Published in final edited form as:

Atherosclerosis. 2009 January ; 202(1): 128–134. doi:10.1016/j.atherosclerosis.2008.03.029.

Inhibition of reactive oxygen species attenuates aneurysm formation in a murine model

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Abstract

Reactive oxygen species (ROS) are increased in human abdominal aortic aneurysms (AAA). NADPH oxidases are the predominant source of superoxide anion (O_2^-) in the vasculature. Inducible nitric oxide synthase (iNOS) produces significant amount of nitric oxide (NO) during inflammatory processes. We hypothesized that ROS produced by NADPH oxidases and iNOS play an important role in aneurysm formation. We examined this hypothesis using selective blockade of NADPH oxidases and iNOS in a murine model of AAA. Mice, including C57BL/6, iNOS knockout (iNOS^{-/-}) mice, and its background matched control (C57BL/6), underwent AAA induction by periaortic application of $CaCl_2$. Aortic diameter was measured at aneurysm induction and harvest. Beginning 1 week prior to aneurysm induction and continuing to aortic harvest 6 weeks later, one group of the C57BL/6 mice were treated with orally administered apocynin (NADPH oxidase inhibitor). Control mice were given water. The mean diameter and change in diameter of each group were compared with concurrent controls. Aortic levels of the NO metabolite, NO_x ($N(NO_2$ and $NO_3)$), are significantly increased in $CaCl_2$ -treated wild type mice. iNOS^{-/-} mice are resistant to aneurysm induction. This is associated with reduced expression of matrix metalloproteinase (MMP)-2 and MMP-9 and decreased production of NO_x in the aortic tissues. Inhibition of NADPH oxidase by apocynin also blocked aneurysm formation. In conclusion, both iNOS deficiency and NADPH oxidase inhibition suppress aneurysm formation in association with decreased NO_x levels. These studies suggest that both the NADPH oxidase and iNOS pathways contribute to ROS production and AAA development.

1. Introduction

Abdominal aortic aneurysms (AAA) are a leading cause of mortality and morbidity in the elderly. AAA are characterized by large numbers of inflammatory cell infiltrates, elevated levels of matrix metalloproteinases (MMPs), and destruction of the elastic media. A number

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of studies have demonstrated that proinflammatory cytokines, such as interleukin (IL)-1 β , tumor necrosis factor (TNF)- α , and interferon (IFN)- γ , can enhance inducible nitric oxide synthase (iNOS) expression in vascular smooth muscle cells (SMC), and other cell types such as macrophages[1–3]. Oxidative stress has been shown to be associated with aortic aneurysm formation in both human tissue and animal models[4–6]. Reactive oxygen species (ROS), including superoxide anion (O₂⁻), hydroxyl radical, and reactive nitrogen species, such as nitric oxide (NO) and peroxynitrite, are increased in human AAA[7], suggesting that ROS are one of the possible contributors to arterial wall degeneration.

All vascular cell types produce ROS primarily via membrane-associated NADPH oxidases, xanthine oxidase (XO), and the multiple isoforms of nitric oxide synthase (NOS). Both NADPH oxidases and XO produce superoxide, whereas inducible NOS (iNOS) produces mainly nitric oxide (NO) during inflammatory processes. ROS have emerged as an important proinflammatory mediator in various inflammatory diseases. ROS are capable of activating matrix degrading proteins, MMPs, and inducing apoptosis of smooth muscle cells. For the activation of MMPs by ROS, various intracellular signaling pathways have been identified. A study by Meli et al had shown that MMP-9 activation associated with polymorphonuclear cells occurs through ROS-dependent pathway[8]. Rajagopalan et al reported that NO_x (NO₂ and NO₃), NO by-products, may accelerate connective tissue destruction by promoting MMP-2 activation[9]. Previous studies have shown that MMP-2 and MMP-9 are required for AAA formation[10,11]. In addition, protein nitration causes oxidative damage to structural connective tissue proteins, such as elastin and collagen[12]. Furthermore, NO and O₂⁻ react to form peroxynitrite (ONOO⁻), which has deleterious effects on the extracellular matrix.

In the present study, we hypothesized that ROS produced by NADPH oxidases, XO, and iNOS, play an important role in aneurysm formation. We examined the ability of a selective blockade of NADPH oxidases, XO, and iNOS to inhibit aneurysm formation in a murine model of AAA.

2. Methods

2.1. Mice

The homozygous inducible nitric oxide synthase (iNOS)-deficient (iNOS^{-/-}) mice on a C57BL/6 background and wild type C57BL/6 were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All mice used were males. All mice were maintained in the pathogen free animal facility at the University of Nebraska Medical Center. All studies were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center.

