The involvement of heme oxygenase 1 but not nitric oxide synthase 2 in a hepatoprotective action of quercetin in lipopolysaccharide-induced hepatotoxicity of d-galactosamine sensitized rats

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ABSTRACT

The objective of this study was to evaluate potential hepatoprotective capabilities of quercetin in relation to its modulation of the HO-1 and NOS-2 activities in an experimental model of fulminant liver failure. Liver insult was induced by in vivo administration of d-galactosamine (d-GalN, 400 mg/kg, i.p.) and lipopolysaccharide (LPS, 10 μg/kg, i.p.). The effects of quercetin (50 mg/kg, i.p) on d-GalN toxicity were evaluated by standard biochemical, RT-PCR and Western blot methods. Administration of d-GalN/LPS combination resulted in significantly higher plasma levels of aminotransferases, as well as increased mRNA and protein expressions of both HO-1 and NOS-2 enzymes. Quercetin exhibited cytoprotective effects on the liver, as evidenced by decreased aminotransferase plasma levels. Additionally, quercetin treatment in d-GalN/LPS treated rats significantly increased HO-1 mRNA and its protein expressions. On the contrary, quercetin did not exhibit any significant effects on the levels of nitrites, and NOS-2 mRNA and protein expressions in d-GalN/LPS treated rats. Quercetin when given alone did not have any significant changes on liver enzymes nor HO-1 and NOS-2 mRNA and protein expressions. It can be concluded that the quercetin's induction of HO-1 and its byproducts, without concomitant NOS-2 activity reduction, is among mechanisms contributing to the hepatoprotective effect in d-GalN/LPS hepatotoxicity.

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1. Introduction

Throughout history it has been known that diets rich in fresh fruits, vegetables and grains have been associated with beneficial health effects. In the past years, polyphenols isolated from these natural food sources have received a great deal of the attention due to their various health benefits. One of these substances is quercetin, a member of flavonoid family, which exists as a glycoside and is commonly found in food sources such as apples, onions, green tea and wine. This flavonoid exhibits anti-inflammatory, antioxidant, anti-aging, anti-proliferative, among other favorable characteristics [1,2].

Small-scale cardiovascular studies testing the potential health benefits of quercetin were among the first to demonstrate its antioxidant potential in humans, where quercetin decreased blood low density lipoprotein levels and regulated blood pressure in hypertensive patients [3,4]. It is hypothesized that quercetin may exhibit its blood pressure lowering effect by enhancement of levels of the potent vasodilator, nitric oxide, by inducing endothelial nitric oxide synthase in spontaneous hypertensive rats [5]. Quercetin exerts its anti-inflammatory effect through inhibition of TNF-α and NF-κB production, which may further reduce oxidative stress markers [6]. Several studies, evaluating quercetin’s effect on
malignant cells, have revealed that this polyphenol increases expression of pro-apoptotic Bcl-2 proteins and it sensitizes malignant cell lines to a death ligand, both of which account for its favorable anti-proliferative properties [7,8]. Moreover, growing scientific evidence attributes oxidative stress and increased reactive oxygen species production as the main mediators of pathology of both acute and chronic liver injury caused by xenobiotics. In regard to the small number of studies done on liver, it is reported that quercetin exhibits its antioxidant properties by increasing the antioxidant status of the hepatocytes, decreasing the levels of pro-oxidant and inflammatory mediators, as well as altering gene expression of a number of antioxidant genes [9,10]. However the exact mechanism and hepatoprotective role of quercetin still remain to be further studied.

