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## Research Paper

## Extracellular but not cytosolic superoxide dismutase protects against oxidant-mediated endothelial dysfunction



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## ABSTRACT

Superoxide ( $O_2^{\bullet-}$ ) contributes to the development of cardiovascular disease. Generation of  $O_2^{\bullet-}$  occurs in both the intracellular and extracellular compartments. We hypothesized that the gene transfer of cytosolic superoxide dismutase (SOD1) or extracellular SOD (SOD3) to blood vessels would differentially protect against  $O_2^{\bullet-}$ -mediated endothelial-dependent dysfunction. Aortic ring segments from New Zealand rabbits were incubated with adenovirus (Ad) containing the gene for *Escherichia coli*  $\beta$ -galactosidase, SOD1, or SOD3. Activity assays confirmed functional overexpression of both SOD3 and SOD1 isoforms in aorta 24 h following gene transfer. Histochemical staining for  $\beta$ -galactosidase showed gene transfer occurred in the endothelium and adventitia. Next, vessels were prepared for measurement of isometric tension in Krebs's buffer containing xanthine. After precontraction with phenylephrine, xanthine oxidase impaired relaxation to the endothelium-dependent dilator acetylcholine (ACh, max relaxation  $33 \pm 4\%$  with XO vs.  $64 \pm 3\%$  without XO,  $p < 0.05$ ), whereas relaxation to the endothelium-independent dilator sodium nitroprusside was unaffected. In the presence of XO, maximal relaxation to ACh was improved in vessels incubated with AdSOD3 ( $55 \pm 2\%$ ,  $p < 0.05$  vs. control) but not AdSOD1 ( $34 \pm 4\%$ ). We conclude that adenoviral-mediated gene transfer of SOD3, but not SOD1, protects the aorta from xanthine/XO-mediated endothelial dysfunction. These data provide important insight into the location and enzymatic source of  $O_2^{\bullet-}$  production in vascular disease.

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## Introduction

Reactive oxygen species (ROS) have an important role in mediating vascular disease in hypertension, diabetes mellitus, and atherosclerosis, as well as in acute conditions such as hypoxia-reoxygenation. One clinically-relevant enzymatic source of ROS is xanthine oxidase (XO). Circulating levels of XO are increased with hypercholesterolemia [1,2]. Moreover, activity of XO is elevated in human atherosclerotic lesions [3–5]. Infusion of a XO inhibitor in humans with cardiovascular disease improves vascular function [6]. The pathophysiologic effects of ROS causing vascular dysfunction can occur through multiple mechanisms. Superoxide anion ( $O_2^{\bullet-}$ ) reacts with endothelium-derived nitric oxide ( $\bullet$ NO) via a radical-radical reaction at a diffusion-limited rate to generate peroxynitrite ( $ONOO^-$ ), a potent oxidant and mediator

of vascular tissue injury [7]. Inactivation of  $\bullet$ NO impairs the ability of vessels to relax normally [8]. Peroxynitrite can oxidize lipoproteins and damage lipid membranes. Oxidation of LDL in the vessel wall by  $O_2^{\bullet-}$  can alter signal transduction and cause cytotoxicity [9]. Excess levels of  $O_2^{\bullet-}$  increase platelet aggregation [10], and the adhesion and migration of monocytes [11]. Cell proliferation and cell death are also influenced by the cellular pro-oxidant state [12]. By activating metalloproteinases,  $O_2^{\bullet-}$  can also modulate matrix degradation [13].

Perhaps the most important cellular mechanism by which to protect against endothelial dysfunction involves the activity of superoxide dismutases (SODs) in the vessel wall [14]. In cardiovascular disease,  $O_2^{\bullet-}$ -generating systems can result in both the intracellular and extracellular generation of  $O_2^{\bullet-}$ . Of the two copper–zinc containing SODs, one resides in the cytoplasm (SOD1) and the second is extracellular and has an affinity for cell-surface heparin sulfate proteoglycans (SOD3) [15]. Adenoviral delivery of SOD3 improves endothelial-dependent relaxation in multiple disease states [16–19]. In contrast, gene transfer of SOD1 restores acetylcholine-dependent vasodilation response in diabetic rabbits [20], whereas arterial relaxation in either hyperlipidemic rabbits or rabbits treated with Ang-II is not improved [21,22]. A limitation of these studies is that  $O_2^{\bullet-}$  localization may be intracellular and/or extracellular.

