Dietary inhibition of xanthine oxidase attenuates radiation-induced endothelial dysfunction in rat aorta

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Departments of 1Biomedical Engineering and 2Anesthesiology/Critical Care Medicine, Johns Hopkins Medical Institutions, Baltimore, Maryland; 3Department of Anesthesiology and Pain Medicine, Yonsei University, Wonju; 4Division of Life Sciences, Kangwon National University, Chuncheon, Korea; and 5Medical Department, Brookhaven National Laboratory, Upton, New York

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Soucy KG, Lim HK, Attarzadeh DO, Santhanam L, Kim JH, Bhunia AK, Sevinc B, Ryoo S, Vazquez ME, Nyhan D, Shoukas AA, Berkowitz DE. Dietary inhibition of xanthine oxidase attenuates radiation-induced endothelial dysfunction in rat aorta. J Appl Physiol 108: 1250–1258, 2010. First published February 18, 2010; doi:10.1152/japplphysiol.00946.2009.—Radiation exposure is associated with the development of various cardiovascular diseases. Although irradiation is known to cause elevated oxidant stress and chronic inflammation, both of which are detrimental to vascular function, the molecular mechanisms remain incompletely understood. We previously demonstrated that radiation causes endothelial dysfunction and increased vascular stiffness by xanthine oxidase (XO) activation. In this study, we investigated whether dietary inhibition of XO protects against radiation-induced vascular injury. We exposed 4-mo-old rats to a single dose of 0 or 5 Gy gamma radiation. These rats received normal drinking water or water containing 1 mM oxypurinol, an XO inhibitor. We measured XO activity and superoxide production in rat aorta and demonstrated that both were significantly elevated 2 wk after radiation exposure. However, oxypurinol treatment in irradiated rats prevented aortic XO activation and superoxide elevation. We next investigated endothelial function through fluorescence measurement of nitric oxide (NO) and vascular tension dose responses. Radiation reduced endothelium-dependent NO production in rat aorta. Similarly, endothelium-dependent vasoconstriction in the aorta of irradiated rats was significantly attenuated compared with the control group. Dietary XO inhibition maintained NO production at control levels and prevented the development of endothelial dysfunction. Furthermore, pulse wave velocity, a measure of vascular stiffness, increased by 1 day postirradiation and remained elevated 2 wk after irradiation, despite unchanged blood pressures. In oxypurinol-treated rats, pulse wave velocities remained unchanged from baseline throughout the experiment, signifying preserved vascular health. These findings demonstrate that XO inhibition can offer protection from radiation-induced endothelial dysfunction and cardiovascular complications.

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gamma radiation. Furthermore, this XO inhibition preserved endothelial function and prevented the development of aortic stiffness after radiation exposure.

MATERIALS AND METHODS

Animals. Sprague-Dawley 4-mo-old male rats (Harlan) were exposed to a single cesium-137 gamma irradiation at either 0 or 5 Gy. The animals were whole body irradiated while being held in a bowl-like container without anesthesia. For inhibition of XO, animals were randomized to receive 1 mM oxypurinol (Oxp; Sigma-Aldrich, St. Louis, MO) in their drinking water (30). Treatment began 1 wk before irradiation and continued until death, at 2 wk after irradiation. Rats were housed at Johns Hopkins Medical Institution with controlled light and temperature conditions and ad libitum feeding and watering. Consumption of normal water and Oxp water per pair of rats was tracked, and no noticeable differences were detected. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Johns Hopkins University.

Aorta preparation. The rats were heparinized 1 h before being euthanized. After death, a maximum length of aorta, from the distal aortic arch to the femoral bifurcation, was isolated and removed. The dissected vessel was immediately placed in ice-cold Krebs buffer (pH 7.4, 95% O2:5% CO2, containing [in mM] 118 NaCl, 4.7 KCl, 25.0 NaHCO3, 1.16 MgSO4, 1.18 KH2PO4, 11.1 D-(+)-glucose, and 3.24 CaCl2). The aorta was then cleaned of additional connective tissue and sectioned into segments of appropriate length. Aortic rings were designated for immediate ex vivo experiments outlined below or snap frozen and stored at -80°C for XO activity analysis.

