Xanthine Oxidase Produces Hydrogen Peroxide which Contributes to Reperfusion Injury of Ischemic, Isolated, Perfused Rat Hearts

James M. Brown, Lance S. Terada, Michael A. Grosso, Glenn J. Whitmann, Stephen E. Velasco, Anita Patt, Alden H. Harken, and John E. Repine
Departments of Surgery and Medicine and the Webb-Waring Lung Institute at the University of Colorado Health Sciences Center, Denver, Colorado 80262

Abstract

Three lines of investigation indicated that hydrogen peroxide (H_2O_2) from xanthine oxidase (XO) contributes to cardiac dysfunction during reperfusion after ischemia. First, addition of dimethylthiourea (DMTU), a highly permeant O_2 metabolite scavenger (but not urea) simultaneously with reperfusion improved recovery of ventricular function as assessed by ventricular developed pressure (DP), contractility ($+dP/dt$), and relaxation rate ($-dP/dt$) in isolated Krebs-Henseleit–perfused rat hearts subjected to global normothermic ischemia. Second, hearts from rats fed tungsten or treated with allopurinol had negligible XO activities (< 0.5 mU/g wet myocardium compared with > 6.0 mU/g in control hearts) and increased ventricular function after ischemia and reperfusion. Third, myocardial H_2O_2-dependent inactivation of catalase occurred after reperfusion following ischemia, but not after ischemia without reperfusion or perfusion without ischemia. In contrast, myocardial catalase did not decrease during reperfusion of ischemic hearts treated with DMTU, tungsten, or allopurinol.

Introduction

Although ischemic heart disease remains a leading cause of death, recent advances in techniques that reestablish blood flow have raised hope regarding improved salvage of ischemic myocardium (1, 2). However, along with these advances has come concern regarding the existence and causes of injury produced by reperfusion. A role for xanthine oxidase (XO)[1]–derived O_2 metabolites (3–5) has been suggested, but a number of crucial aspects remain unknown. In particular, the nature, timing, source, and significance of O_2 metabolites have not been fully determined, in part because of the complexity of

studies in intact animal models and human subjects. We addressed these issues by hypothesizing that generation of hydrogen peroxide (H_2O_2) by XO during reperfusion contributes to cardiac reperfusion injury. We tested this premise in simplified ("blood-free") isolated hearts using dimethylthiourea (DMTU), a highly permeant O_2 metabolite scavenger, infused only during reperfusion (6). We also assessed the effect of inhibiting XO (by HPLC analysis) with tungsten (7–9) or allopurinol (10) on ventricular function after ischemia and reperfusion. In addition, we measured H_2O_2-dependent aminotriazole inactivation of myocardial catalase activity as an indicator of myocardial H_2O_2 production (11, 12).

Methods

Preparation and measurement of injury in isolated hearts. Healthy, male Sprague-Dawley rats (300–325 g) were anesthetized (60 mg/kg i.p. sodium pentobarbital) and heparinized (500 U via right atrium). Hearts were rapidly excised and then perfused (70 mmHg) in retrograde fashion at the aortic root with a Krebs-Henseleit solution containing 1.2 mM [Ca^{2+}], 4.7 mM [KCl], and 25.0 mM [$NaHCO_3$] on a modified Langendorff apparatus (13). A gas mixture (92.5% O_2/7.5% CO_2) was used (Shirley Membrane), which achieved a $Po_2 = 450$, $Pco_2 = 40$, and pH = 7.4. A water-filled latex balloon was placed through the left atrium into the left ventricle, secured with an atrial 6–0 silk purse string suture and adjusted to a left ventricular end diastolic pressure of 6 mmHg. Pacing wires (rate 300 beats per minute; turned off during ischemia) were attached to the pulmonary outflow tract. After equilibration for 15 min, ventricular developed pressure (DP) and the positive ($+dP/dt$) and negative ($-dP/dt$) first derivatives were recorded using direct pressure and differentiator amplifiers. Subsequently, hearts were subjected to global normothermic (37°C waterbath) ischemia (aortic root stopcock) and reperfusion. In some experiments, 19.2 mM DMTU (Alpha Products, Danvers, MA) or equimolar urea (Fisher Scientific, Fair Lawn, NJ) solutions (in 0.9% NaCl) were infused at the aortic root at 0.2 cm³/min simultaneously with reperfusion. No changes in pH or temperature occurred during infusion of DMTU or urea. Other rats were fed a low molybdenum, tungsten-enriched diet (protein impregnated with 0.7 g/kg sodium tungstate, ICN Nutritional Biomedicals, Cleveland, OH) and were given H_2O with 10 ppm tungsten (Sigma Chemical Co., St. Louis, MO) for 3 wk (5, 7–9, 14), or were treated with allopurinol (infused at aortic root, final concentration at 25 µM/liter for 10 min) before ischemia and then continuously during reperfusion.