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In this study, we compared the roles of intracellular and extracellular SOD in protecting against extracellular oxidant-induced endothelial dysfunction. Our data demonstrate that adenoviral transfer of SOD3 but not SOD1 improves vasomotor function following exposure to xanthine/XO. We conclude that, to maintain bioavailability of •NO, SOD must be localized to the site of  $O_2^{\bullet-}$  production rather than •NO production, suggesting that the reaction of •NO with  $O_2^{\bullet-}$  occurs outside the cell.

## Methods

### Animals and tissue preparation

All protocols were approved by the Animal Care and Use Committee at the University of Iowa. New Zealand white rabbits ( $n=17$ , either sex, 2.5–3.0 kg) were sacrificed with an overdose of pentobarbital and the thoracic aorta immediately dissected and placed into ice cold Krebs's bicarbonate buffer of the following composition (in mM) 118.0 NaCl, 4.7 KCl, 2.5  $CaCl_2$ , 1.2  $MgSO_4$ , 25.0  $NaHCO_3$ , 1.2  $KH_2PO_4$ , 5.5 glucose, 0.0025 EDTA. The aorta was trimmed free of fat and loosely adhering connective tissue, and cut into approximately 2.5 mm wide ring segments. Vessel segments were incubated for two hours at 37 °C in 1.0 ml of Dulbecco's Modified Eagle Medium (DMEM) containing 0.1 ml of either replication deficient adenovirus ( $5 \times 10^9$  pfu/ml) or phosphate buffered saline as described below. After two hours, the vessel segments were transferred to DMEM containing 10% bovine serum and placed in an incubator at 37 °C for 24 h.

### Adenoviral-mediated gene transfer

Adenovirus (Ad) with CMV promoters used for this study were obtained from the University of Iowa Gene Vector Core Facility and include Ad $\beta$ gal for expression of the histochemical marker  $\beta$ -galactosidase, AdSOD3 [21] for expression of the human extracellular isoform of SOD, or AdSOD1 [23] for expression of the human copper–zinc cytoplasmic isoform of SOD.

### $\beta$ -galactosidase activity assay

Rings were rinsed in phosphate buffered saline and fixed in 2% paraformaldehyde for 10 min. Segments were then stained in 1 mg/ml of X-gal for 4 h at 37 °C. Tissue was further fixed in 2% paraformaldehyde, imbedded in paraffin, and sectioned for histologic analysis.

### SOD activity assay

SOD activity was measured by the method of NADPH oxidation described by Paoletti and Mocali [24]. Because this method relies on the oxidation of NADPH, it is less prone than other SOD assays to interference by nonspecific reduction from cellular components. Care was taken to insure specimens were free of blood. Aorta from four different rabbits were combined for each intervention (control, Ad $\beta$ gal, AdSOD3, and AdSOD1) and homogenized with a Dounce homogenizer in 50 mM  $KPO_4$  buffer (pH 7.4, 4 °C) containing anti-proteolytics (PMSF 1.0 mM, leupeptin 10  $\mu$ g/ml, aprotinin 10  $\mu$ g/ml, and pepstatin A 10  $\mu$ g/ml, Sigma). The homogenate was sonicated (five second burst times five) and centrifuged at 3000g for ten minutes at 4 °C. Protein concentrations were determined by the Lowry assay. The assay was performed in triplicate and values averaged.