Protein abundance. For protein assessment, aortic rings were homogenized in 200 μL of PBS and total protein concentration was determined using the Bradford method. Aortic homogenates (50 μg) were resolved on 4–20% gradient gel (Bio-Rad Laboratories, Hercules, CA) using SDS-PAGE. The proteins were electrophoretically transferred to a nitrocellulose membrane and blocked using 5% nonfat dry milk solution in TBS containing 0.1% Tween. The blots were incubated with rabbit polyclonal anti-XO or rabbit polyclonal anti-tubulin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with appropriate horseradish peroxidase-conjugated secondary antibodies. The membrane was finally exposed to West Pico chemiluminescent substrate (Pierce Biotechnology, Rockford, IL) and developed on film (GE Healthcare, Piscataway, NJ). It is important to note that the XO antibody employed binds near the NH2 terminus of the xanthine oxidoreductase (XOR) enzyme. Therefore, the resultant immunoblot band intensity is a better indicator of the XDH form than of the XO form (4). For comparison between the resultant immunoblot band intensity is a better indicator of the product, isoxanthopterin. Aortic samples, ground to a powder with a mortar/pestle filled with liquid nitrogen, were collected into Tris·HCl-based buffer (50 mM Tris·HCl, pH 7.5, 0.1 mM EDTA, 10 mM DTT, 0.2 mM PMSF, and 0.25 M sucrose). Approximately 50 μg of protein were mixed with 10 μL saline (XO activity), plus 10 μL methylene blue (XO + XDH activity), plus 10 μL allopurinol (inhibition), in sequential order (Sigma-Aldrich). Fluorescence values were recorded at 10-min intervals after rates were stable. The fluorescence data were corrected for blank controls and calibrated with an isoxanthopterin calibration curve. The isoxanthopterin fluorescence was measured with a fluorescent plate reader ( Molecular Devices Gemini EM) excited at 345 nm and with emissions collected at 390 nm.

ROS production. The production of ROS was quantified with the fluorescent superoxide indicator dihydroethidium (DHE; Invitrogen; Carlsbad, CA). Fluorescence intensity time-lapse images were acquired with an upright microscope (Nikon Eclipse 8i), a ×10 objective, and a mercury lamp using NIS-Elements software (Nikon). The DHE fluorescence was collected at excitation-emission wavelengths of 480/567 nm (10, 54). For each condition mentioned below, time-lapse images were collected over 10 min at 30-s intervals. Aortic rings were cut open and pinned endothelium side up in a petri dish coated with Sylgard 184 (Dow Corning, Midland, MI). The segments were incubated with 5 μM DHE in HEPES buffer [containing (in mM) 136 NaCl, 5.9 KCl, 1.2 MgCl2, 11.6 HEPES, 11.5 dextrose, and 1.66 CaCl2, adjusted to pH 7.4 with NaOH] at 37°C, with 5 min allowed for temperature equilibration before data collection. Two aorta segments per rat were used for DHE measurements. With the first segment, a baseline DHE fluorescence time lapse was acquired. As a negative control for ROS, this aorta was then incubated with 10 μM manganese(III) tetrakis (4-benzoic acid) porphyrin (MnTBAP) and 103 U/ml catalase-polyethylene glycol (PEG-catalase; Sigma-Aldrich) for 15 min, followed by another time-lapse acquisition. The second aortic segment was incubated with 1 mM allopurinol at room temperature for 20 min. This aorta was then incubated with DHE, and the fluorescence intensity was measured as described above. Each aorta was exposed to a final treatment of 1 mM H2O2 as a positive control. As a representation of ROS production rate, the fluorescence intensity rate of change was calculated via the linear least-squares method and normalized to the initial intensity for each data sequence.

