Protective Effects of Topiroxostat on an Ischemia-Reperfusion Model of Rat Hearts

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Background: Ischemia/reperfusion (I/R) injury triggers cardiac dysfunctions via creating reactive oxygen species (ROS). Because xanthine oxidase (XO) is one of the major enzymes that generate ROS, inhibition of XO is expected to suppress ROS-induced I/R injury. However, it remains unclear whether XO inhibition really yields cardioprotection during I/R. The protective effects of the XO inhibitors, topiroxostat and allopurinol, on cardiac I/R injury were evaluated.

Methods and Results: Using isolated rat hearts, ventricular functions, occurrence of arrhythmias, XO activities and thiobarbituric acid reactive substances (TBARS) productions and myocardial levels of adenine nucleotides before and after I/R, and cardiomyocyte death markers during reperfusion, were evaluated. Topiroxostat prevented left ventricular dysfunctions and facilitated recovery from arrhythmias during I/R. Allopurinol and the antioxidant, N-acetylcysteine (NAC), exhibited similar effects at higher concentrations. Topiroxostat inhibited myocardial XO activities and TBARS productions after I/R. I/R decreased myocardial levels of ATP, ADP and AMP, but increased that of xanthine. While topiroxostat, allopurinol or NAC did not change myocardial levels of ATP, ADP or AMP after I/R, all of the agents decreased the level of xanthine. They also decreased releases of CPK and LDH during reperfusion.

Conclusions: Topiroxostat showed protective effects against I/R injury with higher potency than allopurinol or NAC. It dramatically inhibited XO activity and TBARS production, suggesting suppression of ROS generation.

Key Words: Ischemia-reperfusion injury; Rat heart; Reactive oxygen species (ROS); Topiroxostat; Xanthine oxidase

Ischemic reperfusion (I/R) injury is the major cause of cardiac dysfunctions in thrombolytic therapy, percutaneous coronary intervention and coronary bypass surgery in patients with acute myocardial infarction.[1] I/R injury causes cardiac dysfunctions and lethal arrhythmias.[2] Reactive oxygen species (ROS)[3] play important roles in the pathophysiology of I/R injury; excessive ROS leads to loss of mitochondrial membrane potential[4] and release of cytochrome C, which triggers cellular apoptosis. ROS is generated by xanthine oxidase (XO),[5] which can be converted from xanthine dehydrogenase (XDH) via proteolysis during ischemia.[6,7] While XDH is responsible for production of urate from hypoxanthine and xanthine during normoxic conditions, XO generates ROS under not only hypoxic conditions, but also I/R conditions through converting hypoxanthine and xanthine degraded from cellular ATP to urate. Thus, inhibition of XO might reduce ROS production, protect cardiac mitochondria from oxidative damage, and attenuate myocardial I/R injury. Since the introduction of the concept of I/R injury,[8] several lines of evidence support the protective role of XO inhibitors against ischemic damages of the heart. Allopurinol, an authentic XDH and XO inhibitor, is frequently used in the clinical setting for treating hyperuricemia and gout. Manning and Hearse reported that the size of infarction and I/R-induced arrhythmias were reduced by the authentic XO inhibitor,

allopurinol, in rats, rabbits, canines, and pigs.[9] However, allopurinol is a relatively weak XO inhibitor and exerted several off-target effects,[10] which are attributable to its purine and pyrimidine motifs.[11] Thus, it remains unknown whether inhibition of XO could be directly related to cardioprotective actions of allopurinol.

Topiroxostat, a non-purine selective XO inhibitor, is used as a therapeutic agent for hyperuricemia and gout in Japan.[12] Topiroxostat binds to the catalytic sites of XO and XDH, with its adverse effects being less than those of allopurinol.[12] In the present study, we examined the preventive effects of topiroxostat, as well as allopurinol and the antioxidant, N-acetylcysteine (NAC), on I/R-induced cardiac dysfunctions and arrhythmias in isolated rat hearts.

Methods

Isolation and Coronary Perfusion of Rat Hearts
Male Lewis rats (300–350 g, 12-weeks-old) were used.[13] Animals were handled in strict accordance with the Tottori University Guide for the Care and Use of Laboratory Animals. Isolation and coronary perfusion of hearts were conducted as previously described.[14] In brief, after peritoneal injection of heparin (1,000 IU/kg; Shimizu Seiyaku, Shimizu, Japan), rats were anaesthetized by inhalation of isoflurane (3–5%; DS Pharma Animal Health, Osaka, Japan). The heart was rapidly excised, and the aorta was cannulated for retrograde perfusion of coronary arteries with a modified Langendorff perfusion system (model 7523-40; Masterflex, Barrington, IL, USA) at 37°C. Throughout the experiment, the coronary perfusion pressure (CPP) was held at 80 mmHg by adjusting the coronary perfusion flow of modified Tyrode's solution [(in mmol/L) NaCl 144, KCl 5, CaCl₂ 1.5, MgCl₂ 0.9, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 6, and glucose 5; pH 7.4 with NaOH] equilibrated with 100% oxygen. The perfusate was not recirculated.

Ventricular function was assessed by measuring the left ventricular pressure (LVP) with a fluid-filled latex balloon inserted into the left ventricle (LV) through the mitral valves and inflated to give a LV end-diastolic pressure (LVEDP) of 5–7 mmHg. The transducer was connected to a PowerLab/8SP (AD Instruments, Castle Hill, NSW, Australia), and LVP and CPP were measured.

Experimental Protocol for I/R and Administration of Drugs
After 30-min perfusion of the normal Tyrode's solution to allow stabilization of LVP and CPP, hearts were subjected to global ischemia for 45 min by stopping coronary perfusion, followed by reperfusion for 30 min. Topiroxostat (0.3, 3 and 30 μmol/L; Sigma), allopurinol (7, 70 and 1,000 μmol/L; Sigma), or NAC (500 μmol/L; Sigma) were dissolved in Tyrode's solution. Each drug was applied for 10 min before ischemia and for 30 min during reperfusion. In certain experiments, topiroxostat at 3 μmol/L was applied only for 10 min prior to ischemia or only during 30-min reperfusion.

Measurements of LV Functions and Arrhythmias During I/R
We continuously measured the maximum of LVP (LVP$_{max}$), LVEDP, LV developing pressure, and maximum rates of LVP rise (dP/dt$_{max}$) and fall (dP/dt$_{min}$) throughout the experiment. Heart rate was kept at 240 beats/min using a pacing generator (model SEN3201; Nihon Kohden, Tokyo, Japan) as previously described.[15] Ventricular arrhythmias

during reperfusion were evaluated using an electrocardiogram connected to a PowerLab/8SP. The recovery time was determined as the time when I/R-induced arrhythmias disappear after the beginning of reperfusion.

