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Superoxide dismutase mimetic M40403 improves endothelial function in apolipoprotein(E)-deficient mice

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1 Overproduction of superoxide anions in the vascular wall contributes to endothelial dysfunction in vascular disease. A superoxide-generating reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) oxidase has recently been identified as a major source of oxidative radicals in vascular tissues. We studied the effects of a synthetic manganese-containing superoxide dismutase (SOD) mimetic, M40403, on NADPH oxidase-dependent superoxide generation and on endothelial dysfunction.

2 In rat aortic smooth muscle cells, NADPH (100 μM) markedly stimulated superoxide production as detected by lucigenin (5 μM)-enhanced chemiluminescence. M40403 reduced NADPH oxidase-dependent superoxide production in a concentration-dependent manner, with IC_{50} being 31.6 μM. In contrast, native Cu/Zn SOD (up to 300 U ml^{-1}) had no effect. Angiotensin II (100 nM) increased the NADPH oxidase activity by 70%, and treatment with M40403 (10 μM) reduced this increased superoxide to the control level.

3 In aortae from apolipoprotein(E)-deficient mice (apoE^0) with hyperlipidemia and atherosclerosis, superoxide production is largely derived from NADPH oxidase. The attenuation of endothelial nitric oxide vasodilator function parallels the increase in vascular superoxide production at different stages of the disease. Acute incubation of such aortic rings with M40403 significantly suppressed superoxide production and improved endothelium-dependent vasorelaxation to a level comparable to that in wildtype control mice.

4 In summary, the cell-permeable SOD mimetic M40403 was found to reverse endothelial dysfunction in apoE^0 aorta *ex vivo* by decreasing NADPH oxidase-dependent superoxide levels. The advantages of synthetic SOD mimetics over the native Cu/Zn SOD enzyme, such as greater cell permeability and stability, confer significant therapeutic potential in vascular disease.
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Keywords: Apolipoprotein(E)-deficient; atherosclerosis; endothelial dysfunction; NADPH oxidase; nitric oxide; SOD mimetic; superoxide

Abbreviations: ACh, acetylcholine perchlorate; AngII, angiotensin II; apoE^0, apolipoprotein(E)-deficient mouse; CPS, count per second; DETCA, diethyldithiocarbamic acid; tiron, 4,5-dihydroxy-1,3-benzenedisulfonic acid; DMEM, Dulbecco's modification of Eagle's medium; DPI, diphenyleneiodonium chloride; KHB, Krebs–HEPES buffer; L-NAME, N^G-nitro-L-arginine methyl ester hydrochloride; NADPH, reduced β-nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; NOS, nitric oxide synthase; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; 17-ODYA, 17-octadecynoic acid; one-way ANOVA, one-way analysis of variance; RASMC, rat aortic smooth muscle cells; s.e.m., standard error of the mean; SOD, superoxide dismutase; tempo, 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy; U46619, 9,11-dideoxy-9α,11α-methanoepoxyprostaglandin $F_{2\alpha}$; X/XO, xanthine plus xanthine oxidase

Introduction

Increased production of superoxide anions ($O_2^{-\bullet}$) in vascular tissues has been implicated in many cardiovascular diseases such as hypertension, hyperlipidemia and atherosclerosis (Mugge *et al*., 1994; Miller *et al*., 1998; Kerr *et al*., 1999). Although reactive oxygen species may have important roles in various intracellular signal transduction pathways (Kunsch & Medford, 1999), excess superoxide molecules can cause multiple pathophysiological consequences. Superoxide rapidly inactivates endothelium-derived nitric oxide (NO), which is a predominant endogenous vasodilator, leading to decreased NO bioavailability and elevated blood pressure (Zicha *et al*., 2001). Superoxide and the product of its reaction with NO, peroxynitrite, promote lipid peroxidation that contributes to atherosclerotic lesion formation (White *et al*., 1994). Moreover, impairment of NO function by superoxide may result in vascular smooth muscle cell proliferation and migration and expression of a variety of proinflammatory molecules in vascular cells, all of which are operative mechanisms of atherosclerosis and restenosis (Dusting *et al*., 1998).

Recently, a superoxide-producing reduced β-nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase has been identified in vascular tissues (Griendling *et al*., 2000). This enzyme is composed of two cytosolic subunits p47phox and

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p67phox, a cell membrane-bound cytochrome *b*558 which consists of gp91phox (or its homologues) and p22phox, and a small G protein rac. Upon assembly of these subunits in the membrane, this enzyme generates a superoxide by one-electron reduction of oxygen *via* its gp91phox subunit using NADPH or reduced β-nicotinamide adenine dinucleotide (NADH) as the electron donor (Griendling *et al*., 2000). The activity of this enzyme can be increased by angiotensin II (AngII) (Griendling *et al*., 1994), growth factors (Marumo *et al*., 1997) or cytokines (De Keulenaer *et al*., 1998). Emerging evidence has suggested that this NAD(P)H oxidase is the major source of superoxide in vascular tissues (Guzik *et al*., 2000; Paravicini *et al*., 2002) and generation of pathological amounts of superoxide by this enzyme has been implicated in cardiovascular diseases such as atherosclerosis (Yokoyama *et al*., 2000) or hypertension (Morawietz *et al*., 2001).

Metabolism of superoxide largely depends on its dismutation to hydrogen peroxide by endogenous superoxide dismutase (SOD). It has been shown that *in vivo* treatment with modified SOD enzyme improved endothelium-dependent vasorelaxations in atherosclerotic rabbits (Mugge *et al*., 1991). The therapeutic potential of native SOD enzyme is, however, restricted because it is a large peptide with limited stability and permeability, and several cell-permeable small molecule SOD mimetics have been synthesized. M40403 is a low-molecular weight manganese-containing SOD mimetic, which has marked protective effects in inflammation and septic shock by suppressing superoxide generation (Salvemini *et al*., 1999; 2001; Macarthur *et al*., 2000). However, the actions of synthetic SOD mimetics in the context of cardiovascular diseases such as atherosclerosis and hypertension, and in particular their interactions with the vascular NADPH oxidase, have not been well defined. Using cultured rat aortic smooth muscle cells (RASMC) and apolipoprotein(E)-deficient (apoE^0) mice, which developed hyperlipidemia, hypertension, atherosclerosis and endothelial dysfunction (Plump *et al*., 1992; Yang *et al*., 1999; Jiang *et al*., 2001), we investigated (1) whether synthetic SOD mimetic may functionally antagonize the NADPH oxidase activity, and (2) the role of superoxide production in the endothelial NO dysfunction in apoE^0 mice and the efficacy of M40403 *ex vivo*.

