A comparison of reactive oxygen species metabolism in the rat aorta and vena cava: focus on xanthine oxidase

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Szasz T, Thompson JM, Watts SW. A comparison of reactive oxygen species metabolism in the rat aorta and vena cava: focus on xanthine oxidase. Am J Physiol Heart Circ Physiol 295: H1341–H1350, 2008. First published July 25, 2008; doi:10.1152/ajpheart.00569.2008.—Reactive oxygen species (ROS) are important mediators in vascular biology. Venous function, although relevant to cardiovascular disease, is still understudied. We compared aspects of ROS metabolism between a major artery (the aorta) and a major vein (the vena cava, VC) of the rat, with the hypothesis that venous ROS metabolism would be overall increased compared with its arterial counterpart. Superoxide and hydrogen peroxide (H$_2$O$_2$) release in basal conditions was higher in VC compared with aorta. The antioxidant capacity for H$_2$O$_2$ was also higher in VC than in aorta. Exogenous superoxide induced a higher contraction in VC compared with aorta. Because XO seemed a likely source of the higher VC ROS levels, we examined it further and found higher mRNA expression and activity of XO in VC compared with aorta. We also investigated the impact of XO inhibition by allopurinol on aorta and VC functional responses to norepinephrine, ANG II, ET-1, and ACh. Maximal ET-1-mediated contraction was decreased by allopurinol in VC but not in the aorta. Our results suggest that there are overall differences in ROS metabolism between aorta and VC, with the latter operating normally at a higher set point, releasing but also being able to handle, higher ROS levels. We propose XO to be an important source for these differences. The result of this particular comparison may be reflective of a general arteriovenous contrast.

VEINS ARE OFTEN OVERLOOKED when the contribution of the vascular system to systemic pathophysiology is considered. Yet, besides being a passive clearance conduit of every organ and tissue, veins hold most of the circulating blood (∼70%), a volume that can be actively moved by their contractile properties (28). Increases in mean circulatory filling pressure, a measure of venomotor tone (26), have been reported in the developmental stages of several experimental models of hypertension (9, 19, 43), indicating a potential contribution of the venous system in the pathophysiology of hypertension. Veins also develop specific pathologies, such as deep vein thrombosis and chronic venous insufficiency. In addition, venous grafts are used in coronary artery bypass graft (CABG) surgery with different properties and outcomes compared with arteries (21, 30). All these facts point out the need for a better understanding of the basic physiology of the venous system.

Although they possess the same basic three-layer design as arteries, veins are structurally and functionally different from arteries. The smooth muscle component of veins is smaller than that of arteries, and there are overall differences between arteries and veins in their contractile, endothelial, and synthetic properties. Similarly, there are overall differences in the venous response to pathophysiologic conditions that commonly affect arteries, such as atherosclerosis or hypertension, when veins do not undergo the same atheromatous, remodeling, or endothelial dysfunction changes observed in arteries.

Reactive oxygen species (ROS) are now recognized as important factors in the generation and progression of many cardiovascular diseases, with their involvement in vascular pathology supported by a large body of evidence (27). ROS can influence vascular function by modifying numerous important intracellular signaling pathways, as well as by inducing direct damage to cellular structures when an imbalance between production and destruction of ROS occurs (oxidative stress). Arterial ROS production and the contribution of arterial oxidative stress to cardiovascular disease have been extensively studied. However, little is known about venous ROS metabolism (33).

There are only a few reports comparing aspects of ROS metabolism in arteries and veins. One research group has systematically compared ROS metabolism in human arteries and veins (11, 12). In these studies using tissues from CABG patients, no difference was observed in basal ROS production between the human saphenous vein (HSV) and internal mammary artery (IMA). Different protein and mRNA expression of NAPDH oxidase subunits between HSV and IMA were reported, as well as different contributions by NAPDH oxidase and xanthine oxidase (XO) to the total ROS levels (12). We have previously reported increased superoxide-derived H$_2$O$_2$ production by the rat vena cava (VC) compared with the thoracic aorta, as well as differences between arterial and venous contraction to H$_2$O$_2$, potentially explained by differences in arterial and venous K$^+$ channel activity and extracellular Ca$^{2+}$ influx (34, 35).

