Superoxide–lowering therapy with TEMPOL reverses arterial dysfunction with aging in mice

Bradley S. Fleenor, **Douglas R. Seals**, **Melanie L. Zigler**, and **Amy L. Sindler**
Department of Integrative Physiology, University of Colorado, Boulder, CO, USA

Abstract

To test the hypothesis that the antioxidant enzyme superoxide dismutase (SOD) mimetic TEMPOL improves arterial aging, young (Y, 4–6 mo) and old (O, 26–28 mo) male C57BL6 mice received regular or TEMPOL-supplemented (1mM) drinking water for 3 weeks (n=8/group). Aortic superoxide was 65% greater in O ($p<0.05$ vs. Y), which was normalized by TEMPOL. O had large elastic artery stiffening, as indicated by greater aortic pulse wave velocity (aPWV, 508 ± 22 vs. 418 ± 22 AU), which was associated with increased adventitial collagen I expression ($p<0.05$ vs. Y). TEMPOL reversed the age-associated increases in aPWV (434 ± 21 AU) and collagen *in vivo*, and SOD reversed increases in collagen I in adventitial fibroblasts from older rats *in vitro*. Isolated carotid arteries of O had impaired endothelial function as indicated by reduced acetylcholine-stimulated endothelium-dependent dilation (EDD) (75.6 ± 3.2 vs. $94.5 \pm 2.0\%$) mediated by reduced nitric oxide (NO) bioavailability (L-NAME) associated with decreased endothelial NO synthase (eNOS) expression ($p<0.05$ vs. Y). TEMPOL restored EDD ($94.5 \pm 1.4\%$), NO bioavailability and eNOS in O. Nitrotyrosine and expression of NADPH oxidase were ~100–200% greater, and MnSOD was ~75% lower in O ($p<0.05$ vs. Y). TEMPOL normalized nitrotyrosine and NADPH oxidase in O, without affecting MnSOD. Aortic pro-inflammatory cytokines were greater in O ($p<0.05$ vs. Y) and normalized by TEMPOL. Short-term treatment of excessive superoxide with TEMPOL ameliorates large elastic artery stiffening and endothelial dysfunction with aging, and this is associated with normalization of arterial collagen I, eNOS, oxidative stress and inflammation.

Keywords

superoxide dismutase; inflammation; collagen; nitric oxide; glycation endproducts

Introduction

Advancing age is the major risk factor for cardiovascular diseases (CVD) and this is largely attributable to stiffening of the large elastic arteries and the development of vascular endothelial dysfunction (Lakatta 2003; Mitchell *et al.* 2010). Large elastic artery stiffening with aging is mediated in part by molecular changes to the arterial wall including increased collagen I deposition, reductions in elastin and post-translational modifications of these proteins involving the formation of advanced glycation end-products (AGEs) (Lakatta 2003). Vascular endothelial dysfunction develops with aging as a result of reductions in the

Corresponding Author: Bradley S. Fleenor, Ph.D., University of Colorado, 354 UCB, Boulder, CO 80309, Phone: 303-492-3010, Fax: 303-492-6778, bradley.fleenor@colorado.edu.

nitric oxide (NO) bioavailability, as indicated by impaired NO-mediated endothelium-dependent dilation (Celermajer *et al.* 1994) (Taddei *et al.* 1995; d'Uscio *et al.* 1997). The latter may occur with or without reductions in endothelial NO synthase (eNOS) (Spier *et al.* 2004; Durrant *et al.* 2009; Rippe *et al.* 2010), the enzyme responsible for NO production in the vascular endothelium.

The primary mechanism believed to cause arterial dysfunction with aging is oxidative stress, a state in which bioavailability of reactive oxygen species exceeds the capacity of antioxidant defenses, leading to cellular dysfunction and damage. We recently showed increased superoxide production in aorta of old mice using direct measurement with spin trapping and electronic paramagnetic resonance spectroscopy (Rippe *et al.* 2010; Sindler *et al.* 2011) suggesting, along with earlier reports (Hamilton *et al.* 2001; Francia *et al.* 2004; Lesniewski *et al.* 2009; Sindler *et al.* 2009), that this is a key contributor to oxidative stress in arterial aging. Excessive superoxide may cause vascular aging in part by stimulating inflammation, as characterized by increased expression of pro-inflammatory cytokines (Zou *et al.* 2006; Csiszar *et al.* 2008; Ungvari *et al.* 2010; Lesniewski *et al.* 2011; Sindler *et al.* 2011). As such, therapies that reduce superoxide bioavailability have the potential to ameliorate arterial oxidative stress and inflammation, and improve vascular dysfunction with aging.

In the present study, we tested the hypothesis that short-term treatment with TEMPOL, a superoxide dismutase (SOD) mimetic that scavenges superoxide anion (Simonsen *et al.* 2009), would reduce arterial superoxide bioavailability, decrease large elastic artery stiffness, and improve NO-mediated vascular endothelial function in old mice. We also hypothesized that the improvements in arterial function in old mice would be associated with evidence of reduced oxidative stress and inflammation. Finally, we sought to gain preliminary insight into some of the molecular mechanisms by which TEMPOL treatment may mediate these changes.

Results

Animal characteristics

Animal characteristics of the groups (n=8/group) are shown in Table 1. Body mass, heart mass and carotid artery baseline diameter were greater in the old compared with the young control mice (p<0.05). *Ex vivo* carotid artery preconstriction to phenylephrine and arterial blood pressure, the latter measured in a separate cohort of animals (n=3–4/group), did not differ with age. TEMPOL treatment had no effects on any of the aforementioned variables in the young or old mice.

TEMPOL treatment decreases arterial superoxide production in old mice

Aortic superoxide production was greater in the old compared with the young control mice (p<0.05, Figure 1). TEMPOL treatment normalized superoxide production in the old mice without affecting the young mice. These results indicate that TEMPOL is effective in lowering the excessive arterial superoxide production associated with aging.

TEMPOL treatment reduces aortic pulse wave velocity in old mice

Aortic pulse wave velocity was greater in old compared with young control mice (p<0.05, Figure 2). TEMPOL normalized aortic pulse wave velocity in old mice without affecting young mice. These results indicate that superoxide-lowering therapy with TEMPOL reverses age-associated large elastic artery stiffening.

TEMPOL treatment reduces collagen I expression in old mice

Collagen I was greater ($p<0.05$) in the adventitial layer of old compared with young control mice with no age-related changes observed within the medial layer (Figure 3A and B). TEMPOL treatment reversed the age-associated increase of collagen I in the adventitia, while also reducing expression in the media of young and old mice ($p<0.05$).

Elastin was lower in the media, but not in the adventitia, of old compared with young control animals ($p<0.05$, Table 2). TEMPOL reduced elastin in the young animals and further reduced expression in the old mice ($p<0.05$).

