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MOLECULAR MECHANISMS INVOLVED IN THE INDUCTION OF LIVER ISCHEMIC PRECONDITIONING AND POSTCONDITIONING

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PUBLICATIONS

Alchera E, Tacchini L, Imarisio C, Dal Ponte C, De Ponti C, Gammella E, Cairo G, Albano E, Carini R. *Adenosine-dependent activation of hypoxia inducible factor 1 (HIF-1) induces late preconditioning in liver cells.* Hepatology 2008;48:230-239.

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ABBREVIATIONS

PI3K, phosphoinositide 3-kinase; PKB/Akt, protein kinase B; PTEN, phosphatase and tensin homologue deleted from chromosome 10; ERK1/2, extracellular signal regulated kinase 1/2; A2AR, adenosine A2A receptor; I-R, ischemia–reperfusion; HIF-1, hypoxia inducible factor 1; CAIX, carbonic anhydrase IX.

ABSTRACT

Ischemia/reperfusion is the main cause of hepatic damage consequent to temporary clamping of the hepatoduodenal ligament during liver surgery as well as graft failure after liver transplantation (Serracino-Inglott, 2001).
Liver ischemic preconditioning obtained by a transient interruption of blood flow applied before a prolonged ischemic insult confers tissue protection against ischemia-reperfusion injury (Carini, 2003a).
The studies reported in this thesis investigated the molecular mediators of the endogenous cytoprotective systems activated by liver preconditioning and postconditioning in experimental rat liver models and in human liver. The main aims were to establish the efficacy of preconditioning and postconditioning procedures and focalize critical natural targets for future pharmacological or genetic interventions aimed to promote and intensify cytoprotection not only upon surgical procedures, but also in clinical conditions where the application of ischemic preconditioning is precluded, like in xenobiotic induced liver injury.
The study “Adenosine-dependent activation of Hypoxia-Inducible Factor-1 induces late preconditioning in liver cells” examined the possible involvement of the nuclear transcription factor HIF-1 in the development of the delayed phase of hepatic preconditioning. The results obtained indicated that activation of HIF-1 by adenosine-mediated signals is involved in the development of late preconditioning and that the expression of HIF-1–regulated carbonic anhydrase IX contributes to increased hepatocyte tolerance to hypoxia by preventing the alteration of intracellular pH and Na^+ homeostasis that lead ischemic cell death.
The study “Variable activation of phosphoinositide 3-kinase influences the response of liver grafts to ischemic preconditioning” analyzed the intracellular signals activated by ischemic preconditioning in human liver grafts. The data obtained suggested that the contradictory reports on the clinical response of transplanted livers to ischemic preconditioning might be explained by large inter-individual variability in giving an efficient

stimulation of phosphoinositide 3-kinase (PI3K) signaling by ischemic preconditioning in transplanted livers from heart-beating deceased donors.
The study “Pharmacological postconditioning by stimulation of adenosine A2A receptors or by chemical inhibition of PTEN protects rat hepatocytes from different pathogenic insults” evidenced the possibility to induce pharmacological postconditioning in primary rat hepatocytes after the instauration of liver damage of different etiology, such as post-ischemic damage and CCl_4 and menadione intoxication, by activation of common endogenous survival signaling pathways where PI3K and its modulator phosphatase and tensin homologue deleted from Chromosome 10 (PTEN) play a central role. The results also indicated adenosine A2A receptor agonists and PTEN inhibitors as possible useful agents for the pharmacological induction of postconditioning in the liver.

INTRODUCTION

Ischemia-reperfusion injury

Many surgical procedures on the liver require a period of ischemia, especially in extensive hepatic trauma, major hepatic resections or during liver transplantation. On blood restoration the liver is subjected to a further insult, aggravating the injury already caused by ischemia. This is named ischemia-reperfusion (I-R) injury, and is the main cause of both initial poor function and primary non-function of liver allograft (Serracino-Inglott, 2001).

Pathophysiology of ischemia /reperfusion injury

Several factors contribute to hepatic ischemia-reperfusion injury. The lack of oxygen during the ischemic period causes mitochondrial de-energization, adenosine triphosphate (ATP) depletion, and alterations of H^+, Na^+, and Ca^{2+} homeostasis that activate hydrolytic enzymes and impair cell volume regulation (Bronk, 1991; Gasbarrini, 1992; Rosser, 1995; Carini, 1999a). On oxygen readmission, the formation of reactive oxygen species by uncoupled mitochondria promotes oxidative stress and mitochondrial permeability transition (Jassem, 2002). The combination of these events is responsible for cell death by either necrosis or apoptosis (Rosser, 1995). The concomitant activation of Kupffer cells releases reactive oxygen species, nitric oxide, and pro-inflammatory cytokines (TNFα, IL-6, IL-1β, monocyte chemotactic peptide (MCP-1), IL-12, and CXC chemokines) (Lentsch, 2000). Cytokines and increased expression of adhesion molecules (intercellular adhesion molecule 1, vascular cell adhesion molecule 1, and E-selectin) by sinusoidal endothelial cells contribute to the progression of parenchymal injury by promoting liver neutrophil infiltration (Lentsch, 2000). The hepatic production of TNFα also propagates the inflammatory response to other organs, particularly to the lung, causing pulmonary insufficiency (Lentsch, 2000).

Ischemic preconditioning

The recent discovery of the endogenous cellular protective mechanism known as ischemic preconditioning has raised hopes that natural pathways could be activated to help the cells stave off the commitment to death (Murry, 1986).
Ischemic preconditioning refers to a phenomenon in which tissues are rendered resistant to the deleterious effects of I-R by previous exposure to brief periods of vascular occlusion. The protective effects were first described in the myocardium (Murry, 1986), but have been demonstrated also in other organs including the liver (Yellon, 2000).
Ischemic preconditioning has been shown to effectively reduce reperfusion damage during hepatic resection in humans (Clavien, 2003; Petrowsky, 2006) as well as to improve the outcome of hepatic transplants in experimental animals (Carrasco-Chaumel, 2005; Franco-Gou, 2006).

Phases of preconditioning

The protection induced by ischemic preconditioning takes place in two different phases. The first phase, known as “early preconditioning”, immediately follows the preconditioning stimulus and modulates different cellular functions; the second phase, known as delayed or late preconditioning, starts 12-24 hours after the preconditioning stimulus, can last for up to 3-4 days, and is characterized by gene transcription and “de novo” protein synthesis (Bolli, 2000, 2007; Gidday, 2006).
Despite these differences, both phases of preconditioning can be initiated by the same stimuli and partially share the same intracellular signaling pathways (Cohen, 2000; Bolli, 2000).

Mechanisms of liver preconditioning

A number of mechanisms involved in the hepato-protective effects of liver preconditioning have been evidenced (Carini, 2003a). Liver preconditioning protects mitochondria from post-ischemic oxidative damage (Lee, 2005) and preserves mitochondrial redox-state (Glanemann, 2003). Besides these effects on mitochondria, studies by our group have demonstrated that the preconditioning procedures reduce intracellular acidosis and Na^+ accumulation in hypoxic hepatocytes (Carini, 2000a; Carini, 2001a). Na^+ accumulation, upon the hypoxia-induced ATP depletion, results from the combined block of ATP-dependent Na^+ efflux through the Na^+/K^+ ATPase and the activation of Na^+/H^+ exchanger and Na^+/HCO_3^- co-transporter in response to cytosolic acidification (Yadav, 1999). The maintenance of intracellular pH in preconditioned hepatocytes is related to the activation and translocation on plasma membrane of the vacuolar ATPase (V-ATPase). V-ATPase, acting as alternative pH buffering system, allows proton extrusion, avoiding the activation of Na^+-dependent transporters (Carini 2000a, 2004a, 2006). This process increases hepatocyte tolerance to I-R, since intracellular Na^+ accumulation promotes hepatocyte killing during hypoxia and at the beginning of re-oxygenation (Carini, 1999a, 2000b) by impairing volume regulatory mechanisms (Carini 1999b). Preconditioned livers also show a significant reduction of oxidative damage occurring during re-oxygenation (Cavalieri, 2002; Peralta, 2002). This effect can be ascribed to an increased antioxidant capability as well as to a reduced ROS generation by intracellular sources or inflammatory cells. The combined effects on energy preservation, ion homeostasis and oxidative stress can thus explain the reduction of hepatocyte and sinusoidal endothelial cell apoptosis and necrosis observed in preconditioned livers exposed to I-R (Yadav, 1999; Lee, 2005). The hepato-protective effects of preconditioning are not limited to enhanced hepatocyte and endothelial cell resistance to I-R injury, but also involve the reduction of the inflammatory response associated to reperfusion. Ischemic preconditioning, in fact, reduces the

adhesion of leukocytes to sinusoidal endothelial cells, decreasing post-ischemic neutrophil infiltration of the liver (Peralta, 2000; Seraffin, 2004). Moreover, preconditioning attenuates the production of pro-inflammatory cytokines during reperfusion (Yoshizumi, 1998; Peralta, 2000; Serafin, 2004).

Signaling of liver preconditioning

The knowledge of the signal mediators responsible for the production of the preconditioned liver phenotype is still incomplete. The release of adenosine and nitric oxide (NO) by liver cells has been clearly demonstrated to induce the onset of the hepato-protective action of ischemic preconditioning (Peralta, 1997).

The changes of the constitutive proteins involved in the induction of the early resistance of preconditioned rat hepatocytes to hypoxia have been examined in recent works from our laboratory. The complex signal network stimulated in hepatocytes preconditioned with adenosine and NO has been partly enlightened. The interaction of adenosine with adenosine A2A receptors (A2AR) induces the stimulation of G stimulatory protein and the consequent activation of adenylate cyclase and protein kinase A (PKA). PKA phosphorylates A2AR and shifts its coupling to G inhibitory protein and Src kinase thus producing the activation of the survival mediator phosphatidyl-inositide-3-kinase (PI3K). This allows the stimulation of phospholipase C, the recruitment of the specific isoforms delta and epsilon of protein kinase C and the consequent activation of p38 MAPK (Carini, 2001b, 2004b). By stimulating two other different pathways also NO leads to PI3K (Carini, 2006) and p38 MAPK activation (Carini 2003b); one involves guanylate ciclase and cGMP-dependent kinase and the other the small G protein Ras (Carini 2003b, 2006). From these observations emerges the key role played by PI3K and p38 MAPK in mediating the signals leading to the early effects of liver preconditioning. These findings have been also confirmed "in vivo" showing a marked increase in the dual phosphorylation of hepatic p38 MAPK (Teoh, 2002) and demonstrating the

critical role of PI3K in mediating the hepatoprotection in preconditioned liver (Izuishi, 2003) . Moreover, data obtained in isolated hepatocytes have shown that both p38 MAPK and PI3K mediate the protection given by preconditioning on intracellular pH and Na^+ homeostasis promoting protons extrusion through V-ATPase (Carini, 1995, 2004a, 2006).
The intracellular signals responsible for the inhibition of the macrofage/Kupffer cells activation and of the adhesion molecules expression by endothelial cells are still unknown. Not yet clarified is the possible role of the mutual interaction of the different liver cells in producing the preconditioned liver phenotype.

Mechanisms and signaling in the late phase of preconditioning

The knowledge about the late phase of ischemic preconditioning has been deepened especially in the heart. Late preconditioning of the myocardium is a polygenic phenomenon that requires the simultaneous activation of multiple stress-responsive genes (Bolli, 2000). Chemical signals released by a sublethal ischemic stress (such as NO, reactive oxygen species, and adenosine) trigger a complex cascade of signaling events that include the activation of protein kinase C, Src protein tyrosine kinases, and nuclear factor kB and culminates in increased synthesis of inducible NO synthase (iNOS), cyclooxygenase-2, aldose reductase, Mn superoxide dismutase, and probably other cardioprotective proteins (Bolli, 2000).
In the liver the sustained protective effects that allow preconditioning to preserve liver grafts during both warm and cold ischemia and during reperfusion after transplant indicate the induction of newly synthesized protective mediators. Novel data on this aspect are now starting to emerge. In preconditioned livers an increased expression of proteins with potential protective effects (iNOS, antioxidants enzymes, Bcl-2, Bcl-xl and eme-oxygenase-1) has been observed (Lai, 2004; Koti, 2005; Yuan, 2005).

The Hypoxia Inducible Factor 1

Many transcription factors such as Hypoxia Inducible Factor 1 (HIF-1), Nuclear Factor kB (NFkB), Protein Activator 1 (AP-1) and Signal Transducer and Activator of Transcription proteins (STATs) are involved in the general response of cell to stress, in the induction of proliferation and in the modulation of inflammatory reactions (Pahl, 1999; Semenza, 2000; Laderoute, 2002; Mitchell, 2005).

In particular the transcription factor HIF-1, controlling the expression of hundreds of genes (Manalo, 2005), mediates developmental and physiological pathways that either deliver O_2 to cells or allow cells to survive O_2 deprivation, playing an essential role in the maintenance of oxygen homeostasis in metazoan organisms (Semenza, 2004; Brahimi-Horn, 2007). HIF-1 promotes the transcription of genes involved in energy metabolism, angiogenesis, erythropoiesis, glucose and iron transportation, glycolysis, proliferation, survival and migration (Semenza, 2007a).

Pharmacologic agents that activate or inhibit the hypoxia signal transduction pathway may be useful therapies for ischemic and neoplastic disorders respectively (Semenza, 2007b).

HIF-1 is a heterodimer comprising HIF-1α and HIF-1β subunits, both of which are basic helix-loop-helix transcription factors (Wang, 1995; Giordano, 2001). HIF-1β (ARNT) is a nuclear protein that is constitutively expressed and is independent of O_2 tension (Semenza, 2001). HIF-1α, in contrast to HIF-1β, is a cytoplasmic protein responsive to O_2 levels. In well-oxygenated cells, HIF-1α is continuously degraded by the ubiquitin-proteasome system. This degradation process takes place only when certain conserved prolyl residues of HIF-1α are hydroxylated, a modification requiring O_2-dependent enzyme activity (Maxwell, 2001). Only HIF-1α containing modified prolyl sites binds to the von Hippel-Lindau protein, which is the recognition component of an E3 ubiquitin ligase that finally targets HIF-1α for proteasomal degradation. Under hypoxic

conditions, HIF-1α subunits translocate to the nucleus, where they heterodimerize with HIF-1β subunits. The resultant product is an active HIF-1 protein that binds to specific hypoxic response elements present in target genes, ultimately activating transcription of these genes, which encode for erythropoietin, VEGF, various glycolytic enzymes, transferrin, and a variety of other proteins essential for systemic, local, and intracellular homeostasis (Vaupel, 2004).
Although hypoxia is the principal stimulus for HIF-1 stabilization, there is increasing evidence showing that HIF-1 is also implicated in biological functions requiring its activation under normoxic conditions. Amongst others, growth factors, vascular hormones, viral proteins and inflammatory mediators, such as NO and cytokines, are implicated in this normoxic activation (Dery, 2005).
Moreover a recent study by our group shows that in macrophages the formation of adenosine and induction of HIF-1 that occur in response to hypoxia are linked directly and suggest that HIF-1 activation through the adenosine A2A receptors may contribute to the anti-inflammatory and tissue-protecting activity of adenosine (De Ponti, 2007).

The carbonic anhydrase IX

Carbonic anhydrase IX (CAIX) is a major target gene of the transcription factor HIF-1 (Koong, 2000; Wykoff, 2000). Carbonic anhydrases are zinc metalloenzymes that catalyze the reversible hydration of CO_2 to form HCO_3^- and protons (Breton, 2001) . The carbonic anhydrase (CA) gene family includes ten enzymatically active members, which are major players in many physiological processes, including renal and male reproductive tract acidification, bone resorption, respiration, gluconeogenesis, signal transduction, and formation of gastric acid (Sly, 1995). The tumor-associated protein CAIX is an isoenzyme of the CA family whose expression was identified in a large number of human tumors but not in the corresponding normal tissue (Zavada, 1993; Ivanov, 2001). It has been suggested that tumor-associated transmembrane CA isoenzymes

(CAIX and CAXII) may facilitate acidification of the extracellular milieu surrounding cancer cells and in this way promote tumor growth and spread (Ivanov, 1998; Ivanov, 2001; Pouyssegur, 2006). Hypoxia has a profound activating effect on CAIX expression (Svastova, 2004), implicating the transcription factor HIF-1 as a strong regulator of the CAIX promoter (Koong, 2000). The expression of transmembrane CAIX is actually induced under hypoxic conditions in cultured cells and in hypoxic regions of human tumors suggesting the potentially important role of CAIX in cells adaptation to hypoxic conditions (Wykoff, 2000; Ivanov, 2001).
By catalyzing the reversible hydration of carbon dioxide, hypoxia-inducible CAIX activity influences pH in tumors (Ivanov, 2001). Extracellular pH in tumors is acidic due to increasing lactate production by glycolysis (Gillies, 2001), and CAIX and CAXII contribute to maintaining more neutral intracellular pH at the expense of lowering extracellular pH (Pouyssegur, 2006).

Ischemic preconditioning in human liver transplantation

Liver transplantation represents the most effective therapy for patients suffering from acute and chronic end-stage liver disease. In liver transplantation the organ undergoes periods of warm ischemia, during liver harvesting and implantation, but also a cold ischemic phase, during storage in the preservation solution. The injury caused by ischemia and following reperfusion is the main cause of liver graft failure (Jaeschke, 1998; Serracino-Inglott, 2001). Several strategies have been designed to limit this injury and its consequences. These include discarding grafts with severe steatosis (Adam, 1991; D'Alessandro, 1991), optimizing the preservation solution (Belzer, 1988), minimizing the ischemia time (Adam, 1992), and matching the quality of the graft to the status of the recipient (Strasberg, 1994). Recent animal studies have shown that ischemic preconditioning, during which brief exposure to warm ischemia provides robust protection against injury during long periods of ischemia, increases

tolerance to reperfusion injury (Serracino-Inglott, 2001; Selzner, 2003). In human liver transplants ischemic preconditioning is generally obtained by a 10 minutes Pringle maneuver, that consists in clamping the hepatoduodenal ligament with consequent interruption of the hepatic artery and portal vein blood flow, followed by 15 minutes warm reperfusion before starting the cold ischemic phase infusing the preservation solution through the aorta and the portal vein (Clavien, 2000, 2003).

Ischemic preconditioning effectively reduces reperfusion damage during hepatic resections in humans (Clavien, 2003; Petrowsky, 2006). In a prospective randomized study by Clavien et al. the data indicate that the protective effects of ischemic preconditioning correlate with the age of the patients and the volume of the remaining liver left after resection, furthermore they suggest that preconditioning is particularly effective in livers with steatosis and indicate preservation of ATP contents in the liver after reperfusion as a protective mechanism that may explain the failure of the older liver to respond to preconditioning (Clavien, 2003). In experimental studies on animal models ischemic preconditioning has demonstrated to be an effective strategy to improve also the outcome of liver transplants (Carrasco-Chaumel, 2005; Franco-Gou, 2006), but the application of ischemic preconditioning to reduce reperfusion injury after liver transplantation in humans has produced contradicting results (De Oliveira, 2007; Gurusamy, 2008; Desay, 2008). The first study using the model of cadaveric whole liver transplantation to evaluate the effect of ischemic preconditioning of the graft in humans was performed by the group of Azoulay (Azoulay, 2005). In accordance with most animal studies, it showed that ischemic preconditioning protects against ischemia-reperfusion injury as indicated by postoperative lower aspartate aminotransferase levels (Iu, 1987), but didn’t have the same positive impact on liver function, being significantly associated with graft initial poor function (evaluated by prothrombin time and bilirubin level). In another similar study by Cescon et al. (Cescon, 2006) ischemic preconditioning applied to deceased donors had a positive impact on postoperative levels of aminotransferases and was not detrimental for graft viability, but failed

to modify other clinical parameters. A more recent and larger clinical trial (Koneru, 2007) on deceased donor liver transplantation has given a paradoxical result: contrary to expectations ischemic preconditioning of the donor liver increased reperfusion injury (recipients of preconditioned livers had higher aminotransferases levels in the initial postoperative days compared to recipients of non preconditioned livers), but at the same time didn't have any adverse clinical consequences and was also associated with increased systemic levels of the anti-inflammatory cytokine IL-10 and fewer clinically important early rejection.

The phenomenon of ischemic postconditioning

Postconditioning in the heart

In cardiovascular research ischemic postconditioning is defined as an intervention that can be applied at the onset of myocardial reperfusion to reduce myocardial injury. Intervening at time of myocardial reperfusion, following the onset of an acute myocardial infarction, offers a potentially more powerful approach to cardioprotection compared to ischemic preconditioning, that can be applied only when myocardial ischemia can be predicted, as in cardiac bypass surgery (Yellon, 2005). The phenomenon of ischemic postconditioning was introduced in the research field of cardioprotection in 2003 by the group of Vinten-Johansen (Zhao, 2003), when they first noted that interrupting myocardial reperfusion with brief cycles of coronary artery re-occlusions had several beneficial effects including reduction in infarct size, less myocardial oedema, less neutrophil accumulation, reduced apoptotic cell death and improved endothelial function. Since then, the mechanisms underlying ischemic postconditioning in the heart have been intensely investigated.