We now report higher superoxide production by rat VC compared with aorta in basal conditions. Since these observations were made in normal animals, where no oxidative stress is expected, a logical assumption would be that veins, in accordance with higher ROS production, are capable of handling higher levels of ROS due to increased antioxidant defense mechanisms compared with arteries, as well as potentially different downstream pathways. We therefore hypothesize that ROS metabolism is overall increased in the rat VC compared with aorta. In this report, we investigate basic measures of ROS metabolism in aorta and VC. We then
identify XO as a ROS enzyme contributing to the observed differences and examine its potential functional relevance in terms of vascular reactivity.

MATERIALS AND METHODS

Animal use. Normal male Sprague-Dawley rats (250 g; Charles River, Indianapolis, IN) were anesthetized with pentobarbital sodium (60–80 mg/kg ip). Aorta and VC were removed and used in one of the protocols described below. All animal procedures were approved and performed in accordance with regulations of the Institutional Animal Care and Use Committee at Michigan State University.

Superoxide measurements. Studies were performed as previously described (17, 24). Briefly, rings of aorta and VC were cleaned of outer adipose tissue and incubated in a modified Krebs-HEPES buffer [containing (in mM) 20 HEPES, 119 NaCl, 4.6 KCl, 1.0 MgSO4, 7H2O, 0.15 NaH2PO4, 0.4 KH2PO4, 5 NaHCO3, 1.2 CaCl2, and 5.5 dextrose, pH 7.4] at 37°C for 1 h with diethyldithiocarbamate, a superoxide dismutase (SOD) inhibitor. A TD 20/20 luminoimeter (Turner Designs, Sunnyvale, CA) was used. Lucigenin (5 μM, a concentration previously shown to avoid redox cycling; Ref. 22) was added for 10 min, and 10 consecutive luminometer readings (each 30 s long) were taken. 4,5-Dihydroxy-1,3-benzene disulfonic acid (tiron), a superoxide scavenger, was then added for 15 min to assess background, and 10 more consecutive readings were taken. Tissues were blotted dry and weighed. The average of readings with tiron was subtracted from the average of readings without tiron, and the resulting value was used as input in a superoxide standard curve. Results are presented as nanomoles of superoxide per minute per milligram of tissue.

H2O2 measurements. H2O2 production was measured using an Amplex red H2O2 assay kit (Molecular Probes, Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Aortic and VC rings were cleaned of outer adipose tissue and equilibrated for 1 h at 37°C in the same modified Krebs-HEPES buffer (pH 7.4) as detailed for superoxide measurements. They were then incubated with Ultra red working solution (100 μM) at 37°C for 1 h. The supernatant was then transferred to a 96-well plate, and fluorescence emission was measured (excitation, 530 nm; emission, 590 nm) on an AscentFluoroskan plate reader (Thermo Fisher Scientific, Waltham, MA). A H2O2 standard curve constructed on the same 96-well plate was incubated with Ultra red working solution (100 μM) at 37°C at the same time with the tissues and was used to determine H2O2 concentrations from samples. After each experiment, tissue total protein was determined with the Lowry method and used for normalization.

Antioxidant capacity for H2O2. Antioxidant capacity of aorta and VC was measured using the Amplex red H2O2 assay as detailed above. Incubation of aorta and VC rings with Ultra red working solution (100 μM) was performed in the presence of 20 μM H2O2. H2O2 that was quenched by tissue was determined by subtracting the fluorescence values of samples incubated with 20 μM H2O2 from the fluorescence value of 20 μM H2O2 alone, and the value obtained was used as input in the H2O2 standard curve. After each experiment, tissue total protein was determined with the Lowry method and used for normalization.