Expression of AGEs was greater in the adventitial and medial layers of old compared with young control animals ($p<0.05$, Table 2). TEMPOL treatment had no effect on AGEs in either age group.

SOD treatment inhibits superoxide-induced collagen deposition in vitro

Because the only effect of TEMPOL on these proteins that could contribute to reductions in stiffness was the reduction in collagen I, additional experiments were performed in cultured rat aortic adventitial fibroblasts to further link SOD treatment to inhibition of superoxide-induced collagen deposition. Pyrogallol, a superoxide generator, increased collagen I protein in fibroblasts obtained from aorta of young rats by ~100% ($p<0.05$, Figure 3C). Treatment with PEG-SOD completely prevented the induction of collagen by superoxide in these cells. Adventitial fibroblasts cultured from aorta of old rats had greater collagen I protein compared with cells from young animals, and this was reversed with PEG-SOD treatment ($p<0.05$, Figure 3D). Collectively, these observations indicate that inhibiting superoxide bioavailability in vitro, as occurred in the arteries of the old mice with short-term TEMPOL treatment in vivo, prevents and reverses collagen I deposition in cells known to populate the adventitia, i.e., the arterial layer in which collagen expression was normalized by TEMPOL.

TEMPOL treatment ameliorates vascular endothelial dysfunction in old mice

Old animals had lower endothelium-dependent dilation to acetylcholine compared with young control mice ($p<0.05$) due to a smaller NO influence, as indicated by a smaller reduction in endothelium-dependent dilation in the presence vs. absence of the NO inhibitor N-G-nitro-L-arginine methyl ester (L-NAME) (Figure 4 A and B). TEMPOL improved endothelium-dependent dilation to acetylcholine in old mice by restoring NO-mediated dilation, but had no effect in the young animals (Figure 4 A and B). Endothelium-independent dilation to sodium nitroprusside did not differ among the groups (Figure 4C). Old animals had lower eNOS protein in the aorta compared with young control animals (Figure 4D) and this was normalized by TEMPOL treatment. These data demonstrate that TEMPOL restores NO-mediated endothelium-dependent dilation and eNOS in old mice in the absence of improvements in vascular smooth muscle sensitivity to NO.

TEMPOL treatment normalizes vascular oxidative stress and NADPH oxidase in old mice

In aortas of old compared with young mice, nitrotyrosine, a marker of oxidative stress, was ~ 200% greater ($p<0.05$) (Figure 5A), and was associated with an ~100% increase in the p67 subunit of the oxidant enzyme nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) ($p<0.05$) (Figure 5B). TEMPOL normalized aortic nitrotyrosine in old mice and reversed the age-associated increases in aortic NADPH oxidase p67 expression. Protein expression of the antioxidant enzyme manganese SOD was decreased in the aorta of old mice ($p<0.05$), but this was unaffected by TEMPOL treatment (Figure 5C). These results indicate the treatment with TEMPOL reverses vascular oxidative stress in old mice and that this is associated with normalization of expression of the oxidant enzyme NADPH oxidase.

TEMPOL treatment ameliorates vascular inflammation in old mice

Protein expression of the pro-inflammatory cytokines interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor α (TNFα) was increased in the aortas of old compared with young mice ($p<0.05$, Figure 6). TEMPOL normalized expression of these inflammatory cytokines in old mice, while having no effect in the young controls. These observations indicate that short-term suppression of superoxide with TEMPOL ameliorates arterial inflammation with aging.

Discussion

Advancing age is the major risk factor for CVD due primarily to stiffening of the large elastic arteries and development of vascular endothelial dysfunction. Thus, treatments that can prevent or reverse these changes to arteries with aging hold great promise for preventing age-associated CVD. The novel finding of the present study is that short-term treatment with TEMPOL, an exogenous SOD mimetic, normalizes the age-associated increase in arterial superoxide production measured directly by spin trapping and electron paramagnetic resonance spectroscopy, and ameliorates large elastic artery stiffness and carotid artery endothelial dysfunction in old C57BL6 mice. These preclinical findings suggest that TEMPOL may have translational efficacy as a pharmacological intervention for treatment of arterial aging in humans. Our findings also provide preliminary insight into the mechanisms by which TEMPOL may exert these favorable effects on aging arteries, including inhibition of adventitial collagen I deposition, increasing NO bioavailability and reversing vascular oxidative stress and inflammation.

Large elastic artery stiffness

The present findings are consistent with previous work from our laboratory (Sindler *et al.* 2011) and others (Reddy *et al.* 2003) demonstrating increases in aortic pulse wave velocity with aging in mice, reflective of large elastic artery stiffening. Our results extend these earlier observations by demonstrating that short-term treatment with the SOD mimetic TEMPOL completely reverses this age-associated increase in aortic pulse wave velocity. These findings have important implications for aging in humans because elevated aortic pulse wave velocity recently has emerged as a strong independent risk factor for incident CVD in older adults (Mitchell *et al.* 2010). Thus, the present data provide critical preclinical evidence for assessing the efficacy of TEMPOL for treatment of large elastic artery stiffness in middle-aged and older adults.

Our results also provide insight into some of the mechanisms that may be involved. Arterial blood pressure influences aortic pulse wave velocity (Cavalcante *et al.* 2011). However, consistent with previous findings on normotensive animals (Schnackenberg & Wilcox 1999; Gaubert *et al.* 2007; Mariko *et al.* 2011), we observed no significant differences in blood pressure with aging or with TEMPOL, suggesting that blood pressure did not contribute to the increased aortic pulse wave velocity with aging or with the reductions with TEMPOL treatment in old mice. Rather, these data further suggest that changes to the arterial wall were involved.

Structural modifications to the arterial wall involving increased collagen I deposition, reduced elastin expression and cross-linking of these proteins by AGEs are thought to contribute to age-associated stiffening of large elastic arteries (Lakatta & Levy 2003) (Kass *et al.* 2001). As we reported recently (Fleenor *et al.* 2010), expression of collagen I, the major collagen in arteries (Diez 2007), was selectively increased within the arterial adventitia in old compared with young control animals, whereas elastin was reduced specifically in the medial layer with aging (Wang & Lakatta 2002; Csiszar *et al.* 2007;

Fleenor *et al.* 2010). As observed previously (Qiu *et al.* 2007), abundance of AGEs was greater in the aortas of older animals and we extended these findings here by showing that this occurs in both the adventitial and medial layers. The only change in these potential mechanisms with TEMPOL that was directionally associated with the destiffening influence observed was a reduction in adventitial collagen I expression. Although consistent with previous observations that TEMPOL reduces elastin expression in vascular smooth muscle cells (Klemm *et al.* 2011), the TEMPOL-induced decreases in medial elastin found in the present study would, if anything, act to further increase stiffness in the aorta of the old animals, and no effects on AGEs were observed.

