Protective Effects of Baicalin against Ischemia/Reperfusion Injury in Rat Liver

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The cytoprotective properties of baicalin (**1**), a flavonoid glycoside isolated from *Scutellaria baicalensis*, have been investigated against injury to the liver caused by ischemia/reperfusion (I/R). Rats were subjected to 60 min of ischemia followed by 5 h of reperfusion, and **1** was administered intraperitoneally 24 and 1 h before ischemia. Following I/R, the levels of serum alanine aminotransferase and hepatic lipid peroxidation were elevated, whereas the hepatic glutathione content was decreased, with these changes attenuated by **1**. Serum levels of tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 were markedly increased by I/R, but suppressed by **1**. Baicalin attenuated increases in inducible nitric oxide synthase, cyclooxygenase (COX)-2, and TNF receptor 1-associated protein expression and augmented an increase in heme oxygenase-1 (HO-1). The increase in TNF-α, IL-6, and, COX-2 mRNA expression was attenuated by **1**, while the increase in HO-1 mRNA expression was augmented. Nuclear factor-κB nuclear localization was inhibited by **1**, and this compound limited the rate of mitochondrial swelling and the activation of caspases-3 and -8 observed in I/R rats. Rats treated with **1** had markedly fewer apoptotic cells than I/R rats. It was concluded that baicalin (**1**) exhibits antioxidant, anti-inflammatory, and antiapoptotic effects, which protect against hepatocellular I/R-induced damage.

Ischemia/reperfusion (I/R) injury to the liver occurs in many clinical conditions, such as liver transplantation, hepatic failure after shock and liver surgery, trauma, and cancer.[1] Although the nature of I/R injury has been studied extensively, the mechanisms by which organ damage occurs are unclear. A number of studies have suggested that the production of reactive oxygen species (ROS) during I/R of the liver is a major pathophysiological component of acute liver failure. The ROS generated by activated Kupffer cells in the early phase of reperfusion can destroy the cell membranes of parenchymal cells.[2] While necrosis is a major feature of damage in hepatic I/R injury, recent work has demonstrated that apoptosis plays a significant role in cellular damage after I/R injury.[3] The additional release of ROS evokes a secondary inflammatory response by promoting the synthesis of pro-inflammatory mediators that act as second messengers.[4] Endogenous antioxidants such as superoxide dismutase, catalase, and glutathione limit the effects of ROS but can become overwhelmed by the large amounts of ROS.[5]

The roots of *Scutellaria baicalensis* Georgi (Lamiaceae) are used as a traditional Chinese herbal medicine with anti-inflammatory and antibacterial properties and as a component of hepatoprotective herbal mixtures in the Far East.[6] Baicalin (**1**) is an active constituent of *S. baicalensis* roots and has antioxidative and anti-inflammatory activities.[7] This flavonoid glycoside has protective effects against various forms of acute and chronic experimental liver injury induced by carbon tetrachloride, iron overload, acetaminophen, or concanavalin A.[8,9] Recently, our group reported that **1** protects hepatocytes from oxidative damage caused by carbon tetrachloride; this protection is likely due to the induction of heme oxygenase-1 (HO-1) expression and the inhibition of pro-inflammatory mediators.[10] In addition, **1** protects against I/R injury through oxidant scavenging in the cardiomyocytes and brain.[11,12] However, there is no information on the protective effects of **1** against hepatic I/R injury. Therefore, in the present study, the hepatoprotective effects of baicalin (**1**) were examined against I/R injury, particularly on the extent of oxidative damage and apoptosis.

Results and Discussion

Serum Alanine Aminotransferase (ALT) Activity and Histological Analysis. Oxidative damage to biologically active molecules can be inhibited by antioxidants, including flavonoids isolated from a variety of natural sources.[13] Many studies have suggested the beneficial role, as an antioxidant, of baicalin (**1**) in reducing focal cerebral[12] and myocardial[11] I/R injury and hepatotoxin-induced hepatotoxicity.[10,14] However, there is no information on the protective effect of **1** against hepatic I/R injury. An investigation into the main mode of cell death that occurs in vivo after I/R is of clinical importance. During the ischemic period, the supply of glucose and oxygen is blocked and the metabolic and respiratory processes required for energy production are severely compromised, which lowers cellular ATP levels. This is believed to be responsible for the necrosis observed in tissues under I/R.

As shown in Table 1, the serum ALT activity was 30.7 ± 5.1 $U \cdot L^{-1}$ in sham-operated rats. At 5 h after reperfusion, the level of serum ALT released from ruptured necrotic hepatocytes increased to approximately 181-fold that in sham animals. The increase was attenuated significantly by pretreatment with **1**. This was supported by histological findings, as shown in Figure 1, which suggests that the livers from the sham animals had normal lobular architecture and cell structure. However, liver sections isolated after 5 h of reperfusion showed severe necrosis with disintegration of their hepatic cords and neutrophil infiltration. These pathological changes were also ameliorated by pretreatment with **1** (Table 1). These results suggest that **1** offers protection by preserving the structural integrity of the hepatocellular membrane against I/R injury.