Western blot analysis. Proteins were isolated from aorta and VC as previously described (24), and protein concentration was determined using the BCA kit (Sigma-Aldrich, St. Louis, MO). Thirty-five micrograms of protein from each sample and 10 μl of positive control were separated on SDS-polyacrylamide gels and transferred to Immobilon-FL membranes. The membranes were blocked with Li-cor blocker or 5% milk and probed with specific antibodies against XO (Rockland, Gilbertsville, PA), catalase (Abcam, Cambridge, MA), endothelial nitric oxide synthase (eNOS; BD Biosciences, San Jose, CA), Mn-SOD (BD Biosciences), Rac1 (Upstate, Millipore, Billerica, MA), CuZn-SOD (Calbiochem, EMD Biosciences, San Diego, CA), and the 39-kDa subunit of mitochondrial oxidative phosphorylation complex I (Molecular Probes). The appropriate horseradish peroxidase-linked or fluorescent secondary antibodies were added for 1 h at 4°C, followed by visualization with enhanced chemiluminescence or the infrared imaging scanner Odyssey (Li-cor, Lincoln, NE). Gels were stained with Gel Code Blue (Pierce Chemical, Rockford, IL), and bands were reimaged with smooth muscle α-actin antibody (Onecogene, EMD Biosciences, San Diego, CA) to ensure equal protein loading. Band density was quantified using NIH Image version 1.63. Smooth muscle α-actin expression per equal amount of total protein is significantly higher in aortic compared with VC homogenates; therefore, densitometry values were not normalized to α-actin, because this manipulation would have skewed results, biasing expression of proteins of interest toward the VC.

Bright-field immunohistochemistry. Studies were performed as previously described (41) on paraffin-embedded sections (8 μm) of thoracic aorta and VC. Concentrations of primary antibodies used were 1:200 for anti-XO (Rockland) and 5 μg/ml for anti-catalase (Abcam) and anti-Cu-Zn-SOD (Calbiochem). Each experiment included no primary controls for each slide. Sections were photographed using a Spot digital camera on an inverted Nikon light microscope. DNA isolation, reverse transcription, and RT-PCR. Total RNA from ~10-ng sections of aorta and VC was isolated using the MELT total RNA isolation system (Ambion/Applied Biosystems, Austin, TX) and quantified on a Nanodrop spectrophotometer. One microgram of DNA-treated total RNA from each sample was reverse transcribed using an oligo(dT)12-18 primer, dNTP mix, and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer’s protocol. Primers for rat xanthine dehydrogenase (XDH; GeneID 29289, mRNA sequence NM_017154) were designed using the Primer3 software (29) (Whitehead Institute, Cambridge, MA) and synthesized at the Macromolecular Structure, Sequencing and Synthesis Facility at Michigan State University: XO, forward, 5’-GCA-TCCAGACCATACTGAAA-3’, and reverse, 5’-AAATCCGTT-GCGGACAAAC-3’. Primers for rat GAPDH were purchased from SuperArray (Frederick, MD). Relative quantification of XO in report tissues was performed using SYBR green PCR master mix (Applied Biosystems, Foster City, CA) and the respective primers (0.1 μM) on a 7500 Real-Time PCR system (Applied Biosystems).

XO activity assay for urate production. Aorta and VC rings were cleaned in XO assay buffer (50 mM K-phosphate, 0.1 mM EDTA, and 1 mM oxonic acid, pH 7.4), pulverized in liquid nitrogen, solubilized in XO lysis buffer (XO assay buffer containing 10 mM DTT, 1 mM PMSF, and protease inhibitors cocktail), sonicated, and centrifuged (20,800 g, 4°C, 30 min). Equal amounts of supernatant were then incubated with no additions, with 100 μM xanthine, or with 100 μM xanthine and 100 μM allopurinol at 37°C for 30 min. Urate formation was measured spectrophotometrically at 292 nm in quartz cuvettes. The amount of uric acid produced was calculated using a uric acid standard curve. Protein concentration of homogenates was determined using the Bradford assay (Bio-Rad, Hercules, CA). XO activity was reported as the allopurinol-inhibitable uric acid production, normalized for protein.

XO activity assay for H2O2 production. XO H2O2-producing activity was measured using the Amplex red H2O2 assay as detailed above for basal H2O2 production measurements. Aorta and VC rings were incubated with Ultra red working solution (100 μM) in the presence of 100 μM xanthine or in the presence of 100 μM xanthine and 100 μM allopurinol. H2O2 concentrations from samples were calculated using a H2O2 standard curve and were normalized to the total protein content of samples determined using the Lowry method. XO activity was determined by subtracting H2O2 production of samples incubated with 100 μM xanthine (substrate) and 100 μM allopurinol (inhibitor) from the H2O2 production of samples incubated with 100 μM xanthine alone.