Methods

Animals and aortic tissue preparation

All animal studies were carried out in accordance with the guidelines of Howard Florey Institute Animal Ethics Committee and National Health and Medical Research Council of Australia. Male apoE^0 mice with >99% C57BL/6J background were purchased from Animal Resource Centre (Western Australia) at 4 weeks of age and maintained with either a normal diet or a high-fat diet containing 0.15% cholesterol (Harlan Teklad, Madison, WI, U.S.A.). These animals developed hyperlipidemia and atherosclerosis (Jiang *et al*., 2001). Age-matched C57BL/6J mice were used as wildtype controls (WT). Animals were killed at 16 or 28 weeks of age by decapitation under general anesthesia. Abdominal aortae were isolated immediately for vascular reactivity and lucigenin-enhanced chemiluminescence assay.

Aortic smooth muscle cell (SMC) culture

SMCs were isolated from thoracic aorta of male Sprague–Dawley rats by enzymatic digestion with collagenase I (1 mg ml^{-1}), elastase (0.5 mg ml^{-1}) and trypsin (1.25 mg ml^{-1}). Cells were maintained in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% fetal calf serum in a humidified incubator with 5% CO_2 at 37°C. Cells between passages 4 and 10 was used. The purity of the cells was determined as >95% by antismooth muscle α-actin immunocytochemistry.

Lucigenin-enhanced chemiluminescence assay

Lucigenin-enhanced chemiluminescence assay was based on the method described by Ohara *et al*. (1993) with some modifications. Aortic segments of about 0.5 cm long were equilibrated in Krebs–HEPES buffer (KHB) (pH 7.4) comprising (mM): NaCl 98.0, KCl 4.7, $NaHCO_3$ 25.0, $MgSO_4$ 1.2, KH_2PO_4 1.2,$CaCl_2$ 2.5, D-glucose 11.1 and HEPES-Na 20.0 for 60 min. The chemiluminescence was detected with a microplate scintillation counter (Topcount model 9912, Packard) running in single-photon-count mode. Polystyrene 96-well plate (OptiPlate-96 Packard) was loaded with KHB (300 μl in each well) containing 5 μM lucigenin, with or without NADPH (100 μM). The specificity of this assay was confirmed by the concentration-dependent generation of superoxide by xanthine plus xanthine oxidase (X/XO) and its inhibition by Cu/Zn SOD.

To measure superoxide in SMCs, cells were suspended with collagenase I (1 mg ml^{-1}) dissolved in KHB, pelleted by centrifugation (1000 r.p.m. × 4 min) and then resuspended in 0.5 ml KHB containing lucigenin (5 μM) with or without NADPH. Each well was loaded with 300 μl of cell suspension. For various drug treatments, drugs were added to the culture plate at least 40 min before cell collection. To eliminate the influence of endogenous Cu/Zn SOD activity, all aortic segments and SMCs were treated with diethyldithiocarbamic acid (DETCA) (3 mM) for 60 min prior to chemiluminescence assay, unless otherwise mentioned. All drugs, except for DETCA, were also present in the cell suspension throughout the counting. To measure superoxide generation in cell homogenates, confluent SMCs grown in three six-well plates were scraped into 9 ml cold KHB containing 10 μg ml^{-1} leupeptin. Cells were homogenized by sonication on ice and centrifuged at 1000 r.p.m. for 3 min. Chemiluminescence was measured in the supernatant as described in the cell suspension. To measure superoxide generated by X/XO, the assay was carried out in a reaction mixture of 300 μl containing xanthine (100 μM), xanthine oxidase (0.03 U ml^{-1}) and lucigenin (5 μM) in PBS with or without scavenging drugs. The reaction was initiated by the addition of xanthine oxidase into the mixture. As the superoxide signal produced by X/XO was transient, all counting commenced 1 min after xanthine oxidase addition.

Vascular reactivity study

Vascular reactivity was studied in aortic rings (~1.5 mm long) using a four-channel myograph as described previously (Jiang *et al*., 2001). Briefly, rings were mounted onto two parallel stainless-steel pins through the lumen and placed in a chamber

containing 6 ml physiological salt solution, which was maintained at $36 \pm 1^{\circ}C$ and gassed with 5% CO_2 and 95% O_2. After a 20 min period of equilibration, the resting tension was raised stepwise to 5 mN; then a further equilibration period of 20 min was given. The tissues were maximally contracted with the thromboxane analogue 9,11-dideoxy-9α,11α-methanoepoxy-prostaglandin $F_{2\alpha}$ (U46619) (1 μM). Relaxations were induced after the tone had been raised with U46619 to 40–50% of the maximum. In some experiments, M40403 was added 30 min prior to the relaxation experiment and present throughout.

Drugs used

The drugs used were β-Nicotinamide adenine dinucleotide phosphate (NADPH, ICN), 17-octadecynoic acid (17-ODYA), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), U46619 (Calbiochem, U.S.A.), acetylcholine perchlorate (ACh, BDH, England), allopurinol, AngII (Auspep, Australia), diethyldithiocarbamic acid (DETCA) (ICN), 4,5-dihydroxy-1,3-benzenedisulfonic acid (disodium salt, tiron), diphenyleneiodonium chloride (DPI), 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy (tempo, Aldrich), indomethacin, losartan (Merck), leupeptin (ICN), L-phenylephrine hydrochloride, M40403 (MetaPhore Pharmaceuticals, St Louis, MO, U.S.A.), N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME), Cu/Zn SOD, X, XO. Drugs with unspecified source are from Sigma.

Data and statistics

The superoxide production level was expressed as count per second (CPS). Results were normalized to per mg wet tissue (aorta) or to a concentration of 10^6 cell ml^{-1} (cell suspension). Data were presented as mean ± standard error of the mean (s.e.m.). One-way analysis of variance (one-way ANOVA) followed by Tukey's test (for multiple comparisons) or Student's *t*-test (for two groups) was used as appropriate for statistical analysis. A value of $P<0.05$ was regarded as significant. The regression analysis of dose–response curves and calculation of IC_{50} (the molar concentration required to produce 50% inhibition of the enzyme activity) were performed using Prism software (GraphPad).

Results

Superoxide-scavenging efficacy of M40403

The *in vitro* superoxide-scavenging efficacy of M40403 was assessed in a biochemical system comprising X (100 μM) plus XO (0.03 U ml^{-1}) (X/XO). The chemiluminescence signal generated by X/XO was abolished by Cu/Zn SOD (Figure 1a). M40403 suppressed the superoxide generated by X/XO in a concentration-dependent manner, the IC_{50} being 6.3 μM. This is in contrast to the efficacies of the nonspecific superoxide radical scavengers tempo and tiron under the same experimental conditions, the IC_{50} values being 398.1 and 794.3 μM, respectively. The regression analysis of the dose–response curves of M40403, tempo and tiron are shown in Figure 1b.