NO production. The cell-permeable fluorescent probe 4-aminophenylmethylen blue-2',7'-difluorofluorescein diacetate (DAF-FM DA; Invitrogen) was used to quantify endothelium-derived NO. Fluorescence measurements were acquired, and fluorescence rates were calculated as described for DHE, with differences explained below. The DAF fluorescence was collected at excitation-emission wavelengths of 485/510 nm, and time-lapse images were collected over 5 min at 30-s intervals. The segments were incubated with 5 μM DAF-FM DA for 4 min and then rinsed with fresh HEPES buffer. The temperature was increased to 37°C, and 5 min were allowed for equilibration. Baseline DAF fluorescence was measured first, and then the aortas were treated with acetylcholine (ACh; 10–5 M; Sigma-Aldrich) to characterize endothelial NO responsiveness. The change of fluorescence rate in response to ACh was calculated with respect to the baseline rate per vessel.

Vascular tension studies. For analysis of endothelial function and vasoreactivity, aortic rings were suspended from strain gauges in 25-mL organ chambers. The chambers were filled with oxygenated Krebs buffer maintained at pH 7.4 and 37°C. The vessels were incrementally stretched to 3 g for optimized contractility and were precontracted with 10–6 M phenylephrine (PE; Sigma-Aldrich). Cumulative dose responses to ACh or sodium nitroprusside (SNP; 10–9–5 M; Sigma-Aldrich) were obtained to characterize endothelium-dependent and -independent vasorelaxation, respectively. Vasorelaxation is expressed as percent relaxation, calculated as the percent decrease in tension from the PE-induced precontraction.

In vivo vascular stiffness. Pulse wave velocity (PWV) is related to vascular stiffness through a simple approximation of a distensible pipe (Moens–Korteweg equation) (18), and PWV can be measured in vivo. Rats were anesthetized in a closed chamber with isoflurane. Anesthesia was maintained by mask ventilation of 1.5% isoflurane with a coupled charcoal scavenging system. Animals were positioned supine on a temperature-controlled circuit board with limbs taped to electrocardiogram (ECG) electrodes (Indus Instruments). Body temperature was maintained at 37°C. Thoracic and abdominal aortic outflow profiles were captured noninvasively with a 2-mm-diameter, 10-MHz Doppler probe. The distance separating the measurement locations and a simultaneous ECG were also recorded. From these data, aortic PWV was calculated before irradiation and 1 day, 1 wk, and 2 wk postirradiation. Data was captured and analyzed off-line using Doppler Signal Processing Workstation software (Indus Instruments). Just before PWV measurements were taken, the systolic and diastolic blood pressures were measured with a noninvasive tail-cuff sphygmomanometric system (Kent Scientific).
Passive aortic compliance and distensibility. To investigate passive vascular properties, the proximal thoracic aorta was cannulated in a perfusion chamber. The aorta was perfused with oxygenated calcium-free Krebs buffer using a peristaltic pump (Cole-Parmer Instrument), which also continuously monitored perfusion pressure. Pressure was incrementally increased from 10 to 120 mmHg in steps of 10 mmHg, each for 30-s intervals. Vessel outer diameter was simultaneously recorded using microscopic imaging and video dimension analysis (Analog Digital Instruments). Compliance can be described as the ability to stretch and hold volume (V). Since the aortic section is cannulated at fixed length, the relative compliance can be calculated from the relationship between luminal pressure (P) and cross-sectional area (A) or diameter (d), assuming a circular lumen.

\[ \text{Compliance} = \frac{\Delta V}{\Delta P} = \frac{\Delta A}{\Delta P} \approx \frac{\Delta d^2}{\Delta P} \]

To minimize the influence of aortic geometry, the vascular distensibility can be calculated by normalizing the stressed condition (V, A, or d2) to the unstrained condition (V0, A0, or d0) (29).

\[ \text{Distensibility} = \frac{\Delta V}{V_0} \cdot \frac{\Delta A}{A_0} \cdot \frac{\Delta d^2}{d_0^2} \approx \frac{\Delta d^2}{A_0 \cdot d_0} \]

