Potentiation of Nitric Oxide–Mediated Vasorelaxation by Xanthine Oxidase Inhibitors (43982)

YOICHI MIYAMOTO,*[,1,2] TAKAAKI AKAIKE,*[,2] MASAKI YOSHIDA,† SHINGO GOTO,*[,†] HIDECHIKA HORIE,* AND HIROSHI MAEDA*[,3]

Departments of Microbiology and Urology,† Kumamoto University School of Medicine, Kumamoto 860, Japan*

Abstract. **Nitric oxide (NO), now almost synonymous with endothelium-derived relaxing factor (EDRF), reacts with superoxide anion radical (O_2^-) and forms a potentially toxic molecular species, peroxynitrite ($ONOO^-$). Because xanthine oxidase (XO) seems to be a major O_2^--producing enzyme in the vascular system, it is important to clarify the mechanism of XO regulation of NO/EDRF. We first characterized the inhibition of XO *in vitro* by three types of pyrazolopyrimidine derivatives. Kinetic studies indicated that 4-amino-6-hydroxypyrazolo[3,4-*d*]pyrimidine (AHPP) and allopurinol competitively inhibited the conversion of xanthine to uric acid catalyzed by XO, with apparent K_i values of 0.17 ± 0.02 and 0.50 ± 0.03 μ*M*, respectively; alloxanthine inhibited this conversion in a noncompetitive manner with an apparent K_i value of 3.54 ± 1.12 μ*M*. O_2^- generation in the xanthine/XO system assayed by lucigenin-dependent chemiluminescence was suppressed most strongly by AHPP in a dose-dependent fashion; allopurinol itself appears to reduce the enzyme by transfer of an electron to O_2, thus generating O_2^-. AHPP significantly augmented EDRF-mediated relaxation of aortic rings from both rabbits and spontaneously hypertensive rats (SHR) in a dose-dependent manner, whereas allopurinol did not affect the relaxation and only marginal potentiation of the vasorelaxation was observed with alloxanthine. Finally, iv injection of AHPP (50.4 mg/kg; 100 μmol/300 g rat) reduced the blood pressure of SHR rats to 70% of the initial pressure; this pressure is almost the blood pressure of normal rats. Allopurinol (100 μmol/300 g rat; iv) showed transient decrease in blood pressure and moderate reduction of hypertension of SHR (10%) was observed with iv injection of alloxanthine (100 μmol/300 g rat). On the basis of these results, it seems that XO regulates EDRF/NO via production of O_2^-.** [P.S.E.B.M. 1996, Vol 211]

Considerable attention has been focused on multiple functions of a labile inorganic radical species, nitric oxide (NO), in a variety of biological systems (1–5). NO is produced by different isoforms of NO synthase (NOS) in many types of cells, including vascular endothelial cells, vascular smooth muscle cells, activated macrophages, neuronal cells, and glial cells (5). It is well known that a constitutive isoform of NOS (cNOS) is expressed in vascular and neuronal systems and that an inducible isoform of NOS (iNOS) is induced in activated murine macrophages, vascular smooth muscle cells, hepatocytes, and other cells (5–9). Basal release of NO by endothelial cell cNOS in vessel walls activates soluble guanylate cyclase in the smooth muscle cells to regulate vascular tone, blood pressure, and tissue perfusion under physiological conditions (5). On the other hand, it is reported that, under pathological conditions such as endotoxin-related and septic shock, induction of iNOS in the vascular system causes prolonged and excessive biosynthesis of NO, which leads to sustained vasodilation and shock (6–10).

[1] Present address: Tsukuba Research Laboratory, NOF Co., Tokyo, Japan.
[2] Contributed equally to this work.
To whom requests for reprints should be addressed at Department of Microbiology, Kumamoto University School of Medicine, Kumamoto 860, Japan.

Received April 20, 1995. [P.S.E.B.M. 1996, Vol 211]
Accepted December 14, 1995.

0037-9727/96/2114–0366$10.50/0

The biological actions of NO are abrogated *via* a direct reaction with endogenous non–heme- and heme-containing proteins such as hemoglobin (5, 11), with superoxide anion radical (O_2^-) (12–15), and with certain organic nitronylnitroxide radicals that scavenge NO (16). Among these, O_2^- can react with NO very rapidly, yielding a toxic molecular species, peroxynitrite ($ONOO^-$) (14, 17), whereas superoxide dismutase (SOD), which converts O_2^- to H_2O_2 and O_2 (18), would prolong the biological half-life of NO because of removal of O_2^- and hence enhancement of vasorelaxation by endothelium-derived relaxing factor (EDRF)/NO (13). It is thought that one of the major enzymes that produce O_2^- in the vessel walls (endothelial cells) is xanthine oxidase (XO) (19–21). Therefore, it is possible that the EDRF-dependent vasorelaxation is regulated by XO in vascular systems *via* extremely rapid reaction between O_2^- and NO, resulting in a decreased amount of NO/EDRF. The results demonstrated in this paper indicate that specific inhibition of XO potentiates EDRF-induced vasorelaxation *ex vivo* in an organ bath and *in vivo* in spontaneously hypertensive rats (SHR).

Materials and Methods

Reagents. Three types of pyrazolopyrimidine derivatives shown in Figure 1 were used as XO inhibitors. 4-Amino-6-hydroxypyrazolo[3,4-*d*]pyrimidine (AHPP), allopurinal, and lucigenin (bis-*N*-methylacridinium nitrate) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Alloxanthine was provided by NOF Co. (Tokyo, Japan) and was synthesized according to the method described by Robins (22). Xanthine and hypoxanthine were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). These pyrazolopyrimidines and purines were dissolved in 0.25 *N* NaOH at a concentration of 0.1 *M* and were diluted to appropriate concentrations with distilled water or physiological saline. Bovine milk XO was purchased from Boehringer Mannheim GmbH, (Mannheim, Germany). CuZn-SOD and N^{ω}-nitro-L-arginine methyl ester (L-NAME) were obtained from Sigma. All other chemicals and reagents were from commercial sources.