To date, the role of nitric oxide in liver damage remains controversial. It seems that this molecule plays a paradoxical effect in liver injury depending on the experimental conditions and current oxidative status in the cell [11]. Additionally, nitric oxide has been shown to influence the expression of heme oxygenase 1 (HO-1), an antioxidant enzyme that plays an important role in defense against oxidative stress [12–14]. In our previous work, we have addressed the potential cross talk between the HO-1 and nitric oxide synthase 2 (NOS-2) enzyme systems as targets of hepatoprotective agents [15,16]. We further aim to evolve our previous findings regarding the demonstrated hepatoprotective effects of natural products, resveratrol and curcumin, with definite roles of HO-1/NOS-2 hepatotoxicity/hepatoprotection. The present study, therefore, focuses on the evaluation of whether, and how, quercetin influences the activity of HO-1 and NOS-2 in the experimental model of hepatotoxicity, which resembles fulminant hepatic failure. Additionally, given the encouraging reported findings on the cytoprotective properties of quercetin, we would like to evaluate its potential hepatoprotective capabilities in relation to its modulation of the HO-1 and NOS-2 activities in rats.

2. Materials and methods

2.1. Chemicals

Lipopolysaccharide from *Escherichia coli* K-235 (LPS), D-galactosamine hydrochloride (D-GalN), quercetin, that is 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one with HPLC purity of >95%, (M. wt. 302.24), Tris-2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one, lactosamine hydrochloride (D-GalN), quercetin, that is 2-(3,4-dihydroxyphenyl)-3,5,7-trihdroxy-4H-1-benzopyran-4-one with HPLC purity of >95%, (M. wt. 302.24), Tris, 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one with HPLC purity of >95%, (M. wt. 302.24), Tris–HCl, dimethyl sulfoxide, isopropyl alcohol, Tween 20, sodium dodecyl sulphate, ammonium persulfate, methanol, glycine, N,N,N′,N″-tetramethylethylenediamine, 2-mercaptoethanol, bromophenol blue, glyceral, N,N′-methylenebis (acrylamide), NaCl, KCl, Na2HPO4, KH2PO4 ammonium molybdate terahydrate, hydrogen peroxide, acrylamide, filter paper, nitrocellulose membrane, anti-mouse and anti-rabbit IgG (whole molecule) horse radish-peroxidase (HRP) conjugated secondary antibodies, rabbit primary NOS-2 antibody, mouse HO-1 primary antibody and beta-actin primary antibody were purchased from Sigma-Aldrich (Prague, Czech Republic). Non-fat dry milk was from Biotech A.S. (Prague, Czech Republic). NaCl 0.9% w/v i.v. infusion was from Bieffe Medital (Grosotto, Italy). Bio-Rad protein assay dye reagent was from Bio-Rad (Prague, Czech Republic).

2.2. Animals

Male Wistar rats (Velaz, Lysolaje, Czech Republic) of 300–400 g body weight were used in this study. They were given a standard granulated diet and water *ad libitum*. The animals were housed in standard environmental conditions: light (i.e. 12 h light and 12 h dark); temperature (22 ± 2 °C); relative humidity (50 ± 10%). The rats received humane care in accordance with the guidelines and approval by the Ethical Committee of the First Faculty of Medicine, Charles University in Prague, Czech Republic.

2.3. The study design

In the pilot experiments the effect of various doses of quercetin (50–500 μM) on tert-butylhydroperoxide induced toxicity in rat hepatocyte culture was evaluated. The results of these preliminary studies have indicated that 250 μM (57 mg/kg) of quercetin preserved hepatocyte viability and reduced ALT and AST leakage compared to the other doses that were tested (data not shown). The decision to use the dosage of 50 mg/kg in this experiment was also based on the studies of others dealing with quercetin’s cytoprotective effects that include HO-1 induction and consequent NOS-2 reduction [17–19]. Thus we felt that this concentration complements our *in vitro* findings and is the most suitable one to test the aims of our study.

The rats were therefore divided randomly into four groups of six animals each and treated as follows:

- Group 1 — control rats received vehicle solution consisting of physiological solution and dimethyl sulfoxide (DMSO) intraperitoneally (i.p).
- Group 2 — rats were injected i.p. with a dose of 400 mg/kg D-GalN and 10 μg/kg LPS dissolved in physiological solution.
- Group 3 — rats were injected i.p. with a dose of 50 mg/kg of quercetin dissolved in DMSO.
- Group 4 — rats received a combination of 400 mg/kg D-GalN and 10 μg/kg LPS dissolved in appropriate vehicle solution, followed by a treatment dose of 50 mg/kg quercetin one hour later.

