

BASIC RESEARCH STUDIES

Suppression of experimental abdominal aortic aneurysms in mice by treatment with pyrrolidine dithiocarbamate, an antioxidant inhibitor of nuclear factor- κ B

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Objective: Proinflammatory cytokines and matrix metalloproteinases (MMPs) are prominent mediators of the connective tissue destruction that characterizes abdominal aortic aneurysms (AAAs), and nuclear factor (NF)- κ B is a cytokine-responsive transcription factor that promotes macrophage MMP expression. The purpose of this study was to determine whether aneurysmal degeneration is influenced by pyrrolidine dithiocarbamate (PDTC), a pharmacologic inhibitor of NF- κ B.

Methods: Adult male C57BL/6 mice underwent transient elastase perfusion of the abdominal aorta to induce the development of AAAs. Animals were treated every 48 hours by intraperitoneal injection with either saline (n = 34) or PDTC 20 mg/kg (n = 49). Aortic diameter (AD) measurements were used to determine the extent of aortic dilatation before and immediately after elastase perfusion and again at day 14.

Results: All saline-treated mice developed AAAs associated with mononuclear inflammation and destruction of medial elastin (overall increase in AD, mean \pm SEM, 169.1% \pm 7.5%). In contrast, the incidence of AAAs was only 63% in PDTC-treated mice, with a reduction in the overall increase in AD to 109.8% \pm 4.2% ($P < .0001$ vs saline), decreased inflammation, and structural preservation of aortic wall connective tissue. Although aneurysm development in saline-treated mice was associated with a marked increase in aortic tissue NF- κ B and activator protein 1 DNA-binding activities, both activities were substantially reduced in PDTC-treated animals. PDTC-treated mice also exhibited significantly lower serum and aortic wall concentrations of interleukin 1 β and interleukin 6, as well as lower amounts of aortic wall MMP-9, as compared with saline-treated controls.

Conclusions: Treatment with PDTC inhibits elastase-induced experimental AAAs in the mouse, along with suppression of aortic wall NF- κ B and activator protein 1 transcription factor activities, reduced expression of proinflammatory cytokines, and suppression of MMP-9. NF- κ B is therefore a potentially important therapeutic target for the suppression of aneurysmal degeneration. (*J Vasc Surg* 2005;41:479-89.)

Clinical Relevance: Development and progression of human AAAs is associated with inflammation and enzymatic degradation of connective tissue proteins. MMP-9 is one of the enzymes involved in aneurysm disease, and its production may be induced in part by activation of the transcription factor NF- κ B. In this mouse model, treatment with pyrrolidine dithiocarbamate (a pharmacologic inhibitor of NF- κ B) acted to suppress MMP-9 and aneurysm development. It is hoped that treatment strategies that target NF- κ B may eventually be shown to suppress the growth of small aortic aneurysms in patients.

Abdominal aortic aneurysms (AAAs) are characterized by chronic transmural inflammation and destructive re-

modeling of aortic wall connective tissue.¹⁻³ The inflammatory infiltrates in AAAs are dominated by macrophages and T lymphocytes,⁴ which produce and respond to various mediators, including chemokines, cytokines, prostaglandins, and reactive oxygen species (ROS).⁵⁻⁸ Macrophages in aneurysm tissue are also the principal source of matrix metalloproteinases (MMPs), a family of zinc-dependent enzymes capable of degrading elastin and interstitial collagen, the primary structural proteins of the aortic wall.^{9,10} Investigations in several animal models of aortic aneurysm have demonstrated that increased macrophage expression of MMP-9 plays a critical role in the development and progression of AAAs: suppression of aneurysmal degeneration has been observed in animals treated with nonspecific

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MMP inhibitors (eg, doxycycline)¹¹⁻¹⁴ or with local overexpression of tissue inhibitor of metalloproteinases 1¹⁵ and in mice with targeted deletion of the MMP-9 gene.^{16,17} Further understanding of the mechanisms that stimulate and regulate aortic wall expression of MMP-9 is therefore important for the development of novel therapeutic strategies for aneurysmal disease.

MMP-9 is known to exhibit developmental and tissue-specific patterns of expression distinct from those of other MMPs, consistent with unique mechanisms of transcriptional regulation.^{18,19} MMP-9 is encoded by a 7.7-kilobase gene located on mouse chromosome 2, which consists of 13 exons that correspond in size to the human gene.²⁰ The 5'-flanking regions of the mouse and human MMP-9 genes are characterized by a number of conserved transcriptional control elements, including a TATA box, two to four activator protein (AP)-1 sites, four PEA3/ets sites, three Sp1 sites, several CA-repeat microsatellite regions, an AP-2 site, and a nuclear factor- κ B (NF- κ B) binding motif located -527 to -519 base pairs (bp) upstream from the transcription start site.²⁰⁻²⁴ Like the promoters for other MMP genes, the AP-1 site located in closest proximity to the transcription start site (-50 to -44 bp) is thought to be indispensable for basal and cytokine-induced MMP-9 transcription, whereas the NF- κ B and other sites exert additional modulating effects on inducible MMP-9 gene expression.^{18,19} Although the importance of NF- κ B in activating maximal MMP-9 expression has been demonstrated in a variety of normal and transformed cells in vitro,²⁵⁻²⁹ the functional role of this pathway has only recently been examined in animal models of AAAs.^{30,31}

In addition to endogenous mechanisms of regulation,^{32,33} cellular NF- κ B activity can be inhibited by a variety of different drugs, including salicylates, nonsteroidal anti-inflammatory agents, glucocorticosteroids, proteasome inhibitors, antioxidants, and selective peptide antagonists.^{34,35} Pyrrolidine dithiocarbamate (PDTC) is an antioxidant compound that specifically suppresses NF- κ B activity in both cultured cells and in vivo studies.³⁶⁻⁴¹ To assess the functional role of NF- κ B activation in the pathophysiology of aneurysmal degeneration, the purpose of this study was to determine how treatment with PDTC influences the development of experimental AAAs in the mouse.

