ELSEVIER

Clotrimazole Protects the Liver Against Normothermic Ischemia-Reperfusion Injury in Rats

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ABSTRACT

Objective. To investigate the possible antiapoptotic prosurvival role of the pregnane X receptor (PXR) in hepatic ischemia-reperfusion injury in rats using clotrimazole (CTZ), a strong PXR transactivator.

Materials and Methods. Male Sprague-Dawley rats were divided into 3 groups of 6 each: sham-treated, control, and CTZ-treated animals. Control and CTZ-treated animals were subjected to 30 minutes of normothermic ischemia of the whole liver followed by 6 hours of reperfusion. The animals were then killed, and the liver was excised and blood samples collected.

Results. Clotrimazole induced a significant increase in expression of the *CYP3A* gene, indicating PXR transactivation, whereas expression of the antiapoptotic *Bcl-xL* gene was not increased. Serum concentrations of aspartate aminotransaminase and alanine aminotransaminase were lower in CTZ-treated animals than in control animals (difference not significant). Levels of poly(adenosine diphosphate–ribose) polymerase, a caspase-3 substrate, remained significantly higher in the CTZ-treated group compared with controls ($P < .05$). Clotrimazole increased the expression of phospho-p 44/42 extracellular signal-regulated kinase 1,2 ($P < .05$). The gene expression of the *heat shock proteins 27, 70 and 90* was significantly lower in CTZ-treated animals than in controls ($P < .05$).

Conclusion. Clotrimazole-mediated PXR transactivation protects the liver against ischemia-reperfusion apoptosis in rats. Phospho-p 44/42 extracellular signal-regulated kinase 1,2 is activated, whereas gene expression of *heat shock proteins 27, 70, and 90* is downregulated by induction of PXR.

LIVER ISCHEMIA-REPERFUSION (I/R) injury occurs in several clinical conditions including liver trauma, transplantation, and hepatectomy.[1] The inflammatory response that follows I/R and leads to organ dysfunction is caused by hepatocyte necrosis and apoptosis.[1] Necrosis has classically been considered the sole mode of cell death induced by I/R; however, increasing evidence indicates that apoptosis (programmed cell death) also has a critical role.[2]

The pregnane X receptor (PXR) is a nuclear receptor that induces expression of several genes involved in the metabolism and transport of xenobiotics and endobiotics.[3] This receptor binds to molecules of various molecular structures, serving as a xenosensor, and has a pivotal role in protection of cells against xenobiotics and endobiotics.[3] Previous in vitro studies by our group have provided evidence that transactivation of PXR diminishes the rate of spontaneous or provoked apoptosis by inducing the antiapoptotic proteins Bcl-2 and Bcl-xL in human and rat hepatocytes.[4–6] To our knowledge, the role of PXR in regulation of apoptosis has not been studied in vivo. Herein we describe activation of PXR in an in vivo model of liver apoptosis–normothermic I/R of rat whole liver and its

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effects on expression of the antiapoptotic *Bcl-xL* and heat shock protein (*hsp*) genes.

The objective of this study was to demonstrate that a prototypic PXR agonist such as clotrimazole (CTZ) is capable of protecting the rat liver against normothermic I/R injury. The mechanism seems to involve activation of the mitogen-activated protein kinase extracellular signal-regulated kinase 1,2 pathway and decreased expression of heat shock proteins *(hsps) 27, 70 and 90*.

MATERIALS AND METHODS

Animals

All experiments were performed according to our institutional guidelines for the care and use of laboratory animals. The study animals were age-matched male Sprague-Dawley rats (Charles River Laboratories, L'Arbresle, France) weighing 250 to 300 g. Animals were housed in individual polymethyl methacrylate (Plexiglas) cages, with free access to food and water before, during, and after the I/R procedure. Light (from 8:00 AM to 7:00 PM) and room temperature (21 ± 2°C) were kept constant.