Because the results of our in vivo treatment suggested that TEMPOL may have reduced aortic adventitial collagen by reducing superoxide bioavailability, we sought to establish further evidence for this mechanism of action in vitro. We studied fibroblasts given their prevalence in the arterial adventitia and used PEG-SOD to directly scavenge superoxide (Lijnen *et al.* 2008). As we showed recently (Fleenor *et al.* 2010), superoxide generation produced by administration of the agent pyrogallol induced an ~100% increase in collagen I of fibroblasts cultured from aorta of young animals. This was completely prevented by administration of PEG-SOD. To determine if superoxide scavenging could reverse age-associated increases in collagen I expression, fibroblasts from aorta of old rats were treated with PEG-SOD and this normalized collagen I expression to levels observed in cells from young animals. Together, the results of these in vitro experiments support the idea that TEMPOL reduced arterial collagen I expression in old mice by inhibiting the actions of superoxide.

Vascular endothelial dysfunction

Consistent with previous reports from our laboratory (Rippe *et al.* 2010; Sindler *et al.* 2011), we found that endothelium-dependent dilation was impaired in the carotid arteries of old mice due to a reduction in the NO-mediated dilatory component, and that this was associated with a decrease in eNOS protein expression. The results of the present study extend these earlier findings by demonstrating that treatment of excessive superoxide with TEMPOL completely restores endothelium-dependent dilation in old mice by improving NO bioavailability and that this is associated with normalization of eNOS protein. TEMPOL treatment had no effect on endothelium-independent dilation, indicating that the improvements in endothelium-dependent dilation were mediated by increases in endothelial NO production. The data also are consistent with our previous work showing that acute *ex vivo* administration of TEMPOL to carotid arteries of old mice restores endothelium-dependent dilation (Durrant *et al.* 2009; Rippe *et al.* 2010; Lesniewski *et al.* 2011; Sindler *et al.* 2011). Most importantly, these results demonstrate for the first time that treatment of excessive age-associated superoxide may have therapeutic potential to prevent and/or reverse vascular endothelial dysfunction in middle-aged and older humans. This has substantial clinical implications as endothelial dysfunction is considered the primary antecedent to the development of age-related clinical cardiovascular disorders including coronary and peripheral artery disease, hypertension and stroke (Lakatta & Levy 2003).

Oxidative stress and inflammation

Our finding of increased arterial oxidative stress with aging, as indicated by marked increases in aortic nitrotyrosine staining in the old mice, is in agreement with previous reports from our laboratory (Rippe *et al.* 2010; Lesniewski *et al.* 2011; Sindler *et al.* 2011) and others (van der Loo *et al.* 2000; Csiszar *et al.* 2002; Yang *et al.* 2009). Nitrotyrosine is produced by post-translational nitration of tyrosine residues primarily by peroxynitrite, a byproduct of the reaction between superoxide and NO (Radi 2004). As we have reported previously, the greater arterial oxidative stress in our old animals was associated with

increased superoxide production, which was, in turn, associated with increased expression of the superoxide-producing enzyme, NADPH oxidase (Durrant *et al.* 2009; Rippe *et al.* 2010), and reduced expression of the mitochondrial-expressed antioxidant enzyme, manganese SOD (Sindler *et al.* 2011). The present findings show that short-term treatment with TEMPOL normalizes aortic nitrotyrosine staining, superoxide and expression of the p67 subunit of NADPH oxidase 2 without influencing manganese SOD in old mice. Overall, these results are consistent with the concept that TEMPOL reverses age-associated arterial oxidative stress via normalization of superoxide bioavailability. Our data also show for the first time that TEMPOL reduces expression of the major superoxide-producing enzyme in vascular tissue, the p67 subunit of NADPH oxidase 2, and this could contribute to the reduction in superoxide concentrations observed.

Excessive superoxide and oxidative stress are believed to stimulate inflammation with aging (Csiszar *et al.* 2008; Ungvari *et al.* 2010). Consistent with previous reports by our laboratory (Lesniewski *et al.* 2011; Sindler *et al.* 2011) and others(Csiszar *et al.* 2007; Csiszar *et al.* 2004; Zou *et al.* 2006; Ungvari *et al.* 2007), expression of pro-inflammatory cytokines was increased in aorta of old mice in the present study. Here we extend these earlier observations by showing that 3 weeks of treatment with TEMPOL normalizes aortic pro-inflammatory cytokines in old mice. These results demonstrate novel anti-inflammatory effects of TEMPOL in arteries with aging, and suggest therapeutic potential of this compound for treatment of vascular inflammatory disorders including arterial aging.

Conclusions

Short-term treatment with the SOD mimetic TEMPOL normalized arterial superoxide production and ameliorated large elastic artery stiffness and endothelial dysfunction, while reversing oxidative stress and inflammation in old mice. These preclinical findings provide the experimental basis for translational studies aimed at determining the efficacy of TEMPOL in the prevention and treatment of arterial aging in humans.

Experimental procedures

Animals

Young (4–6 months) and old (26–28 months; ~50% survival) male C57BL6 mice were obtained from the National Institute on Aging rodent colony and were fed normal rodent chow *ad libitum*. After an acclimation period of 2 weeks, the young and old mice were divided into two subgroups: control animals continued on regular drinking water whereas the treated animals had TEMPOL supplemented at a dose (1mM) used previously in vivo (Schnackenberg & Wilcox 1999), (Yanes *et al.* 2005). Mice (8/group) were treated for a period of 3 weeks because we recently found that duration of treatment with different pharmacological agent to be effective in lowering superoxide and improving large elastic artery stiffness and EDD in old C57BL6 mice (Sindler *et al.* 2011). All mice were housed in an animal care facility at the University of Colorado at Boulder on a 12 h:12 h light-dark cycle. All animal procedures conformed to the *Guide to the Care and Use of Laboratory Animals* (NIH publication n. 85-23, revised 1996) and were approved by the UCB Animal Care and Use Committee.

Arterial superoxide production

Production of superoxide was measured by electron paramagnetic resonance (EPR) spectrometry as previously described (Rippe *et al.*; Sindler *et al.* 2011). Two-millimeter aortic rings were incubated for 60 min at 37° C in 200 µl of Krebs-HEPES buffer containing 0.55 mmol/L 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (Alexis

Biochemicals) and analyzed immediately on an MS300 X-band EPR spectrometer (Magnettech, Berlin, Germany).

Aortic pulse wave velocity—Aortic pulse wave velocity was measured as described previously (Kim *et al.* 2009; Sindler *et al.* 2011). Mice were anesthetized with 2% isoflurane and placed supine on a heating board with legs secured to ECG electrodes. Aortic velocity was measured with Doppler probes at the transverse aortic arch and abdominal aorta. Pre-ejection time, the time between the R-wave of the ECG to foot of the Doppler signal, was determined for each site. Aortic pulse wave velocity was calculated by dividing the distance between the transverse and abdominal probes by the difference in the thoracic and abdominal pre-ejection times.

