Original Research

Tissue protective effect of xanthine oxidase inhibitor, polymer conjugate of (styrene–maleic acid copolymer) and (4-amino-6-hydroxypyrazolo[3,4-*d*]pyrimidine), on hepatic ischemia–reperfusion injury

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Abstract

The detrimental role of superoxide anion (O_2^-) has been well documented in the pathogenesis of ischemia–reperfusion (I/R) injury. Our and other studies suggested that one critical source of O_2^- generation may be xanthine oxidase (XO). We thus hypothesized that I/R injury could be protected by inhibiting XO activity, which would reduce the amount of O_2^- and hence reduce pathogenic consequences. Among various XO inhibitors, we previously found 4-amino-6-hydroxypyrazolo[3,4-*d*]pyrimidine (AHPP) exhibited potent XO inhibitory activity. Here, we report that the covalent conjugate of AHPP with amphipathic styrene–maleic acid copolymer (SMA-AHPP) showed protective effect against I/R-induced injury in a rat hepatic I/R model. Liver ischemia was induced by occluding both the portal vein and the hepatic artery for 30 min, and followed by reperfusion. SMA-AHPP was administered via the tail vein two hours before ischemia was initiated. A remarkable increase of liver enzymes in plasma (aspartate aminotransferase, AST; alanine aminotransferase, ALT and lactate dehydrogenase, LDH) was detected three hours after reperfusion, whereas prior injection of SMA-AHPP greatly suppressed this increase of AST, ALT and LDH. Moreover, induction of inflammatory cytokines, i.e. tumor necrosis factor-alpha (TNF-α), interleukin-12 (IL-12) and monocyte chemotactic protein-1 (MCP-1) by I/R were significantly inhibited by SMA-AHPP treatment. Accordingly, cytotoxic effect or apoptosis in the liver caused by I/R was clearly reduced by SMA-AHPP pretreatment. Furthermore, thiobarbituric acid-reactive substance assay showed a significant decrease of lipid peroxidation in rat liver after the administration of SMA-AHPP, which is parallel with the decreased XO activity after SMA-AHPP treatment, indicating the involvement of reactive oxygen species generated by XO. In addition, SMA-AHPP was found to bind to albumin, thus to exhibit prolonged *in vivo* (plasma) half-life. These results suggest that SMA-AHPP exerted a potent cytoprotective effect against I/R injury in rat liver, by inhibiting XO activity and the subsequent generation of O_2^-.

Keywords: xanthine oxidase, superoxide, ischemia/reperfusion injury, AHPP, styrene–maleic acid copolymer

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Introduction

Ischemia–reperfusion (I/R) injury is responsible for many fatal diseases such as cardiac infarct, cerebral ischemia or thrombus and limits the therapeutic effect of medical interventions such as organ transplantation;[1] therefore, it becomes a major contributor to the morbidity and mortality of many diseases. Ample reports have shown that reactive oxygen species (ROS), especially superoxide anion (O_2^-), is excessively produced, mostly from xanthine oxidase (XO) in many tissues upon the onset of I/R.[1,2] O_2^- is one of the crucial ROS with highly cytotoxic activity. O_2^- will be subsequently converted to hydrogen peroxide (H_2O_2) by superoxide dismutase in most normal tissues.[3] In the presence of

transition metals (e.g. Fe^{2+}), H_2O_2 will become hydroxyl radicals ($OH^{\cdot}$) that are more reactive than O_2^- or H_2O_2.[2,4–6] ROS can readily cross cellular membranes and cause oxidative damage to DNA, protein and lipid by its direct oxidation. More importantly, besides the direct toxicity of O_2^- and its derivatives, O_2^- reacts more rapidly with nitric oxide (NO), becoming a more toxic species peroxynitrite ($ONOO^-$), by which tissue injury will be exacerbated. Furthermore, NO, $ONOO^-$ and H_2O_2 can enhance the vascular permeability among other vascular mediators, and facilitate inflammatory cell infiltration and cell growth, or induce apoptosis.[7,8] In addition, consumption of NO by reacting with O_2^- as above in vascular luminal endothelial surface will result in vasoconstriction of vascular smooth muscle, and trigger neutrophil adherence and accumulation into the ischemic area, which would exacerbate reperfusion injury.[7,9,10] This situation is most typical during the onset of sickle cell thrombus formation.[11,12] Taken together, XO and O_2^- play a critical role in the pathogenesis of I/R injury.[1]

XO is widely distributed in various tissues, particularly in the intestine, lung and liver.[1] In normal healthy tissues, about 90% of the total activity of this enzyme is present as xanthine dehydrogenase (type D) (XD), which cannot transfer electrons to molecular oxygen to form O_2^-, but it reduces nicotinamide adenine dinucleotide (NAD^+).[13] However, interestingly and importantly, if the tissue is completely ischemic for a very short time, i.e. within 10 s for ileum, eight minutes for the heart and 30 min for the liver, spleen, lung and kidney, there is a rapid increase in XO (type O) activity that is converted from XD, which can use molecular oxygen instead of NAD^+ to generate O_2^-.[1,13] These facts further supported the crucial role of XO in the production of O_2^- following the I/R injury of tissues. As a consequence, potential involvement should be realized in O_2^- generation by XO, and thus XO is considered as the therapeutic target for I/R injury. Under these circumstances, the potential of XO inhibitors for this therapeutic target was examined by many researchers.[14–16]

Among various inhibitors of XO, 4-amino-6-hydroxypyrazolo[3,4-*d*]pyrimidine (AHPP) is one of the most potent inhibitors having apparent K_i values of 0.17 ± 0.02 μmol/L, which is much lower than those of commonly used XO inhibitors, such as allopurinol (0.50 ± 0.03 μmol/L) and alloxanthine (3.54 ± 1.12 μmol/L), suggesting the therapeutic potential of AHPP as an XO inhibitory agent for various diseases.[17] However, the water solubility of AHPP is very poor, which hindered its development as a therapeutic agent. To overcome this drawback, we designed and prepared a polymer-conjugated AHPP by use of a biocompatible amphipathic polymer, copoly(styrene–maleic acid) (SMA), resulting in a highly water-soluble molecule (SMA-AHPP) with similar XO inhibitory activity to free AHPP.[18] Intravenous injection of SMA-AHPP resulted in a prolonged therapeutic effect in spontaneously hypertensive rats, which is a model of primary hypertension caused by the increase of O_2^- and concomitant consumption of NO in vascular luminal surface.[18] In the present report, we describe the protective effect of SMA-AHPP on hepatic I/R injury in a rat model.

