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ISCHEMIA/REPERFUSION INJURY ON MICE STEATOTIC HEPATOCYTES and DIFFERENTIAL EFFECTS OF ADENOSINE A2A AND A1 RECEPTORS STIMULATION

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CO-ORDINATOR

Prof. Emanuele Albano

TUTOR

Prof. Rita Carini

DOCTORATE

CHANDRASHEKAR BANGALORE REVANNA

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ABBREVATIONS

I/R	Ischemia -Reperfusion
NAFLD	Non Alcoholic Fatty Liver Disease
FFA	Free Fatty Acid
ECD	Extended Criteria Donor
OPTN	Organ Procurement and Transplantation Network
IP	Ischemic Preconditioning
PI3K	Phosphatidyl-inositole-3-kinase
HIF-1	Hypoxia-inducible factor 1
ER	Endoplasmic Reticulum
TRAF2	Tumor Necrosis Factor Receptor (TNF-R) Adaptor Factor 2
ASK1	Apoptosis Signal-Regulating Kinase 1
ROS	Reactive Oxygen Species
JNK	C-Jun N-terminal kinase
PI3K	Phosphoinositide 3-Kinase
PKB	Protein Kinase B
HFD	High Fat Diet
Hp	Hepatocytes
SHp	Steatotic Hepatocytes
A2aR	Adenosine 2a Receptor
A1R	Adenosine 1 Receptor
CGS21680	2p-(2-carboxyethyl)-phenyl-amino-50-Nethylcarboxyamido-Adenosine
CCPA	2-Chloro-N6-cyclopenty–l adenosine,
NQDI-1	2,7-Dihydro-2,7-dioxo-3H-naphth-o[1,2,3dequin- oline-1-carboxylic acid ethyl ester.
DCFH-DA	2,7-dichlorofluorescin diacetate
SiRNA	Small interference RNA
ATP	Adenosine Triphosphate
WRT	Wortmannin

1. INTRODUCTION

1.1 ISCHEMIA -REPERFUSION (I/R) INJURY OF THE LIVER

Hepatic ischemia/reperfusion (I/R) injury is defined as the phenomenon during which cellular damage in an organ, caused by hypoxia, is paradoxically exacerbated after the restoration of oxygen delivery (Peralta et al., 2010). It is a dynamic process which involves the two interrelated phases of local ischemic insult and inflammation-mediated reperfusion injury (Zhai et al., 2013). This concept occurs in several organ systems such as the central nervous system, liver, heart, lung, intestine, skeletal muscle and kidney (Eltzschig et al., 2004).

Hepatic I/R injury is a frequent and major complication in clinical practice, which compromise liver function and increases postoperative morbidity, mortality, recovery and overall outcome (Serracino-Inglott et al., 2001). Liver, being an organ with high energy requirements, is highly dependent on oxygen supply and susceptible to hypoxic or anoxic conditions (Teoh et al., 2011).

Extensive researches have investigated the mechanisms responsible for liver damage by I/R. I/R affect liver tissue for the combined alterations occurring during the ischemic period as well as during the reperfusion phase. The lack of oxygen during the ischemic period causes mitochondrial de-energization, ATP depletion and impairment of H^+, Na^+ and $Ca2^+$ homeostasis (Selzner et al., 2007).

Upon oxygen re-admission, the formation of reactive oxygen species (ROS) by uncoupled mitochondria promotes oxidative stress and mitochondrial permeability transition, and results in a decreased capacity to synthesize ATP. These events are responsible for caspase activation, necrosis and apoptosis. Concomitantly, the activation of Kupffer cells releases ROS, nitric oxide (NO) and pro-inflammatory cytokines. The pro-inflammatory cytokines, in concert with the increased expression of adhesion molecules by sinusoidal endothelial cells, promote liver neutrophil infiltration that contributes to the progression of parenchymal injury (Jaeschke et al., 2003; Urakami et al., 2007).

I/R can induce liver dysfunction or failure that is still a significant clinical problem after tissue resection and transplantation surgery. In Europe over 1853 patients are waiting for a liver graft, while only about 1591 liver donors become available per year (European Liver Transplant Registry. 2014: http://www.eltr.org; Euro transplant Annual Report 2013-2014). Such dramatic organ shortage for transplantation, forces consideration of steatotic grafts, however meta-analysis

on total of 1000 patients shows that patients with steatosis have an up to two fold increased risk of postoperative complications, and those with excessive steatosis had an almost three fold increased risk of death (de Meijer et al., 2010).

1.2 NON ALCOHOLIC FATTY LIVER DISEASE (NAFLD)

Non-alcoholic fatty liver disease (NAFLD) is defined when lipids exceed 5% of the total liver weight (Reid et al., 2001). In a subset of the patients, NAFLD further evolves in non-alcoholic steatohepatitis (NASH) characterized by cell death by either apoptosis and necrosis and lobular inflammation with alteration or failure of hepatic functions.

At present, NAFLD/NASH represents the hepatic manifestation of the so called Metabolic Syndrome which is a complex of clinical manifestations associated with obesity and over-weights that includes diabetes, hypertension and hyper triglyceridemia.

1.2.1 PATHOGENESIS AND EPIDEMIOLOGY OF NAFLD

Based on the clinical and experimental data available at the time, the so called 'two hit' model of progressive NAFLD was proposed in 1998. This model considered the development of steatosis to be the 'first hit' increasing the sensitivity of the liver to the putative 'second hits' leading to hepatocyte injury, inflammation and fibrosis. The best candidates for these second hits were considered to be oxidative stress and associated lipid peroxidation and cytokines; principally TNFα. Studies published over the subsequent seven years have led to revisions in this model of pathogenesis, although oxidative stress and cytokines retain a central role. The most important modifications to the model have come from an increased understanding of the sources of oxidative stress and cytokines, in particular the prominent role of insulin resistance, free fatty acids (FFA) and adipose tissue inflammation (Cortez-Pinto et al., 2006). Oxidative stress inhibits the replication of mature hepatocytes which results in expansion of the hepatic progenitor cell (oval cell) population. These cells can differentiate into hepatocyte-like cells, and both oval cell and intermediate hepatocyte-like cell numbers are strongly correlated with fibrosis stage, suggesting that cumulative hepatocyte loss promotes both accumulation of progenitor cells and their differentiation towards hepatocytes (Roskams et al., 2003). In chronic liver injury, the development of fibrosis/cirrhosis is dependent on the efficacy of hepatocyte regeneration, and therefore cell death with impaired proliferation of hepatocyte progenitors represents the proposed third hit in NAFLD pathogenesis (Jou et al., 2008).