After 24 h, the animals were sacrificed and the blood samples were collected. In the next step, the livers were excised, preserved in liquid nitrogen for RT-PCR and Western blot studies.

2.4. Biochemical analyses

Determinations of plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin plasma levels were carried out using Fluitest® GPT ALT, Fluitest® GOT AST and Fluitest® BL-TotAl kits (Analyticon, Lichtenfels, Germany) respectively. Nitric oxide production was determined spectrophotometrically (540 nm) by measurement of one of its stable oxidation products, NO2−, in plasma using Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine, 2.5% H3PO4). The medium nitrite levels were calculated by comparison with a sodium nitrite standard curve. The measurement of catalase in plasma was
performed according to the reaction between $\text{H}_2\text{O}_2$ and molybdenium ammonium as previously reported [20].

2.5. Real-time PCR gene expression measurements

Total RNA isolation was carried out according to the manufacturers’ instructions of the Qiagen® RNeasy plus kit (Bio-Consult Laboratories, Prague, Czech Republic). Subsequently, the reverse transcription from total RNA to cDNA was carried out with universal GeneAmp® RNA PCR kit (Applied Biosystems, Prague, Czech Republic), using a murine leukemia virus (MvLu) as reverse transcriptase. Reverse transcription included the following phases: 10 min at 25 °C for reverse transcriptase enzyme activation, 30 min at 48 °C for PCR amplification and 5 min at 95 °C for denaturation.

The obtained total mRNA was reverse transcribed to cDNA with the help of ABI PRISM 7900, and TagMan® Gene Expression master mix (Applied Biosystems Prague, Czech Republic). Expressions of genes of interest were evaluated using real-time polymerase chain reaction (RT-PCR). The TagMan® Gene Expression Assays Kit (Applied Biosystems Prague, Czech Republic) was used. NOS-2 and HO-1 were used as genes of interest (target genes) and beta-actin gene as a control (housekeeping) gene, using the FAM colored primers and probes. Housekeeping gene-expression was stable and constant during the experiment and was used in comparison with target gene-expression. The conditions for thermal cycling for primer and probes optimization were as follows: 10 min at 90–95 °C for Taq polymerase activation, followed by 15 s at 95–99 °C for DNA denaturation and 1 min at 60 °C for annealing. The obtained Ct values were used in relative quantification of gene expression measurements relative to the endogenous gene control Ct measurements, and the relative gene expression was calculated using the ΔΔCt method [21].

2.6. Western blot analysis of proteins

Isolated liver samples were lysed with lysis buffer (1 M Tris HCl) and homogenized with an electric homogenizer. The samples were then centrifuged for 20 min at 12,000 rpm at 4 °C and supernatant was collected. The cell lysates were heated for 5 min at 95 °C. Protein from the tissue samples (50 μg protein measured by Bradford method) were separated by electrophoresis overnight at 4 °C. Membranes were blocked for 1 h with 5% non-fat milk in Tris-buffered solution, at room temperature. Membranes were then washed in a washing buffer (NaCl, KCl, Na₂HPO₄, KH₂PO₄, Tween, H₂O). They were then incubated with rabbit primary antibody against NOS-2 (1:1000), mouse HO-1 primary antibody (1:500) or mouse beta actin primary antibody (1:5000). After eluting the unconjugated primary antibody with the wash buffer, the membrane was then incubated with corresponding secondary antibody anti-rabbit IgG HRP conjugate (1:20,000) or anti-mouse IgG HRP conjugate (1:100,000). This step was followed by chemiluminescence labeling with Super Signal West Pico Chemiluminescent Substrate (GeneTiCA s.r.o. Prague, Czech Republic) for 2 min. Bands were detected with the use Molecular Imager VersaDoc™ MP 5000 System and analyzed by Quantity One 1-D Analysis Software (Bio-Rad, Prague, Czech Republic). Optical densities of NOS-2 and HO-1 bands were normalized by the corresponding loading control (ACTB) and then to the mean of the corresponding control group.

