Overexpression of Catalase in Vascular Smooth Muscle Cells Prevents the Formation of Abdominal Aortic Aneurysms

Ioannis Parastatidis, Daiana Weiss, Giji Joseph, W. Robert Taylor

Objective—Elevated levels of oxidative stress have been reported in abdominal aortic aneurysms (AAA), but which reactive oxygen species promotes the development of AAA remains unclear. Here, we investigate the effect of hydrogen peroxide (H₂O₂)–degrading enzyme catalase on the formation of AAA.

Approach and Results—AAA were induced with the application of calcium chloride (CaCl₂) on mouse infrarenal aortas. The administration of PEG-catalase, but not saline, attenuated the loss of tunica media and protected against AAA formation (0.91±0.1 versus 0.76±0.09 mm). Similarly, in a transgenic mouse model, catalase overexpression in the vascular smooth muscle cells preserved the thickness of tunica media and inhibited aortic dilatation by 50% (0.85±0.14 versus 0.57±0.08 mm). Further studies showed that injury with CaCl₂ decreased catalase expression and activity in the aortic wall. Pharmacological administration or genetic overexpression of catalase restored catalase activity and subsequently decreased matrix metalloproteinase activity. In addition, a profound reduction in inflammatory markers and vascular smooth muscle cell apoptosis was evident in aortas of catalase-overexpressing mice. Interestingly, as opposed to infusion of PEG-catalase, chronic overexpression of catalase in vascular smooth muscle cells did not alter the total aortic H₂O₂ levels.

Conclusions—The data suggest that a reduction in aortic wall catalase activity can predispose to AAA formation. Restoration of catalase activity in the vascular wall enhances aortic vascular smooth muscle cell survival and prevents AAA formation primarily through modulation of matrix metalloproteinase activity. (Arterioscler Thromb Vasc Biol. 2013;33:2389-2396.)

Key Words: aortic aneurysm ■ catalase ■ hydrogen peroxide ■ inflammation ■ smooth muscle

Abdominal aortic aneurysms (AAA) are permanent dilatations of the aortic wall. The majority are located in the infrarenal aorta between the takeoff of the renal arteries and the iliac bifurcation. Age, male sex, and history of cigarette smoking are the major risk factors for the development of AAA. Although sometimes aneurysms remain stable in size, frequently the aortic diameter increases in size until rupture occurs. Previous studies have demonstrated that aneurysmal dilatation is an active process that involves infiltration of the adventitia and tunica media with inflammatory cells, loss of smooth muscle cells, thinning of the tunica media, and degradation of the extracellular matrix.

Elevated levels of oxidative stress have been reported in aneurysmal tissues. Miller et al demonstrated increased nitrosoimide adenine dinucleotide phosphate oxidase activity, as well as elevated levels of superoxide, lipid peroxidation, and 3-nitrotyrosine in human aortic aneurysmal tissue compared with healthy tissue from the same aorta. The inducible form of nitric oxide synthase, a source of reactive oxygen species (ROS) and reactive nitrogen species, is upregulated in human AAA. Furthermore, decreased antioxidant enzyme activity, including decreased neutrophil catalase activity, and lower levels of α-tocopherol in the plasma of patients with AAA have been described. In animal models, angiotensin II infusion in hyperlipidemic mice, which induces nicotinamide adenine dinucleotide phosphate activity and ROS formation, results in vascular inflammation and aneurysm formation, whereas administration of apocynin (an inhibitor of nicotinamide adenine dinucleotide phosphate oxidases), deletion of p47phox, or vitamin E supplementation attenuates AAA formation.

Although these data have established a firm connection between ROS and AAA development, the identity of the specific ROS responsible for the vascular injury that precedes aneurysmal development, as well as the effect of antioxidant enzymes on aortic dilatation, remains unclear. Interestingly, hydrogen peroxide (H₂O₂) modulates the pathophysiological processes that have been shown to govern AAA formation, such as inflammation, vascular smooth muscle cell (VSMC) death, and matrix metalloproteinase (MMP) activation. Thus, we hypothesized that H₂O₂ causes vascular
injury and promotes AAA formation. Here, using a pharmacological and a genetic approach, we explored the effects of the H$_2$O$_2$-degrading enzyme catalase on vascular wall injury and AAA formation.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
Effects of CaCl$_2$ on Vascular Wall Catalase and H$_2$O$_2$ Fluxes
First, we investigated the effect of vascular injury on aortic wall catalase and H$_2$O$_2$ levels. We observed a sustained decrease in catalase mRNA expression and activity as early as day 3 after injury (Figure 1A and 1B). The levels of H$_2$O$_2$ were also quantified in infrarenal aortas at different time points after exposure to calcium chloride (CaCl$_2$). In agreement with the catalase activity, we found that the H$_2$O$_2$ levels generated in the aortas exposed to CaCl$_2$ peaked on postoperative day 10 (Figure 1C).

Infusion of PEG-Catalase Attenuates CaCl$_2$-Induced Aortic Dilation
To investigate the impact of catalase on the vascular wall injury that precedes AAA formation, aneurysms were induced in wild-type mice, which were infused intravenously with PEG-catalase or saline. The administration of PEG-catalase, unlike infusion of saline, protected from CaCl$_2$-induced aortic dilation and AAA formation (Figure 2D and 2E). Histological examination showed significant dilatation, distortion of the normal architecture, and thinning of the media in aortas exposed to CaCl$_2$ and infused with saline (Figure 2F, top). In contrast, aortas from PEG-catalase–treated mice had normal architecture and preserved tunica media (Figure 2F, bottom). Quantification of the media thickness by hematoxylin and eosin staining confirmed that PEG-catalase was protective against VSMC loss (Figure 2G).