The numerous clinical situations that have been associated with NAFLD can be grouped into 6 etiological groups (Bellentani et al., 2010) (Table 1).

TABLE 1: CLASSIFICATION OF DIFFERENT CAUSES ASSOCIATED WITH NAFLD

Genetic and metabolic diseases	Drugs	Environ-mental	Extra hepatic conditions	Nutritional conditions	Infections
Obesity Diabetes mellitus Hyperlipidemia Wilson disease Lipodystrophy Christian disease-Weber Hemochromatosis Storage disease-cholesterol esters	Corticosteroids Estrogens NSAIDs Calcium-antagonists Amiodarone Tamoxifen Tetracycline's Chloroquine Antiretroviral Perhexiline	Environ-mental toxins	Heart failure Inflammatory bowel disease Bacterial overgrowth syndrome Hypothyroidism Polycystic ovary syndrome Pregnancy Neoplastic diseases	Jejunoileal-bypass Total-parenteral nutrition Prolonged-fasting Protein-malnutrition Carbohydrate-diet	Hepatitis B and C HIV infection

NAFLD is common in Europe and now is the most frequent hepatic lesion in Western countries with prevalence rates reported to be anywhere between 2-44% in the general population and 42.6-69.5% in people with type 2 diabetes and rising up to 90% in morbidly obese individuals (Machado et al., 2006). About 15-20 % patients accounts for the pathological evolution form of NASH from the NAFLD with possible progression to cirrhosis or hepatocellular carcinoma. The more severe and clinically significant form of NASH is less common, affecting an estimated 2–3% of the general population and up to 37% of the morbidly obese (Neuschwander-Tetri et al., 2003). The particular concern with significant implications for future disease burden is the increasing prevalence of NAFLD in children and young adults. Studies have reported a 3% prevalence of

NAFLD in the general pediatric population, rising to 53% in obese children (Tominaga et al., 1995; Franzese et al., 1997). Recent surveys show that the prevalence of NAFLD across the Asia-Pacific region is at least 10%, and in some regions as many as one-third of individuals could be affected (Liu et al., 2012; Amarapurkar et al., 2007). Likewise, the reported prevalence of NAFLD ranges from 16% in Mexico, 23% in Italy, 30% in Israel, and 9.3% in Japan, respectively (Lazo et al., 2008). The epidemiological significance of NAFLD streams from the data published by the United States Centre for Disease Control and Prevention that estimates that about 66% of US adults in are overweight and half of those are obese. The prevalence of obesity is projected to increase in the United States up to 45% by 2025. Similarly, by 2030 the projected percent increase in type 2 diabetes mellitus is 32% in Europe, 72% in the United States, and 150% or greater in sub-Saharan Africa, India, and the Middle East.

As obesity and diabetes are important risk factors for NAFLD, it is likely that the prevalence of NAFLD will rise in the near future to epidemic proportions. A recent prospective cohort study using ultrasound and liver biopsy determined the prevalence of NAFLD in asymptomatic middle-aged patients to be 46.0% (Williams et al., 2011). Although hospital-based studies are flawed because of ascertainment bias, population-based studies using non-invasive imaging studies (e.g., sonography) suffer the poor specificity of sonography for the diagnosis of NAFLD. Recently, magnetic resonance imaging has been used to quantify the extension of hepatic steatosis. And using this technique, it is estimated that 31% of the U.S. population has NAFLD. In contrast, depending on the definition used, between 2.8% and 24% of U.S. adults have NAFLD according to a comprehensive National Health and Nutrition Examination Survey III (NHANES III) data set–based analysis.

1.2.2 MECHANISMS OF STEATOSIS

The accumulation of triglycerides (TG) originating from the esterification of free fatty acids (FFAs) and glycerol within the hepatocyte is a key point for NAFLD onset.

The contributing factors for the accumulation of FFAs within the liver include dietary sources, enhanced lipolysis in the adipose tissue, insulin resistance and “de novo” lipogenesis in the liver (Postic et al., 2008).

Available evidence suggests that fatty liver results from derangements in fatty acid metabolism in both the liver and the adipose tissue consequent to insulin resistance (Fabbrini et al., 2010; Sanyal et al., 2005; Tilg et al., 2008). In fact, insulin resistance promotes lipolysis in the adipose tissue

increasing circulating free fatty acid (FFA) levels and affects hepatocyte FFA metabolism (Tilg et al., 2008). Liver FFA influx through the portal circulation along with decreased FFA oxidation and enhanced "de novo" lipogenesis promote triglyceride accumulation within the hepatocytes (Sanyal et al., 2005).

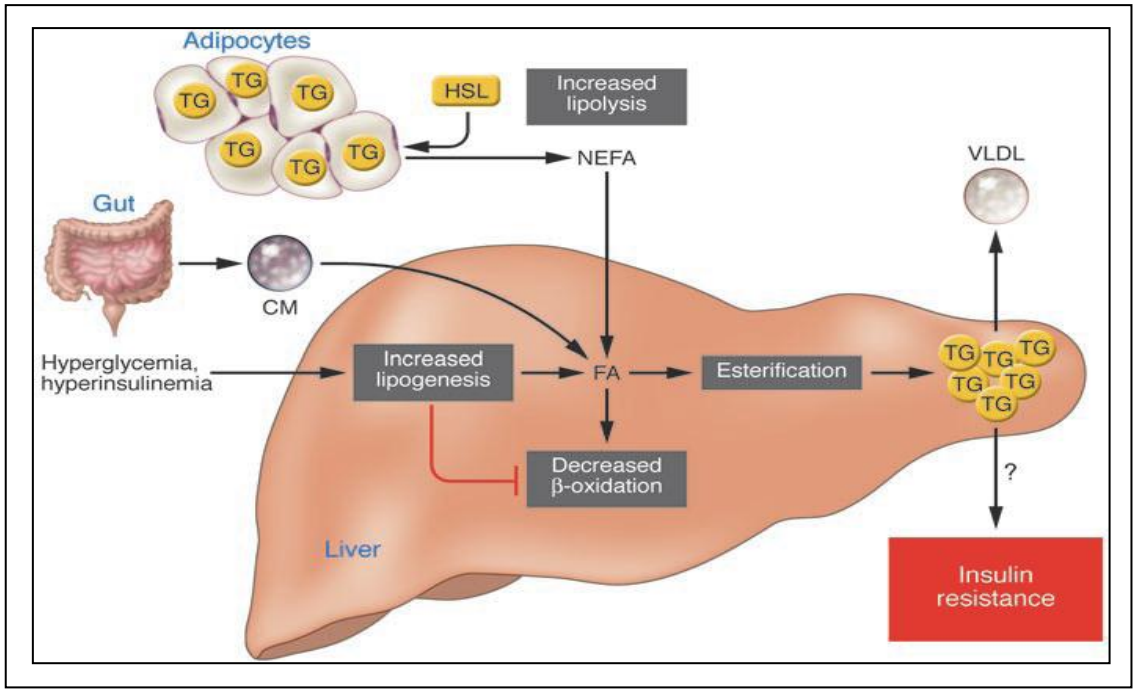

Figure 1: *Schematic mechanisms of metabolic defects leading to the development of hepatic steatosis*