2.7. Statistical analysis

All experiments were performed in four groups of six rats with the reported results stated as ± standard error of mean (SEM). The statistical analysis was performed using one way ANOVA, followed with Tukey–Kramer comparison test. The p-values less than 0.05 were considered significant.

3. Results

3.1. Ameliorative effects of quercetin on d-GalN/LPS hepatotoxicity

The first question that we wanted to address was whether quercetin treatment (i.p., 50 mg/kg) will have ameliorative effects on d-GalN (i.p., 400 mg/kg) and LPS (i.p., 10 μg/kg) model of hepatotoxicity. The results show that male Wistar rats treated with d-GalN and LPS developed acute hepatotoxicity within 24 h of the insult. This can be confirmed by a several-fold increase in the plasma levels of transaminases (AST and ALT) that are indicative of failing liver function. Quercetin alone had no influence on the plasma levels of these two liver enzymes. Interestingly, quercetin (Q) treatment in d-GalN/LPS rats resulted in significant lowering of the ALT (5.6-fold) and AST (5.9-fold) plasma levels, compared to the group receiving the hepatotoxic d-GalN/LPS combination. Thus, this data suggests a clear hepatoprotective effect as a result of quercetin treatment (Table 1).

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<td>Effects of quercetin treatment in lipopolysaccharide-induced hepatitis in d-galactosamine sensitized rats (d-GalN/LPS) on levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and catalase. Control: vehicle only; Q: quercetin 50 mg/kg; d-GalN/LPS: d-galactosamine 400 mg/kg + lipopolysaccharide 10 μg/kg; d-GalN/LPS + Q: combination of d-galactosamine, lipopolysaccharide and quercetin treatment. The results are expressed as means ± SEM, n = 6.</td>
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*a Value significant compared to the control group (vehicle only), p ≤ 0.05.

*b Value significant compared to the respective positive control group (d-GalN/LPS), p ≤ 0.05.
The plasma level measurements of the antioxidant molecule and product of heme degradation pathway, bilirubin, and antioxidant enzyme catalase were used as indicators of the oxidative status. In the groups treated with D-GalN/LPS, we observed significant 6.4-fold increase in bilirubin and 5.5-fold increase in catalase plasma levels. Quercetin treatment had no effect on the levels of these two antioxidants as can be seen by comparable values between D-GalN/LPS and D-GalN/LPS + Q groups (Table 1 and Fig. 1).

3.2. Effect of quercetin on HO-1, NOS-2 mRNA and protein expressions

The second question that we wanted to address in this study was to determine whether, and how, quercetin influences the activity of the potent antioxidant enzyme HO-1, as well as that of the enzyme NOS-2 in D-GalN/LPS model of hepatotoxicity. As postulated, both HO-1 mRNA and protein expressions were significantly increased (30.6-fold and 2-fold respectively, \( p \leq 0.05 \)) in D-GalN/LPS group compared to control (Fig. 2). Conversely, quercetin alone did not exert any significant changes in HO-1 mRNA nor protein expressions, as the levels were comparable to those seen in the control group. However, when given to D-GalN/LPS treated rats, quercetin significantly \( (p \leq 0.05) \) increased the levels of HO-1 mRNA expression (by 2.3-fold) and protein expression (by 1.2-fold) in comparison to the group receiving hepatotoxic D-GalN/LPS combination (Fig. 2). These results clearly indicate the induction of HO-1 under hepatotoxic conditions, where quercetin further increased its activity under hepatotoxic, but not under physiological, conditions.