METHODS

Experimental animals and drug treatment. Adult male C57BL/6J mice were purchased from The Jackson Laboratory (Bangor, Me). Mice in the experimental group (n = 49) were treated by intraperitoneal injection of 0.03 mL of saline solution containing PDTC 20 mg/kg (Sigma Chemical Company, St Louis, Mo), beginning 1 day before elastase perfusion and continuing every 48 hours for the entire 14-day experimental period, on the basis of previous studies showing the effectiveness of PDTC in other mouse models of inflammatory disease at doses ranging from 10 to 100 mg/kg.^{38,41} The control group consisted of 34 mice treated by intraperitoneal injection of saline solution alone according to the same schedule. All experimental proce-

dures were performed according to a protocol approved by the Animal Studies Committee at Washington University School of Medicine.

Elastase perfusion model and aortic diameter measurements. All mice were subjected to transient perfusion of the abdominal aorta with type I porcine pancreatic elastase to induce the development of AAAs, as described in Appendix I (online only) and in previous reports.¹⁶ At the time of elastase perfusion, the preperfusion aortic diameter (AD Pre) was measured with the assistance of an operating stereomicroscope (Leica, Deerfield, Ill) and a calibrated ocular grid, and the postperfusion AD (AD Post) was measured 5 minutes after flow was restored to the lower extremities. Final AD measurements (AD Final) were obtained during repeat laparotomy 14 days after the initial elastase perfusion procedure, before mice were killed and tissue was procured. For each animal, the extent of immediate aortic dilation was calculated as the percentage increase between the preperfusion and postperfusion AD measurements (Δ AD Pre \rightarrow Post), and the extent of interval aortic dilation was calculated as the percentage increase between the postperfusion and final AD measurements (Δ AD Post \rightarrow Final). The extent of overall aortic dilatation was calculated as the percentage increase between the preperfusion and final AD measurements (Δ AD Pre \rightarrow Final). AAAs were defined as an overall extent of aortic dilatation greater than 100%.

Light microscopy. Specimens of the abdominal aorta were excised after systemic perfusion/fixation with 10% neutral buffered formalin (120 mm Hg for 10 minutes) and embedded in paraffin. Cross sections of aortic tissue (5 μ m) were stained with Verhoeff-van Gieson for elastin and examined by light microscopy. Sections from three animals in each group were scored for the extent of inflammation and elastin content by four different observers unaware of the experimental group by using a one- to five-point scale, and the mean \pm SEM histologic scores were determined for comparisons between PDTC- and saline-treated mice by using the unpaired *t* test.

Electrophoretic gel shift assays. Double-stranded oligodeoxynucleotide DNA probes were prepared that corresponded to the murine recognition elements for NF- κ B (sense strand, 5'-AGT TGA GGG GAC TTT CCC AGG C-3') and AP-1 (sense strand, 5'-CGC TTG ATG ACT CAG CCG GAA-3'), along with nonbinding mutant DNA probes as controls (NF- κ B mutant sense strand, 5'-AGT TGA GGc GAC TTT CCC AGG C-3'; AP-1 mutant sense strand, 5'-CGC TTG ATG ACT tgG CCG GAA-3'). Each DNA probe was end-labeled with ³²P-adenosine triphosphate and purified by Sephadex G-25 column chromatography before use. Nuclear protein extracts were prepared from aortic wall specimens and preincubated for 10 minutes at room temperature with 0.25 μ g of poly(dI-dC) in 10 mmol/L Tris-HCl buffer (pH 7.5) containing 50 mmol/L NaCl, 4% glycerol, 0.5 mmol/L dithiothreitol, 0.5 mmol/L ethylenediamine tetraacetic acid (EDTA), and 1 mmol/L MgCl₂, followed by the addition of 25,000 cpm of ³²P-labeled specific DNA probe for 20 minutes at

room temperature. Samples were resolved by electrophoresis on prerun 4% polyacrylamide gels followed by autoradiography. The specificity of protein/DNA interactions was assessed by incubating nuclear protein samples either with excess unlabeled DNA probe (cold competition) or with 32 P-labeled nonbinding mutant DNA probe. For supershift assays, nuclear protein extracts were preincubated (30 minutes at room temperature) with 1 μ g of antibodies recognizing either p65, for NF- κ B, or c-Fos, for AP-1 (Santa Cruz Biochemicals, Santa Cruz, Calif).

Gelatin zymography. Total protein extracts were prepared from aortic tissue samples, mixed with nonreducing sample buffer containing 0.1% sodium dodecyl sulfate (SDS), and electrophoretically resolved through 10% polyacrylamide gels co-polymerized with 1 mg/mL gelatin substrate (Sigma), as described in Appendix I (online only) and in previous reports.¹⁶ The relative molecular weight of each proteolytic band was estimated by the migration positions of known molecular weight standards (Bio-Rad, Richmond, Calif) and authentic 92- and 72-kd gelatinase standards, and the relative amount of each gelatinase activity was estimated by densitometry. To determine whether PDTC had any direct effect on gelatinase activities, various concentrations of PDTC (0 to 100 μ mol/L) were included in the substrate buffer before visualization of proteolytic bands.

Enzyme-linked immunosorbent assays. Aortic protein extracts and serum samples were analyzed by using commercially available enzyme-linked immunosorbent assay kits specific for mouse interleukin (IL)-1 β and IL-6, according to the manufacturer's instructions (R&D Systems, Inc, Minneapolis, Minn). Each of these assays uses a dual-antibody method with a reported sensitivity of 7.8 pg/mL and no significant cross-reactivity with other cytokines. Spectrophotometric optical density measurements were made at 450 nm by using an automated microplate reader (Bio-Tek Instruments, Inc, Winooski, Vt), and the amount of IL-1 β or IL-6 in each sample was determined from linear standard curves by using recombinant mouse proteins.