Measurement of XO Activity
XO activity was measured as previously described.[13] Briefly, frozen myocardium that was immediately collected after 30-min reperfusion was homogenized in ice-cold 50 mmol/L potassium phosphate buffer (pH 7.4) containing 1.0 mmol/L EDTA and a protease inhibitor cocktail. The mixture was centrifuged at 20,000 g for 20 min at 4°C. Supernatants were subjected to the following experiment. An XO substrate, pterin (Sigma) at 50 μmol/L, was added to the phosphate buffer (pH 7.4) and then the enzyme reaction was started by adding each supernatant. XO activity was measured by a spectrofluorometer (model infinite F500; TECAN, Kanagawa, Japan) with excitation at 345 nm and emission at 390 nm for 60 min. To measure both XO and XDH activities, the above reaction was carried out in the absence and presence of 20 μmol/L NAD⁺. The activity of XO was calculated from the standard curve of authentic XO (butter milk, Calbiochem) and was expressed as "μU/mg wet tissue".

Measurement of Thiobarbituric Acid Reactive Substances (TBARS)
Lipid peroxidation in the myocardial tissue was determined by assessing the level of TBARS. The TBARS assay was carried out using a kit (NWK-MDA01; Northwest Life Science Specialties LLC, Vancouver, Canada) according to the manufacturer's instructions. Briefly, the supernatant that was prepared for the XO activity measurement described above was mixed with butylated hydroxytoluene that was solved in ethanol, followed by the addition of phosphoric acid and thiobarbituric acid (TBA). The mixture was placed in an incubator for 60 min at 60°C, and then was centrifuged at 10,000 g for 2 min using a high-speed micro centrifuge (model himac CF16RN; HITACHI, Tokyo, Japan). The absorbance of the supernatant was measured against a reference blank at 532 nm. The concentration of TBARS was calculated from the standard curve of lipid peroxidation and was expressed as "pmol/mg wet tissue".

Measurements of Adenine Nucleotides and Purine Metabolites in Myocardium
Myocardium was collected after 30-min reperfusion and subjected to assays. Amounts of adenine nucleotides and purine metabolites were measured as previously described.[13] In brief, frozen myocardium was homogenized (50% wt/vol) in 10% perchloric acid (PCA) and held on ice for 30 min, followed by the addition of 5% PCA. The homogenates were centrifuged at 10,000 g for 10 min at 4°C. The supernatants were combined and neutralized by the addition of KOH. The final volume was adjusted to 50 mL and filtered through a 0.45 μm Millipore membrane filter (Merck Japan, Tokyo, Japan). The amount of purine metabolites in myocardium was determined by HPLC (L6000 pump, L4000 UV Detector; HITACHI, Tokyo, Japan). To separate nucleotides (adenosine triphosphate [ATP], adenosine monophosphate [AMP], inosine, hypoxanthine, xanthine) from nucleosides, we used a mobile phase buffer containing 150 mmol/L NaH₂PO₄ with pH adjusted to 4.3. Adenosine diphosphate (ADP) and inosine monophosphate (IMP)

Table 1. Effects of 10-min Treatment With Topiroxostat, Allopurinol and NAC on the Cardiac Functions Before I/R

	Control	Topiroxostat (µmol/L)		
		0.3	3	30
	n=6	n=5	n=6	n=6
LVP$_{max}$ (mmHg)	94.8±3.5	100.8±4.7	93.9±4.6	101.1±6.7
LVEDP (mmHg)	5.5±0.5	6.6±0.3	6.4±0.5	5.8±0.4
Developing pressure (mmHg)	89.3±3.9	94.2±4.7	87.5±5.0	95.3±6.8
dP/dt$_{max}$ (mmHg/s)	2,058.6±184.8	2,530.3±125.8	2,201.5±148.3	2,237.9±247.4
dP/dt$_{min}$ (mmHg/s)	−1,610.9±101.6	−1,804.6±56.8	−1,626.5±172.9	−2,014.7±274.2

	Allopurinol (µmol/L)			NAC (µmol/L)
	7	70	1,000	500
	n=6	n=6	n=6	n=6
LVP$_{max}$ (mmHg)	90.2±2.3	105.2±4.6	105.2±5.2	104.8±6.6
LVEDP (mmHg)	7.1±0.3	5.6±0.4	6.2±0.4	6.4±0.4
Developing pressure (mmHg)	83.1±2.6	99.6±4.6	99.0±4.9	98.4±6.3
dP/dt$_{max}$ (mmHg/s)	2,307.1±97.6	2,550.2±155.0	2,097.5±216.1	2,112.3±204.0
dP/dt$_{min}$ (mmHg/s)	−1,587.4±83.0	−1,805.1±57.9	−1,400.1±92.4	−1,470.6±109.9

LVP$_{max}$, peak left ventricular pressure (LVP); LVEDP, left ventricular end-diastolic pressure; dP/dt$_{max}$, maximum rate of rise of LVP; dP/dt$_{min}$, maximum rate of fall of LVP (minimum of the first derivative of LVP during diastole). I/R, ischemia/reperfusion; NAC, N-acetylcysteine.

were then separated using the buffer containing 150 mmol/L NaH$_2$PO$_4$ with pH adjusted to 2.5. All buffers were filtered through 0.45-µm Millipore filters. Nucleotides and nucleosides were identified by comparing their retention times with those of standard samples (ATP, ADP, AMP, IMP, inosine, hypoxanthine, and xanthine [all from Sigma]), and a concentration of each sample was determined on the basis of the peak area using external standards. The amount of purine metabolites in myocardium was expressed as "µmol/g wet tissue".

Measurements of CPK and LDH as Cardiomyocyte Death Markers

To quantify the release of CPK and LDH as cardiomyocytes death markers, the perfusate sample (~0.5 mL) released from coronary perfused hearts was collected at 0.5, 1.0, 1.5, 2.5, 5, 10, 15, 20, 25 and 30 min after the beginning of reperfusion. Both CPK and LDH concentrations in the samples (10 µL each) were determined using an auto-analyzer (model DRI-CHEM7000v; FUJIFILM, Tokyo, Japan).

Statistical Analysis

Comparisons among multiple groups, in the cardiac function, XO activity, concentrations of CPK and LDH, and amounts of myocardium adenine nucleotides and purine metabolites, were determined by one-way analysis of variance with the Tukey-Kramer test. Comparisons of the cardiac functions before and after I/R in each group, and of myocardium TBARS values between I/R (control) and other groups were performed by using an unpaired Student's t-test as appropriate. All data are expressed as the mean±S.E.M.; P<0.05 was considered statistically significant.