Hepatic Lipid Peroxidation and Glutathione Content. Although the underlying mechanisms involved in hepatocyte death after I/R remain elusive, it has long been assumed that overproduction of ROS caused by the sequential combination of hypoxia and reoxygenation is responsible for the induction of cellular injury leading to necrosis. The attack of ROS on the biological membrane can lead to oxidative destruction of the membrane polyunsaturated fatty acids by lipid peroxidation. As shown in Table 2, the malondialdehyde (MDA) level in the liver tissues was 0.34 ± 0.01 nmol·mg $protein^{-1}$ in sham-operated animals. In ischemic animals,

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Table 1. Effect of Baicalin (**1**)[a] on Serum ALT Activity and Histopathologic Damage after Hepatic Ischemia/Reperfusion (means ± SEM, $n = 8$ to 10)

group	ALT ($U \cdot L^{-1}$)	histopathologic damage
sham		
vehicle	30.7 ± 5.1	0.30 ± 0.11
1	42.5 ± 5.2	0.45 ± 0.16
I/R		
vehicle	5554.3 ± 720.1[b]	2.67 ± 0.17[b]
1	2658.1 ± 321.5[b,d]	1.60 ± 0.16[b,c]

[a] Compound **1** (200 $mg \cdot kg^{-1}$) was administered intraperitoneally 24 and 1 h before ischemia. [b] Significantly different ($p < 0.01$) from vehicle-treated sham group. [c] Significantly different ($p < 0.05$) from vehicle-treated I/R group. [d] Significantly different ($p < 0.01$) from vehicle-treated I/R group.

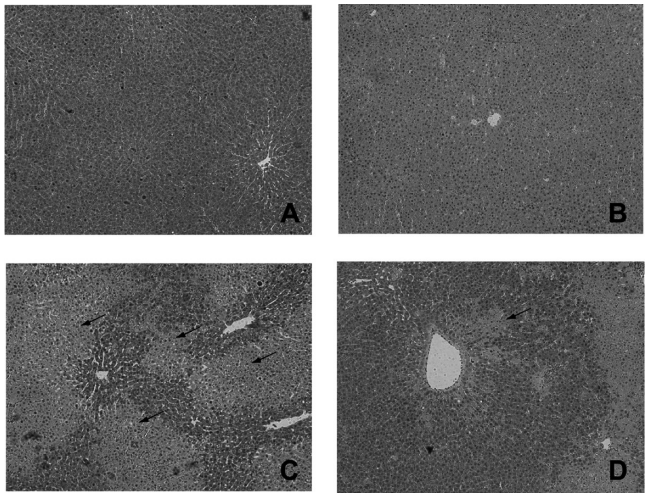

Figure 1. Effect of baicalin (**1**) on the histological changes of rat liver after hepatic ischemia/reperfusion (I/R; original magnification 100×). (A) Vehicle-treated sham group. (B) **1**-treated sham group. (C) Vehicle-treated I/R group: multiple and extensive areas of hepatocyte necrosis distributed through the hepatic parenchyma with liver cell dysplasia. (D) **1**-treated I/R group: minimal hepatocellular necrosis and liver cell dysplasia. The arrows identify necrotic areas.

Table 2. Effect of Baicalin (**1**)[a] on Lipid Peroxidation and Glutathione Content after Hepatic Ischemia/Reperfusion (means ± SEM, $n = 8$ to 10)

group	MDA ($nmol \cdot mg\ protein^{-1}$)	GSH ($\mu mol \cdot g\ liver^{-1}$)
sham		
vehicle	0.34 ± 0.01	5.58 ± 0.21
1	0.35 ± 0.03	5.50 ± 0.49
I/R		
vehicle	0.52 ± 0.03[b]	3.53 ± 0.31[b]
1	0.31 ± 0.04[c]	4.98 ± 0.13[c]

[a] Compound **1** (200 $mg \cdot kg^{-1}$) was administered intraperitoneally 24 and 1 h before ischemia. [b] Significantly different ($p < 0.01$) from vehicle-treated sham group. [c] Significantly different ($p < 0.01$) from vehicle-treated I/R group.

the MDA level increased to approximately 1.6 times that in the sham animals.

Reduced glutathione (GSH) plays an important role as a free radical scavenger to counteract the deleterious effects of ROS. The GSH content in sham-operated animals was 5.58 ± 0.21 $\mu mol \cdot g\ liver^{-1}$. In ischemic animals, the hepatic GSH level markedly decreased after 5 h of reperfusion with a concomitant increase in the level of lipid peroxidation. Treatment with baicalin (**1**) attenuated a decrease in hepatic GSH content and lipid peroxidation during I/R (Table 2).