Statistical analysis. Data are means ± SE, with sample size (n) indicated for each reported value. A value of P < 0.05 was considered statistically significant. Data were analyzed off-line with PRISM data analysis software (GraphPad). Statistical analysis for variance between groups was performed using two-tailed Student’s t-tests. Paired t-tests were employed for comparison of data from the same sample or animal. Unpaired t-tests were used to compare results from different samples or animals. Two-way ANOVA with Bonferroni post tests was employed to compare data sets of multiple time points or dose responses. Vasorelaxation dose-response curves were fitted with the sigmoidal dose-response function through nonlinear regression. The best-fit maximum response and EC50 parameters were also calculated. Comparisons of the best-fit curves and parameter values were accomplished with an F-test.

RESULTS

We measured XOR protein abundance and activity to verify that 5 Gy gamma radiation upregulates XO in rat aorta and to determine the effect of 1 mM Oxp treatment. In aortas from irradiated rats, normalized XDH protein band intensity was greater than that of aortas from sham irradiated rats; however, this increase was not statistically significant (Fig. 1A). Rats receiving Oxp diet and control rats demonstrated nearly identical aortic XDH expression. To measure XO and XDH activity, we used the pterine-based fluorescence assay (Fig. 1, B and C). The aortas of 5 Gy gamma-irradiated rats demonstrated a significantly elevated XO activity compared with controls (7.05 ± 0.595 vs. 4.80 ± 0.498 pmol·min⁻¹·mg⁻¹, P < 0.05). Remarkably, this activation was abolished by Oxp dietary inhibition (2.74 ± 0.467 pmol·min⁻¹·mg⁻¹, P < 0.001) (Fig. 1B). Total XO + XDH activity was not significantly affected by radiation; however, a slight increase was noted. Aortas from 5 Gy irradiated rats on Oxp diet demonstrated significantly lower XO + XDH activity compared with untreated 5 Gy aortas (P < 0.05). Acute XO + XDH inhibition with allopurinol significantly reduced the activities of the 0 Gy, 5 Gy, and 5 Gy + Oxp groups (P < 0.001 vs. respective XO + XDH activity). Endothelium removal in irradiated rat aortas significantly reduced the XO and XO + XDH activities (Fig. 1B), indicating that a substantial portion of XO and XDH activity is confined to the aortic endothelium. Furthermore, the aortic XO:XDH activity ratio was increased by 5 Gy irradiation (P < 0.05), whereas Oxp treatment significantly reduced the ratio (P < 0.001) compared with control (Fig. 1C). These data, when coupled with our XOR immunoblots, imply that radiation-induced XO activation derives from XO:XDH conversion and, potentially, an increased expression of total XOR.
To determine whether radiation-induced XO activation is associated with increased ROS in the rat aorta, we quantified superoxide production with DHE fluorescence. The baseline DHE fluorescence rate was significantly greater in the aortas of 5 Gy irradiated rats compared with the aortas of 0 Gy unirradiated rats \((P < 0.05)\) (Fig. 2A). Aortas from irradiated rats treated with Oxp demonstrated a significantly lower fluorescence rate than aortas from irradiated rats without treatment \((P < 0.05)\). These observations suggest that whole body irradiation elevates ROS in the aorta and that XO inhibition attenuates radiation-dependent ROS generation. When aortas were treated with 10 \(\mu\)M MnTBAP, a superoxide scavenger, and \(10^3\) U/ml PEG-catalase, a catalyst for \(\mathrm{H}_2\mathrm{O}_2\) decomposition, the DHE fluorescence rates were reduced for all animal groups (Fig. 2B). In the aortas of rats receiving 0 and 5 Gy irradiation, the decreases from paired baseline rates were statistically significant \((P < 0.05\) and \(P < 0.01\), respectively). Furthermore, acute treatment of the aorta with 1 mM allopurinol significantly reduced the DHE fluorescence rate in the 5 Gy-exposed rats \((P < 0.01)\) (Fig. 2B). Allopurinol did not significantly alter DHE fluorescence rate in either the 0 Gy rat group or the Oxp diet group. As a positive control, each aorta was exposed to 1 mM \(\mathrm{H}_2\mathrm{O}_2\). The DHE fluorescence responses were similar among all of the tested aortas, regardless of radiation or diet (Fig. 2C), thus validating our assay as a reliable measure of aortic ROS production.

