Protective Role of Hepatocyte Cyclooxygenase-2 Expression Against Liver Ischemia–Reperfusion Injury in Mice

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Liver ischemia and reperfusion injury (IRI) remains a serious clinical problem affecting liver transplantation outcomes. IRI causes up to 10% of early organ failure and predisposes to chronic rejection. Cyclooxygenase-2 (COX-2) is involved in different liver diseases, but the significance of COX-2 in IRI is a matter of controversy. This study was designed to elucidate the role of COX-2 induction in hepatocytes against liver IRI. In the present work, hepatocyte-specific COX-2 transgenic mice (hCOX-2-Tg) and their wild-type (Wt) littermates were subjected to IRI. hCOX-2-Tg mice exhibited lower grades of necrosis and inflammation than Wt mice, in part by reduced hepatic recruitment and infiltration of neutrophils, with a concomitant decrease in serum levels of proinflammatory cytokines. Moreover, hCOX-2-Tg mice showed a significant attenuation of the IRI-induced increase in oxidative stress and hepatic apoptosis, an increase in autophagic flux, and a decrease in endoplasmic reticulum stress compared to Wt mice. Interestingly, ischemic preconditioning of Wt mice resembles the beneficial effects observed in hCOX-2-Tg mice against IRI due to a preconditioning-derived increase in endogenous COX-2, which is mainly localized in hepatocytes. Furthermore, measurement of prostaglandin E\(_2\) (PGE\(_2\)) levels in plasma from patients who underwent liver transplantation revealed a significantly positive correlation of PGE\(_2\) levels and graft function and an inverse correlation with the time of ischemia. Conclusion: These data support the view of a protective effect of hepatic COX-2 induction and the consequent rise of derived prostaglandins against IRI. (HEPATOLOGY 2019;70:650-665).

Liver ischemia and reperfusion (I/R) injury (IRI) remains a serious clinical problem affecting liver transplantation (LT) outcomes. IRI causes up to 10% of early organ failure and predisposes to chronic rejection.1 Moreover, the shortage of donor organs has led to an increased use of marginal livers, which are more susceptible to IRI.2 The mechanisms underlying hepatic IRI are complex. However, it is now becoming clear that reactive oxygen species (ROS) activate redox-regulated

**Abbreviations**: ALT, alanine aminotransferase; AMPK, adenosine monophosphate–activated protein kinase; AST, aspartate aminotransferase; BCL, base class library; CHOP, CCAAT/enhancer binding protein homologous protein; CLEC4F, C-type lectin domain family 4 member F; COX-2, cyclooxygenase-2; DFU, 5,5-dimethyl-3(3-fluorophenyl)-4-(4-methylsulfonyl)phenyl-2(5H)-furanone; EP2/EP4, PGE\(_2\) receptor types 2/4; ERS, endoplasmic reticulum stress; Gpx, glutathione peroxidase; GRP78, glucose-regulated protein 78; Grv, glutathione reductase; hCOX-2-Tg, human COX-2 transgenic (mice); Hmox1, heme oxygenase 1; IkBa, inhibitor of kappa B alpha; IL, interleukin; IPC, ischemic preconditioning; I/R, ischemia/reperfusion; IRI, ischemia/reperfusion injury; JNK, c-jun N-terminal kinase; LC3-I, microtubule associated protein 1 light chain 3; LDH, lactate dehydrogenase; LT, liver transplantation; Ly6G, lymphocyte antigen 6 complex locus G; MnSOD, manganese superoxide dismutase; MPO, myeloperoxidase; NF-κB, nuclear factor kappa B; Nfrp3, NLR family pyrin domain containing 3; NRF2, nuclear factor–erythroid 2–related factor 2; p-, phosphorylated; PC, preconditioning; PGE\(_2\), prostaglandin E\(_2\); ROS, reactive oxygen species; Sod1, cytosolic superoxide dismutase; Sod2, mitochondrial superoxide dismutase; TNF-α, tumor necrosis factor alpha; Wt, wild type.

