Hepatoprotective activity of chrysin is mediated through TNF-α in chemically-induced acute liver damage: An in vivo study and molecular modeling

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Abstract. Chrysin (5,7-dihydroxyflavone) is a naturally occurring flavonoid present at high levels in honey, propolis and numerous plant extracts. Chrysin is known to have hepatoprotective activity, however, the mechanisms by which it exerts this effect remain unclear. In the present study, the effects of chrysin in carbon tetrachloride (CCl4)-induced acute liver damage were investigated and the results used to infer a possible mechanism behind chrysin's hepatoprotective activity. Prior to an intraperitoneal injection of CCl4 (1 ml/kg) to induce acute liver damage, chrysin (50 mg/kg) was administered orally to mice for 7 days. The positive control group was given 50 mg/kg standardized silymarin, a well-studied hepatoprotective flavonoid. Twenty-four h following CCl4 administration, an increase in the activity levels of serum aspartate-aminotransferase and alanine-aminotransferase was found. This was accompanied by extended centrilobular necrosis, steatosis and an altered hepatocyte ultrastructure. In addition, CCl4-induced acute hepatotoxicity was associated with an increase in hepatic tumor necrosis factor-α (TNF-α) and α-smooth muscle actin (α-SMA) protein expression, which was significantly decreased in the livers of mice pre-treated with chrysin (P<0.001), similar to the results of the silymarin pre-treated group (P<0.001). Treatment with chrysin prior to CCl4 exposure significantly reduced the activity of enzymes used as biochemical markers of poor liver function compared with the group which did not receive pre-treatment (P<0.001). In addition, the results of histopathological and electron microscopy liver examination showed chrysin pre-treatment reduced the effects of CCl4 treatment. Molecular modeling results demonstrated that the hepatoprotective activity of chrysin is mediated through TNF-α, as it reduces soluble TNF-α generation via blocking TNF-α-converting enzyme activity. In conclusion, the results of the present study suggest that inflammatory pathways are activated in CCl4-induced acute liver damage, which are ameliorated by chrysin pre-treatment. This indicates that chrysin is a potent hepatoprotective agent, similarly to silymarin at the same dose, which has the potential to be a viable alternative to conventional hepatoprotective treatments.

Introduction

The liver is the primary organ responsible for the metabolism of numerous xenobiotics, including drugs and toxic chemicals (1). Carbon tetrachloride (CCl4), an industrial solvent, cleaner and degreaser, has been used extensively in models of xenobiotic-induced hepatotoxicity (2). CCl4-induced liver damage is characterized by progressive tissue injury, starting with inflammation and followed by necrosis, fibrosis and, finally, cirrhosis (2,3). Acute inflammation triggers further inflammatory processes, initiated by cytokines released from activated Kupffer cells. This represents a key event in the induction of liver damage. Previous studies have demonstrated
that inflammation is initiated by the release of pro-inflammatory mediators, including TNF-α, cyclooxygenase-2 and interleukin-6, in response to oxidative stress conditions, such as those during CCl₄-induced hepatotoxicity, and occurs in parallel with increasing apoptosis (4,5). TNF-α, the primary pro-inflammatory protein synthesized by Kupffer cells, initiates a cascade of cytokines that mediate the inflammatory response (6).

A previous study found an elevated expression of TNF-α in the early phases of liver damage (7). The next stage in CCl₄-induced liver damage is activation of hepatic stellate cells (HSCs) (8). HSCs are myofibroblasts that reside in a heterogeneous cell population originating from liver fibroblasts and bone marrow-derived circulating fibroblasts. Active HSCs are characterized by a high rate of proliferation, migration and contractility (9). In addition, HSCs are the primary producers of α-SMA, which activates the production of transforming growth factor-b1, the primary pro-fibrogenic cytokine (10). These events result in collagen deposition and the release of other matrix proteins into the extracellular space, promoting liver fibrosis.

Natural antioxidants can prevent liver damage by scavenging free radicals and other reactive oxygen species, or by modulation of the inflammatory response (11). Additionally, a previous study has shown that xenobiotic-induced hepatotoxicity is diminished by flavonoids, such as silymarin (12). Silymarin, the primary bioactive compound of Silybum marianum, is a complex mixture of flavonolignans, which has protective effects against xenobiotics, particularly in the liver (13). The hepatoprotective activity of silymarin is a result of its antioxidant properties, lipid peroxidation inhibition and cell membrane preservation (14). Chrysin (5,7-dihydroxy-flavone) is another natural flavonoid. Chrysin has not been as well-studied as silymarin, but is known to be present in high levels in honey, propolis and numerous plant extracts (15). Chrysin has been identified to possess antioxidant (16–18), anti-allergic (19), anti-inflammatory (20), anti-fibrotic (21) and anti-cancer (22,23) properties. However, there are previous reports in the literature regarding the hepatoprotective activity of chrysin, but these did not reveal how its protective activity is initiated in acute liver damage condition.