Results

Effects of Topiroxostat, Allopurinol and NAC on Cardiac Functions Before Ischemia

The LVP$_{max}$, LVEDP, LV developed pressure, dP/dt$_{max}$ and dP/dt$_{min}$ were determined just before ischemia in the absence of any drugs or in the presence of topiroxostat, allopurinol or NAC (**Table 1**). There were no significant differences in LVP$_{max}$, LVEDP, LV developed pressure, dP/dt$_{max}$ or dP/dt$_{min}$ between the control group and any of the drug-treated groups.

Effects of Topiroxostat, Allopurinol and NAC on I/R-Induced Cardiac Dysfunctions

Figure 1A shows effects of the three agents on cardiac functions before and after I/R. There was no significant difference in LVP$_{max}$ before and after I/R in the control group or in those treated with one of the agents at indicated concentrations, except for the 1,000 µmol/L allopurinol-treated group in which LVP$_{max}$ was significantly higher after I/R than before I/R (**Figure 1A-a**). LVEDP increased after I/R in all groups; however, the increments of LVEDP by I/R were smaller in the groups treated with the agents than in the control group (**Figure 1A-b**). LVEDP after I/R was particularly low in the 3 µmol/L topiroxostat-, 1,000 µmol/L allopurinol- and 500 µmol/L NAC-treated groups. The I/R-induced decreases in LV developing pressure were smaller in the groups treated with the agents than in the control group, with no significant decreases for topiroxostat at 0.3 and 3 µmol/L, allopurinol at 1,000 µmol/L, and NAC at 500 µmol/L (**Figure 1A-c**). LV developing pressure after I/R was significantly higher in the 1,000 µmol/L allopurinol- and 500 µmol/L NAC-treated groups than in the control group. **Figure 1A-d** and **1A-e** show the dP/dt$_{max}$ and dP/dt$_{min}$, respectively. In the control group, dP/dt$_{max}$ and |dP/dt$_{min}$| were significantly decreased after I/R. However, the I/R-induced decreases in dP/dt$_{max}$ and |dP/dt$_{min}$| were
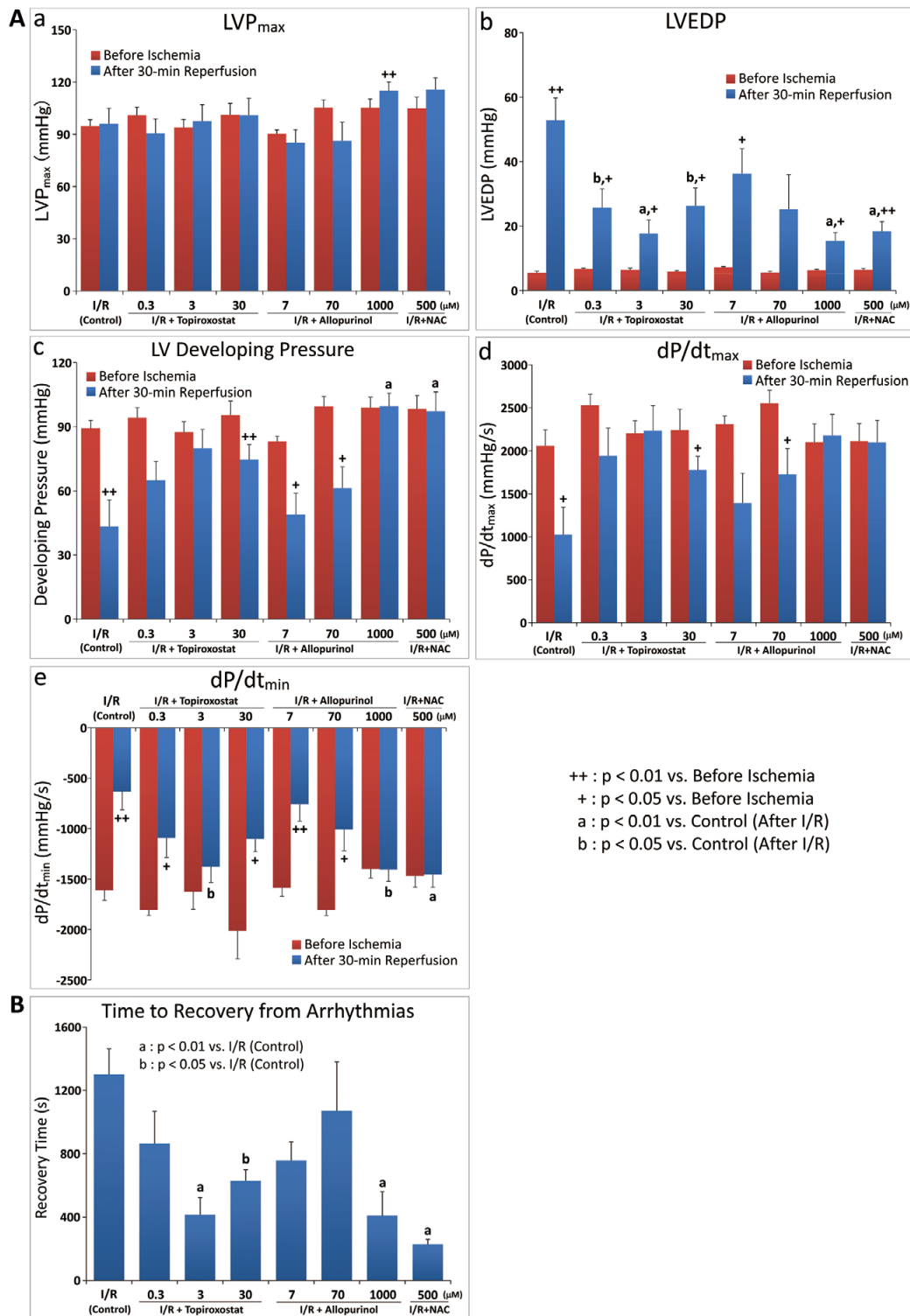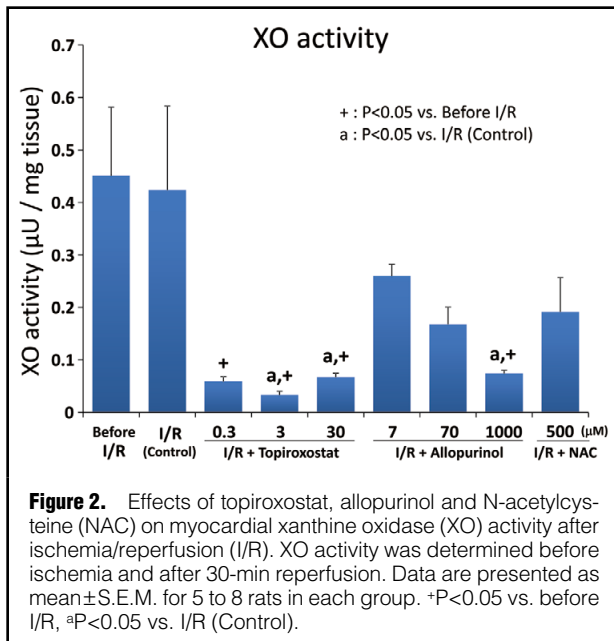
Figure 1. Effects of topiroxostat, allopurinol and N-acetylcysteine (NAC) on ischemia/reperfusion (I/R)-induced left ventricle (LV) dysfunctions and arrhythmias. (**A**) Effects of the agents at indicated concentrations (μmol/L) on the maximum of left ventricular pressure (LVP_{max}) (**a**), LV end-diastolic pressure (LVEDP) (**b**), LV developing pressure (**c**), maximum rate of rise of LVP (dP/dt_{max}) (**d**) and maximum rate of fall of LVP (dP/dt_{min}) (**e**) just before ischemia (red bars) and after 30-min reperfusion (blue bars). Data are presented as mean±S.E.M for 5 to 8 rats in each group. [++]$P<0.01$ vs. before ischemia, [+]$P<0.05$ vs. before ischemia, [a]$P<0.01$ vs. Control (after 30-min reperfusion), [b]$P<0.05$ vs. Control (after 30-min reperfusion). (**B**) Effects on the time to recovery from arrhythmias during reperfusion. The recovery time was determined as the time when I/R-induced arrhythmias disappear after the beginning of reperfusion. Data are presented as mean±S.E.M. for 5 to 8 rats in each group. [a]$P<0.01$ vs. I/R (Control), [b]$P<0.05$ vs. I/R (Control).

