Beneficial effects of rutin and L-arginine coadministration in a rat model of liver ischemia-reperfusion injury

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Acquaviva R, Lanteri R, Li Destri G, Caltabiano R, Vanella L, Lanzafame S, Di Cataldo A, Li Volti G, Di Giacomo C. Beneficial effects of rutin and L-arginine coadministration in a rat model of liver ischemia-reperfusion injury. Am J Physiol Gastrointest Liver Physiol 296: G664–G670, 2009. First published December 24, 2008; doi:10.1152/ajpgi.90609.2008.—Reperfusion following liver ischemia results in oxidative stress leading to liver injury. The aim of this study was to investigate the combined effects of two antioxidant agents, rutin and L-arginine, in rat liver ischemia-reperfusion (I/R). Male Wistar rats were divided into five groups: 1) sham operated, 2) I/R, 3) I/R + rutin, 4) I/R + L-arginine, and 5) I/R + rutin + L-arginine. Plasmatic and hepatic levels of alanine transaminase (ALT), aspartate transaminase (AST), lipid peroxides (LOOH), and thiol groups (RSH) were examined, as well as DNA fragmentation and liver histopathology. Furthermore, to elucidate the pathophysiological processes involved in the antioxidant mechanism(s) of rutin and L-arginine, we assessed the expression of inducible nitric oxide synthase (iNOS) and cytoprotective mechanisms activated during cellular stress, hyperthermic preconditioning, or long-term fasting, exerting antioxidant defenses, and induction of heat shock protein, among which heme oxygenase (HO)-1, playing both key roles in the biochemical cascade of liver injury. Significant increase in plasmatic ALT and AST activities were observed in untreated I/R rats compared with sham-operated animals, whereas treatment with rutin or L-arginine in I/R rats reduced hepatic damage. Interestingly, combined therapy with rutin and L-arginine resulted in a further reduction of plasmatic ALT and AST activities compared with rutin or L-arginine alone. These results were further confirmed by the analysis of DNA fragmentation, LOOH, RSH groups, and liver histopathology, which showed the highest protective effects following the coadministration of rutin and L-arginine. Finally, the combined therapy protocol resulted in a significant induction of liver HO-1 and a concomitant reduction of iNOS expression that may both be responsible for the beneficial effects of the proposed pharmacological protocol.

heme oxygenase; nitric oxide synthase; oxidative stress; total thiol groups; lipid hydroperoxides; DNA damage; polyphenols

HEPATIC ISCHEMIA-REPERFUSION (I/R) injury involves a complex series of processes culminating in hepatocellular injury. I/R injury is a common consequence of hepatic surgery, particularly following hepatotomies and liver transplantation, and is complicated by microcirculatory failure, followed by necrosis and cell death (23, 39). In this context, oxidative damage is thought to play an important role, including the outcome of organ transplantation. I/R is characterized by the formation of reactive oxygen species (ROS) on reintroduction of molecular oxygen to ischemic tissues, and this phenomenon (the sudden reintroduction of blood into ischemic tissue) results in massive tissue disruption, widespread lipid and protein oxidative modifications, enzymes release, reductions in high-energy phosphate stores, mitochondrial injury, release of proinflammatory cytokines, apoptosis, necrosis, and ultimately, organ failure (7, 10). In addition, after reperfusion, blood flow may not return uniformly to all portions of the ischemic tissue, a phenomenon termed as “no-reflow,” which further contributes to cellular injury and necrosis (21). Furthermore, I/R results in cellular injury, triggering a complex series of biochemical events, among which nitric oxide (NO) release seems to play a key role (36). NO, generated by any of the three isoforms of NO synthase (NOS), plays an important role in liver function (16, 28, 40). However, under pathological states, the inhibition of the expression or activity of inducible NOS (iNOS) (38) can ameliorate or prevent liver I/R injury, suggesting that NO generated by iNOS contributes to liver I/R injury.

On the other hand, in response to enhancement of oxidative stress, cells undergo specific changes in enzyme activities, cytoskeletal structure, membrane transport, antioxidant defenses, and induction of heat shock protein, among which heme oxygenase (HO)-1 is believed to play a key role (14, 37). The HO system is among the most critical of the cytoprotective mechanisms activated during cellular stress, hyperthermic preconditioning, or long-term fasting, exerting antioxidant and anti-inflammatory functions, acting as a molecular chaperon, modulating cell cycle, and maintaining microcirculation (3, 9, 20).