1.3 LIVER TRANSPLANTATION AND MARGINAL LIVER AVAILMENT

The lack of available organs for liver transplantation (LT) associated with the increased death rates among patients on most waiting lists for LT has triggered the use of so-called extended criteria donor (ECD) grafts, previously called ''suboptimal grafts''. Among the wide range of these ECD livers, hepatic steatosis is one of the most frequent disorders (Nocito et al., 2006), which is mostly related to an increasing prevalence of NAFLD. The decision to implant or reject a steatotic liver for LT, however, is difficult, due to a risk of impaired graft function or even failure after implantation. How much and what types of fat represent a significant risk for primary non function (PNF) of the graft remains under debate. Ploeg et al originally suggested a classification of fatty change as mild (<30% of visualized hepatocytes involved), moderate (30% to 60 %), and severe (>60%), a system approximately applied by most transplantation Centre's (Ploeg et al, 1993).

The reserves of transplant steatotic livers are based on the strong association with primary non function (PNF) after a period of cold preservation, initially described by Todo (Todo et al., 1989). However, if a valid and standard method of assessment could be developed, it may be possible to maximize the use of fatty livers while simultaneously minimizing their risk to the recipient. In the early 1990s, four studies examined the relationship of fatty change to PNF. (D'Alessandro et al., 1991; Markin et al., 1993; Ploeg et al., 1993). The largest of these assessed 390 frozen section biopsy specimens and found that 13% of grafts showing greater than 30% steatosis showed PNF compared with 2.5% of non steatotic grafts. Progressive deterioration in graft survival was observed from mild to massive steatosis. Thus, it was concluded that grafts with severe steatosis should be discarded, and those with moderate change should be evaluated in conjunction with other criteria, such as the condition of the recipient and availability of organs at that time. The institution involved, in line with most others worldwide, found no contraindication to transplanting livers with minimal change. This concurs with the findings of Ploeg et al, who found PNF rates as high as 80% in severely steatotic organs, but more worryingly, initial poor function rates as high as 30% in moderately steatotic livers.

1.3.1 STEATOTIC GRAFTS

Steatotic grafts are considered a risk factor for dysfunction or even primary non function of liver transplants; grafts with more than 50% fatty infiltration are routinely discarded. Steatosis is typically characterized quantitatively and qualitatively. The quantitative evaluation is based on the percentage of hepatocytes containing cytoplasmic fat inclusions. In the clinical setting, steatosis is usually reported as mild, moderate or severe if, respectively less than 30%, between 30% and 60%, or more than 60% of hepatocytes contain fat vacuoles within the cytoplasm (Nocito et al., 2006; McCormack et al., 2005;Selzner et al., 2001). In addition, fatty infiltration is divided quantitatively into two categories: microsteatosis and macrosteatosis. In microsteatosis (MiS), the cytoplasm of the hepatocytes contains tiny lipid vesicles without nuclear dislocation. MiS are usually encountered in mitochondrial disruption following acute viral, toxin- or drug-induced injury, sepsis and in some metabolic disorders (Silva et al., 2009). Macrosteatosis (MaS) is characterized by a single, bulky fat vacuole in hepatocytes, displacing the nucleus to the edge of the cell. This type is most commonly associated with obesity, diabetes, and hyperlipidemia and alcohol abuse. The underlying pathogenesis is related to an excessive triglyceride accumulation in the liver, mainly due to an increased uptake of fatty acids released from adipose tissue or

augmented by “de novo” synthesis (Nocito et al., 2006; McCormack et al., 2005; Selzner et al., 2001). Additionally, a defective hepatic export caused by reduced lipoprotein synthesis or impaired β-oxidation of fatty acids, further increases hepatic triglyceride content. Hence the use of grafts with MaS has been associated with increased rates of initial poor function (IPF), primary non function (PNF), and poorer outcome (Marsman et al., 1996). Estimation of steatosis using haematoxylin and eosin (H&E)-stained frozen section liver biopsy has been reported to be difficult and subjective (Franzen et al., 2005; Urena et al., 1999). Even Organ Procurement and Transplantation Network (OPTN) data regarding steatosis are recorded in broad ranges and until recently, did not differentiate between macro vesicular and micro vesicular steatosis (Feng et al., 2006). Therefore the reported variability in both the numbers and grading of steatotic donor livers may reflect differences in both qualitative and quantitative evaluations between different Centers (Selzner et al., 2001; Urena et al., 1998). Some experts believe that physical inspection of an expert in assessing the fat content is equivalent to biopsy (Cameron et al., 2006). However, this has not yet been validated and remains largely subjective. Body mass index (BMI) per se correlates weakly with presence and severity of steatosis (Ryan et al., 2002). Imaging studies alone are not proper tools for the accurate quantification of hepatic fat in all donor candidates (Kim et al., 2006). It has been suggested that differential quantification of color pixels in Oil Red O (ORO) stained liver biopsies using a computer methodology yields more objective and consistent estimation of liver fat content compared with visual interpretation of H&E stained sections (Fiorini et al., 2004), although these computer methods determine the total amount of fat and not the size of the fat droplet (i.e., micro vesicular vs. macro vesicular steatosis). Similarly, the additional negative influence of older donor age and hepatic steatosis has been underlined (De Carlis et al., 1999). A large retrospective single-center study has suggested that recurrent hepatitis C is more common in recipients of moderate and severe steatotic donor livers (Verran et al., 2003). Currently, a macro vesicular fat content of 30% in liver graft, a value with a historical basis resting on early nineties’ observations is widely accepted for transplantation (D’Alessandro et al., 1991). Grafts with moderate MaS (30–60%) may be utilized in the absence of additional risk factors in the donor or recipient livers with more than 60%, MaS should probably be excluded (Burke et al., 2004). There are recommendations to allocate livers of different degrees of steatosis based on the Model for End-Stage Liver Disease (MELD) scores of the candidates; these recommendations are however yet to be verified by multivariate analysis (Briceno et al., 2005).

The Organ Transplant Registry update as of 2014 gives information that in the last five years the number of liver donors has decreased at the same time the waiting list for liver transplantation also increased and this disparity between available organs for transplantation and 12 to 24 months waiting list mortality have forced the clinicians to use marginal livers (Steatotic grafts) and even cadaveric grafts for liver transplantation.