Measurement of myocardial XO activity. In separate experiments, hearts were removed from the perfusion apparatus after ischemia, freeze clamped with liquid nitrogen, weighed, and homogenized in buffer containing 50 mM KPO_4, 0.2 M Na_2CO_3/HCO_3 (pH = 10.0), 1

1. *Abbreviations used in this paper:* DMTU, dimethylthiourea; DP, developed pressure; XO, xanthine oxidase.

mM PMSF, 1 mM dithioerythritol, and 0.2 mM EDTA. After centrifugation for 30 min at 45,000 g, supernatants were passed over a buffer-equilibrated G-25 Sephadex column of 4°C (15). Supernatants from allopurinol or control hearts were treated similarly, except that they were not passed over the column to prevent loss of allopurinol into the Sephadex. XO activity was determined by measuring uric acid made in the presence of xanthine and ambient O_2. Calibration standards were prepared using uric acid (5, 10, and 15 μM) or 50 μM xanthine in buffer with PMSF, dithioerythritol, and EDTA. Samples were prepared by adding 1 ml Sephadex effluent to buffer, 50 μM buffer + xanthine, or 50 μM buffer + xanthine + allopurinol. All standards and samples were incubated at 37°C for 1 h and then 1.8 M perchloric acid was added to stop the reaction. After precipitated protein was removed by centrifugation, 10 μl aliquots of supernatants were injected into the HPLC column ([Waters C-18 Resolve; Waters Associates, Millipore Corp., Milford, MA] reverse phase, with a mobile phase of 20 mM KH_2PO_4 plus 3% MeOH at pH 5.0, at a rate of 0.7 ml/min). Uric acid peaks were integrated at 290 nM. Specific activity was expressed as milliunits per gram wet weight of myocardium. One unit of activity equaled one micromole of uric acid made per minute.

Measurement of catalase activity after aminotriazole treatment. In separate experiments, rats were pretreated with aminotriazole (500 mg/kg i.p.) 1 h before anesthetization. After study, hearts were placed immediately in KPO_4 buffer (10 ml of 5 mM at pH = 7.8) and homogenized. Homogenates were centrifuged at 20,000 rpm for 10 min, supernatants were collected, and catalase activities were measured spectrophotometrically (240 nM). After adjusting the absorbance to zero, using empty quartz cuvettes for reference and sample slots, potassium phosphate Triton X buffer (3 ml of 50 mM, pH = 7.0) and H_2O_2 (100 ml of 50 mM KPO_4 buffer + 135 μl of 30% H_2O_2) were added to reference and sample cuvettes, respectively. Absorbance was adjusted to 0.550 U by adding 30% H_2O_2 to the KPO_4/H_2O_2 solution. Standards were prepared by adding a standard catalase solution (75 μl) to each cuvette. Samples were prepared by adding sample supernatants (75 μl) to each cuvette. Absorbance per minute was recorded and catalase activity calculated as units per gram wet weight myocardium.

Analyses of statistical significance (16). Values for ventricular function and catalase activities were compared by analysis of variance and by post hoc Student-Newman-Keuls tests for between group differences. Differences in XO activities were compared by paired t tests.

Results

Effect of DMTU or urea treatment on ventricular function after ischemia and reperfusion. Measurements of ventricular function, including ventricular DP, contractility (+dP/dt), and relaxation rate (−dP/dt), were the same ($P > 0.05$) in all hearts studied before ischemia or ($P > 0.05$) after perfusion for 60 min without ischemia (Fig. 1). However, hearts subjected to ischemia for 20 min and then reperfused for 40 min had decreased ($P < 0.05$) ventricular function compared with hearts studied before ischemia or subjected to perfusion for 60 min without ischemia. In contrast, hearts subjected to ischemia and then treated with DMTU simultaneously with reperfusion had increased ($P < 0.05$) ventricular function compared with hearts subjected to ischemia and then reperfused with urea or no additions.