Serum Tumor Necrosis Factor-α (TNF-α) and Interleukin (IL)-6 Levels and mRNA Expression. During reperfusion, activated Kupffer cells produce a number of signaling molecules that promote inflammatory reactions, which are the main cause of damage to liver cells. TNF-α is a pleiotropic pro-inflammatory cytokine that is produced rapidly by macrophages in response to tissue injury and induces phagocyte oxidative metabolism and nitric oxide (NO) production.[15] Furthermore, TNF-α activates neutrophilic granulocytes and stimulates them to deliver various inflammatory mediators, as an important inflammatory cytokine in I/R injury.[16] IL-6 is also released in vivo during I/R, but this release is delayed compared with TNF-α and IL-1, the serum levels of which rise within minutes of reperfusion.[17] Other studies have shown that hypoxia induces release of IL-6 from cultured lymphocytes, endothelial cells, and myocytes through the activation of nuclear factor-κB (NF-κB).[18] As shown in Table 3, the serum TNF-α and IL-6 levels in sham-operated animals were low. After 5 h of reperfusion, the serum TNF-α and IL-6 levels increased significantly to 6.1 and 7.2 times that observed in sham-operated animals, respectively. This increase was attenuated significantly by baicalin (**1**) pretreatment. Figure 2 shows the TNF-α and IL-6 mRNA expression in liver tissue. After 5 h of reperfusion, the levels of TNF-α and IL-6 mRNA expression increased significantly compared with sham-operated animals. Baicalin inhibited the increase in IL-6 mRNA expression, but did not affect the increase in TNF-α mRNA expression.

Table 3. Effect of Baicalin (**1**)[a] on Serum TNF-α and IL-6 Levels after Hepatic Ischemia/Reperfusion (means ± SEM, $n = 8$ to 10)

group	serum TNF-α ($pg \cdot mL^{-1}$)	serum IL-6 ($pg \cdot mL^{-1}$)
sham		
vehicle	22.9 ± 3.7	27.4 ± 3.9
1	17.8 ± 2.4	37.6 ± 3.8
I/R		
vehicle	139.3 ± 23.1[b]	196.8 ± 20.6[b]
1	64.8 ± 5.9[b,c]	77.4 ± 8.1[b,c]

[a] Compound **1** (200 $mg \cdot kg^{-1}$) was administered intraperitoneally 24 and 1 h before ischemia. [b] Significantly different ($p < 0.01$) from vehicle-treated sham group. [c] Significantly different ($p < 0.01$) from the vehicle-treated I/R group.

Inducible Nitric Oxide Synthase (iNOS) and Cyclooxygenase (COX)-2 Protein and mRNA Expression. NO is a highly reactive oxidant that is produced by parenchymal and nonparenchymal liver cells from L-arginine through the action of NOS. The notion that NO is involved in acute liver injury is based on several observations that toxin-induced hepatic damage is associated with increased NO production by the liver.[19] However, it remains to be determined as to whether or not the augmented production of NO has a protective or deleterious role in the liver. COX-2 is the mitogen-inducible isoform of COX, and it is induced in macrophages by several pro-inflammatory stimuli, such as cytokines and growth factors, leading to COX-2 expression and the subsequent release of prostaglandins. The 5′-promoter region of COX-2 was reported to contain two putative NF-κB binding sites, and NF-κB has been shown to be a positive regulator of COX-2 expression in murine macrophages and human adenocarcinoma cell lines.[20] Chou et al.[21] reported that baicalin (**1**) shows anti-inflammatory and analgesic effects through the inhibition of both important inflammatory mediators and pro-inflammatory cytokines as well as neutrophil infiltration at the site of inflammation. Moreover, **1** suppresses the LPS-induced expression of iNOS and COX-2 in RAW 264.7 mouse macrophages.[22] After 5 h of reperfusion, the levels of iNOS and COX-2 mRNA expression increased significantly compared with sham-operated animals. Baicalin inhibited the increase in COX-2 mRNA expression, but did not affect the increase in iNOS mRNA expression (Figure 2). Similarly, along with the increase in the mRNA expression, the levels of iNOS and COX-2 protein expression increased signifi-