Animals. Female New Zealand White rabbits, weighing 2.5–3.0 kg, were from Japan SLC (Shizuoka, Japan), and male 14- to 18-week-old SHR, weighing 250–300 g, were obtained from Hoshino Experimental Animals, Ltd. (Saitama, Japan).

Measurement of XO-catalyzed uric acid formation and inhibition by pyrazolopyrimidines. Uric acid formation by bovine milk XO was measured spectrophotometrically (23). Briefly, to the cuvette containing XO at 3.33 mU/ml (final concentration) in 10 m*M* sodium phosphate buffer, pH 7.5, graded amounts xanthine and pyrazolopyrimidines were added to start the reaction. The velocity of uric acid formation at 25°C was determined as evidenced by the increase in absorbance at 290 nm.

Measurement of superoxide production. XO-catalyzed O_2^- production was monitored by measurement of lucigenin-dependent chemiluminescence at 25°C. The reaction mixture consisted of 10 μ*M* lucigenin, 20 mU/ml XO, and indicated amounts of xanthine, AHPP, allopurinol, or alloxanthine in 10 m*M* sodium phosphate buffer (pH 7.5). Chemiluminescence of the mixture was measured by using a six-channel luminometer equipped with a data-analyzing system (Model LB 9505C; Labaratorium Berthold AG, Wildbell, Germany). Lucigenin-dependent chemiluminescence observed in this XO system was completely nullified by addition of 100 U/ml of SOD, indicating that the luminescence was attributed solely to the reaction of lucigenin and O_2^-, as reported previously (24).

Bioassay system for EDRF generation. Both rabbits and SHR were anesthetized with sodium pentobarbitone and were exsanguinated. The chest was opened for removal of the thoracic aorta. After excess fat and connective tissue surrounding the vessel were removed, the aorta of rabbits and SHR were cut into rings 5 mm and 2 mm wide, respectively. The rings were mounted vertically in 20-ml organ baths filled with Krebs solution, and isometric tension development was recorded as was reported previously (16). The medium was maintained at 37°C and was bubbled with a gas mixture of 95% O_2/5% CO_2. Tissues were precontracted with 0.15 μ*M* phenylephrine, after which acetylcholine- or ATP-induced relaxation was measured, as were the effects of AHPP, allopurinol, and alloxanthine on the smooth muscle relaxation induced by acetylcholine or ATP.

In some experiments, the effect of AHPP on va-

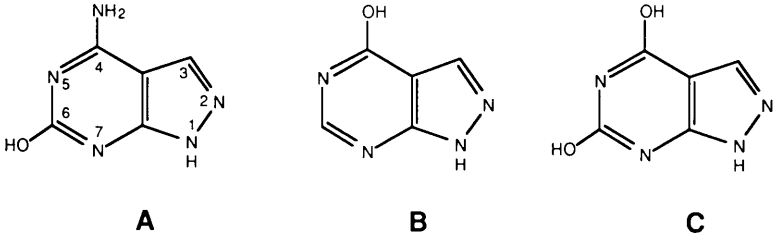

Figure 1. Chemical structure of three pyrazolopyrimidine derivatives used in this experiment. (A) AHPP. (B) Allopurinol. (C) Alloxanthine.

sorelaxation was tested in the presence or absence of hypoxanthine. Also in our study, the vascular tone of the aortic rings was studied in the presence of 3.0 μM indomethacin after phenylephrine-induced contraction of the smooth muscle.

Further, the effect of L-NAME, an NOS inhibitor on vasorelaxation, was examined to confirm the dependence of the effect of XO inhibitors on NO pathway in vascular relaxation.

Blood pressure measurements and administration of XO inhibitors to SHR. An arterial line was placed in the femoral artery of each SHR after the rats were anesthetized with sodium pentobarbitone. Mean arterial pressure and heart rate were continuously monitored by use of a pressure transducer as described (10). After blood pressure and heart rate were stabilized, AHPP, allopurinol, or alloxanthine was infused slowly intravenously (iv) for 5 min at the doses of 50.4, 45.4, and 50.7 mg/kg (100 μmol/300 g rat equivalent, each) in 1 ml of 0.1 N NaOH. The control group received 1 ml of 0.1 N NaOH given in a similar fashion. Blood pressure and heart rate were monitored for 3 hr after injection of AHPP. Blood pH remained unchanged during infusion of the reagent and vehicle.

The effect of administration with L-NAME (2 mg/rat; iv) was also examined in SHR treated with AHPP.

Statistical analysis. Statistical analysis for enzyme kinetic of XO inhibitors was performed as described by Cleland (25), and those for other studies were done by two-tailed t test for unpaired data.

Results

Inhibition of XO-catalyzed uric acid formation by AHPP and other XO inhibitors. The inhibitory potency of AHPP was compared with that of the well-known XO inhibits allopurinol and alloxanthine by measuring the conversion of xanthine to uric acid, which is catalyzed by bovine milk XO. The initial velocity of uric acid formation was measured in the presence of various concentrations of xanthine and the inhibitors. As shown in Figure 2, Lineweaver-Burk analysis indicated that AHPP inhibited XO in a competitive manner with an apparent inhibition constant K_i of 0.17 ± 0.02 μM (value ± SD), which was consistent with that described in a previous report (26). Allopurinol also inhibited the conversion of xanthine to uric acid competitively with an apparent K_i of 0.50 ± 0.03 μM. However, alloxanthine, which is an XO-oxidized product of allopurinol and is known to be a titrating inhibitor of this enzyme (27), showed weaker inhibitory activity (an apparent K_i = 3.54 ± 1.12 μM). Lineweaver-Burk analysis of the reaction with alloxanthine showed that the inhibition of XO was noncompetetive. The K_i value for AHPP was significantly lower than that for allopurinol or alloxanthine ($P < 0.05$; AHPP versus allopurinol and alloxanthine).