Overexpression of Catalase in VSMC Inhibits AAA
To investigate the significance of VSMC-derived catalase on aneurysm formation, we used a transgenic mouse model that overexpresses human catalase specifically in the VSMC, driven by the myosin heavy chain-$\alpha$ promoter (Tgcat-VSMC). The expression of the human catalase transgene in the infrarenal aortas remained relatively stable, despite vascular injury (Figure 3A). This resulted in preserved catalase activity in Tgcat-VSMC aortas. However, aortas from the wild-type littermates had significantly decreased catalase activity after exposure to CaCl$_2$ (Figure 3B). As expected, plasma catalase activity was similar between the 2 groups (Figure 3C). In additional studies, we sought to determine the effect of VSMC-derived catalase on AAA formation. Wild-type and Tgcat-VSMC mice were exposed to CaCl$_2$ and euthanized after 8 weeks. We noted a remarkable reduction in aortic dilation of catalase-overexpressing mice compared with their wild-type littermates (Figure 3D and 3E). In agreement with the PEG-catalase experiment, wild-type mice had dilated, distorted aortas (Figure 3F, top), but the aortas from transgenic animals exhibited preserved architecture (Figure 3F, bottom) with intact tunica media thickness (Figure 3G).

Catalase Attenuates MMP Activity
To delineate the mechanism by which catalase is protective against aneurysmal dilatation, first we examined the expression and activity of extracellular matrix–degrading enzymes between the wild-type and Tgcat-VSMC mice. We found a trend toward higher levels of MMP-9 mRNA at baseline and MMP-2 mRNA on day 10 after surgery in wild-type mice (Figure 1 in the online-only Data Supplement). Similarly, immunohistochemistry demonstrated a more intense staining for MMP2 (Figure 4A, top) and MMP9 (Figure 4A, middle) in the aortic wall of wild-type compared with transgenic mice 10 days after exposure to CaCl$_2$. Furthermore, in situ gelatin zymography in aortas 10 days after surgery showed higher MMP activity in wild-type compared with Tgcat-VSMC mice (Figure 4A, bottom). Addition of 20 mmol/L EDTA suppressed the fluorescent signal, thus demonstrating specificity (Figure 1 in the online-only Data Supplement). Quantification of the fluorescent signal confirmed significantly higher levels of MMP activity in wild-type compared with catalase-overexpressing aortas (Figure 4B). In contrast to MMPs, the cathepsin activity was found to be similar between the 2 groups (Figure 1 in the online-only Data Supplement). We also sought to determine whether infusion of PEG-catalase had a similar effect on extracellular matrix degradation. Wild-type mice were exposed to CaCl$_2$, infused with saline or PEG-catalase, and euthanized 10 days after surgery. In agreement with the previous data, gelatin zymography of aortic lysates showed attenuated MMP activity after infusion of PEG-catalase, but not saline (Figure 4C).
Reduction of Apoptotic Cell Death in Catalase-Overexpressing Aortas

Because loss of VSMC is important in AAA formation, we investigated the effect of VSMC-specific catalase overexpression on apoptotic cell death after vascular injury with CaCl₂. Initially, we used terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining to look for apoptosis in the vessel wall. Apoptotic cells were present in wild-type and Tgcat-VSMC aortas after exposure to CaCl₂ on days 3 and 7 after surgery. We observed apoptotic

Figure 2. Infusion of PEG-catalase (PC) is protective against calcium chloride (CaCl₂)-induced aortic expansion. A, Catalase activity in aortic lysates at baseline and after CaCl₂-induced injury (n=4/group; **P<0.01 vs baseline by 1-way ANOVA). B, Catalase activity in plasma (n=4/group; **P<0.01 vs baseline and vs saline infusion by 1-way ANOVA). C, Aortic hydrogen peroxide (H₂O₂) levels on day 10 after CaCl₂ exposure in mice infused with saline vs PEG-catalase (n=6/group; †P=0.065 by nonparametric Mann–Whitney test). D, Aortic expansion after 10-day infusion with saline or PEG-catalase 8 weeks after surgery (n=5/group; †P=0.04 vs saline infusion by nonparametric Mann–Whitney test). E, Percent change of aortic diameter from baseline of saline vs PEG-catalase–infused mice (n=5/group; †P=0.055 by nonparametric Mann–Whitney test). F, Representative hematoxylin and eosin stain of aortas exposed to CaCl₂ and infused with saline (top) vs PEG-catalase (bottom). G, Quantification of media thickness in aortas of saline vs PEG-catalase–infused mice on postoperative week 8 (**P=0.015 nonparametric Mann–Whitney test; scale bar, 200 µm).