Materials and methods

Materials

AHPP was purchased form Wako Chemical Co (Osaka, Japan). SMA-AHPP was prepared in our laboratory as reported previously.[18] All other chemicals and reagents were from commercial sources.

Animals

Male Wistar rats, weight between 200 and 230 g, were obtained from Kyudo Inc (Kumamoto, Japan). All animals were maintained under standard conditions and were fed water and murine chow *ad libitum*. All experiments were carried out according to the guidelines of the Laboratory Protocol of Animal Handling, Faculty of Pharmaceutical Sciences, Sojo University.

Experimental protocol of hepatic I/R injury

The rats were fasted overnight before the experiment, but were allowed free access to water. Two hours before operation for I/R, saline or SMA-AHPP of different doses was injected via the tail vein. Animals were anesthetized with isoflurane during the operation by use of an anesthesia system (SF-B01, DS Pharma Biomedical Co, Osaka, Japan). After the abdomen was shaved and disinfected with 70% ethanol, a complete midline incision was made. The portal vein and hepatic artery were then exposed and cross-clamped for 30 min with a non-crushing microvascular clip. Subsequently, reperfusion was initiated by removing the clips, and then the abdomen was closed into two layers with 2-0 silk. Rats were kept under warming lamps until they awakened and became active.

Liver enzyme activity in serum

At scheduled times after reperfusion was initiated, rats were sacrificed under anesthesia, and whole blood was withdrawn from the inferior vena cava. Enzyme activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) in serum were determined by AutoAnalyzer system (Hitachi Ltd, Tokyo, Japan). Activities were expressed as international units per liter.

Enzyme-linked immunosorbent assay of tumor necrosis factor-alpha, interleukin-12 and monocyte chemotactic protein-1 in serum

Serum samples from I/R-treated rats were obtained as described above, and the tumor necrosis factor-alpha (TNF-α), interleukin-12 (IL-12) and monocyte chemotactic protein-1 (MCP-1) levels were quantified by using enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instruction. The ELISA kits for TNF-α and IL-12 were from Pierce Biotechnology Inc. (Rockford, IL, USA), and the MCP-1 kit was from Immuno-Biological Laboratories Co, Ltd (Takasaki-Shi, Gunma, Japan).

Preparation of liver tissue sections for histological examination, and detection of apoptosis

The rat liver was removed three hours after I/R and was cut into small tissue blocks ($3 \times 4 \times 5\ mm^3$). Then, the tissue blocks were fixed with 6% buffered neutral formalin solution and then embedded in paraffin as usual. Paraffin sections (6 μm thick) were prepared for each block, among which one section was for histological examination and one section was for detection of apoptosis as described below. The histological examination was performed after hematoxylin and eosin staining as usual.

In situ detection of apoptosis in the liver

Using the above paraffin slice section, *in vivo* induction of apoptosis by I/R with or without SMA-AHPP treatment was determined by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method,[19] with the *in situ* apoptosis detection kit (TACS; Trevigen Inc, Gaithersburg, MD, USA), according to the manufacturer's instruction. TUNEL-positive cells were counted in four different fields per sample, and were expressed per mm^2 of tissue section.

Expression of XO and antioxidative molecule heme oxygenase-1 in the liver tissues

XO mRNA expression was detected by reverse transcription polymerase chain reaction (RT-PCR). Briefly, total RNA from liver tissues was extracted by using Sepasol®-RNA I Super reagent (Nacalai Tesque Inc, Kyoto, Japan), according to the manufacturer's instruction. The nucleotide sequences of the oligonucleotide primers used for PCR of XO were as follows: forward, 5′-GCATGCCAGACCATACTGAA-3′ and reverse, 5′-AAATCCAGTTGCGGACAAAC-3′ to obtain a 120-bp XO cDNA. After an initial denaturing step at 94°C for two minutes, 38 PCR cycles were performed as follows: denaturing for one minute at 94°C, primer annealing for one minute at 56°C and DNA synthesis for one minute at 72°C. The mRNA for glyceraldehyde 3-phosphate dehydrogenase was examined as an inner control, whose primers were as follows: forward, 5′-TCCCAGACCCCATAACAACAG-3′ and reverse, 5′-TGAGGGTGCAGCGAACTTTA-3′, and PCR condition was as follows: denaturation at 95°C for 10 min, followed by 40 cycles of 10 s at 95°C, 15 s at 60°C and 20 s at 72°C. PCR products then underwent electrophoresis on ethidium bromide-stained 1% agarose gels.

The expression of heme oxygenase-1 (HO-1), which is a major antioxidative enzyme in liver especially upon I/R, was measured by Western blot assay. In brief, proteins of liver tissue were extracted by using tissue extraction reagent CelLytic™MT (Sigma, Saint Louis, MO, USA) according to the manufacturer's instruction. Total protein (50 μg each) in tissue homogenates was used for the Western blot assay, with monoclonal antibody of HO-1 (GTS-1, Takara Bio Inc, Otsu, Shiga, Japan).