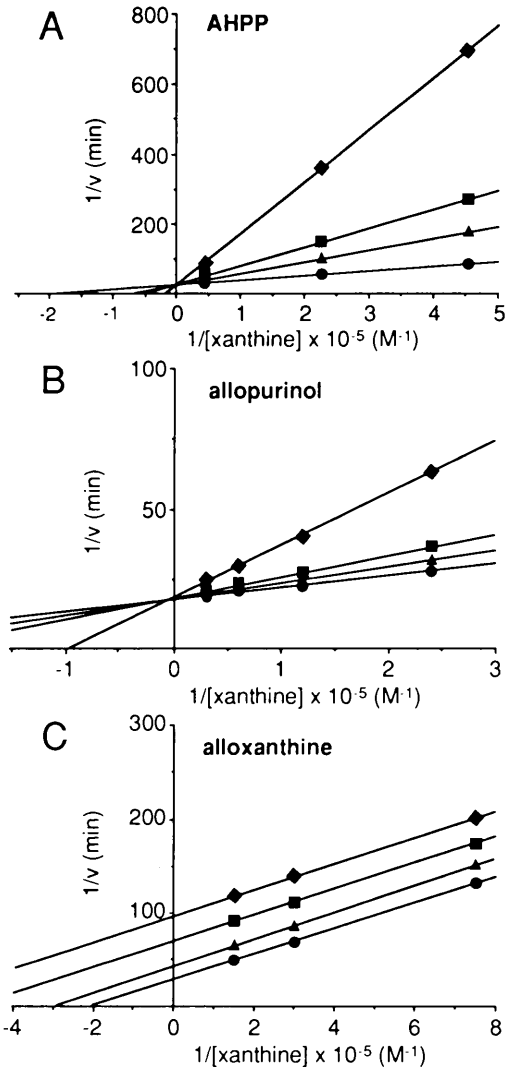

Figure 2. Lineweaver-Burk plot analyses of the inhibitory effects of AHPP (A), allopurinol (B), and alloxanthine (C) on the XO-catalyzed conversion of xanthine to uric acid. Initial velocity of uric acid formation was determined spectrophotometrically. Concentrations of AHPP used were: 0 (●), 0.22 (▲), 0.44 (■), and 1.32 μM (◆); those for allopurinol: 0 (●), 0.5 (▲), 1.0 (■), and 3.3 μM (◆); those for alloxanthine: 0 (●), 1.1 (▲), 3.3 (■), and 11.1 μM (◆). The apparent K_i values for AHPP, allopurinol, and alloxanthine were estimated to be 0.17 ± 0.02, 0.50 ± 0.03, and 3.54 ± 1.12 μM, respectively. Note that the modes of inhibition of AHPP and allopurinol are competitive and that of alloxanthine is noncompetitive. See text for detail.

Inhibition of XO-catalyzed superoxide radical production by AHPP and other inhibitors. XO reduces molecular oxygen species during oxidation of the substrates. More specifically, a reduced form of XO, from which an extra electron is transferred from the substrate xanthine, donates electrons to molecular oxygen to yield O_2^- (and/or H_2O_2), and the enzyme itself returns to its oxidized form of XO (28). Therefore, we monitored O_2^- production by the xanthine/XO system in the presence of AHPP, allopurinol, or alloxanthine by measuring lucigenin-dependent chemiluminescence (Fig. 3, A and B). As described in Ma-

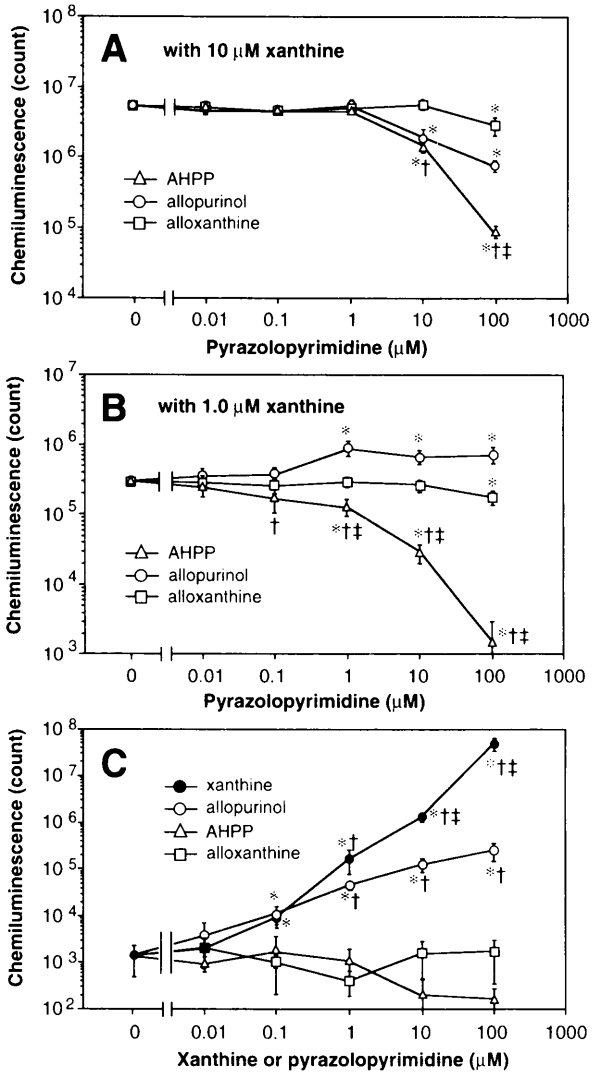

Figure 3. Effects of AHPP, allopurinol, and alloxanthine on O_2^- production by XO determined by lucigenin-dependent chemiluminescence. (A) O_2^- generation in the mixture of XO containing 10 μ*M* xanthine and indicated amounts of various XO inhibitors. (B) The same experiments as in Panel A except that the concentration of xanthine was 1 μ*M*. (C) O_2^- generation in the reaction mixture containing indicated amounts of xanthine, allopurinol, alloxanthine, or AHPP as a substrate for XO. Chemiluminescence responses are based on the integral of photocounting for the initial 10-min reaction period. Results were means ± SD (n = 4). *Significantly different from the control value without inhibitors (A and B) or substrates (C) ($P < 0.05$). † in Panel A and B, $P < 0.05$ between AHPP and alloxanthine (A) and between AHPP and allopurinol (B); ‡ in Panel A and B, $P < 0.05$ between AHPP and both allopurinol and alloxanthine. † in Panel C, significantly different ($P < 0.05$) from AHPP and alloxanthine; ‡ in Panel C, indicates $P < 0.05$ between xanthine and allopurinol.