Figure 3. Catalase overexpression in vascular smooth muscle cells (VSMC) prevents abdominal aortic aneurysm formation. A, Human catalase mRNA expression in wild-type (wt) and Tgcat-VSMC mice over time after injury with calcium chloride (CaCl₂). B, Catalase activity in aortic lysates at baseline and after injury with CaCl₂ (n=3/group; **P=0.02 vs Tgcat-VSMC day 3 by 1-way ANOVA). C, Catalase activity in plasma (n=3/group). D, Aortic diameter in wt and Tgcat-VSMC mice 8 weeks after CaCl₂ exposure (n=10–12; **P<0.001 vs wt week 8 by nonparametric Mann–Whitney test). E, Percent change in aortic diameter from baseline in wt vs Tgcat-VSMC mice (**P<0.001 by nonparametric Mann–Whitney test). F, Representative hematoxylin and eosin stain of wt (top) and Tgcat-VSMC aortas (bottom). G, Quantification of media thickness in wt and Tgcat-VSMC mouse aortas 8 weeks after surgery (**P=0.035 by nonparametric Mann–Whitney test; scale bar, 200 µm).
cells in the intima, media, and adventitia in wild-type aortas, but in catalase-overexpressing aortas the vascular wall was protected from apoptotic death (Figure 5A, top). Similarly, immunohistochemistry for cleaved caspase 3, another marker of apoptosis, showed diminished levels of apoptosis in transgenic compared with wild-type aortas (Figure 5A, bottom). Quantification of TUNEL-positive cells in the media and intima showed remarkably reduced apoptosis in Tgcat-VSMC compared with wild-type mice (Figure 5B). In addition, quantification of the TUNEL-positive area over the total vascular wall area showed higher degree of apoptosis in wild-type compared with Tgcat-VSMC mice (Figure II in the online-only Data Supplement). The levels of tumor necrosis factor α (TNFα), a potent proapoptotic cytokine, were also diminished in the transgenic aortas (Figure 5C). The proliferative response as assessed by 5-bromo-2-deoxyuridine staining was similar between the 2 groups (Figure II in the online-only Data Supplement). Although catalase overexpression in VSMC was clearly protective against apoptosis, infusion of PEG-catalase did not have a significant effect on apoptotic cell death on day 7 after surgery and it did not change the levels of TNFα in the vasculature (Figure II in the online-only Data Supplement).

**Figure 4.** Catalase decreases the activity of matrix metalloproteinase (MMPs). A, Staining for MMP2 (top) and MMP9 (middle) in wild-type (wt; left) and Tgcat-VSMC mice (right) 10 days after chemical injury. Bottom, Representative images of in situ gelatin zymography of a wt (left) and a Tgcat-VSMC aorta (right) on postoperative day 10. B, Percent fluorescent signal over total aortic wall area on postoperative day 10 in wt and Tgcat-VSMC mice exposed to calcium chloride (CaCl$_2$) and wt mice exposed to saline (sham; n=4/group; *P<0.01 by 1-way ANOVA). C, Gelatin zymography in aortic lysates from wt mice exposed to CaCl$_2$, and treated with saline or PEG-catalase (PC) 10 days after chemical injury; scale bar, 50 μm. aMMP indicates activated matrix metalloproteinase; DAPI, 4′,6-diamidino-2-phenylindole; and VSMC, vascular smooth muscle cells.

**Figure 5.** Reduced apoptosis in Tgcat-VSMC aortas. A, Representative images of terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining (top) and cleaved caspase 3 (bottom) in wild-type (wt; left) and Tgcat-VSMC (right) mice on day 7 after surgery. B, Quantification of apoptotic cells in the intima and tunica media of wt and Tgcat-VSMC mice on days 3 and 7 after surgery (*P=0.03 vs Tgcat-VSMC day 7 by 1-way ANOVA). C, Quantification of tumor necrosis factor α (TNFα) mRNA levels in wt and Tgcat-VSMC aortas at baseline (day 0) and days 3 and 10 after surgery (n=4/group; *P=0.015 vs Tgcat-VSMC day 3 by 2-way ANOVA; scale bar, 50 μm). VSMC indicates vascular smooth muscle cells.
Attenuated Inflammatory Response in Tgcat-VSMC

We also investigated the mRNA levels of known and likely inflammatory mediators of AAA formation in response to CaCl₂. We found that TNFα expression in the aortas of Tgcat-VSMC mice was significantly lower on postoperative day 3 compared with wild-type mice (Figure 5C). The levels of transforming growth factor (TGF) β1, osteopontin, and the macrophage chemotactic monocyte chemotactic protein-1 were also attenuated in the transgenic mice (Figure III in the online-only Data Supplement). There was a trend toward lower levels of IL-1β in Tgcat-VSMC mice. We did not observe differences in the mRNA levels of IL-6, intercellular adhesion molecule 1, stromal cell–derived factor 1, IL-1α, and TGFβ1 on postoperative day 3 (data not shown).

To examine the potential role of smooth muscle catalase overexpression in modulating inflammatory cell infiltration into the arterial wall, we stained for the macrophage marker Mac3. We found higher levels of macrophage infiltration in wild-type compared with catalase-overexpressing aortas on postoperative day 7 (Figure IV in the online-only Data Supplement). Quantification of immunohistochemistry with ImageJ confirmed higher degree of macrophage infiltration in wild-type mice (Figure IV in the online-only Data Supplement). Additional studies with immunohistochemistry revealed comparable degree of neutrophilic and lymphocytic infiltration on postoperative days 3 and 10, respectively (Figure IV in the online-only Data Supplement).

Interestingly, contrary to overexpression of catalase in the VSMC, administration of PEG-catalase did not alter the levels of any inflammatory marker, except from TGFβ1, which was remarkably reduced compared with saline controls on day 3 after surgery (Figure V in the online-only Data Supplement). The degree of macrophage infiltration was also comparable between the mice infused with saline and PEG-catalase (Figure V in the online-only Data Supplement).