2.2. Aneurysm induction model

At 8 weeks of age, the mice underwent surgery as described previously[11,13]. Briefly, mice were anesthetized with intraperitoneal 2,2,2-tribromoethanol (Avertin) (Sigma, St. Louis, MO) at the dose of 200 mg/kg before undergoing laparotomy. The abdominal aorta between the renal arteries and bifurcation of the iliac arteries was isolated from the surrounding retroperitoneal structures. The diameter of the aorta was measured with video microscopy in triplicate midway between the renal artery origin and iliac artery bifurcation. After baseline measurements, 0.25 M CaCl₂ was applied to the external surface of the aorta for 15 minutes. The aorta was rinsed with 0.9 % sterile saline and the incision was closed. NaCl (0.9%) was substituted for CaCl₂ in sham control mice. Six weeks later, the mice underwent laparotomy and dissection. Measurements were repeated at the same location in the mid infrarenal aorta. The aorta was collected for zymographic analysis and measurement of NO_x and superoxide levels. For histological studies, the aorta was perfusion-fixed with 10% neutral buffered formalin.

The drug treatment groups began receiving drug at 7 weeks of age, 1 week prior to the initial laparotomy. This was continued until harvest at age 14 weeks. The mice were given acetovanillone (apocynin) (100 mg/kg/day) (Acros Organics, New Jersey) or oxypurinol (10mg/kg/day) (Sigma) in the drinking water. Controls received water only.

2.3. RT-PCR

Aortas from wild type C57BL/6 mice were collected at 6 weeks after treatment with 0.9% NaCl or 0.25 M CaCl₂. Total RNA from aortas was extracted using Trizol reagent (Invitrogen Corp., Carlsbad, CA) and iNOS expression was analyzed by RT-PCR. First-strand cDNA was synthesized by reverse transcription using the first-strand synthesis kit (Invitrogen Corp.) primed with oligo(dT)₂₀. The PCR reaction was performed using Platinum Taq DNA polymerase (Invitrogen Corp.) and primers specific for iNOS. PCR products were analyzed using a 2% agarose gel. As an endogenous reference, the globally expressed housekeeping gene β -actin was used.

2.4. Gelatin zymography

Aortic proteins were extracted as previously described[11]. The protein concentration for aortic proteins was standardized with Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Gelatin zymography was performed as described previously[11] by Longo et al, with 0.8% gelatin in a 10% SDS-polyacrylamide gel. The molecular sizes of gelatinolytic activities were determined using protein standards (Invitrogen Corp.).

2.5. Histology and microscopy

Masson's trichrome staining: After perfusion-fixation with 10% neutral buffered formalin, abdominal aortic tissues were embedded in paraffin and cut into 4 μ m sections. The slides were stained with hematoxylin, Crocein Scarlet, Acid Fuchsin, and Aniline Blue (Sigma). Each staining cycle alternated between fixing and washing procedures. The slides were examined and photographed using light microscopy (Kodak) (40 $\times$).

2.6. Measurement of NO metabolites (NOx)

Before the aortas were collected for measurement of NO metabolites (nitrate and nitrite), mice were fasted for 24 hours. The NO metabolites in aortic tissue were measured by a NOx analyzer (ENO-20, EICOM, Grand Island, NY) based on the Griess reaction.

2.7. Measurement of superoxide anion production

Superoxide anion production was measured using the lucigenin chemiluminescence method (Femtomaster FB12 luminometer, Zytex). Aortas were placed in polypropylene tubes containing NADPH (10 μ M) and dark-adapted lucigenin (5 μ M) and then light emission was recorded over 30-sec intervals for 5 min. Data were corrected for background and expressed as mean light units/min/ μ g of aortic proteins.

2.8. Statistical analysis

Measurements of aortic diameter and level of NOx are expressed as mean value $\pm$ the standard error (SE) of the mean. A paired Student's *t* test was used to compare original and final diameters. A Student's *t* test was used to compare final aortic diameter between groups. Statistical significance was accepted at a *P* < 0.05. The aorta was considered to be aneurysmal when the diameter increased by 50% or more from the baseline measurement.