Thiobarbituric acid-reactive substance assay

Oxidative cellular damage of the liver after I/R with or without SMA-AHPP treatment was further determined by assay of lipid peroxide formation via the thiobarbituric acid (TBA) reaction.[20] Three hours after I/R, the liver tissues of each group were obtained and weighed followed by homogenization and centrifugation. The thiobarbituric acid-reactive substance (TBARS) assay was then carried out. Briefly, tissue homogenates were first prepared in a ratio of 1 g of wet tissue to 9 mL of 1.15% KCl by using a Polytron homogenizer (Littau-Lucerne, Switzerland). Then, 0.1 mL of tissue homogenate was mixed with 0.2 mL of 8.1% sodium dodecyl sulfate, 1.5 mL of 20% acetic acid (pH 3.5) and 1.5 mL of 0.8% aqueous solution of TBA. The mixture was finally made up to 4 mL with distilled water, and incubated at 95°C for 60 min. After cooling with tap water, 1.0 mL of distilled water and 5.0 mL of the mixture of *n*-butanol and pyridine (15:1, v/v) were added, and the mixture was shaken vigorously. After centrifugation at 4000 rpm for 10 min, the absorbance of the organic layer (upper layer) was measured at 532 nm. The concentration of the product, malondialdehyde, was calculated using an extinction coefficient of 1.56×10^5/mol/L/cm of the control malondialdehyde.[21]

Determination of the activity of XO in rat liver tissues

Enzyme activity of XO was determined by quantifying the formation of uric acid from xanthine by measuring the increase in absorbance at 290 nm, ε_{max} of uric acid.[22] Three-hundred mg of liver tissue was homogenized with 1.2 mL of 0.05 mmol/L Tris-HCl (pH 7.5) at 2–4°C and centrifuged at 20,000*g* for 30 min. Then, 0.5 mL of the supernatant was incubated with 0.5 mL of 10 mmol/L xanthine (first dissolved in 0.25 mmol/L NaOH at the concentration of 0.1 mmol/L, then diluted to 10 mmol/L by the above-mentioned Tris-HCl buffer) for 30 min at room temperature (25°C). Then, 0.5 mL of 20% trichloroacetic acid was added to the assay mixture, mixed and centrifuged at 14,000 rpm for five minutes. The absorbance of the supernatant was read at 290 nm against a blank sample consisting of the same assay mixture but without the substrate (using Tris-HCl buffer instead of xanthine). One enzymatic unit of XO was defined as the amount of enzyme that oxidizes in one minute 1 μmol of xanthine to produce 1 μmol of uric acid under the above assay conditions.

Albumin binding assay of SMA-AHPP

To determine apparent molecular size of SMA-AHPP in solution, Sephadex G-100 (Pharmacia LKB, Uppsala, Sweden) gel chromatography of SMA-AHPP with or without bovine serum albumin (BSA) was carried out.

In brief, SMA-AHPP was first dissolved in 0.01 mmol/L phosphate-buffered 0.15 mmol/L saline (pH 7.4) at 10 mg/mL, to which different concentrations of BSA (0–50 mg/mL) were added and incubated for one hour at room temperature. Then, 1 mL of each sample was subjected to the Sephadex G-100 column (size: 85 cm [h] × 1.5 cm [Ø]). The mobile phase was 0.2 mmol/L $NaHCO_3$ (pH 8.2), and each tube of 5 mL fraction was collected, and monitored by absorption at 260 and 280 nm.

Statistical analyses

Student's *t*-test was used to determine the significance between each experimental group. $P < 0.05$ was considered statistically significant.

Results

Amelioration of hepatic I/R injury by SMA-AHPP

The liver injury was evaluated by measurement of extracellular release of the liver enzymes AST, ALT and LDH in serum, in this rat hepatic I/R injury model. As reported, these enzymes increased to a maximum level at three hours after reperfusion;[23] as a result, all experiments were performed at three hours after reperfusion in this study. As shown in Figure 1, I/R procedure caused a large increase of all three liver enzymes, which were above 5–20 times of normal level (AST, ALT and LDH of normal rat serum, i.e. 2537.5 $\pm$ 590.5 versus 109.0 $\pm$ 8.9, 1248.7 $\pm$ 298.9 versus 64.1 $\pm$ 6.1 and 6200 $\pm$ 1551.9 versus 1016.9 $\pm$ 19.6 IU/L, respectively). Pretreatment of SMA-AHPP significantly lowered the elevated levels of AST, ALT and LDH (Figures 1a–c) in a dose-dependent manner. Consistent with these findings, histological examination of the liver tissues after I/R revealed that the hepatic I/R led to apparent necrotic features (Figure 2b). A significant reduction of necrosis was observed in the liver tissues of animals receiving SMA-AHPP treatment (Figure 2c). Similar findings were observed when SMA-AHPP was given simultaneously with I/R treatment to the rats (data not shown). In addition, animals without I/R procedure showed no apparent change of serum AST, ALT and LDH after injection of SMA-AHPP (data not shown), suggesting that SMA-AHPP *per se* appears non-toxic to the liver.

Apoptotic changes in the liver after I/R

To further elucidate the pathological changes of I/R and the possible mechanism of SMA-AHPP against I/R, liver tissues were subjected to apoptosis assay. Apoptosis induced by I/R was clearly detected at three hours after reperfusion, and the pretreatment with SMA-AHPP significantly lowered numbers of apoptotic cells in the liver tissues (Figure 3). The apoptotic change in the liver was correlated well with the profiles of liver enzymes.

Oxidative injury induced by I/R in the liver

To investigate the potential role of O_2^- and other ROS in the hepatic I/R injury, the TBARS assay, which is a standard method for evaluating tissue damage involving lipid peroxidation,[20] was performed. As shown in Figure 4, elevated level of TBARS was observed after I/R in the rat liver tissue. This increased level of TBARS was remarkably inhibited by the treatment of SMA-AHPP, in a dose-dependent manner (Figure 4), which indicated that the liver injury caused by I/R is the consequence of increased generation of ROS in the liver.