Similar Total H₂O₂ Levels in Wild-Type and Tgcat-VSMC Aortas

To assess whether catalase overexpression affected the overall H₂O₂ release in Tgcat-VSMC compared with wild-type aortas, we quantified H₂O₂ fluxes in both groups on days 3, 8, and 10 after surgery with the Amplex Red assay. The levels of H₂O₂ were not statistically different between the 2 groups (Figure 6A). Because endothelial cells are capable of generating significant amounts of H₂O₂, we sought to determine whether there was a compensatory increase in H₂O₂ production by the endothelial layer, as a consequence of enhanced H₂O₂ degradation by the VSMC layer in Tgcat-VSMC mice. To investigate this, wild-type and Tgcat-VSMC mice were exposed to CaCl₂, and the H₂O₂ levels were measured on postoperative day 10 after removing the endothelial layer by scraping. Endothelial denudation resulted in an equal reduction of H₂O₂ levels by 2 pmol/min per aortic ring in both groups (Figure 6B and 6C), which approximates 30% of the total H₂O₂ levels in the infrarenal aortas at this specific time point. This result demonstrates that there is equal endothelial contribution in H₂O₂ levels in both groups.

Next, we sought to determine the expression of important vascular wall redox-regulating enzymes. Wild-type and transgenic mouse aortas had similar levels of the antioxidant enzymes superoxide dismutase 1 and glutathione peroxidase 1 (Figure 6D–6F). The levels of peroxiredoxin 1 and 2 were also comparable between the 2 groups at baseline and after exposure to CaCl₂ (Figure VI in the online-only Data Supplement). Similarly, infusion of PEG-catalase had no effect on the expression of major antioxidant enzymes (Figure VII in the online-only Data Supplement).

The hematoxylin and eosin and the TUNEL assay analysis showed higher survival of VSMC in Tgcat-VSMC compared with wild-type aortas after CaCl₂ injury. This observation suggested that the slightly elevated levels of H₂O₂ in transgenic mice could be explained by an increased number of VSMC in tunica media compared with wild-type mice. Indeed, staining for smooth muscle α-actin showed an intact medial layer in the Tgcat-VSMC aortas (Figure 6H) as opposed to wild-type aortas, in which areas of VSMC loss were evident as early as day 3 after surgery (Figure VIII in the online-only Data Supplement) and were remarkable on postoperative day 10 (Figure 6G). As expected, wild-type aortas exposed to saline rather than CaCl₂ had an intact tunica media similar to the Tgcat-VSMC mice (Figure 6I).

Despite the similar levels of H₂O₂ in the aortas of wild-type and Tgcat-VSMC mice, staining for 4-hydroxy-2-nonenal, a lipid peroxidation product and a marker of oxidant damage in the vascular wall, revealed significantly higher levels in the wild-type (Figure 6J) compared with catalase-overexpressing mice (Figure 6K) 8 weeks after surgery. This observation suggests that the total H₂O₂ levels do not describe accurately the levels of oxidative stress in the vasculature.

Discussion

Oxidative stress has been described in human and animal models of AAA; however, the identity of ROS that mediate the development of aortic dilatation remains elusive. Here, we investigated the effect of catalase, a peroxisomal H₂O₂-scavenging enzyme, on the formation of CaCl₂-induced aortic aneurysms. The significance of catalase on AAA formation has been controversial. Previous work from our laboratory demonstrated that catalase overexpression in the VSMC protected against angiotensin II–induced biomechanical changes that predispose to aortic dilatation.22 Similarly, Grigoryants et al23 demonstrated that tamoxifen upregulated catalase and attenuated elastase-induced AAA in rats. However, Uchida et al24 showed that catalase deficiency had no effect on angiotensin II–induced aortic aneurysms in mice. In the present work, using the CaCl₂ model, we show that catalase expression and activity were substantially decreased early after vascular injury. Reconstitution of catalase activity either via pharmacological administration or via genetic tissue-specific overexpression in the VSMC had a profound protective effect against vascular wall damage and AAA formation. Of note, as opposed to angiotensin II infusion, which raised catalase activity,24 vascular injury with CaCl₂ decreased aortic catalase activity. The differential response highlights the inherent differences in the mechanism of aneurysm induction between the 2 models. Unfortunately, Grigoryants et al23 did not report how the levels of catalase in the elastase model of AAA compare with its baseline levels before surgery.
The degradation of extracellular matrix by proteolytic enzymes, such as MMPs and cathepsins, promotes aneurysm formation. It has been previously shown that the activity of MMPs can be modulated by H$_2$O$_2$ in vitro. It is also possible that the activity of cathepsins can be affected by ROS either directly through cysteine oxidation or indirectly through disruption of the lysosomal membrane integrity by hydroxyl radicals. In our studies, catalase overexpression in the VSMC did not affect the activity of cathepsins at early or late stages after vascular injury. However, we found decreased MMP expression and activity in the aortas of transgenic compared with wild-type mice 10 days after injury. Similarly, infusion of PEG-catalase decreased the gelatinolytic activity in aortic lysates 10 days after exposure to CaCl$_2$. The data suggest that the protective effect of catalase is primarily mediated by modulation of MMP activity.

The overexpression of catalase in VSMC decreased the formation of inflammatory mediators such as TNF$\alpha$ and decreased apoptotic cell death. The antiapoptotic effects were validated with 2 independent methods, staining for TUNEL and cleaved caspase 3. Interestingly, infusion of PEG-catalase failed to decrease TNF$\alpha$ expression and did not protect from apoptosis. Thus, the attenuated apoptosis in the Tgcat-VSMC mice could be, in part, attributed to diminished TNF$\alpha$ expression, because failure to reduce the expression of TNF$\alpha$ by PEG-catalase resulted in lack of antiapoptotic effect.