Effect of tungsten or allopurinol treatment on myocardial XO activity and ventricular function after ischemia and reperfusion. After ischemia for 20 min, hearts from rats treated with tungsten or allopurinol had decreased ($P < 0.05$) XO activities compared with untreated hearts (Fig. 2). In addition, after ischemia for 20 min and reperfusion for 40 min, hearts from rats treated with tungsten or allopurinol had increased ($P < 0.05$) ventricular function compared with untreated hearts (Fig. 3).
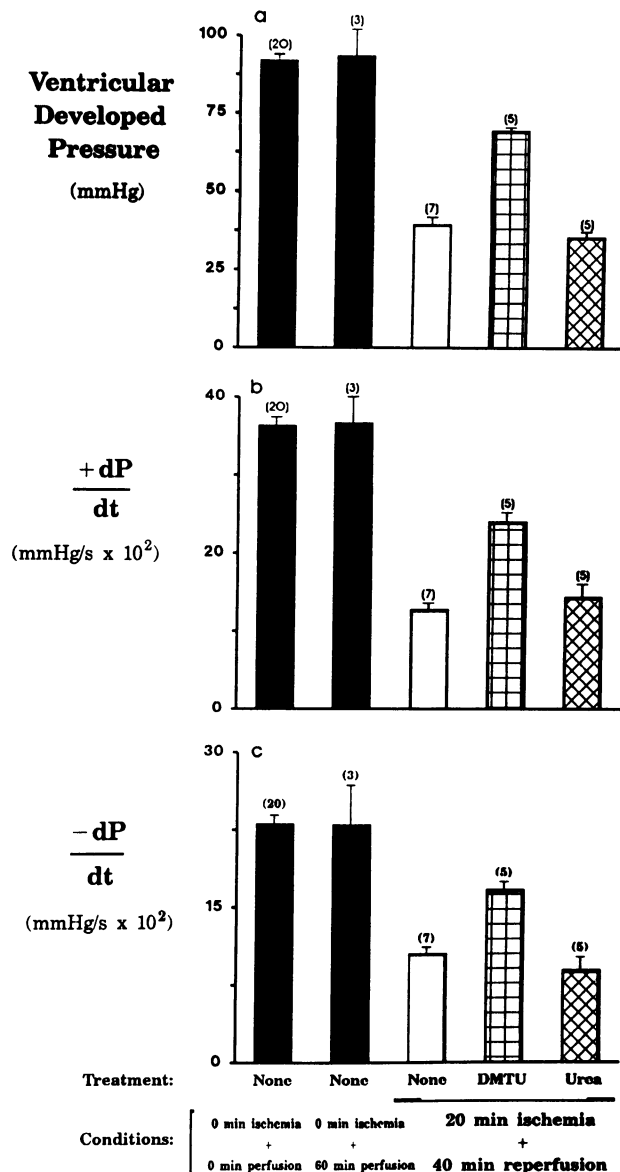
Figure 1. Ventricular function of isolated Krebs-Henseleit perfused rat hearts after global normothermic ischemia for 20 min and reperfusion for 40 min. (*a*) Ventricular DP, (*b*) contractility (+dP/dt), and (*c*) relaxation rate (−dP/dt) were the same ($P > 0.05$) in all hearts (untreated, DMTU, or urea treated) studied before ischemia. In addition, perfusion for 60 min without ischemia did not change ($P < 0.05$) DP, +dP/dt, or −dP/dt from preischemic values. However, after ischemia for 20 min and reperfusion for 40 min, untreated hearts had decreased ventricular function compared with hearts studied before ischemia or after perfusion for 60 min without ischemia. In contrast, after ischemia for 20 min and reperfusion for 40 min, hearts infused with 19.2 mM DMTU (0.2 cm³/min at aortic root) simultaneously with reperfusion had increased ($P < 0.05$) DP, +dP/dt, and −dP/dt values compared with untreated hearts or hearts infused with equimolar urea. Each bar is the mean±1 SE of the number of determinations shown within the parentheses.