Experimental Design and Surgical Procedure

Animals were divided into 3 groups of 6 each. Animals in the drug-treated group received intraperitoneal injections of CTZ (Sigma, l'Isle d'Abeau Chesnes, France), 50 mg/kg/d, diluted in 1 mL of corn oil (Sigma) for 3 days before the I/R procedure; animals in the control group received intraperitoneal injections of corn oil, 1 mL/d, for 3 days before the procedure; and sham-treated animals received nothing. Under light anesthesia, animals were placed on an ad hoc operating table, and the abdomen was prepared with povidone-iodine solution (Betadine; Astra Medica, Merignac, France). A midline laparotomy was performed, and the abdominal cavity was explored to rule out any complications from previous intraperitoneal injections. The vascular and biliary pedicles of the liver were cross-clamped with a nontraumatic clamp (201/A; Moria, Antony, France). The abdominal wall was then closed with 1 layer of running sutures. After 30 minutes of ischemia, the abdominal sutures were removed, and the clamp was released to allow reperfusion of the liver. Six hours later, the animals were killed by exsanguination, and blood samples were obtained from the inferior vena cava for determination of serum transaminase concentrations. The retrieved liver was washed in phosphate-buffered saline solution, and samples were snap-frozen in liquid nitrogen. Animals in the sham group underwent laparotomy for 5 minutes without vascular clamping.

Western Blot Analysis

Liver samples, 100 mg, were homogenized mechanically in 1 mL of hypotonic buffer (HEPES, pH 7.5: 25 mmol/L of magnesium chloride, 5 mmol/L of dithiothreitol, 5 mmol/L of phenylmethylsulfonyl fluoride, 2 mmol/L of pepstatin A, 10 μg/mL of leupeptin, 5 mmol/L of EDTA, 10 μg/mL of aprotinin, and antiphosphatases; Roche Diagnostics, Hoffman-La Roche Ltd, Basel, Switzerland). Protein concentrations were determined with a bicinchroninic acid assay kit (Pierce Biotechnology, Inc, Rockford, Illinois) according to the manufacturer's instructions. Samples were resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylfluoride membrane (Millipore Corp, Billerica, Massachusetts). Membranes were blocked with 5% nonfat skim milk in Tris-buffered saline solution with Tween-20 (TBS-T: 10 mmol/L of Tris, pH 7.5; 140 mmol/L of sodium chloride; and 0.1% Tween-20) for 1 hour at 37°C, washed, and incubated with the corresponding primary antibody in TBS-T containing 3% bovine serum albumin. Membranes were then incubated for 12 hours with the appropriate antibody diluted 1:1000 in TBS: rabbit anti-P-ERK 1,2 (Cell Signaling Technology, Danvers, Massachusetts); mouse monoclonal anti–(adenosine diphosphate–ribose)polymerase (PARP; Becton-Dickinson and Co, Franklin Lakes, New Jersey). The membranes were washed extensively in TBS. Detection was performed using horseradish peroxide–conjugated anti-rabbit or anti-mouse antibodies and enhanced chemiluminescence reagents; the reagents (Millipore Corp) were used according to the manufacturer's instructions. The signal was recorded using a CCD camera (ChemImager 2; SynGene, Frederick, Maryland), and analyzed using Gene Tools software (SynGene).

Real-Time Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was isolated using acid phenol extraction.[7] One microgram of total RNA was reverse transcribed using a kit (SuperScript II; Invitrogen Corp, Carlsbad, California) following the manufacturer's instructions. The resultant complementary DNA was diluted 100-fold (conditions set to obtain 98% efficacy), and for each gene (target genes *Bcl-xL, CYP3A, hsp 27, hsp 60, hsp 70,* and *hsp 90* or reference genes *Pgk1* and ribophorin-encoding genes) and each condition (CTZ, corn oil, or sham), a mixture of Taq polymerase, 6.4 mmol/L of magnesium chloride, deoxynucleotide triphosphate, primer, and the probe (https://www.roche-applied-science.com) was added. The cDNA was then amplified in a thermocycler (LightCycler 480; Roche Applied Science, Penzburg, Upper Bavaria, Germany) for 45 cycles using conditions of 95°C and 60°C for 10 seconds each. Commercially available software (LightCycler 480; Roche Applied Science) was used for relative quantitative analysis.

Measurement of Plasma Transferase Concentrations

Blood samples were centrifuged at 3000 rpm for 15 minutes at room temperature. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) concentrations were measured using an automatic analyzer (AU5400; Olympus Corp, Tokyo, Japan). Values are given as units per liter.

Statistical Analysis

Results are given as mean (SD). The nonparametric Mann-Whitney test was used to compare mean values. $P < .05$ was considered statistically significant.

RESULTS

CTZ Protection Against Normothermic I/R-Induced Apoptosis

Animals treated with CTZ exhibited significantly lower serum concentrations of AST and ALT ($P < .05$) (Fig 1). The caspase target substrate, PARP, an important regulator of apoptosis was significantly decreased in the livers of corn oil–treated animals ($P < .05$) (Fig 2). This demonstrates that less cleavage of this protein occurred after ischemia in CTZ-treated animals.