Figure 2. Effects of topiroxostat, allopurinol and N-acetylcysteine (NAC) on myocardial xanthine oxidase (XO) activity after ischemia/reperfusion (I/R). XO activity was determined before ischemia and after 30-min reperfusion. Data are presented as mean±S.E.M. for 5 to 8 rats in each group. +P<0.05 vs. before I/R, aP<0.05 vs. I/R (Control).

Figure 3. Effects of topiroxostat, allopurinol and N-acetylcysteine (NAC) on the level of myocardial thiobarbituric acid reactive substances (TBARS) after ischemia/reperfusion (I/R). The amount of TBARS in myocardial tissues was determined before ischemia and after 30-min reperfusion. Data are presented as mean±S.E.M. for 3 to 4 rats in each group. aP<0.01 vs. I/R (Control), bP<0.05 vs. I/R (Control).

smaller in the presence of the agents. There were no significant decreases in dP/dt$_{max}$ for topiroxostat at 0.3 and 3μmol/L, allopurinol at 7 and 1,000μmol/L, or NAC at 500μmol/L; no significant changes were found in dP/dt$_{min}$ with 3μmol/L topiroxostat, 1,000μmol/L allopurinol, or 500μmol/L NAC. After I/R, |dP/dt$_{min}$| values were significantly higher in the presence of 3μmol/L topiroxostat, 1,000μmol/L allopurinol, and 500μmol/L NAC, while dP/dt$_{max}$ showed no difference.

Effects of Topiroxostat, Allopurinol and NAC on the Time to Recovery From I/R Arrhythmias
As I/R of the heart is well-known to induce arrhythmias, we studied the effects of topiroxostat, allopurinol and NAC on ventricular arrhythmias induced by I/R. **Figure 1B** shows the time to recovery from arrhythmias in the control, topiroxostat, allopurinol and NAC groups during reperfusion. Reperfusion immediately caused arrhythmias such as ventricular tachycardia. The recovery time was significantly shorter with topiroxostat at 3 and 30μmol/L, allopurinol at 1,000μmol/L, and NAC at 500μmol/L.

Effects of Topiroxostat, Allopurinol and NAC on Myocardial XO Activities After I/R
In order to determine whether XO activity is increased by I/R and how I/R-induced changes in XO activity are affected by topiroxostat or allopurinol, we measured XO activity in the myocardium. **Figure 2** shows myocardial XO activity before and after I/R. I/R did not change the myocardial XO activity in the control group. Myocardial XO activity after I/R was significantly inhibited by topiroxostat at 3 and 30μmol/L and allopurinol at 1,000μmol/L, but not by allopurinol at the lower concentrations or NAC at 500μmol/L, in comparison to the control values before and after I/R. Because XO is converted from XDH, we also determined the ratio of XO activity to the sum of XO and XDH activities; the ratio of XO activity to the total (XO+XDH) activity was 0.61±0.06 before I/R and 0.59±0.04 after I/R.
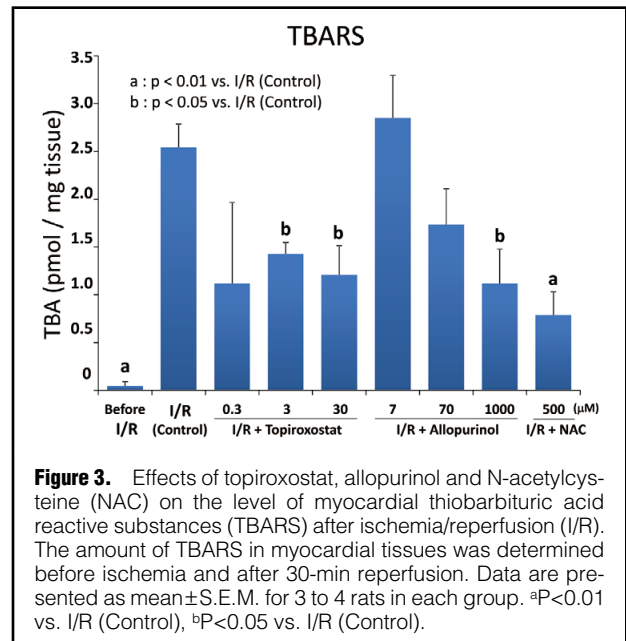
Effects of Topiroxostat, Allopurinol and NAC on Myocardial TBARS After I/R
Lipid peroxidation products, TBARS, were measured as a marker of oxidative injury. **Figure 3** shows productions of myocardial TBARS after I/R in the absence and presence of topiroxostat, allopurinol or NAC. TBARS dramatically increased after I/R in the control. Topiroxostat at 3 and 30μmol/L, as well as allopurinol at 1,000μmol/L and NAC at 500μmol/L, significantly reduced myocardial TBARS productions during I/R.