Statistical analysis. Data are presented as the mean \pm SEM. Between-group comparisons of AD measurements and calculated Δ AD values were made by using the Mann-Whitney *U* test, and the incidence of AAAs was compared by using the Fisher exact test. Mean histologic scores for inflammation and elastin content were compared by using unpaired *t* tests. Densitometry data (gelatin zymography) and serum and aortic tissue cytokine concentrations were compared by using one-way analysis of variance with the Bonferroni multiple comparisons test. All statistical analyses were performed with InStat3 version 3.0a from GraphPad Software, Inc (San Diego, Calif), and *P* values $<$.05 were considered significant.

RESULTS

Treatment with PDTC suppresses development of elastase-induced AAAs. As illustrated in Fig 1, there were no differences in either preperfusion or immediate postperfusion AD measurements between saline- and PDTC-

treated mice, but final AD measurements in the PDTC-treated group were 20% lower than in the saline-treated controls (*P* $<$.0001; Mann-Whitney *U* test). The median extent of aortic dilatation immediately after elastase perfusion was approximately 65% to 70%, with no significant difference between groups (Δ AD Pre \rightarrow Post, mean \pm SEM: PDTC, 66.0% \pm 1.9%; saline controls, 71.7% \pm 2.2%; Mann-Whitney *U* test), and this is consistent with our previous studies using this mouse model (Fig 1, B).^{16,42-44} Although all animals exhibited secondary (interval) dilatation over the 14-day experimental period, the extent of interval dilatation was 53% less in PDTC-treated mice compared with saline-treated controls (Δ AD Post \rightarrow Final: PDTC, 26.3% \pm 1.9%; saline, 56.1% \pm 2.9%; *P* $<$.0001; Mann-Whitney *U* test; Fig 1, C). This difference was further reflected by a 35% decrease in the overall extent of aortic dilation between PDTC-treated mice and saline-treated controls (Δ AD Pre \rightarrow Final: PDTC, 109.8% \pm 4.2%; saline, 169.1% \pm 7.5%; *P* $<$.0001; Mann-Whitney *U* test), along with a significant difference in the incidence of AAAs (PDTC, 31 [63%] of 49 mice; saline, 34 [100%] of 34 mice; *P* $<$.0001; Fisher exact test; Fig 1, D).

By light microscopy, elastase-induced AAAs in the saline-treated control group were characterized by an intense transmural inflammatory response accompanied by destruction of medial elastin (Fig 2). In contrast, the suppression of AAAs in PDTC-treated mice was associated with preservation of the medial elastic lamellae, decreased inflammation, and a dense fibrocollagenous response in the adventitia (Fig 2).

Treatment with PDTC inhibits aortic wall NF- κ B and AP-1 DNA-binding activities. In saline-treated mice, development of elastase-induced AAAs was associated with a marked increase in aortic wall NF- κ B/DNA-binding activity as compared with normal (unperfused) aorta (Fig 3, A, lanes b and c). In contrast, treatment with PDTC was associated with suppression of this NF- κ B/DNA-binding activity to a level similar to that seen in the unperfused normal aorta (Fig 3, A, lane d). The specificity of the DNA-binding activity for NF- κ B was confirmed by preincubation with a 100-fold excess of unlabeled NF- κ B oligonucleotide probe (cold competitor; Fig 3, A, lane e) and by assays conducted with a nonbinding mutant NF- κ B DNA probe (not shown); there was also a shift in the dominant NF- κ B/DNA-binding activity to a slower-migrating species after preincubation with antibody recognizing the p65 NF- κ B subunit (Fig 3, A, lane f). Additional gel-shift assays revealed a substantial increase in AP-1/DNA-binding activity during development of elastase-induced AAAs in saline-treated controls as compared with normal aorta (Fig 3, B, lanes b and c), as well as a pronounced inhibition of aortic wall AP-1/DNA-binding activity in mice treated with PDTC (Fig 3, B, lane d). The specificity of the DNA-binding activity for AP-1 was confirmed by assays conducted with either unlabeled AP-1 DNA probe or with a nonbinding mutant AP-1 DNA probe (not shown), as well as by preincubation with antibody recognizing c-Fos, one of the principal protein components of the AP-1 complex (Fig 3, B, lane e).

A Aortic Diameter Measurements

Treatment	n	AD Pre	AD Post	AD Final
Saline	34	0.54 +/- 0.01 mm (0.53 mm)	0.92 +/- 0.01 mm (0.95 mm)	1.44 +/- 0.03 mm (1.42 mm)
PDTC	49	0.56 +/- 0.01 mm (0.53 mm)	0.92 +/- 0.01 mm (0.95 mm)	1.16 +/- 0.02 mm (1.16 mm)
Mann-Whitney		P = 0.100	P = 0.721	P < 0.0001