In the present study, the hepatoprotective effects of chrysin against acute CCl₄-induced liver damage are investigated and the results used to postulate a possible mechanism by which this occurs. In addition, the interaction between chrysin and TNF-α was evaluated by computational molecular modeling.

Materials and methods

Chemicals and reagents. Chrysin (97%), silymarin (98%) and carboxymethyl cellulose were purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany). Anti-TNF-α and anti-α-SMA, antibodies were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The Novolink Max Polymer Detection System for immunohistochemistry was purchased from Leica Microsystems GmbH (Wetzlar, Germany).

Experimental animals. A total of 50 male CD-1 mice (weight, 20±3 g; age, 8-10 weeks), supplied by the Animal House of the Vasile Goldis Western University of Arad (Arad, Romania) were used in the present study. The animals were maintained in an environment at a constant temperature of 20±1°C and 50±5% humidity, with a 12-h light/dark cycle and ad libitum access to food and water. All experimental procedures were approved by the Ethical Committee of Vasile Goldis Western University of Arad (Arad, Romania).

Treatments and experimental design. A 50 mg/kg body weight dose of chrysin was chosen, as it was previously proven to be protective against oxidative damage caused by toxicants in rodents (24). Silymarin (50 mg/kg) was used as the positive control drug. Chrysin and silymarin were dissolved in 0.5% sodium carboxymethylcellulose (Sigma-Aldrich; Merck Millipore) and given orally. CCl₄ (1.0 ml/kg, in a 1:1 ratio with 50% olive oil), injected intraperitoneally, was used to induce acute liver damage.

The 50 mice were divided into 5 groups of 10 mice. Group 1 (control) received only the vehicle daily for 7 days. Group 2 (CCl₄) received the vehicle daily for 7 days, followed by CCl₄ the next day. Group 3 (chrysin pre-treatment group; CHR+CCl₄) received chrysin for 7 days, followed by CCl₄ the next day. Group 4 (silymarin pre-treatment group; Sy+CCl₄) received silymarin for 7 days, followed by CCl₄ the next day. Group 5 (CHR) group, received chrysin alone for 7 days.

Serum and liver sample collection. The mice were sacrificed with 2.5 ml/l/min isoflurane on day 9 and blood collected from the venae cavae. The collected blood was placed in heparinized tubes and centrifuged at room temperature for 15 min at 366 x g in order to obtain serum samples for biochemical analysis. Liver samples (2-cm samples) were preserved in a buffered formalin solution for histology and immunohistochemistry and glutaraldehyde solution for electron microscopy processing.

Biochemical analysis of the activity of serum markers of hepatic function. The activities of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were evaluated by spectrophotometry using a commercially available detection kits (cat. no. 11876805216; Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions.

Histopathology. Liver sections (5-μm) were deparaffinized and processed routinely for hematoxylin and eosin staining. The extent of CCl₄-induced liver damage was then evaluated by assessing morphological changes to the liver sections. Frozen sections were cut to 8 μm using an MNT cryostat (SLEE medical GmbH, Mainz, Germany), fixed in 10% buffered formaldehyde and stained with Oil Red O according to the manufacturer's instructions. Mounted sample slides were examined under a BX43 light microscope and images captured using an XC30 digital camera (both Olympus Corporation, Tokyo, Japan).

Immunohistochemistry. Immunohistochemistry analysis was performed on paraffin-embedded 4-μm-thick liver tissue sections. Liver sections were deparaffinized in toluene and rehydrated prior to epitope retrieval in Novocastra Epitope
The effects of chrysin on the activities of serum (A) AST and (B) ALT following CCl₄ treatment. Chrysin pre-treatment reduced serum AST and ALT activities. Results are represented as the mean ± standard deviation (n=8). ***P<0.001 vs. the control group; ****P<0.001 vs. the CCl₄ group. AST, aspartate transaminase; ALT, alanine transaminase; CCl₄, carbon tetrachloride; CHR, chrysin; Sy, silymarin.

Statistical analysis. Statistical analysis was performed with a one-way analysis of the variance procedure using Stata software (version 13; StataCorp LP, College Station, TX, USA). P<0.05 was considered to indicate a statistically significant difference.