Effects of Topiroxostat, Allopurinol and NAC on Myocardial Adenine Nucleotides After I/R
Figure 4 shows the amounts of myocardial ATP, ADP, AMP, IMP, inosine, hypoxanthine and xanthine before and after I/R. The levels of myocardial ATP, ADP and AMP were significantly decreased by I/R (**Figure 4A–C**). There was no significant difference in ATP, ADP or AMP levels after I/R between the control group and the topiroxostat, allopurinol or NAC groups. The level of myocardial xanthine was significantly increased by I/R, while myocardial IMP, inosine and hypoxanthine tended to increase with no statistical significance. There was no significant difference in the levels of myocardial IMP, inosine or hypoxanthine after I/R between the control group and the topiroxostat, allopurinol or NAC groups. The levels of myocardial xanthine were significantly lower in the groups treated with topiroxostat at 3 and 30μmol/L, allopurinol at 1,000μmol/L, and NAC at 500μmol/L than in the control group.

Effects of Topiroxostat, Allopurinol and NAC on I/R-Induced Cardiomyocyte Death
We further examined the protective effects of topiroxostat, allopurinol, and NAC against I/R-induced cardiomyocyte death by measuring CPK and LDH as cardiomyocyte death markers in the perfusate. As shown in **Figure 5 (A and C)**, we determined time-dependent changes in the levels of CPK and LDH released from hearts at 0.5, 1.0, 1.5,
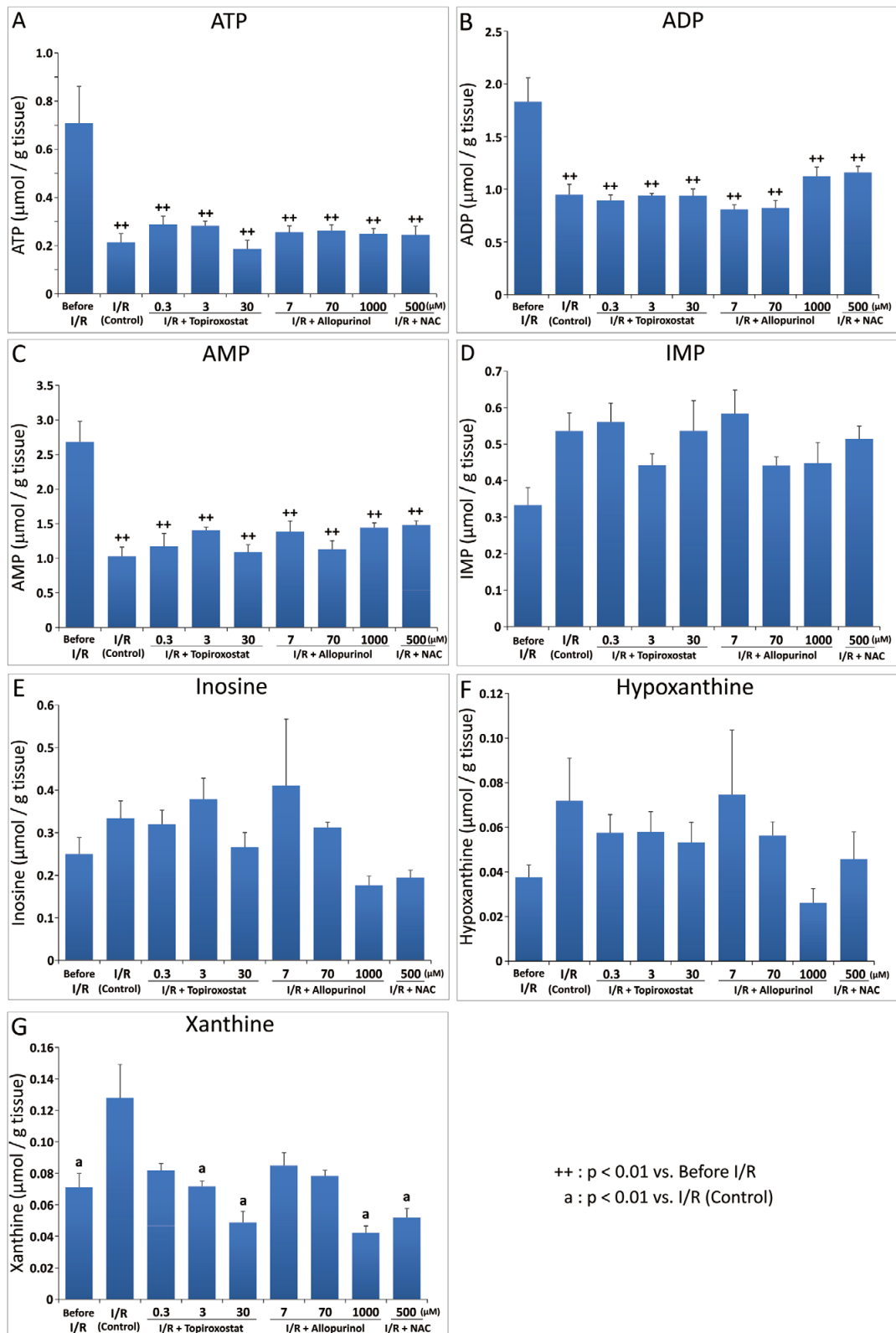
Figure 4.   Effects of topiroxostat, allopurinol and N-acetylcysteine (NAC) on the amounts of adenine nucleotides after ischemia/reperfusion (I/R). The amounts of myocardial adenosine triphosphate (ATP) (**A**), adenosine diphosphate (ADP) (**B**), adenosine monophosphate (AMP) (**C**), inosine monophosphate (IMP) (**D**), inosine (**E**), hypoxanthine (**F**) and xanthine (**G**) were determined before ischemia and after 30-min reperfusion in the absence and presence of topiroxostat, allopurinol or NAC. Data are presented as mean±S.E.M. for 5 to 8 rats in each group. ++P<0.01 vs. before I/R, aP<0.01 vs. I/R (Control).
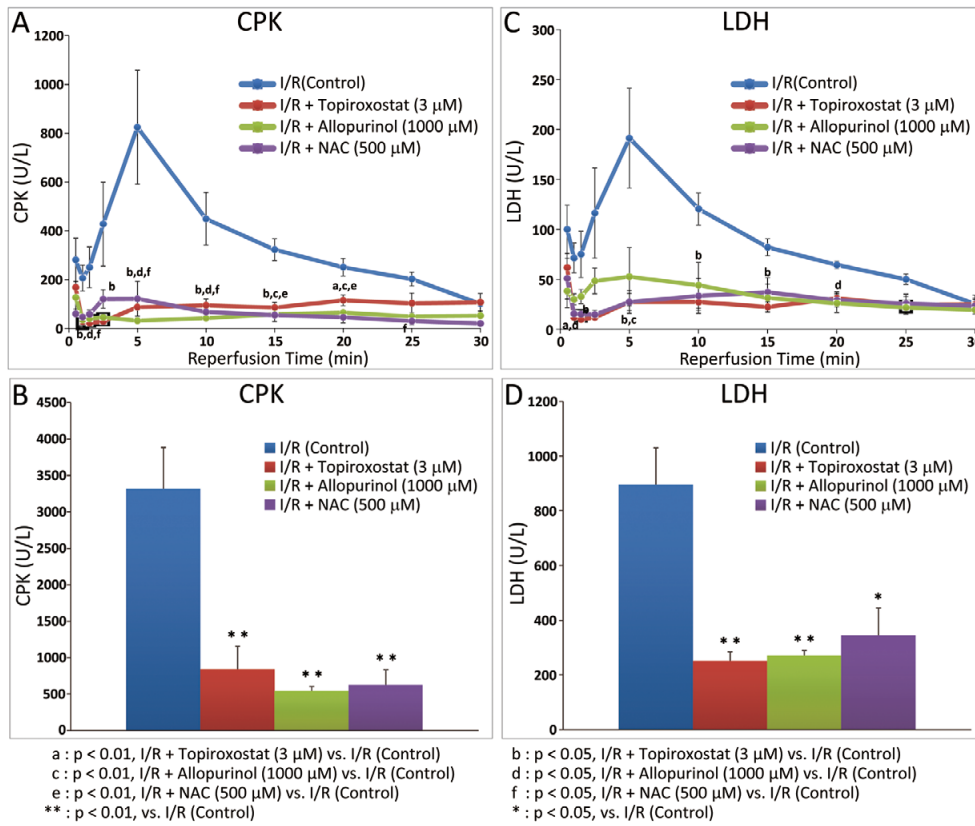
Figure 5. Effects of topiroxostat, allopurinol and N-acetylcysteine (NAC) on the releases of CPK and LDH from the perfused heart during reperfusion. (**A,C**) Time-dependent changes in the concentrations of CPK (**A**) and LDH (**C**) in the perfusate collected from perfused hearts during reperfusion in the absence and presence of topiroxostat, allopurinol or NAC. Data are presented as mean±S.E.M. for 3 to 4 rats in each group. [a]P<0.01, ischemia/reperfusion (I/R)+topiroxostat (3 μmol/L) vs. I/R (Control); [b]P<0.05, I/R+topiroxostat (3 μmol/L) vs. I/R (Control); [c]P<0.01, I/R+allopurinol (1,000 μmol/L) vs. I/R (Control); [d]P<0.05, I/R+allopurinol (1,000 μmol/L) vs. I/R (Control); [e]P<0.01, I/R+NAC (500 μmol/L) vs. I/R (Control); [f]P<0.05, I/R+NAC (500 μmol/L) vs. I/R (Control). (**B,D**) Total amounts of the released CPK (**B**) and LDH (**D**) during reperfusion determined in the absence and presence of topiroxostat, allopurinol or NAC. **P<0.01 vs. I/R (Control), *P<0.05 vs. I/R (Control).