Initially postconditioning appeared to be a relatively passive mechanical process, protecting the heart against the detrimental effects of lethal myocardial reperfusion injury by limiting oxidative stress, reducing calcium

accumulation, maintaining endothelial function and reducing inflammation (Zhao, 2003). However, further studies have evidenced that the protection elicited by postconditioning is mediated by activation of a number of signaling pathways, many of which are fundamental to obtain the cardioprotective effects, and that these pathways are partly common to those recruited by ischemic preconditioning (Hausenloy, 2006). Different studies have demonstrated that the cardioprotective benefits of ischemic postconditioning are dependent on the activation of Akt and Erk1/2 at the immediate onset of myocardial reperfusion (Tsang, 2004; Yang, 2004). This protective role for Akt and Erk1/2 has been confirmed in both diseased and non-diseased animal hearts (Zhu, 2006; Feng, 2006) as well as in human atrial muscle (Sivaraman, 2007).
The mechanisms through which postconditioning activates Akt at the time of reperfusion are not completely defined, but some studies have evidenced that, as it happens in preconditioning, the retention of endogenous adenosine and the stimulation of the adenosine receptors are partly responsible (Yang, 2005; Kin, 2006).

Postconditioning in the liver

The number of studies about postconditioning in the liver is still limited. These works show how the application of ischemic postconditioning during orthotopic liver transplantation in rats through intermittent interruptions of blood flow in the early phase of reperfusion is associated to amelioration of transaminase levels, to inhibition of hepatocellular apoptosis (Sun, 2004; Wang, 2008), to reduction of hepatic tissue damage and hepatocellular lesion, and to improved survival rate (Wang, 2008; Wang, 2009). To date the signal mediators triggered by postconditioning in the liver have not yet been clarified.

GENERAL AIMS

The main aim of the studies reported in this thesis is the investigation of the molecular mediators of the endogenous cytoprotective systems activated by liver preconditioning and postconditioning in experimental rat liver models and in human liver in order to:

- establish the therapeutic efficacy of preconditioning and postconditioning procedures;
- focalize critical natural targets for future pharmacological or genetic interventions aimed to promote and intensify cytoprotection not only upon surgical procedures, but also in clinical conditions where the application of ischemic preconditioning is precluded, like in xenobiotic induced liver injury.

On this general basis, the 3 studies respectively dealt with:

1) Investigate the possible involvement of the nuclear transcription factor HIF-1 in the development of the delayed phase of hepatic preconditioning, examining the capacity of previously characterized triggers and constitutive mediators of liver preconditioning to promote its activation and DNA binding.
2) Examine the role of the central mediator of liver preconditioning in animal models PI3K in influencing the response to ischemic preconditioning of human transplanted livers from deceased donors.
3) Develop a model of liver postconditioning and check the capacity of triggers and mediators of liver preconditioning to be effective, also if activated after a pathogenic insult, to increase hepatocytes resistance not only to ischemia-reperfusion injury, but also to toxic agents as carbon tetrachloride or menadione, employed as model substances of xenobiotic liver damage.

EXPERIMENTAL SECTION

STUDY 1: "Adenosine-dependent activation of Hypoxia-Inducible Factor-1 induces late preconditioning in liver cells"

AIMS: In the liver, late preconditioning improves post-ischemic sinusoidal perfusion, leucocyte infiltration, bile production and aminotransferase release for up to 48 hours after the application of the preconditioning stimulus (Carini, 2003a). The pharmacological induction of these sustained protective effects might be particularly relevant to improve liver surgery, but the knowledge on the factors that modulate gene expression during the late phase of liver preconditioning is still limited. One of the main transcription factors involved in tissue adaptation to ischemia is the Hypoxia Inducible Factor-1 (HIF-1) that binds to the hypoxia responsive elements located in the regulatory region of many genes (Semenza, 2000), influencing cell survival responses by enhancement of tolerance against re-oxygenation injury (Date, 2005). However, HIF-1 can be activated also in non-hypoxic conditions by oxidative stress, growth factors, cytokines and, as recently shown in macrophages, by the stimulation of the adenosine A2A receptors (De Ponti, 2007). Since adenosine is a well established inductor of liver preconditioning, this study aimed to investigate the possible involvement of HIF-1 in the development of the delayed phase of hepatic preconditioning.

CONCLUSIONS: The stimulation of the adenosine A2A receptors triggered late preconditioning through a PI3K dependent and PKC dependent activation of the nuclear transcription factor HIF-1, which in turn induced the expression of carbonic anydrase IX, a transmembrane enzyme that regulates extracellular and intracellular pH (Pouissegur, 2006). Carbonic anydrase IX expression increased hepatocytes tolerance to hypoxia preventing the cytosolic pH alteration and intracellular Na^+ accumulation that lead ischemic cell death. These findings propose HIF-1

as a potential target for the induction of pharmacological late preconditioning in the liver.

Adenosine-dependent activation of Hypoxia-Inducible Factor-1 induces late preconditioning in liver cells

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Abbreviations

A2AR, adenosine A2A receptor; CAIX, carbonic anhydrase IX; EMSA, electrophoretic mobility-shift assay; HIF-1, hypoxia-inducible factor-1; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C.

ABSTRACT

The cellular mechanisms by which ischemic preconditioning increases liver tolerance to ischemia/reperfusion injury are still poorly understood. This study investigated the role of the hypoxia-inducible factor-1 (HIF-1) in the protection associated with the late phase of liver preconditioning. Late preconditioning was induced in primary cultured rat hepatocytes by a transient (10 minute) hypoxic stress or by 15 minutes incubation with the adenosine A2A receptors agonist CGS21680 24 hours before exposure to 90 minutes of hypoxia in a serum-free medium. Late preconditioning induced the nuclear translocation of HIF-1 and the expression of carbonic anhydrase IX (CAIX), a HIF-1-regulated transmembrane enzyme that catalyzes bicarbonate production. Such effects were associated with prevention of hepatocyte killing by hypoxia and the amelioration of intracellular acidosis and Na^+ accumulation. The inhibition of PKC-mediated and PI3-kinase-mediated signals with, respectively, chelerythrine and wortmannin abolished HIF-1 activation and blocked both

CAIX expression and the protective action of late preconditioning. CAIX expression was also prevented by interfering with the transcriptional activity of HIF-1 using a dominant negative HIF-1 subunit. The inhibition of CAIX with acetazolamide or the block of bicarbonate influx with disodium-4-acetamido-4-isothiocyanato-stilben-2,2-disulfonate also reverted the protective effects of late preconditioning on intracellular acidosis and Na^+ accumulation. Conclusion: The stimulation of adenosine A2A receptors induced late preconditioning in liver cells through the activation of HIF-1. HIF-1-induced expression of CAIX increases hepatocyte tolerance to ischemia by maintaining intracellular Na^+ homeostasis. These observations along with the importance of HIF-1 in regulating cell survival indicates HIF-1 activation as a possible key event in liver protection by late preconditioning.

INTRODUCTION

A transient interruption of blood flow confers tissue protection against ischemia-reperfusion injury, a process known as ischemic preconditioning [1,2]. Liver ischemic preconditioning is receiving increasing attention for its capacity to improve reperfusion damage during hepatic surgery and transplantation [3,4]. The protective effects of ischemic preconditioning occur in two phases: an early phase that immediately follows the preconditioning stimulus (early preconditioning) and involves the modulation of different cellular functions and a delayed phase (late preconditioning) that is evident several hours after the preconditioning stimulus and is associated with gene transcription and “de novo” protein synthesis [1,2,5]. In recent years, several studies have characterized the mechanisms responsible for the development of early preconditioning. In the liver, adenosine released by hepatocytes is an important trigger of early preconditioning [6]. By interacting with adenosine A2A-receptors (A2AR), adenosine activates different intracellular signal pathways involving, among others, phosphatidylinositol 3-kinase (PI3K), protein

kinase B (PKB/Akt), the isofoms δ and ε of protein kinase C (PKC δ/ ε) and p38 mitogen activated protein kinase (p38MAPK) [6,7]. However, the factors that modulate the gene expression during the delayed phase of hepatic preconditioning are less well characterized [6]. The hypoxia-inducible factor-1 (HIF-1) is the main transcription factor responsible for the tissue adaptation to ischemia [8,9]. HIF-1 binds to hypoxia responsive elements (HRE) located in the regulatory regions of a vast array of genes that regulate energy metabolism, neovascularization, hematopoiesis and cell migration [9,10]. Moreover, recent evidence points out HIF-1 influence on cell survival responses [8,10]. In particular, the expression in rat cardiomyocytes of stable hybrid forms of HIF-1α prevent cell death induced by simulated ischemia/reperfusion [11], indicating the involvement of HIF-1–regulated genes in enhancing cell tolerance against reoxygenation injury. HIF-1 is a helix-loop-helix transcription factor that requires for its activity the dimerization of HIF-1α and HIF-1ß subunits [9]. HIF-1ß is a constitutively expressed nuclear protein, while HIF-1α is a cytoplasmatic protein that is continuously degraded by oxygen-dependent HIFprolyl-4-hydroxylase and arginyl-hydroxylase factor inhibiting-HIF-1α [9]. Thus, hypoxia prevents HIF-1α hydroxylation allowing its nuclear translocation and the transcription of HIF-1 target genes. However, increasing evidence indicates that HIF-1α can be also activated under nonhypoxic conditions by oxidative stress, growth factors, and cytokines [12]. In particular, we have recently demonstrated that the stimulation of adenosine A2A-receptors triggers an oxygen-independent induction of HIF-1 in macrophages [13]. These observations and the notion that adenosine is a key inducer of liver preconditioning, prompted us to investigate the possible involvement of HIF-1 in hepatic late preconditioning.

MATERIALS AND METHODS

Materials.

CGS21680, acetazolamide, disodium 4-acetamido-4-isothiocyanato-stilben-2,2-disulfonate, gentamycin, penicillin, streptomycin, 8-cyclopently-1,3-dipropylxanthine (DPCPX), VUF5574, PD 98059, H89, chelerythine, wortmannin and dexferroxamine were purchased from Sigma-Aldrich, Milan, Italy. ZM241325 was obtained from Tocris's Cookson Ltd. (Bristol, UK).

Preparation and Treatment of Cultured Hepatocytes.

Liver cells were isolated from the livers of fed male Wistar rats (180-250 g weight) (Harlan-Nossan, Italy) by collagenase perfusion [7] and hepatocytes were purified by centrifugation at 50g for 5 minutes followed by a further 2 minutes of centrifugation at 350g through a layer of Percoll (1.06 final density). Cell purity was assessed according to Benten et al [14]. The use and care of the animals were approved by the Italian Ministry of Health. Hepatocyte suspension (purity >95%) were plated on collagen-coated culture dishes and cultured for 48 hours in Dulbecco's modified Eagle medium (DMEM-HAM F12) containing 10% fetal bovine serum and 1% penicillin/streptomycin and 1% glutamine. Hepatocytes were preconditioned in Krebs-Henseleit-HEPES (KHH) medium by exposure to 10 minutes of hypoxia (95% N2, 5% CO2) followed by 10 minutes of reoxygenation (95% air, 5% CO2) or by 15 minutes of treatment with 1 µmol/L CGS21680. At the end of the preconditioning treatments, the cells were transferred into fresh DMEM-HAM F12 and cultured for 1-24 hours.

The development of late preconditioning was evaluated 24 hours after cell stimulation by assessing hepatocyte tolerance to the killing induced by 90 minutes of incubation in Krebs-Henseleit-HEPES (KHH) buffer (final cell density of 10^6/mL) under a hypoxic atmosphere [7]. Control cells were maintained in normoxic DMEM-HAM F12. The different inhibitors were added 15 minutes before preconditioning and removed 2 hours afterward.

Acetazolamide and disodium 4-acetamido-4-isothiocyanato-stilben-2,2-disulfonate, were added 30 minutes before the hypoxic incubation. The HTC rat hepatoma cell line was obtained from the European Collection of Cell Cultures and cultured in DMEM-HAM F12 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin and 1% glutamine. The preconditioning procedures and treatments were the same as for primary hepatocytes.

Electrophoretic Mobility Shift Assay (EMSA).

Nuclear extracts were prepared as described [15] and aliquots were incubated with γ-[^{32}P] ATP-labeled oligonucleotides (Primm, Milan, Italy) encompassing the binding sites for HIF-1 (5'-AGCGTACGTGCCTCAGGA-3') and oct-1 (5'-TGCGAATGCAAATCACTAGAA-3'), and then electrophoresed and autoradiographed [15]. The specificity of the assay was demonstrated by the disappearance of the signals after the addition of a 50-fold excess of specific and not nonspecific unlabeled oligonucleotides. The quantitative determinations were performed by direct nuclear counting using an InstantImager (Packard Instruments, Milan, Italy), and the values were normalized to the activity of oct-1.

Transient Transfection Assay.

Subconfluent HTC cells in 24-well tissue culture dishes were transiently transfected using the TransIT- (Mirus, Tema Ricerca, Italy) with a 5:1 mixture of pGL3PGK6TKp (100 ng) vector containing a HRE multimer (gift from P.J. Ratcliffe, Wellcome Trust Center for Human Genetics, Oxford, UK) and the pRL-TK reporter vector containing renilla luciferase, which was used to normalize transfection efficiency. After 5 hours, the medium was replaced with fresh medium, and the cells were exposed to 5μM CGS21680. When appropriate, the cells were cotransfected with 1μg of the expression vector pcDNA3ARNTdelta_b (ΔARNT) coding for the dominant negative mutant form of ARNT subunit (obtained from M. Schwarz, University of Tübingen, Germany). After 24 hours, the cells were collected and luciferase activities were measured in a Promega

luminometer using the Dual-Luciferase Reporter Assay System (Promega, Milan, Italy) according to the manufacturer's instructions. The empty vectors showed practically undetectable luciferase activity. All of the transfection experiments were carried out on duplicate plates and repeated at least three times.

Evaluation of HIF-1 Expression.

HIF-1α was determined in nuclear extracts from hepatocytes and HTC cells. Equal amounts of proteins were electrophoresed in acrylamide-SDS gels and electroblotted to Hybond-ECL membranes (Amersham, Milan, Italy). After assessing transfer by means of Ponceau S staining, the membranes were incubated with monoclonal anti-human HIF-1 antibody (H1α67, dilution 1:1000; Novus Biologicals, Littleton, CO). The anti-TFIID antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used to assess equal protein loading. The antigens were detected using an immunodetection kit (ECL Plus; Amersham) and quantified by videodensitometry.

Evaluation of Carbonic Anhydrase IX Expression.

Cell lysates were prepared as described previously [7], were centrifuged 10 minutes at 13,000g. Aliquots (30μg) of the protein extract were electrophoresed on a 10% SDS-polyacrylamide gel and blotted onto nitrocellulose membranes. CAIX was revealed using a policlonal anti-CAIX antiserum (dilution 1:500; Santa Cruz Biotechnology, CA). The anti-actin monoclonal antibody (Sigma-Aldrich, Milan, Italy) was used to assess for equal protein loading. The antigens were detected using an immunodetection kit (ECL Plus; Amersham). The relative intensity of CAIX and actin bands was measured by videodensitometry and the results were expressed as ratios.

Determination of Cell Viability.

Cell viability was estimated by microscope-counting the hepatocyte excluding Trypan blue and by the determination of nuclear fluorescence staining with propidium iodide [7].

Measurement of Cytosolic pH and Intracellular Na^+ Content.

Cytosolic pH and intracellular Na^+ content were evaluated using the respective fluorescent dyes 2,7-bis(carboxyethyl)-5,6-carboxyfluorescein-acetoxymethyl ester (BCECF-AM) and benzofuran isophthalate acetoxymethyl ester (SBFI-AM) (Molecular Probes, Eugene, OR) as previously described [16].

Data Analysis and Statistical Calculations.

The data were expressed as means ± SD. Statistical analysis was performed by Instat-3 statistical software (GraphPad Software Inc., San Diego, CA) using one-way analysis of variance test with Bonferroni's correction for multiple comparisons when more than two groups were analyzed. Distribution normality of all groups was preliminary verified by Kolmogorov and Smirnov test. Significance was taken at 5% level.

RESULTS

The Stimulation of Adenosine A2A Receptors Induces Late Preconditioning in Rat Hepatocytes.

Primary cultured rat hepatocytes were exposed to a transient (10 minute) hypoxic stress followed by the return to normoxic conditions and the development of the late phase of preconditioning was evaluated 24 hours later. Figure 1A shows that late preconditioning significantly increased hepatocyte tolerance against cell killing induced by 90 minutes of hypoxia in a serum-free medium. Previous studies demonstrated the specific role of adenosine A2A receptors (A2AR) in triggering the early phase of hypoxic preconditioning [6,17,18]. In agreement with these results, we also observed that the protection given by late preconditioning was reverted by the specific A2AR antagonist ZM241385 (1 μmol/L). Moreover, late preconditioning was evident 24 hours after a short (15 minute) hepatocyte incubation with the A2AR agonist CGS21680 (1 μmol/L) (Fig. 1A). The tolerance toward hypoxic death in hepatocytes undergoing late preconditioning was associated with the prevention of both intracellular acidosis and Na^+ accumulation (Fig. 2).

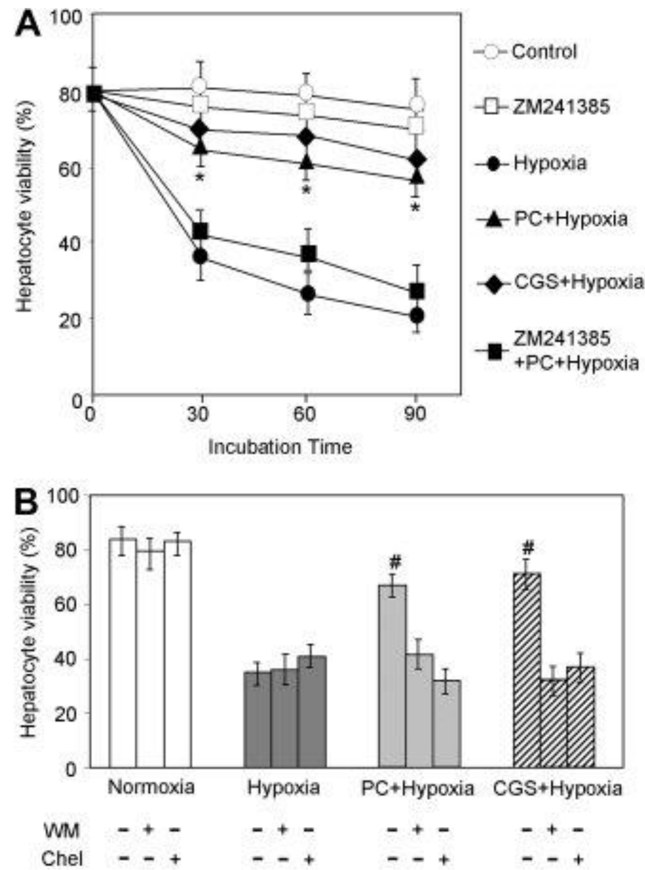

Figure 1. Late preconditioning protects hepatocytes from killing induced prolonged hypoxia: role of adenosine A2A receptors (A2AR) and PI3K- and PKC-mediated signals. Late preconditioning was induced by exposing cultured rat hepatocytes to transient (10 minutes) hypoxic stress (PC) or by 15 minutes incubation with the A2AR agonist CGS21680 (CGS, 1 µmol/L). Preconditioned and non preconditioned hepatocytes were further cultured in normoxic conditions for 24 hours before the exposure to hypoxia in serum-free medium. ZM241385 (1 µmol/L), wortmannin (WM, 250 nmol/L), or chelerythrine (Chel, 50 µmol/L) were added to the culture medium 15 minutes before the preconditioning procedures and removed 2 hours after. The results are means of at least five different experiments ± SD. Statistical significance: *P < 0.001 versus hypoxia or ZM241385+PC+hypoxia; #P < 0.001 versus PC+hypoxia+WM, or PC+ hypoxia+Chel.