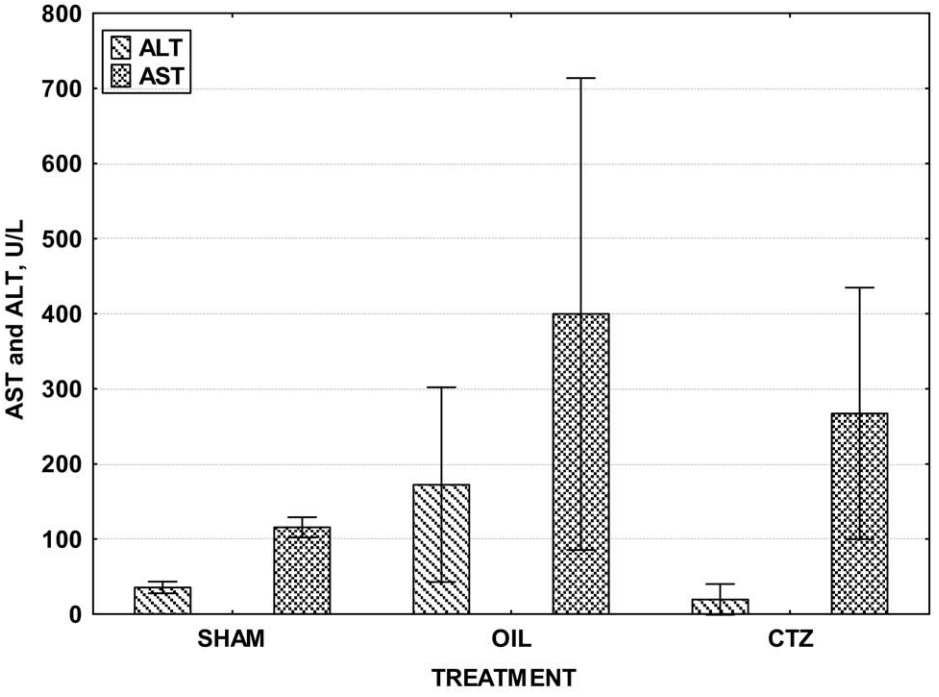

Fig 1. Serum concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) after ischemia-reperfusion of the whole liver. Animals in the clotrimazole (CTZ)–treated group demonstrated lower serum concentrations of ALT and AST; however, the difference was not statistically significant ($P < .05$). OIL = control group (corn oil).

PXR Transactivation and *Bcl-xL gene* Expression

Expression of *CYP3A1,* a known PXR target gene, was investigated to assess PXR transactivation in the mechanism of hepatic protection. The *CYP3A1* messenger RNA, as measured using quantitative real-time reverse transcription–polymerase chain reaction, was expressed 5- to 6-fold in the animals treated with CTZ, which indicates PXR transactivation ($P < .05$) (Fig 3). Inasmuch as we previously showed that activation of PXR promotes hepatocyte survival in vitro through modulation of the antiapoptotic protein Bcl-xL in rats,[4–6] we investigated the level of expression of *Bcl-xL gene* after an apoptotic stimulus such as I/R of the