terials and Methods and reported previously, lucigenin-dependent chemiluminescence is evoked by the direct reaction of O_2^- with lucigenin to form an excited state of *N*-methylacridone (24). No O_2^--scavenging activity by these pyrazolopyrimidines was found (data not shown) by electron spin resonance spin trapping for O_2^- generated in NADPH/cytochrome P-450 systems as described earlier (29).

As shown in Figure 3, A and B, AHPP inhibited O_2^- production in a concentration-dependent manner. Chemiluminescence induced in the reaction of 1 or 10 μ*M* xanthine and 20 mU/ml XO was decreased by almost 100-fold in the presence of 100 μ*M* AHPP. The suppressive effect of allopurinol on O_2^- production by xanthine/XO was weaker than that of AHPP. Namely, chemiluminescence induced with 10 μ*M* xanthine and XO was reduced to only one tenth of the inhibitor-free control in the presence of 100 μ*M* allopurinol; whereas 100 μ*M* AHPP reduced the chemiluminescence response to 1/100 of the inhibitor-free control (Fig. 3A). Allopurinol slightly increased the chemiluminescence at a concentration of 1 μ*M* and above when 1 μ*M* xanthine was used as a substrate (Fig. 3B). Alloxanthine showed very weak inhibitory effect on O_2^- production in the same setting. Therefore, among the three pyrazolopyrimidines tested, AHPP was the most potent inhibitor of O_2^- production by XO.

Because allopurinol is itself a substrate for XO and is converted to alloxanthine (27), O_2^- production caused by XO activity was examined using each of three pyrazolopyrimidines as a substrate. As shown in Figure 3C, allopurinol increased the lucigenin-dependent chemiluminescence in a dose-dependent manner, which indicates that XO could indeed transfer an electron from allopurinol to molecular oxygen. In contrast, AHPP and alloxanthine, both of which possess an oxygenated moiety at carbon position 6 (Fig. 1), did not produce the O_2^--induced chemiluminescence response, probably because of their inability to provide an electron to molecular oxygen *via* XO.

Effects of Different Pyrazolopyrimidines on EDRF-Induced Vasorelaxation *ex Vivo* Using Aorta Rings of Rabbits and SHR. First, the effects of AHPP and allopurinol on vasorelaxation induced by acetylcholine and ATP, which are known to be mediated by EDRF/NO, were examined *ex vivo* using vascular rings of rabbit thoracic aorta. As shown in Figure 4, A and B, AHPP enhanced the vasorelaxation in a dose-dependent manner. In the presence of 300 μ*M* AHPP, the concentrations of acetylcholine and ATP required for 50% relaxation of the aorta strips precontracted by phenylephrine were reduced to one-third and one-fifth of those obtained in the absence of AHPP, respectively. In contrast, allopurinol did not affect the acetylcholine-induced relaxation of the aorta strips (Fig. 4C).

Similarly, AHPP significantly potentiated the EDRF-response induced by acetylcholine in vascular ring of SHR (Fig. 5A). In this bioassay system with SHR aorta, only weak enhancement of the vasorelaxation was obtained with 300 μ*M* alloxanthine, and no appreciable effect on the EDRF response was observed with allopurinol (Fig. 5B). Accordingly, AHPP appears most potent in enhancement of EDRF (NO)–mediated vascular relaxation in the *ex vivo* bioassay