TABLE 2: LIVER DONORS

Liver donors	2010	2011	2012	2013	2014	2013/2014
0-15	66	59	54	53	55	3.8 %
16-55	915	902	838	811	832	2.6 %
56-64	316	318	320	303	335	10.6 %
65+	437	448	430	348	369	6.0 %
Total	1734	1727	1642	1515	1591	5.0 %

TABLE 3 WAITING LISTS

Liver	liver	2588	2530	2327	2041	1853	-3.6%
	liver + kidney	90	72	67	57	55	7.8%
	liver + heart	2	3	2	1	0	-100.0%
	liver + heart + kidney	1	0	0	0	0	0.0%
	liver + heart + lung	0	1	0	0	0	0.0%
	liver + heart + pancreas	1	0	0	0	0	0.0%
	liver + lung	5	1	3	5	6	20.0%
	liver + pancreas	6	6	6	6	3	-50.0%
	liver + pancreas + kidney	2	1	1	1	1	0.0%
Liver	Total	2695	2614	2406	2111	1918	-3.5 %

1.4 ISCHEMIA-REPERFUSION (I/R) INJURY IN STEATOTIC LIVER

Hepatic I/R injury can be categorized into warm and cold ischemia. Warm ischemia occurs in the setting of transplantation, trauma, shock and selective liver surgery, in which hepatic blood supply is temporarily interrupted. It may also occur in some types of toxic liver injury, sinusoidal obstruction and Budd-Chiari syndrome (Fernandez et al., 2012). Cold storage ischemia occurs during organ preservation before transplantation (Nickkholgh et al., 2008; Kupiec-Weglinski et

al., 2005). Mitochondrial dysfunction has been reported after prolonged cold ischemia in steatotic livers (Caraceni et al., 2004) and also low ATP levels in steatotic livers after transplantation (Jimenez-Castro et al., 2011).

Steatotic livers have been reported to be more susceptible to cold ischemia injury (Schemmer et al., 1999; Fukumori et al.,1999) and moderate to severe MaS steatosis has been observed as the leading cause of severe liver preservation injury (Briceno et al., 2005). In one experience with MaS steatotic livers, every additional hour of total ischemia time longer than 10 hours significantly increased the relative risk of graft and patient loss (Salizzoni et al., 2003). This highlights the difficult issue of acceptance steatotic livers previously evaluated and refused by other Centers, as in these cases ischemia times were always much longer.

Steatotic livers have been shown to be more susceptible to IR injury also after transplantation. During cold ischemia, structural changes attributable to the disruption of hepatic microcirculation caused by fat droplets and hepatocellular swelling, results in occlusion of the sinusoids. After reperfusion, loss of viable endothelial cells and activation of Kupffer cells are accentuated over the non steatotic graft. The migration of leukocytes and adherence to the vascular endothelium is an early and key step in I/R injury and is mediated by three classes of adhesion molecules: selectins, integrin, and immune globulins.

The major event of re-perfusion injury in steatotic livers are due to the abnormal accumulation of fat within the cytoplasm of hepatocytes, resulting in increased hepatocellular volume and narrowing of sinusoid, compromising the suitable graft revascularization and viability after transplantation. Moreover, several evidences indicated that an increased sensitivity of fatty hepatocytes to the harmful effects of reactive oxygen species (ROS) plays a pathogenic role in this event (Domenicali et al., 2005). Where Selzner et al showed the increased susceptibility of fatty livers to reperfusion injury is associated with a change in hepatocytes death form, where the lean rat liver had a prevalence of apoptosis death, while steatotic liver had more massive necrosis present after an ischemic insult (Selzner et al., 2000). After major liver resection, steatosis is associated with mortality higher than 14% respect to the 2% using normal liver. Several hypotheses have been suggested to explain the decreased tolerance of steatotic liver to I/R injury compared with normal livers. These include increased lipid peroxidation, neutrophil infiltration, and release of pro-inflammatory mediators and the alteration of micro circulation (Serafin et al., 2002). The end result is multiple alterations in the steatotic liver finally rendering it more susceptible to I/R. The donor medical history factors may enhance pre preservation injury. They

include a history of donor alcohol or drug abuse, presence of a fatty liver, cardiovascular instability after brain death, hypotension during the donor operation, and surgical trauma at the time of harvest.

IR injury is the underpinning of graft dysfunction that is seen in the marginal organ. On restoring the blood supply, the liver is subjected to insult, aggravating injury already caused by the initial ischemia (Clavien et al., 1992; Serracino et al., 2001). I/R injury to endothelial cells disrupts the sinusoidal microcirculation by up-regulating the attraction, activation, adhesion, and migration of neutrophils (polymorph nuclear cells [PMN]) causing local tissue destruction by release of proteases and oxygen-free radicals. I/R in liver transplantation lead to PNF/IPF and increased rejection, and contribute to high morbidity. Preservation injury in liver allografts occurs at four stages: (1) pre preservation injury, (2) cold preservation, (3) rewarming, and (4) reperfusion injury. Cold preservation is also associated with injurious effects. The Kupffer cells, endothelial cells, and Ito cells are more susceptible to cold IR injury as compared with hepatocytes. Sinusoidal endothelial cells undergo apoptosis and coagulated necrosis after cold storage followed by reperfusion of liver grafts (Gao et al., 1998). The sinusoidal cell lining, which is most sensitive to cold ischemia, becomes deficient, exposing the hepatocyte microvilli (Clavien et al., 1998). White blood cells attach where the sinusoidal cells vacated and obstruct the sinusoids and liver blood flow. Additionally, PMNs release numerous mediators, amplifying the inflammatory response (Engler et al., 1983; Varani et al., 1989, Cywes et al., 1993). Platelets, which adhere to the sinusoids, almost immediately on reperfusion aggravate the degree of preservation injury via a mechanism of procoagulant activity and cytokine release, which results in hepatocyte hypoxia (Cywes et al., 1993). Additionally, on reperfusion, Kupffer cells become activated, generating inflammatory mediators such as cytokines and oxygen-derived free radicals, which are injurious to endothelial cells and hepatocytes. The energy stores of the liver (e.g., ATP, glycogen) are depleted, severely compromising hepatocyte function (Lemasters et al., 1995; Kukan et al., 2001). Furthermore, morphologic changes to the endothelial cells are observed, resulting in an endothelin/ nitric oxide imbalance during the reperfusion period, which has been correlated with decreased liver blood flow (Serracino et al., 2001; Clavien et al., 1998; Chazouilleres et al., 1993).