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transcription factors such as nuclear factor-κB (NF-κB), which trigger the secretion of proinflammatory cytokines, leading to tissue injury.\(^\text{(3,4)}\) In response to these cytokines, neutrophils are recruited into the injured tissue, and they further release ROS, cytokines, myeloperoxidase (MPO), and other mediators which aggravate tissue damage.\(^\text{(5,6)}\) Moreover, oxidative stress induces the translocation of the nuclear factor-erythroid 2–related factor (NRF2), and the expression of several antioxidant and detoxification enzymes, such as glutathione-S-transferases, quinine oxidoreductase 1, glutamate cysteine ligase, and heme oxygenase 1, which in turn decrease ROS and reduce oxidative stress.\(^\text{(7)}\)

Additionally, IRI impairs autophagy\(^\text{(8)}\) and activates endoplasmic reticulum stress (ERS).\(^\text{(6,9)}\) Autophagy is a catabolic process mainly regulated by the conjugation of microtubule-associated protein 1 light chain 3 (LC3-I) to the membrane lipid phosphatidylethanolamine to form LC3-II. LC3-II serves as a recognition site for LC3-binding chaperones such as p62 that deliver their cargo to autophagosomes.\(^\text{(10)}\) Then, autophagosomes reach lysosomes and fuse to form autophagolysosomes, allowing the degradation by lysosomal acid hydrolases of their contents.\(^\text{(11)}\) ERS, initiated by a defensive process known as the unfolded protein response (UPR), comprises cellular mechanisms for adaptation of cell survival characterized by translational arrest of protein synthesis. UPR is mediated by the release of intraluminal chaperone glucose-regulated protein 78 (GRP78) from the endoplasmic reticulum sensors (PKR-like eukaryotic initiation factor 2 kinase [PERK], activating transcription factor 6, and the inositol requiring 1alpha). If the cell fails to adapt, oxidative and inflammatory signaling pathways are activated, leading to apoptosis through PERK-mediated CCAAT/enhancer binding protein homologous protein (CHOP) expression and signal-regulated kinase 1/C-jun N-terminal kinase (JNK), among others.\(^\text{(12)}\)

In the liver, and in various tissues, short periods of ischemia protect efficiently against subsequent IRI. This phenomenon, known as ischemic preconditioning (IPC), indicates that a brief ischemic insult triggers a protective biological reaction in the liver which is associated with inhibition of proapoptotic pathways.\(^\text{(13)}\)

Cyclooxygenase (COX)-1 and COX-2 catalyze the first step in prostanoid biosynthesis. COX-1 is constitutively expressed in many tissues, whereas COX-2 is induced by a variety of stimuli\(^\text{(14)}\); and its expression has been detected in several liver pathologies.\(^\text{(15)}\) On the other hand, hepatocyte-specific constitutive expression of COX-2 exerts an efficient...
protection against acute liver injury by an antiapoptotic/antinecrotic effect. Controversial results have been published related to COX-2 and IRI. It was reported that several COX-2 inhibitors protect mice and rats from hepatic IRI. However, Kuzumoto et al. reported that the prostaglandin E2 (PGE2)–PGE2 receptor type 4 (EP4) signaling pathway has an inhibitory role in hepatic IRI, showing that EP4 agonists effectively protect against IRI. Because there is a lack of studies on the effects of hepatic COX-2 induction and prostaglandins during liver IRI, in the present study we have demonstrated that constitutive hepatocyte expression of COX-2 significantly protects against IRI by decreasing inflammation, oxidative stress, apoptosis, and ERS. Moreover, the constitutive hepatocyte expression of COX-2 increases survival and autophagic signaling pathways, suggesting a cytoprotective role for COX-2 in hepatic IRI.

Patients and Methods

PATIENTS

Arterial PGE2 plasma concentration in a cohort of 63 human LTs from cadaveric donors, 60 minutes after reperfusion, was analyzed. This analysis was approved by the Ethics Committee on Clinical Research of the University General Hospital Gregorio Marañón (Madrid, Spain, June 2016). All patients had already signed a previous informed consent allowing the plasma analysis. Early graft function was assessed by the Toronto classification, which accounts for elevation of alanine aminotransferase (ALT), coagulopathy, and bile flow. Patients were classified as good early graft function (grades 1 and 2) and bad early graft dysfunction (grades 3 and 4). Graft ischemia time was recorded in all cases.