Arterial Blood pressure—Systolic and diastolic blood pressure was assessed in a separate cohort of mice (n=3–4/group) using a CODA non-invasive tail-cuff system (Kent Scientific)(Daugherty *et al.* 2009). Mice were placed in restrainers on a warm pad. After 10 minutes, 5 acclimation cycles followed by 20 data collection cycles of blood pressure measurements were recorded. Blood pressure was acquired on 3 consecutive days and averaged. The inter-day coefficient of variation for systolic and diastolic blood pressure was 7 ± 1 and 11 ± 2 mmHg, respectively.

Immunohistochemistry—Immunohistochemistry was performed as previously described (Fleenor *et al.* 2010). Briefly, segments of thoracic aorta were excised and frozen in optimal cutting temperature compound (Fisher Scientific) in liquid nitrogen cooled isopentane. Sections (7 μm) were fixed with acetone and washed in Tris Buffer. All slides were stained in one batch with the Dako EnVision+ System-HRP-DAB kit according to the manufacturer's protocol (Dako). Primary antibodies for collagen type I (1:4000, Millipore), alpha elastin (1:25, Abcam) and AGEs (1:200, GeneTex) were incubated for 1 hour at 4< C. The labeled polymer secondary was applied for 30 minutes and staining was visualized after a 2-minute exposure to diaminobenzidine. Slides were counterstained with hematoxylin to visualize the nuclei that were subsequently dehydrated and cover slipped. Digital photomicrographs were obtained using a Nikon Eclipse TS100 photomicroscope, and quantification was performed with Image-Pro Plus software (Media Cybernetics) as described previously (Fleenor *et al.* 2010)

Primary adventitial fibroblast cell culture—Male Sprague-Dawley rats (6 and 24 months of age) were obtained from the University of Colorado at Boulder breeding colony. Rats were euthanized with an overdose of carbon dioxide. Adventitial fibroblasts were isolated from the aorta as previously described (Pagano 1997) (Fleenor *et al.* 2010). To determine the effects of the superoxide generator pyrogallol (Sigma) and age on collagen production, cells were grown to 90–95% confluence and serum starved for 24 hours prior to experimentation. Cells from young animals were pretreated with polyethylene glycol-superoxide dismutase (PEG-SOD, 20 Units/mL) for 30 minutes followed by a 24 hour pyrogallol (10 μM) treatment. Fibroblasts from old rats were treated with or without PEG-SOD (20 Units/mL) for 24 hours. Western blots were performed as described previously (Fleenor *et al.* 2010; Sindler *et al.* 2011). Antibodies for western blots were collagen type I (1:2000, Millipore) and β-actin (1:1000, Cell Signaling).

Carotid artery vasodilatory responses

Endothelium-dependent and endothelium-independent dilation were determined *ex vivo* in isolated carotid arteries as previously described (Rippe *et al.* 2010; Sindler *et al.* 2011). Briefly, mice were anesthetized using isoflurane and euthanized by exsanguination via cardiac puncture. The carotid arteries were carefully excised, cannulated onto glass

micropipettes and secured with nylon (11-0) suture in myograph chambers (DMT Inc.) containing buffered physiological saline solutions. The arteries were pressurized to 50 mmHg at 37° C and were allowed to equilibrate for 1 h. After submaximal preconstriction with phenylephrine (2 μmol/L), increases in luminal diameter in response to acetylcholine (ACh: 1×10^{-9} - 1×10^{-4} mol/L) with and without co-administration of the NO synthase inhibitor N-G-nitro-L-arginine methyl ester (L-NAME), (0.1 mmol/L, 30 min incubation) were determined. Endothelium-independent dilation was determined by vasodilation in response to sodium nitroprusside (SNP: 1×10^{-10} - 1×10^{-4} mol/L). All pharmacologic agents were added to the abluminal surface of the artery.

All dose response data are presented on a percent basis. Preconstriction was calculated as a percentage of maximal diameter according to the following formula:

$$\text{Preconstriction (\%)} = (D_m - D_b)/D_m \times 100$$

Because of differences in maximal carotid artery diameter between young and old animals, vasodilator responses were recorded as actual diameters expressed as a percentage of maximal response according to the following formula:

$$\text{Relaxation (\%)} = (D_s - D_b)/(D_m - D_b) \times 100$$

Where D_m is maximal inner diameter at 50 mmHg, D_s is the steady-state inner diameter recorded after the addition of drug, and D_b is the steady-state inner diameter following preconstriction before the first addition of drug.

NO-dependent dilation was determined from the maximal EDD in the absence or presence of L-NAME according to the following formula:

$$\text{NO-dependent dilation (\%)} = \text{Maximum dilation}_{ACh} - \text{Maximal dilation}_{ACh + L-NAME}$$

Protein expression

Aortas were used as a surrogate large elastic artery to provide sufficient tissue for analysis of protein expression by Western blot as described previously (Rippe *et al.* 2010; Sindler *et al.* 2011) (Lesniewski *et al.* 2009) (Lesniewski *et al.* 2011). The vessels were excised, cleared of surrounding tissues and frozen in liquid nitrogen before storage at −80° C. The tissue was pulverized over liquid nitrogen and homogenized in ice-cold RIPA lysis buffer containing protease and phosphatase inhibitors [Protease Inhibitor Cocktail Tablet (Roche, Indianapolis, IN, USA) and 0.01% phosphatase inhibitor cocktail (Sigma, St. Louis, MO, USA)]. Ten micrograms of protein was loaded on 4–12% polyacrylamide gels, separated by electrophoresis and transferred onto nitrocellulose membranes for Western blot analysis. Antibodies for Western blot analysis included anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH 1:1000, Cell Signaling), anti-nitrotyrosine (1:100, Abcam) (for 25 and 55 kD band), anti-p67 phox (1:1000, Cell Signaling), anti-MnSOD (1:2000, Stressgen), and anti-endothelial NO synthase (eNOS 1:500, BD Biosciences). Concentrations of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α were determined in aortic whole cell lysates by multiplex ELISA (Searchlight Mouse Inflammatory Cytokine Kit; Aushon Biosystems; Billerica, MA) as previously described (Sindler *et al.* 2011).

Statistics

Results are presented as mean ± SEM. Statistical analysis was performed with SPSS 17.0 software. For the *ex vivo* vasodilatory dose response, group differences were determined by repeated measures ANOVA. A two-way ANOVA was used to analyze pulse wave velocity and histology data. For maximal dilation, protein expression, superoxide production and animal characteristics comparisons between groups were made using ANOVA. Significance was determined using $p<0.05$.