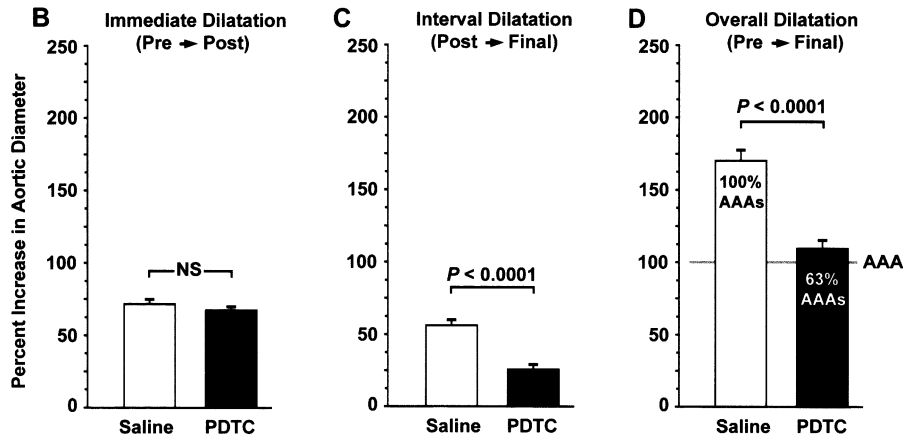


Fig 1. Extent of aortic dilatation. C57BL/6 mice were subjected to transient aortic perfusion with elastase to induce aneurysmal degeneration and were treated every 48 hours with either saline or pyrrolidine dithiocarbamate (PDTC). **A**, Aortic diameter (AD) measurements were obtained before (AD Pre) and immediately after (AD Post) elastase perfusion; final measurements were obtained on day 14 (AD Final). Data shown represent the mean \pm SEM (median values are shown in parentheses). Statistical comparisons between groups were performed with the nonparametric Mann-Whitney *U* test. **B-D**, The extent of aortic dilatation immediately after elastase perfusion (**B**), during the interval between elastase perfusion and death on day 14 (**C**), and the overall extent of dilatation (**D**) were each calculated as percentile changes in AD. Data shown represent the mean \pm SEM. Statistical comparisons between groups were performed with the nonparametric Mann-Whitney *U* test (AAA, Threshold for definition of aortic aneurysm; NS, not significant).

PDTC treatment suppresses aortic wall expression of MMP-9. By gelatin substrate zymography, aneurysm development in saline-treated mice was accompanied by an increase in aortic wall gelatinase activities corresponding to both MMP-2 and MMP-9 (Fig 3, C, lanes 4 to 6). In contrast, metalloproteinase activity corresponding to MMP-9 was significantly less in mice treated with PDTC (Fig 3, C, lanes 7 to 9). Scanning densitometry confirmed a significant decrease in the amount of MMP-9 activity after PDTC treatment compared with saline controls ($P < .05$), but there was no significant difference in the amount of MMP-2 activity (Fig 3, D). There was no evidence that the addition of PDTC to the substrate buffer had any direct inhibitory effects on metalloproteinase activities up to concentrations of 100 μ mol/L (Fig 3, E).

PDTC treatment suppresses aortic wall cytokine production. Immunoassays for IL-1 β and IL-6 protein revealed that both of these cytokines were nearly undetectable in extracts of normal aortic tissue, but aortic wall concentrations of both IL-1 β and IL-6 increased substan-

tially with the development of elastase-induced AAAs in saline-treated mice (Fig 4, A and B). In contrast, the mean aortic wall concentration of IL-1 β was reduced by 66% in PDTC-treated mice (saline, 65.39 ± 15.98 pg/mg protein; PDTC, 22.19 ± 6.52 pg/mg protein; $P < .05$), and the mean concentration of IL-6 was reduced by 67% (saline, 20.45 ± 3.76 pg/mg protein; PDTC, 6.74 ± 2.64 pg/mg protein; $P < .05$). Similar changes in cytokine levels were observed in the systemic circulation: serum IL-1 β levels increased 2.9-fold, and serum IL-6 levels increased 3.3-fold in saline-treated controls (each $P < .05$ vs normal serum; Fig 4, C and D). Mice treated with PDTC exhibited serum IL-1 β levels 42% lower than those of saline-treated controls ($P < .05$), and serum IL-6 levels were reduced by 71% ($P < .05$), to levels equivalent to those found in normal mouse serum.

DISCUSSION

NF- κ B transcription factors play a vital role in cellular responses to immunologic and inflammatory stimuli, isch-

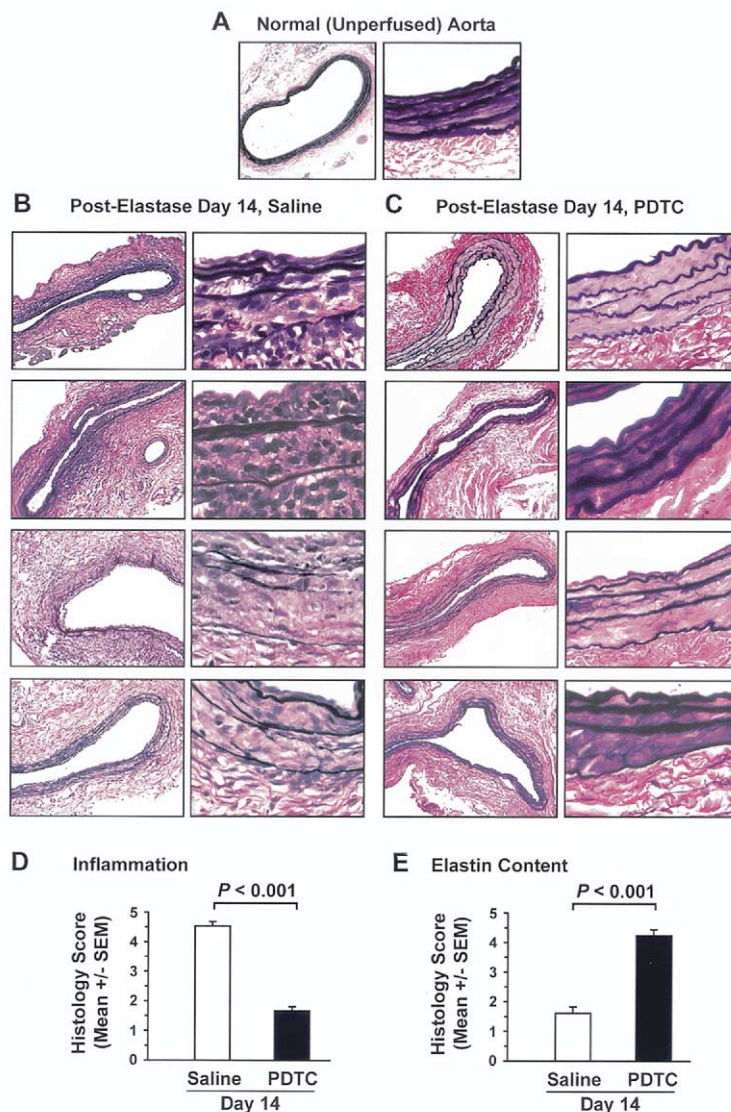


Fig 2. Aortic wall structure. Representative micrographs of aortic wall cross sections taken from normal (unperfused) aorta and aortas from mice 14 days after elastase perfusion and treatment with either saline (control) or pyrrolidine dithiocarbamate (PDTC). All sections were stained with Verhoeff-van Gieson, with low-power views (20 \times) shown on the left and high-power views (100 \times) shown on the right. **A**, Normal aorta. **B**, Day 14 after elastase; saline controls. **C**, Day 14 after elastase; PDTC treatment. **D** and **E**, Histologic sections were scored for the extent of aortic wall inflammation (**D**) and the integrity of aortic elastin (**E**) on a five-point scale, by four different examiners unaware of the treatment groups. Data represent the mean \pm SEM of histologic scores for three different animals in each group. Comparisons were made with the unpaired (two-tailed) Student *t* test.