Effect of DMTU, urea, tungsten, or allopurinol treatment on myocardial catalase activities in aminotriazole-treated rats subjected to ischemia and reperfusion. Catalase activities were the same in hearts perfused for 60 min without ischemia or subjected to ischemia for 20 min without reperfusion (Fig. 4). However, when subjected to ischemia for 20 min and then
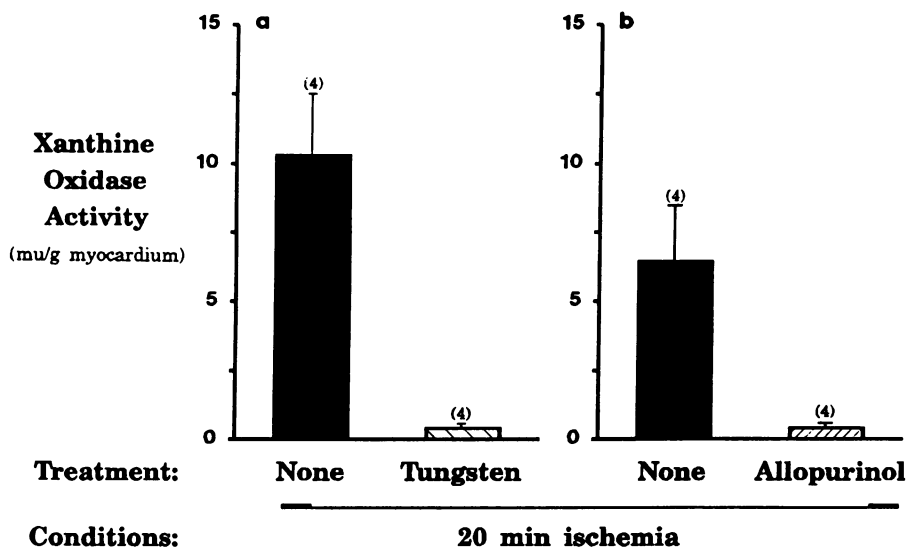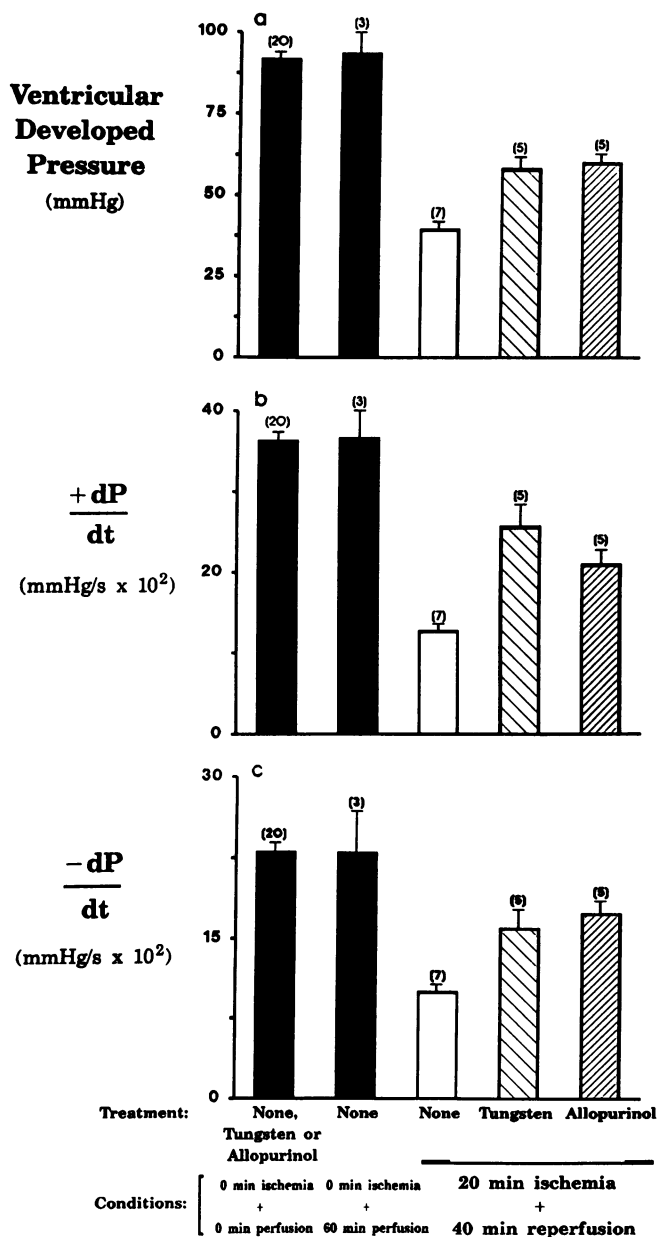
Xanthine Oxidase Activity
(mu/g myocardium)