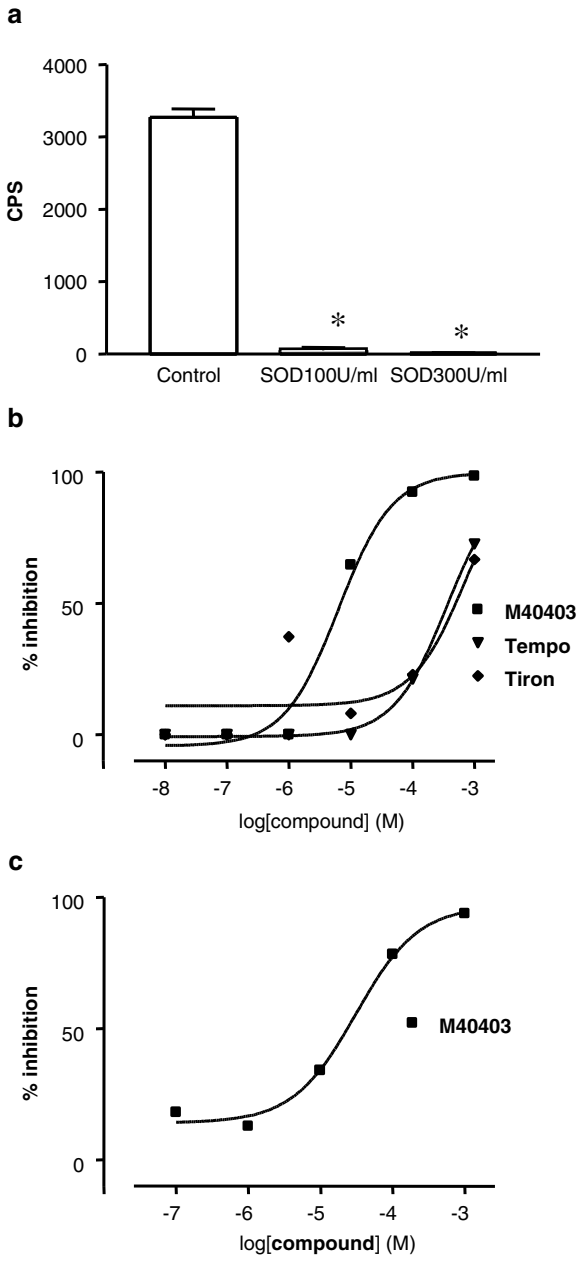

Figure 1 (a) Effects of Cu/Zn SOD on superoxide generated with xanthine (100 μM) plus xanthine oxidase (0.03 U ml^{-1}) (X/XO) detected by lucigenin-enhanced chemiluminescence. Results were expressed in CPS as mean ± s.e.m., $*P<0.05$, $n=4$. (b) Regression analysis of the inhibitory effects of M40403, tempo and tiron on superoxide production in the X/XO system. (c) Regression analysis of the effect of M40403 on NADPH (100 μM)-stimulated superoxide production in RASMCs pretreated with DETCA (3 mM). In (b) and (c) each point represents the average of four measurements.

Effects of M40403 on NADPH-induced superoxide in RASMCs

In cultured RASMCs, superoxide could not be detected either under resting conditions or after inactivation of endogenous SOD by treatment with DETCA (3 mM). In DETCA-treated cells, NADPH (100 μM) stimulated superoxide production; the absolute magnitude (CPS) of the chemiluminescence signal is similar to that generated with X/XO. The superoxide produc-

tion was inhibited by the NADPH oxidase inhibitor DPI, but not by the nitric oxide synthase (NOS) inhibitor L-NAME, the XO inhibitor allopurinol, the cyclooxygenase inhibitor indomethacin or the cytochrome *P*450 inhibitor 17-ODYA (Figure 2a). NADPH-stimulated superoxide production was suppressed by M40403 in a concentration-dependent manner, with IC_{50} being 31.6 μM (Figure 1c). Pretreatment with AngII for 4 h significantly increased NADPH oxidase activity, an effect that was blocked by the AngII type-1 (AT_1) receptor antagonist losartan (Figure 2b). Incubation with DPI and M40403 also suppressed NADPH-stimulated superoxide in AngII-treated cells (Figure 2b).

To elucidate whether the native Cu/Zn SOD has similar effects as M40403 on NADPH-stimulated superoxide in RASMCs, we measured the superoxide production in cell suspensions that were not pretreated with DETCA. Treatment with Cu/Zn SOD at 100 or 300 U ml^{-1} had no effect on the superoxide production in intact cells (Figure 3a). In SMC homogenates, the superoxide production in the presence of NADPH was not affected by Cu/Zn SOD treatment either, whereas M40403 reduced the superoxide level in a concentration-dependent manner (Figure 3b).

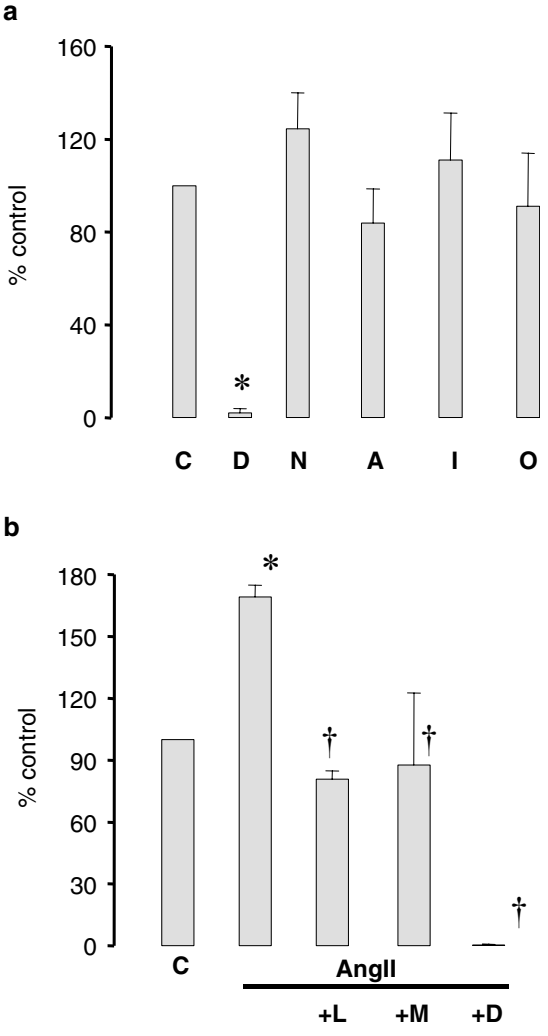

Figure 2 (a) The effects of DPI (D, 10 μm), L-NAME (N, 200 μM), allopurinol (A, 100 μM), indomethacin (I, 30 μM) and 17-ODYA (O, 10 μM) on NADPH-stimulated superoxide production in RASMCs pretreated with DETCA; (b) the effects of AngII (100 nM) alone or in the presence of losartan (L, 10 μM), M40403 (M, 10 μM) and DPI (D, 10 μM) on NADPH-stimulated superoxide production in RASMCs. Results are converted to percentage of control (C). Data are mean ± s.e.m. *$P<0.05$ vs control; †$P<0.05$ vs AngII, $n=3-10$.