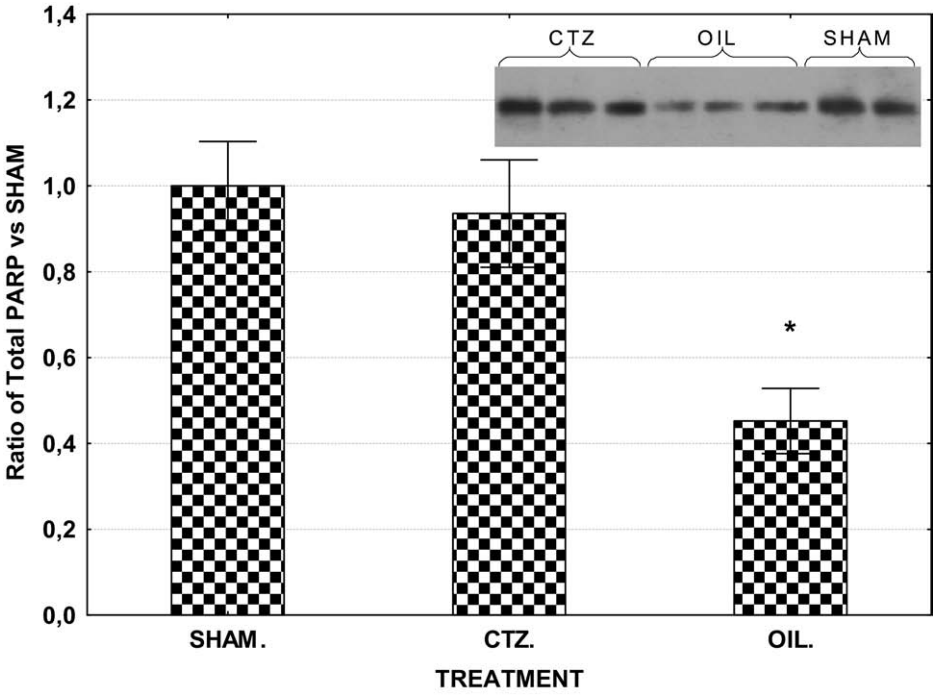

Fig 2. Western blot analysis of the poly(adenosine diphosphate-ribose)polymerase (PARP) concentration in liver tissue after ischemia-reperfusion. The caspase target substrate PARP is highly expressed in the liver of clotrimazole (CTZ)–treated animals compared with control animals ([corn] OIL). Data are expressed as the ratio between groups undergoing liver ischemia (CTZ and OIL) and untreated animals (SHAM group). *$P < .05$, SHAM vs OIL and CTZ vs OIL.

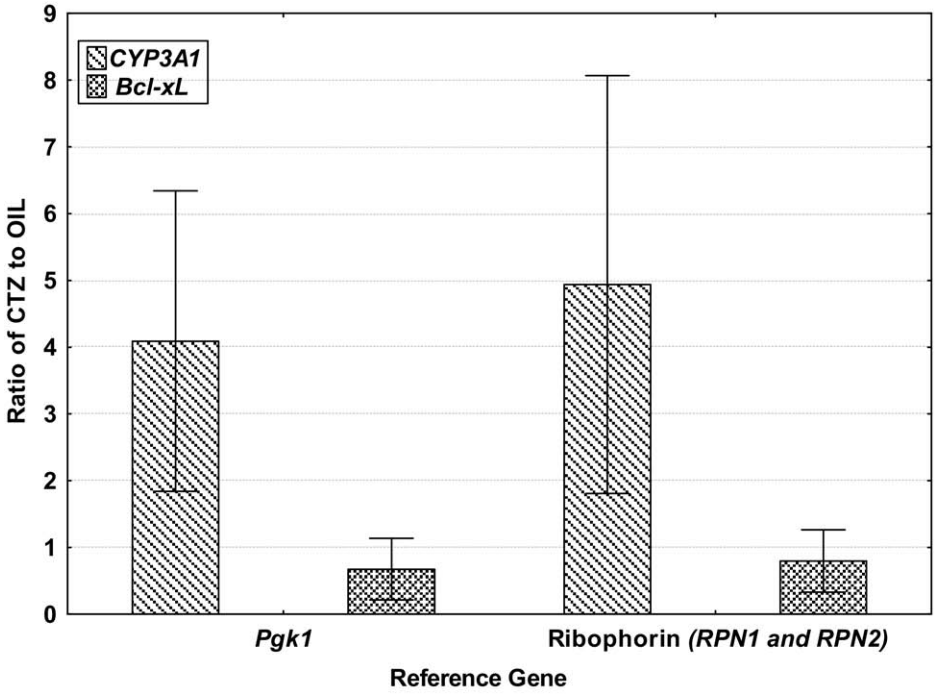

Fig 3. Quantitative polymerase chain reaction analysis of *CYP3A1* and *Bcl-xL* gene expression in liver tissue after ischemia-reperfusion of the whole liver. Clotrimazole (CTZ) induced expression of *CYP3A1,* a known pregnane X receptor target gene, in the liver in CTZ-treated animals, whereas no expression of *CYP3A1* was detected in the liver in control animals ([corn] OIL). Expression of the *Bcl-xL* gene was not significantly increased by CTZ treatment. The target genes (*CYP3A1* and *Bcl-xL*) are normalized to a reference gene (*PgK1* and ribophorin-encoding genes (RPN1 and RPN2)), and expressed as the mean (SD) ratio between the groups of animals undergoing liver ischemia treated with CTZ to OIL and the untreated group (SHAM).

liver. We observed no increase in expression of the *Bcl-xL* gene after treatment with CTZ (Fig 3).