Acknowledgments

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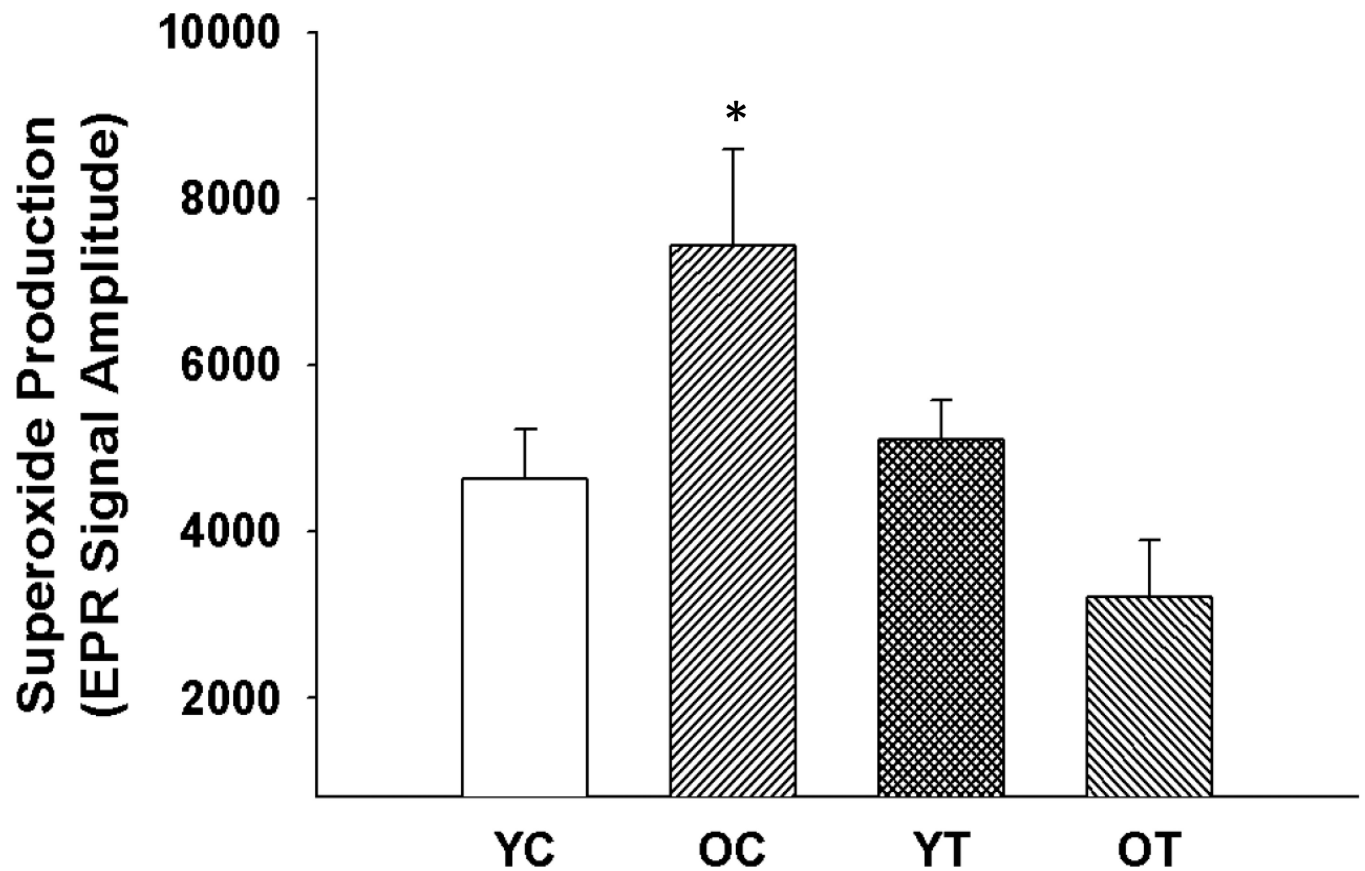
Figure 1. Aortic superoxide production