We next investigated whether radiation-induced XO activation and ROS production could affect NO bioavailability by measuring NO production in aortic samples using a DAF-FM fluorescence assay. ACh-stimulated NO, as measured by fluorescence rate increase from baseline slope \((127 \pm 7.47\%)\). Aortas of the 5 Gy gamma-irradiated rats did not produce a detectable change in response \((91.7 \pm 9.02\%)\). Dietary XO inhibition significantly elevated this response compared with untreated 5 Gy irradiated rats \((168 \pm 31.3\%, P < 0.05)\), suggesting a protection of endothelial NO responsiveness (Fig. 3). In a separate study, we found that NOS inhibition with \(\text{N}^\text{G}-\text{nitro-\text{l-arginine methyl ester (L-NAME; Sigma-Aldrich) treatment yielded significantly attenuated baseline rates, yet responses to an exogenous NO donor, SNP, were comparable (data not shown). These control experiments validate our present results with regard to endothelium-derived NO production.}

We next wanted to determine whether the diminished NO responsiveness we observed would affect endothelium-dependent regulation of vascular tone. As expected, the exposure of rats to 5 Gy gamma radiation markedly impaired aortic endothelium-dependent vasorelaxation. The percent relaxation responses to ACh were significantly attenuated compared with the responses in control rats \((P < 0.001\) for \(10^{-7}\) to \(10^{-5}\) M ACh) (Fig. 4A). Irradiated rats treated with Oxp demonstrated significantly improved relaxation responses compared with the untreated irradiated rat responses \((10^{-5}\) M ACh response: 110 \(\pm\) 9.17\% vs. 84.3 \(\pm\) 0.617\%, \(P < 0.001\)). The best-fit dose-response curves \((P < 0.001)\) and maximum responses \((5\) Gy vs. 0 Gy and \(5\) Gy + Oxp, \(P < 0.001\); 0 Gy vs. \(5\) Gy + Oxp, \(P < 0.01\)) were significantly different between the groups, but the EC\(_{50}\) values were not statistically different. In contrast to ACh-dependent relaxation, endothelium-independent vasorelaxation induced by SNP was not different between the groups (Fig. 4B). This suggests that smooth muscle sensitivity to exogenous NO is not affected by 5 Gy gamma radiation, which further implies that the observed effect of radiation on vascular tone could be due to a diminished NO bioavailability.

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**Fig. 2.** Dietary Oxp treatment protects against radiation-induced ROS generation in rat aorta. A: baseline superoxide production is represented as the untreated, normalized dihydroethidium (DHE) fluorescence rate. 0 Gy, \(n = 4\); 5 Gy, \(n = 5\); 5 Gy + Oxp, \(n = 6\). *\(P < 0.05\) by unpaired Student’s \(t\)-test. B: acute inhibition of XO was achieved with 1 mM allopurinol. Treatment with \(10 \mu\)M manganese(III) tetrakis (4-benzoic acid) porphyrin and \(10^3\) U/ml polyethylene glycol-catalase served as a negative control (neg. control) condition for ROS. Values are means \(\pm\) SE; for untreated vs. allopurinol: 0 Gy, \(n = 4\); 5 Gy, \(n = 5\); 5 Gy + Oxp, \(n = 5\). *\(P < 0.05\); **\(P < 0.01\) by paired Student’s \(t\)-test, compared with untreated rate of the same radiation/diet group. C: all of the aortas were treated with 1 mM \(\mathrm{H}_2\mathrm{O}_2\) as a positive control condition. Values are means \(\pm\) SE; 0 Gy, \(n = 7\); 5 Gy, \(n = 9\); 5 Gy + Oxp, \(n = 9\).
function is predominantly endothelium dependent. The magnitudes of the PE-induced preconstriction used to calculate percent relaxation were not different between groups, for both the ACh (Fig. 4C) and SNP (Fig. 4D) dose responses. Finally, the endothelium-denuded aortas did not demonstrate substantial relaxation in response to ACh (data not shown).