Phospho-p44/42 ERK 1,2 and hsps 27, 60, 70, and 90

Because it has been proposed that ERK 1,2 activation promotes survival in models of hepatic injury,[8] we investigated the expression of phospho-p44/42 ERK 1,2 in the liver after I/R injury. Expression of phospho-p44/42 ERK 1,2 was significantly increased in the liver in animals treated with CTZ ($P < .05$), which suggests that CTZ protects the liver against I/R-induced apoptosis via activation of phospho-p44/42 ERK 1,2 (Fig 4). Induction of stress response (heat

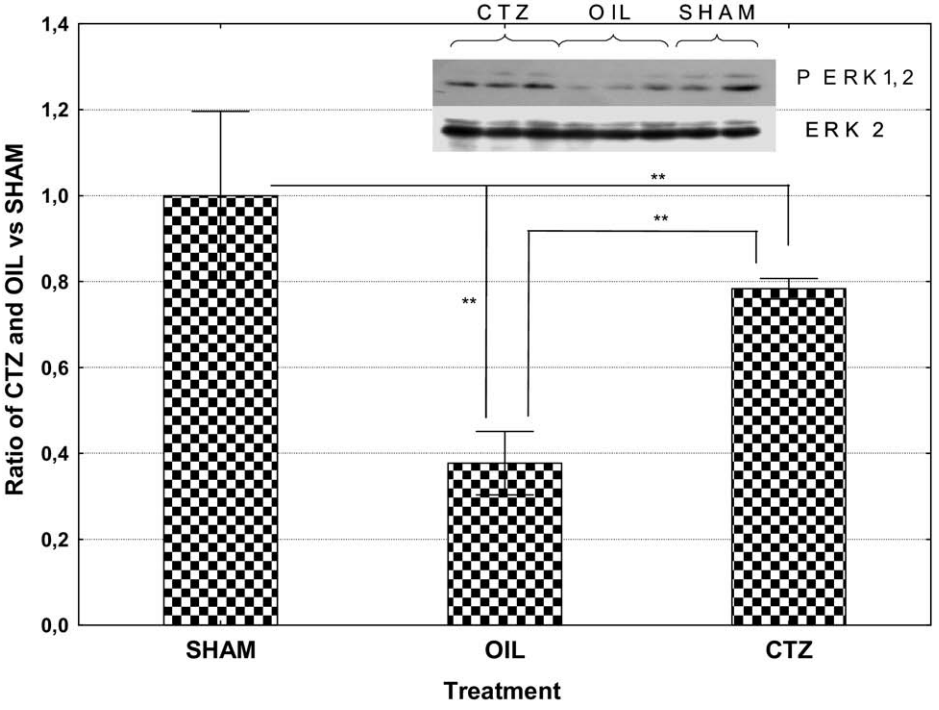

Fig 4. Western blot analysis of ischemia-reperfusion–induced phosphorylation of P-42/44 ERK in liver tissue. Clotrimazole (CTZ)–treated animals demonstrated a significant increase in expression of the antiapoptotic pro-survival P-41/44 ERK compared with control animals ([corn] OIL). ERK-2 is used as loading control. Data are expressed as the ratio between the groups undergoing liver ischemia (CTZ and OIL) and the untreated group (SHAM). *$P < .05$.