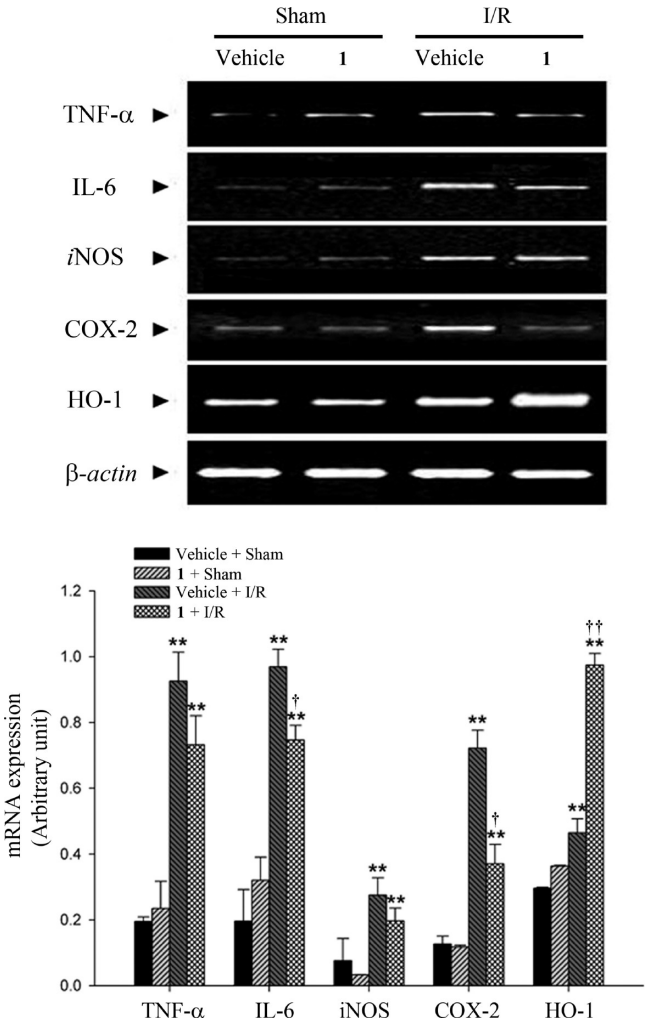

Figure 2. Effect of baicalin (**1**) on TNF-α, IL-6, iNOS, COX-2, and HO-1 mRNA expression after hepatic ischemia/reperfusion (I/R). Values are means ± SEM for 8−10 rats per group. **Significantly different ($p < 0.01$) from vehicle-treated sham group. †, ††Significantly different ($p < 0.05$, $p < 0.01$) from vehicle-treated I/R group.

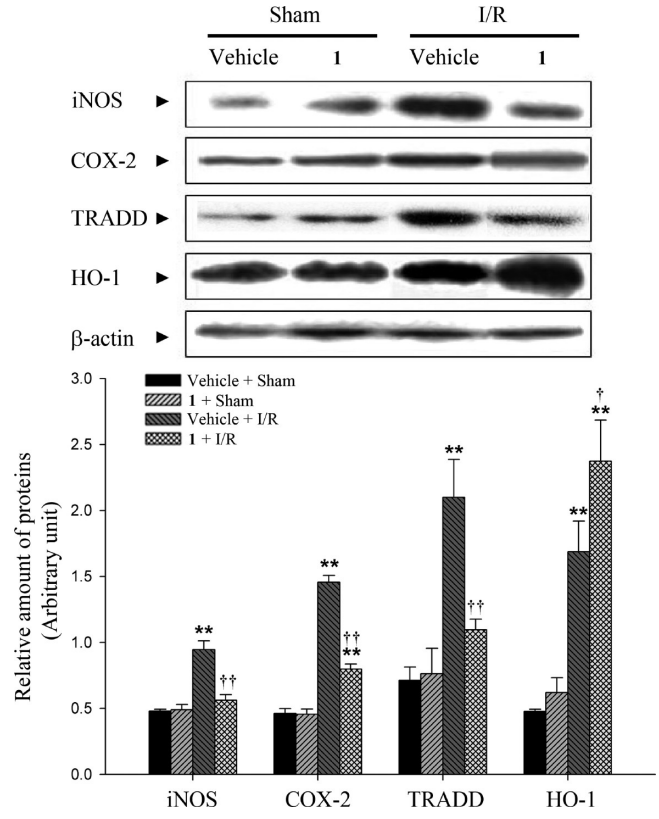

Figure 3. Effect of baicalin (**1**) on iNOS, COX-2, TRADD, and HO-1 protein expression after hepatic ischemia/reperfusion (I/R). Values are means ± SEM for 8−10 rats per group. **Significantly different ($p < 0.01$) from vehicle-treated sham group. †, ††Significantly different ($p < 0.05$, $p < 0.01$) from vehicle-treated I/R group.

cantly compared with those in sham-operated animals after 5 h of reperfusion. Baicalin reduced iNOS and COX-2 expression (Figure 3).

Heme Oxygenase-1 (HO-1) Protein and mRNA Expression. The I/R-induced liver inflammatory process is regulated not only by pro-inflammatory mediators that initiate and exacerbate inflammation but also by anti-inflammatory mediators that resolve inflammation. HO-1 is a cytoprotective enzyme that is induced by a variety of stimuli such as cytokines, heavy metals, and oxidants. Several studies have shown that up-regulation of HO-1 expression by inducers, such as hemin or cobalt protoporphyrin, dramatically attenuates pathological activities including inflammation and vascular proliferation and decreases chronic transplant rejection.[23,24] Previous studies have reported that HO-1 induction inhibits inflammatory cytokines such as TNF-α and attenuates iNOS expression, and also exerts important functions in cellular defense against oxidative insult.[25] Among the various attempts made previously to attenuate I/R injury, one of the possible approaches for protecting a reperfused organ is to enhance cell defenses by inducing endogenous defense mechanisms that are selectively engaged in restoring cellular homeostasis and function. In this regard, modulation of HO-1 activity has emerged as a promising target for hepatoprotection.[26] In addition, a range of dietary and naturally occurring antioxidants, including baicalin (**1**), are considered to be beneficial in several experimental models because of the induction of HO-1.[27] In the present study, the levels of HO-1 mRNA and protein expression markedly increased within 5 h after reperfusion. Interestingly, treatment with **1** augmented increases in HO-1 mRNA and protein expression (Figures 2 and 3). These findings suggest that a strong induction response of HO-1 by **1** results in the protection of liver cells from I/R-induced oxidative cellular injuries.