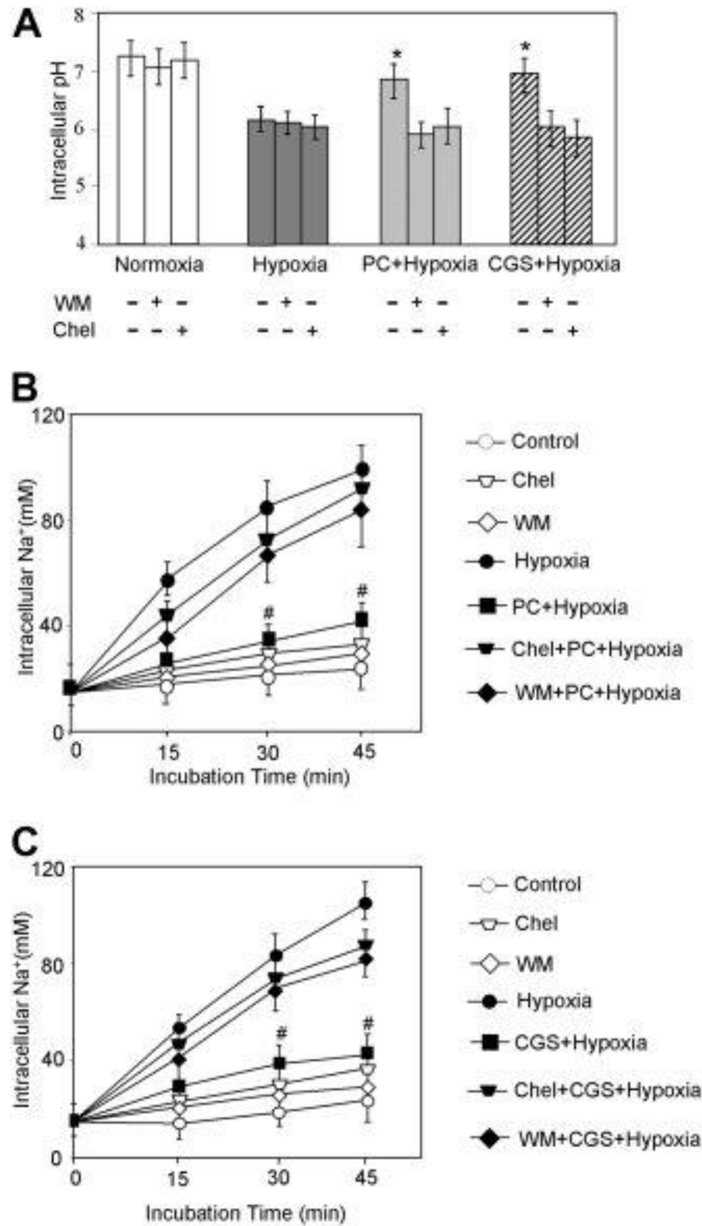

Figure 2. Late preconditioning prevents intracellular acidosis and Na^+ accumulation in ischemic hepatocytes. Late preconditioning was induced by hypoxic stress (PC) or by 15 minute incubation with CGS21680 (1 µmol/L; CGS). Preconditioned and non preconditioned hepatocytes were further cultured in normoxic conditions for 24 hours before the exposure to hypoxia. Wortmannin (WM, 250 nmol/L) or chelerythrine (Chel, 50 µmol/L) were was added to the culture medium 15 minutes before the preconditioning procedures and removed 2 hours after. (A) Intracellular pH was measured following 30 minutes of cell incubation in hypoxic or normoxic conditions. The changes in intracellular Na^+ were measured following the induction of late preconditioning by (B) hypoxic stress or (C) CGS21680. The results are means of at least four different experiments ± SD. Statistical significance: *P < 0.05 versus PC+hypoxia+WM or PC+hypoxia+Chel; # P < 0.002 versus hypoxia, PC+hypoxia+WM or PC+hypoxia+Chel.

The Stimulation of Adenosine A2A Receptor Activates HIF-1 in Preconditioned Hepatocytes.

EMSA assays using nuclear extracts of hepatocytes preconditioned by a transient (10 minutes) hypoxic stress revealed an increase in the DNA binding of HIF-1 2-3 hours after the preconditioning stimulus (Fig. 3). The effect of preconditioning on HIF-1 activity was comparable to that induced by 3 hours of incubation under hypoxia or by the iron chelation with desferrioxamine (Fig. 3), a well-known HIF-1 inducer [19]. In the same assays the complex that migrated faster than HIF-1α represented constitutive factors closely related or identical to the transcription factors ATF-1 and CREB-1 [20] that have been previously shown to be induced by hypoxia, iron chelation, or adenosine [21,22]. HIF-1 activation in preconditioned hepatocytes was not the response to the transient oxygen deprivation because no HIF-1 DNA binding was appreciable in cells exposed to 30 minutes of hypoxia (Fig. 3). HIF-1 induction by hypoxic preconditioning was abolished by the A2AR antagonist ZM241385 (Fig. 3) that did not affected HIF-expression induced by 3 hours of hypoxia or desferrioxamine (not shown). Conversely, A2AR stimulation by CGS21680 promoted HIF-1 DNA binding in oxygenated cells mimicking the effects of preconditioning (Fig. 3). The specificity of HIF-1 DNA binding was confirmed by competition experiments using unlabeled oligonucleotides (Fig. 3). Immunoblotting of nuclear proteins from cells exposed to hypoxic preconditioning or CGS21680 confirmed an increase in the nuclear content of HIF-1 protein which paralleled with HIF-1 DNA binding (Fig. 4A,B). Also in these experiments, the A2AR antagonist ZM241385 prevented the nuclear translocation of HIF-1α (Fig. 4C), whereas the block of adenosine A_1 and A_3 receptors with, respectively, DPCPX (100 μmol/L) or VUF5574 (100 μmol/L) was ineffective (Fig. 4E). The capacity of A2AR to trigger nonhypoxic HIF-1 response in hepatocytes was confirmed by in vivo experiments where CGS21680 was administered (0.5 mg/kg body weight, intraperitoneally) [23] to rats 3 hours before liver cell preparation. Figure 4F shows that HIF-1 nuclear content in hepatocyte isolated from

CGS21680-treated rat increased by about five-fold as compared to hepatocytes from untreated rats.

Studies in vascular smooth muscle cells have shown that the oxygen-independent activation of HIF-1 by angiotensin II involves transcriptional/translational mechanisms depending on the PI3K/Akt-dependent and PKC-dependent signals [24]. Because both these kinases are involved in the transduction of A2AR-mediated signals in hepatocytes [7,16,17], we have explored their role in HIF-1 activation by preconditioning. Figure 4C,D show that the inhibition of PI3K with wortmannin (250 nmol/L) or the block of PKC with chelerythrine (50 μmol/L) abolished the nuclear translocation of HIF-1 induced by either hypoxic preconditioning or CGS21680. No effect was observed by blocking ERK1/2 with PD98059 (20 μmol/L), whereas the inhibition of PKA with H89 (0.1 μmol/L), previously shown to affect PI3K activation by A2AR [7], prevented HIF-1 nuclear translocation (Fig. 4E). Wortmannin and chelerythrine also reverted the protective effects of late preconditioning against hypoxic injury (Figs. 1B and 2).

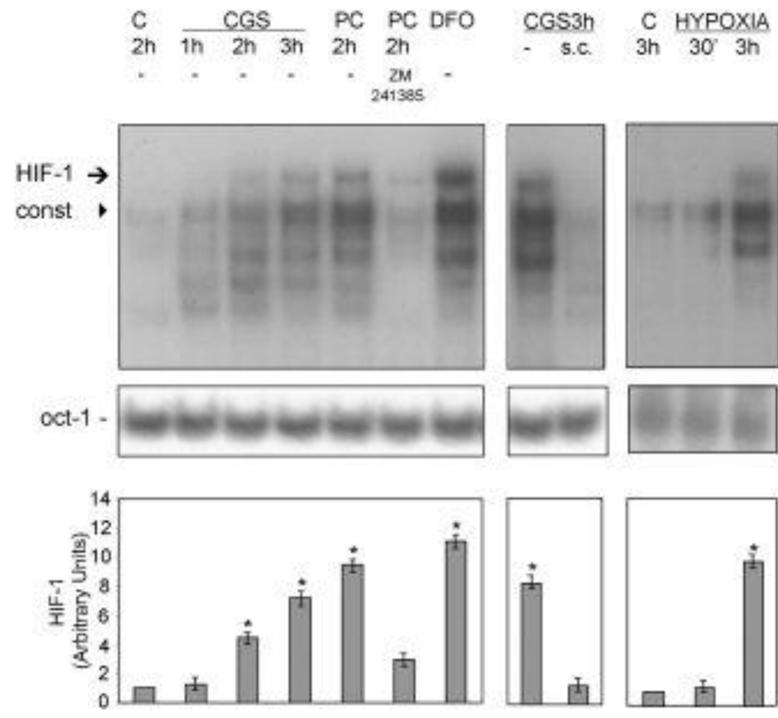

Figure 3. Late preconditioning promotes HIF-1 DNA binding through the stimulation of adenosine A2A receptor. EMSA analysis of the HIF-1 binding activity in nuclear extracts from untreated hepatocytes (C), hepatocytes treated with CGS21680 (CGS, 1 µmol/L), or exposed to preconditioning by a 10-minute hypoxic stress (PC). As positive controls, HIF-1 DNA binding was assessed in hepatocytes incubated for 3 hours under hypoxia or for 20 hours with desferrioxamina (DFO, 100 µmol/L). ZM241385 (1 µmol/L), was added to the cell culture medium before PC. The arrow indicates the inducible HIF-1 complex, whereas the arrowhead indicates the constitutive complex (const). s.c. indicates specific competition of the 3 hour CGS sample with 50-fold excess of unlabeled specific oligonucleotides. The binding activity of the constitutively expressed transcription factor oct-1 was used to assess equal loading. The bars indicate the fold difference ± SD in relation to untreated control. The results are representative of three independent experiments. Statistical significance: *P < 0.001 versus C, CGS 1h, PC2h+ZM241385, CGS 3h+s.c.

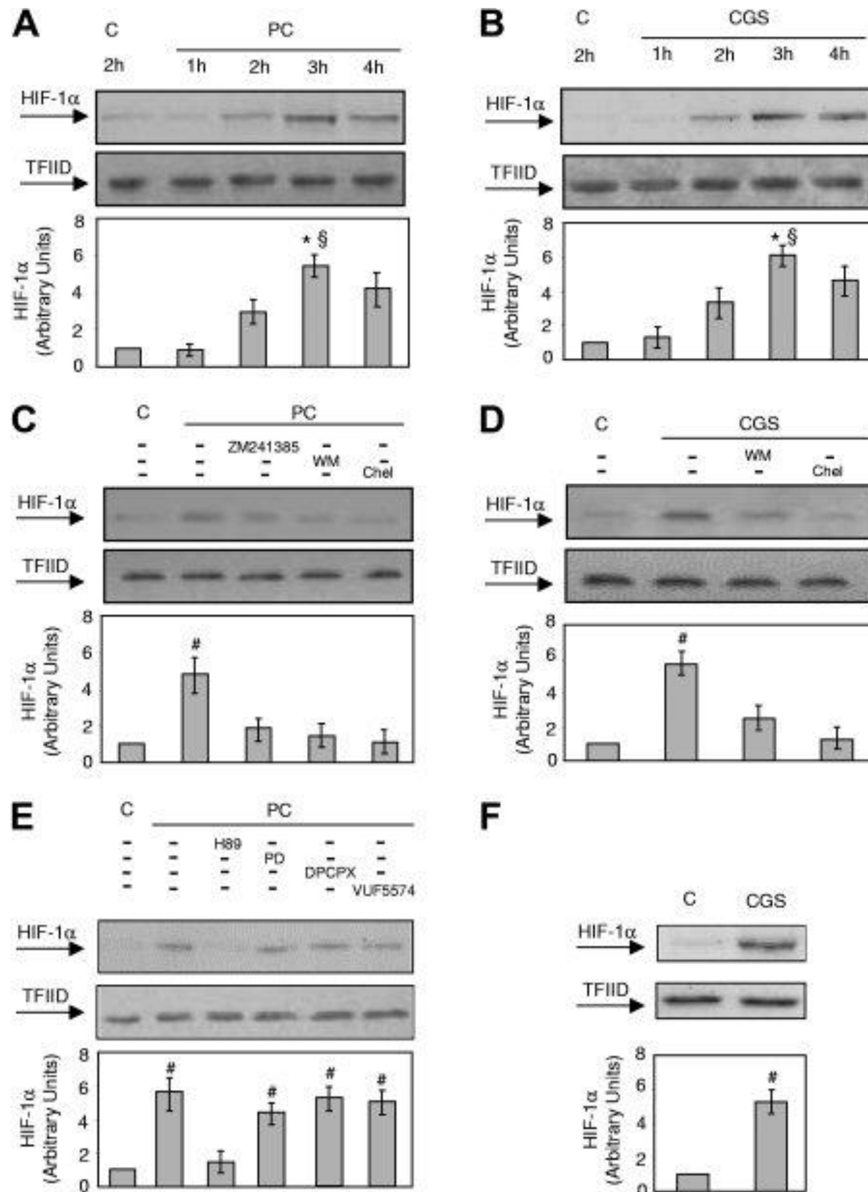

Figure 4. Late preconditioning increases the nuclear translocation of HIF-1. Hepatocytes were preconditioned by transient hypoxic stress (PC) or by treatment with CGS21680 (1 μmol/L; CGS). Preconditioned and untreated cells (C) were then cultured in normoxic conditions for 1-4 hours. (A, B) Time course of the nuclear accumulation of HIF-1α protein following the different preconditioning treatments. (A,B) Effect of blocking different adenosine receptors or protein kinases on the nuclear localization of HIF-1α in preconditioned hepatocytes. ZM241385(1 μmol/L), DPCPX (100 μmol/L), VUF5574 (100 μmol/L), wortmannin (WM, 250 nmol/L), chelerythrine (Chel, 50 μmol/L), PD98059 (20 μmol/L), or H89 (0.1 μmol/L) were added 15 minutes before hepatocyte preconditioning. Preconditioned and control cells (C) were then cultured in normoxic conditions for 3 hours. (F) Effect of in vivo administration of CGS21680 on the nuclear translocation of HIF-1α in isolated hepatocytes. Rats received CGS21680 (0.5 mg/kg body weight) intraperitoneally 3 hours before hepatocyte preparation. HIF-1α protein levels were evaluated by immunoblot analysis of nuclear extracts from primary hepatocytes. TFDII was used as a loading control. The values are expressed as arbitrary units after normalization at 1 to the control samples. The results are means of at least four different experiments ± SD. Statistical significance: *P < 0.002 versus C, CGS 1h or PC 1h; §P < 0.05 versus CGS 2h or PC 2h; #P < 0.002 versus C, CGS+WM, CGS+Chel, PC+ ZM241385, PC+WM or PC+Chel.

HIF-1-Induced Carbonic Anhydrase IX Expression in Preconditioned Hepatocytes.

Carbonic anhydrase (CAIX) is a transmembrane enzyme that catalyses the conversion of carbon dioxide and water to carbonic acid and is a major target of HIF-1 activity [25,26]. This prompted us to investigate CAIX as a marker of HIF-1-mediated action in hepatocytes undergoing late preconditioning. Immunoblotting revealed that control rat hepatocytes expressed negligible levels of CAIX (Fig. 5A). Conversely, 2-4 hours after hypoxic preconditioning or transient incubation with CGS21680 CAIX expression increased in a time-dependent manner up to 24 hours (Fig. 5A,B). The block of A2AR, PI3K and PKC with, respectively, ZM241385, wortmannin and chelerythrine, abolished CAIX expression in preconditioned hepatocytes (Fig. 5C,D). Parallel experiments showed that the stimulation of the HTC rat hepatoma cell line with CGS21680 (5 μmol/L) induced the nuclear accumulation of HIF-1 and the expression of CAIX with a kinetic comparable to that observed in preconditioned primary hepatocytes (Fig. 6A,B). To obtain further insight into the role of HIF-1 in CAIX induction by hepatic preconditioning, HTC cells were transfected with a luciferase reporter gene controlled by a DNA fragment containing multiple consensus HREs, previously shown to drive HIF-1-dependent transcription [27]. The expression of the reporter gene increased more than 2.5-fold in response to CGS21680 addition (Fig. 6C). Such an effect was almost abolished upon cotransfection with a plasmid expressing a dominant negative form of HIF-1β subunit (HRE/ΔARNT) (Fig. 6C), that forms a heterodimer with HIF-1, but cannot bind to DNA [15]. The transfection with the dominant negative HIF-1β also prevented the expression of CAIX in HTC cells exposed to CGS21680 (Fig. 6D), confirming that HIF-1 was involved in CAIX induction by preconditioning.

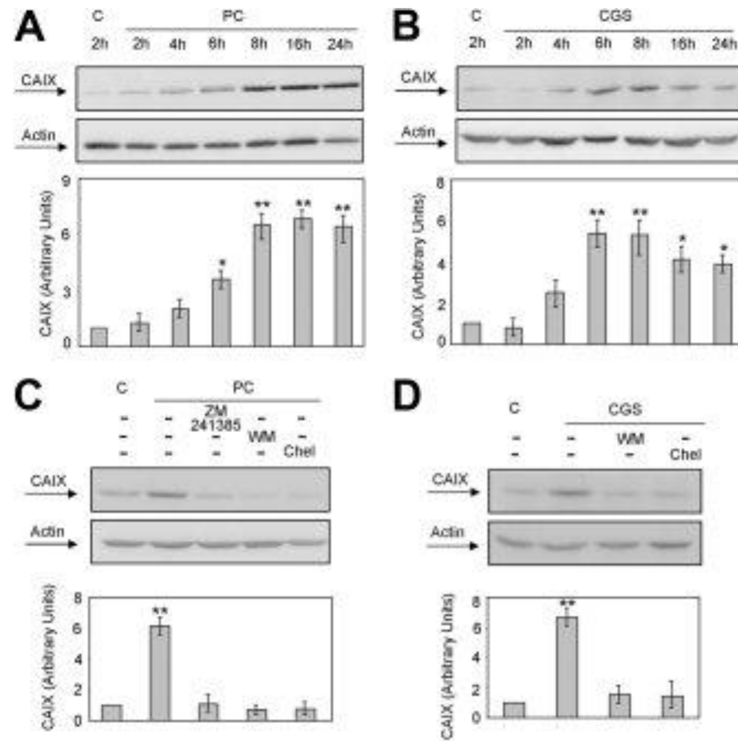

Figure 5. Late preconditioning induces CAIX expression in hepatocytes. Hepatocytes were preconditioned by transient hypoxic stress (PC) (A, C) or with CGS21680 (CGS, 1 µmol/L) (B, D). Preconditioned and control cells (C) were then cultured in normoxic conditions for (C, D) 8 hours or (A, B) up to 24 hours. ZM241385(1 µmol/L), wortmannin (WM, 250 nmol/L), chelerythrine (Chel, 50 µmol/L) were added before preconditioning. CAIX expression was evaluated by western blotting. The relative intensity of CAIX and actin related bands was measured by videodensitometry and the results were expressed as ratios after normalization at 1 of the control. The results are means of at least four different experiments ± SD. Statistical significance: *$P < 0.05$ versus C (A), PC 2h, PC 4h; or versus CGS 6h or CGS 8h (B); **$P < 0.001$ versus C, PC 2h, PC 4h, PC 6h (A), or C, CGS 2h, CGS 4h (B) or versus PC+ZM241385, PC+WM, or PC+chel (C); or versus C, CGS+WM or CGS+chel (D).