Mean electron paramagnetic resonance signal for superoxide in aortic rings from young and old control (YC and OC) and young and old TEMPOL-supplemented (YT and OT) mice. Values are mean ± SEM. (n = 4 – 8 per group) *p < 0.05 for OC vs. YC, YT and OT.
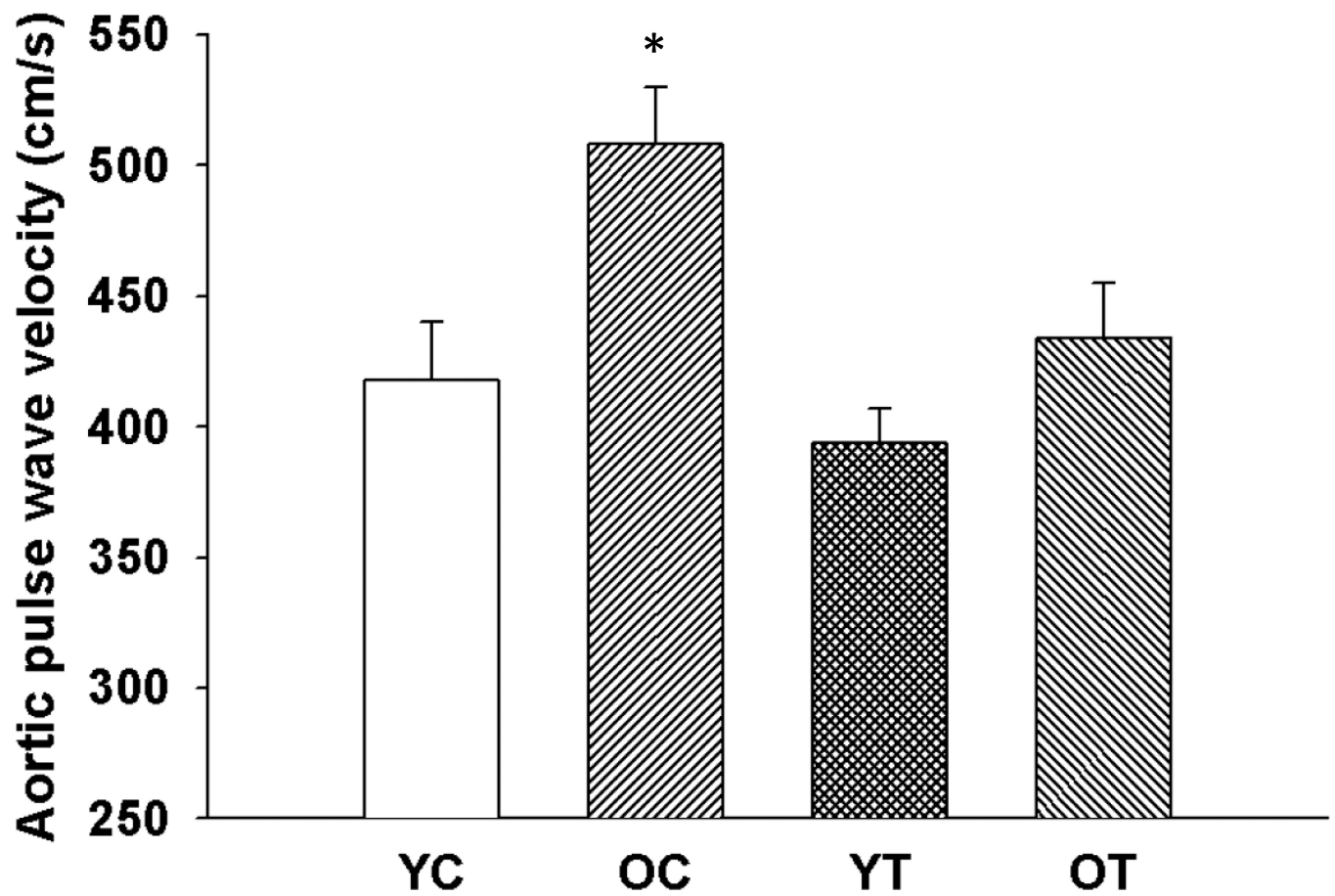
Figure 2. Large elastic artery stiffness
Aortic pulse wave velocity in young and old control (YC and OC) and young and old
TEMPOL-supplemented (YT and OT) mice. Values are mean ± SEM. (n = 5 – 7 per group)
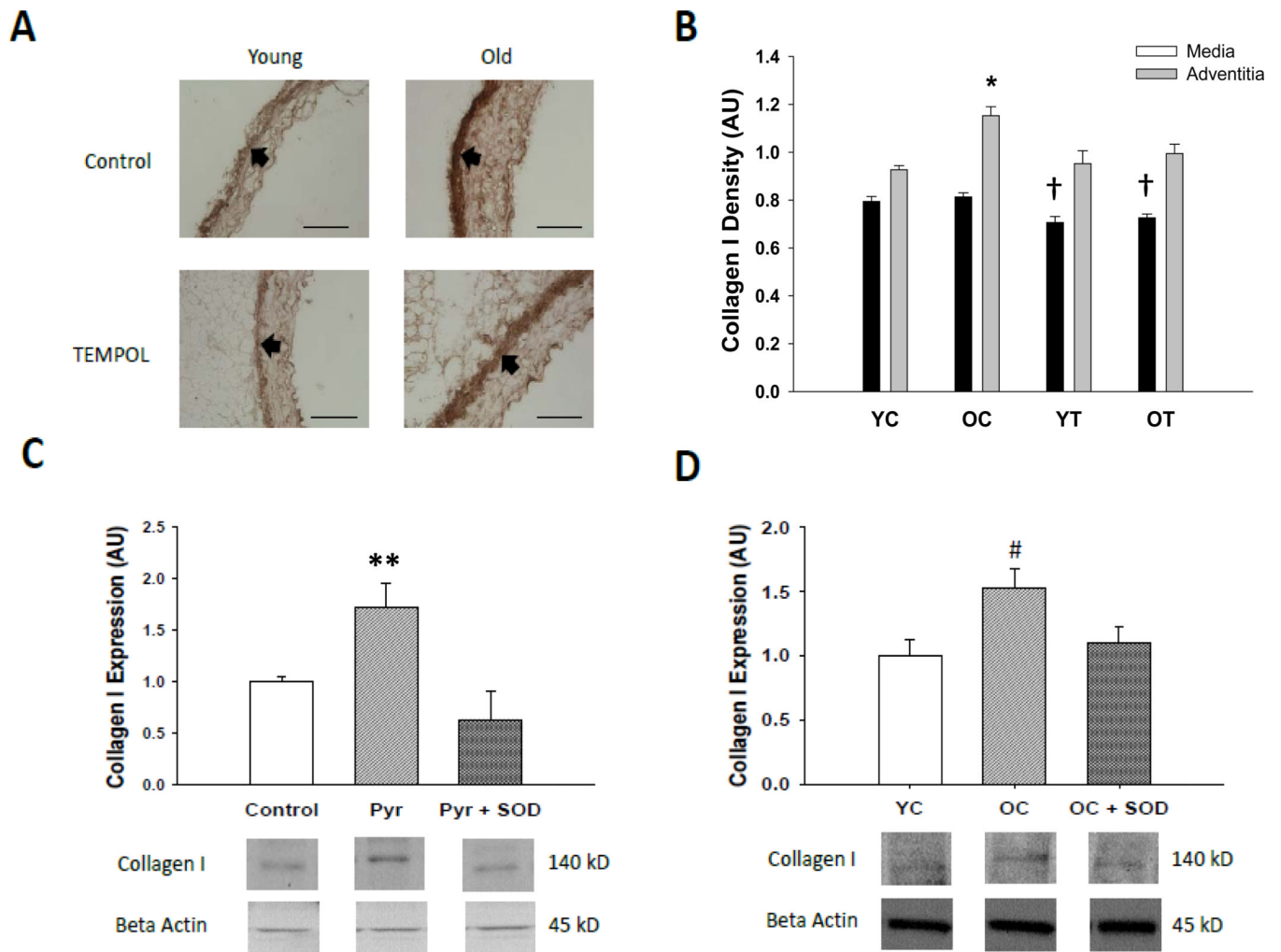*p < 0.05 for OC vs. YC, YT and OT.