ANIMAL EXPERIMENTATION

Male 12-week-old human COX-2 transgenic mice (hCOX-2-Tg) and their wild-type (Wt) siblings were used. The mice were divided randomly into four groups: (1) sham operation group, (2) hepatic I/R group, (3) IPC (Wt PC) group, and (4) 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulfonyl)phenyl-2(5H)-furanone (DFU)–treated group (Wt PC DFU and hCOX-2-Tg DFU). Animals were anesthetized with isoflurane 1.2%-2%, and a model of segmental (70%) warm hepatic I/R protocol was assessed. Reperfusion was initiated by removal of the clamp for 4 and 24 hours. For IPC experiments, Wt mice were subjected to 20 minutes of ischemia followed by 30 minutes of reperfusion prior to 90 minutes of ischemia and 4 hours of reperfusion. For DFU treatment, Wt PC and hCOX-2-Tg mice were injected intraperitoneally with 5 mg/kg DFU in dimethyl sulfoxide (DMSO) 3 days and 45 minutes before the PC or hepatic ischemia, respectively. Control mice were treated with DMSO. The animals were sacrificed, and livers were removed, rapidly snap-frozen in liquid nitrogen, stored at –80°C, and collected in a solution containing 30% sucrose in phosphate-buffered saline or fixed in 10% buffered formalin. Plasma was obtained from the inferior cava vein. All animal experimentation was controlled following the recommendations of the Federation of European Laboratory Animal Science Associations on health monitoring, European Community Law (2010/63/UE), and Spanish law (R.D. 53/2013) with approval of the Ethics Committee of the Spanish National Research Council, Spain.

WHOLE TRANSCRIPTOME ANALYSIS

Total RNA was obtained using the mirVana miRNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA), prepared for sequencing using the Illumina TruSeq RNA Sample Prep Kit v2 according to the manufacturer’s instructions, and sequenced on a HiSeq 4000 platform (Macrogen, Geumchon-gu, Seoul, Korea). The Illumina HiSeq 4000 generated raw images using HCS (HiSeq Control Software v3.3) for system control and base calling through an integrated primary analysis software called RTA (Real Time Analysis, v2.7.3). The Base Class Library (BCL) binary was converted into FASTQ using the Illumina package bcl2fastq (v2.17.1.14). Trimmed 100-bp sequenced paired-end reads were mapped to a reference genome with HISAT2. After the read mapping, Stringtie was used for transcript assembly. An expression profile was calculated for each sample and transcript/gene as fragments per kilobase of transcript per million mapped reads.
DATA ANALYSIS

Data are expressed as means ± SE. Statistical significance was tested by one-way analysis of variance, followed by Bonferroni’s test. Analysis was performed using the statistical software GraphPad Prism 5. For patients, the Kolmogorov-Smirnov test was used to assess the normality of the distribution of numeric variables. To compare means between independent samples, the Mann-Whitney rank-sum test was used. To assess a significant relationship between two variables, Spearman’s correlation was calculated. Data were analyzed using SPSS IBM for Windows statistical package, version 24 (SPSS Inc., Chicago, IL). For all statistical tests, \( P < 0.05 \) was considered significant.

Results

HEPATIC COX-2 EXPRESSION PROTECTS TRANSGENIC MICE FROM IRI-INDUCED LIVER DAMAGE

We used our previously described transgenic mouse model, hCOX-2-Tg, which constitutively expresses human COX-2 in hepatocytes under the control of the human apolipoprotein E promoter containing the hepatic control region.\(^{(23)}\) The expression of human COX-2 in these livers is comparable to the endogenous levels reached in regenerating liver after partial hepatectomy.\(^{(24)}\) No changes in COX-2 mRNA or protein levels were found in hCOX-2-Tg mice after 90 minutes of ischemia and 4 or 24 hours of reperfusion (Supporting Fig. S1B; Fig. 1A). Furthermore, Cox-1 mRNA and COX-1 protein levels were maintained without changes in all conditions (Supporting Fig. S1A; Fig. 1A).

PGE\(_2\) levels were, as expected, significantly higher in hCOX-2-Tg when compared to Wt mice, even when there was a clear tendency to increase PGE\(_2\) hepatic levels in Wt mice 24 hours post-IRI (Fig. 1B). Liver sections were stained with hematoxylin/eosin and then evaluated by an experienced liver pathologist. Extensive parenchymal areas of necrosis and vascular congestion were observed in Wt mice at 4 and 24 hours after IRI. Necrotic hepatocytes were characterized by condensation of chromatin, swelling of organelles, karyolysis, and cell rupture. These pathological features were significantly attenuated in hCOX-2-Tg hepatocytes (Fig. 1C).

Elevated serum levels of aminotransferases such as ALT and aspartate aminotransferase (AST) reflect the degree of hepatic cell damage. IRI resulted in a marked elevation of serum ALT and AST levels, but they were significantly lower in hCOX-2-Tg mice after 4 hours of reperfusion (Fig. 1D). Lactate dehydrogenase (LDH) activity was measured as a necrotic marker.\(^{(25)}\) The increase in LDH activity after IRI was much lower in hCOX-2-Tg mice at 4 and 24 hours (Fig. 1D), confirmed by quantification of the necrosis grade (Fig. 1E).