3. Results

3.1. Role of iNOS in AAA formation

To examine the contribution of iNOS in murine AAA, expression levels of iNOS in CaCl₂-treated wild type mice were compared to NaCl-treated controls using RT-PCR. Inducible NOS mRNA were significantly increased in aneurysmal CaCl₂-treated aorta relative to non-aneurysmal NaCl-treated controls (Figure 1a), corroborating the findings in human AAA tissues[4]. Therefore, iNOS may play a role in the development of AAA but these data only confirm an association. To test the hypothesis that iNOS has a causal role in AAA, iNOS deficient (iNOS^{-/-}) mice and their corresponding wild type mice, C57BL/6, were used to examine the response to calcium chloride aneurysmal induction. Six weeks after periaortic application of CaCl₂, iNOS^{-/-} mice showed a relatively small increase (31%±6.7) in aortic diameter with only 20% developing aneurysms (> 50% increase in aortic diameter). These values are compared to the wild type mice where the increase in aortic diameter was two fold greater (61% ±6.4) and 63% developed aneurysms (Table 1). Masson's trichrome staining of aortic sections from CaCl₂-treated wild type mice showed disruption and fragmentation of medial elastic lamellae (Figure 1c). This damage was attenuated with decreased elastin fragmentation and damage to the medial elastic lamellae in CaCl₂-treated iNOS^{-/-} mouse aorta (Figure 1e). The aortic wall of the NaCl-treated control mice from both groups was intact without damage (Figure 1b&d). These results demonstrate that iNOS plays a causal role in aneurysm formation.

3.2. Effect of iNOS on MMP-2 and MMP-9 activation

Our previous results demonstrated that MMP-2 and MMP-9 are required for aneurysm formation. We further hypothesized that iNOS may affect MMP-2 and MMP-9 activation and expression in aorta. MMP-2 and MMP-9 expression in aortas was analyzed by gelatin zymography. The activation and expression of MMP-2 and MMP-9 in a CaCl₂-treated iNOS^{-/-} mouse aorta was less than that observed in wild type mice (Figure 1f). This result suggested that an iNOS affects MMP-2 and MMP-9 expression and activation and one mechanism through which iNOS might impact AAA.

3.3. Aortic NO metabolites levels in aneurysm formation

NO metabolites (NOx), nitrite and nitrate, can react with the matrix proteins, such as elastin and collagen, to produce damaging effects. NOx levels in aortic tissues were measured at six weeks after aneurysm induction. All mice were fasted 24 hours before the aortas were taken. NOx levels in CaCl₂-treated wild type mice were significantly increased compared with CaCl₂-treated iNOS^{-/-} mice (p<0.05) (Table 2). There is no significant difference in NOx levels between NaCl-treated and CaCl₂-treated iNOS^{-/-} mice. The data indicate that iNOS contributes significantly to tissue NOx levels and suggests that NOx may contribute to matrix degradation and aneurysm development.

3.4. Role of NADPH oxidase and XO in AAA formation

To examine the role of superoxide produced by NADPH oxidase and XO in AAA formation, wild type mice were treated with apocynin (100 mg/kg/day), a specific inhibitor of NADPH oxidases or oxypurinol (100 mg/kg/day), a specific inhibitor of XO. Apocynin and oxypurinol were given orally through the drinking water. Treatment began a week before aneurysm induction until mouse harvest. Control group mice were given untreated drinking water. We found that six weeks after periaortic application of CaCl₂, apocynin-treated mice showed a nominal increase (6.2%) in aortic diameter compared with their wild type controls (53%) (Figure 2a). Masson's trichrome staining of aortic sections from CaCl₂-treated wild type mice showed disruption and fragmentation of medial elastic lamellae (Figure 2b), whereas only mild

damage was present in the medial elastic lamellae in apocynin-treated CaCl_2 -induced mouse aorta (Figure 2c). Furthermore, MMP-2 and MMP-9 levels were decreased in apocynin treated mice compared with controls (Figure 2d). In contrast, mice treated with oxypurinol developed aneurysms similar to untreated control mice (data not shown). These results demonstrate that apocynin, an NADPH oxidase inhibitor, but not oxypurinol, a xanthine oxidase inhibitor, suppresses experimental aortic aneurysm formation. In order to be assured of the mechanism through which apocynin is working, we determined superoxide levels in apocynin-treated and untreated wild type mouse aortas. We found that superoxide levels were relatively, although not statistically significant, lower in apocynin-treated mouse aortas compared to untreated mouse aortas (Figure 3) ($p=0.2671$). These findings are consistent with an important role for NADPH oxidase as a source of oxygen radicals that promote aneurysm formation.

