections and placed on a glass slide. DHE (2 μmol/L) was topically applied, and slides were incubated for 30 minutes in a humidified chamber at 37°C. Images were obtained with a Bio-Rad laser scanning confocal microscope. AAA and NA sections were processed and imaged in parallel (excitation at 488 nm) at identical laser settings.

Superoxide levels were also measured by lucigenin-enhanced chemiluminescence as described previously. Aortic segments were placed in PBS and lucigenin (5 μmol/L), and after 2 minutes of dark adaptation, relative light units (RLU) emitted were measured for 5 minutes in an FB12 luminometer (Zylux Corp). To assess NAD(P)H oxidase activity, NADH (0.1 mmol/L) or NADPH (0.1 mmol/L) was added to the PBS-lucigenin containing the vessel segment, and O$_2^-$ levels were measured. Some segments were preincubated with polyethylene-glycolated superoxide dismutase (SOD, 250 U/mL) or the flavin inhibitor diphenylene iodonium (DPI, 0.1 mmol/L). Surface areas were measured for each segment to allow normalization for tissue size.

**Determination of Lipid Peroxidation**

Segments of tissues were washed with cold PBS to remove red blood cells and homogenates (10% [wt/vol] in PBS) prepared by using a Tempset homogenizer (Vertis). Lipid peroxidation products in homogenates were determined by measuring thiobarbituric acid–reactive substances (TBARS) with the use of a fluorometric assay. A separate test for the formation of conjugated dienes was also performed by using a spectrophotometric assay (234 nm). Concentration of conjugated dienes was calculated by using the molar extinction coefficient of 28 500 and is expressed as nanomoles per gram of tissue. Values of TBARS are expressed as nanomolar equivalents of malondialdehyde per gram of tissue.

**Statistical Analyses**

All data are expressed as mean±SEM. Differences between mean values were analyzed by Student t tests or by one-way ANOVA combined with the Student-Newman-Keuls multiple comparison test. Values of P≤0.05 were considered to be statistically significant.

**Results**

Atherosclerotic changes (ie, neointimal proliferation and foam cell formation) were observed in AAA and adjacent NA segments, but extensive medial degeneration was observed predominately in the aneurysmal tissues. In addition, localized inflammatory cell infiltrates were frequently observed in the AAA tissues.

To examine the distribution and cellular sources of O$_2^-$, AAs and adjacent NAs were incubated with the oxidative fluorescent dye DHE. There were diffuse increases in O$_2^-$ levels, as measured by DHE fluorescence, throughout the wall of AAA segments, particularly within the medial layer (Figure 1, n=7). In addition, AAA tissues frequently contained regions of inflammatory cell infiltration associated with increased levels of O$_2^-$ (Figure 2). Immunohistochemis-

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*Figure 1.* Superoxide levels are increased in AAA. Segments of aorta procured from the same patient undergoing elective AAA repair were sectioned, incubated with the oxidative fluorescent dye DHE, and examined by confocal microscopy. Cellular O$_2^-$ levels are increased in AAA tissues (right) compared with adjacent NA tissue (left). Ad indicates adventitia; M, media; and IEM, internal elastic membrane. Relative differences in fluorescence between AAA and NA segments are representative of studies performed in 7 patients.

*Figure 2.* Association of O$_2^-$ with inflammatory infiltrate and disruption of medial SMCs and elastin in AAA. Adjacent sections of AAA were examined for histology (hematoxylin and eosin staining, left), for O$_2^-$ (DHE staining, bottom insert), or for elastin (Verhoeff–van Gieson stain, right). A higher magnification of the inflammatory infiltrate is also shown (top insert). Although increases in O$_2^-$ were observed throughout the AAA tissues, focal inflammatory infiltrates were associated with localized increases in O$_2^-$, disruption of medial SMC organization, and loss of elastic fibers. The arrow denotes the inflammatory infiltrate. M indicates medial layer; V, vasa vasorum within the adventitial layer. Images are representative of those obtained from 4 patients.
from patients without AAA. Superoxide levels were >10-
fold higher in AAA segments compared with NA segments
(n=6, Figure 3A).