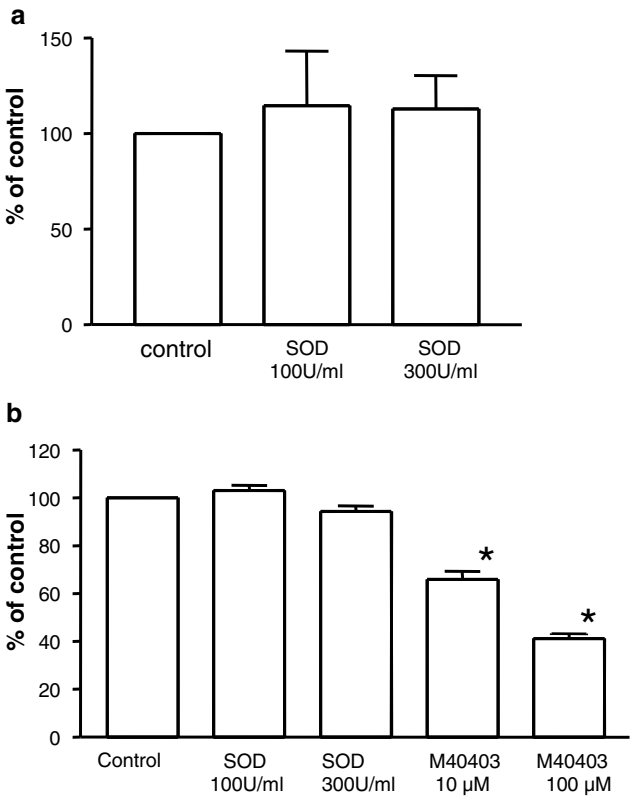

Figure 3 (a) Effects of Cu/Zn SOD on NADPH-stimulated superoxide in intact RASMCs (without DETCA pretreatment), $n=3$. (b) Effects of SOD and M40403 on NADPH-stimulated superoxide in RASMC homogenates (without DETCA pretreatment). Results are expressed as percentage of control (C) and data are mean ± s.e.m. *$P<0.05$ vs control, $n=5-6$.

Superoxide generation in mouse aorta and effects of M40403

In aortic rings from 28-week-old WT and apoE^0 mice (fed a normal diet) pretreated with DETCA (3 mM), superoxide could be detected under basal conditions. Inclusion of NADPH (100 μM) further stimulated this superoxide production, but there was no difference in the basal or NADPH-stimulated superoxide between WT and apoE^0 (Table 1). To clarify the enzymatic source of the superoxide anion in the aorta, we examined the effects of various enzyme inhibitors in aortic rings from apoE^0 mice. Both the basal and NADPH-stimulated superoxide productions were blocked by DPI (5 μM), but not significantly affected by L-NAME (200 μM), allopurinol (100 μM), indomethacin (30 μM) or 17-ODYA (10 μM) (Figure 4a–d). Similar as in RASMCs, Cu/Zn SOD treatment had no significant effect on the superoxide level in either the absence or presence of NADPH in these aortic rings (not pretreated with DETCA) (Figure 4e,f).

In aortic rings from 28-week-old apoE^0 mice (fed a high fat diet) pretreated with DETCA, both the basal and NADPH-stimulated superoxide productions were significantly elevated as compared to WT control and apoE^0 on a normal diet

Table 1 Effects of M40403 (10 μM) on basal and NADPH-stimulated superoxide production in mouse abdominal aorta

	Superoxide (CPS mg^{-1})	n
−NADPH		
WT control	41±8	10
apoE^0/normal diet	53±10	13
apoE^0/high-fat diet	102±12^{ab}	8
apoE^0/normal diet+M40403 (10 μM)	16±3^b	9
apoE^0/high-fat diet+M40403	15±2^c	7
+NADPH (100 μM)		
WT control	311±125	10
apoE^0/normal diet	180±43	13
apoE^0/high-fat diet	1198±245^{ab}	7
apoE^0/normal diet+M40403	43±11^b	9
apoE^0/high-fat diet+M40403	112±12^c	7

All tissues were pretreated with DETCA. $^aP<0.05$ vs WT; $^bP<0.05$ vs apoE^0/normal diet; $^cP<0.05$ vs apoE^0/high-fat diet.