Intensive research efforts have been focused on the amelioration of various pathophysiological components of I/R injury to limit the extent of tissue injury and necrosis. In this regard, there is increasing interest in the protective, “in vivo” effects of substances supplied with diet against oxidative damage. Several free radical scavengers have been evaluated in I/R injury (12, 37). In particular, previous studies have demonstrated the antioxidant properties of rutin and the implication of L-arginine in NO-mediated pathways (24, 25, 33). The aim of the present study was therefore to evaluate the effects of coadministration of rutin and L-arginine on hepatic structural and functional parameters in rats submitted to an experimental model of hepatic I/R.

MATERIALS AND METHODS

Chemicals. Rutin and L-arginine were purchased from Sigma Aldrich (St. Louis, MO), aspartate transaminase (ALT) and aspartate transaminase (AST) kits were obtained from Chematil (Rome, Italy),
and the QIAMP DNA mini kit was purchased from Qiagen (Milan, Italy). Monoclonal HO-1 and endothelial NOS (eNOS) antibodies were obtained from Stressgen Biotechnologies (Victoria, BC, Canada). iNOS antibody and secondary horseradish peroxidase-conjugated anti-mouse antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The enhanced chemiluminescence system for developing immunoblots and nitrocellulose membranes was purchased from Amersham (Milan, Italy). All other chemicals were purchased from Merck (Frankfurt, Germany).

**Animals and surgical procedure.** All the experimental procedures reported in this study were conducted in conformity with principles in the care and use of animals and the protocol used was approved by the National Institutional Animal Care and Use Committee of University of Catania, Italy. Male Wistar rats (body weight: 200–220 g) were fed with balanced diet and kept in temperature (20°C)- and humidity (50%)-controlled rooms. Pharmacological treatments were performed as follows: rutin (30 mg/kg ip) was administered once a day for 3 days before I/R; l-arginine (400 mg/kg ip) was administered immediately after ischemia. The animals fasted for 12 h before experiments but were allowed free access to water. Rats were randomly divided into five groups: 1) sham operated, 2) I/R, 3) I/R + rutin, 4) I/R + l-arginine, and 5) I/R + rutin + l-arginine.

Animals underwent normothermic ischemia by selective occlusion of the portal vein and hepatic artery for 30 min by using vascular clips with ethidium bromide (46). The gel was then photographed under ultraviolet luminescence. In these conditions, damaged DNA appears as a ladder consisting of DNA fragments, whereas intact DNA is high molecular weight and does not migrate very far into the gel.

**Histopathological observations.** Liver tissue was fixed in 10% buffered formalin for 24 h and then embedded in paraffin, and 10-μm sections were stained with hematoxylin and eosin.

**Western blotting.** Liver homogenates were collected for Western blot analysis, and protein levels were visualized by immunoblotting with antibodies against HO-1, eNOS, or iNOS. Briefly, aliquots of homogenate containing 50 μg of proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. To block non-specific binding sites, we blocked the membranes by incubation overnight with 5% nonfat dry milk in 10 mM Tris·HCl (pH 7.4)-150 mM NaCl-0.05% Tween 20 (TBST) buffer at 4°C. After being washed with TBST, the membranes were incubated with a 1:1,000 dilution of anti-HO-1 antibody or a 1:500 dilutions of anti-eNOS or anti-iNOS for 1 h at room temperature with constant shaking. The filters were then washed and subsequently probed with horseradish peroxidase-conjugated anti-mouse IgG for HO-1 at a dilution of 1:2,000 or anti-rabbit for eNOS or iNOS at a dilution of 1:20,000. Chemiluminescence detection was performed using an enhanced chemiluminescence detection kit according to the manufacturer’s instructions. Protein content was evaluated according to the method of Lowry (29).

**Statistical analysis.** One-way analysis of variance (ANOVA) followed by Bonferroni’s t-test was performed to estimate significant differences among groups. Data are means ± SD, and differences between groups were considered to be significant at P < 0.05.

**RESULTS**

**Determination of plasma ALT and AST activities.** Our results demonstrated significant alterations in hepatic functions in untreated I/R rats compared with sham-operated animals; in fact, significant increases in plasmatic ALT and AST activities were observed (Fig. 1). These enzymes are specific markers of liver injury and are released from the liver following a stressful insult. Treatments with rutin and l-arginine significantly reduced plasmatic ALT and AST activity in I/R rats, whereas no effects were observed in sham-operated rats. In particular, pretreatment with rutin was more efficient than pretreatment with l-arginine in reducing ALT and AST activities of I/R rats. Interestingly, rats treated with both molecules showed a further significant reduction in plasmatic ALT and AST activities with respect to I/R rats treated with rutin or l-arginine alone. No significant modifications were observed in treated sham-operated rats.