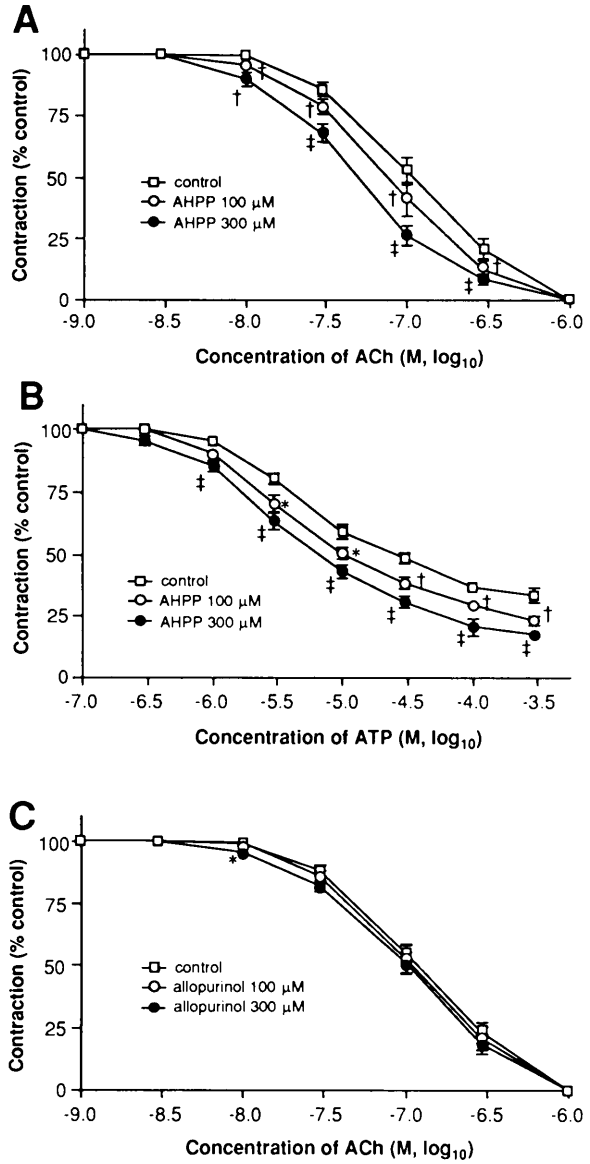

Figure 4. Inhibitory effects of AHPP and allopurinol on acetylcholine- and ATP-induced relaxation of rabbit thoracic aorta rings. Tissues were precontracted with 0.15 μ*M* phenylephrine, after which indicated concentrations of acetylcholine (ACh) (A and C) or ATP (B) were added. AHPP (A and B) and allopurinol (C) were added immediately after the addition of phenylephrine. Data are means ± SD (n = 4). *·†·‡The value with each XO inhibitor is significantly different from the control value without inhibitors: *$P < 0.05$; †$P < 0.025$; ‡$P < 0.01$. See text for details.

system. All of the vasorelaxation with or without XO inhibitors were almost completely abrogated by an NOS inhibitor L-NAME (Fig. 5A).

More importantly, the potentiation of vasorelaxation by AHPP was strongly inhibited by hypoxanthine an endogenous substrate for XO in a concentration-dependent fashion as shown in Figure 5, C and D. Hypoxanthine itself had no effect on acetylcholine-induced vasorelaxation in the aorta rings of SHR (data not shown).

Effect of AHPP on blood pressure of SHR. The SHR is used as an animal model for essential hypertension, and we examined the effect of XO inhibitors on the blood pressure of the SHR. As demonstrated in Figure 6A, the blood pressure of the rats started to decline immediately after injection of AHPP, and the gradual trend continued to 70% of the initial blood pressure without any appreciable change in heart rate within 80 min after intravenous injection of AHPP at a dose of 50.4 mg/kg (100 μmol/300 g rat). Treatment with allopurinol at a dose of 45.4 mg/kg (100 μmol/300 g rat) showed transient decrease in blood pressure for the initial 10-min period after allopurinol injection. The blood pressure of the rats was reduced moderately only by 10% after administration with 50.7 mg/kg of alloxanthine (100 μmol/300 g rat).

Moreover, the blood hypotension in SHR observed with AHPP treatment was completely nullified by iv injection of L-NAME (2 mg/rat in 0.2 ml of saline) (Fig. 6B).

Discussion

An important role for O_2^- generation produced by XO has been well documented in the pathogenesis of a number of disorders including ischemia-reperfusion and inflammatory injury (30). We previously reported the pathogenic role of O_2^- produced by XO in influenza virus infection in mice (31, 32). In contrast, it was recently demonstrated that O_2^- reacts with NO rapidly and hence removes NO from the milieu (12–14). XO is one of the major enzymes that produce O_2^- in vascular systems (19–21). It is therefore conceivable that vascular endothelial XO may regulate EDRF-dependent vasorelaxation indirectly *via* its O_2^--producing potency.

In this report, we describe an enhancing effect of AHPP on EDRF-dependent vasorelaxation *ex vivo* and *in vivo*. In contrast to AHPP, allopurinol, a well-known pyrazolopyrimidine-type XO inhibitor, did not affect EDRF actions in the same experimental settings. The different effects of these two XO inhibitors might be attributed to their difference not only in the XO-inhibitory efficacy (K_i) of each inhibitor but also in their potency in suppressing O_2^- production by the enzyme.

An oxidized form of XO is reduced by substrates such as xanthine and hypoxanthine, and is converted to a form that in turn transfers electrons to oxygen to generate O_2^- and/or H_2O_2 (28). Because the mechanism of inhibition by AHPP and alloxanthine was reported to be binding to stabilize the reduced form of the enzyme (27, 33–35), AHPP was expected to inhibit XO in the same fashion as alloxanthine.

We found that alloxanthine inhibited uric acid formation by XO in an noncompetitive manner, whereas AHPP was a competitive inhibitor for XO (Fig. 2). It appears that this difference in mode of inhibition by AHPP and alloxanthine may be due to the distinct