We continued to investigate the effects of chronic XO inhibition through measurements of PWV, an in vivo measure of aortic stiffness. It is now well established that increased vascular stiffness signifies declining vascular function and is associated with elevated cardiovascular risk (53). In control rats, no significant changes in PWV were observed over the experimental time course (Fig. 5). At 1 wk postradiation, PWV in irradiated rats was significantly higher than PWV in the control group (4.93 ± 0.210 vs. 4.06 ± 0.102 m/s, P < 0.001) and remained elevated at 2 wk (Fig. 5A). Irradiated rats treated with Oxp demonstrated significantly lower PWV compared with untreated irradiated rats at all time points (P < 0.001), and the values were not different from those of control rats. The PWV of individual rats within a radiation and diet group generally followed the trend of the group mean and were consistent between animals (Fig. 5B). Blood pressure measurements were not affected by radiation dose or diet (2-way ANOVA, P = not significant) (Table 1).

Finally, we observed a noticeable decrease in passive aortic compliance (Fig. 6A) and distensibility (Fig. 6B) of irradiated rats. Oxp treatment produced significant elevation in both these parameters (P < 0.05), implying reduced passive stiffness of the thoracic aorta. The matching trends of compliance and distensibility were due to the similar unstressed outer diameter values used to calculate distensibility from compliance (0 Gy, 2,220 ± 113 μm; 5 Gy, 2,170 ± 70 μm; 5 Gy + Oxp, 2,180 ± 125 μm). Therefore, changes of vessel wall composition and not geometric remodeling may be the primary contributor to radiation-induced passive vascular stiffness.

**DISCUSSION**

In the present study we verified our previously published data of radiation-induced XO activation (42). The novel findings presented in this report are that chronic oral XO inhibition protects endothelial function and reduces vascular stiffness by attenuating aortic ROS production and enhancing endothelial NO bioavailability in rats exposed to whole body gamma irradiation.
radiation. This implies that XO is a critical target for vascular
radiation injury.

In this study we demonstrated that dietary Oxp treatment
significantly impairs XO activity and provides vascular radi-
oprotection. Oxypurinol and allopurinol inhibit XO or XDH by
competing with natural substrates, xanthine and hypoxanthine
(4). As a result, oxypurinol was an ideal inhibitor for the
purpose of our study because it directly inhibits XO, indepen-
dently of any upstream factors. Minhas et al. (30) implemented
a nearly identical oxypurinol diet to reverse cardiac remodeling
in rats with cardiomyopathy. In agreement with our findings,
they reported that Oxp diet reduced the elevated XO activity in
heart failure rats. They also demonstrated that Oxp treatment
did not alter the activity or the protein subunits of NADPH
oxidase, another major enzymatic source of vascular ROS. We
are not aware of any reports that would indicate that Oxp or
allopurinol inhibit additional ROS-producing enzymes, other than
XO. It should be mentioned that Oxp and allopurinol act as
antioxidants at high concentrations (31). However, the oral dose
of Oxp and the acute doses of allopurinol (10 μM for aorta
homogenates and 1 mM for intact aorta) administered in our study
are considerably below doses necessary for antioxidant effect.
Based on average body mass and water consumption, we calcu-
lated that our rats received ~6 mg·kg⁻¹·day⁻¹ Oxp. It was

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**Systolic blood pressure, mmHg**

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**Diastolic blood pressure, mmHg**