Because ROS (e.g. O_2^-) generated from XO is considered the major cause of the oxidative injury, XO expression and activity in liver tissue after I/R and with/without SMA-AHPP treatment was measured. As shown in Figure 5, there was no significant change of XO mRNA expression (Figure 5a), but significantly increased XO enzymatic activity (Figure 5b) in liver tissues were found after I/R, which is well in agreement with previous reports.[1,13] Moreover, SMA-AHPP treatment did not affect XO expression significantly; however, it greatly decreased the XO activity in liver tissue (Figure 5b). These findings strongly suggested that SMA-AHPP, as the inhibitor of

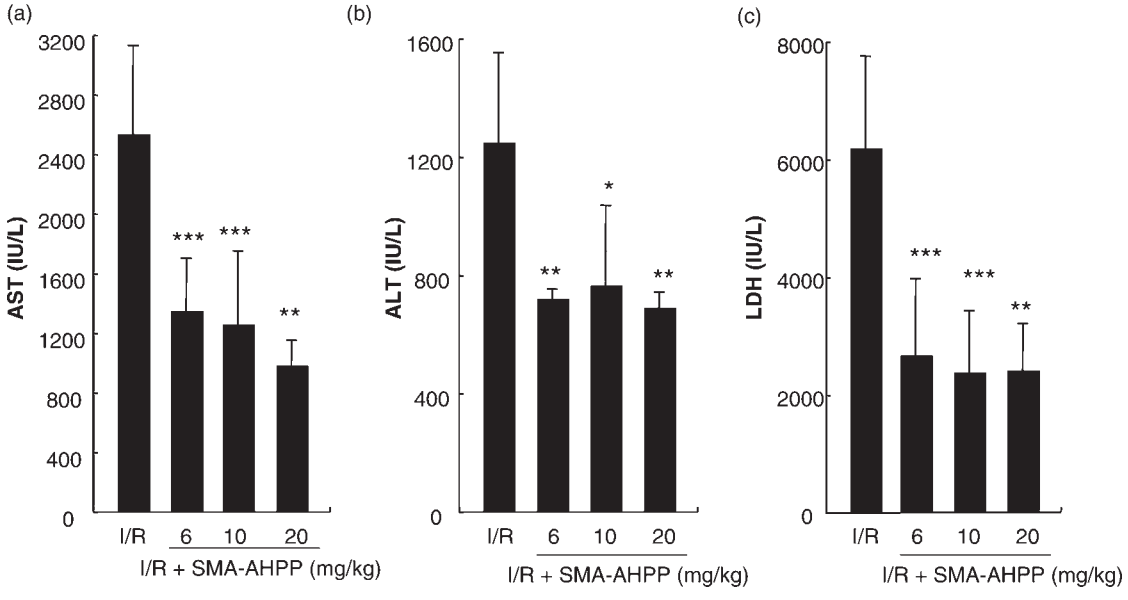

Figure 1 Effect of styrene–maleic acid copolymer (SMA)-4-amino-6-hydroxypyrazolo[3,4-*d*]pyrimidine (AHPP) on hepatic ischemia–reperfusion (I/R) injury as evaluated by changes in serum levels of aspartate aminotransferase (AST) (a), alanine aminotransferase (ALT) (b) and lactate dehydrogenase (LDH) (c). Ischemia was induced by occluding both the portal vein and hepatic artery for 30 min followed by reperfusion. SMA-AHPP was administered via tail vein two hours before ischemia. Data are means $\pm$ standard error ($n = 4–5$). $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.005$, versus the group of I/R alone

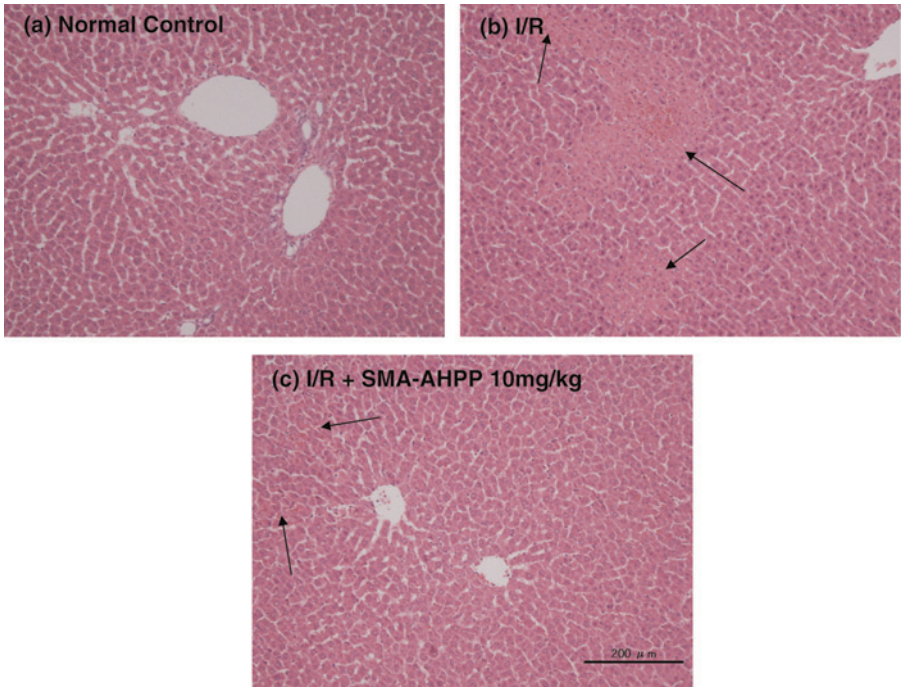

Figure 2 Histological examination of rat liver tissue after ischemia–reperfusion (I/R) injury with or without styrene–maleic acid copolymer (SMA)-4-amino-6-hydroxypyrazolo[3,4-*d*]pyrimidine (AHPP) treatment. (a) Normal rat liver tissue, (b) rat liver tissue after I/R and (c) treatment with 10 mg/kg SMA-AHPP before I/R. Arrows indicate necrosis of the liver tissue