emia/reperfusion injury, and oxidant stress.^{32,33} NF- κ B is normally sequestered in the cytoplasm as an inactive complex of p50 and/or p65 protein subunits, where it is bound to a member of the endogenous inhibitor of NF- κ B (I κ B) family. Cellular activation by lipopolysaccharide, cytokines, or oxidative stress leads to serine phosphorylation of I κ B, whereupon I κ B is ubiquitinated and targeted for degradation in the proteasome. Upon release from I κ B, active NF- κ B complexes rapidly translocate to the cell nucleus,

where they bind to specific DNA elements (κ B sites) found within the promoter regions of many different genes; this results in activation of gene transcription. Previous studies indicate that NF- κ B activation can be inhibited by PDTC and other antioxidant agents to reduce expression of NF- κ B-responsive gene products, including IL-1 β , IL-6, and metalloproteinases (eg, MMP-1, MMP-3, and MMP-9), and that treatment with PDTC improves outcomes in experimental inflammatory conditions, ischemia/

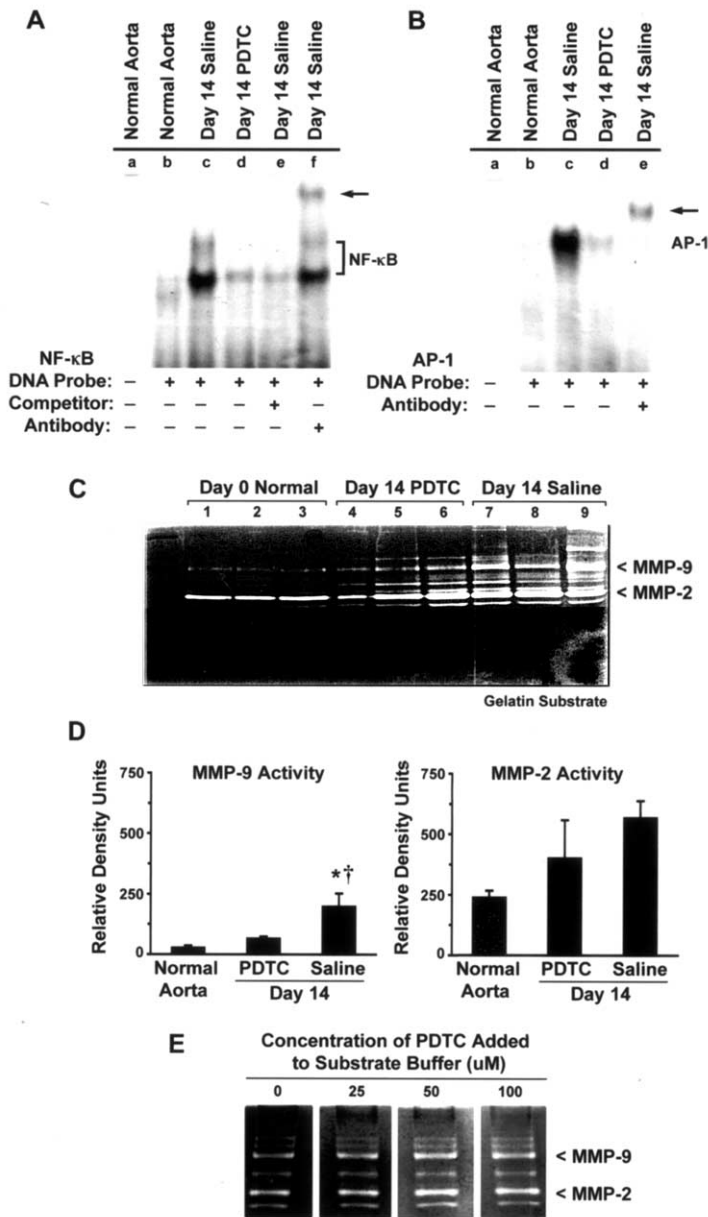


Fig 3. Aortic wall nuclear factor- κ B (*NF- κ B*), activator protein (*AP*)-1, and metalloproteinase (*MMP*) activities. **A and B,** Gel-shift assays for *NF- κ B* (**A**) and *AP*-1 (**B**). DNA-binding activities were performed with nuclear protein extracts from normal aorta and aortas from mice 14 days after elastase perfusion and treatment with either saline or pyrrolidine dithiocarbamate (*PDTC*). Control incubations included an excess of unlabeled DNA probe (competitor) and supershifts with antibodies recognizing p65 for *NF- κ B* or *c-Fos* for *AP*-1 (*arrows*). **C,** Gelatin zymograms performed with total protein extracts showing increased proteinase activity corresponding to *MMP*-2 and *MMP*-9 14 days after elastase perfusion in saline-treated controls, but showing diminished *MMP* activities in animals treated with *PDTC* (triplicate samples shown). **D,** Scanning densitometry of bands corresponding to *MMP*-9 and *MMP*-2 in gelatin zymograms, demonstrating a significant reduction in the amount of *MMP*-9 activity in aortic extracts from *PDTC*-treated mice compared with saline-treated controls. Data shown represent the mean \pm SEM ($n = 3$ for each group). Statistical comparisons were made with one-way analysis of variance with the Student-Newman-Keuls multiple comparisons test ($*P < .05$ vs normal aorta and $\dagger P < .05$ vs *PDTC*-treated group). **E,** Gelatin zymograms containing *MMP*-2 and *MMP*-9 activities (aortic extracts from day 14 saline-treated mice) were incubated in vitro with substrate buffers containing varying concentrations of *PDTC*; they demonstrated no direct inhibition of gelatinase activities.