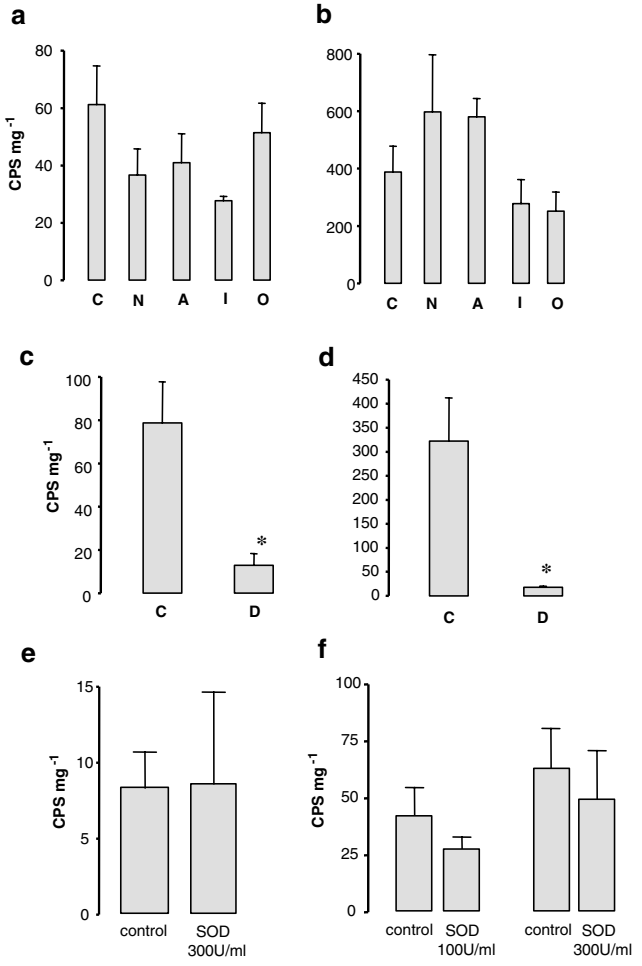

Figure 4 Effects of L-NAME (N, 200 μM), allopurinol (A, 100 μM), indomethacin (I, 30 μM) and 17-ODYA (O, 10 μM) (a,b), DPI (D, 5 μM) (c,d) and Cu/Zn SOD (e,f) on the superoxide production in aortic rings from 28-week-old apoE^0 (normal diet fed) mice, in the absence (a,c,e) or presence (b,d,f) of NADPH (100 μM) stimulation. Tissues were pretreated with DETCA (3 mM) except for (e) and (f). Results are expressed as CPS per mg wet tissue. Data are mean±s.e.m. C, control; $*P<0.05$ vs control, $n=4$.

(Table 1). Acute *ex vivo* incubation with M40403 (10 μM) significantly reduced the superoxide levels in these tissues (Table 1).

Vascular superoxide generation and endothelial NO function

We have previously shown that in the aorta of both WT and apoE^0 mice, endothelium-dependent relaxations induced by ACh could be blocked by the NOS inhibitor L-NAME (Jiang *et al*., 2001), suggesting that this response is mediated entirely by endothelial NO. In pilot studies, we found that ACh-induced relaxations in the aorta from 28-week-old apoE^0 (normal diet) were not different from those in WT control (data not shown). In contrast, ACh-induced vasorelaxations were significantly reduced in tissues of high-fat diet-fed apoE^0 mice as compared to WT (see Figure 6), while the relaxations induced by the NO donor *S*-nitroso-*N*-acetylpenicillamine were not significantly different (Jiang *et al*., 2001). These data suggest that the increased vascular superoxide level in apoE^0 mice (high fat) compromises endothelial NO function. We further studied endothelial function and vascular superoxide generation in apoE^0 mice (high fat) at different ages (16 and 28 weeks). There was an inverse association between the ACh-induced vasorelaxation and the vascular superoxide level (without NADPH) or the NADPH oxidase activity (with NADPH) (Figure 5). A correlation analysis within tissue was not possible because the superoxide level and vascular reactivity cannot be measured in a single tissue.

Effects of M40403 on endothelial function

In aortic rings from 28-week-old apoE^0 (high fat) mice, the endothelium-dependent relaxations induced by ACh (30–300 nM) were significantly reduced as compared to age-matched WT controls (Figure 6). Acute incubation of the apoE^0 aorta with M40403 (10 μM) improved ACh-induced relaxations to a level comparable to those in WT (Figure 6). M40403 at the same concentration had no significant effect in aortic rings from WT mice (Figure 6). To confirm that superoxide affects the basal bioavailability of endothelial NO, the vasoconstrictor response induced by phenylephrine (1 μM) was studied in the presence and absence of superoxide generated exogenously. X/XO significantly increased phenylephrine-induced contraction, and this was abolished by M40403. Blockade of the NO-cyclic GMP pathway with L-NAME plus the soluble guanylate cyclase inhibitor ODQ markedly increased contractions, and X/XO had no further effect (Figure 7).

Discussion

NADPH oxidase is expressed in vascular smooth muscle. The expression of the NADPH oxidase subunits p22phox, p47phox and the gp91phox homologues nox1 and nox4 has been identified in human and rat smooth muscle cells (Ushio-Fukai *et al*., 1996; Patterson *et al*., 1999; Lassegue *et al*., 2001). We have now showed that NADPH stimulated superoxide generation in RASMC and this was totally blocked by the flavin-binding NADPH oxidase inhibitor DPI, but was not affected by inhibitors of other potential superoxide-generating enzymes, including NOS, XO, cyclooxygenase or cytochrome P450

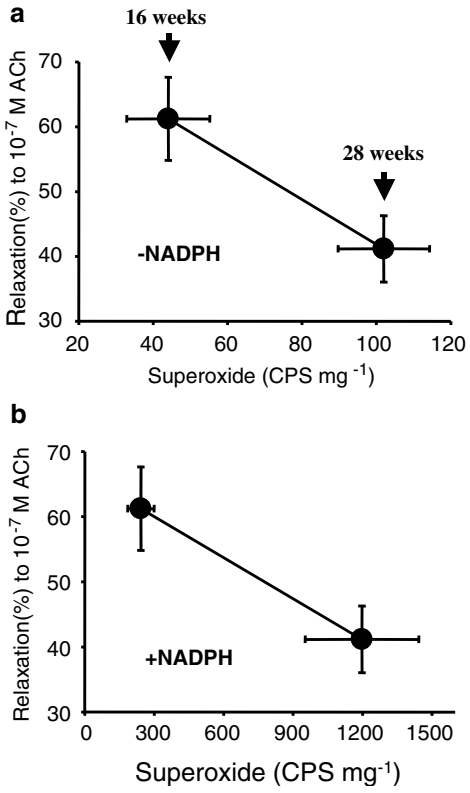

Figure 5 Dynamic changes in the endothelial NO function (assessed as ACh-induced vasorelaxation) and the vascular superoxide production in the absence (a) or presence (b) of exogenous NADPH (100 μM) in isolated abdominal aorta from apoE^0 (high fat diet treated) mice at 16 or 28 weeks of age ($n=4-8$). There is an obvious trend that the increase in vascular superoxide level or NADPH oxidase activity is accompanied by the deterioration of ACh-induced vasorelaxation. At 28 weeks, both of the endothelium-dependent relaxation and the superoxide production/NADPH oxidase activity are significantly different from those at 16 weeks ($P<0.05$).

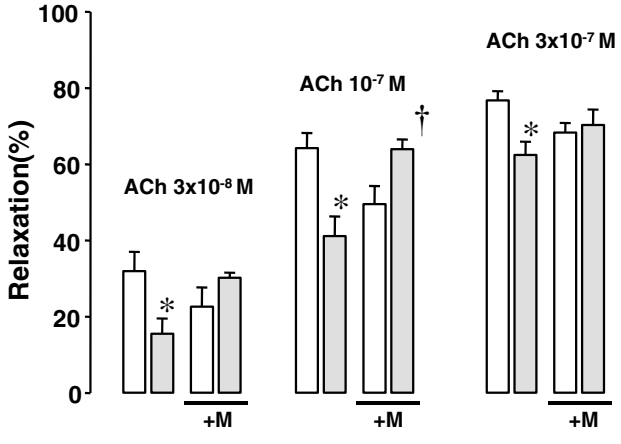

Figure 6 Acute effects of M40403 (10 μM) on ACh-induced vasorelaxations in the abdominal aorta from 28-week-old WT (open bars) and apoE^0 (high fat, closed bars) mice. Relaxations are expressed as the percentage reduction of U46619-induced tone. Data are mean$\pm$s.e.m. $^*P<0.05$ vs WT; $^\dagger P<0.05$ vs without M40403, $n=7-13$.