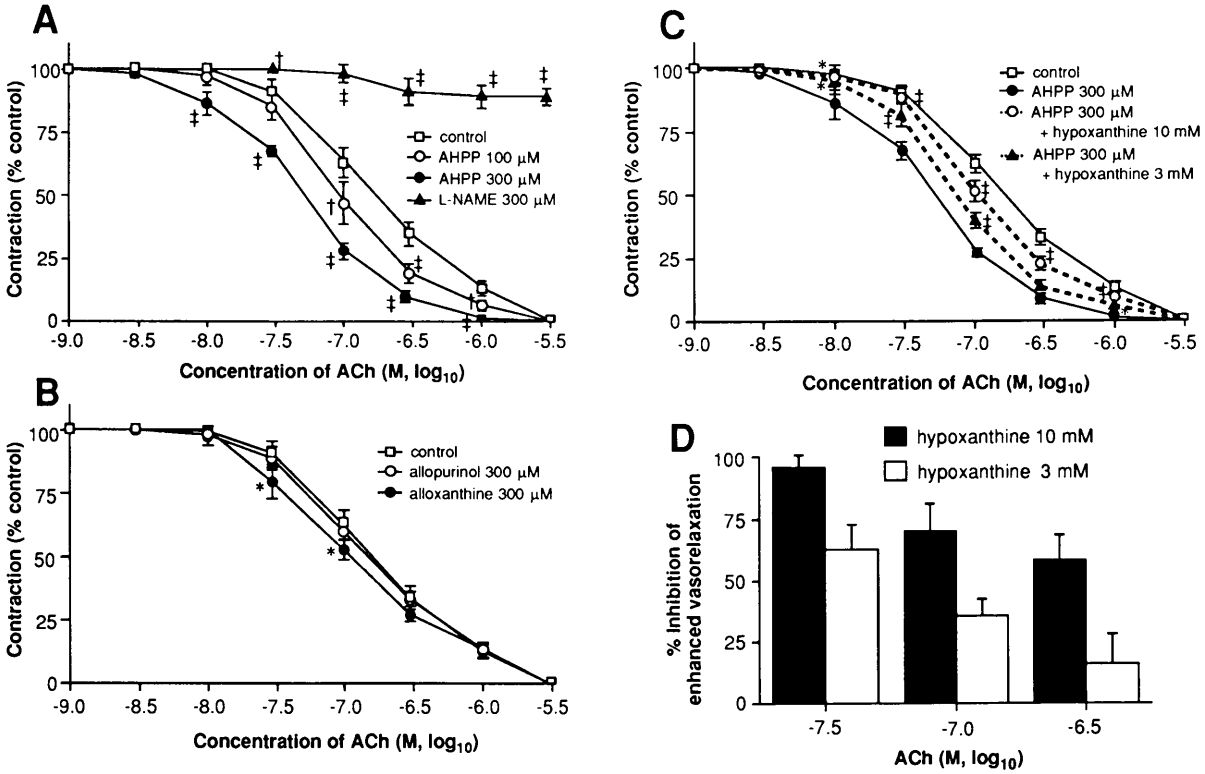

Figure 5. Inhibitory effects of AHPP, allopurinol, and alloxanthine on acetylcholine-induced relaxation of the aorta rings of SHR. The relaxation of vascular rings were obtained with indicated concentrations of acetylcholine (ACh) with or without AHPP (A), allopurinol (B), and alloxanthine (B) in a same manner as in Figure 4. Similarly, the effect of L-NAME (300 μ*M*) on the ACh-induced vasorelaxation was tested in the presence of AHPP (300 μ*M*) (A). (C) The effects of hypoxanthine on enhanced vasorelaxation by 300 μ*M* AHPP. Dose-dependent inhibition of the AHPP-potentiated vasorelaxation with hypoxanthine (3 and 10 m*M*) are clearly shown in (D). Data are means ± SD (n = 4). *·†·‡The value with each XO inhibitor is significantly different from the control value without inhibitors: *$P < 0.05$; †$P < 0.025$; ‡$P < 0.01$.

binding character of each compound to the molybdenum catalytic center of XO. It should be noted, however, that the inhibitory potency of AHPP against XO is 21 times stronger than that of alloxanthine. In fact, the inhibition of O_2^- production by AHPP was much higher than that by allopurinol and alloxanthine. Alloxanthine suppressed O_2^- production only marginally (Fig. 3, A and B), whereas allopurinol enhanced O_2^- generation by XO (Fig. 3, B and C). The enhancement of O_2^- production from XO by allopurinol can be explained by the fact that allopurinol is oxidized to alloxanthine and thus an electron is transferred to O_2 producing O_2^- by XO, if hypoxanthine and xanthine, which are more preferred endogenous substrates for XO than allopurinol, does not compete the reaction of allopurinol with XO.

Further, the regulatory function of XO on EDRF/NO in blood vessels was investigated by testing the effect of AHPP on vasorelaxation *ex vivo* and *in vivo*. Results were obtained in experiments using aorta rings as shown in Figure 4 and 5. AHPP, which is a more effective inhibitor of XO-produced O_2^- generation than are allopurinol and alloxanthine, significantly enhanced EDRF/NO action in the organ bath study, whereas allopurinol had no appreciable effect and alloxanthine did so only weakly (Fig. 4C and 5B).

Similar results were observed in experiments *in vivo* using SHR. Specifically, iv injection of AHPP to SHR resulted in 30% reduction of hypertension and to almost the normal level of blood pressure (Fig. 6). In a separate experiment, AHPP was given to the SHR orally at a dose of 100 mg/kg, and the change in blood pressure was monitored in the tail artery in the awake state of the animals by a sphygmomanometer. The results showed again significant improvement of blood hypertension: 30% reduction of blood pressure was obtained 4 hr after administration of AHPP (data not shown). More importantly, the hypotensive effect of AHPP was completely abrogated by the treatment of SHR with L-NAME (Fig. 6B), indicating that reduction of blood pressure produced by AHPP was mediated by the L-arginine–dependent NO synthesis pathway.

Although alloxanthine treatment of SHR produced moderate decrease in blood pressure by 10%, its hypotensive effect was much less potent than that of AHPP. Allopurinol, that showed very little enhancement of EDRF response in organ bath study induced a transient reduction of blood pressure just after its iv injection. The hypotensive effect of allopurinol, however, did not continue longer than 20 min after the administration. Therefore, it appears that the *in vivo*