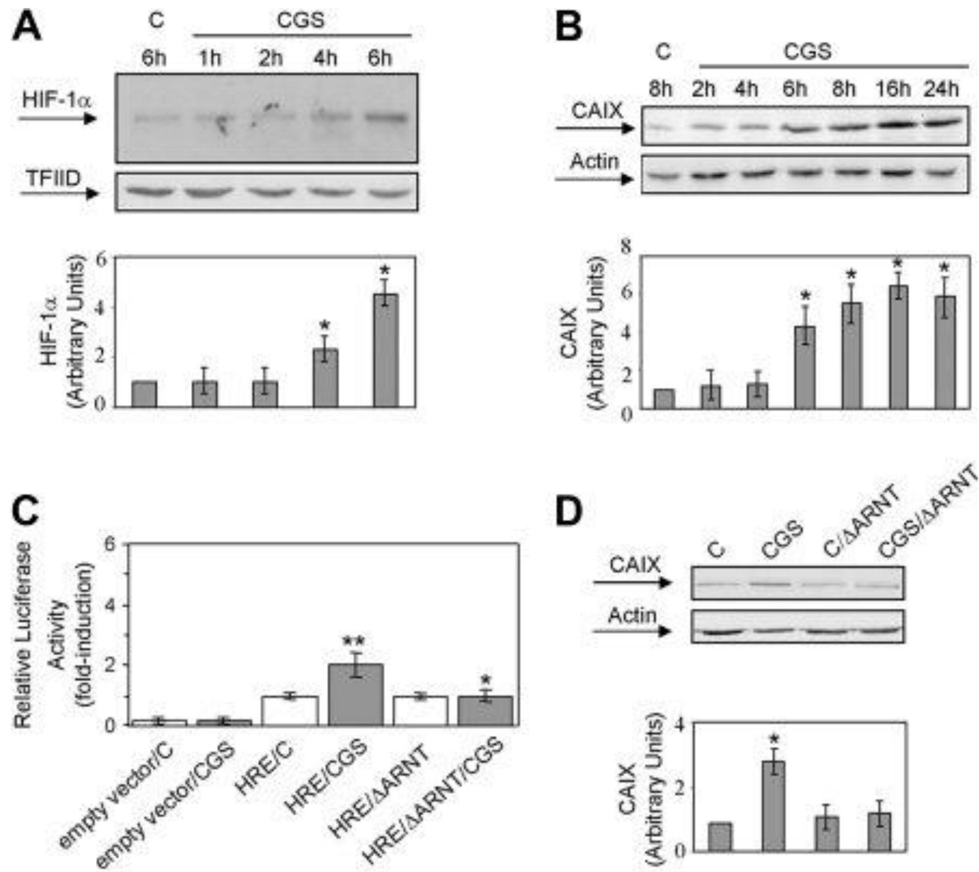

Figure 6. HIF-1-mediated CAIX induction in HTC cell lines. (A) Immunoblotting of the nuclear content of HIF-1α protein in control (C) or CGS21680-treated (5 μmol/L; CGS) HTC cells. Equal amounts of proteins, as assessed by using TFIID as loading control. The values are expressed as arbitrary units after normalization at 1 for the control sample and are the means of at least three different experiments ± SD. Statistical significance: *P < 0.004 versus controls. (B) CAIX expression was evaluated by immunoblotting in control (C) or CGS21680-treated (5 μmol/L; CGS) HTC cells. The relative intensity of CAIX and actin related bands was measured by videodensitometry and the results were expressed as ratios after normalization at 1 of the control. The results are means of at least four different experiments ± SD. Statistical significance: *P < 0.002 versus controls. (C) The HTC cells were transiently transfected with the empty pGL2 basic vector (empty vector) or a construct in which luciferase is controlled by an HRE multimer (HRE), and were left untreated (C), or exposed to CGS 21680 (5 μmol/L; CGS) for 20 hours. When appropriate, they were also cotransfected with an expression vector coding for a dominant negative mutant of the constitutive HIF-1β subunit (ΔARNT). The cells were cotransfected with a control vector containing the Renilla luciferase gene. The chemioluminescence, corrected for transfection efficiency on the basis of Renilla luciferase activity, was normalized to 1 in comparison to the activity in untreated cells. Mean values ± SD of three independent experiments. **P < 0.001 versus empty vector/C or empty vector/CGS; *P < 0.004 versus HRE/CGS. (D) CAIX expression in HTC cells cotransfected with an expression vector coding for a dominant negative mutant of the constitutive HIF-1β subunit (ΔARNT). The results are means of at least three different experiments ± SD. Statistical significance: *P < 0.002 versus C, C/ΔRNT or ΔRNT/CGS.

Carbonic Anhydrase IX Expression Mediates the Protective Effects of Late Preconditioning Against Hypoxic Injury.

Several studies have proposed a role of CAIX in regulating extracellular and intracellular pH [10,25,26,28]. Thus, we investigated the possible contribution of CAIX in mediating the protective effects of late preconditioning against hypoxic injury. Figure 7 shows that blocking CAIX with acetazolamide (100 µmol/L) reverted the protection induced by late preconditioning toward intracellular acidosis and cell death. Intracellular Na^+ accumulation was also evident in preconditioned hepatocytes receiving acetazolamide (Fig. 7C). Acetazolamide did not affect intracellular pH, Na^+ homeostasis and cell viability in control hepatocytes exposed to normoxic or hypoxic conditions. The protective action of late preconditioning was also abolished by inhibiting hepatocyte bicarbonate uptake through the Cl^-/HCO_3^- exchanger using disodium 4-acetamido-4’-isothiocyanato-stilben-2,2’-disulfonate (SITS) (50 µmol/L) (Fig. 7). This indicated that in hepatocytes undergoing late preconditioning the combined activities of CAIX and Cl^-/HCO_3^- exchanger were responsible for maintaining intracellular H^+ and Na^+ homeostasis during hypoxia.

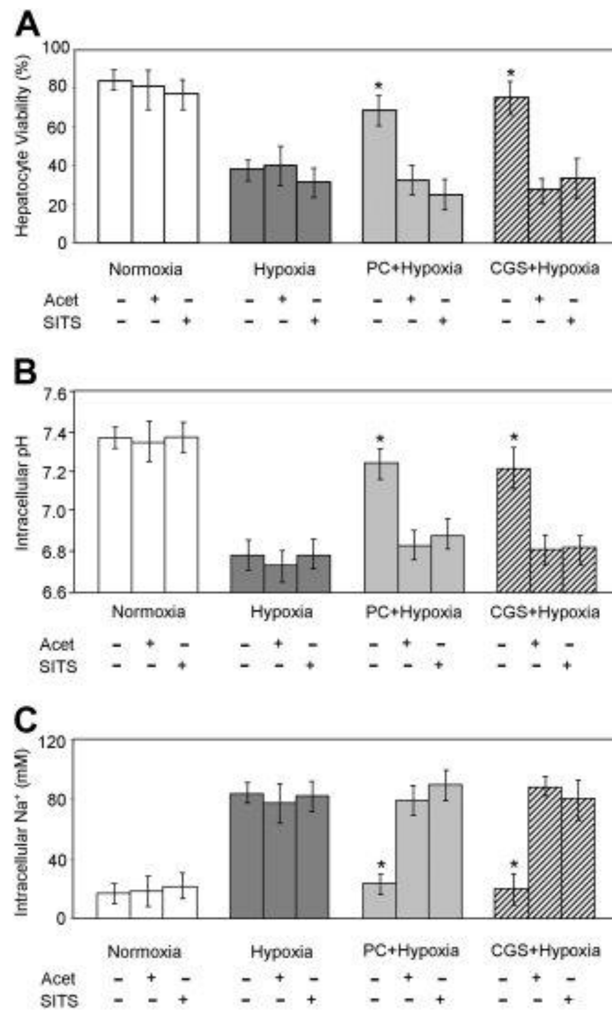

Figure 7. CAIX mediates the protective effects of late preconditioning against hypoxic injury. Late preconditioning was induced by hypoxic stress (PC) or by 15 minutes of incubation with CGS21680 (1 µmol/L; CGS). Preconditioned and control cells (C) were cultured for 24 hours in normoxic conditions. CAIX inhibitor acetazolamide (Acet, 100 µmol/L) or the Cl^-/HCO_3^- exchanger blocker SITS (50 µmol/L) were added 30 minutes before the hypoxic incubation. (A) Hepatocyte viability was evaluated after 60 minutes of incubation in normoxic or hypoxic conditions. Results are mean of at least four independent experiments. Statistical significance: $^*P < 0.001$ versus preconditioned cells+inhibitors. (B) Intracellular pH was evaluated after 30 minutes of incubation in normoxic or hypoxic. Results are the mean of at least three independent experiments. Statistical significance: $^*P < 0.04$ versus preconditioned cells+inhibitors. (C) Intracellular Na^+ concentration was evaluated after 30 minutes of incubation in normoxic or hypoxic conditions. Results are the mean of at least three independent experiments. Statistical significance: $^*P < 0.002$ versus preconditioned cells+inhibitors.

DISCUSSION

The protective action associated with the late phase of preconditioning is well-documented in the heart and the brain [2,5]. In the liver, late preconditioning can be induced by transient ischemia/reperfusion, short-term hyperthermia, oxidative stress, and atrial natriuretic peptide[6] and is associated with the induction of a number of cytoprotective genes, including inducible nitric oxide synthase (iNOS), Bcl-2, Bcl-XL, heat shock proteins, and heme-oxygenase-1 [6,29,30]. Hepatic late preconditioning ameliorates postischemic sinusoidal perfusion, leukocyte infiltration, bile production and aminotransferase release up to 48 hours after the application of the preconditioning stimulus [6]. Because of its sustained duration, the pharmacological induction of late preconditioning might be particularly relevant to improve liver surgery [3,4]. However, the present knowledge of the mechanisms involved is quite preliminary.

A role for HIF-1 in preconditioning has emerged from studies in rodent brain showing that 1-6 hours of nonlethal hypoxia followed by 24 hours of reoxygenation induce the expression of elevated levels of HIF-1 protein and of HIF-1-regulated genes that are associated with the prevention of the injurious effects of a subsequent ischemia [31,32]. Moreover, Cai and coworkers have recently reported the complete loss of the cardioprotection by ischemic preconditioning in mice with partial deficiency of HIF-1 [33]. In hepatocytes, HIF-1 DNA binding is evident 2 hours after the preconditioning stimulus in parallel with the increase in the nuclear content of HIF-1α. HIF-1 activation is abolished by blocking adenosine A2A receptors, while the stimulation of oxygenated hepatocytes with the A2A receptors agonist CGS21680 mimics the action of the transient hypoxia. This indicates that HIF-1 stimulation during hepatic preconditioning involves oxygen-independent mechanisms triggered by adenosine-mediated signals. Adenosine is a well recognized mediator of hepatic preconditioning [17,18] and enhances liver tolerance to ischemia/reperfusion [34]. The capacity of adenosine to promote the oxygen-independent activation of HIF-1 in hepatocytes is consistent with

recent observations concerning the role of adenosine in triggering HIF-1 activity in human macrophages and glioblastoma cells [13,35,36]. Growing evidence indicates that growth factors, cytokines and vascular hormones can lead to an oxygen-independent induction of HIF-1 in many cell types [12]. Differently from the stabilization of HIF-1α occurring in response to oxygen deprivation, the mechanisms implicated in such an oxygen-independent induction of HIF-1 involve an increase in the translation of HIF-1α mRNA that shifts the balance between the synthesis and the degradation towards an accumulation of HIF-1α [12]. Studies from several laboratories, have identified the role of PI3K, PKB/Akt, mTOR, and p70S6 in regulating HIF-1 mRNA translation through the phosphorylation of the S6 protein in the 40S ribosomal units [12]. Moreover, Page and co-workers [24] have shown that PKC-dependent signals are also responsible for an increased transcription of HIF-1α mRNA in vascular smooth muscle cells stimulated with angiotensin II. It is noteworthy, that both adenosine-induced and angiotensin II-induced stimulation of HIF-1 are evident after 2 hours from the addition of the agonist and require the transduction of PI3K/Akt-dependent and PKC-dependent signals. These analogies, along with the notion that hepatocyte adenosine A2A receptors are coupled with both PI3K and PKCδ/ε signalling [7,16,17], suggest the possibility that the adenosine-mediated activation of HIF-1 might involve an increased transcription/translation of HIF-1α (Fig. 8).

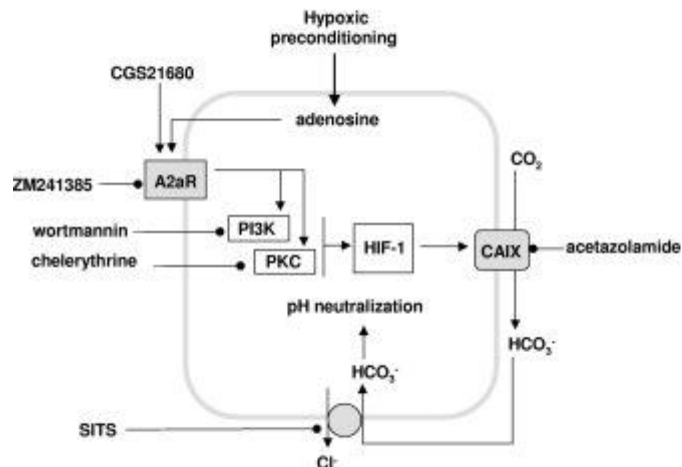

Figure 8. Molecular mechanisms involved in the prevention of hypoxic injury in rat hepatocytes exposed to late preconditioning. By inducing the autocrine stimulation of the adenosine A2A receptors (A2aR) hypoxic preconditioning promotes a PI3K-dependent and PKC-dependent activation of the nuclear transcription factor HIF-1. In turn, HIF-1 induces the expression of carbonic anhydrase IX (CAIX) that converts CO_2 in bicarbonate in the extracellular milieu. Bicarbonate is transported into the hepatocytes through the Cl^-/HCO_3^- exchanger and neutralizes the intracellular acids, thus maintaining cytosolic pH and preventing Na^+ accumulation. The inhibitors used to elucidate the various steps are indicated.

The role of HIF-1 in protecting liver cells against ischemic damage is also consistent with recent observations concerning the capacity of HIF-prolyl-hydroxylase inhibitors in preventing stroke [2] and myocardial infarction [37]. In this study, we observed that the activation of HIF-1 triggered by the stimulation of adenosine A2A receptors is associated with an increased expression of carbonic anhydrase IX (CAIX). CAIX is normally expressed in the gastrointestinal tract where facilitates H^+/CO_2^- transport coupled to gastric secretion [38]. Under normoxic conditions CAIX expression is negligible or absent in the tissues outside the gastrointestinal tract, but it is strongly induced by hypoxia [26,28]. CAIX activity has been proposed to play a role in the acidification of extracellular milieu as well as in maintaining intracellular pH via bicarbonate supply [25,26,28]. CAIX transcription is tightly regulated by HIF-1 and in vitro studies have shown that CAIX can be used as a tool for monitoring HIF-1 activity [26]. Accordingly, we report that in liver cells CAIX expression is prevented by overexpressing a defective HIF-1β subunit that interfere with HIF-1 binding

to DNA. In agreement with the role of CAIX in regulating cellular pH, CAIX induction in preconditioned hepatocytes ameliorates intracellular acidosis induced by ischemia by promoting bicarbonate uptake through the Cl^-/HCO_3^- exchanger (Fig. 8). We have previously shown that the activation of acid buffering systems (Na^+/H^+ exchanger and Na^+/HCO_3^- cotransporter) in response to the intracellular acidosis causes an irreversible accumulation of Na^+ that precipitates osmotic hepatocyte lysis by altering the cell volume regulation [39]. In this contest, the agents that prevent intracellular acidosis or Na^+ overload protect against hepatocyte death [40,41]. We now demonstrate that CAIX induction during the late phase of hepatic preconditioning ameliorates intracellular Na^+ overload and prevents hepatocyte killing by hypoxia. This mechanism is consistent with the role of CAIX in the development of cancer cell resistance to hypoxia [42]. Nonetheless, the importance of HIF-1 activation by preconditioning can have a broader significance in ameliorating hepatic reperfusion injury; Plock and co-workers have recently reported that the activation of HIF-1 in mouse livers exposed to moderate hypoxia up-regulates cytoprotective genes and prevents hepatocyte apoptosis induced by Fas ligand [43].

In conclusion, our results show that: (1) the activation of HIF-1 by adenosine-mediated signals is involved in the development of the late phase of hepatic preconditioning; (2) the expression of HIF-1-regulated CAIX contributes to increased hepatocyte tolerance to hypoxia by preventing the alteration of intracellular pH and Na^+ homeostasis that lead ischemic cell death. These observations, along with the capacity of HIF-1 to down-modulate cell responses to proapoptotic signals [2,9,10,43] suggest HIF-1 as potential target for the pharmacological induction of late preconditioning in the liver.

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pathways up-regulate cytoprotective genes in the murine liver. J Hepatol 2007; 47: 538-545.

STUDY 2: "Variable activation of phosphoinositide 3-kinase influences the response of liver grafts to ischemic preconditioning"

AIMS: Many reports have been published on the outcome of the application of liver preconditioning on human liver transplants, leading to conflicting results (Azoulay, 2005; Cescon, 2006; Koneru, 2007). In this work the intracellular signals activated by ischemic preconditioning in transplanted livers from heart-beating deceased donors were investigated to get some insights in the possible reasons of failure of ischemic preconditioning to protect liver grafts against reperfusion injury.

CONCLUSIONS: Ischemic preconditioning of livers obtained from deceased donors effectively stimulated PI3K mediated protective intracellular signals only in half of the grafts and this variable response was associated to concomitant lowering of PTEN. The contradictory reports on the clinical response of transplanted livers to ischemic preconditioning might be explained by the observed large inter-individual variability to obtain an efficient stimulation of PI3K signalling by ischemic preconditioning and indicate the necessity to work out alternative methods, possibly using pharmacological agents, to increase the efficacy of ischemic preconditioning of livers obtained from deceased donors.

Variable activation of phosphoinositide 3-kinase influences the response of liver grafts to ischemic preconditioning

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Abbreviations: ALT, alanine aminotranspherases; AST, aspartate aminotranspherases; ICU, intensive care unit; IPC, ischemic preconditioning; MELD, model for end-stage liver disease; OLT, orthotopic liver transplantation; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase tensin-homologues deleted from chromosome 10; PKB-Akt, protein kinase B; PRBC, packed blood cells.

ABSTRACT

Background/Aims

The efficacy of ischemic preconditioning (IPC) in preventing reperfusion injury in human liver transplants is still questioned. Phosphoinositide-3-kinase (PI3K) is essential for IPC development in rodent livers. This work

investigates whether PI3K-dependent signals might account for the inconsistent responses to IPC of transplanted human livers.

Methods

Forty livers from deceased donors were randomized to receive or not IPC before recovery. PI3K activation was evaluated in biopsies obtained immediately before IPC and 2 h after reperfusion by measuring the phosphorylation of the PI3K downstream kinase PKB/Akt and the levels of the PI3K antagonist phosphatase tensin-homologue deleted from chromosome 10 (PTEN).

Results

IPC increased PKB/Akt phosphorylation ($p = 0.01$) and decreased PTEN levels ($p = 0.03$) in grafts, but did not significantly ameliorate post-transplant reperfusion injury. By calculating T_{2h}/T_0 PKB/Akt phosphorylation ratios, 10/19 (53%) of the preconditioned grafts had ratios above the control threshold (IPC-responsive), while the remaining nine grafts showed ratios comparable to controls (IPC-non-responsive). T_{2h}/T_0 PTEN ratios were also decreased ($p \leq 0.03$) only in IPC-responsive grafts. The patients receiving IPC-responsive organs had ameliorated ($p \leq 0.05$) post-transplant aminotransferase and bilirubin levels, while prothrombin activity was unchanged.

Conclusions

Impaired PI3K signaling might account for the variability in the responses to IPC of human grafts from deceased donors.

1. INTRODUCTION

Ischemia-reperfusion injury is the most common cause of primary graft non-function or initial poor function occurring in the patients undergoing liver transplantation [1]. The frequency and the severity of liver graft reperfusion injury largely depends upon the length of cold ischemia as well as upon the use of marginal livers [2,3]. This latter aspect is a matter of growing concern considering that the shortage of organs increasingly

compels the use of marginal livers [4]. This situation has stimulated the development of surgical and pharmacological strategies aimed to reduce reperfusion damage of liver grafts. Among these strategies, ischemic preconditioning has received increasing attention. Ischemic preconditioning defines the capacity of a transient (5–10 min) ischemic stress followed by reperfusion to increase tissue tolerance to a subsequent severe ischemia/reperfusion injury [5,6]. Ischemic preconditioning has been shown to effectively reduce reperfusion damage during hepatic resection in humans [7,8] as well as to improve the outcome of hepatic transplants in experimental animals [9,10]. However, the application of ischemic preconditioning to the transplantation of human livers from deceased donors has given conflicting results with some studies reporting improvements in post-operative aminotransferase release, inflammation markers and benefits in the graft survival, whereas others were unable to detect appreciable differences between the preconditioned and the control groups or even showed a worsening of reperfusion injury [11-16]. Experimental studies demonstrate that the protective action of hepatic ischemic preconditioning results from the activation of a complex network of intracellular signals that modulate inflammatory reactions as well as hepatocyte capacity to preserve energy functions, ion homeostasis and anti-apoptotic responses [17]. Among the signals activated by ischemic preconditioning, those involving phosphoinositide-3 kinase (PI3K) and the down stream serine-threonine protein kinase B (PKB-Akt) are of particular interest [18,19]. PI3Ks are a family of intracellular signal transducers characterized by the capacity of generating phosphatidylinositol (3,4,5)-triphosphate (PIP3) that in turn acts as a second messenger activating several kinases implicated in the regulation of cell proliferation, survival and metabolism [20]. The importance of PI3K in preventing hepatic injury has emerged from a number of observations showing that PI3K-mediated signals are important in preventing hepatocytes apoptosis as well as in ameliorating liver reperfusion injury [21-23]. Consistently, recent studies have shown that ischemic preconditioning activates PI3K signalling in rodent livers, while

the block of this kinase abolishes the protective action of preconditioning both in isolated hepatocytes and in the whole organs [18,19].
These observations prompted us to investigate whether PI3K activity might be implicated in causing the inconsistent responses to ischemic preconditioning observed in transplanted human livers.