Apart from suppressing MMP activity, both genetic and pharmacological catalase administration decreased the expression of TGF$\beta$1 in the vasculature. The effect of TGF$\beta$ on aneurysm formation is controversial. It has been reported that it promotes the formation of thoracic aneurysms in mouse models of Marfan syndrome, although it seems to be protective.
against angiotensin II–induced aortic dilatation. The significance of TGFβ in CaCl2-induced aneurysms is understudied. One report suggested that TGFβ may decrease CaCl2-induced aneurysms in mice. Here, we show that TGFβ1 levels were elevated after CaCl2 injury and catalase administration significantly lowered its levels. However, it remains unclear whether the change in TGFβ1 expression induced by catalase is incidental or contributes to the protection against aortic dilatation in the CaCl2 model.

Interestingly, although catalase overexpression in the VSMC and pharmacological administration of PEG-catalase reduced the activation of MMPs, the antiapoptotic and anti-inflammatory effects observed in the transgenic model were not evident after PEG-catalase infusion. The administration of PEG-catalase is a short-term event and affects multiple tissues and cell types. However, in the transgenic mice catalase is overexpressed specifically in the VSMC since birth. This chronic overexpression can potentially alter the phenotype and the way VSMC respond to inflammatory stimuli. Although this is an intriguing observation, investigation of the effects of chronic overexpression of catalase on VSMC phenotype is beyond the scope of this article.

Despite the profound protective effect of catalase on AAA formation, infusion of PEG-catalase at 10,000 U/kg per day only modestly decreased the H2O2 release. Furthermore, we could not detect differences in the H2O2 levels between the wild-type and Tg(VSMC) mice. This is not an entirely unexpected finding. First, catalase is specifically expressed in the peroxisomes, and although it is essential for H2O2 detoxification produced in the peroxisomes as a consequence of fatty acid oxidation, its role in maintaining the total cellular H2O2 levels is limited. Other more abundant enzymes with lower Km values for H2O2, such as the peroxiredoxins and glutathione peroxidases, are the main regulators of cellular H2O2 levels. Second, a compensatory increase in the expression of peroxidases, a marker of oxidant-induced damage in the extracellular H2O2 levels. Despite the common knowledge that H2O2 moves freely across membranes, emerging data suggest a selective regulation of H2O2 fluxes by the aquaporin-3 channel. Thus, the extracellular H2O2 levels do not necessarily reflect the intracellular levels. Furthermore, as suggested by 4-hydroxy-2-trans-nonenal staining, the formation of lipid peroxidation products, a marker of oxidant-induced damage in the aortic wall, was significantly attenuated in the catalase-overexpressing mice. This observation raises the possibility that extracellular H2O2 levels alone do not reflect accurately the degree of oxidative damage in the vasculature. Finally, in a seminal work published previously, a strong overexpression of human catalase specifically in mitochondria resulted in a 50-fold increase in catalase activity and prolonged the animal life span. However, only a modest reduction in H2O2 levels was detected in isolated mitochondria.

In summary, in this work we demonstrate for the first time that catalase activity in the aorta drops significantly after chemical injury with CaCl2. Restoring catalase activity decreases the activation of MMPs and protects from AAA formation. This finding suggests that altered H2O2 signaling as a consequence of decreased catalase activity in the vasculature could be an early insult that leads to aortic dilatation.

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Disclosures

None.

References

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Significance
Although oxidative stress has been firmly connected with aortic aneurysm disease, the identity of the specific reactive oxygen/nitrogen species and antioxidant enzymes that predispose to the development of aortic dilatation remains elusive. In this work, we demonstrate for the first time that catalase activity is decreased and hydrogen peroxide levels rise early after vascular injury before the development of aortic dilatation. Furthermore, we show that restoration of catalase activity in the aortic wall inhibits the degradation of extracellular matrix by matrix metalloproteinases and subsequently prevents the development of aortic aneurysms.
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Supplemental methods

Chemicals

Unless specified all chemicals were purchased from Sigma (Sigma, St. Luis, MO). The solutions, such as ethanol, methanol and acetic acid were purchased from Fisher (Fisher Scientific, Pittsburg, PA).

Mice

Eight to 10 week old male mice, all on the C57Bl6 background were used. The C57Bl6 mice infused with PEG-catalase were purchased from Jackson laboratories (Jackson Laboratories, Bar Harbor, ME). For the rest of the experiments we used an established colony of catalase over-expressing mice and their wild type littermates generated and maintained in our laboratory. These mice over-express the human catalase gene specifically in the VSMCs, driven by the myosin heavy chain α promoter (Tg\textsuperscript{cat-VSMC}) and have been described previously\textsuperscript{1}.

Induction of AAA

AAA were induced with the application of CaCl\textsubscript{2} on the infrarenal aorta as described previously\textsuperscript{2}. Briefly, 8-10 week old male mice on the C57Bl6 background were used. Mice were anesthetized with 1-2% isoflurane, the aorta was isolated from the inferior vena cava (IVC) using fine forceps, and a sterile gauze soaked in 0.25M sterile CaCl\textsubscript{2} was applied on its external surface for 15 minutes. A picture of the aorta was taken with a digital camera connected to the surgical microscope. The aortic diameter was measured in the midline between the renal arteries and the iliac bifurcation in live animals with a digital caliper and with image J (NIH) after taking a picture just prior to the application of the gauze and 8 weeks later before euthanizing each mouse. Buprenorphine at 0.05 mg/kg was used for post-operative analgesia. All the procedures were approved by the institutional animal care and use committee.