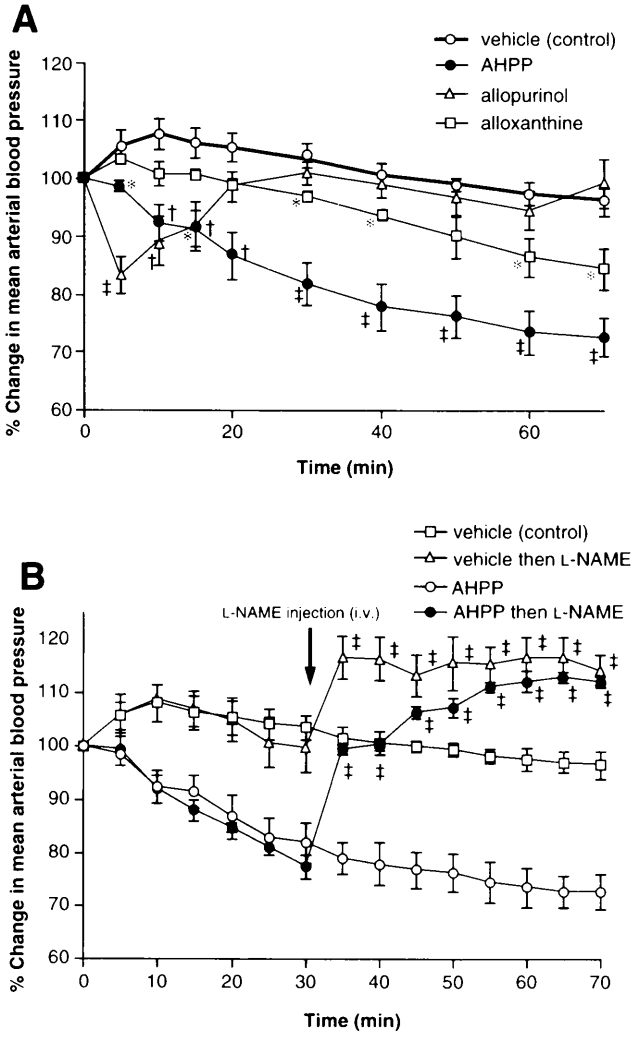

Figure 6. Effects of intravenous (i.v.) administration of AHPP, allopurinol and alloxanthine on the blood pressure of SHR (A), and the effect of iv injection of L-NAME on reduction of hypertension induced by AHPP in SHR (B). XO inhibitors were injected *via* the jugular vein, after which mean arterial blood pressure was continuously measured. Data are expressed as means ± SE (n = 8 for each group) of percent change in mean arterial blood pressure after injection of XO inhibitors or vehicle. *P < 0.05; †P < 0.025; ‡P < 0.01; control (vehicle) versus XO inhibitor–treated group (A), and L-NAME–treated versus nontreated groups (B). See text for details.

hypotensive activity of these three XO inhibitors observed in SHR fairly correlates with *ex vivo* EDRF potentiation as well as XO inhibitory potential of the inhibitors, and is consistent with their suppressive potentials against O_2^- generation by XO *in vitro*.

It has recently been demonstrated that XO localizes not only in the cytosol of vascular endothelial cells but also on the surface of endothelial cells (19–21). In addition, it was recently reported that EDRF functions in the vascular system of SHR (36), and that not only alloxanthine but also a chemically modified recombinant SOD, which exhibited high binding affinity to the vascular wall, could decrease the blood pressure of the SHR (37). Besides, it is recently reported that a synthetic SOD mimics intensified EDRF activity mediated by NO in the rat aortic vascular rings and exhibits the hypotensive effect in rats (38). These findings were substantiated by our result that AHPP significantly decreased the blood pressure of the SHR to an almost normal blood pressure (fig. 6).

On the basis of these results, it is concluded that XO may be one of the most important regulatory enzymes for EDRF/NO in vascular endothelial systems. The present finding suggests the possibility of the development of a new class of antihypertensive drug, in addition to previously known hypotensive drugs such as angiotensin-converting enzyme (ACE) inhibitors, calcium antagonists, and β-blocker.

It has been suggested that interaction of NO with O_2^- and the formation of chemically more reactive product $ONOO^-$ may be a toxic principle in the pathogenesis of various inflammatory disorders (39). This notion is strongly supported by our recent observation on influenza virus pneumonia in mice, in which $ONOO^-$ generation in the lung appears to be involved in its pathogenesis (40). Therefore, a specific and potent XO inhibitor such as AHPP might be beneficial not only for potentiation of EDRF activity of NO but also for prevention of $ONOO^-$-mediated cytotoxicity.

We thank Ms. Judith B. Gandy and Ms. Rie Yoshimoto for editorial work and typing, respectively. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

1. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the regulation of arterial smooth muscle by acetylcholine. Nature **288**:373–376, 1980.
2. Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc Natl Acad Sci USA **84**:9265–9269, 1987.
3. Palmer RMJ, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature **327**:524–526, 1987.
4. Shibuki K, Okada D. Endogenous nitric oxide release required for long-term synaptic depression in the cerebellum. Nature **349**:326–328, 1991.
5. Moncada S, Palmer RMJ, Higgs EA. Nitric oxide: Physiology, pathophysiology and pharmacology. Pharmacol Rev **43**:109–142, 1991.
6. Radomski MW, Palmer RMJ, Moncada S. Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. Proc Natl Acad Sci USA **87**:10043–10047, 1990.
7. Busse R, Mulsch A. Induction of nitric oxide synthase by cytokines in vascular smooth muscle cells. FEBS Lett **275**:87–90, 1990.
8. Thiemermann C, Vane J. Inhibition of nitric oxide synthesis reduces the hypotension induced by bacterial lipopolysaccharides in the rat in vivo. Eur J Pharmacol **182**:591–595, 1990.
9. Kilbourn RG, Gross SS, Jubran A, Adams J, Griffith OW. N^G-Methyl-L-arginine inhibits tumor necrosis factor-induced hy-