### Isometric force measurement

Vasomotor reactivity of rabbit aorta was measured by determination of isometric force, 24 h after virus incubation. Ring segments were

mounted in organ baths containing 10 ml of Krebs's buffer containing xanthine ( $10^{-4}$  M) and catalase (500 U/ml) at 37 °C and continuously gassed with 20%  $O_2$ , 5%  $CO_2$ , and 75%  $N_2$ . Rings were stretched to an optimal resting tension of 6 g as determined by repeated administration of KCl (75 mM). Vessels were equilibrated for 30 min and then constricted twice with KCl (75 mM). After the segments were washed, phenylephrine was added to achieve a tension of 50–100% of the maximal contraction to KCl. Xanthine oxidase (XO, Sigma) was added to the buffer for generation of  $O_2^{\bullet-}$  immediately prior to performing a concentration response to the endothelium-dependent vasodilator acetylcholine (ACh,  $10^{-9}$ – $10^{-5}$  M) or the endothelium-independent dilator sodium nitroprusside (SNP,  $10^{-9}$ – $10^{-5}$  M). Although in some vessels the addition of XO produced an increase in tension, this increase was not significant.

Based on our findings that 5 U/L of XO moderately impaired relaxation to ACh without affecting the response to SNP, protocols examining vasomotor responses following gene transfer were performed with 5 U/L of XO. Only one concentration-dose response curve was performed in each vessel. In this way, abnormalities in relaxation could not be attributed to prior exposure to xanthine/XO.

### Data analysis

Results are expressed as mean  $\pm$  standard error. For the ring studies, data were obtained in duplicate for each intervention (control, Ad $\beta$ gal, AdSOD3, and AdSOD1) and averaged such that  $n$  is equal to the number of animals studied. Relaxations are the percent change from the precontracted tension. Dilator responses are compared among groups using a two factor repeated measures ANOVA with a Bonferroni correction for multiple comparisons. A non-linear curve fit (3 parameter with a Hill slope of 1.0) was used to determine the maximal and EC50 relaxation (GraphPad Prism for Windows). Statistical significance was accepted if the null hypothesis was rejected at  $p < 0.05$ .

## Results and discussion

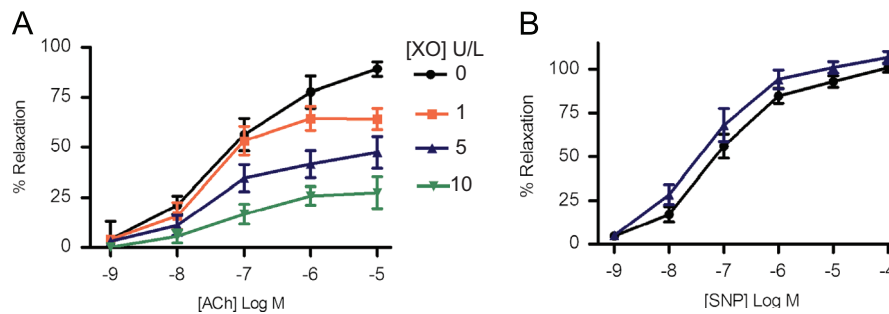
### Effect of xanthine and xanthine oxidase on relaxation of aorta

We adapted a previously described model whereby incubation of rabbit aortic ring segments in the presence of xanthine/XO impairs endothelial-dependent relaxation [2]. After contraction with phenylephrine (mean  $75 \pm 4\%$ ), increasing concentrations of the endothelium-dependent dilator ACh produced a dose-dependent relaxation (Fig. 1A). Addition of XO immediately prior to ACh impaired relaxation. Our protocol for oxidant production was designed such that generation of  $O_2^{\bullet-}$  occurred simultaneously with ACh-induced release of •NO. The maximal relaxation at  $10^{-5}$  M ACh was  $88 \pm 4\%$  for control,  $61 \pm 7\%$  for 1 U/L XO,  $50 \pm 8\%$  for 5 U/L XO, and  $32 \pm 5\%$  for 10 U/L XO ( $p < 0.05$  for each XO dose vs. control). These results confirmed that oxidants derived from XO mediate vascular dysfunction in a concentration-dependent manner [2].