4. Discussion

Abdominal aortic aneurysms (AAA) represent a life-threatening condition characterized by diffuse inflammatory infiltration, extracellular matrix degradation, and dilatation of the aortic wall. While the specific factors initiating chronic inflammation in AAA remain unknown, enhanced vascular reactive oxygen species (ROS) production have been associated with chronic inflammation, atherosclerosis, and human abdominal aortic aneurysms[7,14,15]. ROS are a collection of oxygen-derived molecules such as NO and superoxide. A number of specific enzymes can generate intracellular ROS, including xanthine oxidase (XO), NOS, and the NADPH oxidases. Our present studies demonstrate the causal roles of ROS produced by iNOS and NADPH oxidases in the oxidative stress and matrix degradation that lead to aneurysm formation. A deficiency in iNOS or inhibition of NADPH oxidase leads to decreased ROS production in the aortic tissues. Importantly, decreased ROS levels are directly correlated with inhibition of matrix destruction and aneurysm formation.

Inducible NOS is widely expressed in diverse cell types under transcriptional regulation by inflammatory mediators[16,17] and has been implicated in the pathogenesis of many disorders including atherosclerosis, stroke, and arthritis[18–20]. In the present study, we found that CaCl_2 -induced mouse aneurysmal degeneration was associated with increased expression of iNOS mRNA in the aortic wall. We further tested the effect of iNOS in aneurysm formation by using iNOS knockout mice. Deficiency of iNOS partially attenuated aneurysm formation. This is in contrast to previous studies reported by Lee and colleagues[21] that indicated that iNOS does not play a necessary role in the aneurysm pathology in the elastase-induced murine aneurysm model. This discrepancy is likely attributable to the use of different murine aneurysm models (ie, elastase-induced murine aneurysm model and CaCl_2 -induced murine aneurysm model). The elastase-induced murine model of AAA relies on an initial pressure mediated mechanical injury followed by an inflammatory injury from infusion of pancreatic elastase. As such, this model may be more representative of the growth, rather than initiation, phase of an aneurysm. The CaCl_2 -induced model depends on an inflammatory response to the local application of CaCl_2 to the adventitial arterial surface. Thus, in this latter model, the expansion begins de novo from the inflammatory response. Both models involve a local arterial injury followed by inflammatory cell invasion, increased expression of MMPs, matrix destruction, and a corresponding increase in vessel diameter, recapitulating the key features of human aneurysms. Our findings are in agreement with studies done previously by Johanning et al showing[22] that pharmacologic inhibition of iNOS limited expansion of elastase-induced aneurysm in rats.

Human AAA are associated with increased local activity of NADPH oxidases and XO[23, 24]. We examined the role of superoxide production by NADPH oxidase and XO in AAA formation using a specific inhibitor of NADPH oxidases, apocynin, and a specific inhibitor of XO, oxypurinol. We found that apocynin, but not oxypurinol, significantly inhibits mouse

aneurysm expansion. These data suggest that NADPH oxidases are the major source of superoxide generation in the aortic tissue and play an important role in aneurysm formation. The profound inhibition of aneurysm formation by NADPH oxidase inhibition suggests a more important and direct role on aneurysm formation than iNOS where aortic expansion is limited to a lesser degree. The results are supported by the observation that SMC, resident mesenchymal cells, and macrophages, the major inflammatory infiltrates in aneurysmal aortic wall, express high levels of NADPH oxidases[25].

ROS play an important role in cardiovascular homeostasis and inflammatory conditions. We further investigated NO production in the aorta. The concentrations of nitrite and nitrate (NO_x), the stable end products of NO, were significantly elevated in CaCl₂-treated aneurysmal tissues. As expected, this increase in NO_x levels was inhibited by iNOS gene deletion. The data suggest that enhanced NO production in aneurysmal tissues is mainly catalyzed by iNOS. Furthermore, the aortic superoxide production by NADPH oxidases was measured. Superoxide production was not significantly inhibited by apocynin. It is important to point out that the absolute amount of superoxide production was significantly reduced in apocynin treated mice prior to normalizing with protein concentrations, because the aortas from untreated CaCl₂-induced aneurysm mice were larger and had higher protein content than apocynin treated mice.

Several studies have shown that ROS can induce the expression of matrix-degrading enzymes such as MMP-2 and MMP-9[8,9]. MMP-2 and MMP-9 degrade various extracellular matrix proteins and play an important role in AAA formation[11,26]. We, therefore, investigated whether NADPH oxidase inhibitor, apocynin, XO inhibitor, oxypurinol, or iNOS deficiency inhibited the activities of MMP-2 and MMP-9 in the aortic tissues. As expected, CaCl₂-treatment markedly induced MMP-2 and MMP-9 activity in wild type mouse aorta. This increase in MMP activity was inhibited by apocynin treatment and iNOS gene deletion, but not oxypurinol treatment. The findings demonstrate one of the mechanisms through which ROS inhibition blocks aneurysm formation.