1.5 STRATEGIES OF PROTECTION

1.5.1 ISCHEMIC PRECONDITIONING (IP)

The term ischemic preconditioning (IP) refers to the increase in tissue tolerance to ischemia/reperfusion (I/R) damage that can be induced by the pre-exposure to brief periods of ischemia followed by re-oxygenation (Yellon et al., 2005). This phenomenon was first described by Murry in the myocardium (Murry et al., 1986), but was subsequently observed in many other tissues (Yellon et al, 2005). In liver, studies in rodents have shown that 10 min interruption of blood supply followed by 10 min reperfusion reduces hepatic injury and inflammation during a subsequent extended period of I/R. Recent studies have shown that the effects of IP can be observed also by the application of a brief ischemia during the reperfusion period. This phenomenon, known as post-conditioning, demonstrates that the mechanisms of hepatoprotection induced by ischemic preconditioning may also act when hepatic damage has already started and suggests the possibility to activate these mechanisms also in case of liver damage different from I/R when surgical application of IP is not practicable.

The protection induced by IP 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 is known as delayed or late preconditioning; it starts 12-24 hours after the pre-conditioning stimulus, can last up to 3-4 days, and is characterized by gene transcription and "de novo" protein synthesis (Peralta et al., 1997). Despite these differences, both phases of preconditioning can be initiated by the same stimuli and partially share the same intracellular signal pathways.

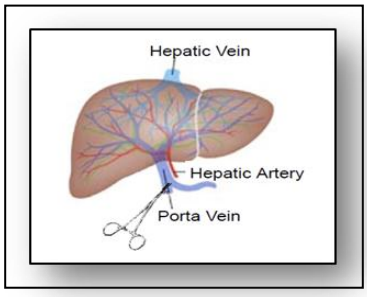

Figure 2: *Liver preconditioning with a brief cycle of ischemia-reperfusion (I/R) (10 minutes of ischemia + 10 minutes of reperfusion) prevents hepatocyte cell death induced by a subsequent prolonged ischemia*.

"In vivo" and "in vitro" studies have clearly established that the onset of IP is triggered by the production of adenosine and by the subsequent stimulation of adenosine A2a receptor (Peralta et al., 1997; Nakayama et al., 1999; Hart et al., 2008; Carini et al., 2000; Carini et al., 2001). This was confirmed in our Laboratory with experiments using primary rat hepatocytes pre-conditioned with 10 minutes of hypoxia plus 10 minutes of re-oxygenation. In this model, the released adenosine to extra-cellular space induced hepatocyte protection by the autocrine stimulation of A2a receptors.

Surgical ischemic preconditioning raised hopes that it could be applied to patients to prevent the side-effect of major liver surgery, but the first application of IP in clinical trials have given conflicting results and in some cases IP did not afford protection and in some cases its protective effects were extremely variable. These contrasting outcomes of the clinical studies, the different protocols of IP application in humans, and the variable clinical settings have not allowed a definitive demonstration of the benefit of the clinical application of IP (Amador et al., 2007; Azoulay et al., 2005; Cescon et al., 2006; Koneru et al., 2007; Franchello et al., 2009; Jassem et al., 2006). Hence this observation has inhibited now, the routine use of IP in human liver surgery and has indicated the need of more efficient approaches to activate IP in patients.

In this regard, the pharmacological induction of liver preconditioning by targeted activation of one or more of the critical molecular mediators of IP may represent a more reliable technique to activate the intrinsic system of hepatoprotection in patients.

1.5.2 ADENOSINE AND ADENOSINE RECEPTORS

Adenosine is an endogenous purine nucleoside that modulates many physiological processes. Extracellular adenosine concentrations in normal cells are approximately 300 nM but these concentrations are elevated quickly during tissue damage and inflammatory reactions.

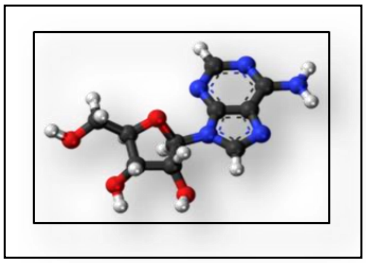

Figure 3: *Adenosine molecular structure.*

The released adenosine interacts with different subtype of adenosine receptors that modulate cell protection, inflammation and immunological responses. There are four kinds of adenosine receptors (ARs) A1, A2A, A2B and A3 that are of purinergic class and G protein coupled receptor. ARs have traditionally been classified based on their differential coupling to adenylyl cyclase to regulate cyclic AMP levels. The A1 and A3aRs are coupled to Gi proteins, while A2aAR and A2b AR are coupled to Gs proteins (Fredholm et al., 2011). Therefore, activation of the A2A and A2B ARs increase cyclic AMP production, resulting in activation of protein kinase A (PKA) and phosphorylation of the cyclic AMP response element binding protein (CREB). In contrast, activation of the A1 and A3AR inhibits cyclic AMP production and decreases PKA activity and CREB phosphorylation (Cunha et al., 2001; Fredholm et al., 2011; Paes-De-Carvalho et al., 2002).

The animal studies have, all together, shown that a brief ischemic stress induces a profound phenotypic modification that makes liver cells resistant to damage, and inhibits hepatic inflammatory reactions. Despite the nearly 25 year's research on liver IP, the knowledge of the proteomic modifications responsible for its production is still poor. To date a well-established notion is the role of the adenosine A2a receptor (A2aR) as a first inductor of rodent liver preconditioning. Since from the early study of Peralta et al (Peralta et al., 1997), a number of