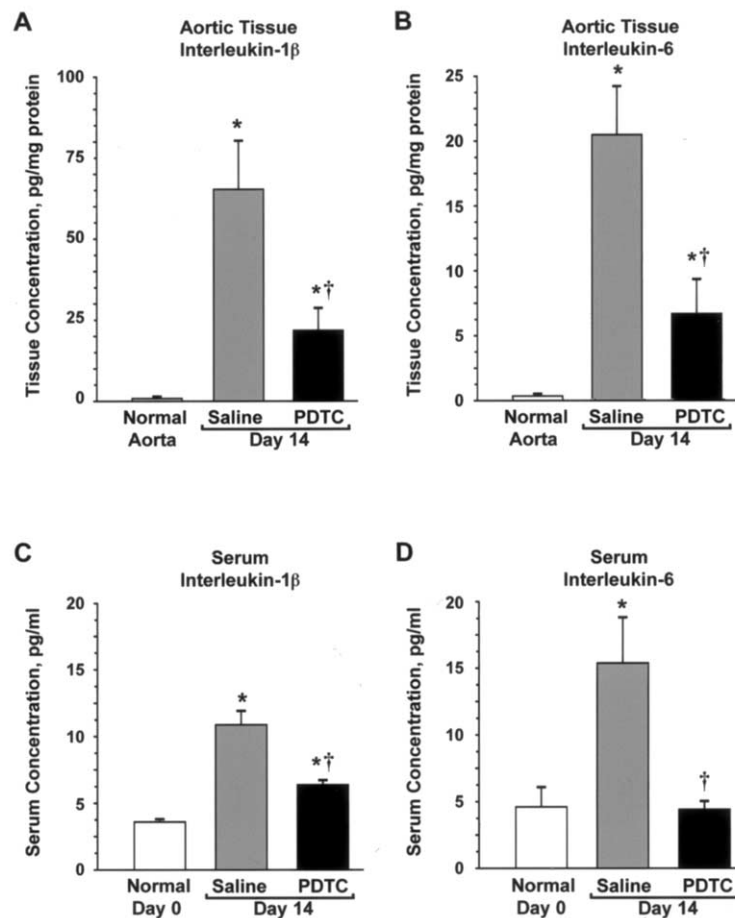


Fig 4. Aortic tissue and serum cytokine levels. Interleukin-1 β and interleukin-6 concentrations were measured by enzyme-linked immunosorbent assay in aortic tissue extracts (**A and B**) and serum (**C and D**) for normal (unperfused) mice and mice treated with either saline or pyrrolidine dithiocarbamate (*PDTC*) for 14 days after elastase perfusion. Data shown represent the mean \pm SEM ($n = 5$ for all groups). Statistical comparisons were made with one-way analysis of variance with the Bonferroni multiple comparisons test (* $P < .05$ vs normal and $\dagger P < .05$ vs day 14 saline-treated controls).

reperfusion injury, and systemic sepsis.³⁶⁻⁴¹ In this study, we demonstrated for the first time that treatment of mice with PDTC suppresses the development of experimental aortic aneurysms in an elastase-induced mouse model. Furthermore, the aneurysm-suppressing effects of PDTC were accompanied by diminished aortic tissue NF- κ B and AP-1 DNA-binding activities; inhibition of aortic wall IL-1 β , IL-6, and MMP-9 expression; and marked reductions in aortic wall inflammation and connective tissue destruction. These findings provide support for the functional importance of oxidative stress and sustained NF- κ B activation within aortic wall tissue during the development of AAAs and suggest that NF- κ B plays a central role in orchestrating the deleterious tissue responses that underlie progressive aneurysmal degeneration.

Electrophoretic gel-shift assays demonstrated increased activation of NF- κ B and AP-1 transcription factor activities within aortic wall tissue during the development of elastase-

induced AAAs and showed that treatment with PDTC was associated with inhibition of both NF- κ B and AP-1. Although signal-dependent activation of transcription factors is often an early and transient event in stimulated cells, the upregulation of NF- κ B and AP-1 observed in day 14 AAAs indicates that activation of these transcription factors is likely a sustained response during the pathophysiologic development of aortic aneurysms. Gel-shift assays demonstrating that treatment with PDTC inhibited NF- κ B activity were also consistent with the known and expected effects of this compound, and, to the extent that these findings reflect overall transcription factor activity in the dominant cell types present in aneurysm tissue, effective inhibition of NF- κ B and AP-1 most likely occurred in macrophages, the most prevalent cell type in these lesions at the 14-day interval examined. Furthermore, the observed reduction in aortic wall expression of IL-1 β , IL-6, and MMP-9 is consistent with this interpretation, because mac-

rophages are also the dominant cellular sources of these molecules in the elastase-induced mouse model of AAAs. It is nonetheless clear that gel-shift assays performed on nuclear protein extracts from aortic tissue reflect the sum of protein/DNA-binding interactions from a complex mixture of cells; thus, the results of these experiments are not as easily interpreted as those of studies performed in isolated cell types, and our data cannot distinguish among events occurring in infiltrating cell types (macrophages and lymphocytes), resident aortic wall mesenchymal cells (smooth muscle cells and fibroblasts), or circulating progenitor cells that may accumulate in damaged aortic tissue. Further study will therefore be needed to elucidate the time course of aortic wall NF- κ B activation in elastase-induced AAAs, the cell types in which NF- κ B activation is most prominent, and the potential resolution of NF- κ B activation that might occur with stabilization of the aneurysm wall at later time intervals.