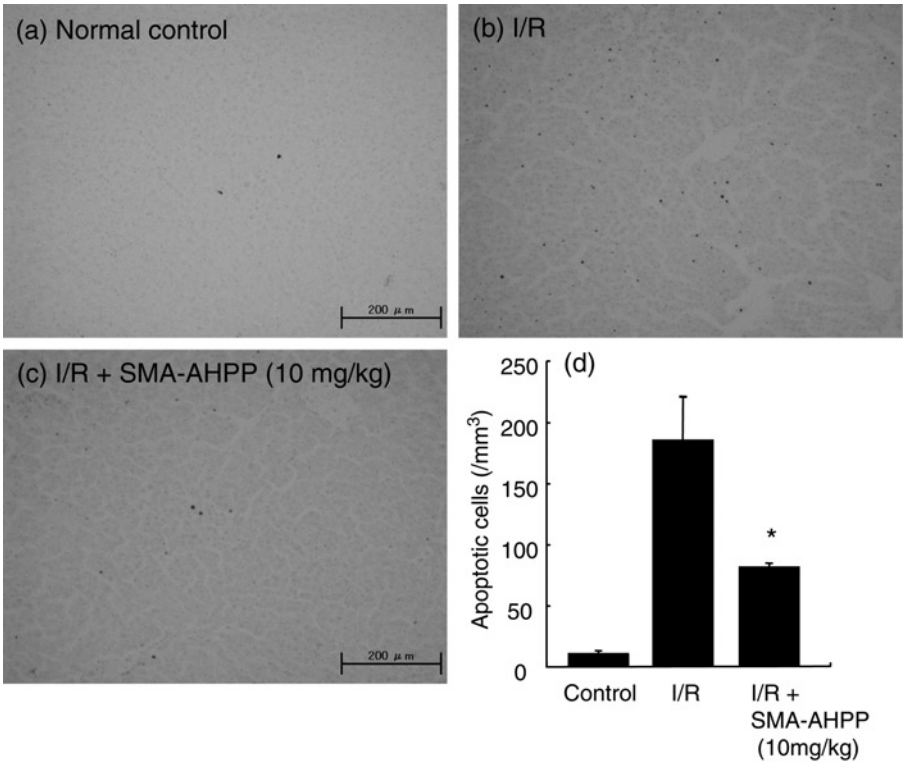

Figure 3 Effect of styrene–maleic acid copolymer (SMA)-4-amino-6-hydroxypyrazolo[3,4-*d*]pyrimidine (AHPP) on apoptotic change in the liver induced by ischemia–reperfusion (I/R). Rats with I/R were treated with SMA-AHPP in the same manner as described in Figure 1. At three hours after reperfusion was initiated, liver tissues were collected and paraffin sections were prepared for an apoptosis assay; apoptotic change was determined by the TUNEL method with an apoptotic detection kit. (a) Normal control (without I/R and SMA-AHPP treatment), (b) I/R group and (c) I/R with SMA-AHPP (10 mg/kg) treatment. Quantification of apoptotic cells after I/R with/without SMA-AHPP treatment was shown in (d). Data are means $\pm$ standard error ($n = 4–5$). $^{*}P < 0.005$, versus the group of I/R alone

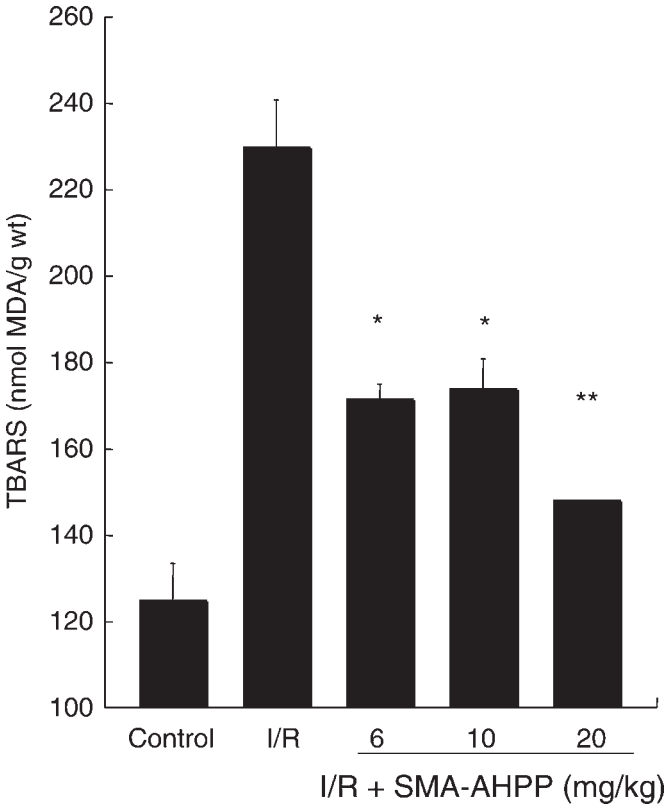

Figure 4 Thiobarbituric acid reactive substances (TBARS) in rat liver after ischemia–reperfusion (I/R) injury with/without styrene–maleic acid copolymer (SMA)-4-amino-6-hydroxypyrazolo[3,4-*d*]pyrimidine (AHPP) treatment. The protocol of I/R and SMA-AHPP treatment is the same as described in Figure 1. At three hours after reperfusion was initiated, rats were killed and liver tissues were collected for TBARS assay. Data are means ± standard error ($n = 4–5$). $^{*}P < 0.001$, $^{**}P < 0.0001$, versus the group of I/R alone