Inhibitor of NF-κB-α (IκB-α) Degradation and NF-κB/p65 Nuclear Localization. NF-κB is a ubiquitous transcription factor that plays a critical role in the regulation of the inducible gene expression in immune and inflammatory responses. Most inhibitors of NF-κB activation mediate their effects through suppression of phosphorylation and degradation of IκB-α.[28] The present results showed that NF-κB/p65 subunit nuclear localization markedly increased after reperfusion (Figure 4). This is consistent with other reports, which suggest that free radicals regulate a series of signal transduction pathways and activate NF-κB during the acute phase of I/R.[29] Baicalin (**1**) represents a class of antioxidants closely associated with an ability to inhibit both NF-κB activation and the inflammatory response associated with its activation.[30] Baicalin inhibited the nuclear localization of the NF-κB/p65 subunit and the degradation of IκB-α (Figure 4).

TNF Receptor 1-Associated Protein (TRADD), Mitochondrial Swelling, Caspases-3 and -8 Activities, and Apoptotic Cell Death. Recent studies have suggested that mitochondrial dysfunction caused by ROS is involved in the processes of both necrotic and apoptotic cell death. The ATP depletion that occurs during ischemia leads to a loss of the mitochondrial transmembrane potential. This is associated with Ca^{2+} overload and causes the opening of the mitochondrial permeability transition (MPT) pore. Once the MPT pores are opened, the mitochondria undergo rapid

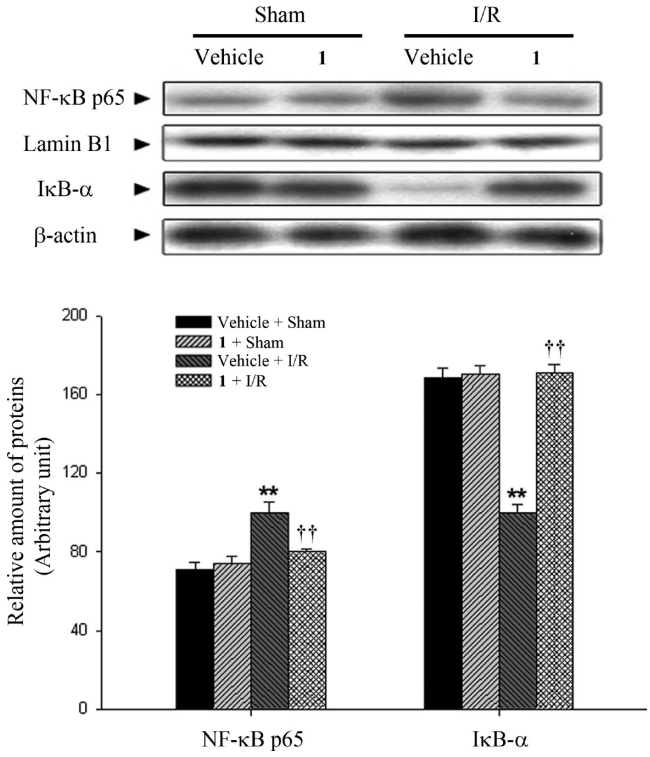

Figure 4. Effect of baicalin (**1**) on NF-kB nuclear localization after hepatic ischemia/reperfusion (I/R). Nuclear NF-κB/p65 subunit and cytoplasmic IκB-α in liver tissure were assessed by western blot analysis. Density values of nuclear NF-κB/p65 subunit and IκB-α were normalized to the nuclear lamin B1 and cytosolic β-actin, respectively. Values are means ± SEM for 8−10 rats per group. **Significantly different ($p < 0.01$) from vehicle-treated sham group. ††Significantly different ($p < 0.01$) from vehicle-treated I/R group.

swelling and rupture due to ion overload and a hyperosmotic effect. The cytochrome *c* localized at the intermembrane space is then released into the cytosol, which then activates caspase-9. This, in turn, activates caspase-3, which then causes DNA fragmentation.[31]

Recent studies have shown that TNF-α and its coupling with TNF-R are responsible for hepatocyte apoptosis induced by various kinds of stresses, including viral hepatitis, alcoholic hepatitis, fulminant hepatic failure, and I/R injury.[32] Coupling of TNF-α to TNF-R1 triggers apoptosis by binding with the adaptor proteins TRADD and Fas-associated death domain, which facilitates recruitment and activation of caspase-8.[33] Caspase-8 then triggers the activation of caspase-3 through multiple apoptosis signal pathways, and this process is known to be essential for TNF-mediated apoptotic signaling during I/R injury.[34] As shown in Figure 3 and Table 4, I/R caused an obvious disruption of the mitochondrial structure, which was characterized by an increase in the swelling rate. The mitochondria from rats pretreated with baicalin (**1**) showed a lower level of mitochondrial swelling. After 5 h of reperfusion, the levels of TRADD protein expression and caspase-3 and -8 were significantly higher after 5 h of reperfusion, and these increases were inhibited by pretreatment with **1**. The biochemical parameters for apoptotic cell death paralleled the morphological changes observed with TUNEL staining. A large number of TUNEL-positive hepatocytes were observed in liver tissues obtained 5 h after reperfusion. However, few TUNEL-positive hepatocytes were observed in livers treated with **1** (Figure 5). This observation indicates that the protective effect of **1** might be related to the TNF-mediated apoptotic pathway. Overall, these results suggest that **1** suppresses the mitochondrial permeability transition pore opening, which may contribute to the antiapoptotic properties of this compound.