potension: Implication for the involvement of nitric oxide. Proc Natl Acad Sci USA **87**:3629–3632, 1990.
10. Yoshida M, Akaike T, Wada Y, Sato K, Ikeda K, Ueda S, Maeda H. Therapeutic effect of imidazolineoxyl *N*-oxide against endotoxin shock through its direct nitric oxide-scavenging activity. Biochem Biophys Res Commun **202**:923–930, 1994.
11. Kosaka H, Watanabe M, Yoshihara H, Harada N, Shiga T. Detection of nitric oxide production in lipopolysaccharide-treated rats by ESR using carbon monoxide hemoglobin. Biochem Biophys Res Commun **184**:1119–1124, 1992.
12. Blough NV, Zafiriou OC. Reaction of superoxide with nitric oxide to form peroxynitrite in alkaline aqueous solution. Inorg Chem **24**:3502–3504, 1985.
13. Gryglewski RJ, Palmer RMJ, Moncada S. O_2^- is involved in the breakdown of endothelium-derived vascular relaxing factor. Nature **320**:454–456, 1986.
14. Huie RE, Padmaja S. The reaction of NO with superoxide. Free Radic Res Commun **18**:195–199, 1993.
15. McCall TB, Boughton-Smith NK, Palmer RMJ, Whittle BJR, Moncada S. Synthesis of nitric oxide from L-arginine by neutrophils. Release and interaction with superoxide anion. Biochem J **261**:293–296, 1989.
16. Akaike T, Yoshida M, Miyamoto Y, Sato K, Kohno M, Sasamoto K, Miyazaki K, Ueda S, Maeda H. Antagonistic action of imidazolineoxyl *N*-oxides against endothelium-derived relaxing factor/'NO through a radical reaction. Biochemistry **32**:827–832, 1993.
17. Radi R, Beckman JS, Bush KM, Freeman BA. Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. J Biol Chem **266**:4244–4250, 1991.
18. Klug-Roth D, Fridovich I, Rabani J. Pulse radiolytic investigation of catalyzed disproportionation. Mechanism for bovine superoxide dismutase. J Am Chem Soc **95**:2786–2790, 1973.
19. Jarasch E-D, Grund C, Bruder G, Heid HW, Keenan TW, Franke WW. Localization of xanthine oxidase in mammary-gland epithelium and capillary endothelium. Cell **25**:67–82, 1981.
20. Jarasch E-D, Bruder G, Heid HW. Significance of xanthine oxidase in capillary endothelial cells. Acta Physiol Scand (Suppl) **548**:39–46, 1986.
21. Adachi T, Fukushima T, Usami Y, Hirano K. Binding of human xanthine oxidase to sulphated glycosaminoglycans on the endothelial-cell surface. Biochem J **289**:523–527, 1993.
22. Robins RK. Potential purine antagonists. I. Synthesis of some 4,6-substituted pyrazolo[3,4-*d*]pyrimidines. J Am Chem Soc **78**:784–790, 1956.
23. Xanthine oxidase (milk). In: Worthington Enzyme Manual. Worthington Biochemical, Freehold, NJ, pp30–31, 1972.
24. Takahashi K, Akaike T, Sato K, Mori K, Maeda H. Superoxide anion generation by Pacific oyster (*Crassostrea gigas*) hemocytes: Identification by electron spin resonance spin trapping and chemiluminescence analysis. Comp Biochem Physiol **105B**:35–41, 1993.
25. Cleland WW. Statistical analysis of enzyme kinetic data. Methods Enzymol **63**:103–138, 1979.
26. Feigelson P, Davidson JD, Robins R. Pyrazolopyrimidines as inhibitors and substrates of xanthine oxidase. J Biol Chem **226**:993–1000, 1957.
27. Robins RK, Revanker GR, O'Brein DE, Springer RH, Novinson T, Albert A, Senga K, Miller JP, Streeter DG. Purine analog inhibitors of xanthine oxidase—Structure-activity relationships and proposed binding of the molybdenum cofactor. J Heterocyclic Chem **22**:601–634, 1985.
28. Nishino T, Schopfer LM, Massey V. The reactivity of chicken liver xanthine dehydrogenase with molecular oxygen. J Biol Chem **264**:2518–2527, 1989.
29. Sato K, Akaike T, Suga M, Ando M, Maeda H. Generation of free radicals from neocarzinostatin mediated by NADPH/cytochrome P-450 reductase via activation of enediyne chromophore. Biochem Biophys Res Commun **205**:1716–1723, 1994.
30. McCord JM. Oxygen-derived free radicals in postischemic tissue injury. N Engl J Med **312**:159–163, 1985.
31. Akaike T, Ando M, Oda T, Doi T, Ijiri S, Araki S, Maeda H. Dependence on O_2^- generation by xanthine oxidase of pathogenesis of influenza virus infection in mice. J Clin Invest **85**:739–745, 1990.
32. Maeda H, Akaike T. Oxygen free radicals as pathogenic molecules in viral diseases. Proc Soc Exp Biol Med **198**:721–727, 1991.
33. Whitaker JR. Mechanisms of oxidoreductases. In: Richardson T, Finley JW, Eds. Chemical Changes in Food During Processing. New York: Van Nostrand Reinhold Company, pp121–176, 1985.
34. Massey V, Komai H, Palmer G, Elion GB. On the mechanism of inactivation of xanthine oxidase by allopurinol and other pyrazolo[3,4-*d*]pyrimidines. J Biol Chem **245**:2837–2844, 1970.
35. Nishino T, Tsushima K. The mode of inhibition of chicken liver xanthine dehydrogenase by pyrazolo[3,4-*d*]pyrimidines. Yokohama Med Bull **29**:1–4, 1978.
36. Aisaka K, Mitani A, Kitajima Y, Ishihara T. Pressor effect of N^G-monomethyl-L-arginine in SHR. Jpn J Pharmacol **54**:461–463, 1990.
37. Nakazono K, Watanabe N, Matsuno K, Sasaki J, Sato T, Inoue M. Does superoxide underlie the pathogenesis of hypertension? Proc Natl Acad Sci USA **88**:10045–10048, 1991.
38. Kasten TP, Settle SL, Misko TP, Riley DP, Weiss RH, Currie MG, Nickols GA. Potentiation of nitric oxide-mediated vascular relaxation by SC52608, a superoxide dismutase mimic. Proc Soc Exp Biol Med **208**:170–177, 1995.
39. Crow JP, Beckman JS. The role of peroxynitrite in nitric oxide–mediated toxicity. In: Koprowski H, Maeda H, Eds. The Role of Nitric Oxide in Physiology and Pathophysiology. Current Topics in Microbiology and Immunology. Berlin: Springer-Verlag, Vol **196**: pp57–73, 1995.
40. Akaike T, Noguchi Y, Ijiri S, Setoguchi K, Suga M, Zheng YM, Dietzschold B, Maeda H. Pathogenesis of influenza virus-induced pneumonia: Involvement of both nitric oxide and oxygen radicals. Proc Natl Acad Sci USA 1996 (in press).