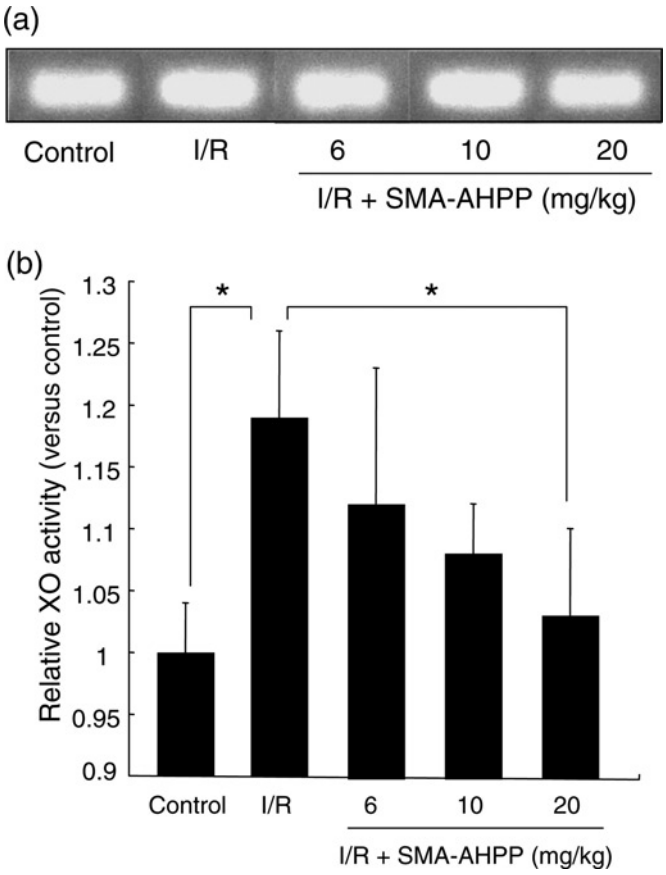

Figure 5 mRNA expression and enzymatic activity of xanthine oxidase (XO) in liver tissues after ischemia–reperfusion (I/R) with/without styrene–maleic acid copolymer SMA-4-amino-6-hydroxypyrazolo[3,4-*d*]pyrimidine (AHPP) treatment. The protocol of I/R and SMA-AHPP treatment is the same as described in Figure 1. At three hours after reperfusion was initiated, rats were killed and liver tissues were collected for detecting mRNA levels of XO (a) and measuring XO enzymatic activity (b). Data are means ± standard error ($n = 4–8$). $^{*}P < 0.05$

XO, reduced the production of ROS by suppressing XO activity, thus decreasing the oxidative injury to liver tissue triggered by I/R.

We further investigated the protein expression of one of the major antioxidative enzyme involved in I/R, i.e. HO-1,[24] whose expression in the liver was remarkably increased by I/R; however, no significant change was found after SMA-AHPP treatment (data not shown).

Change of inflammatory cytokine levels in the serum after I/R with or without SMA-AHPP treatment

A variety of inflammatory cytokines (e.g. TNF-α and IL-12) are known to be induced by I/R, which are important mediators of inflammation or host responses upon I/R.[25,26] We thus investigated the levels of TNF-α and IL-12 in the serum after I/R, and with/without SMA-AHPP. As shown in Figures 6a and b, production of IL-12 and TNF-α in the serum were increased at three hours after I/R. However, upon SMA-AHPP treatment, the elevations of TNF-α and IL-12 levels were significantly attenuated, whereas the levels of TNF-α and IL-12 in the serum of normal rats without I/R were not detectable.

It is also known that ROS is strongly associated with the increases of the pro-inflammatory chemokine MCP-1 levels.[27] We thus measured MCP-1 levels in rat plasma in our experimental setting. Similar to the results of TNF-α and IL-12, I/R significantly increased the MCP-1 levels, which were remarkably lowered by SMA-AHPP treatment (Figure 6c).

Apparent molecular weight of SMA-AHPP

It is known that SMA has an albumin binding property.[28–31] We thus anticipate that SMA-AHPP will show a macromolecular nature *in vivo* after binding to serum albumin. To clarify the *in vivo* behavior of SMA-AHPP, Sephadex G-100 gel chromatography of SMA-AHPP in the presence or absence of BSA was carried out. As shown in Figure 7, when added with different concentrations of BSA, the peak corresponding to SMA-AHPP decreased gradually in a dose-dependent manner. Meanwhile, a peak with the molecular size slightly larger than BSA was observed (Figure 7a), suggesting the formation of SMA-AHPP/BSA complex. Based on this result (Figure 7a), we plotted a linear curve of BSA concentration versus % bounded SMA-AHPP (Figure 7b), by which we calculated that for one BSA molecule, about 8.4 molecules of SMA-AHPP were bound.