Table 4. Effect of Baicalin (**1**)[a] on Mitochondrial Permeability and Caspase-3, and -8 Activities after Hepatic Ischemia/Reperfusion (I/R; means ± SEM, $n = 8$ to 10)

group		initial rate of swelling ($\Delta A \times 10^{-2} \cdot min^{-1} \cdot mg$ protein^{-1})	caspase-3 activity (% of vehicle-treated I/R)	caspase-8 activity (% of vehicle-treated I/R)
sham	vehicle	0.0080 ± 0.0092	51.7 ± 1.5	45.6 ± 3.3
	1	0.0070 ± 0.0016	51.0 ± 2.4	49.6 ± 2.1
I/R	vehicle	0.0311 ± 0.0070[c]	100.0 ± 20.8[c]	100.0 ±15.8[b]
	1	0.0094 ± 0.0019[e]	64.7 ± 4.9[e]	48.7 ± 9.2[d]

[a] Compound **1** (200 mg·kg^{-1}) was administered intraperitoneally 24 and 1 h before ischemia. [b] Significantly different ($p < 0.05$) from vehicle-treated sham group. [c] Significantly different ($p < 0.01$) from vehicle-treated sham group. [d] Significantly different $p < 0.05$) from vehicle-treated I/R group. [e] Significantly different ($p < 0.01$) from vehicle-treated I/R group.

In conclusion, it has been demonstrated that baicalin (**1**) affords hepatoprotection against I/R injury by reducing hepatocyte cell death and the expression of inflammatory genes through suppression of oxidative stress and NF-κB nuclear localization. The present results also suggest that, as a downstream effector of **1**, HO-1 mediates the protective effect of this flavonoid glycoside against I/R-induced liver injury. Therefore, pretreatment with **1** might be useful as a potential pharmacological maneuver for attenuating the level of I/R injury.

Experimental Section

Hepatic Ischemia Procedures. Male Sprague−Dawley rats (270−300 g; Dae Han Biolink, Co., Ltd., Eumsung, Korea) were fasted for 18 h before experiments and were provided with tap water ad libitum. This study was approved by the Animal Care Committee of Sungkyunkwan University, Korea, and all animals were handled according to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health guidelines. The rats were anesthetized intraperitoneally with ketamine (55 mg·kg^{-1}) and xylazine (8 mg·kg^{-1}), and the body temperature was maintained at 37 °C throughout the anesthesia using a heating pad. A midline incision was made to the abdomen, and the left branches of the portal vein and hepatic artery were clamped to induce complete ischemia of the median and left lobes of the liver. The right lobes remained perfused to prevent venous congestion of the intestine. After 60 min of ischemia, the clamp was removed to allow reperfusion. The wound was closed in layers using silk sutures, and the animals were returned to their cages and allowed to recover from the anesthesia. The sham control animals were prepared in a similar manner except that a clamp was not placed on the left and median lobes. The rats were sacrificed 5 h after reperfusion, and blood samples were taken from the abdominal aorta. The blood samples were allowed to coagulate and then centrifuged to obtain the serum. Ischemic liver tissues were collected at the same time. The liver tissue was immediately analyzed for any histological changes (aliquots from the left lobe), and blood was examined for serum ALT activity and TNF-α and IL-6 levels. The remaining major portions of the liver tissues were frozen immediately in liquid nitrogen and kept at −75 °C until biochemical analysis.

Administration of Baicalin (1). Baicalin (**1**; 5,6-dihydroxyflavone 7-*O*-β-D-glucopyranoside; 95% purity; Sigma Chemical Company, St. Louis, MO), suspended in 10% Tween 80−phosphate-buffered saline, was administered intraperitoneally (200 mg·kg^{-1}), 24 and 1 h before the sustained ischemia. The dose was selected on the basis of previous studies on the suppression of the in vivo expression of pro-inflammatory mediators in mice challenged with carbon tetrachloride.[10] Rats were divided into four treatment groups: (a) vehicle-treated sham, (b) **1**-treated sham, (c) vehicle-treated ischemic, and (d) **1**-treated ischemic.

Serum ALT Activity. The serum ALT activity was determined by standard spectrophotometric procedures using a ChemiLab ALT assay kit (IVDLab Co., Ltd., Uiwang, Korea).

Histological Analysis. Liver tissues were removed from a portion of the left lobe, fixed immediately in 10% neutral buffered formalin, embedded in paraffin, and cut serially into 5 μm sections. The hematoxylin and eosin-stained sections were evaluated using an optical microscope (Olympus Optical Co., Tokyo, Japan). The histological