To determine whether the observed levels of $O_2^-$ were
sufficient to induce oxidative stress in aneurysmal tissues,
segments of AAA and NA tissues were assayed for the
formation of TBARS. Compared with levels in NA tissues,
levels of TBARS in AAA tissues were increased $\approx$3-fold
(Figure 3B). The formation of conjugated dienes, another
marker of lipid peroxidation, was similarly increased nearly
3-fold in AAA compared with NA tissues (Figure 3B). In
addition, we performed immunostaining with an antibody
against nitrotyrosine, which is a marker for amino acid
oxidation induced by several oxidant species, including
peroxynitrite, the highly reactive product of $O_2^-$ and NO.
Nitrotyrosine immunostaining was markedly enhanced in
AAA compared with NA segments (Figure 4). Collectively,
these results suggest that tissue damage due to oxidative
stress is enhanced in AAA compared with NA tissues.

A plasma membrane–associated NAP(P)H oxidase has
been proposed as a major source of $O_2^-$ in vascular cells.15–18
An NAD(P)H oxidase inhibitor, DPI (0.1 mmol/L), reduced
$O_2^-$ levels in AAA tissue by $\approx$60%, as measured by
lucigenin-enhanced chemiluminescence ($2103\pm895$ RLU • 5
min$^{-1}$ • mm$^{-2}$, n=4). In addition, generation of $O_2^-$ in
response to NADH (0.1 mmol/L) or to NADPH (0.1 mmol/L)
was 3-fold greater in AAA segments compared with NA
segments (n=5, Figure 5A). Furthermore, the increases in
lucigenin-enhanced chemiluminescence in AAA in response
to NADH and NADPH were attenuated by treatment with
DPI. Next, we examined protein expression of p47phox and
p22 phox , 2 subunits of the phagocyte and vascular NAD(P)H
oxidase.19,20 Immunostaining demonstrated markedly in-
creased expression of p47phox and p22phox throughout the AAA
segments (Figure 5B). These findings suggest that NAD(P)H
oxidase activity was increased in AAA and contributed to the
increased production of $O_2^-$.

Increased expression of NAD(P)H oxidase and increased
$O_2^-$ levels were not limited to regions of inflammatory cell
infiltration but were present diffusely throughout the medial
layer (Figure 6, and online Figure).

**Discussion**

The major finding of the present study is that $O_2^-$ levels are
increased in AAA, thereby leading to a generalized increase
in oxidative stress within the vessel wall. The increased
oxidative stress could, in turn, promote remodeling of the
extracellular matrix and apoptosis of SMCs. These findings
may have important implications regarding the mechanisms of aneurysmal formation and progression.

Numerous studies published over the past decade support the view that inflammation plays a key role in the pathogenesis of AAA.2–7 On the basis of previous reports, inflammatory responses may promote aneurysmal formation through several potential mechanisms. First, infiltrating leukocytes are major sources of MMPs and serine proteases, such as tissue plasminogen activator. These proteases degrade structural proteins, such as elastin, collagen, and laminin, thereby weakening the aortic wall.5,7,21 Inhibitors of MMPs prevent aneurysmal degeneration and rupture.22 Second, infiltrating immune cells can induce the death of vascular SMCs in AAAs through induction of the Fas and perforin pathways9 and through cyclooxygenase-2.10,23,24 Induction of cyclooxygenase-2 leads to augmented production of prostaglandin E2, which, in turn, reduces SMC proliferation and viability through direct25 and cytokine-mediated10 mechanisms. Enhanced destruction of structural proteins and a reduced synthetic capacity resulting from SMC apoptosis likely act together to progressively weaken the vessel wall, resulting in dilatation. Our findings indicate that $O_2^-$ is locally increased in AAA segments, associated with inflammatory infiltrates and disruption of SMCs and elastin fibers, suggesting that ROS could contribute to these processes.

The possibility that levels of ROS might be locally elevated in AAA and contribute to the pathogenesis of the disease was suggested from several observations. First, infiltrating inflammatory cells produce ROS and generate proatherogenic factors, which augment the production of ROS by SMCs.26,27 Second, ROS have been reported to activate MMPs11 and to inhibit plasminogen activator inhibitor-1, which, in turn, could promote extracellular matrix degradation.26 Moreover, ROS induce the apoptosis of vascular SMCs,12,28 which is a recently described feature of human AAA.8–10 In fact, generation of ROS is required for the induction of apoptosis by diverse proinflammatory factors, including cytokines and p53.28,29 Third, human AAAs have reduced levels of ascorbic acid and antioxidant enzyme activities.30 Furthermore, plasma levels of vitamin E were found to be markedly reduced in patients with AAA but not in patients with coronary artery disease in the absence of AAA.31 These intriguing observations are consistent with our data, which demonstrate that the magnitude of oxidative stress is considerably greater in AAA compared with atherosclerotic, but nonaneurysmal, aorta.