Table 2. Left Ventricle Functions Determined Just Before Ischemia in the Control Group and the Groups With Topiroxostat Administered Throughout the Experiment, Only for 10 min Prior to Ischemia, and Only During 30-min Reperfusion

	I/R (Control)	I/R+Topiroxostat (3 μmol/L) before ischemia and during reperfusion	I/R+Topiroxostat (3 μmol/L) only for 10 min before ischemia	I/R+Topiroxostat (3 μmol/L) only for 30 min during reperfusion
	n=5	n=5	n=4	n=4
LVP$_{max}$ (mmHg)	96.9±3.9	96.0±2.3	94.2±3.8	90.2±7.8
LVEDP (mmHg)	5.1±0.5	5.1±0.7	4.7±0.9	5.3±0.3
Developing pressure (mmHg)	91.08±4.2	90.8±2.9	89.5±4.5	84.8±7.9
dP/dt$_{max}$ (mmHg/s)	2,229.9±115.4	2,721.8±160.0	2,998.9±81.3	2,784.5±217.8
dP/dt$_{min}$ (mmHg/s)	−1,557.1±79.5	−1,598.6±118.5	−1,742.4±190.9	−1,615.0±312.4

Abbreviations as in Table 1.

2.5, 5, 10, 15, 20, 25 and 30 min after the beginning of reperfusion. Topiroxostat (3 μmol/L), allopurinol (1,000 μmol/L) and NAC (500 μmol/L) significantly reduced the concentrations of CPK and LDH in the samples collected from hearts during reperfusion. As shown in **Figure 5 (B and D)**, we also measured the area under the time-dependent curves of CPK and LDH concentrations to estimate the