Results and Discussion

Serum ALT and AST activity. The results of 7 days of chrysin administration on serum ALT and AST activities in mice from various treatment groups are shown in Fig. 1. Serum AST and ALT activity was significantly increased (P<0.001) 24 h following CCl₄ treatment (CCl₄ group) compared with the control group. Pre-treatment with chrysin (CHR+CCl₄)
significantly decreased serum aminotransferase activity compared with the CCl4 group (P<0.001). This result was similar to that of the silymarin pre-treated group (Sy+CCl4; P<0.001 compared with the CCl4 group). The group treated with chrysin and not CCl4 showed no increase in ALT and AST activity compared with the control group.

It is well known that the hepatotoxic agent CCl4 is metabolized to a trichloromethyl radical (CCl3•), which interacts with oxygen to form more free radicals, which damage the liver. Increased membrane permeability from hepatocyte injury leads to leakage of liver enzymes, which damage the liver. The present study found a significant increase in serum AST and ALT activity in mice following CCl4 administration (P<0.001). Chrysin (50 mg/kg) pre-treatment significantly decreased serum AST and ALT activity in CCl4-treated mice (P<0.001), which indicates that chrysin preserves the structural integrity of membranes. This is consistent with the finding of a previous report (16).

Histopathology. The effect of chrysin on histological changes and lipid accumulation in the liver was investigated (Figs. 2 and 3). Evaluation of liver tissues by light microscopy identified that, compared with the normal liver architecture of the control group (Figs. 2A and 3A), livers of the CCl4 group showed necrotic changes to hepatocytes, which were particularly pronounced in the centrilobular area (Fig. 2C). In addition, inflammatory cell infiltration (Fig. 2C) and microvesicular steatosis of the hepatocytes (Fig. 3C) were detected in the CCl4 group. The group pre-treated with chrysin prior to CCl4 injection (CHR+CCl4; Figs. 2D and 3D), showed a significant reduction in hepatocellular necrosis and steatosis compared with the CCl4 group (P<0.001; Figs. 2F and 3F). This reduction was greater than that seen in the silymarin group (Figs. 2E and 3E and F). The liver morphology of the group treated with chrysin alone (Figs. 2B and 3B) was comparable with that of the control (Figs. 2A and 3A).

Increased deposition of neutral lipids in hepatocytes is caused by an imbalance between the gain and loss of fatty acids, and the synthesis and excretion of triglycerides (30). This imbalance can be induced by exposure to CCl4, causing micro- and macro-vesicular steatosis (30). The reduction in liver triglyceride accumulation seen in the group pre-treated with chrysin suggests that this flavonoid is able to restore the lipid balance in hepatocytes and thus hepatic function.
Liver expression of TNF-α and α-SMA proteins. The effect of chrysin on liver TNF-α and α-SMA expression, which is elevated by CCl₄, a proinflammatory and profibrotic agent, is shown in Figs. 4 and 5. Significantly increased TNF-α (Fig. 4C) and α-SMA (Fig. 5C) expression was observed in the CCl₄ group (both P<0.001 compared with the control), particularly in the areas surrounding the centrilobular veins, forming bridges between neighboring veins. TNF-α and α-SMA expression was significantly decreased in the livers of chrysin pre-treated mice (Figs. 4D and 5D) compared with the CCl₄ group (P<0.001; Figs. 4F and 5F). This decrease was greater than that seen with silymarin pre-treatment (Figs. 4E and 4F, and 5E and F). No expression of TNF-α or α-SMA was detected in the group treated with chrysin alone (Figs. 4B and 5B) or the control group (Figs. 4A and 5A).

In the present study, chrysin pre-treatment reduced CCl₄-induced TNF-α expression. TNF-α is a pro-inflammatory cytokine produced by Kupffer cells, which has been found to be elevated in acute liver diseases and following exposure to hepatotoxic chemicals, including CCl₄ (31,32). In this study, the reduction in TNF-α expression caused by chrysin suggests that it serves an important role in attenuating the CCl₄-induced inflammatory cascade in the liver. In agreement with the findings of the current study, Ai et al (33) determined potential inhibition of the pro-inflammatory TNF-α pathway by other flavonoids.

Strong hepatic inflammatory responses are accompanied by the necrosis of large areas and the formation of bridges between centrilobular veins (34), causing extended damage to the liver parenchyma. In the present study, these changes were observed in the CCl₄ group and were reduced by chrysin pre-treatment.