ALTERATION OF GENE EXPRESSION PROFILE AFTER IRI

Murine whole transcriptome sequencing was performed in order to examine the different gene expression profiles in the hCOX-2-Tg mice after IRI (90 minutes ischemia, 4 hours reperfusion). A total of 70 genes showed significant differential expression (false discovery rate ≤0.05, log\(_2\) ≥2; Fig. 2A). Among these genes, 18 were up-regulated and 52 were down-regulated (Fig. 2B). To elucidate the roles of differentially expressed genes, gene ontology analysis was used to analyze the biological functions of those genes (Fig. 2C; Supporting Table S4). The results indicated that several pathways were consistently modified after IRI between mouse models, such as biological processes related with the response to stress and inflammation. Taking into account that inflammation and oxidant stress are the disease mechanisms most commonly implicated in hepatic IRI\(^{(5,6)}\), we decided to validate and to study in depth these two pathways.

COX-2 EXPRESSION REDUCED HEPATIC INFLAMMATION IN THE IRI MODEL

Proinflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β) are released by activated Kupffer cells and induce hepato-cellular and endothelial injuries. Furthermore, the NLR family pyrin domain containing 3 (NLRP3) inflammasome controls caspase-1 activity and IL-1β release and is activated in IRI by ROS production.\(^{(26)}\) Constitutive COX-2 expression reduced the hepatic mRNA levels of these proinflammatory cytokines, suggesting a protective role of COX-2 in IRI.
Fig. 1. hCOX-2-Tg mice are protected against I/R-induced liver damage. Wt and hCOX-2-Tg mice were subjected to a warm hepatic ischemia for 90 minutes and to reperfusion (I/R) for 4 or 24 hours. (A) Representative western blot of murine COX-1 and human COX-2 proteins in liver extracts. (B) PGE$_2$ levels measured by enzyme immunoassay in liver from hCOX-2-Tg versus Wt mice. (C) Representative images of hematoxylin/eosin-stained, paraffin-embedded liver sections from Wt and hCOX-2-Tg mice after sham or I/R at 4 and 24 hours. Necrotic areas are marked with asterisks and vascular congestion with arrows. (D) Plasma levels of ALT, AST, and LDH activities from Wt and hCOX-2-Tg mice. (E) Necrosis grade assessed by histological examination. Scores used were 0, none; 1, mild lesion (0%-20% of necrosis); 2, moderate lesion (20%-40% of necrosis); 3, severe lesion (>40% of necrosis). Data are expressed as means ± SE (n = 5-8 per group). *P < 0.05 versus Wt-sham, #P < 0.05 versus Wt-I/R. Abbreviation: GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
cytokines including Nlrp3 inflammasome. However, Il-10 expression, an anti-inflammatory interleukin, was increased in hCOX-2-Tg mice (Fig. 3A). Consistently, plasmatic levels of IL-1β, IL-6, and TNF-α reached higher levels at 24 hours of reperfusion, while they were significantly lower in hCOX-2-Tg versus Wt (Fig. 3B). Our previous data showed lower levels of inflammatory markers and reduced hepatic leukocyte recruitment and infiltration in hCOX-2-Tg mice upon different liver injuries. (16,27) There was no evidence of neutrophil accumulation after 4 hours of reperfusion. Tissue MPO activity, an index of neutrophil infiltration, was increased at 24 hours of reperfusion in Wt mice (Fig. 3C). Additionally, at this time point, immunohistochemical analysis showed lower levels of infiltrating lymphocyte antigen 6 complex locus G–positive (Ly6G+) cells (a neutrophil plasma membrane biomarker) when comparing hCOX-2-Tg versus Wt I/R groups (Fig. 3D).

NF-κB not only is an important mediator of the inflammatory response but its signaling pathway is a key component in the progression of I/R-derived injury. (28) To know whether NF-κB is involved in the regulation of inflammation in our model, nuclear p65 and phosphorylated cytosolic inhibitor of kappa B alpha (pIκBα) protein levels were analyzed by Western blot in Wt and hCOX-2-Tg liver subjected to IRI. We found in Wt IRI mice an important increase in nuclear p65 localization and in pIκBα protein levels in hepatocytes, suggesting a strong NF-κB pathway activation, whereas this effect was attenuated in hCOX-2-Tg mice (Fig. 3E,F).