2. EXPERIMENTAL PROCEDURES

2.1. Study design

The effect of ischemic preconditioning of liver grafts from deceased donors was investigated in 40 patients receiving orthotopic liver transplantation (OLT) from January 2006 to November 2007 at the Liver and Multiorgan Transplant Unit of the University of Bologna, Italy. Male and female subjects aged >18 years and undergoing primary whole OLT were selected for the study and alternatively randomized to receive (IPC) or not (Controls) ischemic preconditioning before organ procurement. Patients requiring re-transplantation and recipients of split liver transplantation were excluded from this study. All the grafts were obtained from donors under the condition of haemodynamic stability during the stay in the intensive care unit (ICU) (i.e. absence of cardiac arrests and/or of hypotension episodes with systolic arterial blood pressure <70 mm Hg for >30 min). The cases of multivisceral, intestinal or pancreas donation were excluded. After confirmation of graft viability by abdominal inspection, 500 IU/kg b.wt. of heparin was administered intravenously. In the IPC group, a 10-min Pringle maneuver was performed by vascular clamping followed by 15-min of warm reperfusion before starting cold ischemia using 5 l of cold Celsior solution (Sangstat® Europe, Lyon, France) infused through the aorta and one litre through the portal vein. The livers were recovered according to the conventional technique [24]. All OLT procedures were performed with preservation of the retrohepatic inferior vena cava and with sequential portal and arterial reperfusion. No induction immunosuppression was used.

We obtained paired ≥10 mm deep wedge or through-cut biopsies from the left hepatic lobe at the opening of the peritoneal cavity in the donor, and 2 h after portal revascularization in the recipient from both groups. Liver specimens were divided into two fragments that were either snap-frozen in n-methylbutane pre-cooled in liquid nitrogen or fixed in formalin.
The study was planned in accordance with the 1975 Declaration of Helsinki and was preliminary approved by the local Ethics Committee.

2.2. Analysis of PI3K-mediated signals

Liver fragments (0.4–0.5 mg) were homogenized in 2 mL of ice-cold lysing buffer containing 2 mmol/L Hepes buffer pH 7.4, 10% Glycerol, 50 mmol/L NaCl, 5 mmol/L EDTA, 2 mmol/L EGTA, 1 mmol/L $ZnCl_2$, 50 mmol/L ammonium molybdate, 1% NP-40, 1 mmol/L Na_3VO_4, 0.2 mmol/L phenylmethylsulphonyl fluoride, 10 mmol/L sodium fluoride, 0.5 mM dithiothreitol, 1 µg/mL leupeptin, 1 µg/mL pepstatin, 1 µg/mL trypsin inhibitor, 1 µg/mL aprotinin. After centrifugation at 14,000g for 10 min the supernatant was diluted with 0.5 mL of Laemmli's buffer and proteins content was determined by Lowry's method as modified by Peterson [25]. Aliquots (40 µg protein) were submitted to electrophoresis on a 10% SDS–polyacrylamide gel and then transferred to nitrocellulose membranes. The membranes were sequentially probed with rabbit polyclonal antibodies against (Ser^{473})Phospho-PKB/Akt and PKB/Akt (Cell Signaling Technology, Beverly, MA, USA) or with rabbit polyclonal antibodies against PTEN (Santa Cruz Biotecnology, Santa Cruz CA, USA) and anti-β-actin antibodies (Sigma, St. Louis, MO, USA). The antibody binding was revealed by horseradish peroxydase conjugated anti-rabbit immunoglobulins (Biorad, Hercules, CA, USA) using Western Lightning Chemiluminescence Reagent Plus (Perkin–Elmer, Boston, MA, USA) and X-ray film (Estman-Kodak, Rochester, NY, USA). The relative intensity of the bands was measured by videodensitometry and the results were expressed as ratios.

2.3. Evaluation of liver biopsies

Five micron-thick liver sections stained with haematoxylin/eosin were for macrovescicular steatosis, hepatocyte necrosis, portal inflammation and fibrosis as previously reported [26].

2.4. Statistical analysis

Statistical analysis was carried out with the SPSS software packaging (SPSS, Inc., Chicago, IL). Differences between continuous variables were evaluated with the Mann–Whitney U test for inter-group comparisons and with the Wilcoxon test for related samples for intra-group comparisons. Differences between categorical variables were calculated with the χ^2 test or Fisher's exact test. Statistically significance was taken at 0.05.

3. RESULTS

3.1. Surgical procedures and tolerance to ischemic preconditioning

Twenty donors were randomized to receive ischemic preconditioning (IPC), while conventional organ procurement was performed in the remaining 20 donors. Only dopamine and/or norepinephrine were used as vasopressors in the entire series. During the study one patient in the IPC group was discharged because liver extracts were unsuitable for further analysis. The characteristic of the donors allocated to either the IPC or the control group are reported in Table 1. The two groups were comparable for all the parameters examined. None of the grafts had macrovescicular steatosis >30%. Ischemic preconditioning was well tolerated in all cases with no significant deterioration of haemodynamic parameters of the donor. Ischemic preconditioning did not jeopardize the viability of other abdominal or thoracic organs. The recipient profiles and the operative parameters of the IPC and control groups are reported in Table 2. Following transplantation aspartate – (AST), alanine – (ALT) aminotranspherases, total bilirubin and prothrombin values were comparable in the IPC and control groups (Table 2). None of the patients

in the IPC group and only one (5%) in the control group experienced primary graft non-function (p = 1.0). Two patients in the IPC group (10.5%) and none in the control group also had initial poor function (p = 0.2). Altogether, one patient (5.3%) in the IPC group and 1 (5%) in the control group required re-transplantation (p = 1.0). The causes of re-transplantation were initial poor function in the IPC group and primary graft non-function in the control group. The median ICU stay was also not significantly modified by ischemic preconditioning (Table 2). The median follow-up of the entire population was 23 months (range: 0.9–26). Three (15.8%) patients receiving ischemic preconditioning and two (10%) controls died during the follow-up (p = 0.6). The causes of death in the IPC group were recurrence of hepatocellular carcinoma (2 cases) and HCV recurrence (1 case), while infections were the causes of both death among the controls. Overall patient and graft survival rates at one-year were, respectively, 95% and 89% in the IPC group, and 90% and 90% in the control group (p = 0.5 and p = 0.3, respectively).

Table 1. Baseline characteristics of the 39 deceased donors receiving (IPC) or not (Cont) ischemic liver preconditioning.

	IPC (n = 19)	Cont (n = 20)	p
Donor profile			
Gender (M/F)	7/12	12/8	0.1
Age (years)	68 ± 10 (34–79)	66 ± 15 (34–85)	0.8
BMI	26 ± 3 (22–32)	25 ± 3 (20–31)	0.1
Diabetes	0 (0%)	2 (10%)	0.4
Cause of death			
Cerebral hemorrhage	16 (84%)	15 (75%)	0.5
Cranial trauma	1 (5%)	4 (20%)	
Other	2 (11%)	1 (5%)	
Use of norepinephrine	4 (21%)	6 (30%)	0.7
ICU stay (days)	3.2 ± 2.2 (1–9)	4.6 ± 3.6 (1–13)	0.3
AST (U/l)	32 ± 12 (9–55)	32 ± 19 (15–88)	0.4
ALT (U/l)	28 ± 19 (12–77)	37 ± 50 (9–238)	0.9
Total bilirubin (mg/dL)	0.7 ± 0.4 (0.2–1.8)	0.7 ± 0.6 (0.2–2.9)	0.5
Na^+ (mEq/L)	152 ± 9 (136–166)	146 ± 8 (134–163)	0.1
Macrosteatosis	12 (63%)	8 (40%)	0.1
Macrosteatosis >10%	7 (37%)	5 (25%)	0.4
Necrosis	0 (0%)	0 (0%)	–
Portal inflammation	10 (53%)	7 (35%)	0.2
Portal fibrosis	11 (58%)	13 (65%)	0.6

Data are means ± SD. Range values are reported under parenthesis. BMI, body mass index; ICU, intensive care unit; AST, aspartate aminotranspherases; ALT, alanine aminotranspherases; Na^+, serum sodium level.

Table 2. Demographic, clinical and follow-up characteristics of the 39 recipients receiving liver that were submitted (IPC) or not (Cont) to ischemic preconditioning before graft recovery.

	IPC (n = 19)	Cont (n = 20)	p
Patient profile			
Gender (M/F)	15/4	14/6	0.7
Age (years)	52 ± 10 (23–65)	53 ± 9 (27–67)	0.8
Indication for OLT			
HCC on cirrhosis	9 (47%)	6 (30%)	0.4
Postnecrotic viral cirrhosis	7 (37%)	6 (30%)	
Cholestatic cirrhosis	2 (11%)	1 (5%)	
Alcoholic cirrhosis	1 (5%)	3 (15%)	
Other	0 (0%)	4 (20%)	
MELD score	23 ± 10 (10–41)	23 ± 10 (8–46)	0.8
Operative parameters			
Operation time (min.)	400 ± 82 (225–600)	406 ± 161(255–915)	0.4
Total ischemia time (min.)	357 ± 63 (259–485)	361 ± 70 (285–569)	0.9
Warm ischemia time (min.)	39 ± 17 (20–76)	42 ± 14 (21–76)	0.4
Hypotension episodes*	0 (0%)	0 (0%)	–
Use of adrenaline	6 (32%)	6 (30%)	0.9
PRBC transfusion (L)	3.9 ± 2.7(1.0–11.5)	3.4 ± 3.6 (0–1.4)	0.2
Follow-up parameters			
AST (U/L)			
Day 1	389 ± 220 (112–865)	885 ± 905 (87–2930)	0.1
Day 3	406 ± 725 (59–3321)	405 ± 937 (41–3418)	0.6
Day 7	47 ± 19 (24–89)	50 ± 22 (20–101)	0.7
ALT (U/L)			
Day 1	412 ± 280 (81–1030)	679 ± 649 (58–2340)	0.4
Day 3	593 ± 580 (82–2696)	848 ± 937 (41–3418)	0.6
Day 5	191 ± 145 (40–744)	224 ± 187 (39–840)	0.4
Bilirubin (mg/dL)			
Day 1	5.1 ± 3.1 (1.5–11.9)	5.3 ± 4.5 (0.7–16.4)	0.6

Data are means ± SD. Range values are reported under parenthesis.

MELD, Model for End-Stage Liver Disease; ICU, intensive care unit; AST, aspartate aminotranspherases; ALT, alanine aminotranspherases. Prothromin activity is exressed as INR. PRBC, packed red blood cells.

* Hypotension episodes are defined as systolic arterial pressure values <70 mm Hg for >30 min after graft reperfusion.

3.2. Estimation of PI3K activation by ischemic preconditioning

The activation of the phosphoinositide 3-kinase (PI3K) dependent pathway by ischemic preconditioning was evaluated by Western blotting in the liver biopsies obtained immediately before preconditioning (T_0) and at 2 h from reperfusion by measuring the (Ser^{473})phosphorylation of PKB/Akt, a protein kinase down stream to PI3K, as well as the decline of the PI (3)P phosphatase, phosphatase tensin homologue deleted from chromosome 10 (PTEN), a key negative regulator of PI3K activity [27]. PKB/Akt phosphorylation (Fig. 1A/B) was significantly increased 2 h after reperfusion in both preconditioned (mean ± SD: 0.27 ± 0.21 vs. 0.59 ± 0.38; $p = 0.003$) and control livers (mean ± SD: 0.32 ± 0.25 vs. 0.62 ± 0.37; $p = 0.01$). However, by calculating the ratio between the individual phosphorylation values (Fig. 1C) at 2 h and those at T_0, a significant increase in PKB/Akt phosphorylation T_{2h}/T_0 ratio index (mean ± SD: 1.75 ± 0.92 vs. 4.37 ± 4.30; $p = 0.018$) was evident following ischemic preconditioning. In the control grafts PTEN levels (Fig. 2A) were not significantly different before and 2 h after reperfusion (mean ± SD: 0.30 ± 0.32 vs. 0.26 ± 0.36). Conversely, a significant lowering of PTEN (Fig. 2B) was evident 2 h after reperfusion in preconditioned livers (mean ± SD: 0.26 ± 0.27 vs. 0.17 ± 2.1; $p = 0.03$). Here again, by calculating the T_{2h}/T_0 ratios of the individual PTEN values (Fig. 2C), we observed that ischemic preconditioning significantly decreased PTEN expression in grafted livers (mean ± SD: 0.94 ± 0.36 vs. 0.65 ± 0.31; $p = 0.011$). A significant inverse correlation ($r = -0.477$; $p = 0.045$) was also evident between the individual values of PTEN and PKB/Akt ratios. This indicated that the transduction of PI3K-mediated signals induced by IPC requires the lowering of PTEN.

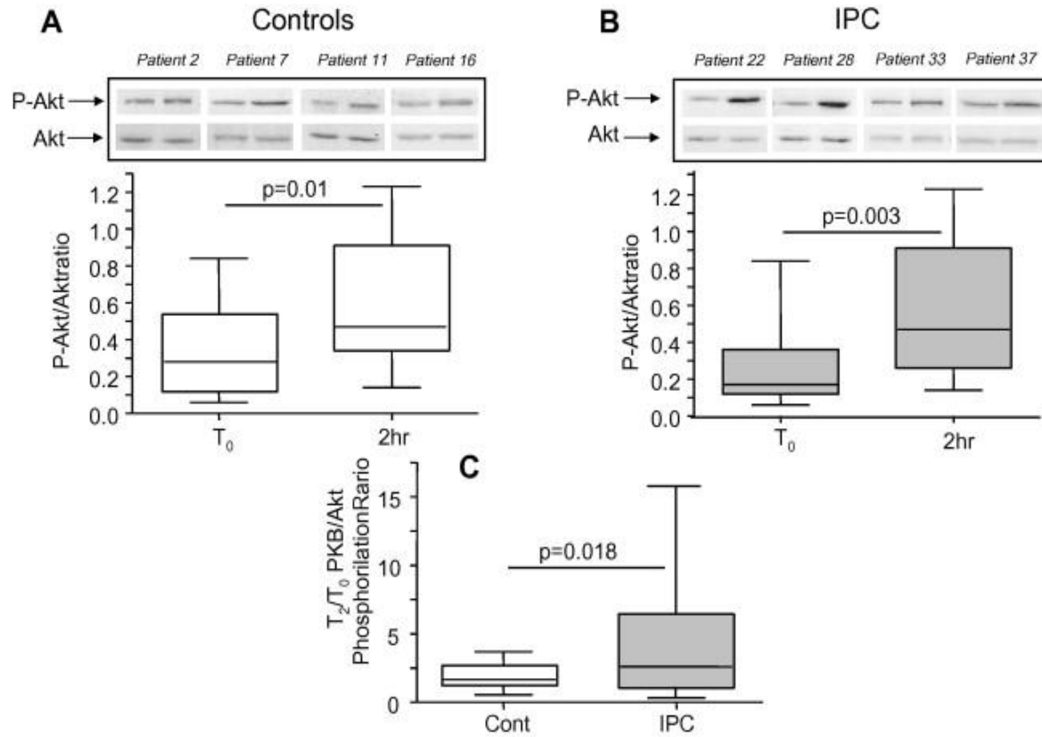

Figure 1. PKB/Akt phosphorylation in transplanted liver grafts exposed or not to ischemic preconditioning. (A and B) PKB/Akt phosphorylation was evaluated by Western blotting in liver biopsies obtained immediately before preconditioning (T_0) and at 2 h after reperfusion (2 h) from 19 grafts exposed to ischemic preconditioning (IPC) and 20 control livers. The individual phosphorylation values were expressed as ratios between intensities of the phosphorylated (P-Akt) and non-phosphorylated (Akt) PKB/Akt bands. Statistical significance was calculated by paired T-tests. (C) PKB/Akt phosphorylation index was calculated by the ratio between the individual phosphorylation values at 2 h and those at T_0 (T_{2h}/T_0 ratio) in the two experimental groups. In all the panels the boxes include the values within 25th and 75th percentile, while the horizontal bars represent the median. Vertical error bars represent 5% to 95% confidence intervals.

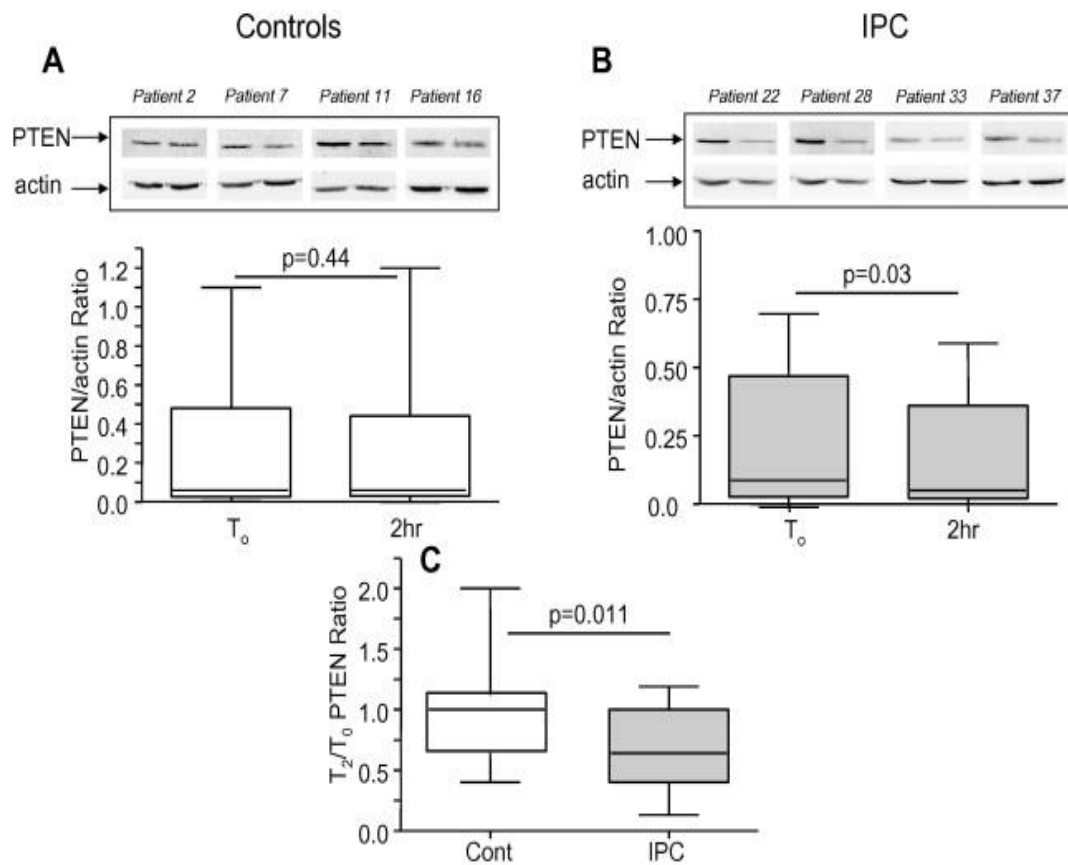

Figure 2. Phosphatase tensin homologues deleted from chromosome 10 (PTEN) levels in transplanted liver grafts exposed or not to ischemic preconditioning. (A and B) PTEN was evaluated by Western blotting in liver biopsies obtained immediately before preconditioning (T_0) and at 2 h after reperfusion (2 h) from 19 grafts exposed to ischemic preconditioning (IPC) and 20 control livers. The individual values were expressed as ratios between intensities of the PTEN bands and those of reference actin bands. Statistical significance was calculated by paired T-tests. (C) PTEN expression index was calculated by the ratio between the individual PTEN values at 2 h and those at T_0 (T_{2h}/T_0 ratio) in the two experimental groups. In all the panels the boxes include the values within 25th and 75th percentile, while the horizontal bars represent the median. Vertical error bars represent 5% to 95% confidence intervals.