Isolated tissue bath contractility. Rings of aorta and VC were removed and cleaned of outer adipose tissue in physiological salt solution (PSS) containing (in mM) 130 NaCl, 4.7 KCl, 1.18 KH2PO4, 25 NaHCO3, 1.25 CaCl2, 0.5 MgCl2, and 10 d-glucose, pH 7.4.

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1.17 MgSO₄·7H₂O, 14.8 NaHCO₃, 5.5 dextrose, 0.03 CaNa₂EDTA, and 1.6 CaCl₂, pH 7.2. Rings were mounted in warmed (37°C), aerated (95% O₂-5% CO₂) PSS in isolated tissue baths (30 ml) for measurements of changes in isometric force (PowerLab; ADInstruments, Colorado Springs, CO). The initial contraction to 10 μM α-adrenergic agonist [phenylephrine (PE) for aorta, norepinephrine (NE) for VC] was used to verify viability of tissues and normalize any further isometric contractions. As previously described, the maximal contractions induced by PE (10 μM) and NE (10 μM) are similar in aorta (42). However, PE does not induce reproducible contractions of VC, and NE was used instead in this tissue. Endothelial integrity was tested in each experiment by measuring relaxation to ACh (10 μM) from the contraction to EC₅₀ PE (aorta) or to 20 μM PGF₂α (VC). In inhibition experiments, parallel tissues were incubated with 100 μM allopurinol or vehicle (DMSO) for 1 h before cumulative concentration-response curves to NE, ANG II, ET-1, or ACh were performed. Relaxation curves to ACh were performed on tissues contracted with 20 μM PGF₂α. No significant difference between tissues incubated with vehicle and allopurinol was observed in terms of changes from the baseline during incubation or contraction to PGF₂α.

Data analysis. Data are means ± SE. When comparing groups, the appropriate Student’s t-test or ANOVA analysis was performed. In all cases, a P value of ≤0.05 was considered statistically significant.

RESULTS

Basal production of superoxide and H₂O₂ was higher in VC compared with aorta from normal rats. Superoxide production was measured through lucigenin-enhanced chemiluminescence in rat aorta and VC tissues (Fig. 1A). Normalized to tissue weight, basal superoxide production was higher in the VC compared with aorta (in nmol superoxide·min⁻¹·mg tissue⁻¹: aorta = 0.04 ± 0.006, VC = 0.08 ± 0.016, P = 0.027). H₂O₂ production was measured using Amplex red fluorescence in rat aorta and VC tissues (Fig. 1B). Normalized to protein content, basal H₂O₂ production was higher in VC compared with aorta (in nM H₂O₂/μg protein: aorta = 0.10 ± 0.05, VC = 3.06 ± 0.79, P = 0.023).

Antioxidant capacity for H₂O₂ was higher in the VC compared with aorta. As a measure of antioxidant capacity, we tested the ability of normal aorta and VC to quench exogenous H₂O₂ by measuring the decrease in H₂O₂ concentration in the presence of tissue, using the Amplex red H₂O₂ assay. The VC consumed significantly more exogenous H₂O₂ per amount of protein compared with the aorta (in nM H₂O₂ consumed/μg protein in the presence of 20 μM H₂O₂: aorta = 8.25 ± 0.29, VC = 40.73 ± 2.71, P < 0.01) (Fig. 1C).

Protein expression of XO, CuZn-SOD, and catalase was higher in VC compared with aorta. To begin to understand the mechanisms that determine the difference in basal ROS levels and antioxidant capacity between aorta and VC, we first performed a protein expression analysis of proteins involved in the generation and/or destruction of ROS. Western blots displayed in Fig. 2 were performed using protein isolates of aorta and VC from normal rats and were followed by band densitometry analysis. The expression of XO, a cytosolic ROS generator (Fig. 2A), catalase, a major H₂O₂-metabolizing enzyme (Fig. 2B), and CuZn-SOD, the cytoplasmic isoform of SOD (Fig. 2C), was significantly increased in VC compared with aorta (in arbitrary densitometry units; X: aorta = 75 ± 8, VC = 246 ± 19, P < 0.01; CuZn-SOD: aorta, 376 ± 22, VC = 534 ± 21, P < 0.01; catalase: aorta = 280 ± 17, VC = 469 ± 13, P < 0.01).