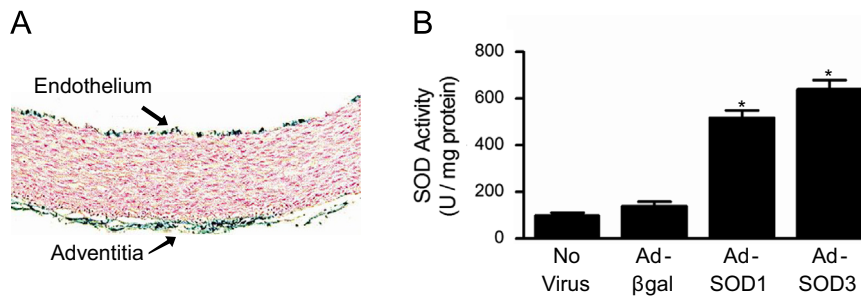
To confirm that the observed impairment in dilation was endothelium specific, we examined the relaxation of rabbit aorta to SNP. Increasing concentrations of SNP produced a dose-dependent relaxation that was not affected by XO (Fig. 1B, relaxation at  $10^{-5}$  M SNP is  $100 \pm 5\%$  for control and  $97 \pm 4\%$  for 5 U/L XO,  $p=NS$ ,  $n=5$ ). Based on these data, 5 U/L of XO was used in subsequent protocols to produce free radical-mediated impairment in relaxation.

### Gene transfer of SOD to rabbit aorta increases SOD expression and activity

Following gene transfer of  $\beta$ -galactosidase into vessel segments, histochemical staining demonstrated  $\beta$ -galactosidase in the endothelium and adventitia, indicative of efficient gene transfer (Fig. 2A).



**Fig. 1.** Xanthine oxidase-derived  $O_2^{\bullet-}$  impairs endothelial-dependent relaxation of rabbit aorta. (A) Dose-dependent relaxation to endothelium-dependent dilator acetylcholine (ACh) in the absence or presence of increasing concentrations of XO as indicated. (B) Dose-dependent relaxation to endothelium-independent dilator sodium nitroprusside (SNP) in the absence (black line) or presence (blue line) of 5 U/L XO. ( $n=8-12$ ) \* $p < 0.05$  vs. control (no XO). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



**Fig. 2.** Adenoviral-mediated gene transfer of SOD3 or SOD1 increases total SOD activity in rabbit aorta. (A)  $\beta$ -galactosidase activity as determined by X-gal staining of aortic segments exposed to Ad $\beta$ gal. Blue staining denotes  $\beta$ -galactosidase activity, which is limited to the endothelium and adventitial layers. (B) SOD activity in rabbit aorta one day after adenoviral-mediated gene transfer of SOD1 or SOD3. Values are the mean of three assays performed on combined homogenate from four aortae. \* $p < 0.05$  vs. Ad $\beta$ gal.

Others have provided evidence that SOD3 and SOD1 properly bind to the membrane and localize to the cytosol, respectively, when expressed *in vivo* using these adenoviral constructs [17,25,26]. To confirm gene transfer of SOD3 and SOD1 produced functional antioxidant protein in the vessel segments, SOD activity was determined by measuring NADPH oxidation. Total SOD activity in vessels after transduction of SOD was approximately 4–6 times higher than in non-transduced vessels (Fig. 2B).