sum of the released CPK and LDH, and found that topiroxostat (3 μmol/L), allopurinol (1,000 μmol/L) and NAC (500 μmol/L) significantly reduced the CPK and LDH releases from hearts during reperfusion.
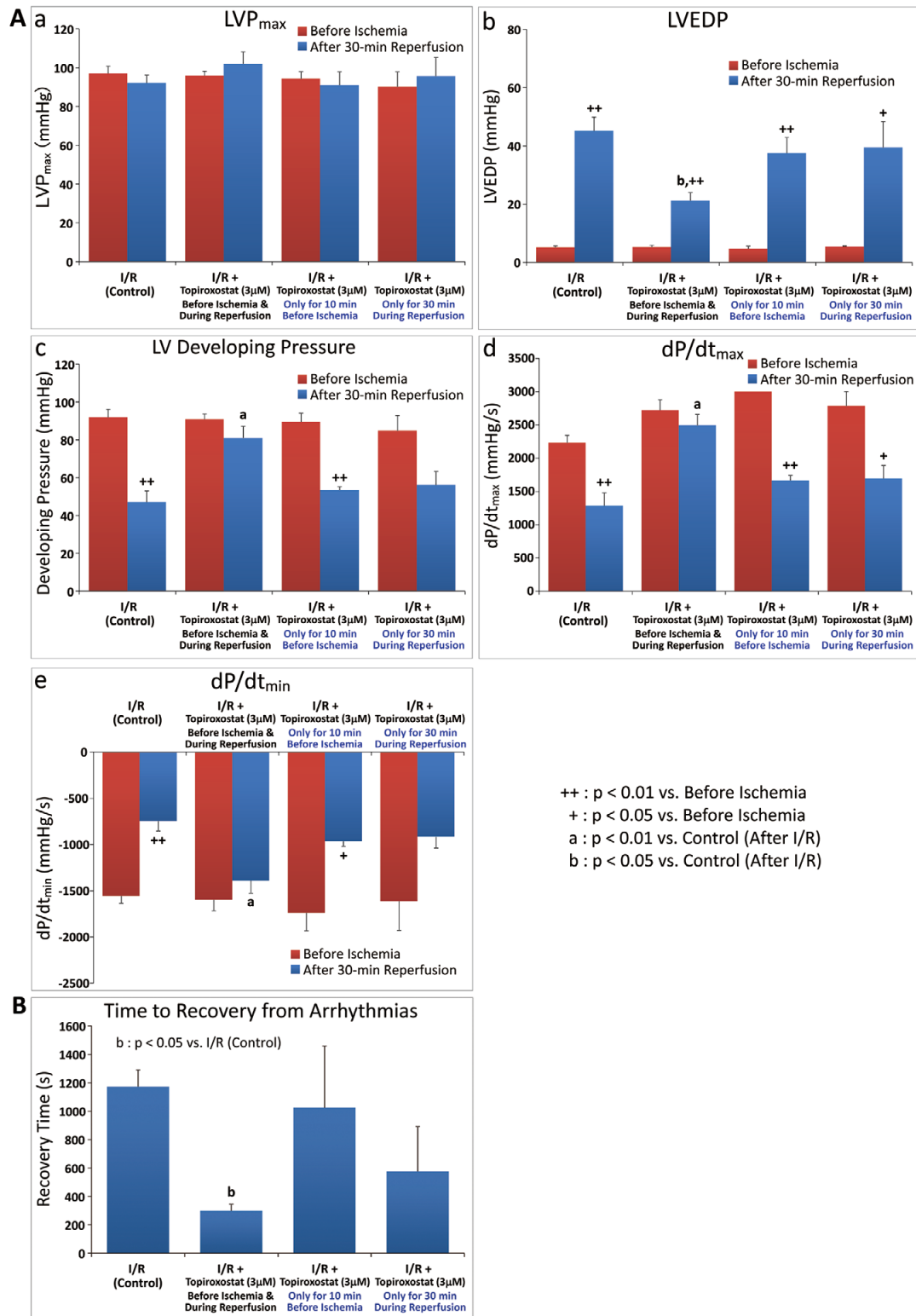
Figure 6. Effects of topiroxostat administered only for 10 min prior to ischemia or only during 30-min reperfusion on ischemia/reperfusion (I/R)-induced left ventricle (LV) dysfunctions and arrhythmias. (**A**) Effects on the maximum of left ventricular pressure (LVP$_{max}$) (**a**), LV end-diastolic pressure (LVEDP) (**b**), LV developing pressure (**c**), maximum rate of rise of LVP (dP/dt$_{max}$) (**d**), and maximum rate of fall of LVP (dP/dt$_{min}$) (**e**) before ischemia (red bars) and after 30-min reperfusion (blue bars). Data are presented as mean±S.E.M. for 4 to 5 rats in each group. [++]P<0.01 vs. before ischemia, [+]P<0.05 vs. before ischemia, [a]P<0.01 vs. Control (after 30-min reperfusion), [b]P<0.05 vs. Control (after 30-min reperfusion). (**B**) Effects on the time to recovery from arrhythmias during reperfusion. Data are presented as mean±S.E.M. for 4 to 5 rats in each group. [b]P<0.05 vs. I/R (Control).

Effects of Topiroxostat Administered Only Before Ischemia or Only During Reperfusion on I/R-Induced Cardiac Dysfunctions and Arrhythmias

Heart muscle can be protected from the injury during severe and prolonged ischemia by pretreatment with ischemia for a few minutes.[16] This ischemia preconditioning can be mimicked by pharmacological agents such as the potassium channel opener, diazoxide,[17] and the inhalation anesthetic, isoflurane.[18] Because the protective action against I/R injury of topiroxostat may also be due to pharmacological preconditioning, we tested the cardioprotective action of topiroxostat (3 μmol/L) against I/R injury in the different ways of its administration; that is, whether it is effective in preventing I/R-induced cardiac dysfunctions and arrhythmias when administered only for 10 min before ischemia or only during 30-min reperfusion. **Table 2** shows the cardiac functions right before ischemia of the control group and the groups treated with topiroxostat during the entire experimental period, only for 10 min prior to ischemia and only during 30-min reperfusion, demonstrating that there was no significant difference in cardiac functions before ischemia among the 4 groups. **Figure 6** shows the cardiac functions before and after I/R, and the time to recovery from arrhythmias during reperfusion in the 4 groups. Topiroxostat treatment during the entire experimental period significantly reduced LVEDP, and increased LV developing pressure, dP/dt_{max} and $|dP/dt_{min}|$ after 30-min reperfusion, although topiroxostat did not improve any parameters of cardiac functions when administered only prior to ischemia or only during reperfusion (**Figure 6A**). As shown in **Figure 6B**, the time to recovery from arrhythmias during reperfusion was significantly shortened by topiroxostat treatment during the entire experimental period, but not by the treatment only prior to ischemia or only during reperfusion.

Discussion

In the present study, we found that: (1) pretreatment with topiroxostat at a clinical concentration of 3 μmol/L alleviated the I/R-induced LV dysfunctions and shortened the duration of arrhythmias during reperfusion, with these actions of topiroxostat at the clinical concentration comparable with those of allopurinol at the concentration much higher than its clinical concentrations (**Figure 1**); (2) topiroxostat within the clinical concentration of 3 μmol/L inhibited myocardial XO activities along with a reduction of TBARS productions after I/R, while allopurinol exerted the same effects only at the highest concentration (**Figures 2 and 3**); (3) topiroxostat, allopurinol and NAC decreased the myocardial xanthine level after I/R without significant changes in myocardial ATP, ADP, AMP, IMP, inosine or hypoxanthine (**Figure 4**). Topiroxostat, allopurinol and NAC significantly decreased the releases of CPK and LDH from perfused hearts during reperfusion (**Figure 5**); and (4) topiroxostat did not show the protective action against I/R injury when administered only prior to ischemia or only during reperfusion (**Figure 6**).