Although our studies focused on $O_2^-$, it is likely that elevated levels of $H_2O_2$, as well as secondary radical species such as hydroxyl radicals, are also present in AAA. Furthermore, expression of the inducible form of NO synthase by macrophages and cytokine-activated SMCs in AAA could generate large quantities of NO, which might also contribute to tissue damage. NO synthase is also a potential source of $O_2^-$.$^3$ Enhanced nitrotyrosine immunostaining in AAA segments suggests that reactive nitrogen species are locally increased in AAA. Therefore, the milieu of AAA would appear to be optimal for the formation and propagation of injurious oxidant species. However, it must be emphasized that the present study was performed on AAA at an advanced
stage of dilation. Whether oxidative stress is also increased in smaller subclinical aneurysms is unknown.

The cellular sources of $O_2^-$ in AAA appear to be resident vascular cells and infiltrating inflammatory cells. An NAD(P)H oxidase functions in SMCs and phagocytes to generate $O_2^-$. That SMCs are contributing to increased $O_2^-$ levels in AAA is based on 3 observations. First, $O_2^-$ levels are diffusely increased throughout the medial layer in AAA, as detected by DHE (Figure 1), and are not limited to areas of macrophages or monocytes (see online Figure). Second, NADH-stimulated $O_2^-$ is increased in AAA (Figure 5A). The phagocyte form of the oxidase predominantly uses NADPH, whereas the vascular oxidase uses NADH and NADPH as substrates.33,34 Third, increased expression of NAD(P)H oxidase subunits in AAA was not confined to infiltrating inflammatory cells but was observed diffusely throughout the media (Figures 5B and 6).

A number of factors could contribute importantly to the production of ROS by SMCs in AAA. For example, angiotensin II has a proinflammatory effect in the aortas of hypercholesterolemic mice and induces the formation of tensin II has a proinflammatory effect in the aortas of human AAA with adjacent NA tissue, thus controlling for the variables inherent to studies of patients with vascular disease. Although the present findings clearly indicate that ROS and oxidative stress are locally enhanced in AAA, they do not prove a pathogenic role in the initiation or progression of the disease process. Investigation of the role of oxidative stress in the pathogenesis of AAA would require a large clinical trial that activate proteolysis are increased in abdominal aortic aneurysms. Arterioscler Thromb Vasc Biol: 1995;15: 1145–1151. 11. Mohazzab KM, Kaminski PM, Wolin MS. NADH oxidoreductase is a unique feature of the present study is the comparison of human AAA with adjacent NA tissue, thus controlling for the variables inherent to studies of patients with vascular disease. Previous studies of human AAA have obtained aortas from autopsy or organ donor patients for comparison. Patients with AAA have a very high incidence of hypertension and atherosclerosis, which can independently induce alterations in aortic structure and function. Such confounding factors were eliminated in the present study, which, in turn, enabled us to establish with certainty that $O_2^-$ production and oxidative stress are locally enhanced in AAA. To our knowledge, this is the first study in which AAA segments were directly compared with adjacent NA segments obtained from the same patients.

Although the present findings clearly indicate that ROS and oxidative stress are locally enhanced in AAA, they do not prove a pathogenic role in the initiation or progression of the disease process. Investigation of the role of oxidative stress in the pathogenesis of AAA would require a large clinical trial using effective antioxidant therapy to be conducted over several years. Considering the magnitude of oxidative stress in AAA and the limited efficacy of nutrient antioxidants, such as vitamins E and C, the latter agents might be of little or no benefit in treating AAA. Moreover, our demonstration of an increased expression of NAD(P)H oxidase in the aneurysmal tissues suggests that targeted inhibition of this enzyme may be a promising approach in the treatment of AAA. Thus, the present findings suggest that additional studies to evaluate the role of oxidative stress in the pathogenesis of AAA are warranted.

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Increased superoxide in the medial layer (top panel, DHE staining) is not limited to areas of inflammatory infiltrate (bottom panel, NCL-MACRO immunostaining) in AAA tissue. The arrow denotes the inflammatory infiltrate. Sections are from the same AAA segment and representative of 3 patients.