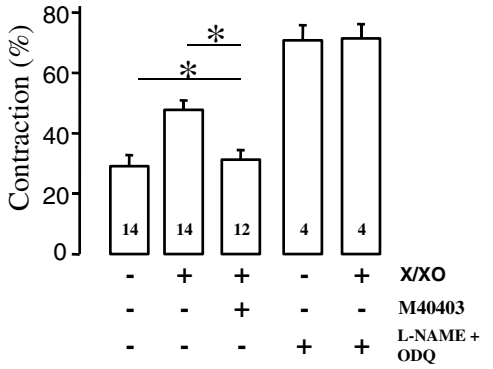

Figure 7 Effect of basally released endothelial NO, as assessed by contractile responses to phenylephrine (1 μM), in normal aortic rings from WT mice. Exogenous superoxide was generated by xanthine (10 μM) plus xanthine oxidase (0.01 U ml^{-1}) combination (X/XO). All tissues were preincubated with DETCA (3 mM). Some tissues were pretreated with M40403 or L-NAME plus ODQ. Contractions are expressed as percentage of the responses induced by U46619 (1 μM). Numbers in the bar indicate n of experiments. Data are mean$\pm$s.e.m. $^*P<0.05$.

(Munzel *et al*., 1999). In the absence of substrate (NADPH), superoxide production was undetectable even after the inactivation of endogenous Cu/Zn SOD. Together with the observation that supplementation with NADPH dramatically enhanced the superoxide level in mouse aortic rings, it is suggested that physiologically, the availability of substrate appears to be a key factor that limits superoxide production by this enzyme.

M40403, whose superoxide-scavenging efficacy is much higher than the nonspecific superoxide scavengers tiron and tempo as determined in the cell-free system, effectively suppressed superoxide production in RASMC stimulated by NADPH, whereas exogenous native Cu/Zn SOD had little effect at concentrations that are high enough to abolish X/XO-produced superoxide of a similar level. Similar findings have also been obtained in isolated rings of apoE^0 mouse aorta. These observations are in agreement with several previous studies showing that native SOD had limited efficacy in reversing redox-sensitive cellular events (Arai *et al*., 1998; Pueyo *et al*., 2000), while polyethylene-glycolated SOD (Mugge *et al*., 1991), which has a longer half-life and higher cellular permeability, and adenovirus-mediated SOD gene-transfer (Arai *et al*., 1998; Zanetti *et al*., 2001), which induced intracellular SOD protein expression, are effective. The contrast between the effects of SOD and M40403 indicates that the smooth muscle NADPH oxidase may generate superoxide intracellularly, and the poor cell permeability of SOD limits its direct therapeutic efficacy in cardiovascular disease. However, the lack of effect with Cu/Zn SOD in contrast to M40403 on NADPH-stimulated superoxide was also found in smooth muscle cell homogenates, where SOD has free access to the cytosolic components. The mechanism of this ineffectiveness of SOD is unknown. In neutrophils, it has been proposed that the membrane-bound subunits (gp91phox and p22phox) reside in the membrane of certain intracellular compartments, and the intracellularly produced superoxide is released to the extracellular environment through the orifice formed by fusion of the compartments with the plasma membrane (Kobayashi *et al*., 2001). Although the exact process of superoxide generation by the NADPH oxidase in smooth muscle cells is unclear, it is conceivable that subcellular

compartmentalization of the enzyme may hinder the access of Cu/Zn SOD to the generated superoxide anions.

Several studies have demonstrated that proatherogenic stimuli such as AngII and proinflammatory cytokines may increase the activity of NADPH oxidase in vascular cells. For instance, in vascular SMC, AngII (Lassegue *et al*., 2001) and tumor necrosis factor-α (De Keulenaer *et al*., 1998) stimulate superoxide generation, and these effects appear to be associated with upregulated expression of the subunits of NADPH oxidase. In this study, we found that preincubation with AngII produced about two-fold increase in NADPH oxidase activity, and this effect was mediated by the AT_1 receptor. To further test the efficacies of M40403 in pathophysiological situations, we examined the effects of M40403 in AngII-treated cells. Coincubation with M40403 greatly reduced the increase in superoxide production by AngII, comparable with the effect of losartan. Taken together, our results indicate that M40403 can functionally antagonize the NADPH oxidase activity in smooth muscle cells under both normal and pathological conditions.

Endothelial NO function is compromised in $apoE^0$ mice, and this is consistent with observations in other species that endothelial dysfunction is a prominent manifestation of early vascular changes in hyperlipidemia and atherosclerosis (Dusting *et al*., 1998). The role of superoxide in the pathogenesis of endothelial dysfunction in $apoE^0$ mice has been investigated by several groups. Laursen *et al*. (2001) showed that there is an increase in superoxide production in $apoE^0$ aorta. Moreover, d'Uscio *et al*. (2001) demonstrated that the endothelial dysfunction is partly reversed by another SOD mimetic Mn(III)tetrakis(4-benzoic acid)porphyrin (MnTBAP) (d'Uscio *et al*., 2001), suggesting that superoxide is involved in the development of endothelial dysfunction. We have now analyzed the relation between the time courses of changes in vascular superoxide production and the endothelial function in this model. Clearly, the decrease in endothelial function is closely associated with a concomitant elevation in vascular superoxide production, and decreased endothelial function was accompanied by an increase in the enzymatic activity of NADPH oxidase. All of these data indicate that NADPH oxidase-derived superoxide anions in the vascular wall have an important role in the impaired endothelial NO function in $apoE^0$ mice. It should be noted that the cellular origin of superoxide in intact blood vessel rings might be diverse. In $apoE^0$ aorta, a potential source of superoxide is phagocytic NADPH oxidase in macrophages (foam cells), which comprise the major cellular component of atherosclerotic lesions. Although the present study did not distinguish the origins of superoxide in $apoE^0$ vessels, recent findings that the superoxide production in vascular tissues is impaired in p47phox knockout mice but not altered in gp91phox (a subunit present in phagocytes and endothelial cells but not smooth muscle cells) knockouts suggest that cells other than phagocytes may also be important in inducing oxidative stress in the vessel wall (Barry-Lane *et al*., 2001; Souza *et al*., 2001).