No difference was observed between the aortic and VC protein expression of other important sources of vascular ROS: the endothelial isoform of nitric oxide synthase, eNOS; the mitochondrial isoform of SOD, Mn-SOD; the small GTPase component of NADPH oxidase, Rac1; and the 39-kDa subunit of mitochondrial oxidative phosphorylation complex I, the most important mitochondrial ROS production site (data not shown). Use of a number of other commercially available antibodies for other ROS metabolizing enzymes was attempted without success against other subunits of NADPH oxidase (Nox2/gp91phox, p67phox, p22phox, p40phox), other isoforms of NOS [inducible (iNOS) and neuronal NOS (nNOS)], cyclooxygenase (COX2), and lipooxygenase (5LOX).

Protein expression of XO, CuZn-SOD, and catalase by tissues was confirmed by immunohistochemical analysis of rat aorta and VC cross sections (Fig. 3). Qualitatively, the XO and catalase staining appeared to be more intense and more diffuse in VC compared with aorta sections. CuZn-SOD staining did not appear to be different between aorta and VC sections.
In light of this protein expression analysis, from among the three ROS enzymes with higher expression in VC compared with aorta (XO, CuZn-SOD, and catalase), we chose XO, the only superoxide generator, as a likely candidate to explain the observed higher basal ROS production by VC compared with aorta. In the following experiments, we therefore measured XO mRNA expression, activity, and functional impact on vascular contractility.

XO mRNA expression was higher in VC compared with aorta. Because circulating XO can bind to vascular endothelium (see Discussion), we tested whether vascular XO is also locally produced by performing RT-PCR for XO mRNA. Importantly, XO mRNA was present in both aortic and VC samples. In addition, relative quantification of XO to GAPDH mRNA (Fig. 4A) showed that XO mRNA expression was higher in the VC compared with the aorta (change over GAPDH, expressed as $2^{-\Delta Ct}$; aorta = 0.13 ± 0.04, VC = 0.47 ± 0.04, P < 0.0001).

XO activity was higher in VC compared with aorta. XO protein activity was measured through two independent assays. First, the formation of urate, the final product of XO-catalyzed reactions, was determined spectrophotometrically from tissue homogenates in the presence of the XO substrate xanthine. The signal measured in the presence of the XO inhibitor allopurinol (100 μM) was considered as nonspecific and was subtracted from the total. The resulting XO activity, expressed as the allopurinol-inhibitable uric acid production in the presence of xanthine, was normalized to the total protein content of samples. XO activity was higher in VC compared with aorta samples (in nM urate/μg protein: aorta = 17.7 ± 7.1, VC = 44.1 ± 9.4, P = 0.01) (Fig. 4B).

Second, the formation of H$_2$O$_2$ from whole tissues in the presence of xanthine was measured fluorometrically using the Amplex red assay. Allopurinol was again used to subtract the non-XO-derived H$_2$O$_2$ formation. XO activity expressed as allopurinol-inhibitable H$_2$O$_2$ production was higher in VC compared with aorta (in nM H$_2$O$_2$/μg protein: aorta = 0.07 ± 0.02, VC = 1.36 ± 0.58) (Fig. 4C).

Xanthine/XO, a superoxide-generating system, induced a greater concentration-dependent contraction in VC compared with aorta. ROS may alter several vascular functions, including the most relevant from our point of view, the contractile function. To compare the potential functional impact of ROS on intact rat aorta and VC tissues, we exposed these vessels to extraneous superoxide in isolated tissue baths. Aorta and VC endothelium-intact rings both contracted in a concentration-dependent manner to exogenous superoxide, generated by the addition of increasing amounts of XO to 200 μM xanthine in the tissue bath (Fig. 5). This contraction was larger in the VC compared with the aorta when normalized to an α-adrenergic-mediated response (3 × 10$^{-2}$ U/ml XO, 200 μM xanthine; VC = 196 ± 41% and aorta = 17.5 ± 7.5% of the initial NE/PE-mediated contraction).