Figure 3. Collagen I protein expression
(A) Representative immunohistochemistry images of collagen I in thoracic aortic sections from young and old control (YC and OC) and TEMPOL (YT and OT) supplemented mice. Arrows demarcate the medial-adventitial border; bar = 100 μm. (B) Collagen I protein in thoracic aortic sections of young and old control (YC and OC) and TEMPOL supplemented (YT and OT) mice. (C) Effects of pyrogallol (Pyr, 10 μM), polyethylene glycol conjugated superoxide dismutase (PEG-SOD, 20 U/mL) and the combination on collagen I adventitial fibroblasts from aorta of young rats. (D) Collagen I expression in adventitial fibroblasts from young and old control rats (YC and OC) and effects of PEG-SOD (20 U/mL) in fibroblasts from old rats (OC + SOD). Values are mean ± SEM. (n = 5 – 7 per group) * $p < 0.05$ for Adventitia OC vs. Adventitia YC, Adventitia YT and Adventitia OT. † $p < 0.05$ for Media YT vs. Media YC and Media OC, Media OT vs. Media YC and Media OC. ** $p < 0.05$ for Pyr vs. control, Pry + SOD. # $p < 0.05$ for OC vs. YC and OC + SOD.
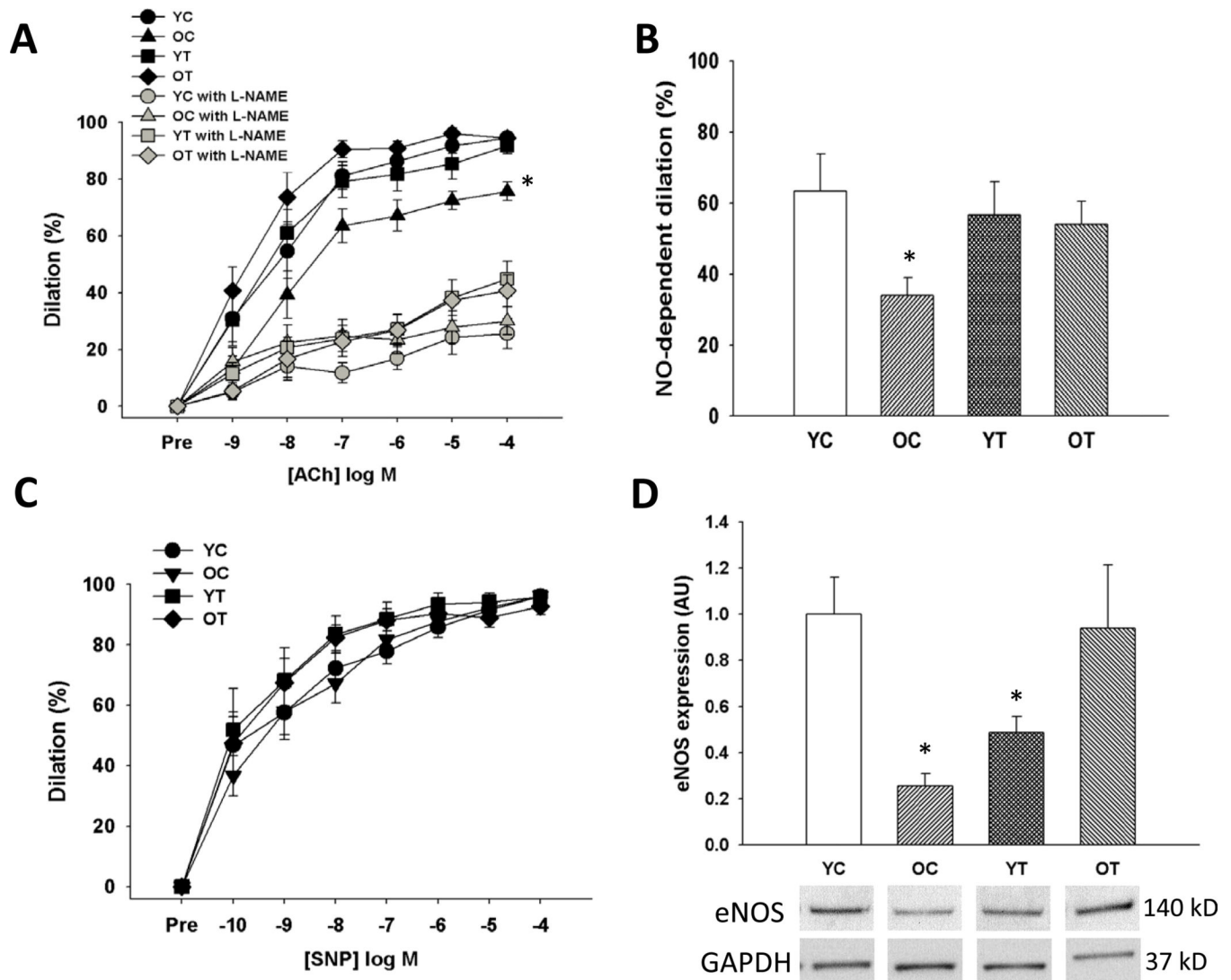
Figure 4. Nitric oxide (NO)-mediated endothelium-dependent dilation
(A) Endothelium-dependent dilation to acetylcholine (ACh) alone and in the presence of the endothelial NO synthase (eNOS) inhibitor N-G-nitro-L-arginine methyl ester (L-NAME) in young and old control (YC and OC) and young and old TEMPOL-supplemented (YT and OT) mice. (B) NO-dependent dilation (max dilation$_{ACh}$ – max dilation$_{ACh + L-NAME}$). (C) Endothelium-independent dilation to sodium nitroprusside (SNP). (D) Aortic protein expression of eNOS expressed relative to GAPDH and normalized to YC mean value. Representative western blot images below. Values are mean ± SEM. (n = 7–8 per group) *p < 0.05 for OC vs. YC, YT and OT.
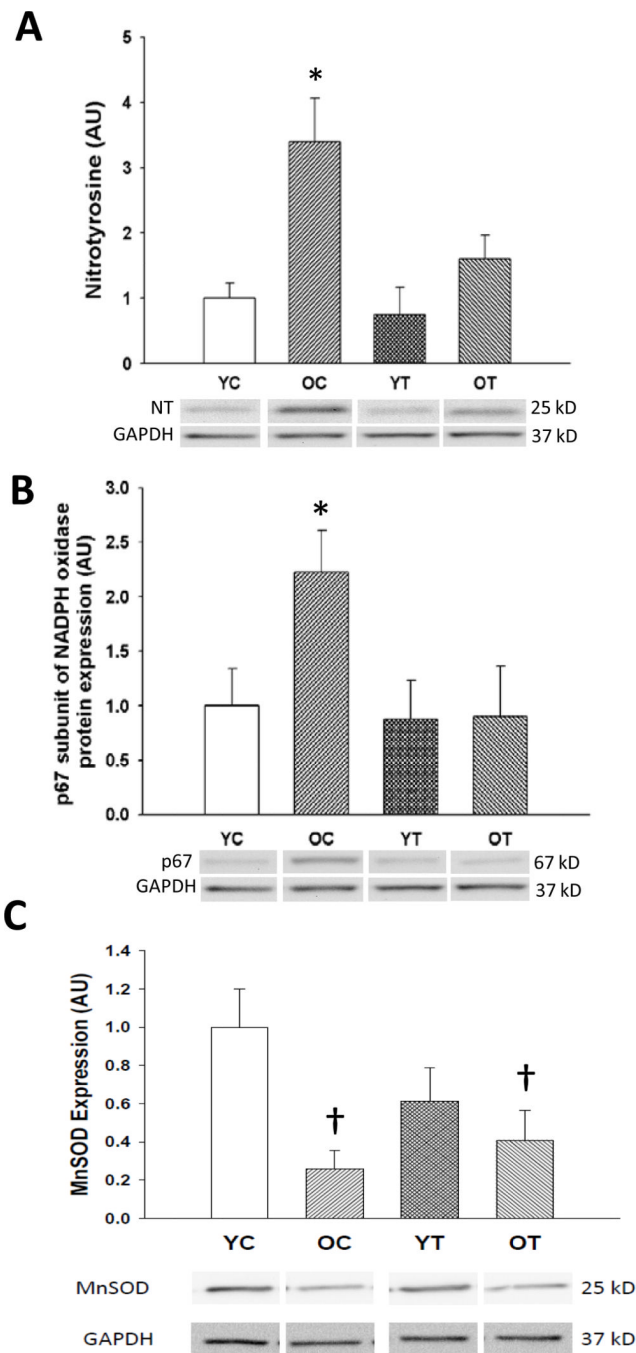
Figure 5. Oxidative stress
(A) Nitrotyrosine abundance, (B) p67 subunit of nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase (NADPH oxidase), and (C) manganese superoxide dismutase (MnSOD) in aorta of young and old control (YC and OC) and young and old TEMPOL-supplemented (YT and OT) mice. Data are expressed relative to GAPDH and normalized to YC mean value. Representative Western blot images below. Values are mean ± SEM. (n = 4–8 per group) * $p < 0.05$ for OC vs. YC, YT and OT. † $p < 0.05$ for OC vs. YC and OT vs. YC.
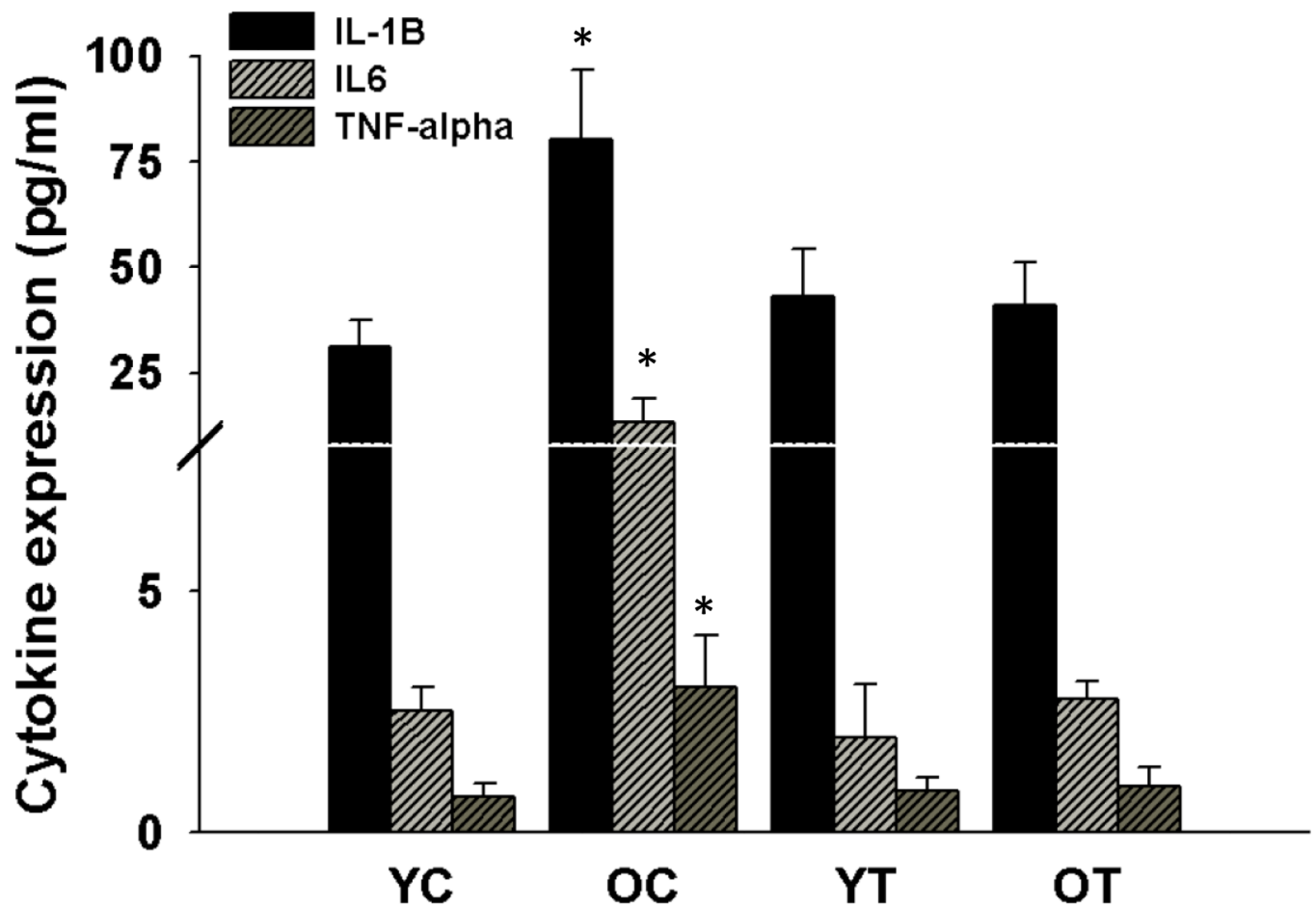
Figure 6. Pro-inflammatory cytokines
Interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) in aorta from young and old control (YC and OC) and young and old TEMPOL-supplemented (YT and OT) mice. Values are mean ± SEM. (n = 7–8 per group) * $p < 0.05$ for OC vs. YC, YT and OT.