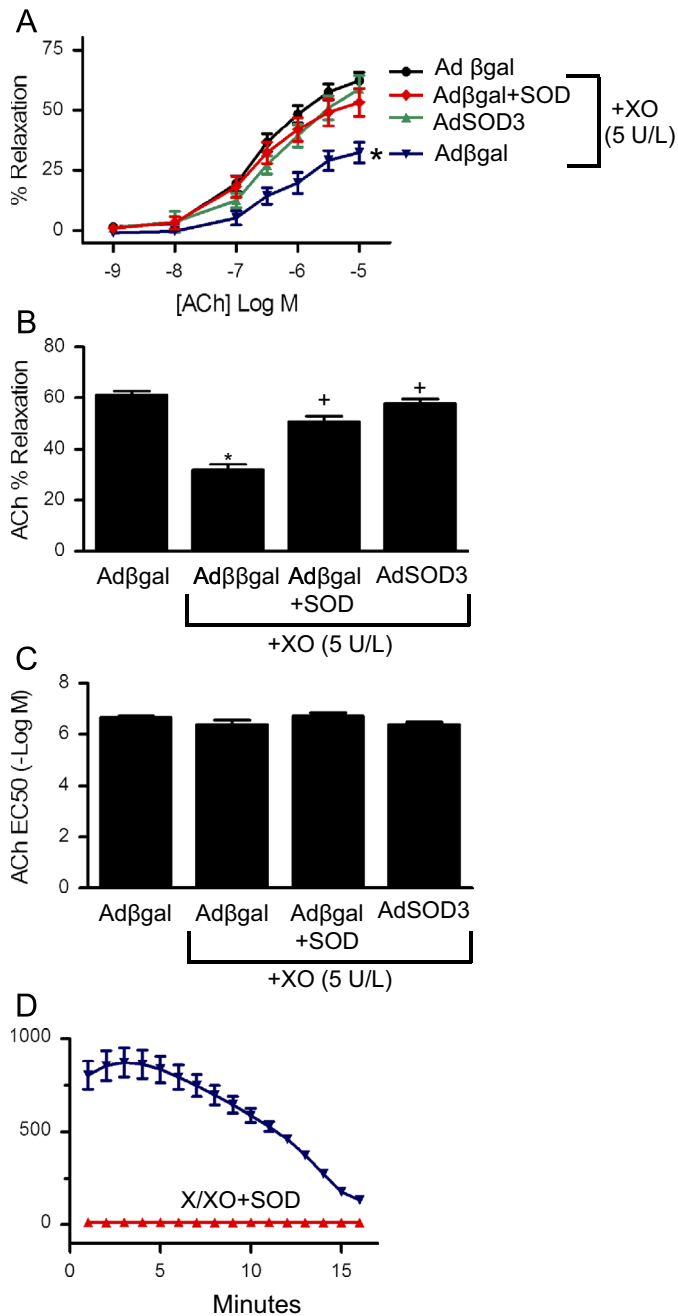
#### Effects of gene transfer of SOD on superoxide mediated vasomotor dysfunction

We next examined the ability of gene transfer of SOD in aorta to protect against oxidant-mediated abnormal relaxation. Following gene transfer of  $\beta$ -galactosidase, the concentration-dependent relaxation to ACh was impaired in the presence of XO (Fig. 3A). Overexpression of SOD3 in the presence of XO restored relaxation to control levels observed in the absence of XO. The maximal relaxation of control vessels was markedly reduced by XO, whereas gene transfer of SOD3 restored relaxation to ACh (Fig. 3B). There was no difference in the EC<sub>50</sub> between groups (Fig. 3C). Bovine SOD added exogenously to the circulating buffer prior to addition of XO improved relaxation to ACh as compared to aorta in XO without SOD (Fig. 3A, C). This concentration of exogenous SOD was sufficient to inhibit  $O_2^{\bullet-}$  accumulation by xanthine/XO detected by lucigenin-enhanced chemiluminescence (Fig. 3D). In contrast to overexpression of SOD3, ACh-induced relaxation of vessels overexpressing SOD1 was unchanged from control vessels after XO (Fig. 4). As in Fig. 3, bovine SOD in the presence of XO restored relaxation to ACh (Fig. 4). In all groups, relaxation to the endothelium-independent dilator SNP was similar (Fig. 5). These data indicate that extracellular, but not cytosolic,

SOD improved endothelium-dependent dilation during generation of  $O_2^{\bullet-}$  by xanthine and XO.