Furthermore, D-GalN/LPS challenge resulted in a significant 12.3-fold increase in NOS-2 mRNA and 2.4-fold increase in NOS-2 protein expressions in comparison to the control group \( (p \leq 0.05) \) (Fig. 3). Likewise, when compared to the control (vehicle only) group, addition of quercetin treatment to D-GalN/LPS treated rats resulted in significant 10-fold increase in NOS-2 mRNA and 2-fold increase in NOS-2 protein expressions \( (p \leq 0.05) \). Interestingly, however, there was no significant difference in NOS-2 mRNA and protein expressions between the D-GalN/LPS and D-GalN/LPS + Q groups, despite the notice of slight decrease in these levels upon addition of quercetin (Fig. 3). Moreover, quercetin did not show to have any influence on NOS-2 mRNA and protein expressions when given under normal physiological conditions (Fig. 3). Additionally, the levels of nitrites measured in plasma closely correlated to both NOS-2 mRNA and protein expression in the tested groups as was expected (Fig. 4).
4. Discussion

Quercetin, a widely distributed dietary flavonoid has been shown to exert several hepatoprotective properties including its strong antioxidant potential in ameliorating alcoholic liver disease, anti-HBV activity, downregulation of expression of inflammatory mediators in cirrhotic rats and induction of cell death in hepatoma cell lines [22–27]. Despite the known aforementioned hepatoprotective properties of quercetin, many questions remain to be answered in relation to its cytoprotection involving induction of HO-1 and NOS-2 enzymes. In our previous studies on dietary polyphenols, resveratrol and curcumin, we have indicated that these agents may exert their hepatoprotective properties by induction of HO-1 and concomitant reduction of NOS-2 activities. This means that there is a kind of crosstalk between the two protein molecules including other cascade of effects which led to cytoprotection [15,16]. Thus the main goal of the current study was to assess potential hepatoprotective effects of quercetin in regard to its modulation of these two inducible enzymes that play important roles in oxidative stress.

In reference to the various mechanisms of hepatocellular damage of differing etiologies, oxidative stress is recognized as the most prominent and the most important mechanism resulting in a physiological misbalance of redox status of the cell favoring reactive oxidative species production and ensuing cellular injury [28,29]. Combination of D-GalN/LPS is a valuable hepatotoxic model that resembles clinical hepatitis, where oxidative stress plays a major role [30]. In short, D-galactosamine inhibits protein synthesis by depleting uridine triphosphate pool, resulting in early generation of reactive oxygen species and apoptosis [31–33]. On the other hand, lipopolysaccharide increases the release of pro-inflammatory cytokines (mainly TNF-α), induces the release of reactive nitrogen species and inflammatory prostaglandins due to the activation of NOS-1 and cyclooxygenase 2 (COX-2) respectively [34–36]. In the present study administration of D-GalN/LPS caused an elevation of plasma aminotransferase (ALT, AST) levels that demonstrate failing liver function, as well it resulted in an increase in the catalase levels indicative of increased physiological antioxidant status of the cell. Quercetin treatment effectively improved D-GalN/LPS induced liver damage by lowering ALT and AST levels. However, the plasma level of catalase was not altered by quercetin treatment, indicating that increased antioxidant status of the cell was maintained in order to combat oxidative stress induced by D-GalN/LPS. For instance, the presence of catalase in the mitochondria of hepatocytes has shown to have important implications in the prevention of ROS generation and consequent activation of pro-apoptotic pathways induced by TNF-α [37].
Furthermore, the pathophysiological conditions of the liver involving oxidative stress initiate upregulation of HO-1 and increase in products of heme degradation pathway [13,38]. Among these products, biliverdin/bilirubin and CO are the key mediators of HO-1 mediated cytoprotection for the reason that they help restore intracellular homeostatic balance under oxidative stress conditions and suppressing inflammation through downregulation of pro-inflammatory mediators [38–41]. In agreement with our previous findings, administration of d-GalN/LPS toxicity resulted in higher levels of HO-1 gene and protein expressions, as well as a significant increase in bilirubin [30]. The augmented levels of bilirubin are of importance since this potent antioxidant ameliorates oxidative stress by scavenging peroxyl radicals, consequently preventing oxidation of fatty acids and proteins. Moreover, quercetin treatment resulted in a further increase of HO-1 gene and protein activities in d-GalN/LPS + Q rats, which is in accordance to other studies demonstrating hepatoprotective effect of quercetin by induction of HO-1 [38]. However, this increase in HO-1 activity was not paralleled by a simultaneous increase in bilirubin plasma levels. This could be explained by a postulated fine tune in bilirubin production, as the beneficial effects of this potent antioxidant is bypassed at higher serum levels resulting in cytotoxicity that targets the central nervous system [42].