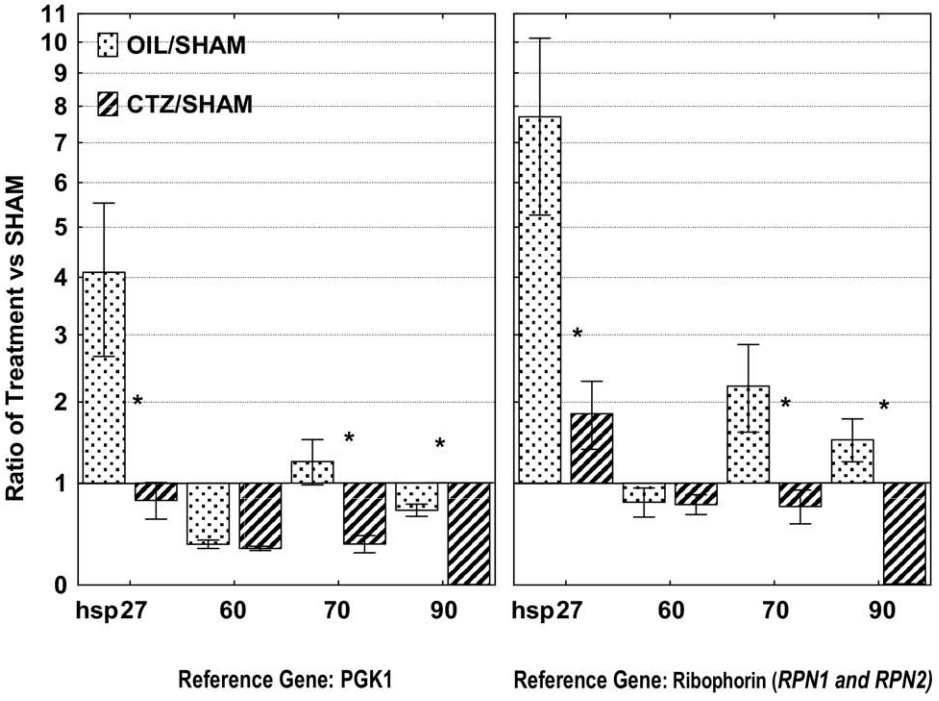

Fig 5. Quantitative polymerase chain reaction analysis of *hsp 27, hsp 60, hsp 70,* and *hsp 90* gene expression in liver tissue after ischemia-reperfusion of the whole liver. Clotrimazole (CTZ) downregulated expression of the *hsp 27, hsp 70,* and *hsp 90* genes, whereas expression of *hsp 60* was not affected. Expression of target genes (*hsp 27, hsp 60, hsp 70,* and *hsp 90*) is given as the mean (SD) ratio between the groups of animals undergoing liver ischemia (CTZ and [corn] OIL) and the untreated group (SHAM). The ratio is then normalized to a reference gene (PgK1 or ribophorin-encoding genes *(RPN1 and RPN2)* *$P < .05$.

shock) proteins is involved in the process of apoptosis[9]; thus, we examined the effect of I/R injury on expression of *hsp 27, 70, 60, and 90 genes* in the liver. Expression of the *hsp 27, hsp 70,* and *hsp 90* genes was decreased in the liver in animals treated with CTZ (Fig 5); however, the level of hepatic expression of *hsp 60* mRNA was not affected by treatment with CTZ ($P < .01$). These data suggest that CTZ liver protection against I/R-induced apoptosis involves decreased expression of *hsp 27, 70 and 90 genes*.

DISCUSSION

Previous in vitro studies performed in our laboratory have shown that PXR modulates liver apoptosis.[4,5] In human beings and in rats, activation of PXR promotes hepatocyte survival in vitro via modulation of the antiapoptotic proteins Bcl-2 and Bcl-xL.[6] In the present study, treatment with CTZ, a known PXR transactivator, induced hepatocyte protection against normothermic I/R-induced injury, as indicated by the decreased serum concentrations of transaminases and significantly lower concentrations of cleaved PARP, which is a substrate of activated executioner caspase 3 and a marker of apoptosis.[10]

Treatment with CTZ activated PXR, as demonstrated by increased expression of *CYP3A,* a known target of PXR and an indicator of PXR transactivation.[11] However, expression of the *Bcl-xL* gene, which codes for the antiapoptotic protein Bcl-xL, was not increased.

We also investigated the effect of CTZ on regulation of ERK 1,2, a protein of the mitogen-activated protein kinase family. We observed a significant increase in expression of ERK 1,2, the activated form that has an antiapoptotic prosurvival effect against I/R-induced liver apoptosis.[8]

Induction of hsps is a highly conserved mechanism that protects various cell types from diverse physiologic and environmental stresses. The (hsps) serve as molecular chaperones that facilitate the folding of enzymes and other proteins into functional conformations. They may also be involved in regulation of the process of apoptosis.[9] Treatment with CTZ was associated with decreased gene expression of *hsp 27, hsp 70,* and *hsp 90* but not *hsp 60.* Expression of *hsp 90* was extremely decreased by treatment with CTZ compared with expression of other studied HSPs (Fig 5). Heat shock protein is able to bind the proapoptotic protein twice daily and to maintain it in an inactive form.[12] This model may explain the prosurvival antiapoptotic effects of *hsp 90* depletion after treatment with CTZ.

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