Although ischemic preconditioning significantly increased PI3K response, we observed a large inter-individual variability in PKB/Akt T_{2h}/T_0 phosphorylation ratio among the preconditioned grafts (Fig. 3A). Taking as a cut-off the 95th percentile of the PKB/Akt T_{2h}/T_0 phosphorylation ratios in the control organs (2.8) we observed that 10 out of 19 (53%) of the preconditioned grafts had PKB/Akt ratios above the control threshold (IPC-responsive), while in the remaining 9 PKB/Akt the ratios were comparable to controls (IPC-non-responsive) (Fig. 3A). As expected, the mean of PKB/Akt T_{2h}/T_0 phosphorylation ratios in the IPC-responsive grafts (7.4 ± 3.9) was significantly ($p < 0.001$) higher than that in both IPC-non-responsive (0.97 ± 0.41) and control livers (1.75 ± 0.92), while no differences were evident between the two latter groups (Fig. 3A). This behavior was not due to pre-transplant stimulation of this kinase, as the T_0 values of PKB/Akt phosphorylation were not significantly different in IPC-responsive and non-responsive livers (0.16 ± 0.09 vs. 0.33 ± 0.24; $p = 0.07$) (Fig. 3B). Pre-transplant PTEN levels were also comparable in the two groups (0.26 ± 0.28 vs. 0.27 ± 0.26; $p = 0.93$) (Fig. 4A). A post-transplant increase in PKB/Akt phosphorylation was evident only in IPC-responsive grafts (0.87 ± 0.30 vs. 0.26 ± 0.11; $p = 0.0001$) (Fig. 3C). Although post-transplant PTEN was not significantly decreased in IPC-responsive livers (0.13 ± 0.19 vs. 0.22 ± 0.23; $p = 0.34$) (Fig. 4B), by comparing T_{2h}/T_0 PTEN ratios we observed that IPC-responsive livers had ratios (0.43 ± 0.28) significantly lower ($p = 0.027$ and $p = 0.004$, respectively) than both IPC-non-responsive (0.83 ± 0.26) and control organs (0.94 ± 0.36) while no difference ($p = 0.37$) was evident between IPC-non-responsive livers and non-preconditioned control organs (Fig. 4C). Altogether these results indicated that ischemic preconditioning effectively stimulated PI3K-mediated protective intracellular signals in only half of the grafts obtained from deceased donors and that the failure to lower PTEN might possibly account for such a variable response.

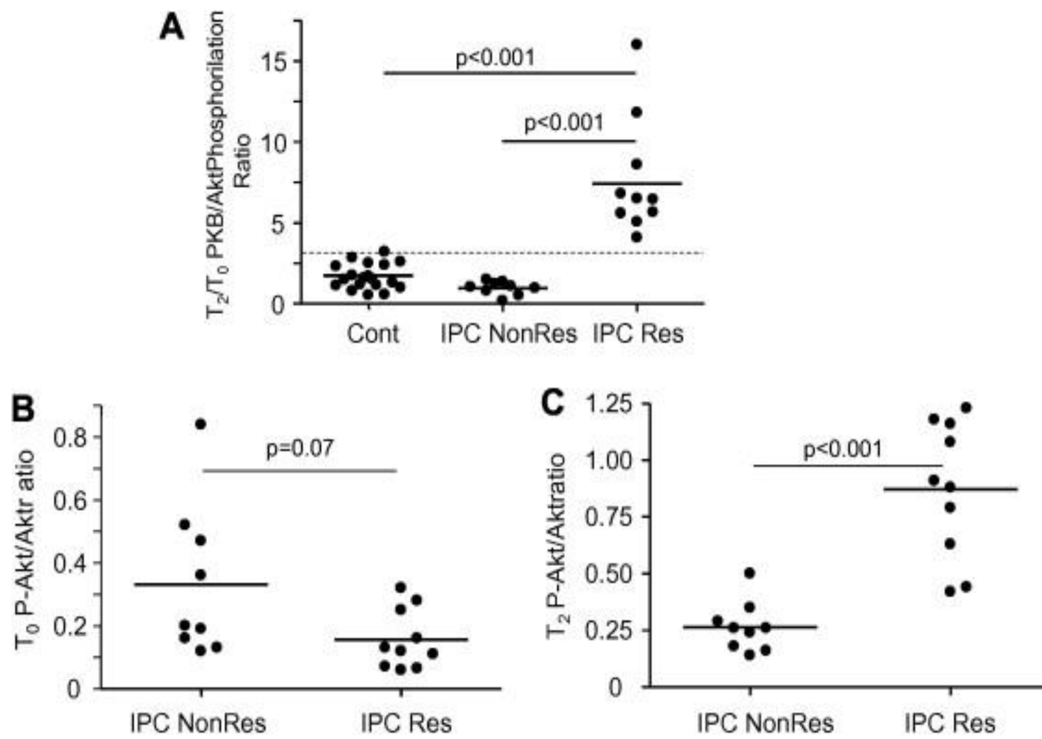

Figure 3. Inter-individual variability of PKB/Akt activation in graft livers submitted to ischemic preconditioning (IPC). (A) Individual values of PKB/Akt phosphorylation ratios in control and IPC organs. The dotted line is the cutoff value for PKB/Akt phosphorylation ratios calculated at the 95th percentile of the control values. Based on this value the IPC liver were sub-grouped as they responded (IPC Res; n = 10) or not (IPC NonRes; n = 9) to ischemic preconditioning. The horizontal bars represent the medians. (B and C) Individual values of PKB/Akt phosphorylation at, respectively, T_0 and T_{2h} in the grafts from of responder donors (IPC Res; n = 10) or non-responder (IPC NonRes; n = 9) to ischemic preconditioning.

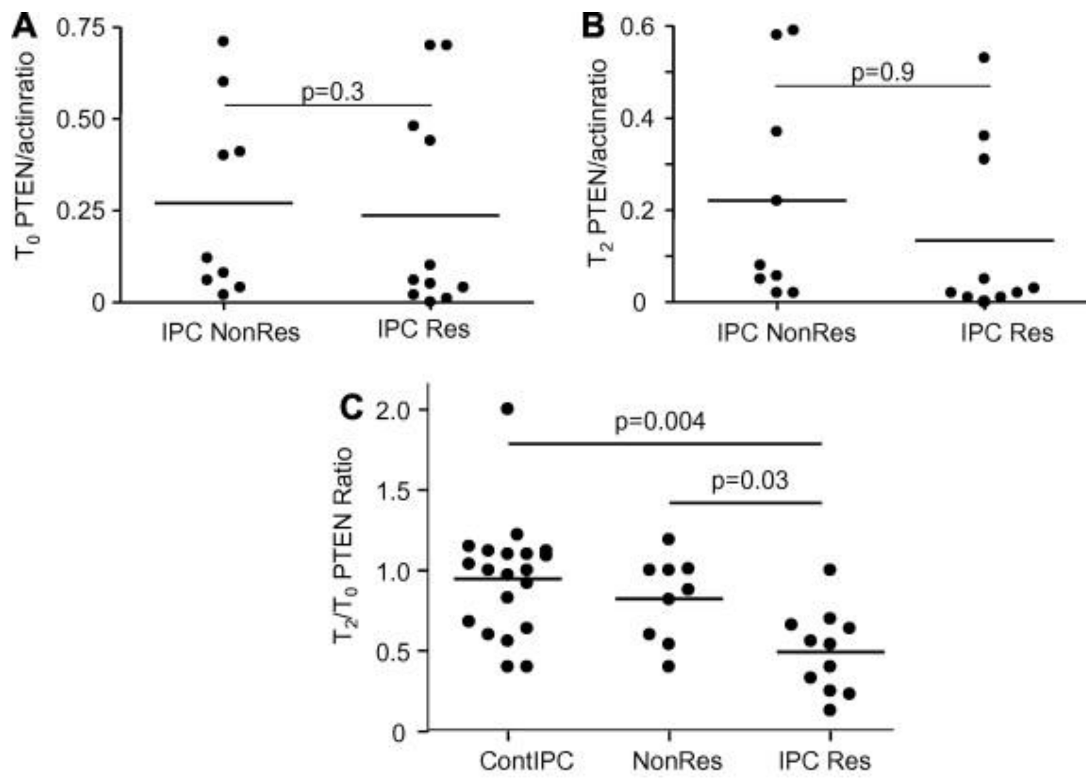

Figure 4. Inter-individual variability of the PTEN expression in graft livers submitted to ischemic preconditioning (IPC). (A and B) Individual values of PTEN expression at, respectively, T_0 and T_{2h} in the grafts from of responder donors (IPC Res; n = 10) or non-responder (IPC NonRes; n = 9) to ischemic preconditioning. (C) T_{2h}/T_0 ratios of PTEN expression in transplanted livers obtained from untreated deceased donors (Cont) or donors responder (IPC Res; n = 10) or non-responder (IPC NonRes; n = 9) to ischemic preconditioning. The horizontal bars represent the medians.

3.3. Consequence of PI3K activation by ischemic preconditioning on liver graft tolerance to ischemia/reperfusion injury

When the donor characteristics, recipient profiles and operative variables referring to IPC-responsive and IPC-non-responsive livers were compared no significant differences were observed between the two groups, except for a higher ($p = 0.04$) use of adrenaline to support haemodynamic parameters during transplant in the patients receiving IPC-non-responsive grafts (Table 3). AST and ALT values 24 h and 48 h after reperfusion were significantly lower ($p \leq 0.02$ and $p < 0.04$, respectively) in the patients transplanted with IPC-responsive organs than in those with IPC-non-responsive organs (Fig. 5). During the follow-up bilirubin clearance was improved in the patients with IPC-responsive grafts and the values reached statistical significance ($p \leq 0.04$) from day 4 up to day 7 (Fig. 5). There was no appreciable difference in the prothrombin activity (Fig. 5). The stay in ICU was not significantly different ($p = 0.5$) between IPC-responsive (mean ± SD: 6.4 ± 5.7; range 2–22) and non-responsive groups (mean ± SD: 9.8 ± 12.2; range 1–41). The two patients receiving preconditioned livers who had initial poor function were both in the IPC-non-responsive group. However such a prevalence was not statistically significant ($p = 0.2$).

Table 3. Demographic and clinical characteristics of the OLT recipients receiving preconditioned liver sub-grouped according to the response to the PI3K mediated signals induced by IPC.

	IPC responders (n = 10)	IPC non-responders (n = 9)	p
Patient profile			
Gender (M/F)	9/1	6/3	0.3
Age (years)	Mean: 54 ± 13 (23–65)	Mean: 51 ± 6 (44–63)	0.1
Indication for OLT			
HCC on cirrhosis	7 (70)	2 (22)	0.1
Postnecrotic viral cirrhosis	2 (20)	5 (56)	
Cholestatic cirrhosis	1 (10)	1 (11)	
Alcoholic cirrhosis	–	1 (11)	
MELD score	21 ± 10 (10–39)	26 ± 10 (13–41)	0.3
Donor profile			
Gender (M/F)	3/7	4/5	0.6
Age (years)	72 ± 6 (60–79)	65 ± 12 (34–76)	0.3
BMI	26 ± 3 (22–32)	26 ± 3 (22–30)	1.0
Cause of death			
Cerebral hemorrhage	10 (100%)	7 (78%)	0.9
Cranial trauma	0 (0%)	1 (11%)	
Other	0 (0%)	1 (11%)	
Use of norepinephrine	4 (40%)	0 (0%)	0.08
ICU stay (days)	3.8 ± 2.7 (1–9)	2.6 ± 1.4 (1–6)	0.2
AST (U/l)	31 ± 15 (9–55)	32 ± 9 (22–48)	0.7
ALT (U/l)	21 ± 9 (13–37)	36 ± 25 (12–77)	0.1
Total bilirubin (mg/dL)	0.5 ± 0.2 (0.2–0.8)	0.8 ± 0.4 (0.5–1.8)	0.1
Na^+ (mEq/L)	151 ± 10 (136–166)	152 ± 8 (141–165)	0.6
Macrosteatosis	7 (70%)	5 (56%)	0.6
Macrosteatosis >10%	4 (40%)	3 (33%)	1.0
Necrosis	0 (0%)	0 (0%)	–

The data are means ± SD. Range values are reported under parenthesis.

MELD, Model for End-Stage Liver Disease; BMI, body mass index; ICU, intensive care unit; PRBC, packed red blood cells.

⁎ Hypotension episodes are defined as systolic arterial pressure values <70 mm Hg for >30 min after graft reperfusion.

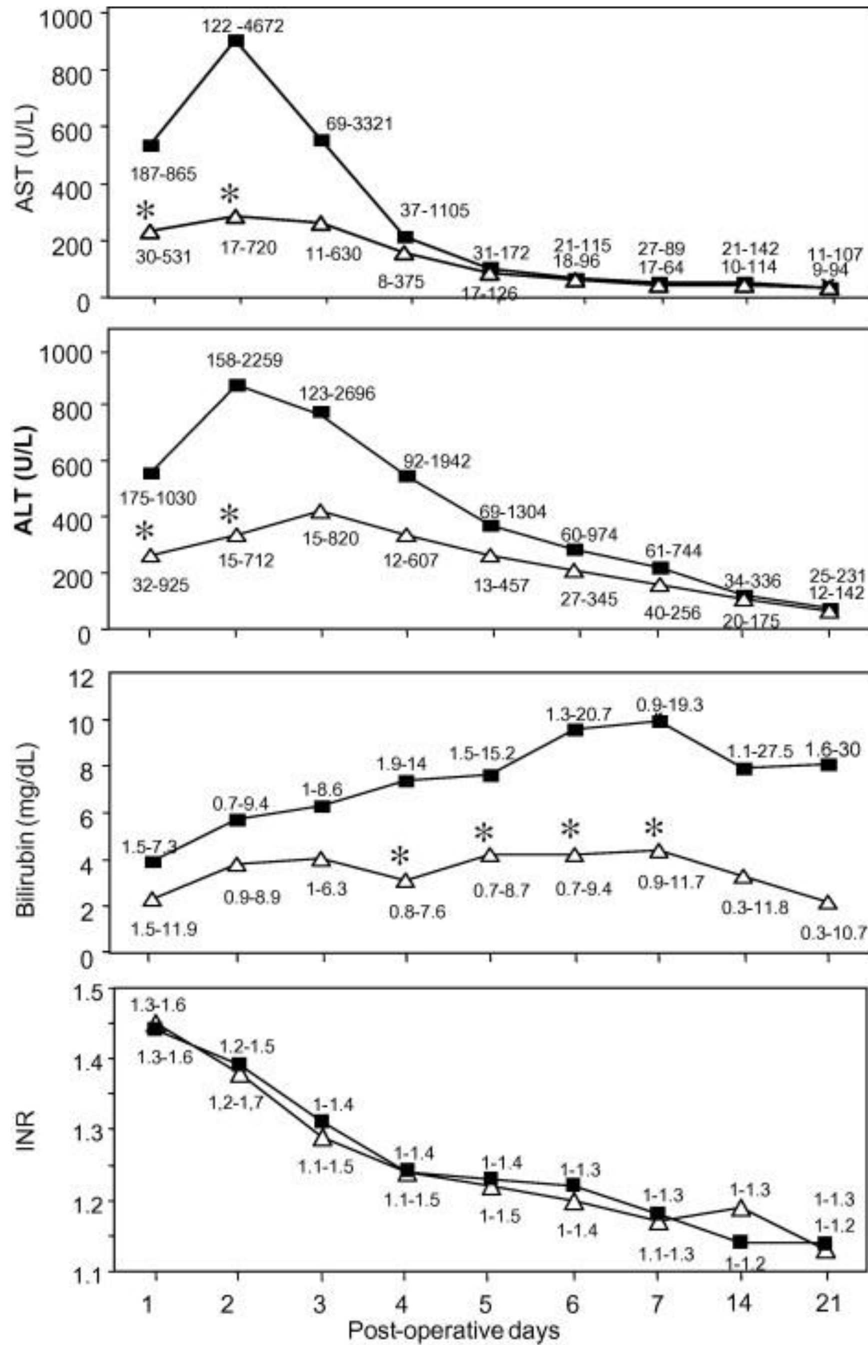

Figure 5. Post-operative changes in serum aspartate (AST) and alanine (ALT) amino transferases, total bilirubin and prothrombin activity (INR) after 24 h from liver grafts reperfusion and during the first 21 days of follow-up in transplanted patients receiving liver grafts responders (open symbols; n = 10) or non-responders (filled symbols; n = 9) to ischemic preconditioning. The points represent the median values. The range values are also reported for each point. The asterisks indicate statistical significant differences ($p < 0.05$) versus IPC-non-responders.

4. DISCUSSION

As outlined by some recent reviews, the use of ischemic preconditioning to ameliorate reperfusion injury following human liver transplantation has given conflicting and inconclusive results [28-30]. The combined analysis of the data available to date indicates that liver ischemic preconditioning before graft recovery does not improve post-operative aminotransferase release, hepatocyte apoptosis and inflammatory infiltrates [29,30]. Similarly, no significant effects have been observed in the incidence of primary graft non-function and initial poor function or in the median stay of transplanted patients in intensive care units [29,30].

In line with these conclusions, the present report fails to show improvement of aminotransferase levels in the subjects receiving preconditioned grafts. This is in contrast with our previous findings in a larger series of patients in whom ischemic preconditioning significantly lowered aminotransferase release up to the third day of follow-up [13]. Such a discrepancy can be due to the fact that post-reperfusion AST and ALT in the non-preconditioned controls included in the present study are much lower than those of the control group of our previous report. This suggests the possibility that the severity of reperfusion injury in control grafts might influence the possibility to detect statistically significant improvements of aminotransferases following ischemic preconditioning. In agreement with our previous observations [13], also in this new series of patients ischemic preconditioning does not influence the post-operative course of bilirubin and prothrombin activity as well as the incidence of primary graft non-function and initial poor function.

To get some insight into the possible causes of the failure of ischemic preconditioning to protect liver grafts against reperfusion injury we have investigated some of the intracellular signals that are associated with the development of the hepato-protective effects of ischemic preconditioning in experimental systems [17]. Recent studies have shown that the activation of phosphoinositide-3 kinase (PI3K) and its downstream effector

PKB/Akt play a key role in the onset of ischemic preconditioning in experimental models of reperfusion injury in different tissues [18,19,31,32]. In accordance with the observations in rodents [18,19], ischemic preconditioning of human livers increases PKB/Akt phosphorylation 2 h after the graft reperfusion. Such an effect is associated with the lowering of the liver content of the phosphatase tensin homologue deleted from chromosome 10 (PTEN) a negative regulator of PI3K activity. PTEN is a dual protein/lipid phosphatase that by dephosphorylating PI(3,4,5)P3 blocks the transduction PI3K-dependent signals to downstream kinases including PKB/Akt [27]. PTEN is present ubiquitously in cells and its activity is reflected by its cellular levels [27,33]. A decline of both PTEN levels and activity occurring in parallel with the activation of PKB/Akt has been recently reported in preconditioned myocardium [34], indicating that ischemic preconditioning modulates both positive and negative regulators of PI3K-dependent signaling. A deeper analysis of PKB/Akt activation in preconditioned human livers revealed a large inter-individual variability, suggesting that in some of the grafts ischemic preconditioning fails to promote the transduction of PI3K-mediated signals. At the moment the causes of such variability are unclear. Donor characteristics and operative parameters are comparable between grafts responsive or non-responsive to the preconditioning stimuli. Nonetheless, we have observed that PTEN is lowered only in IPC-responsive livers that show sustained PKB/Akt activation. PTEN is a constitutively active phosphatase that is regulated by “de novo” transcription as well as by the combination of protein degradation, phosphorylation and oxidation [33]. Experiments in both the myocardium and the brain have shown that the lowering of PTEN is required for the full transduction of PI3K-dependent signals triggered by ischemic preconditioning [27,34,35]. Moreover, pharmacological inhibitors of PTEN prevent ischemic injury in rat brain [36,37]. Thus, a failure to downmodulate PTEN might be responsible for the lack of PKB-Akt activation in the grafts non-responsive to ischemic preconditioning. Recently, Vinciguerra and co-workers have reported that steatosis downregulates PTEN in human livers [38]. Nonetheless, the extension of

steatosis is not different between our IPC-responsive and non-responsive grafts. At present, we cannot exclude that other mechanisms might account for the lack of PKB-Akt activation in IPC-non-responsive livers. In particular, the observation that the T_0 values of PKB-Akt phosphorylation tend to be higher in IPC-non-responsive livers suggests the possibility that the basal activity of the PI3K/PKB-Akt pathway might influence the response of the same kinases to the signals triggered by ischemic preconditioning.
Whatever might be the cause for the variability in PI3K-mediated signals in preconditioned human livers, it is important to note that the patients receiving grafts with an effective PI3K response show significantly less aminotransferase release 24 and 48 h after reperfusion and a better clearance of bilirubin during follow-up. The use of adrenaline to support haemodynamic parameters during transplant was also less prevalent in these patients. Recently, Amadour and co-workers [15] have shown that ischemic preconditioning decreases hepatocyte apoptosis and induces the hypoxia-inducible factor-1α (HIF-1α) in human transplanted livers. It is noteworthy, that by phosphorylating several pro-apoptotic proteins, PKB/Akt plays an important role in preventing apoptosis [20]. Moreover, we have recently shown that PI3K is involved in the oxygen-independent stimulation of HIF-1α activity induced by both hypoxic preconditioning and adenosine A2A agonists in hepatocyte and human macrophages [39,40]. Altogether these observations support the importance of PI3K-mediated signals in preventing reperfusion injury in human livers.

