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Iron overload-induced rat liver injury: Involvement of protein tyrosine nitration and the effect of baicalin

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ABSTRACT

Baicalin has been reported to protect against liver injury in iron-overload mice, however, the mechanisms underlying the hepatoprotective properties of baicalin are poorly understood. In this study, we systematically studied the protective effect of baicalin on iron overload induced liver injury, as well as the underlying mechanism based on nitrative stress in rat model. We found that when iron overload rats (500 mg iron/kg) were fed baicalin-containing diet (0.3% and 1% w/w) for 45 days, baicalin dose dependently protected against iron overload induced liver injury, including alleviation of hepatic pathological damage, decrease of SOD activity, iron content, carbonyl content, and the thiobarbituric acid-reactive substances level in hepatic tissues. It also increased serum iron content, SH content and GPx activity, decreased serum ALT and AST activities. Immunohistochemistry and immunoprecipitation analysis revealed that baicalin could also inhibit iron overload induced protein tyrosine nitration in liver. Moreover, in iron overload rat liver, we found that baicalin decreased the iron overload increased level of glutathione-S-transferases (GSTs) expression, oxidation and nitration. These results suggest that not only oxidative stress, but also nitrative stress, is involved in iron overload induced liver injury, and the underlying mechanism might partially relate to the involvement of GSTs expression and post-translational modification. Baicalin can effectively prevent iron overload caused abnormality and can be a candidate medicine for iron overload diseases.

1. Introduction

Iron is ubiquitous in cells and is essential for its normal functioning. But without adequate management, excess amount of free iron may cause progressive damage to hepatic, endocrine, and cardiac organs, significantly affect overall survival. Liver is the main storage organ of iron in the body, so it is the principal target most likely to be afflicted by iron overload (Papanastasiou et al., 2000). The consequences of hepatic iron overload have been documented in patients with hemochromatosis (HH) (Allen, 2010), hepatocellular necrosis (Toyokuni, 2011) and fibrosis (Ramm and Ruddell, 2005), and even to hepatocellular carcinoma (Kew, 2009).

Free radical-mediated hepatocellular damage is the first and most thoroughly studied mechanism proposed to explain the hepatotoxicity of excessive iron. In iron overload animal and patients, excessive iron facilitates the formation of reactive oxygen and nitrogen species (such as hydroxyl radicals, superoxide anion, and nitric oxide) that disrupt the redox balance of the cells and cause chronic oxidative/nitrosative stress (Toyokuni, 2011). Thus, iron-induced injury may be partially through tyrosine nitration.

Glutathione-S-transferases (GSTs) are a family of principal hepatic phase II biotransformation enzymes which play an important role in protecting tissues from oxidative stress (Hayes et al., 2005). The level of GSTs expression is considered to be an important factor to protect organs against the deleterious effect of toxicants (Hayes and Pulford, 1996; Hayes et al., 2005). Moreover, a growing body of literature has reported that GSTs are important target for reactive oxygen species and reactive nitrogen species, based on emerging evidence indicating changed GSTs activity during liver injury at the conditions with increased reactive oxygen species/reactive nitrogen species production (Ji et al., 2006).

Baicalin (Fig. 1) is the major active component of the root of *Scutellaria baicalensis* Georgi that has many important characteristics, i.e. anti-oxidant (Gao et al., 1999), anti-inflammatory (Xue et al., 2006), strong affinity to iron ions (Yoshino and Murakami, 1998). It is used as a constitute of some hepato-protective herb mixture such as Xiaochaihu-tang (Sho-saiko-to) in China and Japan (Ohta et al., 1997; Shimizu et al., 1999; Taira et al., 2004). Park et al. (2008) indicated that baicalin protects hepatocytes oxidative damage

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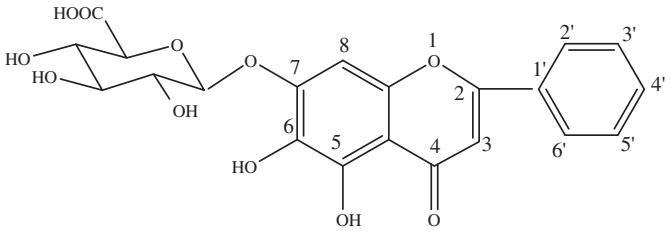


Fig. 1. The structure of baicalin.

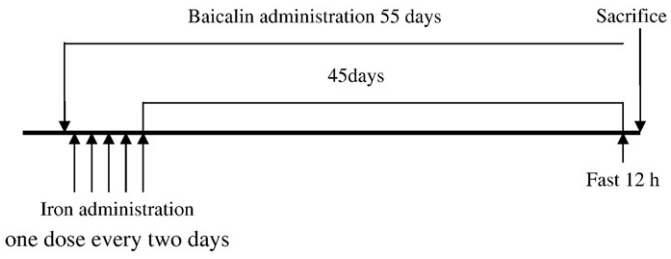
by induction of HO-1expression and inhibition of the proinflammatory mediators. Our previous study found that dietary supplementation of baicalin could effectively decrease iron content and increase antioxidant status in iron overload mouse liver (Zhang et al., 2006; Zhao et al., 2005). These results suggest that baicalin might be used as a medicine for iron overload disease. In order to further confirm the medicinal usage and the mechanism of baicalin on iron overload induced liver injury, in this paper, we used rat iron overload model, to study the hepato-protective effect of baicalin and the mechanisms based on protein oxidation and nitration.