PEG-catalase infusion

In some experiments, mice were subjected to CaCl\textsubscript{2} surgery and infused intravenously with saline or PEG-catalase at a rate of 10,000 Units/Kg/day with an osmotic mini pump (Alzet, Cupertino, CA) connected to the jugular vein through a sterile catheter (Alzet, Cupertino, CA). The duration of PEG-catalase administration was 8 weeks, from 2 days prior to surgery until post-operative week 8. Because the maximum
duration of pump infusion is 4 weeks, the pumps were replaced with new ones after 28
days. In other experiments, wild type mice were first exposed to CaCl₂, and PEG-
catalase or saline was infused from post-operative day 2 to post-operative day 12.

**H₂O₂ quantification**

H₂O₂ levels were measured using the Amplex Red assay (Life Technologies,
Grand Island, NY). At each designated time point the infrarenal aortas were harvested
and cleared from the surrounding fatty tissue on ice, cut into 2 mm rings and incubated
for 50 min in Krebs Ringer's Phosphate Glucose (KRPG) buffer pH 7.4 at 37 °C. A H₂O₂
standard curve was generated with each assay. The levels of H₂O₂ were normalized per
2 mm aortic ring and expressed as pmoles/min/aortic ring.

**Catalase activity**

Catalase activity was measured in plasma and in aortas by quantifying the
amount of H₂O₂ degradation over time using an Amplex Red based assay following the
manufacturer’s instructions (Life Technologies, Grand Island, NY). The aortas were
washed extensively with 0.1 M Tris pH 7.4 before measuring catalase activity in order to
remove completely the red blood cells, which contain high concentrations of catalase.
Catalase activity in aortas was normalized per mg wet weight. For plasma catalase
activity we used dilutions 1/25 in 0.1 M Tris pH 7.4. The blood was withdrawn from the
right ventricle using a large bore needle to avoid hemolysis. The results obtained with
this method were further confirmed using a different catalase activity assay (Cayman
Chemical Company, Ann Arbor, MI), which utilizes the ability of catalase to oxidize
methanol to formaldehyde.

**Histology**

Hematoxylin & Eosin, Verhoeff-Van Gieson, Masson’s Trichrome, and Picrosirius
Red staining were performed using standard protocols. Quantification of media
thickness was done from hematoxylin and eosin stained sections on post-operative
week 8. The media thickness was measured in each section at 3, 6, 9 and 12 o'clock
under 100x magnification using image J (NIH, Bethesda, MD).

**Zymography**
For *in situ* gelatin zymography 7 µm frozen sections were prepared and stored at -80 °C until use. 1% LGT agarose (Sigma, St. Luis, MO) was prepared in Phosphate Buffered Saline (PBS) with and without 20 mmol/L EDTA. DQ Gelatin (Life Technologies, Grand Island, NY) was dissolved to 1 mg/ml in water and mixed 1:10 (vol/vol) with 1% LGT agarose. Forty (40) µl of the final solution was pipetted on each section, the slides were coverslipped and incubated for 10 min at 4 °C and then transferred into a 37°C humid chamber for overnight incubation. Quantification of the fluorescent signal was performed using Image J (NIH, Bethesda, MD). For *in situ* elastin zymography, initially 1% LGT agarose was prepared in 50 mmol/L phosphate, 20 mmol/L EDTA buffer pH 5.5. DQ-elastin (Life Technologies, Grand Island, NY) was dissolved to 1mg/ml in water and diluted 1:10 with 1% LGT agarose/phosphate/EDTA solution in the presence or absence of 1 mmol/L of the cathepsin inhibitor N-Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone (Life Technologies, Grand Island, NY). Forty (40) µl of the final solution was applied on each section, agarose was gelled with a brief incubation at 4 °C and the sections were finally incubated at 37 °C for 48 hrs. Images were acquired with fluorescent microscopy.

Gelatin zymography was performed as described by Godin et al. 3 Briefly, mice were perfused with saline, the infrarenal aortas were dissected and placed in -80 °C until use. Each aorta was homogenized on ice with 150 µl lysis buffer (50 mM Tris, 150 mM NaCl, 10 mM CaCl$_2$, 0.1% SDS, 0.5% Brij 35, 1% Triton X-100, 0.05% azide pH 7.4), the lysates were spun at 10000xg for 15 min at 4 °C and the supernatants were collected. Protein concentration was specified using the Bradford method. Twenty (20) µg of lysates were loaded on each lane of a premade 10% zymogram gel (Bio-Rad, Hercules, CA), then SDS was exchanged with Triton X-100 for 1 hr at room temperature and the gel was incubated with zymogram development buffer (Bio-Rad, Hercules, CA) for 2 days at 37 °C with slight rocking. Next, the gel was stained with Colloidal Brilliant Blue (Bio-Rad, Hercules, CA) for 45 min and destained with destaining buffer (60% water, 30% methanol, 10% acetic acid) for 1 hr.

**Immunohistochemistry and Western Blotting**

Paraffin embedded aortic sections were stained using antibodies for cleaved caspase 3 (9661S, dilution 1:50, Cell Signaling Technology, Inc. Danvers, MA), the macrophage marker mac3 (cat#550292, dilution 1:100, Pharmingen, San Diego, CA) the lymphocyte marker CD3 (Ab5690, dilution 1:100, Abcam, Cambridge, MA), the lipid peroxidation produce HNE (cat# 393207, dilution 1:100, Millipore, Billerica, MA), and smooth muscle α-actin (A5691 dilution 1:50, Sigma, St. Luis, MO). Frozen sections were used to stain for neutrophils (Ab2557, dilution 1:100, Abcam, Cambridge, MA).
and smooth muscle α-actin (A5691, dilution 1:50, Sigma, St. Luis, MO) using standard protocols.