In conclusion, the evaluation of PI3K signaling induced by ischemic preconditioning in graft livers from deceased donor shows a large inter-individual variability that might reflect an insufficient capability of the organs obtained from clinically dead patients to express a fully efficient preconditioning machinery when exposed to a transient ischemic stress. Such variability might likely explain the conflicting reports concerning the clinical response of transplanted livers to ischemic preconditioning. Furthermore, these results point to the urgent need to devise alternative

methods, possibly using pharmacological agents, to induce effective preconditioning in the livers obtained from deceased donors.

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STUDY 3: "Pharmacological postconditioning by stimulation of adenosine A2A receptors or by chemical inhibition of PTEN protects rat hepatocytes from different pathogenic insults"

AIMS: Recent data show that ischemic postconditioning of the liver through brief interruptions of blood flow in the early phase of reperfusion improves post-transplantation liver functionality in rats, but the signal mediators involved in this process have not yet been clarified. The possibility to activate in the intrinsic mediators of liver protection not only prior (preconditioning mediators) but also after (postconditioning mediators) the beginning of a pathogenic insult would have important clinical outcomes. This study investigated in primary rat hepatocytes the pharmacological induction of hepatic postconditioning on different models of injury by the use of the adenosine A2A receptor agonist CGS21680, previously characterized as an effective stimulant of hepatic preconditioning, or by inhibition of PTEN, the major suppressor of PI3K/Akt survival signalling.

CONCLUSIONS: Our results have evidenced the possibility to induce pharmacological postconditioning in hepatocytes after the instauration of liver damage of different etiology, such as post-ischemic damage and CCl_4 and menadione intoxication, by activation of common endogenous survival signaling pathways where PI3K and its modulator PTEN play a central role. The possibility to face hepatic insults of different etiology when the hepatic damage is already started has important clinical outcomes, broadening the relevance of pharmacological postconditioning to a spectrum of conditions were surgical preconditioning is not possible or appropriate. The data obtained also indicated adenosine A2A receptor agonists and PTEN inhibitors as possible useful agents for the pharmacological induction of postconditioning in the liver.

Pharmacological postconditioning by stimulation of adenosine A2A receptors or by chemical inhibition of PTEN protects rat hepatocytes from different pathogenic insults

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Abbreviations: PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homologue deleted from chromosome 10; PKB-Akt, protein kinase B; ERK1/2, extracellular signal regulated kinase 1/2.

ABSTRACT

Background/Aims: The possibility to activate in the intrinsic mediators of liver protection not only prior (preconditioning) but also after the beginning of a pathogenic insult (postconditioning) would have important clinical outcomes. This study investigates the pharmacological induction of postconditioning in primary hepatocytes by the use of the adenosine A2A receptor agonist CGS21680, previously characterized as an effective stimulant of hepatic preconditioning, or by inhibition of PTEN, the major suppressor of PI3K/Akt survival signalling.

Methods: Post-ischemic damage was reproduced by using primary rat hepatocytes cold-stored in ViaSpan® organ preservation solution and then re-oxygenated in warm Krebs-Henseleit buffer. Xenobiotic damage was provoked treating hepatocytes with 172 μMol/L CCl_4 or 50 μMol/L menadione. Postconditioning was induced by the addition of 5 μMol/L CGS21680 or by the addition of the PTEN inhibitor bpV(HOpic) 100 nMol/L .

Results: Postconditioning with CGS21680 or bpV(HOpic) significantly reduced death of hepatocytes upon re-oxygenation or treatment with CCl_4 or menadione. Such effect was associated to the activation of the PI3K target Akt and of ERK1/2 kinase. Chemical inhibition of PI3K or ERK1/2 function by respectively wortmannin or PD98059 reverted the protection given by CGS21680 or bpV(HOpic).

Conclusions: Postconditioning is an effective strategy to prevent early re-oxygenation and xenobiotics induced injury in hepatocytes and adenosine A2A receptor agonists and PTEN inhibitors represent possible useful agents for the pharmacological induction of postconditioning in the liver.

INTRODUCTION

Liver ischemic preconditioning obtained by a transient interruption of blood flow applied before a prolonged ischemic insult confers tissue protection against ischemia/reperfusion injury [1,2,3]. This procedure is gaining increasing importance in the field of liver surgery and transplantation [4,5,6,7] and the mechanisms underlying this phenomenon have been studied and characterized in both “in vivo” [8,9,10,11] and “in vitro” models [12]. The intracellular mediators of liver ischemic preconditioning have been partially elucidated and involve, among the others, the adenosine A2A receptors which activate a cascade including the phospho-inositide-3 kinase (PI3K) and its downstream effector PKB/Akt [13,14], two central mediators of cell survival processes [15,16]. Such effect is associated with lowering of the liver content of PTEN (phosphatase and tensin homologue deleted from Chromosome 10) [17], a negative regulator of PI3K activity. PTEN blocks the transduction of PI3K-dependent signals to downstream kinases including PKB/Akt by dephosphorylating phosphatidylinositol-triphosphate PI(3,4,5)P3 [18]; PTEN is ubiquitously expressed in cells and its intracellular level reflects its grade of activity [18,19].

A new phenomenon, termed ischemic postconditioning, has been described in the heart and consists in the application of subsequent brief

cycles of ischemia during the reperfusion period after the sustained ischemic episode. This procedure results in a reduction of myocardial injury during the reperfusion phase, limiting infarct size with comparable effects to those of heart ischemic preconditioning [20-22].

Two recent studies have shown that the application of ischemic postconditioning on the liver through intermittent interruptions of blood flow in the early phase of reperfusion improves post-transplantation liver functionality in rats, but the signal mediators involved in this process have not yet been clarified [23, 24].

The possibility that critical mediators of liver preconditioning could be activated not only prior but also after the ischemic insult would have important clinical outcomes, offering the possibility to act effectively also when the hepatic damage is already started.

This prompted us to investigate the possible recruitment of the preconditioning mediators upon re-oxygenation in an in vitro model of post-ischemic injury either by the use of the adenosine A2A receptor agonist CGS21680, previously characterized as an inductor of hepatic preconditioning, or blocking the action of PTEN phosphatase through the administration of the inhibitor bpV(HOpic).

The pharmacological stimulation of intracellular signals able to activate endogenous hepatoprotective systems even after a pathogenic insult could be useful not only in ischemia/reperfusion injury, but also to face hepatic insults of different etiology that are responsible for the induction of acute liver failure “in vivo”.

In this study we tested also if the stimulation of adenosine A2A receptors or the inhibition of PTEN are able to protect the hepatocytes from the damage induced by carbon tetrachloride (CCl_4) exposure and by menadione intoxication, employed as model substances responsible for xenobiotic liver injury.

Carbon tetrachloride is an organic compound causing acute and chronic poisoning, with toxic effects on liver, kidneys and nervous system. In the liver CCl_4 induces extensive lipid peroxidation and gives rise to massive necrosis via oxidative stress [25, 26].

Menadione (2-methyl naphthoquinone) is a precursor to various types of vitamin K, but it isn't generally used as a nutritional supplement because it's a superoxide generator and at high doses it causes oxidant-induced cell death [27,28].

MATERIALS AND METHODS

Materials

Collagenase (Type IV), *N*-(2-hydroxyethyl)-piperazine-*N*'-(2-ethanesulfonic acid) (HEPES), DMEM culture medium, propidium iodide, Trypan blue, CGS21680, wortmannin, PD98059 were purchased from Sigma Chemical Co (St. Louis, MO, USA); bpV(HOpic) was purchased from EMDbiosciences (Darmstadt, Germany); carbon tetrachloride was obtained from Riedel De-Haen (Hanover, Germany); Percoll was obtained from GE Healthcare Bio-Sciences (Uppsala, Sweden); ViaSpan® Solution was obtained from Bristol-Myers Squibb (Garden City, NY). All the other chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany).

Hepatocytes isolation preservation and treatments

Primary rat hepatocytes were isolated by collagenase liver perfusion of male Wistar rats (180-250 g weight) (Harlan, Italy) and purified by centrifugation at 50*g* for 5 minutes followed by a further 2 minutes centrifugation at 350*g* through a layer of Percoll. Cell purity was assessed according to Benten et al.[29]. Cell viability, estimated at the beginning of experiments, ranged between 82% and 90%. The use and care of the animals were approved by the Italian Ministry of Health.

Isolated hepatocytes suspended in DMEM culture medium were re-suspended in ViaSpan® solution (University of Wisconsin solution without additives) at 4°C. Hepatocytes suspended in ViaSpan® solution (10^6/ml cell density) were kept for 24 hours at 4°C in hypoxic atmosphere (95% N_2 - 5% CO_2). After 24 hours hepatocytes were re-oxygenated in Krebs-

Henseleit-HEPES buffer (containing 20 nmol/L HEPES) at pH 7.4 and 25°C with 10^6/ml final cell density. Re-oxygenation was performed fluxing the incubation flasks with 95% air – 5% CO_2 gas mixture.
2p-(2-carboxiethyl) phenyl-amino-5'-N-ethylcarboxyamido-adenosine (CGS21680) 5 µMol/L was added to the medium at the beginning of re-oxygenation; the inhibitors wortmannin 500 nMol/L, dipotassium-bisperoxo-(5-hydroxypyridine-2-carboxyl)-oxovanadate (bpV(HOpic)) 100 nMol/L and PD98059 20 µMol/L were also added at the beginning of re-oxygenation.
Damage by CCl_4 was obtained incubating freshly isolated hepatocytes suspended in KHH buffer in the presence of 10 µl of CCl_4 placed in the centre well of 50 ml Erlenmeyer flasks to give a final concentration in the incubation medium of approximately 172 µMol/L [30]. CGS21680 5 µMol/L or bpV (HOpic) 100 nMol/L were added to the medium 15 minutes after the beginning of exposure to CCl_4; wortmannin 500 nMol/L and PD98059 20 µMol/L were administered simultaneously to CCl_4.
Menadione intoxication was induced by the addition of menadione 50 µMol/L to freshly isolated hepatocytes suspended in KHH medium; CGS21680 5 µMol/L or bpV (HOpic) 100 nMol/L were administered 5 minutes later whilst wortmannin 500 nMol/L and PD98059 20 µMol/L were added at the same time of menadione.

Determination of cell viability

Cell viability was estimated by microscope-counting the hepatocytes excluding Trypan blue and by the determination of nuclear fluorescence staining with propidium iodide according to the method described by Gores et al. [31] using a Becton-Dickinson FACScan analyzer (Becton-Dickinson, San Jose, CA) and the Cellquest software program (Becton-Dickinson, San Jose, CA).

Analisys of PKB/Akt and ERK1/2 phosphorylation state and PTEN expression

Protein extracts were electrophoresed on a 10% SDS-polyacrylamide gel and blotted onto nitrocellulose membranes. Protein expression was revealed using specific antibodies against Akt, phospho Akt, ERK1/2, phospho ERK1/2 (Cell Signalling Technology, Beverly , MA) and PTEN (Santa Cruz Biothecnology, Santa Cruz, CA). The anti-actin monoclonal antibody Sigma Chemical Co (St. Louis, MO, USA) was used to assess for equal protein loading. The antigens were detected using an immunodetection kit (Western Lightning Chemiluminescence Reagent Plus, Perkin Elmer, MA). The relative intensity of bands was measured by videodensitometry using the VersaDoc 3000 quantitative imaging system (BioRad, Hercules, CA) and analyzed with the Quantity One software (BioRad, Hercules, CA). The results were expressed as ratios.

Data analysis

The data were expressed as mean ± standard deviation (SD). Statistical analysis was performed by Instat-3 statistical software (GraphPad Software Inc, San Diego, CA) using 1-way ANOVA test with Bonferroni's correction for multiple comparisons when more than 2 groups were analyzed. Distribution normality of all the groups was preliminarily verified by Kolmogorov and Smirnov test. Significance was taken at 5% level.

RESULTS

To evaluate the effects of pharmacological postconditioning on post ischemic reperfusion injury we have set up an experimental model of ischemia/reperfusion damage. Freshly isolated rat hepatocytes were suspended in ViaSpan® organ preservation solution and kept for 24 h at 4°C in hypoxic atmosphere to reproduce the cold ischemic phase of liver graft preservation. The reperfusion phase was then mimicked suspending the hepatocytes in oxygenated Krebs-Henseleit buffer, and

postconditioning was induced by the addition of CGS21680 (5 μMol/L) at the beginning of re-oxygenation.

After 24 hours of hypoxic cold storage hepatocytes showed decreased viability if compared to freshly isolated hepatocytes (mean±SD: 71±4,5 vs 85±3,6). The beginning of re-oxygenation in Krebs-Henseleit buffer was associated to an immediate loss of cell viability of about 15%. Viability then further decreased in the following two hours to approximately 20% (Fig.1A). Cells treatment with the inductor of liver preconditioning CGS21680 reduced significantly hepatocytes death upon progression of re-oxygenation, keeping average hepatocytes viability around 40% (Fig.1A). The development of the protective effect of postconditioning by CGS21680 was associated with an increased phosphorylation in Ser^{473} of the PI3K effector PKB/Akt (Fig.1B). Co-treatment of postconditioned hepatocytes with the PI3K inhibitor wortmannin abolished the effect of CGS21680 on PKB/Akt phosphorylation (Fig.1B). The presence of wortmannin at the beginning of re-oxygenation reverted also the protection given by CGS21680, whilst wortmannin alone did not modify control cells viability (Fig.1A).

Western blotting analysis of PTEN phosphatase expression showed a significant decreased level of PTEN in hepatocytes treated with CGS21680 (Fig.1C). This prompted us to investigate if we could achieve a protective action on hepatocytes viability increasing the intracellular level of PI(3,4,5)P3 by PTEN inhibition. Cells treatment with the PTEN inhibitor bpV(HOpic) (100 nMol/L) reduced hepatocytes death upon progression of re-oxygenation with a similar outcome to that obtained with CGS21680 (Fig.2A). Administration of bpV(HOpic) also induced a strong phosphorylation in Ser^{473} of PKB/Akt (Fig.2B). The blockage of PI3K with wortmannin abolished the effects of bpv(HOpic) on PKB/Akt phosphorylation (Fig.2B) and on hepatocytes protection (Fig.2A).

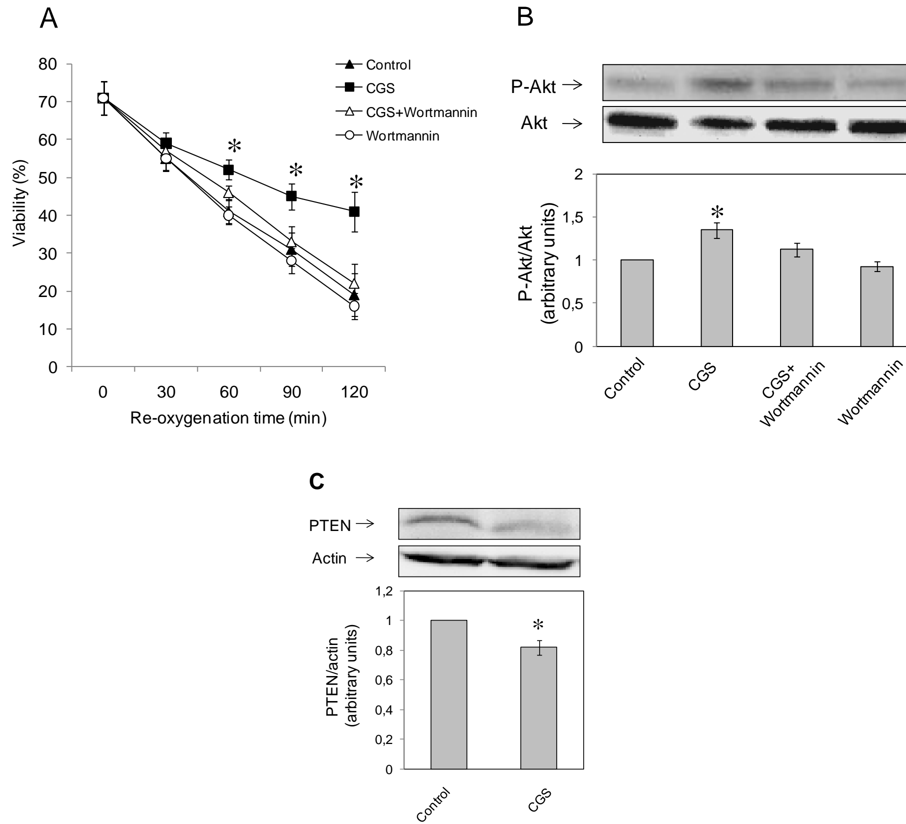

Figure 1. Effects of hepatocytes pharmacological postconditioning by CGS21680 in simulated post-ischemic damage.

Post-ischemic damage was reproduced by using primary rat hepatocytes cold-stored in organ preservation solution and then re-oxygenated in warm Krebs-Henseleit buffer. CGS21680 (CGS; 5 μMol/L) was added at the beginning of re-oxygenation; wortmannin (500 nM/L) was administered simultaneously to CGS21680. (A) Viability of hepatocytes was evaluated for up to 120 min re-oxygenation. Results are mean of 4 different experiments. Statistical significance: *$p<0.05$ vs control or cells treated with wortmannin. (B) Activation of PKB/Akt was estimated by western blotting using antibodies against phospho(Ser^{473}) Akt and Akt, 15 min after treatment with CGS21680. Relative intensity of phosphorylated and non-phosphorylated bands was measured by videodensitometry and the results were expressed as ratios after normalization at 1 of the control. Results are mean of 4 different experiments. Statistical significance: *$p<0.05$ vs control or cells treated with wortmannin. (C) Expression of PTEN was estimated by western blotting 15 min after treatment with CGS21680. Relative intensity of bands was measured by videodensitometry and values were expressed as ratios between the intensity of PTEN band and reference actin band after normalization at 1 of the control. Results are mean of 4 different experiments. Statistical significance: *$p<0.05$ vs control.

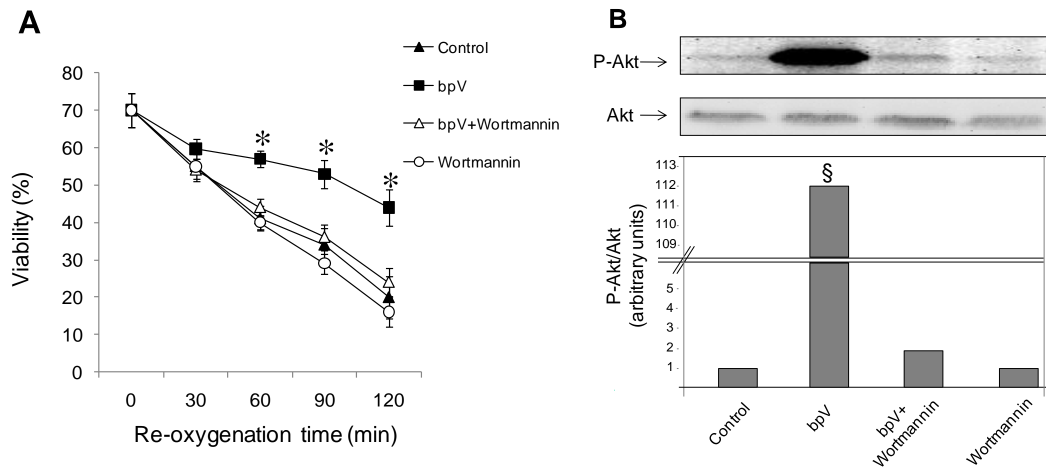

Figure 2. Effects of hepatocytes pharmacological postconditioning by bpV(HOpic) in simulated post-ischemic damage.

Post-ischemic damage was reproduced by using primary rat hepatocytes cold-stored in organ preservation solution and then re-oxygenated in warm Krebs-Henseleit buffer. bpV(HOpic) (bpV; 100nMol/L) was added at the beginning of re-oxygenation; wortmannin (500 nM/L) was administered simultaneously to bpV. (A) Viability of hepatocytes was evaluated for up to 120 min re-oxygenation. Results are mean of 4 different experiments. Statistical significance: $^{*}p<0.05$ vs control or cells treated with wortmannin. (B) Activation of PKB/Akt was estimated by western blotting using antibodies against phospho(Ser^{473}) Akt and Akt, 15 min after treatment with bpV(HOpic). Relative intensity of phosphorylated and non-phosphorylated bands was measured by videodensitometry and the results were expressed as ratios after normalization at 1 of the control. Results are mean of 4 different experiments. Statistical significance: $^{§}p<0.001$ vs control or cells treated with wortmannin.

Further immunoblotting analysis were performed to assess the possible activation by CGS21680 and bpV(HOpic) of other kinases known to mediate survival signals. As shown in Fig.3A and 3B CGS21680 and bpV(HOpic) increased the phosphorylation of p42/44 extracellular signal regulated kinase ERK1/2, while p38 MAP kinase phosphorylation state wasn't significantly changed (data not shown). Blocking ERK1/2 activation by the use of PD98059, the specific upstream ERK1/2 MEK1 inhibitor, reverted the effect of CGS21680 and bpV(HOpic) on ERK1/2 phosphorylation (Fig. 3A and 3B) and reduced the protection on hepatocytes viability (Fig. 3C).