The apoptotic process is a consequence of reperfusion, which marks the onset and progression of hepatocellular damage. (29) We have described an anti-apoptotic role for hepatic COX-2 expression (23,30); to further explore the protective effect of COX-2, caspase-3 activity and some key apoptotic/antiapoptotic protein levels were measured. Caspase-3 activity increased only in Wt mice after IRI (Supporting Fig. S2A). Moreover, an important increase in the BCL-2-associated X protein/B-cell lymphoma 2 (BAX/BCL-2) ratio was found in Wt IRI mice at 24 hours of reperfusion. This effect was attenuated
FIG. 3. Analysis of proinflammatory cytokines and liver inflammation in Wt and hCOX-2-Tg mice after I/R injury. (A) Hepatic mRNA levels of proinflammatory markers (Il-1β, Tnfa, Il-6, Nlrp3, and Il-10) in sham or I/R conditions. (B) Plasmatic levels of IL-1β, TNF-α, and IL-6 were assessed by Luminex analysis. (C) MPO activity measured in liver extract and (D) representative immunohistochemistry for Ly6G + staining from Wt and hCOX-2-Tg mice after I/R. (E) Representative immunofluorescence merged images of p65 (red), albumin or CLEC4F (green), and 4´,6-diamidino-2-phenylindole (blue) from Wt and hCOX-2 Tg mouse livers after I/R at 4 hours. Specific nuclear staining is marked with arrowheads. (F) Representative western blots and densitometric analysis of NF-κB (p65) and pIKBα protein levels in nuclear and cytosolic extracts, respectively. Data are expressed as means ± SE (n = 5–6 per group). *P < 0.05 versus Wt-sham, **P < 0.05 versus Wt-I/R. Abbreviations: DAPI, 4´,6-diamidino-2-phenylindole; Sh, sham.
in hCOX-2-Tg IRI mice (Supporting Fig. S2B). Additionally, after IRI an increase in pAKT/AKT and in phosphorylated adenosine monophosphate–activated protein kinase (pAMPKα/AMPKα ratios were observed in hCOX-2-Tg when compared to Wt mice (Supporting Fig. S2C). DFU treatment reversed the increase in pAKT/AKT and in pAMPKα/AMPKα ratios (data not shown).

IRI is associated with the generation of ROS and other oxidative stress–related molecules. In this regard, some COX-2-derived prostanoid actions were associated with an enhanced antioxidant response. The expression of several antioxidant genes is shown in Fig. 4A. The mRNA levels of Nrf2, heme oxygenase 1 (Hmox1), glutathione peroxidase (Gpx), glutathione reductase (Gsr), cytosolic superoxide dismutase (Sod1), and mitochondrial superoxide dismutase (Sod2) were increased in hCOX-2-Tg mice after 4 hours of IRI versus Wt IRI. To gain insight into the mechanisms involved, nuclear extracts were prepared to quantify the protein levels of NRF2. Nuclear localization of NRF2 increased in hCOX-2-Tg after IRI (Fig. 4B), and mainly hepatocyte location was corroborated by immunofluorescent staining (Fig. 4C). In this sense, we use albumin as a specific hepatocyte marker and C-type lectin domain family 4 member F (CLEC4F) as a Kupffer cell marker. Moreover, the activities of antioxidant enzymes were measured, and there was an increase in manganese superoxide dismutase (MnSOD), GSR, and GPX activities at 4 hours in hCOX-2-Tg mice after IRI (Fig. 4D; Supporting Table S1). Indeed, MnSOD protein levels were already increased in hepatocytes from hCOX-2-Tg in sham conditions. This difference was maintained at 4 or 24 hours of IRI (Supporting Fig. S3A,B).

Dihydroethidium staining was performed as an indirect measurement of ROS production. ROS levels were significantly increased in Wt IRI condition at 4 hours (Fig. 4E; Supporting Fig. S3C). These results were confirmed by the measurement of lipid peroxidation (LPO) levels. LPO increased by IRI only in livers of Wt mice (Supporting Fig. S3D), suggesting a diminished ROS production in hCOX-2-Tg mice. In line with this, total glutathione content was decreased after IRI but to a lesser extent in hCOX-2-Tg. Also, the ratio of oxidized/total glutathione, evaluated as an intracellular redox status marker, was significantly higher in Wt IRI mice after 4 hours (Fig. 4F). Overall, these data are compatible with an attenuated oxidative stress in hCOX-2-Tg IRI mice when compared to Wt IRI.