Western blotting for superoxide dismutase 1 and glutathione peroxidase 1&2 was performed using rabbit polyclonal antibodies against superoxide dismutase 1 (sc-11407, dilution 1:200, Santa Cruz Biotechnology, Inc. Dallas, TX), and glutathione peroxidase ½ (sc30147, dilution 1:100, Santa Cruz Biotechnology, Inc. Dallas, TX) using standard protocols. The antibodies were incubated overnight. The secondary antibody was a polyclonal anti-rabbit antibody conjugated with HRP (Bio-Rad, Hercules, CA) and used in dilution 1/2500. Films were developed using ECL.

**Apoptosis & Proliferation**

We used Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling (TUNEL) staining on paraffin embedded sections to assess for apoptotic cell death following the manufacturer’s protocol (Roche Applied Science, Indianapolis, IN). Images were acquired with fluorescent microscopy. To quantify apoptotic cells we counted the number of TUNEL positive cells in the intima and media per aortic section under 100x magnification using ImageJ (NIH, Bethesda, MD). In addition, we quantified the TUNEL positive area over the total vessel area using image J (NIH, Bethesda, MD).

To assess for proliferation, mice were injected with 100 mg/Kg solution of 5-bromo-2'-deoxyuridine (BRDU) in saline for 2 consecutive days before they were euthanized. Staining for proliferating cells were performed in paraffin sections using an antibody against BRDU (Ab1893, Abcam, Cambridge, MA). Quantification of proliferation was performed by counting the number of BRDU positive cells per aortic section, as well as by calculating the percent BRDU positive area using image J (NIH, Bethesda, MD).

**Real time PCR**

Animals were euthanized with CO₂ at the designated time point and perfused with cold saline. The infrarenal aortas were harvested, quickly cleaned from the surrounding fat tissues and snapped frozen in liquid nitrogen until use. The mRNA was isolated using an RNA isolation kit (Qiagen, Valencia, CA) and RT reactions were carried out from 0.5 µg of starting mRNA material using Superscript III (Life Technologies, Grand Island, NY). Following purification of cDNA with a commercial kit (Qiagen, Valencia, CA), real time PCR reactions were performed 18s, TNFα, OPN, MCP-1, CD68, IL-1β, TGFβ1, TGFβ2, MMP-2, MMP-9, IL-6, ICAM-1, SDF-1, catalase, Superoxide Dismutase 1, glutathione peroxidase 1, peroxiredoxin1, and peroxiredoxin2 using mouse specific primer mix (Qiagen, Valencia, CA). Human catalase primer mix was also purchased.
from Qiagen. Copy number for each marker was normalized per $10^6$ copies of the housekeeping gene 18s.

**Statistical analysis**

Data are presented as mean±SEM. Due to the relatively small number of animals the nonparametric Mann-Whitney test was used to examine statistical significance in this study. For multiple groups comparison one way ANOVA was employed. To compare multiple groups at multiple time points two way ANOVA was performed. Statistical tests were performed using GraphPad Prism v.5.00 (GraphPad Software, San Diego CA) and SPSS v.20 (IBM Corporation, Armonk, NY).
Supplemental references


Supplemental figures

Supplemental Figure I. Detection of ECM degrading enzymes (a) MMP-2 mRNA levels and (b) MMP-9 mRNA levels at baseline (day 0), and on day 3 & 10 after CaCl₂ exposure (n=4-5, † p=0.07 vs. Tg<sup>cat-VSMC</sup> on day 10 by two way ANOVA, ‡p=0.09 vs. Tg<sup>cat-VSMC</sup> on day 3 by two way ANOVA), (c) <em>in situ</em> gelatin zymography in a wild type aorta 10 days after CaCl₂ exposure, (d) the same aorta in the presence of the MMP inhibitor EDTA. <em>In situ</em> elastin zymography to detect cathepsin activity 10 days after CaCl₂ exposure in a wild type aorta in the absence (e) or presence (f) of a cathepsins inhibitor. Cathepsin activity in a catalase over-expressing aorta on day 10 after surgery in the absence (g) and presence (h) of a cathepsins inhibitor (scale bar: 50 µm).

Supplemental Figure II. (a) Quantification of apoptosis in wild type and Tg<sup>cat-VSMC</sup> aortas on day 7 after injury using the ratio of TUNEL positive area over total vascular wall area, (b) assessment of the proliferative response in wild type and Tg<sup>cat-VSMC</sup> aortas on day 7 after surgery by counting the number of BRDU positive cells, (c) quantification of cellular proliferation in wild type and transgenic mice on day 7 after injury using the ratio of BRDU positive over total vessel wall area, (d) apoptosis in the aortic wall of wild type mice exposed to CaCl₂ and treated with saline or PEG-catalase (PC) by counting the number of TUNEL positive cells per aortic section, (e) apoptosis in the aortic wall of wild type mice exposed to CaCl₂ and treated with saline or PEG-catalase (PC) using the ratio of TUNEL positive area over total vessel wall, (f) TNFα mRNA levels in the aortas of wild type animals exposed to CaCl₂ and infused with saline or PEG-catalase at baseline (day 0) and on day 3&10 after surgery (* p=0.02 using the non-parametric Mann-Whitney test).