As for the second enzyme of interest, NOS-2, its gene and protein expressions and the nitrite levels were significantly increased by d-GalN/LPS treatment, which can be attributed to the reported LPS-induced NO production [34,43]. Interestingly, in contrast to our previous experience with dietary polyphenols and those of others [15,16,44,45], quercetin did not have a significant reducing effect on NOS-2 activity or nitrate levels in d-GalN/LPS treated rats. It has been reported that quercetin mediates its cytoprotective actions by suppressing NO production due to induction of HO-1 [44–46]. However, considering the results of this study it could be postulated that there seems to be an alternative mechanism of HO-1 induction protection that is not related directly to NOS-2 activity reduction in this model of hepatotoxicity among other possible mechanisms. One possible explanation is that the products of HO-1 pathway, bilirubin and CO are mainly responsible for the quercetin mediated cytoprotection in this model given their aforementioned cytoprotective actions.

Furthermore, the mechanisms of HO-1 cytoprotection have been attributed in some models to be a result of upregulation of anti-inflammatory mediators such as IL-10 and IL-13, and inhibition of production of pro-inflammatory cytokines such as TNF- α [47,48]. Through inhibition of COX-2 activity, the IL-10 is responsible for the decrease in production of inflammatory prostaglandin E2 that contributes to much of the damage due to LPS insult [49]. However, until now not much is known whether there exists a potential cross-talk between these two enzymes. On the other hand, the relationship between COX-2 and NOS-2 is such that peroxynitrite radicals resulting from increased NOS activity are able to inhibit the activation of COX-2 by tyrosine nitration [36]. Thus, high NOS-2 activity could contribute to reduction of pro-inflammatory damage caused by COX-2.

Another presumption is that quercetin was extensively metabolized into its metabolite quercetin-3’-sulfate, which has been not been shown to possess NOS-2 and NO lowering properties [45,50]. Furthermore, it is known that quercetin, resveratrol, curcumin and related natural and synthetic compounds are SIRT1 activators. The latter is a NAD+ dependent deacetylase responsible for multiple beneficial effects [51]. SIRT1 activation could be achieved by several pathways through stimulation and/or inhibition of mediators, thus a direct effect on NOS-2/NO level due to quercetin treatment was not demonstrated. Moreover, an important finding is that quercetin administration to healthy rats without the presence of a toxicant did not produce any significant changes on the tested parameters. It could be assumed, therefore, that there exist different regulation mechanisms which are responsible for the differences seen under physiological versus pathological conditions.

In our previous findings dealing with dietary hepatoprotective substances we have found that the dosage of 100 mg/kg curcumin and 2.3 mg/kg of resveratrol has ameliorated d-GalN/LPS induced hepatotoxicity [15,16]. In this study, 50 mg/kg was shown to be cytoprotective in d-GalN/LPS model, thus being more potent than curcumin, but less potent than resveratrol.

In conclusion, the results of the present study indicate that quercetin’s significant induction of HO-1 alone, without concomitant NOS-2 activity reduction, might be sufficient in combating cellular damage induced by d-GalN/LPS toxicity which resembles fulminant hepatitis. However, we must remain cautious in regard to many studies demonstrating various cytoprotective effects of quercetin, as this dietary polyphenol can also be toxic in some conditions. Such is the example of its potent inhibiting effect on topoisomerase II that may cause double-strand DNA lesions at topoisomerase binding sites including the MLL gene, which can lead to the development of secondary leukemias [52,53]. Nonetheless, given the wide availability of dietary quercetin as well as its cytoprotective properties in various disease models, the importance of this flavonoid in amelioration of hepatic diseases should be further studied.

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