The effect of pharmacological postconditioning with CGS21680 and bpV(HOpic) was then investigated on two other different models of liver injury that aimed to reproduce the hepatic effects of CCl_4 exposure and menadione intoxication.

The administration of CGS21680 or bpV (HOpic) to freshly isolated rat hepatocytes suspended in Krebs-Henseleit buffer after 15 minutes of exposure to CCl_4 or after 5 minutes treatment with menadione was able to reduce hepatocytes death in the following two hours compared to untreated cells (Fig. 4A and 5A). This effect was associated with increased PKB/Akt phosphorylation (Fig. 4B ,4C, 5B and 5C). The addition to the medium of wortmannin at the beginning of CCl_4 or menadione exposure eliminated the protection induced by CGS21680 or bpV(HOpic) (Fig. 4D and 5D). An increased phosphorylation of ERK1/2 kinase following CGS21680 and bpV(HOpic) treatment was present also in CCl_4 and menadione injury (Fig. 4E, 4F, 5E and 5F) and ERK1/2 blockage by PD98059 reverted CGS21680 and bpV(HOpic) induced hepatocytes resistance to CCl_4 and menadione toxicity (Fig. 4D, 5D).

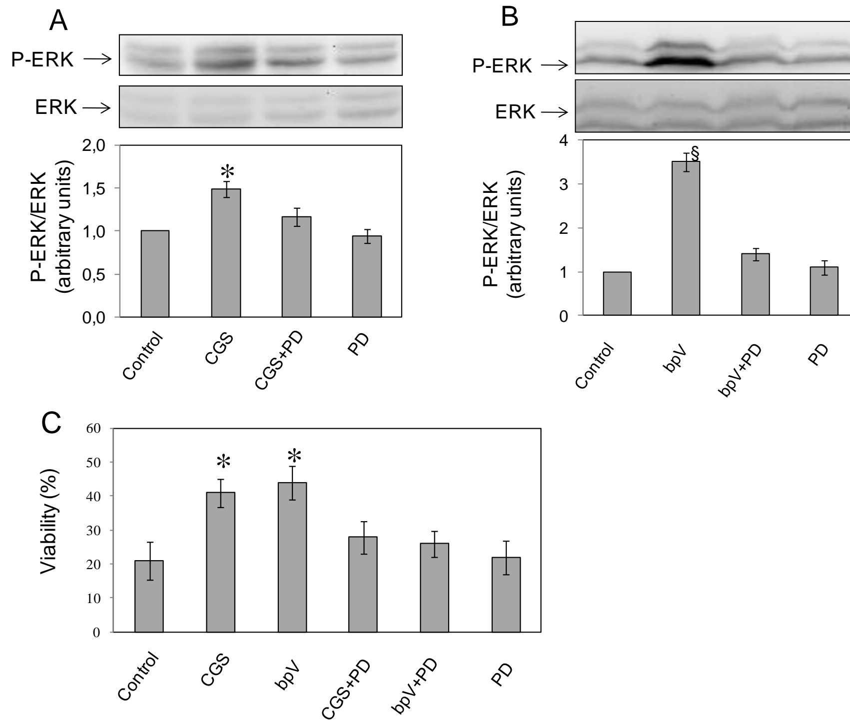

Figure 3. Role of ERK1/2 activation by CGS21680 and bpV(HOpic) postconditioning in simulated post-ischemic damage.
Post-ischemic damage was reproduced by using primary rat hepatocytes cold-stored in organ preservation solution and then re-oxygenated in warm Krebs-Henseleit buffer. CGS21680 (CGS; 5 µMol/L), bpV(HOpic) (bpV; 100nMol/L) and PD98059 (PD 20 µMol/L) were added at the beginning of re-oxygenation. (A&B) Activation of ERK1/2 was estimated by western blotting using antibodies against phospho-ERK and ERK, 15 min after treatment with CGS21680 (A) or bpV(HOpic) (B). Relative intensity of phosphorylated and non-phosphorylated bands was measured by videodensitometry and the results were expressed as ratios after normalization at 1 of the control. Results are mean of 4 different experiments. Statistical significance: *$p<0.05$ versus control or cells treated with PD (A); $^{§}p<0.005$ versus control or cells treated with PD98059 (B). (C) Viability of hepatocytes postconditioned by CGS21680 or bpV(HOpic) is shown at 120 min incubation in oxygenated conditions in presence or absence of the inhibitor PD98059. Results are mean of 4 different experiments. Statistical significance: *$p<0.05$ versus control or cells treated with PD98059.

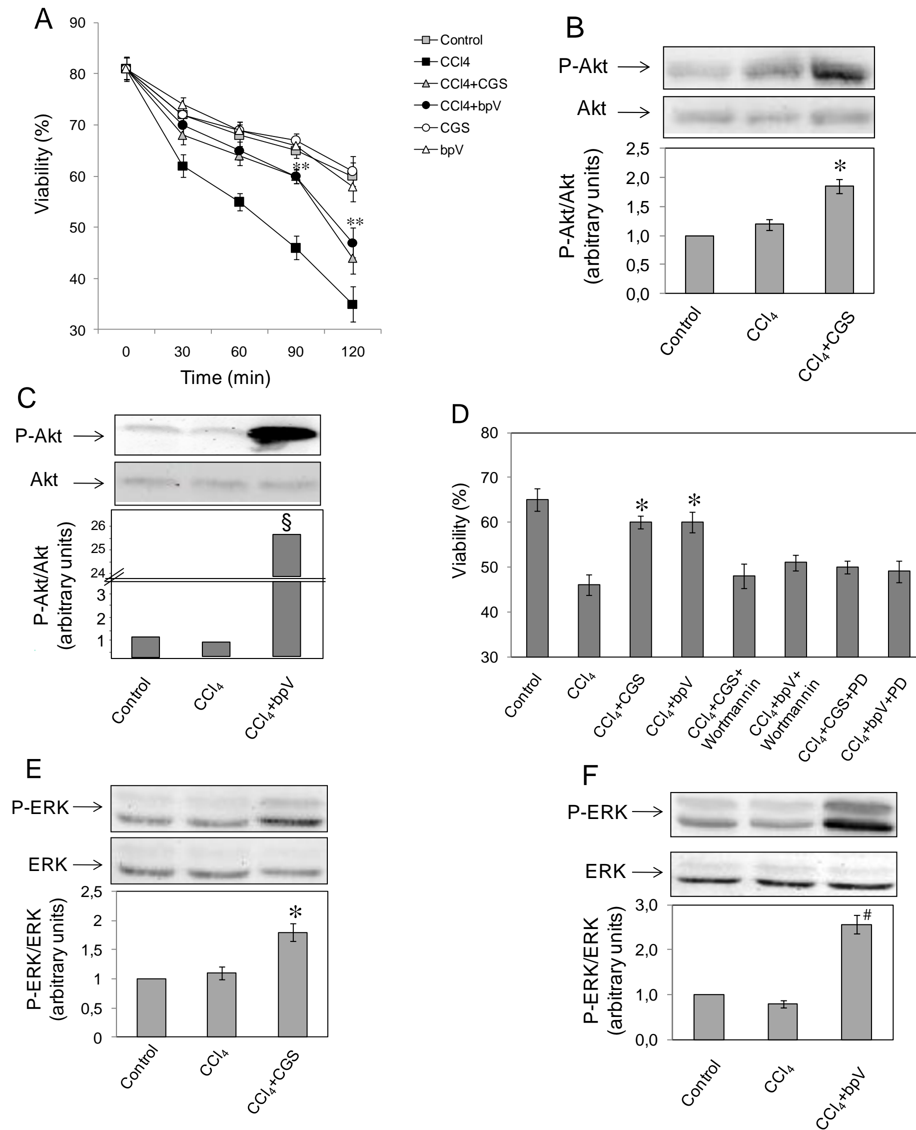

Figure 4. Effects of hepatocytes pharmacological postconditioning by CGS21680 and bpV(HOpic) in CCl_4 induced damage.

Damage by CCl_4 was obtained incubating hepatocytes in presence of 172 µMol/L CCl_4; CGS21680 (CGS; 5 µMol/L) or bpV(HOpic) (bpV; 100 nMol/L) were added to the medium 15 minutes after the beginning of exposure to CCl_4; wortmannin (500 nMol/L) and PD98059 (PD; 20 µMol/L) were administered simultaneously to CCl_4. (A) Viability of hepatocytes was evaluated for up to 120 min treatment with CCl_4. Activation of PKB/Akt upon CGS21680 (B) or bpV(HOpic) (C) and activation of ERK1/2 upon CGS21680 (E) or bpV(HOpic) (F) was estimated by western blotting using antibodies against phospho(Ser^{473}) Akt and Akt and against phospho-ERK and ERK respectively, 15 min after treatment with CGS or bpV. Relative intensity of phosphorylated and non-phosphorylated bands was measured by videodensitometry and the results were expressed as ratios after normalization at 1 of the control. (D) Viability of hepatocytes is also shown at 90 min incubation with CCl_4 in presence or absence of the inhibitors wortmannin and PD98059.

All the results are mean of 4 different experiments. Statistical significance: *$p<0.05$ versus CCl_4 (A), versus control or CCl_4 (B), versus CCl_4 or cells receiving wortmannin or PD (D), versus control or CCl_4 (E); $^{\S}p<0.001$ versus control or CCl_4 (C); $^{\#}p<0.005$ versus control or CCl_4 (F).

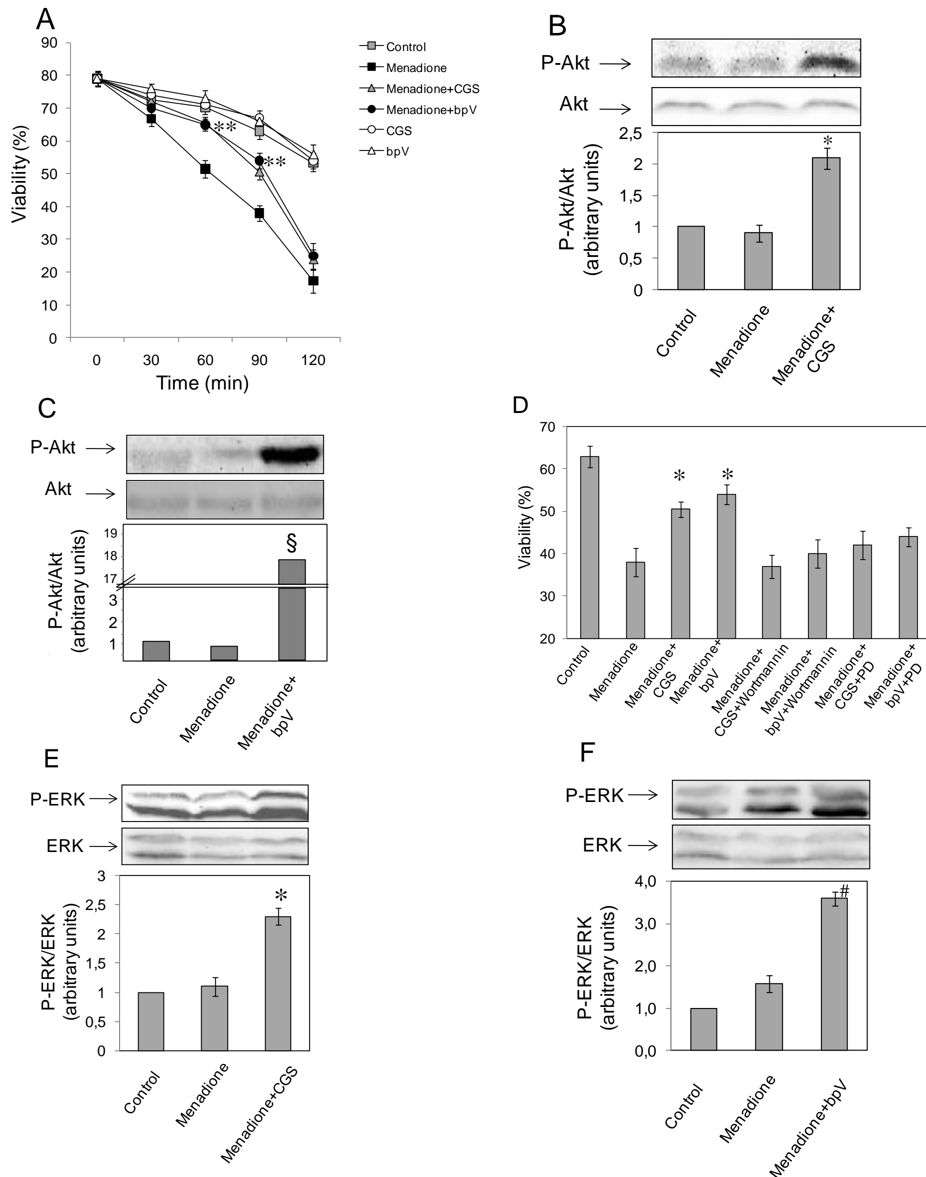

Figure 5. Effects of hepatocytes pharmacological postconditioning by CGS21680 and bpV(HOpic) in menadione induced damage.

Damage by menadione was obtained incubating hepatocytes in presence of 50 μMol/L menadione; CGS21680 (CGS; 5 μMol/L) or bpV(HOpic) (bpV; 100 nMol/L) were added to the medium 5 minutes after menadione; wortmannin (500 nMol/L) and PD98059 (PD; 20 μMol/L) were administered simultaneously to menadione. (A) Viability of hepatocytes was evaluated for up to 120 min treatment with menadione. Activation of PKB/Akt upon CGS21680 (B) or bpV(HOpic) (C) and activation of ERK1/2 upon CGS21680 (E) or bpV(HOpic) (F) was estimated by western blotting using antibodies against phospho(Ser^{473}) Akt and Akt and against phospho-ERK and ERK respectively, 15 min after treatment with CGS21680 or bpV(HOpic). Relative intensity of phosphorylated and non-phosphorylated bands was measured by videodensitometry and the results were expressed as ratios after normalization at 1 of the control. (D) Viability of hepatocytes is shown at 90 min incubation with menadione in presence or absence of the inhibitors wortmannin and PD98059.

All the results are mean of 4 different experiments. Statistical significance: *p<0.05 versus menadione (A), versus control or menadione (B), versus menadione or cells receiving wortmannin or PD98059 (D), versus control or menadione (E); §p<0.001 versus control or menadione (C); $^{\#}$p <0.01 versus control or menadione (F).

DISCUSSION

The technique of ischemic postconditioning has been demonstrated to be an effective strategy to reduce reperfusion injury in the myocardium [22, 32-34], but to date the efficacy of postconditioning in the liver has been tested only in limited studies. These works show how the application of ischemic postconditioning through brief cycles of ischemia during the reperfusion phase of orthotopic liver transplantation in rats is associated to amelioration of transaminase levels, inhibition of hepatocellular apoptosis, reduction of hepatic tissue damage and improved survival rate [23, 24].

Previous studies on liver ischemic preconditioning have demonstrated how this protective phenomenon can be induced not only applying a short ischemic period before a prolonged ischemia [2,6,8,10], but also through the direct stimulation of the adenosine A2A receptors [8,9,13,14], and that the development of preconditioning is associated with increased PKB/Akt phosphorylation in experimental models [13,35] as well as in the human liver during the reperfusion phase [17].

Here we showed that the pharmacological stimulation of hepatocytes adenosine A2A receptors was able to activate the intrinsic mediators of liver protection also at the beginning of re-oxygenation after hypoxic cold storage, in an experimental model that mimicked ischemia/reperfusion in liver transplantation, inducing the phenomenon of liver postconditioning.

The data obtained on cell viability demonstrated the involvement of the central mediator of liver preconditioning PI3K [14] also in the induction of postconditioning, as the specific PI3K inhibitor wortmannin abolished the hepatoprotective action of the adenosine A2A receptor agonist CGS21680 and the development of protection was associated with increased Ser^{473} phosphorylation of PKB/Akt, employed as marker of PI3K activity. These results are in agreement with studies on the myocardium where ischemic postconditioning activates the PI3K-Akt pathway at the time of reperfusion [20,32,33].

Postconditioning of hepatocytes with CGS21680 was also associated with decreased expression of PTEN phosphatase, a negative regulator of PI3K activity [18]. A decline of both PTEN levels and activity occurring in parallel

with the activation of PKB/Akt has been recently reported in preconditioned myocardium [36], and in human transplanted liver the development of protection by ischemic preconditioning has been shown to be dependent on the effective capacity of preconditioning to down modulate PTEN [17]. Our findings thus indicate that, similarly to preconditioning, also liver postconditioning is modulated by both positive and negative regulators of PI3K-dependent signals.

Interestingly our data also showed that pharmacological inhibition of PTEN with bpV(HOpic) was able to induce hepatoprotective effects, reproducing the cytoprotection obtained by adenosine A2A receptors stimulation. The chemical inhibitor bpV(HOpic) belongs to a compound of vanadate derivatives known for being competitive reversible inhibitors of protein tyrosine phosphatases at micromolar concentrations, but specific and effective PTEN inhibitors in the nanomolar concentration range [37]. In our experiments the specific inhibition of PTEN by bpV(HOpic) was confirmed by the strong increased phosphorylation of PKB/Akt, a downstream target of PTEN dependent signaling [38]. The protective role of PTEN chemical inhibition reported in our study is in agreement with recent researches indicating that PTEN inhibitors are able to prevent ischemic injury in rat brain [39,40]. Moreover a study on the myocardium by Cai et al. has evidenced that ischemic preconditioning-induced cardioprotection is lost in mice with immunoproteasome subunit low molecular mass polypeptide-2 deficiency, which is responsible for the degradation of specific proteins including PTEN, whilst, on the contrary, pretreatment of these mice with bpV(HOpic) reduces myocardial infarct size and improves cardiac function [41].

Concomitantly to the abolishment of PTEN inhibitory action on PI3K signaling, hepatocytes treatment with CGS21680 and bpV(HOpic) triggered the activation of the MEK1-ERK1/2 kinase cascade, which is known for mediating survival signals in the myocardium at the time of reperfusion after both ischemic preconditioning [42,43] and postconditioning [44]. The abrogation of CGS21680 and bpV(HOpic)-induced protection in presence of the pharmacological inhibitor of MEK1

PD98059 indicates in the activation of MEK1-ERK1/2 axis an additional hepatoprotective effect of CGS21680 and bpV(HOpic).

Finally we have demonstrated that pharmacological postconditioning can effectively delay hepatocytes death also after the onset of other types of harmful conditions, such as CCl_4 or menadione intoxication, by activating the same protective mediators.

All together our results have evidenced the possibility to induce pharmacological postconditioning in hepatocytes after the instauration of liver damage of different etiology by activation of common endogenous survival signaling pathways where PI3K and its modulator PTEN play a central role. This wider spectrum of efficacy increases the clinical relevance of pharmacological postconditioning broadening its applicability to a range of conditions were surgical preconditioning is not possible or appropriate. Moreover our data indicate adenosine A2A receptor agonists and PTEN inhibitors as possible useful agents for the induction of pharmacological postconditioning in the liver.

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CONCLUSIONS

Understanding the proteomic features of the physiological and pathological phenotype is necessary to identify molecular targets for therapeutic interventions aimed to restore the physiological condition after a pathological insult. Ischemic preconditioning is an inducible natural system able to improve tissues resistance to death by the production of a preconditioned phenotype that is resistant to a subsequent lethal stress. The knowledge of the proteomic changes associated to the production of liver preconditioned phenotype is still limited. The results presented in this thesis have for the first time:

1. Elucidated intracellular mechanisms involved in the delayed protection of preconditioned hepatocytes (Alchera et al. Hepatology 2008 Jul;48:230-9).
2. Indicated the possible explanation of the contrasting results of the mechanical application of ischemic preconditioning in human transplanted livers (Cescon et al. Journal of Hepatology 2009 May;50:937-47).
3. Shown the possibility to induce pharmacological postconditioning of rat hepatocytes by activation of the adenosine A2A receptors and inhibition of PTEN (Dal Ponte et al. Journal of Hepatology; manuscript submitted)
4. Identified new mediators of hepatic preconditioning: HIF, CAIX, PTEN, ERK1/2.
5. Shown the efficacy of pharmacological postconditioning to reduce the damage induced by the xenobiotic agents CCl_4 and menadione (Dal Ponte et al. Journal of Hepatology; manuscript submitted).
6. Identified in PI3K, Akt and PTEN three critical mediators of human liver preconditioning.

These data, besides giving new insides in the molecular mechanisms of liver preconditioning, offer the rational basis to develop new pharmacological strategies for the induction of preconditioning in patients.

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