Suppression of AAAs by treatment with PDTC was associated with preservation of aortic wall elastin and an enhanced fibrocollagenous response within the adventitia. Although PDTC did not completely prevent inflammatory cell infiltration into the elastase-injured aorta, its use was associated with a significant decrease in inflammation and reduced aortic expression of MMP-2 and MMP-9, two of the most prominent elastin-degrading metalloproteinases normally present in AAAs. Although PDTC is known to have metal ion-chelating effects and MMPs are zinc-dependent enzymes, *in vitro* experiments with gelatin zymography revealed no direct inhibition of metalloproteinase activities by PDTC. Because pharmacologic inhibition or gene targeting of these MMPs also leads to suppression of AAAs, the observed preservation of aortic wall connective tissue seems most consistent with a PDTC-mediated reduction in NF- κ B-stimulated macrophage MMP expression. We also observed that treatment with PDTC inhibited IL-1 β and IL-6 production in elastase-induced AAAs. Because the IL-1 β and IL-6 genes are both highly responsive to activation by NF- κ B, it is most likely that PDTC inhibited these cytokines through an NF- κ B-related effect. Because IL-1 β and IL-6 are also known to induce AP-1 and MMP gene expression, decreased cytokine expression might have contributed to the overall suppression of MMP production. Indeed, the importance of these cytokines in experimental aneurysmal degeneration is illustrated by our recent findings that mice with targeted disruption of either IL-1 type I receptor or IL-6 genes are resistant to the development of elastase-induced AAAs (Thompson et al, unpublished data, 2004). PDTC-mediated inhibition of NF- κ B may therefore have had a potent synergistic effect in suppressing macrophage MMP-9 production by direct inhibition of NF- κ B and by indirect inhibition of cytokine-induced AP-1 activation.

Several recent investigations have highlighted the importance of oxidative stress and ROS in the pathophysiology of AAAs.^{8,45,46} Because PDTC is an antioxidant, as well as a specific inhibitor of NF- κ B activation, we cannot exclude the possibility that PDTC exerted its aneurysm-

suppressing effects in part through inhibition of ROS-induced events. However, recent evidence indicates that PDTC suppresses NF- κ B through mechanisms that are independent of its antioxidant activity.⁴⁷ PDTC treatment was also specifically associated with reduced NF- κ B DNA-binding activity and reduced AP-1 activity; because PDTC has actually been reported to activate AP-1 in other systems,⁴⁸⁻⁵⁰ this suggests that inhibition of NF- κ B superseded any activation of AP-1 that might otherwise have occurred within the elastase-injured mouse aorta. Moreover, because it has been shown that NF- κ B activation is required for maximal induction of MMP-9 transcription in some experimental systems,²⁵⁻²⁸ these considerations emphasize the important role played by NF- κ B in MMP-9 induction *in vivo*.

Two recent reports provided the first direct evidence that NF- κ B activation plays a role in experimental AAAs. For example, Nakashima et al³⁰ demonstrated nuclear localization of p65 protein (activated NF- κ B) in adventitial macrophages in human AAA tissues and then used an elastase-induced rat model of AAAs combined with periaortic administration of a decoy oligodeoxynucleotide to block NF- κ B and its transcriptional activities. These experiments demonstrated effective inhibition of AAAs, along with suppression of aortic wall MMP-9 expression, as a consequence of NF- κ B inhibition. More recently, Lawrence et al³¹ used a similar elastase-induced rat model to examine the effects of treatment with the immunosuppressant drug rapamycin. They also observed a significant reduction in aortic dilatation, as well as suppression of NF- κ B activity and inhibition of MMP-9 production. Our study therefore confirms the hypothesis that NF- κ B is a pivotal molecular mediator in aneurysm disease, using a different pharmacologic strategy, and reinforces the specific role of NF- κ B in regulating MMP-9 expression during aortic wall inflammatory responses. Our results also support the notion that therapeutic approaches targeting NF- κ B may be an especially promising strategy to achieve suppression of aneurysmal degeneration.

APPENDIX I. Extended methods

Elastase perfusion model. Mice weighing 20 to 25 g were anesthetized with 55 to 60 mg/kg intraperitoneal sodium pentobarbital, and a laparotomy was performed under sterile conditions. The abdominal aorta was isolated with the assistance of an operating stereomicroscope (Leica), and the preperfusion AD was measured with a calibrated ocular grid. After temporary ligatures were placed around the proximal and distal aorta, an aortotomy was created at the bifurcation by using the tip of a 30-gauge needle. A heat-tapered segment of PE-10 polyethylene tubing was introduced through the aortotomy and secured, and the aortic lumen was perfused for 5 minutes at 100 mm Hg with saline containing type I porcine pancreatic elastase (0.414 U/mL; Sigma). After the perfusion catheter was removed, the aortotomy was repaired without constriction of the lumen, and the AD Post was measured 5 minutes

after flow was restored to the lower extremities. Animals were allowed free access to food and water for 14 days.

Light microscopy. Specimens of the abdominal aorta were excised after systemic perfusion/fixation with 10% neutral buffered formalin (120 mm Hg for 10 minutes) and embedded in paraffin. Cross sections of aortic tissue (5 μ m) were stained with Verhoeff-van Gieson for elastin and examined by light microscopy. The extent of aortic wall inflammation and the integrity of aortic wall elastin were each estimated with histologic scoring by using sections taken from mice 14 days after elastase perfusion and treatment with either saline or PDTC. Sections from three animals in each group were scored for the extent of inflammation and elastin content by four different observers who were unaware of the experimental group. The scoring system for aortic inflammation was based on a one- to five-point scale, with 1 representing no inflammation and 5 representing a severe transmural inflammatory response. A similar one- to five-point scale was used to score aortic wall elastin content, with 1 representing no detectable elastin and 5 representing normal-appearing elastic lamellae. Individual histologic scores for each animal were based on the average scores obtained by examination of four high-power (100 \times) fields per examiner, and the average scores for three animals in each experimental group were used to determine the mean \pm SEM histologic scores for that group. Comparisons of the mean inflammation and elastin content scores between PDTC- and saline-treated mice were made by using the unpaired *t* test.