Acute treatment with M40403 improved the endothelium-dependent relaxation in $apoE^0$ aorta to a level comparable to that in wildtype controls. The specificity of the effect of M40403 on superoxide is confirmed by the observation that exogenously generated superoxide diminishes the basal release of NO in normal vessels (indicated by increased contractions to phenylephrine and its sensitivity to the blockade of NO pathway), and this action is also prevented by M40403 pretreatment. Such observations are consistent with those reported by Laursen *et al*. (2001) showing that acute treatment with liposome-entrapped SOD normalized the endothelium-dependent relaxation in $apoE^0$ aorta. The mechanisms of the impairment in endothelial function in the $apoE^0$ model are controversial and complex. Apart from the inactivation of NO by superoxide, it has been suggested that the product of the reaction between these two species, peroxynitrite, may facilitate the degradation of the NOS cofactor tetrahydrobiopterin, leading to reduced NO production (Laursen *et al*., 2001). Whatever the mechanism, suppression of vascular superoxide generation would confer a protective effect on endothelial NO function. On the other hand, others have reported that endothelial NOS (eNOS) activity is reduced in high-fat-fed $apoE^0$ mice aorta as compared to WT mice, whereas the protein level of eNOS is not changed. This alteration was not observed in our study or that of d'Uscio (d'Uscio *et al*., 2001), where the impairment in endothelium-dependent vasorelaxation was largely prevented by acute superoxide dismutation. The reason for this discrepancy is unclear.

In summary, we have demonstrated that the manganese-based, cell-permeable SOD mimetic M40403 improved the endothelial NO function in $apoE^0$ aorta *via* a functional antagonism of the vascular NADPH oxidase activity. Clearly, synthetic SOD mimetics have therapeutic potential for improving endothelial function in cardiovascular disorders associated with elevated vascular oxidative stress.

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References

ARAI, T., KELLY, S.A., BRENGMAN, M.L., TAKANO, M., SMITH, E.H., GOLDSCHMIDT-CLERMONT, P.J. & BULKLEY, G.B. (1998). Ambient but not incremental oxidant generation effects intercellular adhesion molecule 1 induction by tumour necrosis factor alpha in endothelium. *Biochem. J.,* **331,** 853–861.

BARRY-LANE, P.A., PATTERSON, C., VAN DER MERWE, M., HU, Z., HOLLAND, S.M., YEH, E.T. & RUNGE, M.S. (2001). p47phox is required for atherosclerotic lesion progression in ApoE(−/−) mice. *J. Clin. Invest.,* **108,** 1513–1522.

DE KEULENAER, G.W., ALEXANDER, R.W., USHIO-FUKAI, M., ISHIZAKA, N. & GRIENDLING, K.K. (1998). Tumour necrosis factor alpha activates a p22phox-based NADH oxidase in vascular smooth muscle. *Biochem. J.,* **329,** 653–657.

D'USCIO, L.V., BAKER, T.A., MANTILLA, C.B., SMITH, L., WEILER, D., SIECK, G.C. & KATUSIC, Z.S. (2001). Mechanism of endothelial dysfunction in apolipoprotein E-deficient mice. *Arterioscler. Thromb. Vasc. Biol.,* **21,** 1017–1022.

DUSTING, G.J., FENNESSY, P., YIN, Z.L. & GUREVICH, V. (1998). Nitric oxide in atherosclerosis: vascular protector or villain? *Clin. Exp. Pharmacol. Physiol.,* **25** (Suppl)**,** S34–S41.

GRIENDLING, K.K., MINIERI, C.A., OLLERENSHAW, J.D. & ALEXANDER, R.W. (1994). Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ. Res.,* **74,** 1141–1148.

GRIENDLING, K.K., SORESCU, D. & USHIO-FUKAI, M. (2000). NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ. Res.,* **86,** 494–501.

GUZIK, T.J., WEST, N.E., BLACK, E., MCDONALD, D., RATNATUNGA, C., PILLAI, R. & CHANNON, K.M. (2000). Vascular superoxide production by NAD(P)H oxidase: association with endothelial dysfunction and clinical risk factors. *Circ. Res.,* **86,** E85–E90.

JIANG, F., GIBSON, A.P. & DUSTING, G.J. (2001). Endothelial dysfunction induced by oxidized low-density lipoproteins in isolated mouse aorta: a comparison with apolipoprotein-E deficient mice. *Eur. J. Pharmacol.,* **424,** 141–149.

KERR, S., BROSNAN, M.J., MCINTYRE, M., REID, J.L., DOMINICZAK, A.F. & HAMILTON, C.A. (1999). Superoxide anion production is increased in a model of genetic hypertension: role of the endothelium. *Hypertension,* **33,** 1353–1358.

KOBAYASHI, T., TSUNAWAKI, S. & SEGUCHI, H. (2001). Evaluation of the process for superoxide production by NADPH oxidase in human neutrophils: evidence for cytoplasmic origin of superoxide. *Redox. Rep.,* **6,** 27–36.

KUNSCH, C. & MEDFORD, R.M. (1999). Oxidative stress as a regulator of gene expression in the vasculature. *Circ. Res.,* **85,** 753–766.

LASSEGUE, B., SORESCU, D., SZOCS, K., YIN, Q., AKERS, M., ZHANG, Y., GRANT, S.L., LAMBETH, J.D. & GRIENDLING, K.K. (2001). Novel gp91(phox) homologues in vascular smooth muscle cells : nox1 mediates angiotensin II-induced superoxide formation and redox-sensitive signaling pathways. *Circ. Res.,* **88,** 888–894.

LAURSEN, J.B., SOMERS, M., KURZ, S., MCCANN, L., WARNHOLTZ, A., FREEMAN, B.A., TARPEY, M., FUKAI, T. & HARRISON, D.G. (2001). Endothelial regulation of vasomotion in apoE-deficient mice: implications for interactions between peroxynitrite and tetrahydrobiopterin. *Circulation,* **103,** 1282–1288.

MACARTHUR, H., WESTFALL, T.C., RILEY, D.P., MISKO, T.P. & SALVEMINI, D. (2000). Inactivation of catecholamines by superoxide gives new insights on the pathogenesis of septic shock. *Proc. Natl. Acad. Sci. U.S.A.,* **97,** 9753–9758.