**Determination of LOOH and RSH levels.** Liver damage was also confirmed by a significant increase of plasmatic and hepatic LOOH levels in I/R rats (Fig. 2). Treatment with rutin or l-arginine efficiently counteracted the increased lipoperoxidation in I/R rats; however, pretreatment with rutin was more efficient than pretreatment with l-arginine. Surprisingly, coadministration of rutin and l-arginine did not further reduce LOOH levels compared with rutin-treated animals.

Hepatic damage was also evaluated by RSH determination. Plasmatic and hepatic RSH levels were significantly decreased in untreated I/R rats with respect to controls, whereas treatment with rutin or l-arginine inhibited both plasmatic and liver decreases of RSH in I/R rats (Fig. 3). The coadministration of rutin and l-arginine did not further induce significant modification with respect to I/R rats treated with rutin or l-arginine alone.
DNA fragmentation. Our results (Fig. 4) showed a marked DNA laddering induced by ischemia compared with control. Consistent with the animals’ oxidative status, rutin-, L-arginine-, and rutin + L-arginine-treated animals showed a marked decrease in DNA laddering.

HO-1 and iNOS protein expression. Our data showed a significant increase of HO-1 expression in I/R rats compared with sham-operated animals. Treatment with rutin, L-arginine, or the two drugs together induced the expression of HO-1 in both sham-operated and I/R animals (Fig. 5).

No significant difference in eNOS expression was observed in the I/R group with respect to controls, whereas in I/R-treated rats, a significant increase compared with the I/R group was detected (Fig. 6A). By contrast, iNOS expression was increased in the I/R group compared with controls; interestingly, rutin and/or L-arginine administration attenuated this increase (Fig. 6B).

Histopathological observations. Figure 7 shows representative photomicrographs of hepatic histopathology. I/R lobes demonstrated hepatic sinusoid congestion, necrosis of the hepatocytes, and neutrophil infiltration (Fig. 7E). Livers of sham-operated rutin- and/or L-arginine-treated rats (Fig. 7B–D) were characterized by normal hepatic architecture and appeared similar to those of the control group (Fig. 7A). The lobes of I/R rats treated with rutin (Fig. 7F), L-arginine (Fig. 7G), and the two drugs together (Fig. 7H) demonstrated intact hepatic architecture, focal sinusoid congestion, and only isolated areas of necrosis.

DISCUSSION

Liver transplantation is an ultimate effective treatment for end-stage liver disease. The transplantation procedure obligates cold preservation and warm reperfusion of liver grafts, resulting in some degree of cold I/R injury in all liver grafts. In addition, because of the recent shortage of cadaveric organ donors, the donor pool has been expanded to meet the growing demands with the use of older, steatotic, prolonged cold storage, or non-heart-beating liver grafts. These “marginal” organs are more susceptible to severe cold I/R injury, resulting in the delayed or primary nonfunction rate, infection, renal failure, and allograft rejection. Thus amelioration of hepatic graft preservation injury may lead to improved short- and long-term transplant outcomes.

Oxidative damage is thought to play an important role in I/R injury, including the outcome of organ transplantation. Ischemia leads cells to rapidly manifest distinct biochemical, structural, and functional alterations causing hepatocellular dysfunction. It has been suggested that I/R triggers a series of reactions mainly in the organ that is clamped and reperfused, and these reactions elicit a systemic inflammatory response by the release of cytokines and inflammatory mediators (tumor necrosis factor-α, interleukin-6, platelet-activating factor, leukotrienes, and NO) that cause the formation of oxygen ROS with consequent oxidative stress (8, 13, 18, 45). In this regard, several studies have demonstrated that I/R is associated with protein oxidative modifications, mitochondrial injury, and lipid peroxidation; this last is an autocatalytic mechanism leading to
oxidative destruction of the cellular membranes that, in turn, can lead to the production of toxic, reactive metabolites and cell death (18). However, at present there is no treatment available to prevent hepatic I/R injury, and even then, only a better comprehension of mechanisms of hepatic I/R injury could improve orthotopic liver transplantation results.