Treatment: **None** **Tungsten** **None** **Allopurinol**

Conditions: **20 min ischemia**

Figure 2. Myocardial XO activity (milliunits per gram myocardium) after global normothermic ischemia for 20 min (before reperfusion). (a) Treatment with tungsten or (b) allopurinol decreased ($P < 0.05$) myocardial XO activities. Each bar is the mean±1 SE of the number of determinations shown within the parentheses.

Ventricular Developed Pressure
(mmHg)

$$\frac{+dP}{dt}$$
(mmHg/s x 10^2)

$$\frac{-dP}{dt}$$
(mmHg/s x 10^2)

Treatment: **None, Tungsten or Allopurinol** **None** **None** **Tungsten** **Allopurinol**

Conditions:
0 min ischemia	0 min ischemia	20 min ischemia
+	+	+
0 min perfusion	60 min perfusion	40 min reperfusion

reperfusion for 40 min, catalase activities were decreased ($P < 0.05$) in untreated or urea-treated hearts compared with DMTU-, tungsten-, or allopurinol-treated hearts.

Discussion

Our results provide evidence that H_2O_2 is generated during reperfusion of ischemic, isolated, perfused rat hearts. Specifically, we found that catalase activities decreased in hearts from aminotriazole-treated rats during reperfusion after ischemia, but not in hearts subjected to ischemia without reperfusion or perfusion without ischemia. Since aminotriazole is highly specific for the first intermediate in the reaction of catalase with H_2O_2 (compound I), assessing H_2O_2-mediated inactivation of catalase reflects H_2O_2 generation in biological systems (11–13). Moreover, addition of DMTU only during reperfusion not only prevented decreases in cardiac function, but also prevented H_2O_2-dependent decreases in myocardial catalase activity after reperfusion of ischemic hearts. Although DMTU can react with H_2O_2, hydroxyl radical ($\cdot$OH), or hypochlorous acid (HOCl) in vitro (6, 17), the additional finding that DMTU decreased H_2O_2-mediated inactivation of catalase in hearts from aminotriazole-treated rats further suggests that H_2O_2 participates in cardiac reperfusion injury. Moreover, $\cdot$OH is usually generated from H_2O_2 in vivo (3); a contribution from HOCl seems unlikely since production of HOCl from H_2O_2