Chronic or severe inflammation can stimulate a fibrotic response, characterized by an irreversible decline in liver function (35). In the present study, chrysin pre-treatment reduced the activation of HSCs, as determined by α-SMA expression and the progression of acute hepatic damage into liver fibrosis. α-SMA is considered an important factor in the development of liver fibrosis and is thus a marker for HSC (the primary producers of α-SMA) activation and fibrous tissue deposition. Therefore, the expression of α-SMA is a useful marker for monitoring the efficacy of hepatoprotective therapy. The
results of the present study identified that α-SMA expression in liver tissue from the CCl₄ group was significantly increased compared with the control group (P<0.001). A previous study reported that a reduction in α-SMA expression was accompanied by a decrease in the quantity of activated HSCs (36). The results of the present study showed that α-SMA expression in CCl₄-injured livers was reduced by chrysin, which indicates that chrysin deactivates HSCs.

**Electron microscopy.** Electron microscopy revealed a normal hepatocyte ultrastructure in the control group (Fig. 6A) and chrysin alone (Fig. 6B), with regularly shaped nuclei and rough endoplasmic reticuli (rER), and few lipid globules. However, hepatocytes of the CCl₄ group showed lipid globule accumulation, organelle degeneration and proliferation of smooth ER (sER) vesicles (Fig. 6C). Pre-treatment with chrysin markedly reduced lipid globule enlargement and quantity (Fig. 6D), similarly to the silymarin pre-treated group (Fig. 6E).

The results of the present study identified that organelle and cytoplasmic structures were protected against hepatotoxic effects of CCl₄ by chrysin pre-treatment including membranes preservation. A previous study demonstrated that membrane damage causes alterations in lipoprotein and lipid droplet accumulation in hepatocytes (37). Lipid accumulation is accompanied by dilatations and focal breaks of rER cisternae, likely due to membrane structure damage caused by lipid peroxidation (38). In addition, a previous study showed that the flavonoids provided protection against free radicals generated by xenobiotic biotransformation and lipid peroxidation (36).

Numerous xenobiotics are metabolized in oxidase chain reactions, including that of the cytochrome P450 system. Located primarily in centrlobular hepatocytes, cytochrome P450 enzymes are associated with drug-induced sER proliferation (39). In the present study, sER proliferation was evident in electron microscopy micrographs of the CCl₄ treated group. Furthermore, chrysin reduced CCl₄-induced effects on hepatocyte ultrastructure, similarly to silymarin at the same dose.

**Molecular modeling.** The present study demonstrated that the hepatoprotective activity of chrysin is mediated through TNF-α. Chrysin pre-treatment significantly reduced CCl₄-induced TNF-α protein expression (P<0.001). This indicates that chrysin may modulate TNF-α processing, reducing soluble TNF-α generation. TNF-α is synthesized as a membrane-anchored precursor and the soluble form of TNF-α is released into the extracellular space through limited proteolysis by the zinc-endopeptidase TACE (40). TACE is a
multi-domain peptidase consisting of an extracellular region, a transmembrane helix and an intracellular C-terminal tail. The extracellular region of TACE comprises an N-terminal pro-domain, a 259 amino acid residue catalytic domain and a disintegrin-like cysteine-rich domain (40). The catalytic domain of TACE recognizes the pro-TNF-α cleavage site (Ala76-Val77) to generate TNF-α.

The present study investigated the theory of chrysin interacting with the TACE active site using the FlexX program. The crystal structure of TACE in complex with the inhibitor IK682 (PDB ID: 2FV5) (25) was used for a docking study (results shown in Fig. 7), which was validated by a re-docking procedure. FlexX reliably reproduced the crystallographic orientation of bound IK682 with an RMSD of 2.2 Å (Fig. 7). Comparison of the pharmacophoric features of the ligand-enzyme interaction in FlexX revealed the most favorable binding energy solution (Fig. 7A), which elucidated the pharmacophoric interaction for TACE inhibition as observed in the crystal structure (Fig. 7B). Therefore, FlexX accurately docked IK682 within the TACE active site (Fig. 7, upper right).

This methodology was then applied to investigate docking of chrysin within the TACE active site.

