Protection Against Hepatic Ischemia-reperfusion Injury in Rats by Oral Pretreatment With Quercetin

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Objective   To investigate the possible protection provided by oral quercetin pretreatment against hepatic ischemia-reperfusion injury in rats. **Methods**   The quercetin (0.13 mmol/kg) was orally administrated in 50 min prior to hepatic ischemia-reperfusion injury. Ascorbic acid was also similarly administered. The hepatic content of quercetin was assayed by high performance liquid chromatography (HPLC). Plasma glutamic pyruvic transaminase (GPT), glutamic oxaloacetic transaminase (GOT) activities and malondialdehyde (MDA) concentration were measured as markers of hepatic ischemia-reperfusion injury. Meanwhile, hepatic content of glutathione (GSH), activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and xanthine oxidase (XO), total antioxidant capacity (TAOC), contents of reactive oxygen species (ROS) and MDA, DNA fragmentation were also determined. **Results**   Hepatic content of quercetin after intragastric administration of quercetin was increased significantly. The increases in plasma GPT, GOT activities and MDA concentration after hepatic ischemia-reperfusion injury were reduced significantly by pretreatment with quercetin. Hepatic content of GSH and activities of SOD, GSH-Px and TAOC were restored remarkably while the ROS and MDA contents were significantly diminished by quercetin pretreatment after ischemia-reperfusion injury. However, quercetin pretreatment did not reduce significantly hepatic XO activity and DNA fragmentation. Ascorbic acid pretreatment had also protective effects against hepatic ischemia-reperfusion injury by restoring hepatic content of GSH, TAOC and diminishing ROS and MDA formation and DNA fragmentation. **Conclusion**   It is indicated that quercetin can protect the liver against ischemia-reperfusion injury after oral pretreatment and the underlying mechanism is associated with improved hepatic antioxidant capacity.

Key words: Quercetin; Ischemia-reperfusion injury; Lipid peroxidation; Liver

INTRODUCTION

Flavonoids are polyphenolic compounds that occur ubiquitously in fruits, vegetables and some herbs. Over 4 000 different forms of flavonoids have been identified and they have been demonstrated to possess an array of biological actions, including antioxidant, antiinflammatory, antiviral, antiproliferative, anticarcinogenic and metal-chelating properties[1]. Epidemiological evidences have suggested an inverse relationship between dietary intake of flavonoids and the risk of coronary heart disease and certain types of cancers[2-5]. Quercetin, a major representative of flavonols, is a potent antioxidant and can quench various free

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radicals and protects the low-density lipoproteins against oxidation *in vitro*[6-8]. The antioxidant activity of quercetin is three times higher than that of ascorbic acid as measured by ferric reducing/antioxidant power (FRAP) assay[9]. The intestinal absorption of quercetin has been well demonstrated in animals and humans[10-13]. The results of our previous studies indicated that quercetin could be absorbed extensively in duodenum, jejunum, ileum and colon after in situ perfusion in rats[14]. Moreover, quercetin could increase total plasma antioxidant capacity (TAOC) of rats within 1 h after oral administration[15]. Recently, Crespy *et al*. reported that quercetin could even be absorbed from rat stomach[16]. The study of Mojzis *et al*. showed that oral pretreatment with quercetin (100 mg/kg) was gastroprotective in rats with ischemia-reperfusion-induced gastric mucosal injury[17].

The liver plays a fundamental role in the metabolism of endogenous and exogenous substances, including flavonoids. Many hepatic diseases are associated with reactive oxygen species (ROS) induced damage since the liver is highly susceptible to the chain reaction of lipid peroxidation under aerobic conditions[18,19]. It has been documented that hepatic ischemia-reperfusion injury is associated with excessive ROS production and administration of antioxidants such as ascorbic acid or α-tocopherol has been shown to be effective in reducing hepatic ischemia-reperfusion injury[20, 21]. As a powerful antioxidant, whether quercetin is hepatoprotective in ischemia-reperfusion condition remains to be clarified. In the present study, the hepatic ischemia-reperfusion injury was induced in rats and the possible protection provided by quercetin pretreatment was investigated.