Table 1

Animal characteristics

	YC	OC	YT	OT
Body mass (g)	28 ± 1	34 ± 1*	29 ± 1	33 ± 1*
Heart mass (mg)	146 ± 4	184 ± 4*	143 ± 3	194 ± 8*
Carotid artery lumen diameter (μm)	404 ± 5	455 ± 8*	400 ± 4	454 ± 4*
Carotid artery constriction to phenylephrine				
Absolute (μm)	339 ± 6	366 ± 7*	331 ± 12	352 ± 10*
Relative (%)	82 ± 1	81 ± 1	82 ± 2	80 ± 2
Blood pressure (mmHg)				
Systolic	100 ± 2	96 ± 2	99 ± 2	95 ± 4
Diastolic	70 ± 3	66 ± 4	70 ± 3	66 ± 4

Values are mean ± SEM.

*
$p < 0.05$ for OC vs. YC and YT, OT vs. YC and YT. YC, Young control; OC, Old control; YT, Young TEMPOL; OT, Old TEMPOL

Table 2

Elastin protein and advanced glycation endproducts in aorta

	YC	OC	YT	OT
Elastin				
Media (AU)	0.681 ± 0.04	0.632 ± 0.02 [*]	0.632 ± 0.02 [†]	0.571 ± 0.01 [*†]
Adventitia (AU)	0.599 ± 0.02	0.596 ± 0.02	0.587 ± 0.01	0.582 ± 0.02
AGEs				
Media (AU)	0.618 ± 0.01	0.692 ± 0.03 [*]	0.642 ± 0.03	0.713 ± 0.07 [*]
Adventitia (AU)	0.718 ± 0.03	0.789 ± 0.01 [*]	0.645 ± 0.04	0.756 ± 0.04 [*]

Values are mean ± SEM.

[*] $p < 0.05$ for OC vs. YC and YT, OT vs. YC and YT.

[†] $p < 0.05$ for YT vs. YC and OC, OT vs. YC and OC. AGEs, advanced glycation end-products; YC, Young control; OC, Old control; YT, Young TEMPOL; OT, Old TEMPOL