HEPATOCELLULAR COX-2 EXPRESSION ATTENUATES ERS AND ACTIVATES AUTOPHAGY AFTER IRI

It has been suggested that impaired autophagy increased hepatocellular damage during the reperfusion period. Therefore, we analyzed p62 and LC3-II protein levels. p62 protein levels were decreased in hCOX-2-Tg after IRI, whereas LC3-II and autophagy-related gene 7 levels increased (Fig. 5A-C). Overall, these data suggest an improvement of the autophagic process in hCOX-2-Tg liver after IRI. This effect was also observed using electron microscopy because we detected more double membrane structures (autophagosomes) in hCOX-2-Tg than in Wt after IRI (Fig. 5D).

Next, we examined if the changes in the autophagic process found during IRI were accompanied by activation of the ERS signaling pathway. Significant increases of GRP78 (at 24 hours), CHOP (at 4 and 24 hours), and pJNK (at 4 hours) were found in Wt mice after IRI versus the corresponding hCOX-2-Tg mice (Fig. 5E,F; Supporting Fig. S2C), suggesting an attenuated activation of the ERS signaling in these mice livers. Moreover, DFU treatment increased JNK phosphorylation in hCOX-2-Tg mice (data not shown).

IPC ATTENUATED I/R DAMAGE IN WT MICE AND WAS ASSOCIATED WITH ENDOGENOUS COX-2 INDUCTION

To check the possible role of endogenous COX-2 induction in the protective effect observed after IRI, we performed IPC in the Wt mice and inhibition of COX-2 activity in Wt IPC and hCOX-2-Tg mice with the COX-2–specific inhibitor DFU. When compared to Wt IRI, Wt IPC exhibited a decrease in the necrosis grade and vascular congestion, whereas DFU treatment led to a significantly higher necrosis grade in Wt IPC and hCOX-2-Tg IRI mice (Fig. 6A,B). These effects were reflected in the plasma levels of ALT, AST, and LDH in accordance with the necrosis grade (Fig. 6C). Consistently, increased levels of PGE2 were
**FIG. 4.** Hepatic hCOX-2 expression leads to increased antioxidant defenses and decreased I/R-derived oxidative stress. (A) mRNA levels of Nrf2 and antioxidant genes (Hmox1, Gpx, Gsr, Sod1, and Sod2) in liver extracts from sham or I/R at 4 hours. (B) Analysis of NRF2 nuclear expression levels in liver extracts from Wt and hCOX-2-Tg mice after I/R at 4 hours. (C) Representative merged images of NRF2 (red), albumin or CLEC4F (green), and 4′,6-diamidino-2-phenylindole (blue) immunostaining in liver from Wt and hCOX-2-Tg mice after I/R at 4 hours. Specific nuclear staining is marked with arrowheads. (D) Manganese superoxide dismutase activity assessed by nitro blue tetrazolium chloride reduction inhibition assay in native polyacrylamide gel electrophoresis. (E) Representative images of dihydroethidium staining in liver sections from Wt and hCOX-2-Tg mice in sham or I/R at 4 hours. (F) Total glutathione content and oxidized glutathione/total glutathione ratio. Data are expressed as means ± SE (n = 4–8 mice per group). *P < 0.05 versus Wt-sham, †P < 0.05 versus Wt-I/R. Abbreviations: a.u., arbitrary units; DAPI, 4′,6-diamidino-2-phenylindole; GSSG/GSHt, oxidized glutathione/total glutathione ratio; Sh, sham.
observed in the Wt IPC group and in hCOX-2-Tg mice when compared to Wt IRI animals, while DFU decreased these levels in both conditions (Fig. 6D). Higher PGE$_2$ levels were associated with more pronounced changes in mRNA and protein levels of endogenous COX-2 in Wt IPC versus Wt mice after IRI (Fig. 6E). Moreover, we confirmed that endogenous COX-2-induced expression is mainly in the hepatocytes (Fig. 6F). These data confirm the specificity of COX-2-dependent PGE$_2$ involvement in the protective effect.