Lipid peroxidation, as a free radical-generating system, has been suggested to be closely related to I/R-induced tissue damage, and LOOH levels may represent a good indicator of the rate of lipoperoxidation. Consistent with previous observations, results obtained in the present study confirm that plasmatic and hepatic LOOH levels significantly increase in postischemic reperfusion (18). Our results also demonstrate that rutin and L-arginine cause a significant decrease in LOOH levels, suggesting that the protective effect of these drugs may be due, in part, to their scavenger capacity (32, 33).

The role of oxidative stress in liver damage induced by I/R is further confirmed by our results showing significant decreases in plasmatic and hepatic RSH levels in I/R rats. These data are consistent with previous observations reporting a significant difference in RSH levels between control and I/R groups (18). Glutathione provides major protection in oxidative injury by participating in a cellular system of defense against oxidative damage. Several reports have indicated that tissue injury induced by various stimuli are coupled with glutathione depletion (18), and the maintenance of high levels of glutathione is essential to reduce oxidative stress during organ reoxygenation (4, 5, 11). Therefore, the decrease in glutathione levels during I/R was probably due to its consumption during oxidative stress. In this study, oxidative stress involvement was also confirmed by an increase in DNA fragmentation in I/R groups with respect to the control group. Results regarding plasmatic levels of liver transaminases (AST and ALT) confirm that reperfusion results in massive tissue disruption with enzyme release. However, increased plasmatic AST and ALT activities and LOOH levels after I/R injury were significantly reduced by treatment with rutin and L-arginine; these treatments were also able to inhibit plasmatic and hepatic decreases in RSH levels and to protect DNA from fragmentation.

Our results suggest that rutin and L-arginine provide protection in postischemic reperfusion injury by participating in the cellular defense systems against oxidative damage. In addition,
rutin is an efficient antioxidant, free radical scavenger and has been shown to quench the hydroxyl radical, superoxide anion radical, and peroxynitrite anion (33).

The enhancement of oxidative stress is accompanied by the induction of “heat shock proteins” such HO-1 or HSP32, the rate-limiting enzymes that degrade heme protein to equimolar amounts of carbon monoxide and bilirubin with a concomitant release of iron. In addition, HO plays a critical role in the regulation of cellular heme-dependent enzyme concentrations (26, 27).

Two HO isoforms have been identified, and among them, the inducible isoform HO-1 is found ubiquitously in all organs.

Fig. 6. Immunoblotting of inducible (iNOS) and endothelial nitric oxide synthase (eNOS) levels in all groups. Densitometric results are expressed as arbitrary units corresponding to signal intensity present on the autoradiographs. Values are means ± SD of 4 experiments performed in duplicate. *P < 0.001.

with the exception of the adult brain and is rapidly and transiently expressed after various stimuli including hyperthermia, hypoxia, radiation, and I/R injury. For this reason, HO-1 is considered one of the most sensitive indicators of cellular stress (1, 41). However, the transcriptional regulation of the HO-1 gene by oxidants led to the hypothesis that HO-1 provides a cellular defense mechanism during oxidative stress (18, 23). The specific mechanism by which HO-1 can mediate cytoprotective function is not clear, but by-products generated during the heme catabolism have been suggested as potential protective mediators, in addition to the degradation of potentially pro-oxidant and cytotoxic heme protein (6, 17). The effects of HO-1 overexpression were recently studied in organ transplantation. Strikingly, studies have shown that upregulated HO-1 expression exerts important adaptive antioxidant and anti-inflammatory functions in cellular protection from pathophysiological conditions, including graft rejection and I/R injury (19).

In the present study, an increase in the expression of the HO-1 in groups treated with rutin and/or L-arginine with respect to untreated I/R rats and the control group was observed; the treatment with rutin and/or L-arginine, inducing an increase of HO-1 expression, allows us to suggest that the observed protective effect of rutin and/or L-arginine administration might be partially due to the ability of these drugs to induce HO-1 expression.

Although the majority of in vitro and in vivo studies conducted have attributed the protective effect of rutin to its chemical reactivity toward free radicals and its capacity to prevent the oxidation of important intracellular components, Motterlini et al. (34) recently suggested a possible novel aspect in the mode of action of polyphenol compounds; that is, the ultimate stimulation of the HO-1 pathway is likely to account for the established and powerful antioxidant/anti-inflammatory properties of these polyphenols (42).