Figure 3. Ventricular function after global normothermic ischemia for 20 min and reperfusion for 40 min. (a) Ventricular DP, (b) contractility (+dP/dt), and (c) relaxation rate (−dP/dt) measurements were the same ($P > 0.05$) in all hearts (untreated, tungsten, or allopurinol treated) studied before ischemia. In addition, perfusion for 60 min without ischemia did not change ($P > 0.05$) DP, +dP/dt, or −dP/dt from values obtained for hearts studied before ischemia. After ischemia for 20 min and reperfusion for 40 min, untreated hearts had decreased ($P < 0.05$) DP, +dP/dt, or −dP/dt values compared with values obtained for hearts studied before ischemia or hearts perfused for 60 min without ischemia. In contrast, after ischemia for 20 min and reperfusion for 40 min, hearts from rats that had been pretreated with tungsten or allopurinol had increased ($P < 0.05$) DP, +dP/dt, and −dP/dt compared with hearts from rats that received no treatment. Each bar is the mean±1 SE of the number of determinations shown within the parentheses.
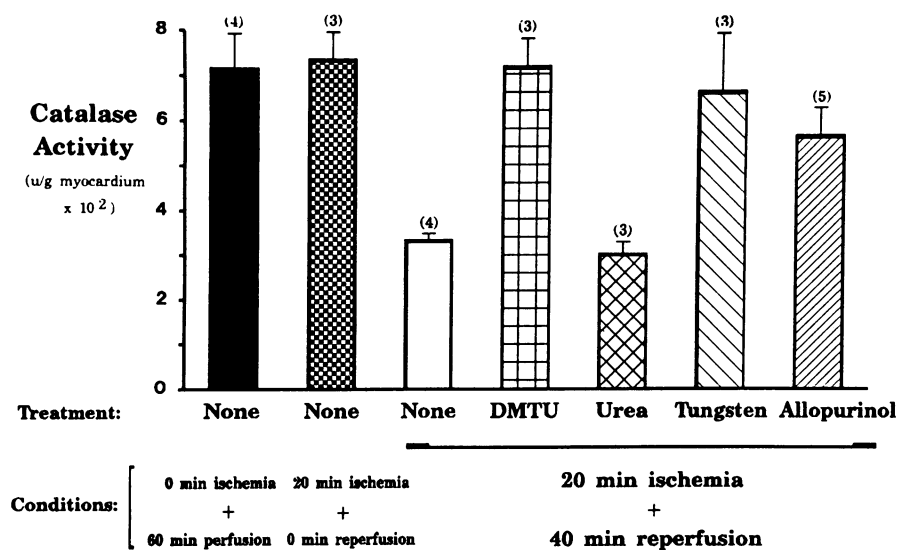
Figure 4. Myocardial catalase activities measured after ischemia and reperfusion in rats pretreated with aminotriazole (Units per gram myocardium $\times 10^2$). Myocardial catalase levels were the same ($P > 0.05$) in hearts perfused for 60 min without ischemia or hearts subjected to ischemia for 20 min without reperfusion. However, catalase activities were less ($P < 0.05$) in hearts subjected to ischemia for 20 min and then reperfusion for 40 min than in hearts studied after perfusion for 60 min without ischemia or after ischemia for 20 min without reperfusion. In contrast, after ischemia for 20 min and reperfusion for 40 min, hearts treated with DMTU simultaneously with reperfusion had catalase activities that were increased ($P < 0.05$) compared with untreated or urea-treated hearts. Similarly, after ischemia for 20 min and reperfusion for 40 min, hearts from rats that had been treated with tungsten or allopurinol had catalase activities which were increased ($P < 0.05$) compared with levels found in untreated hearts. Moreover, after ischemia for 20 min and reperfusion for 40 min, catalase activities were the same ($P > 0.05$) in DMTU-, tungsten-, or allopurinol-treated hearts compared with hearts subjected to perfusion for 60 min without ischemia or ischemia for 20 min without reperfusion. Each bar is the mean±1 SE of the number of determinations shown within the parentheses.

requires neutrophil myeloperoxidase (18) and essentially no neutrophils are present in the isolated Krebs-Henseleit perfused heart. Thus, our findings suggest that H_2O_2 is made during reperfusion and that H_2O_2 contributes either directly or indirectly to the development of cardiac dysfunction after ischemia.

Our study also indicates that XO generates H_2O_2 during reperfusion of ischemic rat hearts. Parallel decreases in myocardial XO activity, ventricular dysfunction, and H_2O_2 generation occurred in tungsten- or allopurinol-treated rats subjected to ischemia and then reperfusion. Tungsten is a relatively specific inhibitor that inactivates xanthine, aldehyde, and sulfite oxidases by interfering with their molybdenum-dependent active site (7–9). By comparison, allopurinol and its metabolite oxipurinol are potent, specific competitive inhibitors of XO (10), but not sulfite or aldehyde oxidases. Accordingly, measurable inhibition of XO by either tungsten or allopurinol treatment, in conjunction with improved function and lessened myocardial H_2O_2 generation, focuses on XO as the predominant source of H_2O_2 production during reperfusion after ischemia. Furthermore, because isolated hearts contain virtually no circulating blood elements, participation of O_2 metabolites from neutrophils, platelets, or other blood sources is unlikely.

Finally, DMTU, tungsten, or allopurinol treatment prevented H_2O_2-dependent decreases in catalase after reperfusion, but did not completely eradicate ventricular dysfunction after reperfusion. Thus, cardiac dysfunction following reperfusion after ischemia may also depend on mechanisms that are independent of processes involving H_2O_2 made by XO.

Acknowledgments

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