In aorta incubated in xanthine/XO, gene transfer of SOD3 only partially restored relaxation to ACh. Although greater vascular levels of SOD, through increased transgene expression, may have further improved ACh-induced dilation, this is unlikely since vessels incubated in AdSOD3 relaxed similarly to vessels incubated in exogenous SOD (500 U/ml). These observations suggest that the inability of SOD to completely restore dilator responses of vessels in xanthine/XO results from non-superoxide mediated mechanisms. This hypothesis is consistent with studies showing hydrogen peroxide contributes to vascular injury caused by xanthine/XO [27].

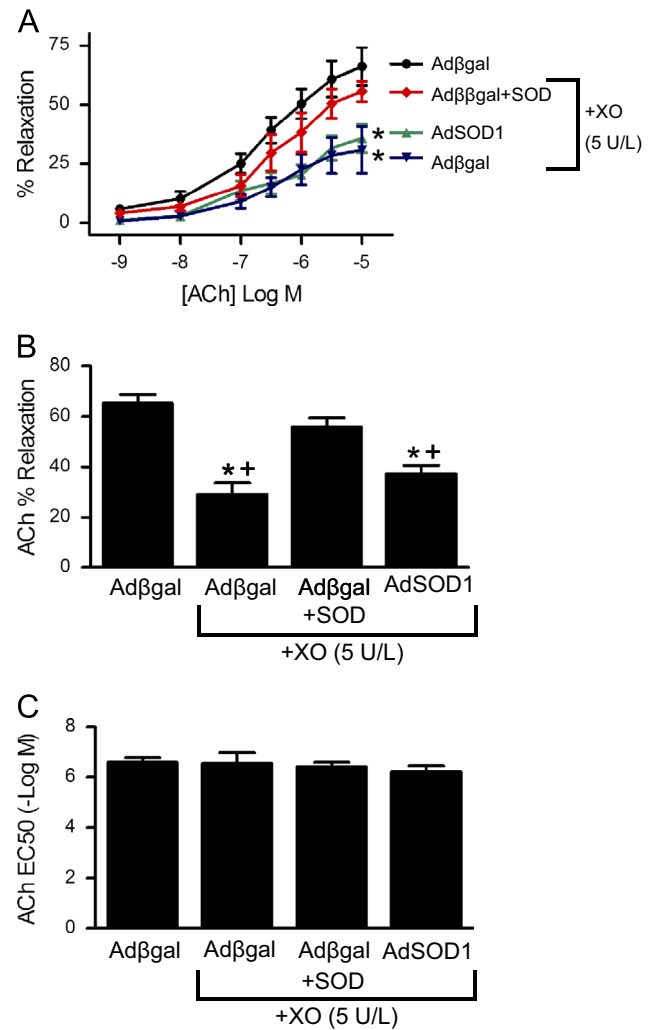
The observation that gene transfer of SOD3 but not SOD1 improved relaxation of aorta during generation of  $O_2^{\bullet-}$  can be explained by the cellular compartmentalization of the antioxidants. Despite relatively similar SOD activity following gene transfer of the two SOD isoforms, SOD1 is localized to the cytosol and therefore has limited access to  $O_2^{\bullet-}$  generated in the extracellular space [15]. In contrast, since SOD3 is primarily bound to extracellular proteoglycans, it has greater access to  $O_2^{\bullet-}$  generated by xanthine/XO. A limitation of this interpretation is based on the concentration of XO at the endothelial surface via its reversible interaction with glycosaminoglycans [28]. In this way, SOD3 may displace XO and reduce its surface concentration, thereby limiting endothelial dysfunction independent of its antioxidant effect. In addition to XO, multiple other cellular enzyme systems have been implicated in vascular disease, including NADPH oxidases, uncoupled endothelial nitric oxide synthase (eNOS), mitochondrial respiratory chain, and arachidonic acid metabolizing enzymes. Our model specifically examines the ability of SOD1 vs. SOD3 in restoring endothelial function in the presence of extracellular XO and do not necessarily address the contribution of these other enzymes that generate intracellular  $O_2^{\bullet-}$ .