MATERIALS AND METHODS

Animals and Experimental Protocol

Male Wistar rats, weighing 220-260 g (obtained from the Animal Center, Academy of Chinese Military Medical Sciences, Beijing, China), were housed individually in stainless-steel wire cages and allowed free access to tap water and a cereal-based diet provided by the animal care unit of the Institute. The ambient temperature was held constant between 22℃ and 25℃ and cycles of light and dark were alternated every 12 h. After being acclimated for 5 days, the rats were randomly assigned to four groups, i.e., sham-operated group, ischemia-reperfusion group, ischemia-reperfusion plus quercetin group and ischemia- reperfusion plus ascorbic acid group. The quercetin (Acros Organics, NJ, USA) or ascorbic acid was dissolved in dimethylsulfoxide (DMSO) and administrated by gavage at a dose of 0.13 mmol/kg (equivalent to 40.0 mg/kg quercetin or 22.9 mg/kg ascorbic acid) to the rats in the ischemia-reperfusion plus quercetin or ascorbic acid group in 50 min before the ischemia-reperfusion operation. The dosage of quercetin administrated was proved to be effective in increasing plasma TAOC of rats after oral administration in our previous experiment[15]. The rats in the sham-operated group and the ischemia-reperfusion group were administrated only with the same volume of vehicle (DMSO).

Liver Ischemia-Reperfusion Injury

All rats were fasted overnight before ischemia-reperfusion operation. The abdomen was opened through a midline incision under sodium pentobarbital anesthesia. All vessels (hepatic artery, portal vein and bile duct) were occluded for 30 min and then released for 30 min reperfusion. Blood and liver samples were collected for the assessment of liver injury and lipid peroxidation. During the ischemia and reperfusion period, the abdomen was closed

and kept warm. The rats in the sham-operated group only underwent operation, without ischemia and reperfusion treatments.

Plasma Aminotransferases Activities and Malondialdehyde Concentration

The plasma glutamic pyruvic transaminase (GPT) and glutamic oxaloacetic transaminase (GOT) activities were assayed using commercial assay kits (Zhongsheng High-Tech Bioengineering Company, Beijing, China). The plasma malondialdehyde (MDA) concentration was measured by thiobarbituric acid reactive species (TBARS) assay[22]. The value of TBARS was expressed as mol MDA/L.

Hepatic Quercetin and Ascorbic Acid Contents

The liver tissue was homogenized in 9 volumes of iced-cold 0.9% NaCl. The quercetin content was determined by a high performance liquid chromatography (HPLC) procedure as reported previously by Crespy *et al*.[23] A dinitrophenylhydrazine method was used to analyze hepatic ascorbic acid content[24].

Hepatic Xanthine Oxidase, Superoxide Dismutase, Glutathione Peroxidase Activities, Glutathione and ROS Contents and TAOC

The activities of xanthine oxidase (XO), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and content of glutathione (GSH) in liver homogenates were measured using commercial assay kits (Jiancheng Bioengineering Institute, Nanjing, China). 2',7'-dichlorofluorescin (DCFH, Sigma, St. Louis, MO, USA) was used as a probe to assay ROS content based on a procedure previously described by LeBel *et al*.[25] The results were expressed as relative fluorescent intensity (RFI) to the blank. The FRAP assay was applied to determine the TAOC of liver homogenates[26]. One unit of TAOC was equal to 0.01 increase in absorbance of the reaction mixture at 520 nm per milliliter or milligram protein per minute under 37℃ incubation. The FRAP reagent kit was also provided by Jiancheng Bioengineering Institute (Nanjing, China). The protein content of liver homogenates was measured by Bradford method[27].