In conclusion, our data demonstrate that molecules endowed with the exception of the adult brain and is rapidly and transiently expressed after various stimuli including hyperthermia, hypoxia, radiation, and I/R injury. For this reason, HO-1 is considered one of the most sensitive indicators of cellular stress (1, 41). However, the transcriptional regulation of the HO-1 gene by oxidants led to the hypothesis that HO-1 provides a cellular defense mechanism during oxidative stress (18, 23). The specific mechanism by which HO-1 can mediate cytoprotective function is not clear, but by-products generated during the heme catabolism have been suggested as potential protective mediators, in addition to the degradation of potentially pro-oxidant and cytotoxic heme protein (6, 17). The effects of HO-1 overexpression were recently studied in organ transplantation. Strikingly, studies have shown that upregulated HO-1 expression exerts important adaptive antioxidant and anti-inflammatory functions in cellular protection from pathophysiological conditions, including graft rejection and I/R injury (19).

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In conclusion, although HO-1 hyperexpression can be considered a marker of cellular stress, this enzyme also can be regarded as a potential therapeutic target in a variety of oxidant- and inflammatory-mediated diseases. Indeed, the induction of HO-1 has been suggested to have protective effects against oxidative stress and to represent an adaptive cellular defense mechanism (34). Moreover, emerging evidence suggests that NO has an important role in the ischemia injury, too. iNOS is triggered in many cell types by cytokines, such as tumor necrosis factor-α, generating large amounts of NO (19), the role of which in liver pathophysiology still remains controversial. Several reports have suggested that moderate levels of NO, generated by eNOS, may be beneficial for its vasodilator action, whereas high levels of NO, produced by iNOS, interacting with superoxide anion can produce the peroxynitrite anion, a potent oxidant associated with pathological liver conditions (2, 35).

Moreover, NO, generated by iNOS, induces leukocyte adhesion, inflammatory cell infiltration, and parenchyma cell dysfunction (4). In fact, overexpression of iNOS has been correlated with several acute and chronic diseases (19). Because recent studies reported that NO levels are able to modify HO-1 expression (25), in this study we also evaluated the dynamic interplay between HO-1 and NOS expression.

In our study, we observed a significant increase in iNOS expression in the I/R group with respect to controls, and rutin and/or L-arginine administration attenuated this increase. No significant difference in eNOS expression was observed in the I/R group with respect to controls; whereas in postischemic reperfused and treated rats, a significant increase compared with the I/R group was detected.

Data obtained in this study show that increased HO-1 expression inhibited iNOS expression; according to previous studies, this result supports the concept that modulation of iNOS expression may be one of the mechanisms of HO-1 action (19), and then the protective effects of rutin could be due to its scavenger capacity and its ability to induce HO-1 expression and inhibit iNOS expression.

The protective effect of L-arginine from the injurious effects of I/R may be due to antioxidant properties of eNOS-derived NO (22, 43). NO, in fact, is known to inhibit ROS-mediated reactions, and it has been suggested that the protective effects found in a variety of conditions are due to the ability of NO to detoxify ROS such as O2−, OH·, and/or ferryl hemoprotein (43). This might be one of the main causes for significantly decreased hepatic LOOH levels in I/R rats treated with L-arginine with respect to untreated I/R rats.

Another mechanism by which eNOS-derived NO may exert protection in our model of I/R is vasodilatation and enhanced perfusion of the postischemic tissue (43). In fact, recently it was reported (43) that eNOS-derived NO may counteract the physiological effect of endothelin-1 (ET-1), a potent and long-lasting vasoconstrictive peptide. Other mechanisms by which eNOS-derived NO may protect the liver from hepatic I/R-induced injury may be related to its inhibition of platelet aggregation and adhesion as well as attenuation of endothelium-leukocyte interactions, all of which may be beneficial to reduce hepatic I/R injury (30). The protective effect of rutin and/or L-arginine was confirmed by histological findings; in fact, lobes of ischemic rats treated with the two drugs showed intact hepatic architecture, focal sinusoid congestion, and only spotty necrosis.

In conclusion, our data demonstrate that molecules endowed with free radical scavengers, antioxidant, and HO-1 upregulation may provide potent protection against I/R hepatic injury; in addition, this study documents the potential utility of HO-1-inducing agents to prevent I/R injury, suggesting that L-arginine and rutin may be used as potential therapeutic agents against I/R-induced oxidative remote organ damage.

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