MARUMO, T., SCHINI-KERTH, V.B., FISSLTHALER, B. & BUSSE, R. (1997). Platelet-derived growth factor-stimulated superoxide anion production modulates activation of transcription factor NF-κB and expression of monocyte chemoattractant protein 1 in human aortic smooth muscle cells. *Circulation,* **96,** 2361–2367.

MILLER JR, F.J., GUTTERMAN, D.D., RIOS, C.D., HEISTAD, D.D. & DAVIDSON, B.L. (1998). Superoxide production in vascular smooth muscle contributes to oxidative stress and impaired relaxation in atherosclerosis. *Circ. Res.,* **82,** 1298–1305.

MORAWIETZ, H., WEBER, M., RUECKSCHLOSS, U., LAUER, N., HACKER, A. & KOJDA, G. (2001). Upregulation of vascular NAD(P)H oxidase subunit gp91phox and impairment of the nitric oxide signal transduction pathway in hypertension. *Biochem. Biophys. Res. Commun.,* **285,** 1130–1135.

MUGGE, A., BRANDES, R.P., BOGER, R.H., DWENGER, A., BODE-BOGER, S., KIENKE, S., FROLICH, J.C. & LICHTLEN, P.R. (1994). Vascular release of superoxide radicals is enhanced in hypercholesterolemic rabbits. *J. Cardiovasc. Pharmacol.,* **24,** 994–998.

MUGGE, A., ELWELL, J.H., PETERSON, T.E., HOFMEYER, T.G., HEISTAD, D.D. & HARRISON, D.G. (1991). Chronic treatment with polyethylene-glycolated superoxide dismutase partially restores endothelium-dependent vascular relaxations in cholesterol-fed rabbits. *Circ. Res.,* **69,** 1293–1300.

MUNZEL, T., HINK, U., HEITZER, T. & MEINERTZ, T. (1999). Role for NADPH/NADH oxidase in the modulation of vascular tone. *Ann. NY. Acad. Sci.,* **874,** 386–400.

OHARA, Y., PETERSON, T.E. & HARRISON, D.G. (1993). Hypercholesterolemia increases endothelial superoxide anion production. *J. Clin. Invest.,* **91,** 2546–2551.

PARAVICINI, T.M., GULLUYAN, L.M., DUSTING, G.J. & DRUMMOND, G.R. (2002). Increased NADPH oxidase activity, gp91phox expression, and endothelium-dependent vasorelaxation during neointima formation in rabbits. *Circ. Res.,* **91,** 54–61.

PATTERSON, C., RUEF, J., MADAMANCHI, N.R., BARRY-LANE, P., HU, Z., HORAIST, C., BALLINGER, C.A., BRASIER, A.R., BODE, C. & RUNGE, M.S. (1999). Stimulation of a vascular smooth muscle cell NAD(P)H oxidase by thrombin. Evidence that p47(phox) may participate in forming this oxidase *in vitro* and *in vivo*. *J. Biol. Chem.,* **274,** 19814–19822.

PLUMP, A.S., SMITH, J.D., HAYEK, T., AALTO-SETALA, K., WALSH, A., VERSTUYFT, J.G., RUBIN, E.M. & BRESLOW, J.L. (1992). Severe hypercholesterolemia and atherosclerosis in apolipoprotein E- deficient mice created by homologous recombination in ES cells. *Cell,* **71,** 343–353.

PUEYO, M.E., GONZALEZ, W., NICOLETTI, A., SAVOIE, F., ARNAL, J.F. & MICHEL, J.B. (2000). Angiotensin II stimulates endothelial vascular cell adhesion molecule-1 *via* nuclear factor-κB activation induced by intracellular oxidative stress. *Arterioscler. Thromb. Vasc. Biol.,* **20,** 645–651.

SALVEMINI, D., MAZZON, E., DUGO, L., RILEY, D.P., SERRAINO, I., CAPUTI, A.P. & CUZZOCREA, S. (2001). Pharmacological manipulation of the inflammatory cascade by the superoxide dismutase mimetic, M40403. *Br. J. Pharmacol.,* **132,** 815–827.

SALVEMINI, D., WANG, Z.Q., ZWEIER, J.L., SAMOUILOV, A., MACARTHUR, H., MISKO, T.P., CURRIE, M.G., CUZZOCREA, S., SIKORSKI, J.A. & RILEY, D.P. (1999). A nonpeptidyl mimic of superoxide dismutase with therapeutic activity in rats. *Science,* **286,** 304–306.

SOUZA, H.P., LAURINDO, F.R., ZIEGELSTEIN, R.C., BERLOWITZ, C.O. & ZWEIER, J.L. (2001). Vascular NAD(P)H oxidase is distinct from the phagocytic enzyme and modulates vascular reactivity control. *Am. J. Physiol. Heart Circ. Physiol.,* **280,** H658–H667.

USHIO-FUKAI, M., ZAFARI, A.M., FUKUI, T., ISHIZAKA, N. & GRIENDLING, K.K. (1996). p22phox is a critical component of the superoxide-generating NADH/NADPH oxidase system and regulates angiotensin II-induced hypertrophy in vascular smooth muscle cells. *J. Biol. Chem.,* **211,** 23317–23321.

WHITE, C.R., BROCK, T.A., CHANG, L.Y., CRAPO, J., BRISCOE, P., KU, D., BRADLEY, W.A., GIANTURCO, S.H., GORE, J., FREEMAN, B.A. & TARPEY, M.M. (1994). Superoxide and peroxynitrite in atherosclerosis. *Proc. Natl. Acad. Sci. U.S.A.,* **91,** 1044–1048.

YANG, R., POWELL-BRAXTON, L., OGAOAWARA, A.K., DYBDAL, N., BUNTING, S., OHNEDA, O. & JIN, H. (1999). Hypertension and endothelial dysfunction in apolipoprotein E knockout mice. *Arterioscler. Thromb. Vasc. Biol.,* **19,** 2762–2768.

YOKOYAMA, M., INOUE, N. & KAWASHIMA, S. (2000). Role of the vascular NADH/NADPH oxidase system in atherosclerosis. *Ann. NY. Acad. Sci.,* **902,** 241–247 discussion 247–248.

ZANETTI, M., SATO, J., KATUSIC, Z.S. & O'BRIEN, T. (2001). Gene transfer of superoxide dismutase isoforms reverses endothelial dysfunction in diabetic rabbit aorta. *Am. J. Physiol. Heart Circ. Physiol.,* **280,** H2516–H2523.

ZICHA, J., DOBESOVA, Z. & KUNES, J. (2001). Relative deficiency of nitric oxide-dependent vasodilation in salt- hypertensive Dahl rats: the possible role of superoxide anions. *J. Hypertens.,* **19,** 247–254.

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