Hepatic DNA Fragmentation

A procedure described by McCabe *et al*. was followed to quantify hepatic DNA fragmentation[28]. Briefly, the liver tissue was passed through a cell dissociation sieve (Sigma, St. Louis, MO, USA) and suspended in a lysis buffer composed of 5 mmol/L Tris, 20 mmol/L EDTA and 0.5% Triton X-100 for 15 min. The intact DNA (pellet) was separated from the fragments (supernatant) by centrifugation. The resulting pellets and supernatants were resuspended in TE buffer (10 mmol/L Tris, 1 mmol/L EDTA) and added with 10% trichloroacetic acid (TCA). The 10% TCA precipitates were mixed with 5% TCA and placed in a 100℃ water bath for 15 min. The supernatants after final centrifugation were reacted with the diphenylamine reagent overnight at 30℃. The absorbance was determined spectrophotometrically at 600 nm. Results are calculated with the following formula:

DNA fragmentation (%) = [(absorbance of the supernatant)/(absorbance of the supernatant + absorbance of the pellet)]×100.

Statistical Analysis

Data are expressed as $\bar{x} \pm s$. Analysis of variance was used to make statistical comparison

with post hoc Newman-Keuls test.

RESULTS

Trace amount of quercetin could be detected in the liver of sham-operated rats (19.10 nmol/100g wet weight, average of 5 samples). It was increased by approximately 4.13 times (78.91 nmol/100g wet weight, average of 5 samples) in 50 min after oral administration of 0.13 mmol/kg quercetin compared to that in the sham-operated group. The area of an unidentified peak eluted after quercetin on the chromatogram of liver homogenates (Fig. 1) was also increased significantly after quercetin administration. Hepatic ascorbic acid content in the ischemia-reperfusion plus ascorbic acid group was increased by approximately 1.14 times in 50 min after oral ascorbic acid administration compared to that in the sham-operated group.
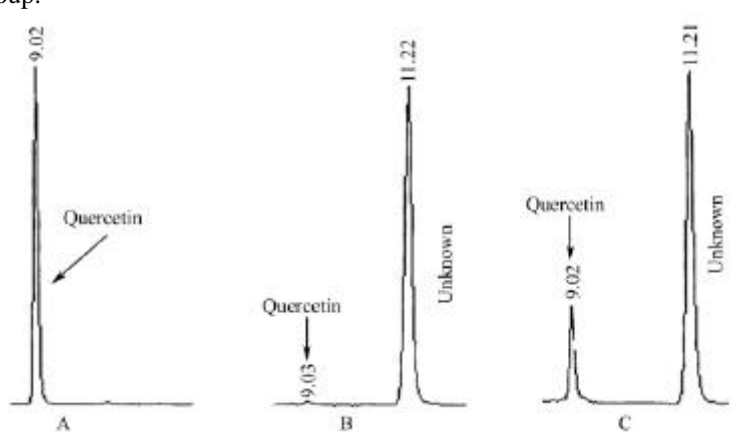

FIG.1. Representative chromatograms of quercetin standard (A) and liver homogenates of sham-operated group (B) and ischemia-reperfusion plus quercetin group (C).

After hepatic ischemia-reperfusion injury, both plasma GPT, GOT activities and MDA concentration were increased significantly (Table 1). Oral pretreatment with quercetin reduced significantly the increases in plasma GPT, GOT activities and MDA concentration by 18.39%, 11.79%, and 19.78% respectively. Oral pretreatment with ascorbic acid produced nearly similar protective action (12.47%, 16.34%, and 22.02%, respectively).

TABLE 1

Effects of Quercetin Pretreatment on Plasma GPT, GOT Activities and MDA Concentration in Hepatic Ischemia-Reperfusion Rats

Group	n	GPT (U/L)	GOT (U/L)	MDA (μmol/L)
Sham-operated	6	43.63 ± 5.84	144.47 ± 24.00	8.83 ± 1.73
Ischemia-reperfusion	6	292.23 ± 26.41[**]	297.30 ± 21.50[**]	16.03 ± 1.83[**]
Ischemia-reperfusion plus quercetin	6	238.30 ± 13.43[**##]	262.25 ± 17.55[**#]	12.86 ± 1.92[**#]
Ischemia-reperfusion plus ascorbic acid	6	255.78 ± 9.68[**##]	248.71 ± 7.65[**##]	12.50 ± 1.67[**##]

Note. GPT: glutamic pyruvic transaminase; GOT: glutamic oxaloacetic transaminase; MDA: malondialdehyde. Values are expressed as $\bar{x} \pm s$ and analyzed by Analysis of Variance with post hoc Newman-Keuls test. [**]: $P<0.01$ vs sham-operated group. [#]: $P<0.05$, [##]: $P<0.01$ vs ischemia-reperfusion group.