2. Materials and methods

2.1. Animals

The experiment procedure referred to our previous study (Zhang et al., 2006; Zhao et al., 2005). Briefly, thirty-six male Wistar rats, initially weighing 180.4 ± 7.8 g, were used. Rats were randomly divided into six groups. Five groups received 5 doses (one dose every two days) of 30 (group 150), 60 (group 300), 100 (group 500), 100, 100 mg/kg (i.p. iron-dextran–saline), respectively, the other group received the same volume of saline as a blank (group B). The group B, 150, 300 and 500 were fed commercial animal chaw, the other two groups were fed with the same animal chow supplemented with 0.3% (Low baicalin group, LBa) and 1% (High baicalin group, HBa) of baicalin (wt/wt), respectively, which started from the day before the first iron-dextran injection until the day before animals were sacrificed. The amount of other flavonoids in animal food was not considered. Each group of rats were housed in a large plastic cage and given free access to food and tap water. All groups of animals were kept at 25 ± 2 °C under a 12-h dark/light cycle. The scheme of animal treatment is shown in Scheme 1. Animal care in this study conformed to the NIH Guide for Care and Use of Laboratory Animals (NIH publication 86–23, revised 1986).

After 45 days of the last iron-dextran injection, rats were fasted overnight. They were anesthetized with ethyl ether, and blood was collected into plastic tubes containing heparin sodium and livers were quickly removed. The blood was centrifuged at 5000 *g* for 15 min and the plasma isolated from each rat was used. Three samples from each group were used for histology and histochemistry. The liver was perfused with 4 °C saline to exclude blood cells and finally blotted on filter paper. All samples were stored at −80 °C.



Scheme 1. Scheme of the treatment.

2.2. Liver histology and immunohistochemistry

Liver sections were stained with hematoxylin–eosin (H&E) and prussian blue for pathological evaluation and iron deposition, respectively. Immunohistochemical staining for 3-nitrotyrosine (3-NT) adducts was performed by using anti-3-NT adducts IgG (Upstate, Lake Placid, NY), followed by a rabbit ABC staining system (Santa Cruz Biotechnology, Santa Cruz, CA).

2.3. Analysis of serum iron concentration, aspartate aminotransferase, alanine aminotransferase, total iron binding capacity and transferrin saturation

Serum iron concentration was determined by the generation of an iron–ferrozine colored complex (Galleano and Puntarulo, 1992). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity in serum were measured using the commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Total iron binding capacity of serum which estimates the degree of serum iron saturation was determined according to Ramsay (1952). Iron transferrin saturation was determined from the ratio of serum iron and total iron binding capacity.

2.4. Analysis of oxidative stress biomarkers in liver

Iron content in the liver digested sample was measured spectrophotometrically at 535 nm, after reacted with 2 mM bathophenanthroline disulfonic acid (Brumby and Massey, 1967). Lipid peroxidation estimated as thiobarbituric acid-reactive substances was measured following the method described by Buege and Aust (1978). The carbonyl content was measured as Peterson (1977) described. Sulfhydryl contents were measured spectrophotometrically at 412 nm using 5, 5-dithiobis (2-nitrobenzoic) acid (DTNB) (Sedlak and Lidsay, 1968). The activities of glutathione peroxidase (GPx) and superoxide dismutase (SOD) were measured according to the method introduced by Reglero et al. (2009).

2.5. Determination of NO_3^- in urine

Rats were moved to metabolism cages on day 20, given free access to water, and 4 h urine of every rats were collected. The urine was centrifuged at 3000 *g* for 10 min at room temperature. After removing the precipitation, the supernatant was injected to capillary electrophoresis. The capillary was employed with total and effective length 60.2 and 50 cm, respectively. The capillary cassette temperature was controlled at 25 °C and UV detector was set at 214 nm. The electrokinetic injection of samples onto the capillary was done at an applied negative voltage of −5 kV for 12 s, and the analysis was done at an applied negative voltage of −25 kV.

2.6. GSTs activity

GSTs activity with 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate was determined according to the method of Habig and Jakoby (1981).

2.7. Immunoprecipitations and Western blotting

For immunoprecipitation experiments, 100 μg of protein extracts was resuspended into immunoprecipitation buffer (50 mM Tris–HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA and 0.1% NP-40 including protease inhibitors) and then incubated with 2 μg of antibody against 3-NT or GSTs at 4 °C overnight. Immune complexes were collected by using protein A/G plus-Agarose for 3–5 h at 4 °C and washed four times with immunoprecipitation buffer. The immunoprecipitated proteins