In summary, we report that iNOS deficiency and inhibition of NADPH oxidase activity protects aortas from AAA formation in a CaCl₂-induced murine aneurysm model. These findings are consistent with the concept that oxidative stress plays a causal role in the pathogenesis of AAA formation. Furthermore, these studies suggest that early inhibition of iNOS-induced NO production and of superoxide production catalyzed by NADPH oxidases may be protective from the development of aortic aneurysm.

Acknowledgements

This study was supported by National Institute Health grant 5R01HL62400-02 and American Heart Association grant 974015N (BTB).

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Figure 1.

a) Expression of iNOS. Six weeks after 0.9% NaCl or 0.25 mol/L CaCl₂ treatment, WT mouse aortas were harvested. Total RNA from each aorta was extracted and iNOS mRNA levels was examined by RT-PCR. Beta-actin was used as the internal standard. The gel is representative of three separate experiments; c-e) Histological changes in mouse aorta by trichrome staining. One example of NaCl or CaCl₂ treatment of C57BL/6 WT controls (b & c) and C57BL/6 iNOS^{-/-} (d & e) mice is shown. The staining is representative of three to five samples with similar results. The matrix damage is profound in the CaCl₂-treated wild type mice. Matrix destruction in the iNOS^{-/-} is significantly less than that observed in the WT controls; f) Gelatin zymographic analysis of latent and processed MMP-2 and MMP-9 in the mouse aorta. Six weeks after 0.9% NaCl or 0.25 mol/L CaCl₂ treatment, abdominal aortas from C57BL/6 (WT) and iNOS^{-/-} mice were harvested. Aortic proteins were extracted and separated by

electrophoresis on a 10% SDS-PAGE containing 0.8% gelatin. The gel shown is representative of three trials with similar results.

Figure 2.

Effect of apocynin treatment on the aneurysm development in CaCl_2 -induced aneurysm model. a) Aortic diameter before (open bar) and 6 weeks (hatched bar) after CaCl_2 -aneurysm induction in C57BL/6 wild type mice without (Control) or with (Apocynin) apocynin treatment; b–c) Histological changes in mouse aorta 6 weeks without or with apocynin treatment using trichrome staining, untreated (b) and apocynin-treated (c) aorta from C57BL/6 mice. Each staining represents three to four samples with similar results. d) gelatin zymographic analysis of latent and processed MMP-2 and MMP-9 in apocynin-treated (Apocynin) or untreated (Control) in the CaCl_2 -treated wild type mouse aorta. The gel shown is representative of three trials with similar results.

Figure 3.

Effect of apocynin on aortic superoxide production in CaCl₂-aneurysm induced C57BL/6 mice. Superoxide production catalyzed by NADPH oxidase was measured using lucigenin chemiluminescence method. Data were corrected for background and expressed as mean light units/min/μg of aortic protein.

Table 1 Changes in aortic diameter in C57BL/6 and iNOS^{-/-} mice after treatment of NaCl and CaCl₂

	C57BL/6		iNOS ^{-/-}	
Treatment	NaCl	CaCl ₂	NaCl	CaCl ₂
Number	7	8	11	9
Pre (μm)	532 ± 8.3	521 ± 13.6	525 ± 12.6	524 ± 10.1
Post (μm)	562 ± 15.8	833 ± 14.8 [*]	563 ± 14.3	685 ± 26.4 ^{*#}
% of increase	5.4	61.0	7.1	30.7
% of aneurysm	0	63	0	20

Aortic diameters were measured before NaCl or CaCl₂ incubation (Pretreatment) and at sacrifice (Post-treatment). Measurements of aortic diameter were expressed as mean ± SE. The percent increase was represented as a percent compared with pre-treatment.

* P < 0.01, Student's *t* test, compared to pretreatment value.

# P < 0.05, compared to CaCl₂-treated control. The development of an aneurysm was defined as at least a 50% increase relative to the original diameter of aorta.

Table 2 Aortic NO₂ & NO₃ levels (μM) in NaCl-and CaCl₂-treated C57BL/6 and iNOS^{-/-} mice

Treatment	C57BL/6		iNOS ^{-/-}	
	NaCl	CaCl ₂	NaCl	CaCl ₂
Number	5	5	4	4
NO _x (μM)	6.2 ± 0.8	9.8 ± 1.2 [*]	6.8 ± 2.3	6.1 ± 2.2

Measurements of aortic levels of NO_x (NO₂ & NO₃) were expressed as mean ± SE.^{*} P < 0.01, Student's *t* test, compared to NaCl-treated control and CaCl₂-treated iNOS mice.