studies "*in vivo*" and "*in vitro*" have in fact clearly established that the brief ischemic stress triggers the onset of IP by inducing the release of adenosine in the extracellular space with subsequent stimulation of the A2aR of liver cells. By using primary rat hepatocytes preconditioned with a brief hypoxia-reoxygenation in the last years we have begun to analyse the signal network that following A2aR stimulation induces hepatocyte resistance hypoxic damage (Figure 4). This network involves Gs protein, adenylate cyclase and protein kinase A (PKA) that phosphorylates ADR2A and shifts its coupling to Gi protein and Src kinase and activates phosphatidyl-inositole-3-kinase (PI3K) and its downstream effector Akt. This allows the stimulation of phospholipase C, the recruitment of the specific isoforms δ and ε of protein kinase C (PKC) and the activation of p38 MAPK. Full activation of preconditioning responses also needs an A2R-induced down modulation of inhibitory enzymes of PKC and PI3K. Hypoxic preconditioning as well as A2aR stimulation, in fact, induces a RhoA-GTPase-dependent inhibition of the diacylglycerol kinases θ thus increasing diacylglycerol (DAG) and sustaining activation of the DAG-dependent PKC δ and ε. A2aR also induces the degradation of the PI3K inhibitor, phosphatase and tensin homologue deleted from chromosome 10 (PTEN) by an NADPH oxidase-dependent mechanism, allowing the maintenance of the PI3K-dependent signals (Alchera et al., 2010). These observations represent the first data on the modulation of constitutive hepatocyte signal proteins upon hypoxic preconditioning and their role in hepatocyte resistance to hypoxia. The knowledge on the induction of gene expression and protein synthesis in preconditioned liver is even lower. "In vivo" studies have shown that liver IP is associated with a stimulation of nuclear factor-κB (NF-κB) activity in the ischemic phase and its inhibition during hepatic reperfusion. The pro regenerative and protective effect of liver preconditioning are instead associated to the activation of the signal transducer and activator of transcription (STAT)/IL6 axis. Hypoxia-inducible factor 1 (HIF-1) is the main regulator of tissue adaptation to oxygen deprivation and it is found increased in human transplanted livers exposed to IP. Consistently the delayed protective effect of hepatocyte preconditioning is related to an A2aR/PKC/PI3K-dependent non hypoxic HIF-1 activation and to the consequent production of its target protein, carbonic anhydrase IX (Alchera et al., 2015).

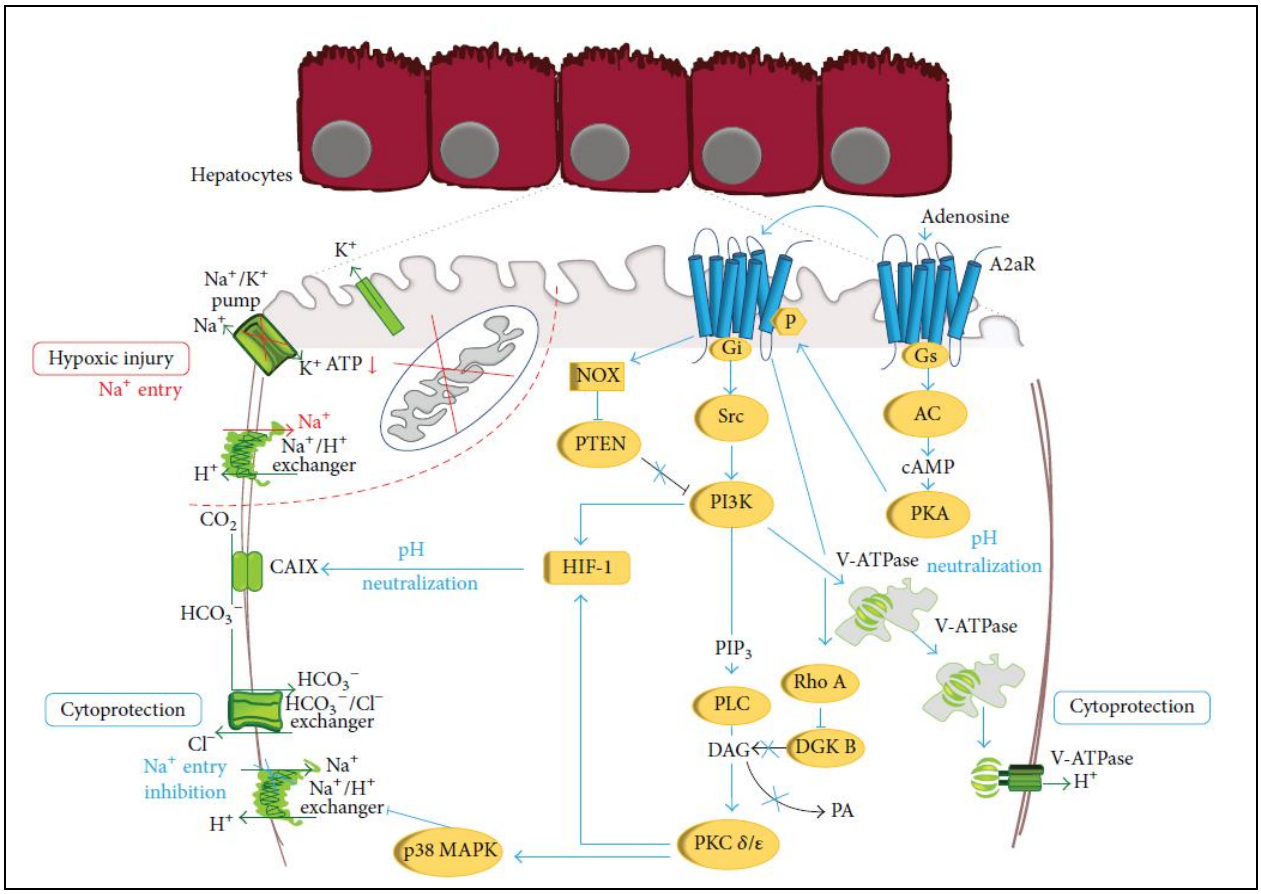

Figure 4: *Molecular mechanisms involved in hypoxic injury of primary rat hepatocytes and their protection upon A2aR stimulation. Hypoxic damage: ATP depletion causes intracellular acidosis, inhibition of the Na^+/K^+ ATPase, and activation of the Na^+/H^+ exchanger with an increase in intracellular Na^+ content and activation of the K^+ channel. For A2aR protection: A2aR stimulation induces the sequential activation of PKA, Gs and Gi protein, Src, PI3K, PLC, PKC δ, and ε and p38 MAPK. A2aR also inhibits the negative regulators of PKC and PI3K, DGK, and PTEN. PI3K activates V-ATPase that maintains intracellular pH avoiding the activation of the Na^+/H^+ exchanger and Na^+ overload. PI3K and PKC δ and ε activate HIF with production of CAIX. CAIX converts CO_2 into bicarbonate that enters into hepatocyte through the $Cl^-/HCO3^-$ exchanger. This neutralizes intracellular pH without activation of the Na^+/H^+ exchanger and the consequent Na^+ increase. (Alchera et al. Biomed Res Int. 2015)*