Allopurinol did not change vascular reactivity of the aorta but inhibited the maximal ET-1-mediated contraction in the VC. To assess the potential involvement of XO-mediated ROS production on vascular reactivity, we tested the effects of the XO inhibitor allopurinol on vascular responses to several agonists in isolated tissue bath experiments (Fig. 6). We used three agonists that induce contraction of both aorta and VC: NE, as a generic α-adrenergic agonist, and ANG II and ET-1,
peptides commonly associated with ROS production in vascular tissues. We also used ACh as a relaxing agent and a general measure of endothelial function. Allopurinol (100 μM) did not modify the contraction or relaxation of normal aorta rings to any of these agonists. As previously reported, ANG II- and ET-1-induced contractions were more efficacious in VC compared with aortic rings, when reported as a percentage of the initial contraction to 10 μM NE (VC) or PE (aorta).
allopurinol (100 μM) did not have an effect on the NE-induced contraction or the ACh-induced relaxation in the VC, and the trend for a decreased ANG II-induced contraction in its presence was not statistically significant. However, allopurinol significantly reduced the maximal contraction to ET-1 (10^{-7} M) in the VC (VC vehicle = 553 ± 68% compared with VC allopurinol = 384 ± 44% of initial NE-mediated contraction).

**DISCUSSION**

In the present study we have compared an artery and a vein in terms of their ROS metabolism. The male Sprague-Dawley rat thoracic aorta and caudal vena cava tissues were chosen as model tissues, because while still generally reflecting the states of the arterial and venous system, respectively, they are also large enough to allow for experimental manipulation.

**Basal superoxide and H_2O_2 production in rat aorta and VC.** We observed that in normal conditions, production of two major ROS, superoxide and H_2O_2, is higher in VC compared with aorta. We measured superoxide in the presence of SOD inhibition so that only differences in production, not destruction, of superoxide would be taken into account. We have previously shown that the majority of H_2O_2 production from aorta and VC is superoxide derived, because this production is almost abolished by SOD inhibition (35). Another study that has compared ROS production in the HSV with that in the IMA from CABG patients did not find significant differences in superoxide production between these two vessels (12). Porcine normal and grafted venous superoxide production was higher than that in normal and grafted arteries, respectively (30). We also have observed higher superoxide production by the superior mesenteric vein compared with the superior mesenteric artery (data not shown). Whether our results are vascular bed specific or reflect a general artery-vein difference remains to be established.

**Antioxidant capacity for H_2O_2 of rat aorta and VC.** ROS production by living cells is normally counterbalanced by the activity of several antioxidant defense mechanisms such as SODs, catalase, or glutathione peroxidases. To compare the status of these mechanisms between aorta and VC, we exposed vessels to exogenous H_2O_2 and assessed how much of that H_2O_2 was metabolized in the presence of tissue. We chose H_2O_2 and not superoxide for this purpose because H_2O_2 is more stable, capable of freely passing cellular membranes, easier to generate at definite concentrations, and also more reliable to measure. We used H_2O_2 at a high enough concentration to produce sufficient fluorescence such that its decrease in the presence of tissue would still be measurable with our method. However, this concentration (20 μM) may not reflect a physiological situation (~2.5 μM in normal plasma; Ref. 2), but rather mimic a state of oxidative stress. We have observed a higher consumption of H_2O_2 by the VC compared with the aorta, indicating a higher activity of antioxidant defense mechanisms, and possibly reflecting the increased ROS destruction in normal VC balancing the increased ROS production. However, in our results, the VC-to-aorta ratio of ROS generation does not numerically match the ratio of ROS destruction, raising the question whether this is indeed a perfect balance or whether the VC is in fact exposed to oxidant stress. This method does not discriminate among specific antioxidant mechanisms, although catalase is likely to be more important than glutathione peroxidase at high H_2O_2 concentrations due to its higher K_m (44). This would be consistent with the higher catalase expression we observed in VC compared with aorta.

**Fig. 5.** Cumulative XO concentration-response curves for rat Ao and VC rings in the presence of 200 μM xanthine, represented as a percentage of the initial contraction to 10 μM phenylephrine (PE) or norepinephrine (NE) for Ao and VC, respectively. Data points represent means ± SE for n = 6–13. *P < 0.05.
Different protein expression of major ROS metabolizing systems between aorta and VC. ROS metabolism is a highly complex biological pathway with many important enzymatic and nonenzymatic players involved. For its careful evaluation, the protein expression analysis performed presently (Fig. 2) is by no means complete. Because tools were unavailable, we could not confidently assess the expression of different Nox isoforms and various subunits of NADPH oxidase, a major ROS producer repeatedly proven as important in vascular biology. In human vessels, protein as well as mRNA expression of NADPH oxidase subunits was reported as significantly different between the saphenous vein and IMA. In addition, the enzymatic activity of NADPH oxidase seemed to be contributing to total superoxide levels in a higher degree in the vein.