TABLE 2

Effects of Quercetin Pretreatment on Hepatic Contents of MDA, GSH, ROS, Activities of Related Enzymes, DNA Fragmentation and TAOC
in Hepatic Ischemia-Reperfusion Rats

Group	n	MDA (nmol/mg Pr.)	SOD (NU/mg Pr.)	XO (U/g Pr.)	GSH (mg/g Pr.)	GSH-Px (U/g Pr.)	TAOC (U/mg Pr.)	ROS (RFI)	DNA Fragmentation (%)
Sham-operated	6	22.93±2.80	66.71±6.62	3.67±0.56	10.84±0.69	51.24±10.54	2.72±0.38	76.14±10.66	6.89±0.51
Ischemia-reperfusion	6	36.98±6.63**	56.92±6.64*	4.69±0.51**	8.44±1.17**	35.44±12.18*	2.02±0.34**	127.08±13.48**	11.65±1.06**
Ischemia-reperfusion Plus Quercetin	6	30.25±2.20**#	65.90±5.52#	4.26±0.27**	10.02±0.69#	48.77±7.19#	2.42±0.18*#	99.05±16.82**##	12.37±1.60**
Ischemia-reperfusion Plus Ascorbic Acid	6	28.56±5.71**#	60.65±7.56	4.19±0.56*	10.95±1.34##	42.26±15.43	2.53±0.32*#	99.58±14.39**##	8.71±0.99**##

Note. MDA: malondialdehyde; SOD: superoxide dismutase; XO: xanthine oxidase; GSH: glutathione; GSH-Px: glutathione peroxidase; TAOC: total antioxidant capacity; ROS: reactive reactive oxygen species; RFI: relative fluorescent intensity. Values are expressed as $\overline{x} \pm s$ and analyzed by Analysis of Variance with post hoc Newman-Keuls test. *: $P<0.05$, **: $P<0.01$ vs sham operated group; #: $P<0.05$, ##: $P<0.01$ vs ischemia-reperfusion group.

As shown in Table 2, hepatic GSH content and TAOC were decreased significantly, while ROS and MDA contents were increased remarkably in the ischemia-reperfusion group compared to that in the sham-operated group. Hepatic XO activity was significantly higher while SOD and GSH-Px activities were significantly lower in the ischemia-reperfusion group than in the sham-operated group. Hepatic ischemia-reperfusion injury also induced a significant increase in DNA fragmentation in the liver. Oral pretreatment with quercetin restored significantly the hepatic GSH content, SOD and GSH-Px activities and TAOC in hepatic ischemia-reperfusion rats. The increased hepatic ROS and MDA contents after ischemia-reperfusion injury were also reduced remarkably by quercetin pretreatment. No significant declines in hepatic XO activity and DNA fragmentation were observed in the ischemia-reperfusion plus quercetin group. In contrast, oral pretreatment with ascorbic acid significantly restored hepatic GSH content and TAOC, and decreased hepatic ROS and MDA contents after ischemia-reperfusion injury. A significant reduction in hepatic DNA fragmentation was found after ascorbic acid administration. However, no significant effect was noted for ascorbic acid pretreatment in hepatic SOD and GSH-Px activities after ischemia-reperfusion injury.

DISCUSSION

It has been demonstrated that quercetin is extensively metabolized in the small intestine and liver. No free aglycone can be recovered in plasma[11-13]. Therefore, it is not certain whether orally taken quercetin can act as an antioxidant *in vivo*. In the present study, we showed that hepatic content of quercetin was increased remarkably in 50 min after oral administration in rats. Thus, it can be expected that orally administered quercetin is partly available for the liver and can play a role as an antioxidant in oxidatively stressed liver. The results of the current study also confirmed that quercetin was metabolized after absorption since the area of an unidentified HPLC peak eluted after the quercetin on the chromatogram of liver homogenates was increased remarkably after oral quercetin administration. The metabolites of quercetin had been demonstrated to retain their antioxidant properties to some extent, which would contribute partly to the increased antioxidant capacity in the liver after oral quercetin administration[29].