Different radiation and diet treatments are not a significant source of variation for systolic or diastolic blood pressure. XO, xanthine oxidase; NW, normal water
diet; Oxp, 1 mM oxypurinol diet. Rats and sample sizes correspond with those described in Fig. 4. P = not significant, by 2-way ANOVA.
mechanism of conversion by radiation is not completely understood, but Srivastava et al. (46) provided experimental evidence that irreversible proteolytic cleavage of XDH is responsible. They demonstrated that the serine protease inhibitor PMSF inhibited radiation-induced increases of XO activity in mouse liver, whereas the reducing agent DTT did not. Along these lines, Kennedy et al. (23) found that the Bowman-Birk inhibitor, a serine protease inhibitor, protected against radiation-induced cytotoxicity and carcinogenic transformation in cultured epithelial cells. Another potential mechanism for XO and XDH activation is increased protein expression. Our observations that radiation increased XO and XDH activity (Fig. 1B) and increased XDH protein abundance (Fig. 1A) support this option. Strong correlations between XDH regulation and inflammation also have been reported (9, 17, 19, 36), and inflammation is a recognized consequence of radiation exposure (3, 20, 28). In fact, it was recently proposed that chronic inflammation and oxidative stress contribute to late developing radiation injury (38, 55). Studies of rat lung tissue (19), rat pulmonary endothelial cells (9), and human mammary epithelial cells (36) all suggest that inflammatory cytokines elevate XDH gene expression and protein abundance. Whereas initial XO activation is likely due to proteolytic cleavage of XDH, prolonged activation may be partially supported by enhanced protein expression in response to inflammatory signals. Further investigation of XO and XDH protein abundance and gene expression is required to confirm this.

Biological injury generated by radiation is widely attributed to increased oxidative stress. Accordingly, we demonstrated that superoxide production in the aorta is significantly amplified 2 wk after whole body irradiation (Fig. 2). However, dietary XO inhibition abolished the superoxide increase. These data support our findings of radiation-induced XO activation, which strongly suggest that oxidative stress following radiation exposure derives from XO. Our aortic ROS measurements in the presence of acute allopurinol further support this statement. The aorta from 5 Gy irradiated rats was the only group that responded to allopurinol with a significant decrease of superoxide production rate (Fig. 2B). Thus we conclude that XO is the primary source of oxidative stress following radiation exposure and that XO inhibition significantly impairs this response.

In the endothelium, ROS is often correlated with diminished NO bioavailability, resulting in endothelial dysfunction (13, 16, 26, 52). We demonstrated that radiation significantly reduces endothelium-derived NO in response to ACh (Fig. 3). This result indicates that a single exposure of gamma radiation produces sustained endothelial dysfunction. Our findings of augmented XO activity and ROS production suggest that XO-generated superoxide contributes to the reduced endothelial NO bioavailability. When irradiated rats received oxypurinol treatment, their aortic NO production rate response was significantly greater than that of irradiated rats without treatment (Fig. 3), further implicating XO in endothelial dysfunction. A reduction of NO availability can be caused by direct NO scavenging or by attenuated NO production, both of which can be regulated by ROS (13, 16, 52). Additional studies of radiation-induced oxidative stress and NO production enzymes, such as endothelial nitric oxide synthase (eNOS), are necessary to determine the exact mechanism of persistent decreases in NO bioavailability.

Vascular tension response to endothelial agonists or antagonists is an established measurement of endothelial function. In agree-
ment with our findings (Fig. 4A), numerous studies have identified impaired endothelium-dependent vascular relaxation after radiation treatment. Soloviev et al. (40) found ACh-induced vasorelaxation to be depressed in the aorta of gamma-irradiated rabbits at 9 and 30 days postradiation, in a radiation dose-dependent manner. In addition, when the antioxidant, α-tocopherol acetate, was administered orally to the rabbits at 1 h postradiation, the resulting vasorelaxation was restored to that of controls. The finding of antioxidant-derived radioprotection was repeated in a 10 Gy exposure, vitamin C-treated rat model (35). We identified a principal source of radiation-induced oxidants in our finding that chronic XO inhibition improves endothelium-dependent vasorelaxation. In our earlier finding with acute XO inhibition, the vasorelaxation of 5 Gy gamma-irradiated aorta was significantly greater after short Oxp incubations (42). Consequently, Oxp treatments could be applicable as both proactive and reactive radiation incident therapies. It remains to be seen whether chronic XO inhibition is still effective when initiated immediately after radiation exposure.