Fig. 6. Cumulative concentration-response curves to NE (A and E), ACh (B and F), ANG II (C and G), and ET-1 (D and H) of rat Ao (A–D) and VC rings (E–H) in the presence of allopurinol (100 μM) or vehicle (DMSO), represented as a percentage of the initial contraction to 10 μM PE or NE for Ao and VC, respectively, or to 20 μM PGF2α in the case of ACh. Data points represent means ± SE for the n represented on each graph. *P < 0.05.
than in the artery, whereas the opposite was observed for XO (12). Also absent from our results are an entire range of other ROS metabolizing systems that also may contribute to the differences observed in ROS levels, such as other mitochondrial ROS production sites (complex III), uncoupling proteins, other isoforms of NO synthase (nNOS, iNOS), lipoxygenase, cyclooxygenase, myeloperoxidase, heme oxygenase, the extracellular isoform of SOD, and glutathione peroxidases. Moreover, any protein expression analysis does not entirely reflect protein activity in living tissues. Therefore, to fully explore arterial versus venous ROS metabolizing systems, more studies would be necessary. A comparison of SOD expression in HSV and IMA reported similar expression of the cytosolic (CuZn-SOD) and mitochondrial (Mn-SOD) isoforms (11). To our knowledge, however, there are no other studies comparing the arterial and venous expression of eNOS, an enzyme that can modify ROS levels by producing NO that reacts with superoxide to produce peroxynitrite and also by generating superoxide when becoming uncoupled. We did not find differences in the expression of eNOS between aorta and VC, correlated with the similar endothelium-dependent relaxation found in these tissues in normal conditions (data not shown). However, from the differences we did observe in protein expression between aorta and VC, only XO, as a superoxide producer, would help explain the higher superoxide levels found in basal conditions in veins. Furthermore, CuZn-SOD, a cytosolic enzyme that converts superoxide to H$_2$O$_2$, and catalase, responsible for further degrading H$_2$O$_2$, are more heavily expressed in the vein, potentially as a consequence of the higher superoxide/ H$_2$O$_2$ levels.

Higher VC XO mRNA, protein expression, and activity compared with aorta. XO is a cytosolic enzyme in the purine degradation pathway, converting hypoxanthine to xanthine and xanthine to uric acid, with the formation of superoxide/H$_2$O$_2$ as by-products (4). XO previously has been demonstrated as capable of generating ROS in vascular tissues (3, 7). Its importance in cardiovascular disease is illustrated by various animal and human studies demonstrating beneficial effects of XO inhibition in hypertension, coronary artery disease, or cardiomyopathies by lowering blood pressure (23, 32), decreasing end organ damage (15, 25), and improving endothelial function (6, 25) and heart function (25). Circulating XO binds to glucosaminoglycans on the vascular endothelium, still retaining specific activity (13). The results presented in Fig. 4A represent a novel finding in that they demonstrate for the first time that blood vessels produce the XO mRNA locally and that binding of circulatory XO to endothelial cells is not the only mechanism responsible for the detection of XO activity in these tissues. We observed a higher protein and mRNA expression, as well as a higher activity of XO in VC than in aorta from normal rats. It should be noted that our mRNA and protein expression studies do not distinguish between xanthine dehydrogenase (XDH) and XO, the two isoforms of xanthine oxidoreductase (XOR). These isoforms are generated by post-translational modifications (4); therefore, they have the same mRNA source; because of the small difference in protein structure between XDH and XO, antibodies will also recognize both isoforms. However, our XO activity assays specifically assess the enzyme activity that uses oxygen (XO), because they are performed in the absence of NAD$^+$, the substrate for XDH. The differences in XO expression between the vein and the artery could be mediated by a number of factors and processes regulating XO expression, such as cytokines or oxygen tension (3). An interesting report in bovine aortic endothelial cells, which appears to be consistent with our findings, has implicated a feed-forward mechanism by which increases in H$_2$O$_2$ stimulate the conversion of XDH to XO, potentially leading to even higher H$_2$O$_2$ levels generated by XO (20).