It is well-known that ischemia followed by reperfusion leads to increased ROS production and results in oxidative stress[19-21]. We have successfully developed hepatic ischemia-reperfusion injury in rats in the present study, as indicated by increased plasma GPT and GOT activities and both plasma and hepatic MDA concentrations, as well as decreased hepatic SOD and GSH-Px activities and GSH content. Hepatic XO activity was also increased significantly after ischemia-reperfusion injury in this study. The activation of XO activity is considered a key event in the ischemia-reperfusion injury because XO is capable of catalyzing the univalent reduction of molecular oxygen to the superoxide anion radical. The superoxide anion radical is further converted to H_2O_2 spontaneously or by catalysis of SOD. In the step followed, the extremely reactive OH is formed via Fenton reaction or Haber-Weiss reaction, which initiates free radical chain reactions in cell membranes[18-21, 30]. We measured the ROS production by using DCFH as a probe, which is sensitive to ROS[25]. The results confirmed that hepatic ROS production was increased significantly after ischemia-reperfusion injury. Meanwhile, the TAOC of the liver was also declined significantly as measured by FRAP assay.

Antioxidants such as ascorbic acid, -tocopherol and cyaniding 3-*O*- -D-glucoside are protective against hepatic ischemia-reperfusion injury[20, 21, 31]. It is well-known that ascorbic

acid can be synthesized in rat liver and is not an essential nutrient for rats. However, Tsuda *et al*. noted that hepatic ascorbic acid content was significantly lowered by hepatic ischemia-reperfusion injury[31]. Takayama *et al*. demonstrated that supplementation of ascorbic acid was beneficial in protecting against hepatic ischemia-reperfusion injury[20]. We confirmed the protective action of ascorbic acid against hepatic ischemia-reperfusion injury in this study. The quercetin administrated at the same dosage produced a similar protection based on the changes of plasma GPT and GOT activities after ischemia- reperfusion injury in rats. Both quercetin and ascorbic acid were effective in decreasing ROS production, restoring GSH content, reducing MDA content and increasing TAOC in the ischemia-reperfusion liver. However, ascorbic acid pretreatment did not improve SOD and GSH-Px activities remarkably while quercetin pretreatment could protect both SOD and GSH-Px activities significantly in the ischemia-reperfusion liver. It seems impossible that the protection provided by quercetin is associated with a direct action on SOD and GSH-Px by quercetin since Duarte *et al*. showed that hepatic GSH-Px activity was not changed significantly after *in vitro* incubation of liver homogenates with quercetin up to 10 mol/L[32]. We consider that quercetin may possibly bind to SOD and GSH-Px and protect them directly against ROS attack because quercetin has a great affinity for proteins[11, 33].

Chang *et al*. demonstrated that the XO activity could be inhibited by quercetin via competing with the substrate, xanthine *in vitro*[34], whereas Nagao *et al*. reported mixed-type inhibition of XO activity by quercetin *in vitro*[35]. However, the results of our study did not show a significant inhibition of hepatic XO activity after quercetin administration. More quercetin may be needed to be administrated because the reported IC50 for quercetin ranged from 0.44 mol/L to 7.23 mmol/L[34, 35].

The DNA damage secondary to the oxidative stress is accompanied by nuclear fragmentation, a phenomenon characterizing the apoptotic type of cell death[36]. Our data also showed that hepatic DNA fragmentation was increased remarkably after ischemia-reperfusion injury. Pretreatment with ascorbic acid was protective against ischemia-reperfusion induced DNA fragmentation. Unfortunately, quercetin pretreatment appeared to be not effective. We do not have a clear explanation for this discrepancy.

In conclusion, the data from this study clearly show that quercetin can be absorbed partly in aglycone form to the liver after oral administration and is protective against hepatic ischemia-reperfusion injury. The underlying mechanism is associated with decreased ROS production, restored GSH content and improved antioxidant enzymes activity in the liver.

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