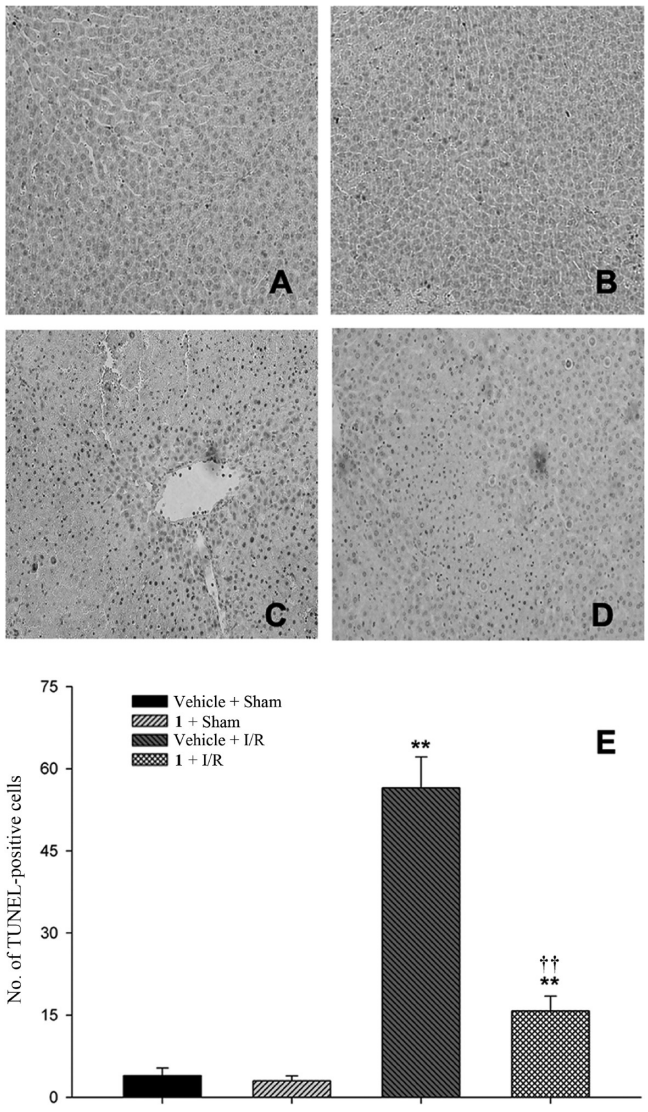

Figure 5. TUNEL stained histology of serial sections of rat liver after hepatic ischemia/reperfusion (original magnification ×200). (A) Vehicle-treated sham group. (B) Baicalin (**1**)-treated sham group. (C) Vehicle-treated I/R group: a large number of TUNEL-positive cells were detected. (D) **1**-treated I/R group: only a few TUNEL-positive cells were detected. (E) TUNEL-positive hepatocytes. Numbers are apoptotic cells in randomly chosen 400× histological fields. **Significantly different ($p < 0.01$) from vehicle-treated sham group. ††Significantly different ($p < 0.01$) from vehicle-treated I/R group.

changes were evaluated at 200× magnification by a point-counting method for severity of hepatic injury using an ordinal scale, according to the method reported by Camargo et al.[35]

The stained sections were graded as follows: grade 0, minimal or no evidence of injury; grade 1, mild injury consisting of cytoplasm vacuolation and focal nuclear pyknosis; grade 2, moderate to severe injury with extensive nuclear pyknosis, cytoplasmic hypereosinophilia, and loss of intercellular borders; grade 3, severe necrosis with disintegration of hepatic cords, hemorrhage, and neutrophil infiltration. Apoptotic cells were detected by TUNEL staining with a commercially available kit (In situ Apoptosis Detection Kit; TaKaRa Co., Shiga, Japan). Under the microscope, the number of TUNEL-positive cells in 400× histological fields was counted per liver section.

Hepatic Lipid Peroxidation and Glutathione Content. The steady-state level of MDA, which is the end-product of lipid peroxidation, was analyzed in the liver homogenates by measuring the level of thiobarbituric acid-reactive substances spectrophotometrically at 535 nm, as described by Buege and Aust,[36] using 1,1,3,3-tetraethoxypropane (Sigma) as the standard. After precipitation with 1% picric acid, the total glutathione level was determined in the liver homogenates using yeast-glutathione reductase, 5,5′-dithiobis(2-nitrobenzoic acid), and NADPH, at 412 nm. The oxidized glutathione (GSSG) level was measured by the same method in the presence of 2-vinylpyridine, and the reduced glutathione level was determined as the difference between the total glutathione and GSSG levels.[37]

Serum TNF-α and IL-6 Levels. TNF-α and IL-6 levels were quantified using commercial TNF-α and IL-6 ELISA kits (eBiosciences Co., San Diego, CA), respectively.

Western Blot Immunoassay. Freshly isolated liver tissue was homogenized in lysis buffer. Nuclear protein was isolated from fresh rat livers using NE-PER Nuclear and Cytoplasmic Extraction Reagents, according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL). A 20 μg sample of protein from each liver homogenate and nuclear and cytosolic fraction was loaded per lane on 10% polyacrylamide gels for electrophoresis. The proteins were then transferred to nitrocellulose membranes and incubated at room temperature for 60 min in buffer containing 5% dried skimmed milk to block nonspecific binding. Bands were detected immunologically using polyclonal antibodies against rat iNOS, COX-2, HO-1, NF-κB/p65, IκB-α, and lamin B1, or monoclonal antibody against rat β-actin. The binding of all antibodies was detected using an ECL detection system (iNtRON Biotechnology Co., Ltd., Seongnam, Korea), according to the manufacturer's instructions. The intensity of the immunoreactive bands was determined using a densitometric analysis program.