The cytoprotective effects of adenosine during preconditioning are only part of the effects of adenosine. A separate bulk of researches have indeed demonstrated that adenosine dramatically increases at extracellular levels during tissue damage, ischemia and inflammation and by interacting with one or more of the four adenosine receptors (A1, A2a, A2b, A3), elicits autocrine and paracrine modulation not only of cell survival but also of inflammatory and immunological reactions (Fredholm et al., 2007; Hasko et al., 2008). Several studies show that adenosine may play pro-inflammatory or anti-inflammatory role depending on the type of adenosine receptor is engaged. Interestingly, some findings indicate that the different adenosine receptors might have dissimilar or even opposite effects. Well characterized is the pro-inflammatory activity of A1 and A2b receptor and in contrast, the immune suppressive action of the A2a receptor. A1R exerts a pro-inflammatory response by enhancing phagocytosis (Salmon et al., 1993), promoting chemotaxis (Schnurr et al., 2004; Rose et al., 1988) and enhancing neutrophils adherence to endothelium during inflammatory process (Cronstein et al., 1992). By contrast A2aR have a major role in suppressing immune response. Engagement of A2aR inhibits neutrophils adherence to endothelium during inflammation (McColl et al., 2006) and inhibits the activation of neutrophils, monocytes platelets and T-cells (Sullivan et al., 2001; Cooper et al., 1995; Koshiba et al., 1997). In animal models, A2aR-agonists can prevent lethal response to bacterial LPS and sepsis (Sullivan et al., 2004; Mazar et al., 2005). In macrophages, A2aR mediate inhibition of TNF-alpha and augment IL-10 production (Haskò et al., 2000; Ryzhov et al., 2008; Nemeth et al., 2005).

The adenosine receptors have contradictory effects on liver steatosis and lipotoxicity. In A1 KO mice ethanol-induced hepatic steatosis is reduced compared to WT mice, indicating a pro-steatotic action of A1 (Peng et al., 2009). On the other hand, recent studies in our laboratory have shown the protective effect of A2aR stimulation in lipoapoptotic liver presence (Imarisio et al., 2012).

As described above, adenosine and ARs play a dynamic role in regulating normal cell physiology and also act as modulators in disease processes. A better understanding of the functions of these receptors, especially the newly identified receptor homomers and heteromers, could stimulate development of new therapies for the treatment of diseases.

1.6 CYTOTOXICITY SIGNAL MEDIATORS

1.6.1 ENDOPLASMIC RETICULUM (ER) AND ER STRESS

The endoplasmic reticulum (ER) is a central organelle of each eukaryotic cell responsible for protein folding, maturation, quality control and trafficking. Proteins of the plasma membrane, secreted proteins as well as proteins of the Golgi apparatus and lysosomes fold into their tertiary and quaternary structure in the ER. The ER is the major signal transducing organelle that senses and responds to changes of the homeostasis (Voeltz et al., 2002). Conditions interfering with the function of ER are collectively called ER stress. ER stress is induced by accumulation of unfolded protein aggregates (unfolded protein response, UPR) or by excessive protein traffic usually caused by viral infection (ER overload response, EOR). In eukaryotic cells, UPR -caused by formation of unfolded protein aggregates -uses an evolutionarily conserved signaling pathway during which the signal of unfolded proteins activates a set of ER-located sensors (Zhang et al., 2011).

The adaptive UPR comprises signal transduction pathways initiated by ER proximal UPR transmembrane proteins: inositol-requiring kinase 1 (IRE1α), activating transcription factor 6 (ATF6), and double-stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK) in an attempt to restore homeostasis and normal ER functions (Schroder et al., 2005). These UPR transducer proteins are negatively regulated by the chaperone GRP78/BIP (immunoglobulin heavy chain binding protein) in unstressed or healthy ER at their luminal ends (amino terminal), however, increase in unfolded proteins causes dissociation of 78-kD glucose-regulated/binding immunoglobulin protein Grp78/BIP thereby releasing the inhibition and thus eliciting the response to stimuli that divert ER chaperones to misfolded proteins, IRE1, PERK and ATF6 initiate signal transduction processes. These events promote the expression of genes required to fold newly synthesized proteins and to degrade the unfolded proteins. Moreover, homeostasis and normal ER function are restored. However, when injury is excessive, the same ER stress signal in response transduction pathways can also induce cell death (Bertolotti et al., 2000; Pfaffenbach et al., 2011) (Figure 5).

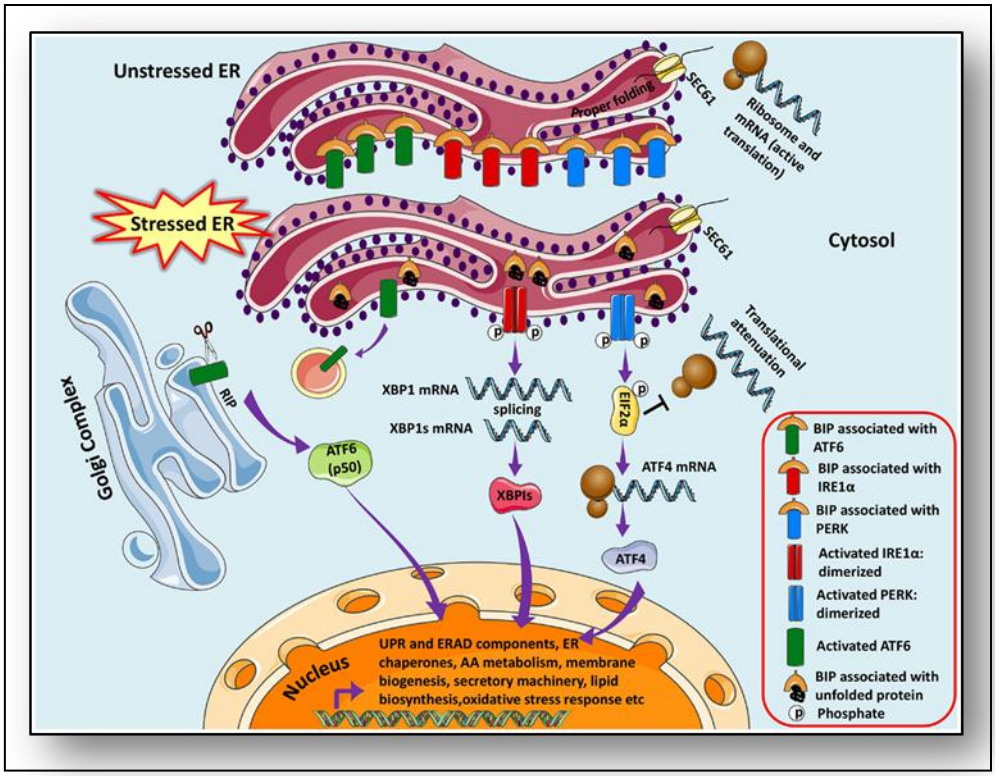

Figure 5: *Unfolded protein response pathways*.