Molecular docking of chrysin revealed that chrysin favorably binds to the active site of TACE, with an estimated binding energy of -23.5 kJ/mol. In addition, chrysin was found to dock close to the catalytic region of TACE. The lowest energy docking solution revealed that the chromone moiety of chrysin, composed of A-, B- and C-rings, was oriented towards the catalytic zinc residue in the hydrophobic S1 sub-pocket of TACE, with the B-ring deep in the hydrophobic S3 sub-pocket (Fig. 8A). This binding of chrysin is non-planar, with the B-ring tilted ~45˚ in respect to the chromone plane (Fig. 8A). Fig. 8A shows essential interactions of chrysin with key catalytic residues of TACE. The catalytic zinc residue of TACE is coordinated by three imidazole nitrogen atoms of His-405, His-409 and His-415. The 7-OH group of TACE-docked chrysin was 4.5 Å away from the catalytic zinc and thus able to coordinate with it. This interaction may displace the ‘catalytic’ water molecule from the active site of the enzyme, explaining the inhibition of TACE activity.

Figure 5. Effect of chrysin on the expression and distribution of α-SMA protein in the liver of CCl4-treated mice. (A) Control group, (B) CHR group, (C) CCl4 group, (D) CHR+CCl4 group and (E) Sy+CCl4 group. Scale bar, 200 µm. (F) Quantification of α-SMA staining intensity. Results are represented as the mean ± standard deviation (n=5). ***P<0.001 vs. the control group; ###P<0.001 vs. the CCl4 group. α-SMA, α-smooth muscle actin; CCl4, carbon tetrachloride; CHR, chrysin; Sy, silymarin.
of TACE activity by chrysin. In addition, the 5-OH group of chrysin forms a hydrogen bond with the carboxylate oxygen of Glu-406 in TACE, which acts as a base during catalysis (41). Furthermore, the 7-OH group of chrysin forms a hydrogen bond with Pro-437 of TACE, which serves a key role in reversing the Met-turn produced by Tyr-433, Val-434, Met-435 and Tyr-436 to form the outer wall of the S1 crevice (39). The hydrogen bonding seen positioned the outer wall loop of the S1 sub-pocket in such a way that it essentially blocked the cavity opening. All these interactions stabilize the TACE-chrysin closed complex.

Fig. 8B represents the surface of the chrysin-bound TACE active site, colored according to B-factor value (flexibility). Chrysin was found to bind deep inside the hydrophobic cavity and the flexible Met-turn closes the cavity opening, which is bridged by Ala-439 and Leu-348. The essential pharmacophoric interaction of TACE inhibition by chrysin is shown in Fig. 8C. Excluding the two hydrogen bonds (described above), the majority of the interactions were hydrophobic. The hydrophobic B-ring of chrysin is oriented inside the S3 sub-pocket framed by a number of hydrophobic residues, including Leu-401, Val-402, Ile-438, Ala-439 and Val-440.

In conclusion, the results of the present study suggest that inflammation signaling pathways were activated in the pathogenesis of CCl₄-induced acute hepatic damage and that this could be counteracted with seven days of chrysin pre-treatment. In addition, the hepatoprotective activity of chrysin was identified to be mediated through TNF-α, via chrysin reducing soluble TNF-α generation via inhibiting TACE. Furthermore, chrysin was demonstrated to be a potent hepatoprotective agent, with similar effects to silymarin at the same dose, and should be investigated as a targeted drug to maintain a healthy liver and prevent liver damage.

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Figure 7. Validation of the FlexX docking method through re-docking of the inhibitor IK682 into the crystal structure (PDB ID: 2FV5) of tumor necrosis factor-α converting enzyme (TACE). (A) Pharmacophoric features of TACE interaction with inhibitor IK682, obtained using the FlexX docking method. (B) Pharmacophoric interaction of TACE with inhibitor IK682, as observed in the crystal structure. Top right, structure of the inhibitor IK682, obtained from docking with the orientation observed in the crystal structure. Amino acid names are abbreviated using standard International Union of Pure and Applied Chemistry convention. Green lines represent hydrophobic interactions, while dashed lines represent hydrogen bonds and metal interactions.

Figure 8. Structure of the lowest energy docking solution of the tumor necrosis factor-α converting enzyme (TACE)-chrysin complex obtained from molecular modeling. (A) Interactions of docked chrysin within the TACE binding site. Chrysin is rendered as a stick representation, TACE is rendered as a ribbon representation and hydrogen bonds indicated by dashed lines. (B) Orientation of docked chrysin within the active site of TACE. The active site is represented in surface mode and colored according to B-factor value (flexibility), with blue to green to red signifying increasing flexibility. (C) The essential pharmacophoric interactions of TACE inhibition by chrysin. Hydrogen bonds are indicated by dashed lines. Amino acid names are abbreviated using standard International Union of Pure and Applied Chemistry convention. Green lines represent hydrophobic interactions, while dashed lines represent hydrogen bonds.
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