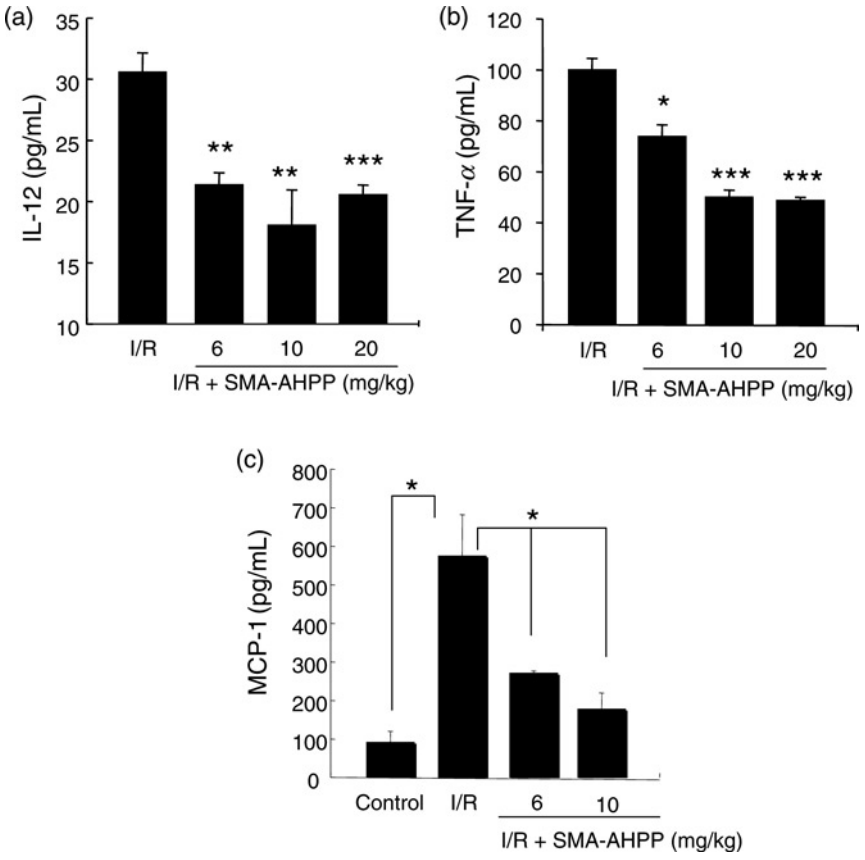

Figure 6 Induction of interleukin-12 (IL-12) (a), tumor necrosis factor-alpha (TNF-α) (b) and monocyte chemotactic protein-1 (MCP-1) (c) by ischemia–reperfusion (I/R) injury and the protective effect of styrene–maleic acid copolymer (SMA)-4-amino-6-hydroxypyrazolo[3,4-*d*]pyrimidine (AHPP). The protocol of I/R and SMA-AHPP treatment is the same as described in Figure 1. At three hours after reperfusion was initiated, rats were killed and serum was collected for measuring IL-12, TNF-α and MCP-1 by using enzyme-linked immunosorbent assay kit. Data are means $\pm$ standard error ($n = 4$–5). $^{*}P < 0.05$, **, $P < 0.0005$, $^{***}P < 0.0001$, versus the group of I/R alone (control)

Discussion

The present study demonstrated that the administration of a macromolecular, water-soluble XO inhibitor SMA-AHPP exerted a potent cytoprotective effect against hepatic I/R injury in rats, as evidenced by the low level of liver enzyme in serum, decreased number of apoptotic cells in the liver and suppressed inflammatory cytokine levels in serum. The cytoprotective effects of SMA-AHPP can be attributed primarily to the inhibition of ROS (e.g. O_2^-) production from XO, which was supported by the findings of the decreased XO activity and the subsequent decreased levels of tissue peroxidation in the liver after SMA-AHPP treatment as assessed by the TBARS assay.

Excessive production of ROS is considered to be a critical factor in I/R injury-caused tissue damage and cell death, as a result of oxidative cellular damage.[1,2,4–6] XO, which plays a crucial role in excessive generation of ROS, is an iron-containing metalloflavoprotein, which catalyzes the two-step oxidation of purines, such as hypoxanthine or xanthine, to uric acid. During this reaction, molecular oxygen is used as an electron acceptor, and ROS including O_2^- and H_2O_2, to a lesser extent, are produced subsequently.[3] In this report, we found a significant increase of XO activity in rat liver upon I/R (Figure 5); further we described that a four- to five-fold increase of lipid peroxidation was observed in rat liver tissues after I/R (Figure 4). These findings further support the above-mentioned notion. Accordingly, the liver function was significantly improved (Figure 1) and apoptotic cell death in the liver was lowered considerably (Figure 3).

AHPP was first reported to be a potent competitive inhibitor of XO by us, and was developed as an antihypertensive agent.[17] Compared with other commonly used XO inhibitors, such as alloxanthine and allopurinol, AHPP interacts with XO in a similar manner to alloxanthine.[17,32] However, its inhibitory potency against XO is up to 21 times stronger than alloxanthine, probably due to the higher binding constant of AHPP to the molybdenum containing catalytic center of XO.[17,32] Moreover, the inhibitory activity of AHPP is about three times stronger than allopurinol, a well-known competitive inhibitor of XO.[17] Allopurinol has been used for the treatment of gout, and it was also used for I/R injury and inflammatory diseases involving excess generation of O_2^-.[14–16,33] However, the effect of allopurinol is paradoxical: it serves as the XO inhibitor at lower or moderate concentrations, whereas at high concentrations, it behaves as the substrate of XO and generates O_2^- by XO.[17] It is thus problematic and dangerous to use allopurinol at higher dose unless dosing is strictly

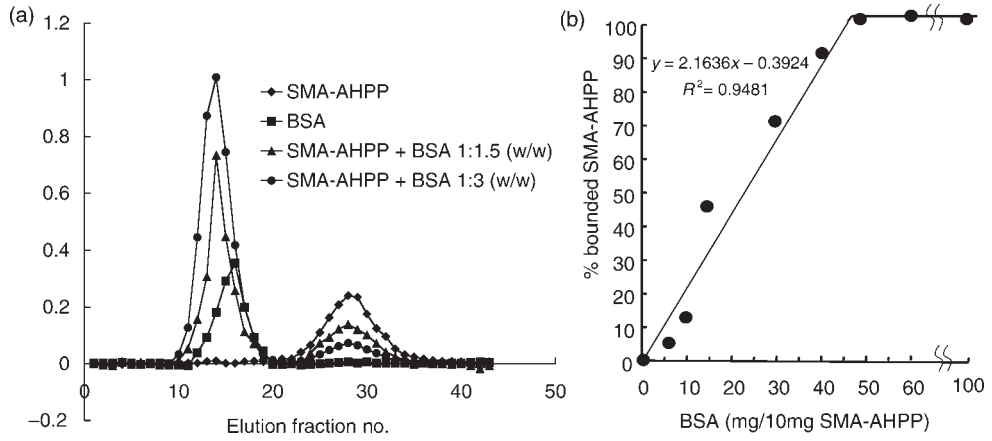

Figure 7 Size exclusion chromatography of styrene–maleic acid copolymer (SMA)-4-amino-6-hydroxypyrazolo[3,4-*d*]pyrimidine (AHPP) conjugate in the presence and absence of bovine serum albumin (BSA). (a) Gel chromatography of SMA-AHPP with/without BSA was performed using a Sephadex G-100 column (size: 85 cm [h] × 1.5 cm [Ø]) with the mobile phase of 0.2 mmol/L $NaHCO_3$ (pH 8.2). (b) BSA concentration versus % bounded SMA-AHPP curve for calculating the average amount of SMA-AHPP binding to each BSA molecule, and thus the molecular weight of SMA-AHPP–BSA complex was obtained

monitored or regulated. Hence, we anticipate AHPP may be a superior therapeutic agent because of its higher and irreversible XO inhibitory activity, and it may be used for any O_2^--related diseases, including I/R injury, viral infectious diseases, hypertension and inflammatory diseases.[1,17,34–37]