**Modulation of vascular smooth muscle contraction by ROS.** The combination of xanthine with XO has long been used as a laboratory tool to produce superoxide. Some researchers have suggested that superoxide can enter living cells through Cl$^-$ channels (14). However, the accepted view in the ROS field is that because it carries a negative charge, superoxide does not cross membranes. Therefore, we can assume that contraction induced by extracellular xanthine/XO is mediated either by extracellular superoxide or by H$_2$O$_2$ as the product of superoxide degradation, a longer-lived molecule that can freely diffuse across membranes. This contraction is indeed parallel to the H$_2$O$_2$ contraction observed previously in the same tissues (35). The mechanisms for the direct contractile effects of ROS are complex, involving many different signaling pathways, such as mitogen-activated protein kinase (MAPK) or Rho kinase, as well as Ca$^{2+}$ channel activation and interference with endothelium-released NO (1, 5). In our experiments, removal of endothelium did not alter aortic contraction to exogenous superoxide (data not shown). The fact that the contraction induced by xanthine/XO was higher in VC would suggest that any alteration of ROS metabolism is likely to have a more important impact on venous rather than arterial contraction.

Smooth muscle contraction signaling pathways may be modulated by ROS in many ways. Some contracting agents such as ANG II are widely known as being capable of inducing superoxide release (10). General ROS scavengers, catalase, or specific ROS enzyme inhibitors have variably decreased the arterial contractile response induced by agonists such as NE, ANG II, or ET-1 (18, 31, 38). We asked specifically whether XO-produced ROS could contribute to the normal contraction induced by NE, ANG II, or ET-1 and normal relaxation induced by ACh. Inhibition of XO activity with allopurinol did not alter any of the aortic responses to these agonists (Fig. 6). However, the maximal ET-1-mediated contraction of the VC was significantly decreased in the presence of allopurinol. There seem to be fundamental arterial-venous differences in the endothelin system, such as receptor expression and interaction (37), processing of endothelin-related peptides (42), or functional responses to endothelin peptides and ET receptor agonists (36, 40). ET-1, a potent vasoconstrictor, also stimulates superoxide production. Similarly to the ANG II-induced superoxide production, the mechanism mostly responsible for this increase in superoxide release seems to be NADPH oxidase activation (17). Although we did not see effects on ANG II-induced contraction in our study, long-term exposure to ANG II can also induce XO activation in cultured endothelial cells (16). The data presented in this report suggest that XO also may contribute to ET-1-mediated vasoconstriction in a way that it does not in the aorta. A limitation of our study is that we relied heavily on the use of allopurinol for XO inhibition for both our activity and contractility experiments. We chose allopurinol, as opposed to oxypurinol, its metabolite, or other purine or nonpurine XO inhibitors, because allopurinol is by far the most widely used in vivo and in vitro XO inhibitor,
ROS IN RAT AORTA AND VENA CAVA

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and at the concentration used in our experiments (100 μM), it should be effective in blocking XO activity, given its IC50 value of <10 μM cited in various reports (reviewed in Ref. 4). XO may theoretically influence endothelial function in at least two ways: first, by producing superoxide that can interact with NO and therefore decrease relaxation, and second, by producing urate itself that is an antioxidant capable of improving endothelial function (39), although many contradicting reports exist suggesting urate is a detrimental factor in cardiovascular function (8). We were expecting allopurinol therefore to modify the relaxation induced by ACh. The fact that we did not observe any changes in the ACh-induced relaxation of either aorta or VC in the presence of allopurinol does not exclude a potential involvement of XO in endothelial function to be unmasked by pathophysiological conditions.

Conclusions. There are significant differences in ROS metabolism between the normal rat aorta and VC. We have observed higher ROS production and antioxidant capacity, higher expression of major ROS metabolizing enzymes (XO, CuZn-SOD, and catalase), and higher contraction induced by ROS in the VC compared with the aorta. We attribute at least a part of these differences to the higher expression and activity of XO in the VC compared with the aorta. XO activity seems to be involved in the ET-1-mediated contraction in the VC but not in the aorta. The relevance of these results in the context of venous function will ultimately be revealed by inhibition of XO activity in vivo.

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References


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