Cardiac dysfunction induced by I/R is characterized by the elevation of LVEDP, reductions of LV developing pressure, dP/dt_{max} and $|dP/dt_{min}|$, and long-lasting arrhythmias. ROS determined by NMR was generated immediately (within 2 min) after I/R in our previous study.[13] In the present study, pretreatment with the antioxidant, NAC, improved cardiac functions after I/R with a reduction of myocardial TBARS, suggesting an involvement of ROS in the I/R injury characterized by the LV dysfunctions.

ROS is generated by various enzymes such as XO, NO synthase, cyclooxygenase, the mitochondrial electron transport chain enzyme, and NADPH oxidase.[5] Cardiac dysfunctions and arrhythmias after I/R are known to be attributable to ROS generated by XO. It is generally accepted that XO is present in human cardiac myocytes,[19] whereas immunochemistry of human tissues have revealed that XO is mainly localized in vascular endothelium and smooth muscle cells.[13] Cardiac ischemia causes the conversion of XDH to XO, subsequent increases in XO activity, and accumulation of its substrate,[6] leading to the production of ROS during the reperfusion stage.[20] The increase in XO activity has been proposed to be a key event in the generation of free radicals during I/R.[21,22] In the present study, XO activity or the ratio of XO activity to the sum of XO and XDH activities, was not affected by I/R. In contrast, myocardial xanthine significantly increased, and hypoxanthine tended to increase after I/R injury, suggesting that an increase in the substrates of XO is responsible for the generation of free radicals during I/R injury. It has been reported that pretreatment with allopurinol at 500 μmol/L directly suppresses ROS production and improves cardiac functions after I/R.[13] In the present study, pretreatment with topiroxostat at a clinical concentration of 3 μmol/L, as well as allopurinol at 1,000 μmol/L, alleviated cardiac dysfunctions after I/R. Taken together, our study suggests that XO-derived ROS can be involved in cardiac dysfunctions after I/R.

It is interesting that topiroxostat improved cardiac functions after I/R within a range of clinical concentrations (0.93–7.1 μmol/L), while allopurinol exerted significant effects only at 1,000 μmol/L, a concentration much higher than its clinical concentrations (7 μmol/L or less). Allopurinol is a relatively weak XO inhibitor in vitro, with IC_{50} values of 0.2–50 μmol/L.[11] In the present study, allopurinol at 1,000 μmol/L, but not at 70 μmol/L, inhibited myocardial XO activity and improved cardiac functions after I/R. Allopurinol has multiple actions such as direct free radical scavenging action,[19,23] copper chelation action,[10] inhibition of lipid peroxidation,[24] induction of heat shock factor expression,[25] Ca^{2+}-sensitizing action,[26] and antioxidant effects.[27] In contrast, topiroxostat is reported to show the mechanism-based and structure-based inhibition of XO without any inhibitory actions on other enzymes. Matsumoto et al[28] reported that topiroxostat competitively inhibited XO, with an IC_{50} value of 5.7 nmol/L; 2 orders of magnitude smaller than that of allopurinol. Pretreatment with topiroxostat at the clinical concentration significantly diminished cardiac dysfunctions and facilitated recovery from arrhythmias after I/R, with these actions accompanied by inhibition of myocardial XO. Thus, the protective effects of topiroxostat at the clinical concentration could be attributed to its inhibitory action on XO. Nonetheless, the protective effects of topiroxostat appeared to be greater at 3 μmol/L than at the higher concentration of 30 μmol/L, possibly reflecting other additional actions of the agent independent of that on XO.

It is well-known that I/R decreases the ATP level but increases ADP and AMP levels, resulting in a reduction of cardiac energy charge. Inhibition of converting hypoxanthine to xanthine and xanthine to uric acid by allopurinol was reported to result in accumulation of hypoxanthine and xanthine in the myocardium; this in turn activates

salvage from hypoxanthine to IMP, leading to preservation of adenine nucleotides, which can prevent post-ischemic LV dysfunctions.[7] This mechanism may contribute to cardiac protection by XO inhibitors. In the present study, however, either topiroxostat or allopurinol did not increase ATP, ADP or AMP, suggesting that the protective actions of topiroxostat or allopurinol on the cardiac function during I/R do not involve the preservation of adenine nucleotides. This discrepancy might be attributable to different experimental protocols such as the duration of ischemia. In the present study, effects of ischemia for 45 min followed by reperfusion for 30 min was examined, while effects of shorter durations of ischemia (10–15 min) were evaluated in previous studies.[29–31] Further studies are needed to clarify an involvement of energy preservation in the preventive effects of topiroxostat as well as allopurinol on myocardial dysfunctions after I/R.

Topiroxostat as well as allopurinol and NAC significantly reduced the release of CPK and LDH from perfused heats with I/R injury, suggesting that these agents prevent the cardiomyocyte death and exert the protective effects against I/R injury.

As described in the Results section, the ischemia preconditioning to prevent severe cardiac I/R injury[16] can be mimicked by pharmacological agents such as potassium channel activators and anesthetics. Therefore, it is interesting to clarify whether topiroxostat produces the pharmacological preconditioning against cardiac I/R injury. In the present study, topiroxostat administered only prior to ischemia or only during reperfusion did not show cardioprotective actions against I/R injury of the heart, suggesting that both pretreatment before ischemia and treatment during reperfusion are needed for topiroxostat to exert the preventive action on I/R injury. Topiroxostat administered before ischemia did not produce the pharmacological preconditioning against I/R injury.

Although precise mechanisms of the protective action of topiroxostat against I/R injury remain to be elucidated, its clinical benefits are obvious. In patients suffering from chronic heart failure, allopurinol improved endothelial functions and survival rates;[32] in this study, however, allopurinol was found to show beneficial effects only at the highest concentration of 1,000 μmol/L, which is much higher than its clinical concentrations. Thus, a more potent XO inhibitor is desirable. The minimum and maximum doses of topiroxostat prescribed for patients with hyperuricemia and gout are 40 and 160 mg/day, respectively. According to the report by Sugiyama et al,[33] the minimum and maximum plasma concentrations of topiroxostat was estimated to be 0.93 and 7.1 μmol/L, respectively, which are reached by 20 and 180 mg/day oral administration. In the present study, topiroxostat at 3 μmol/L, within its clinical concentrations, improved cardiac functions and facilitated recovery from arrhythmias after I/R. Because topiroxostat is 2 orders of magnitude more potent in XO inhibition than allopurinol,[28] and showed the protective actions on cardiac functions after I/R at its clinical concentration of 3 μmol/L in the present study, topiroxostat is expected to exhibit beneficial effects in clinical setting. In conclusion, this is the first report demonstrating the preventive actions of topiroxostat at clinical concentrations on I/R-induced cardiac dysfunctions and arrhythmias.

Acknowledgments

Conflicts of Interest

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