In addition, our vascular tension results demonstrated that endothelium-independent relaxation is not greatly affected by radiation (Fig. 4B). Although other studies support this finding (35, 49), Soloviev et al. (40) observed less endothelium-independent relaxation at 9 days postradiation. However, the relaxation fully recovered after 30 days. In addition to the endothelium, the vascular smooth muscle also may be a target of early radiation injury. For example, it was previously reported (41) that smooth muscle vasoconstriction sensitivity to KCl and PE was increased after irradiation. In our vascular tension experiments, aortic vasoconstriction in response to $10^{-6}$ M PE was not significantly different between radiation groups (Fig. 4, C and D). Thus the vasorelaxation dose responses are not significantly affected by the smooth muscle preconstriction in our study. All these findings strongly suggest that radiation-induced vascular injury is primarily endothelium dependent.

Vascular stiffness is emerging as a reliable independent predictor of cardiovascular disease (53). In vivo vascular stiffness is regulated by both endothelial function and vascular remodeling (12, 34, 43, 51). Considering our findings of endothelium-dependent relaxation (Fig. 4A) and passive aortic stiffness (Fig. 6), both factors appear to be contributing to increased vascular stiffness in response to irradiation. As discussed above, NO bioavailability is significantly attenuated (Fig. 3), and this decrease is associated with XO-derived superoxide (Fig. 2). Thus NO available from physiological shear stress and eNOS activators in the rat will be reduced, resulting in less active vasodilation and greater apparent vascular stiffness. Consequently, the protection that Oxp treatment affords to endothelial function after a radiation incident is extended to vascular stiffness, as our data show (Fig. 5). In addition, changes in passive aortic compliance and distensibility indicate aortic remodeling (12, 34, 50, 51). The nearly identical compliance and distensibility trends indicate that aorta geometries of the different rat groups are similar, at least with respect to unstressed diameter (Fig. 6). Therefore, radiation-induced vascular remodeling is expected to arise from vascular wall composition changes. Our results suggest that XO inhibition impairs vascular remodeling after radiation exposure, which assists in maintaining aortic stiffness.

Blood pressure also can affect vascular stiffness measurements (1), but radiation did not appear to modulate blood pressure in this study (Table 1). Mean arterial blood pressure is highly dependent on small resistance arteries and is less dependent on large conduit arteries, such as the aorta. The predominant vasodilation mechanism of resistance vessels is endothelium-derived hyperpolarizing factor (EDHF) (21, 33). It was previously reported (40) that radiation does not significantly affect EDHF-dependent relaxation. In large conduit arteries the dominant vasorelaxation mechanism is NO release, and our data convincingly demonstrate impaired NO responsiveness in the aorta of irradiated rats. Thus it is likely that radiation predominantly affects large conduit vessels, rather than small resistance arteries. This would partially explain the stable blood pressure in the presence of endothelial dysfunction and aortic stiffening.

In conclusion, XO is a major regulator of vascular damage following radiation exposure. We have provided compelling evidence that amplified XO activity elevates ROS production, which directly limits NO bioavailability and impairs endothelial function. An additional consequence of XO activation is aortic stiffening. However, chronic XO inhibition effectively protects against radiation-induced vascular injury. Accordingly, XO inhibition is an attractive therapy to increase the safety of medical radiation procedures and occupations associated with high radiation risk.

DISCLOSURES
No conflicts of interest are declared by the author(s).

REFERENCES