To assess the contribution of endogenous COX-2 to the protection against IRI, the expression profile of inflammatory and antioxidant genes was analyzed;
FIG. 6. Ischemic PC induces Cox-2 mRNA and PGE₂ production attenuating I/R-induced liver damage. (A) Representative images of hematoxylin/eosin-stained, paraffin-embedded liver sections from Wt, Wt PC, DFU-treated Wt PC (Wt PC DFU), hCOX-2-Tg, and DFU-treated hCOX-2-Tg mice (hCOX-2-Tg DFU) after I/R at 4 hours. Necrotic areas are marked with asterisks and vascular congestion with arrows. (B) Necrosis grade assessed by histological examination. Scores used were 0, none; 1, mild lesion (0%-20% of necrosis); 2, moderate lesion (20%-40% of necrosis); 3, severe lesion (>40% of necrosis). (C) Plasma levels of ALT, AST, and LDH activities. (D) PGE₂ levels were measured by enzyme immunoassay in Wt, Wt PC, DFU-treated Wt PC, hCOX-2-Tg, and DFU-treated hCOX-2-Tg mice after I/R at 4 hours. (E) Mouse endogenous Cox-2 mRNA and protein levels in Wt and Wt PC mice after I/R at 4 hours. (F) Representative immunofluorescence merged images of COX-2 (red), albumin or CLEC4F (green), and 4′,6-diamidino-2-phenylindole (blue) from Wt and Wt PC mouse livers after I/R at 4 hours. Hepatocyte staining is marked with arrowheads. Data are expressed as means ± SE (n = 5-8 per group). *P < 0.05 versus Wt-sham, †P < 0.05 versus Wt-I/R, ‡P < 0.05 versus Wt-PC, ††P < 0.05 versus hCOX-2-Tg-I/R. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; FI, Fold induction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; m, mouse; Sh, sham.
and as shown in Fig. 7A, IPC decreased *Il-1b*, *Il-6*, *Tnfa*, and *Nlrp3* expression and increased *Il-10* in Wt IPC when compared to Wt IRI, resembling the constitutive COX-2 expression phenotype. The expression of antioxidant genes was increased in Wt mice subjected to IPC surgery; however, DFU treatment had no effect on these genes, probably because the decrease in PGE\(_2\) was not sufficient to suppress the modulation of these antioxidant genes (Fig. 7B).

**ELEVATED PLASMATIC PGE\(_2\) LEVELS CORRELATE WITH AN IMPROVEMENT OF LIVER FUNCTION AFTER LT**

To evaluate the contribution of PGE\(_2\) dynamics in a clinical setting, we analyzed arterial plasma PGE\(_2\) concentration in a cohort of 63 patients after receiving LT. Arterial PGE\(_2\) levels 60 minutes after reperfusion were significantly higher in patients with good graft
function compared to patients with poor graft function (479 [335-748] versus 276 [148-579] pg/mL) (Fig. 8A). Additionally, there was a significant inverse correlation between PGE$_2$ levels and graft ischemia time (Fig. 8B) as well as with the time of hospital stay (Fig. 8C).

**Discussion**

We used hCOX-2-Tg mice to investigate the role of COX-2 in the outcome of liver after IRI. Our data demonstrate that hCOX-2-Tg mice were significantly less susceptible to liver IRI. hCOX-2-Tg mice showed reduced ALT, AST, and LDH levels and necrosis grade after IRI, which indicates a lesser liver damage when compared to Wt animals. Moreover, IPC attenuated IRI damage in Wt mice through the induction of endogenous COX-2 expression, which is mainly localized in hepatocytes. Our observation that expression of COX-2 confers a protective role in liver suffering IRI is supported by the fact that DFU treatment restores most of the IRI parameters in both Wt IPC and hCOX-2 Tg mice. These results are also supported by other publications, in which COX-2 expression was beneficial in rat liver IRI$^{(20,33)}$ and in the immediate human postsurgical LT period.$^{(34)}$

Consistent with previous reports$^{(16,27)}$ constitutive hepatic COX-2 expression leads to lower plasmatic levels of proinflammatory cytokines (i.e., IL-1β, IL-6, and TNF-α). hCOX-2-Tg mice presented an attenuated inflammatory response with lower levels of proinflammatory markers and higher levels of IL-10, leading to a diminished hepatic IRI. Our in vivo data support (1) an attenuated inflammatory response, at least in part, by decreasing IRI-dependent NF-κB activation; (2) an antiapoptotic effect of COX-2, as deduced by the decrease of caspase-3 activation after IRI; and (3) an improved antioxidant response to oxidative stress and decreased ROS production induced by COX-2 after IRI.