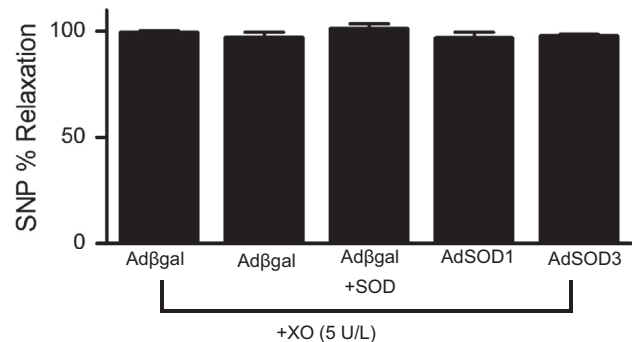
**Fig. 3.** Gene transfer of SOD3 protects aorta against XO-mediated vasomotor dysfunction. Following gene transfer of SOD3, (A) dose-dependent relaxation to ACh in the absence or presence of XO was determined. Gene transfer of  $\beta$ -galactosidase serves as negative control and exogenously applied bovine SOD (500 U/ml) serves as positive control. ( $n=6-10$ ) \* $p < 0.05$  vs Ad $\beta$ gal without XO. (B) Maximal relaxation to ACh based on non-linear curve fit of data in (A). \* $p < 0.05$  vs Ad $\beta$ gal without XO; + $p < 0.05$  Ad $\beta$ gal with XO. (C) EC50 relaxation to ACh based on non-linear curve fit of data in (A). (D) Lucigenin-enhanced chemiluminescence of xanthine/XO in the presence or absence of bovine SOD ( $n=3$ );  $p < 0.05$ .

## Conclusion

In summary, our data demonstrate that adenoviral mediated gene transfer of either SOD3 or SOD1 to rabbit aorta increases antioxidant activity, but only SOD3 protects against xanthine/XO-induced endothelial dysfunction. Selective overexpression of SOD to intracellular or extracellular compartments provides important insights into the location and enzymatic source of  $O_2^{\bullet-}$  production in vascular disease. Our data are particularly interesting when



**Fig. 4.** Gene transfer of SOD1 does not protect against XO-mediated vasomotor dysfunction. Following gene transfer of SOD1, (A) dose-dependent relaxation to ACh in the absence or presence of XO was determined as described for Fig. 3. \* $p < 0.05$  vs Ad $\beta$ gal without XO. (B) Maximal relaxation and (C) EC50 to ACh based on non-linear curve fit of data in (A). \* $p < 0.05$  vs Ad $\beta$ gal without XO; + $p < 0.05$  Ad $\beta$ gal+SOD+XO.



**Fig. 5.** Relaxation to SNP is preserved in the presence of xanthine-XO. Relaxation response to  $10^{-5}$  M SNP following gene transfer and treatment with XO. Gene transfer of  $\beta$ -galactosidase serves as negative control and exogenously applied bovine SOD (500 U/ml) serves as positive control. ( $n=6-10$ ).

considering that both XO and SOD3 can be internalized into the cell via endocytosis [29,30]. The lack of protection with SOD1 provides strong evidence that the interaction of  $^*NO$  with  $O_2^{\bullet-}$  occurs either outside of the cell, or potentially in an endocytic vesicle. XO is implicated as the source of  $O_2^{\bullet-}$  in the vascular

dysfunction associated with ischemia/reperfusion, diabetes, and atherosclerosis [31–33], denoting the relevance of our studies with xanthine/XO. Whereas oxypurinol and allopurinol improve vascular relaxation in humans [6], this strategy of inhibiting XO-derived  $O_2^{\bullet-}$  is most effective in patients that are hyperuricemic [34]. By contrast, SOD3 is protective in the pathogenesis of ischemia/reperfusion injury, atherosclerosis, and hypertension [15]. Thus, our data suggest that selective targeting of extracellular  $O_2^{\bullet-}$ , i.e. with SOD3, may be broadly applicable to multiple cardiovascular disease settings.

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