Reverse Transcription Polymerase Chain Reaction (RT-PCR). Reverse transcription of the total RNA extracted from the tissue samples was carried out in order to synthesize first-strand cDNA using the oligo $(dT)_{12-18}$ primer and TaKaRa RNA PCRTM AMV Kit (Ver. 3.0: TaKaRa Co.). The PCR reaction was performed with a diluted cDNA sample in a 20 μL reaction volume. The final reaction concentrations were as follows: primers, 10 μM; dNTP mix, 250 μM; ×10 PCR buffer; and Ex Taq DNA polymerase, 0.5 U per reaction. The gene-specific primers are listed in Table S1, Supporting Information. RT-PCR was carried out with an initial denaturation step at 94 °C for 5 min and a final extension step at 72 °C for 7 min using a GeneAmp 2700 thermocycler (Applied Biosystems, Foster City, CA). The amplification cycling conditions were as follows: 30 cycles of 30 s at 94 °C, 30 s at 56 °C, and 60 s at 72 °C for TNF-α; 40 cycles of 30 s at 94 °C, 45 s at 65 °C, and 45 s at 72 °C for IL-6; 32 cycles of 45 s at 94 °C, 45 s at 65 °C, and 60 s at 72 °C for iNOS; 40 cycles of 30 s at 94 °C, 30 s at 65 °C, and 60 s at 72 °C for COX-2; 30 cycles of 30 s at 94 °C, 30 s at 65 °C, and 45 s at 72 °C for HO-1; 25 cycles of 30 s at 94 °C, 30 s at 62 °C, and 60 s at 72 °C for β-actin. After RT-PCR, 10 μL samples of the PCR products were visualized by ultraviolet illumination after electrophoresis through 1.5% agarose gel and ethidium bromide staining. The intensity of each PCR product was analyzed semi-quantitatively using a digital camera (DC120, Eastman Kodak, New Haven, CT) and analysis software.

Isolation of Liver Mitochondria and Cytosolic Fraction. The liver mitochondrial fraction was prepared using the method reported by Johnson and Lardy.[38] Briefly, the tissues were homogenized on ice using a Teflon pestle homogenizer in medium containing 250 mM sucrose, 10 mM Tris-HCl, and 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.2 at 4 °C. The homogenate was centrifuged at 600*g* for 10 min, and the supernatant was centrifuged for 5 min at 15000*g* to obtain the mitochondrial pellet. The mitochondrial pellet was washed with medium containing no added EDTA and then centrifuged for 5 min at 15000*g*, producing a final pellet containing approximately 50 mg protein·mL^{-1}. The 15000*g* supernatant was centrifuged at 100000*g* for 30 min. The resulting supernatant was used as the particulate-free cytosolic fraction. Protein content was estimated by the dye-binding assay of Bradford.[39]

Mitochondrial Swelling. The rate of mitochondrial swelling, which indicates the level of the MPT, was determined from the change in the absorbance of a mitochondrial suspension at 520 nm as reported by Elimadi et al.[40] Briefly, the liver mitochondria (4 mg) were isolated from animals in each experimental group, and 4 mL of phosphate buffer (pH 7.2) containing 250 mM sucrose, 5 mM KH_2PO_4, and 1 μM rotenone at 25 °C was added. The resulting suspension (1.8 mL) was then added to both the sample and reference cuvettes. Succinate (6 mM) was added to the sample cuvette only. The cuvettes were scanned at a wavelength of 520 nm.

Measurement of Caspases-3 and -8 Activities. Caspase activities were measured using an in vitro fluorogenic peptide substrate, Ac-Asp-Glu-Val-Asp *p*-nitroanilide for a caspase-3 substrate peptide and Ac-Ile-Glu-Thr-Asp *p*-nitroaniline for a caspase-8 substrate peptide (Bio-Mol, Plymouth Meeting, PA), according to the procedure reported by Morin et al.[41] The liver tissue (1 g) was homogenized in 6 mL of buffer containing 25 mM Tris, 5 mM $MgCl_2$, 1 mM ethylene glycol-bis[aminoethyl ether]-*N*,*N*,*N*,*N* tetraacetic acid (EGTA), and 50 μL of protease inhibitor cocktail (Sigma). The homogenate was centrifuged at 600*g* for 10 min. The supernatant was centrifuged again for 15 min at 40000*g*, and the resulting supernatant was collected to determine the caspases-3 and -8 activities. Dithiothreitol (10 mM) was added immediately to the samples before freezing. The caspases-3 and -8 activities were assayed in a total volume of 100 μL. Thirty micrograms of the cytosolic protein was incubated in buffer containing 30 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid], 0.3 mM EDTA, 100 mM NaCl, 0.15% Triton X-100, and 10 mM dithiothreitol. The samples were incubated at room temperature for 15 min. The caspase reaction was then started by adding 200 μM DEVD-AFC, and the resulting mixture was incubated at 37 °C. The change in fluorescence (excitation at 400 nm and emission at 490 nm) was monitored after 120 min.

Statistical Analysis. All results are presented as means ± SEM. The overall significance of the data was examined by one-way analysis of variance. The differences between the groups were considered statistically significant at a $p < 0.05$ with the appropriate Bonferroni correction made for multiple comparisons.

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Supporting Information Available: Table of PCR primers used. This material is available free of charge via the Internet at http://pubs.acs.org.

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