Interestingly, Hamada et al.$^{(35)}$ reported that global COX-2 deficiency enhances T helper 2 immune function and impairs neutrophil recruitment in liver IRI. Nevertheless, the same authors reported that selective deletion of myeloid cell–derived COX-2 failed to ameliorate liver IRI and developed hepatic extensive

![FIG. 8. Liver graft function with respect to the level of PGE$_2$ measured in R60 plasma. (A) PGE$_2$ levels measured by enzyme immunoassay were analyzed in arterial plasma obtained from 63 patients receiving LT collected 60 minutes after reperfusion. Graft function was evaluated during the first 3 days after LT; the Mann-Whitney rank-sum test was used to analyze the relationship between PGE$_2$ levels and early graft dysfunction: NO, good graft function (grade 1 or 2); YES, poor graft function (grade 3 or 4) (**P = 0.026). (B,C) To assess a significant relationship between PGE$_2$ levels and ischemia time (B) or length of hospital stay (C), Spearman’s correlation was calculated, showing a significant inverse correlation between the two parameters.](image)
necrosis and leukocyte infiltration. These authors concluded that selective myeloid COX-2 gene inactivation, which is the major source of COX-2 activity in nonparenchymal hepatic cells, has no effect on the progression of liver damage in response to IRI. However, inhibition of inducible PGE2 synthase attenuates reperfusion injury through inactivation of Kupffer cells and neutrophils, indicating a crucial role for prostaglandins in hepatic IRI. In our experimental model, the results clearly support that constitutive expression of COX-2 in hepatic parenchymal cells leads to a diminished neutrophil infiltration in liver after IRI as indicated by Ly6G+ immunostaining and MPO activity.

In hepatic IRI, a dead liver cell is usually present in a necrotic form, but malfunctioning programmed cell death is also observed in the hepatic IRI process. We found an attenuated response in the BAX/BCL-2 ratio and caspase 3 activity in hCOX-2-Tg mice after IRI, in agreement with the reported antiapoptotic effect of COX-2 activity. The phosphatidylinositide 3-kinase/AKT and AMPK pathways play a central role in integrating diverse survival signals. It is known that the AKT pathway is a target of prostaglandins and that AKT phosphorylation is enhanced in the liver of hCOX-2-Tg mice, reinforcing the survival pathways. The increase in the pAKT/AKT ratio in hCOX-2-Tg mice after IRI may be due to a direct AKT activation through COX-2-dependent PGE2 acting through EP2/EP4 Gβγ dimers, as reported by Rizzo. It has been described that the activation of both AKT and AMPK is part of a protective mechanism to prevent IRI. Our results agree with this notion, showing increases not only in the pAKT/AKT ratio but also in the pAMPK to AMPK ratio in hCOX-2-Tg mice after IRI.

Ke et al. have reported that NRF2 has a protective role against hepatocellular damage induced by ischemia. In fact, pretreatment with an NRF2 inducer resulted in partial attenuation of hepatic IRI in rats. It has been described that some COX-2 metabolites can induce a nuclear translocation of NRF2 and even reduce ROS levels in a model of IRI. Our results show a clear induction of Nrf2, Hmox1, Sod1, and Sod2 expression in hCOX-2-Tg mice after IRI when compared to Wt with a concomitant increase in the nuclear protein levels of NRF2 and in the activities of antioxidant enzymes, hence leading to induction of an antioxidant response. This pattern was mimicked by IPC in Wt mice.

Several reports have suggested that IRI is characterized by defective liver autophagy. In this work we show an impaired autophagic flux after IRI in Wt mice. The decreased autophagy was attenuated in hCOX-2-Tg IRI, in agreement with previous works that reported in other cells autophagy induction through up-regulation of COX-2 and PGE2 production or that mouse PGES-2 down regulation is associated with autophagy inhibition and enhanced apoptosis.

Recent experimental data suggest the involvement of ERS in several liver diseases, including IRI. As a proof of concept, chemical chaperones such as 4-phenylbutyrate are able to protect against IRI by inhibition of ERS. Our data show that after IRI in Wt mice, an impaired autophagy associated with increased ERS after IRI occurs, involving an increase in ERS markers, such as GRP78, CHOP, and pJNK. Again, constitutive hepatocyte COX-2 expression attenuates ERS and activates autophagy after IRI.

From a translational point of view, we evaluated the relationship between PGE2 levels in plasma and the clinical data of patients undergoing LT. Interestingly, clinical data obtained in the context of LT showed a significant association between arterial PGE2 levels and early graft function, evaluated by a well-known scale that has been employed in previous publications. Additionally, ischemia time was inversely associated to PGE2. These results are clearly in consonance with the experimental results obtained in transgenic animals in which overexpression of COX-2 has a protective effect in several experimental settings. Our results suggest that, even in the physiological range levels, prostaglandins may have a protective role in patients receiving LT.

Overall, the data support beneficial and protective effects of hepatocyte COX-2 induction and its dependent prostaglandins during IPC as a key hepatic mechanism triggered against I/R-derived damage and might provide evidence-based support for further therapeutic interventions after liver injury.
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