Previously, however, one drawback of AHPP which hampered its clinical development was its poor water solubility. AHPP can only be dissolved weakly in strong alkaline, and is also difficult to dissolve in most organic solvents. Therefore, systemic administration of AHPP becomes almost impossible. To overcome this drawback, we recently developed a macromolecular conjugate of AHPP with SMA copolymer, which showed high water solubility with a saturated concentration of 22 mmol/L in distilled water or physiological saline, meanwhile possessing comparable inhibitory activity against XO to native AHPP.[18] Intravenous injection of SMA-AHPP did not induce any apparent side-effects, for example, loss of body weight (data not shown). Our previous study also showed that intravenous or oral administration of SMA-AHPP up to the dose of 100 mg/kg did not show any adverse harmful effect to the rats,[18] all of which suggest the safety of this agent.

In addition, as reported previously, SMA-AHPP also showed an albumin binding character, similar with our previous reports of SMA polymers as well as the micellar formulation of SMA with many drugs.[28–31] In this instance, SMA-AHPP will have an apparent molecular weight of ~69 kDa in blood circulation upon binding with plasma albumin, thus behaving as a macromolecule. We further clearly demonstrated this character of SMA-AHPP using Sephadex G-100 gel chromatography as shown in Figure 7. In parallel to the increased amount of BSA, the amount of free SMA-AHPP decreased gradually in response to the concentrations of BSA, while a peak indicating SMA-AHPP/BSA complex appeared with the molecular size slightly larger than BSA (Figure 7a). According to the linear curve of BSA concentration versus % bounded SMA-AHPP (Figure 7b), we found that about 8.4 molecules of SMA-AHPP was bound to one molecule of BSA, which will form a macromolecular agent of about 96 kDa.

It is well known that macromolecular drugs show various beneficial characteristics, including prolonged *in vivo* half-life, and thus higher AUC (area under concentration/time curve), decreased kidney clearance and selective accumulation and retention in the solid tumor and inflammatory site. This unique phenomenon of macromolecules was coined 'enhanced permeability and retention effect' (EPR effect).[38–43] Accordingly, many macromolecular drugs were developed recently based on EPR effect, not only in the laboratory stage, but also now in the clinical stage.[38–43] This treatise may be found in various reviews.[41,42,44] Thus, SMA-AHPP conjugate not only solved the problem of water solubility, but it also exhibited improved *in vivo* pharmacokinetics after binding to the plasma albumin, which resulted in a remarkable protective effect against hepatic I/R injury (Figures 1–3), by targeted inhibition of XO activity, more preferably at the inflammatory site.

Regarding the pathological process of I/R injury and the involvement of SMA-AHPP, XO activity greatly increased during I/R (Figure 5b), which is well in agreement with previous reports,[1,2,13–16] and thus increased O_2^- triggered oxidative cell damage (e.g. lipid peroxidation; Figure 4) and induced apoptotic cell death (Figure 3). SMA-AHPP suppressed XO activity in liver tissue significantly; however, it did not affect the expression of XO. Thus the potential therapeutic mechanism of SMA-AHPP against I/R may be mostly due to the direct inhibition of O_2^- production by inhibiting XO activity, which in turn attenuated the subsequent pathological changes, i.e. apoptosis, induced by ROS. Moreover, HO-1 is also known as a key antioxidative enzyme involved in I/R, which is upregulated during I/R to protect cells against I/R injury;[24] further induction of HO-1 by its inducers such as hemin, cobalt protoporphyrin may thus become a therapeutic to I/R, which warrants further investigation. However, in this study, we did not find the association of SMA-AHPP with HO-1 expression (data not shown), suggesting that SMA-AHPP fulfills the

protective effect of I/R through an HO-1-independent, but an XO activity-dependent pathway.

Furthermore, SMA-AHPP significantly inhibited the production of inflammatory cytokines, e.g. TNF-α, IL-12 and MCP-1, in the serum (Figure 6). These findings suggested that inflammation is probably one of the major pathological changes in the late stage of I/R; they are, at least in part, elicited by ROS (e.g. O_2^-) that was induced by XO, which is the major effector molecule in the early stage of the pathological changes in I/R. Indeed, in a recent report, Matsuzawa *et al.*[45] demonstrated that ROS was required for inducing apoptosis signal-regulating kinase 1, which is crucial for mammalian innate immunity to induce the production of cytokines and inflammatory responses. It is thus reasonable that ROS generated by XO in I/R process may trigger the inflammatory reactions, which could be suppressed by SMA-AHPP treatment (Figure 6).

In conclusion, SMA-AHPP is newly developed as a water-soluble polymer conjugate drug that exhibits potent XO inhibitory activity. SMA-AHPP showed a superior physiochemical and pharmacological property compared with native AHPP, and it exerted remarkable protection against hepatic damage upon I/R injury (Figures 1–3), via inhibiting XO activity and thus decreasing ROS levels in the liver (Figures 4 and 5). In addition, SMA-AHPP also showed potential effects on inhibiting inflammation (Figure 6) triggered by I/R, which is another major cause of tissue damage induced by I/R. Accordingly, the data described herein suggest the potential of SMA-AHPP as a useful agent for treating I/R injury; moreover, the application of SMA-AHPP may be extended to many other XO/O_2^--related diseases, such as inflammatory diseases, viral infections, hypertension as well as gout, which warrants further investigations.

Author contributions: All authors participated in the interpretation of the studies and analysis of the data and review of the manuscript; JF, TS and HQ conducted the animal experiments and biological assays, GYB conducted the albumin binding assay by Sephadex G-100 chromatography, GYB and AKI contributed to the synthesis of SMA-AHPP and JF and HM designed the experiments and wrote the manuscript. The authors thank Mr Nakamura for his excellent technique supports.

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