Supplemental Figure III. mRNA levels of the inflammatory markers (a) TGFβ1, (b) MCP-1 and (c) OPN in the aortas of wild type and Tg<sup>cat-VSMC</sup> mice after CaCl₂ exposure. (n=3-5/group, * p<0.05 and † p = 0.1 vs. Tg<sup>cat-VSMC</sup> on day 3 by two way ANOVA).

Supplemental Figure IV. Staining for the macrophage marker mac3 on day 7 after CaCl₂ in a wild type mouse (a) and a catalase over-expressing mouse (b) (Quantification of mac3 positive signal using image J reveals higher levels of macrophage infiltration in wild type compared to Tg<sup>cat-VSMC</sup> mice (n=5/group, * p=0.015 by non-parametric Mann-Whitney test) (c) Neutrophil detection by immunohistochemistry in wild type (d) and Tg<sup>cat-VSMC</sup> (e) mice 3 days after CaCl₂ exposure. Identification of lymphocytes using the marker CD3 (arrowheads) in wild type (f) and Tg<sup>cat-VSMC</sup> (g) mice on day 10 after CaCl₂ surgery (scale bar: 50 µm).

Supplemental Figure V. (a) mRNA levels of the inflammatory markers TGFβ1 in the aortas of wild type mice at baseline (day 0) and after CaCl₂ exposure and treatment with
saline or PEG-catalase (n=5/group, * p<0.001 vs. Tg\textsuperscript{cat-VSMC} on day 3 by two way ANOVA), (b) staining for the macrophage marker mac3 of a wild type aorta exposed to CaCl\textsubscript{2} and infused with saline 7 days after surgery, (c) staining for the macrophage marker mac3 of a wild type aorta exposed to CaCl\textsubscript{2} and infused with PEG-catalase 7 days after surgery (scale bar: 50 µm).

**Supplemental Figure VI.** mRNA levels of the H\textsubscript{2}O\textsubscript{2} degrading enzymes peroxiredoxin 1 (a) and peroxiredoxin 2 (b) in the aortas of wild type and catalase transgenic mice at baseline (day 0) and over time after CaCl\textsubscript{2} exposure (n=5/group).

**Supplemental Figure VII.** mRNA levels of the anti-oxidant enzymes superoxide dismutase 1 (a) glutathione peroxidase 1 (b), peroxiredoxin 1 (c), and peroxiredoxin 2 (d) in the aortas of wild type mice at baseline (day 0) and after CaCl\textsubscript{2} exposure and treatment with saline or PEG-catalase (n=5/group).

**Supplemental Figure VIII.** Staining for smooth muscle α-actin (SMA) 3 days after CaCl\textsubscript{2} surgery. (a) Early loss of VSMC in wild type aortas is apparent on day 3 after chemical injury (arrowheads), whereas (b) catalase over-expressing VSMC are resistant against the chemical injury and the aortic wall appears intact (scale bar: 50 µm).
Supplemental Figure I

(a) MMP-2 mRNA expression over post-op days for wt and Tg\textsuperscript{cat-VSMC}.

(b) MMP-9 mRNA expression over post-op days for wt and Tg\textsuperscript{cat-VSMC}.

(c) wt control with MMP activity.

(d) wt control with MMP activity in the presence of EDTA.

(e) wt control with cathepsin activity.

(f) wt control with cathepsin activity in the presence of inhibitor.

(g) Tg\textsuperscript{cat-VSMC} with cathepsin activity.

(h) Tg\textsuperscript{cat-VSMC} with cathepsin activity in the presence of inhibitor.

†, ‡ indicate statistical significance.
Supplemental Figure II

(a) % TUNEL positive area
(b) # proliferating cells
(c) % BRDU positive area
(d) # apoptotic cells
(e) % TUNEL positive area
(f) TNFa mRNA copies/10^6 18S

wt Tg^{cat-VSMC}
Supplemental Figure III

(a) TGFβ1 mRNA copies/10^6 18S RNA

(b) MCP-1 mRNA copies/10^6 18S RNA

(c) OPN mRNA copies/10^6 18S RNA

Post-op day
Supplemental Figure IV

Neutrophils

DAPI

Tg^{cat-VSMC}

wt

Tg^{cat-VSMC}

% macrophage infiltration

wt Tg^{cat-VSMC}

0 5 10 15

Mac3

DAPI

Neutrophils

DAPI

CD3

DAPI

Supplemental Figure IV
Supplemental Figure V

(a) Graph showing TGFβ1 mRNA copies vs. post-op day for wt+saline and wt+PC groups. The graph indicates a significant increase in copies at post-op day 3 for wt+saline compared to wt+PC.

(b) Images showing Mac3 and DAPI staining for wt+saline.

(c) Images showing Mac3 and DAPI staining for wt+PC.
Supplemental Figure VI

(a) PRXD1 mRNA copies / 10^6 18s
(b) PRXD2 mRNA copies / 10^6 18s

post-op day
Supplemental Figure VII

**SOD1 mRNA**
- wt+saline
- wt+PC

**GPX1 mRNA**
- wt+saline
- wt+PC

**PRXD1 mRNA**
- wt+saline
- wt+PC

**PRXD2 mRNA**
- wt+saline
- wt+PC

*post-op day*
Supplemental Figure VIII

Panel a: wt

Panel b: Tg^{cat-VSMC}

The images show immunofluorescence staining for SMA (red) and DAPI (blue) in tissue sections. Panel a is labeled as wt, and panel b as Tg^{cat-VSMC}.