Electrophoretic gel shift assays. To evaluate activation of NF- κ B and AP-1 transcription factors during the development of AAAs, nuclear protein extracts were prepared from aortic wall specimens obtained before or 14 days after elastase perfusion in PDTC- or saline-treated mice. A DNA probe corresponding to the murine NF- κ B/c-Rel recognition element was prepared that consisted of a double-stranded oligodeoxynucleotide with 5'-AGT TGA GGG GAC TTT CCC AGG C-3' as the top strand and 5'-GCC TGG GAA AGT CCC CTC AAC T-3' as the complementary strand. As a control, a nonbinding mutant NF- κ B probe was prepared with 5'-AGT TGA GGc GAC TTT CCC AGG C-3' as the top strand and 5'-GCC TGG GAA AGT CgC CTC AAC T-3' as the complementary strand. A DNA probe was also prepared to correspond to the murine AP-1 recognition element: it consisted of a double-stranded oligodeoxynucleotide with 5'-CGC TTG ATG ACT CAG CCG GAA-3' as the top strand and 5'-TTC CGG CTG AGT CAT CAA GCG-3' as the complementary strand, and a nonbinding mutant AP-1 probe was prepared with 5'-CGC TTG ATG ACT tgG CCG GAA-3' as the top strand and 5'-TTC CGG Cca AGT CAT CAA GCG-3' as the complementary strand. Each DNA probe was end-labeled by using T4 polynucleotide kinase in the presence of 32 P-adenosine triphosphate and purified by Sephadex G-25 column chromatography before use. Nuclear protein samples (10 μ g in a 10- μ L reaction volume) were preincubated for 10 minutes at room temperature with 0.25 μ g of poly(dI-dC) in 10 mmol/L Tris-HCl

buffer (pH 7.5) containing 50 mmol/L NaCl, 4% glycerol, 0.5 mmol/L dithiothreitol, 0.5 mmol/L EDTA, and 1 mmol/L MgCl₂, followed by the addition of 25,000 cpm of 32 P-labeled specific DNA probe and incubation for 20 minutes at room temperature. Samples were resolved by electrophoresis (200 V for 4 hours) on prerun 4% polyacrylamide gels followed by autoradiography. To assess the specificity of protein/DNA interactions, nuclear protein samples were incubated with a 100-fold excess of unlabeled DNA probe (cold competition) or with labeled nonbinding mutant DNA probes. For supershift assays, before incubation with the corresponding DNA probe, the nuclear protein extracts were incubated for 30 minutes at room temperature with 1 μ g of antibodies recognizing the NF- κ B p65 subunit or with antibodies recognizing c-Fos for AP-1 (Santa Cruz Biochemicals).

Gelatin zymography. To assess aortic wall expression of MMP-2 and MMP-9, frozen aortic tissue samples were pulverized under liquid nitrogen and extracted in ice-cold 50 mmol/L Tris-HCl buffer (pH 7.5) containing 1.0 mol/L NaCl, 2.0 mol/L urea, 0.1% EDTA, and 0.1% (wt/vol) Brij-35. After centrifugation at 10,000*g* for 1 hour at 4°C, the supernatant was dialyzed overnight against 50 mmol/L Tris HCl (pH 8.0), 1.0 mol/L NaCl, and 20 mmol/L CaCl₂ by using a 12,000 to 14,000 molecular weight cutoff membrane. The total protein concentration of each sample was determined by using an assay kit purchased from Bio-Rad (Hercules, Calif). Protein-normalized samples were mixed with nonreducing sample buffer containing 0.1% SDS and were then electrophoretically resolved through 10% polyacrylamide gels co-polymerized with 1 mg/mL gelatin substrate (Sigma). Gels were washed free of SDS with 2.5% Triton X-100 and incubated overnight at 37°C in a substrate buffer containing 50 mmol/L Tris-HCl (pH 8.5), 5 mmol/L CaCl₂, and 0.02% NaN₃. After staining with 0.1% Coomassie blue R-250 in 40% methanol/10% acetic acid, gelatinase activities were observed as clear bands against a dark background of intact substrate. The relative molecular weight of each proteolytic band was estimated by the migration positions of known molecular weight standards (Bio-Rad, Richmond, Calif) and authentic 92- and 72-kd gelatinase standards, and the relative amount of each gelatinase activity was estimated by densitometry. To determine whether PDTC had any direct effect on gelatinase activities, various concentrations of PDTC (0 to 100 μ mol/L) were included in the substrate buffer before visualization of proteolytic bands.

Enzyme-linked immunosorbent assays. To compare systemic and aortic wall cytokine production during aneurysm development in PDTC- and saline-treated mice, serum and aortic protein extracts were analyzed with commercially available enzyme-linked immunosorbent assay kits specific for mouse IL-1 β and IL-6 (R&D Systems). Each of these assays uses a dual-antibody method with a reported sensitivity of 7.8 pg/mL and no significant cross-reactivity with other cytokines. Spectrophotometric optical density measurements were made at 450 nm by using an automated microplate reader (Bio-Tek Instruments), and

the amount of IL-1 β or IL-6 in each sample was determined from linear standard curves by using recombinant mouse proteins.

Statistical analysis. Data are presented as the mean \pm SEM. Between-group comparisons of AD measurements and calculated Δ AD values were made with the Mann-Whitney *U* test, and the incidence of AAAs was compared by using the Fisher exact test. Mean histologic scores for inflammation and elastin content were compared by using unpaired *t* tests. Densitometry data (gelatin zymography) and serum and aortic tissue cytokine concentrations were compared by using one-way analysis of variance with the Bonferroni multiple comparisons test. All statistical analyses were performed with InStat3 version 3.0a from Graph-Pad Software, and *P* values $<.05$ were considered significant.

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