It has been demonstrated that the UPR is a fundamental intracellular signal transduction response that is critical for health and disease. ER stress and other cellular stress responses, such as inflammation and oxidative stress, are integrated in many pathophysiological processes. The ER stress response has recently been recognized in a wide spectrum of experimental models of liver injury, which is an emerging field of interest in the pathogenesis of nearly all types of human liver disease. In the liver, hepatocytes, similar to other secretory cells, are rich in ER. Due to its high capacity for protein synthesis, the UPR/ER stress response plays important roles in both preventing and mediating pathological changes in various liver diseases (Zhang et al., 2014).

NAFLD implies that the ability to resolve ER stress has been compromised. Particularly, recent research demonstrated that ER stress and the UPR signaling are critically involved in the initiation and progression of nonalcoholic fatty liver disease (NAFLD). Under metabolic stress conditions, the UPR regulates transcriptional and translational programs that are associated with hepatic steatosis and inflammation; the major characteristics of NAFLD (Pagliassotti et al., 2012).Toxic lipids such as free fatty acids, diacylglyceride, phospholipids and free cholesterol activate several cellular stress pathways. The maintenance of ER function requires high concentrations of intra-ER

Ca^{2+}, which is actively controlled by sarcoplasmic (endo) reticulum Ca^{2+}-ATPase (SERCA). Free cholesterol accumulation triggers ER stress by altering the critical free cholesterol-to-phospholipid ratio of the ER membrane, which is needed to maintain its fluidity. Among the ER enzymes, SERCA ATPase is particularly sensitive to ER membrane cholesterol contents that can inhibit SERCA conformational changes and activity. Such changes induce a decrease in physiologically high intra-ER Ca^{2+}.

The presence of ER stress and activation of the UPR in chronic diseases such as obesity is one of the most important factors for disease progression in NASH along with hepatocyte apoptosis and hepatic stellate cell (HSC) or Kupffer cell activation.

Moreover, in liver cells, ER response is involved in hepatic ischemia-reperfusion injury (I/R) that promotes protein unfolding and hence triggering as documented by the activation of XBP1 and ATF6 in the parenchyma of livers (Brenner et al., 2013). Accumulating evidence suggests perturbations at the ER as a novel sub cellular effector, possibly involved in promotion of cell death in various pathologies including the pathophysiology of organ preservation.

1.6.2 TUMOR NECROSIS FACTOR RECEPTOR (TNF-R) ADAPTOR FACTOR 2 - TRAF2

The tumor necrosis factor (TNF) receptor adaptor factor (TRAF) family of proteins plays a pivotal role in different biological processes, including immunity, inflammation and apoptosis. The mammalian TRAF family comprises seven members: TRAF1 though TRAF7. Among these, TRAF2 and TRAF6 have been most extensively studied. In particular, TRAF2 associates, directly or indirectly, with members of the TNF receptor (TNFR) super family, including TNFR1 and TNFR2, RANK (a receptor that mediates differentiation and maturation of osteoclasts), and CD40 (a receptor important for the proliferation and activation of B cells). TRAF2 play a central role in the cellular response to stress and cytokines via their regulation of stress kinases, resulting in the activation of key transcription factors, including NF-κB, c-Jun and ATF2 (Xia et al., 2005). The function of TRAF2 and TRAF5 is best characterized in TNFR1 signaling, whereas TRAF6 and TRAF3 have been extensively studied in IL-1R or TLR signaling, TRAF3 has been demonstrated to be critical for virus-induced activation of IRF3-IRF7 and interferon production.

It has been known that ER stress can lead to altered lipid metabolism and hepatic steatosis. In particular, the IRE1α-XBP1 pathway has been reported to be required for the maintenance of

hepatic lipid homeostasis under ER stress conditions (Zhang et al., 2014). The association of ER stresses signaling and hepatic steatosis has been proven through the IRE1α/XBP1 pathway and the ER protein translocation pathway. When IRE1α is activated induces the unconventional splicing of the mRNA encoding X-box-binding protein 1 (XBP-1). The cytosolic domain of activated IRE1 binds TRAF2 and triggers the activation of the c-Jun N-terminal kinase (JNK), MAPK p38 and caspase-12. Fumihiko et al demonstrated that stress-induced oligomerization and activation of IRE1 could lead to clustering of TRAF2 that is bound to the COOH-terminal cytoplasmic portion of the IRE1, one of the ER transmembrane proteins involved in initiating signals from the ER. (Urano et al., 2000).

Mediating cellular response to ER stress has been proposed based upon the observation that ectopic expression of a domain negative mutant of TRAF2 lacking the N terminus Ring finger domain blocks ER stress-induced NF-κB and JNK/SAPK activation, and the mouse embryonic fibroblast derived from TRAF2 knock-out mice failed to activate NF-κB following ER stress (Mauro et al., 2006). TRAF2 is not only investigated in a contest of steatotic liver injury. In literature is not well know the exact relationship of ER stress-mediated cell death and cold I/R injury in liver transplantation, but results presented implicate ER stress on a broad scale as an important factor in this injury (Mosbah et al., 2012), shown that activation of the IRE-1 pathway regulates pro-apoptotic responses by activation of stress kinase JNK and mitogen-activated protein kinases. Finally, it has been demonstrated that the use of specific inhibitors of ER stress could represent effective strategies to reduce hepatic I/R injury (Peralta et al., 2010).

1.6.3 APOPTOSIS SIGNAL-REGULATING KINASE 1 (ASK1)

All living organisms are exposed to numerous physicochemical stressors during their lifetime and appropriate responses to stress at the cellular level are essential for the maintenance of homeostasis. The mitogen-activated protein kinase (MAPK) cascades are thought to be crucial among the major signaling pathways that regulate cellular stress responses. Apoptosis signal-regulating kinase 1 (ASK1) is a member of the mitogen activated protein kinase kinase kinase (MAP3K) family that activates downstream MAP kinases (MAPKs). MAPKs control a wide variety of cellular functions, including proliferation, differentiation, migration and apoptosis (Hattori et al., 2009).

ASK1 is activated in response to various stresses, such as oxidative stress, ER stress, calcium overload and inflammatory signals including those induced by tumor necrosis factor α (TNFα) lipopolysaccharide (LPS), and Ang II (Fig.6) (Hayakawa et al.,2012). Activated ASK1 in turn activates the downstream p38 and JNK pathways and induces various cellular responses, including cell death, inflammation, differentiation and survival ASK1 is involved in the IRE1α pathway, one of the adaptive ER proximal UPR transmembrane proteins. IRE1a collaborates with adaptor-like tumor necrosis factor receptor (TNFR)-associated factor2 (TRAF2) and recruits ASK1 that has been shown to relay various stress signals to the downstream activating among others Jun N-terminal kinase (JNK) and p38 MAP kinase (Derijard et al., 1995; Nishitoh et al., 2002).

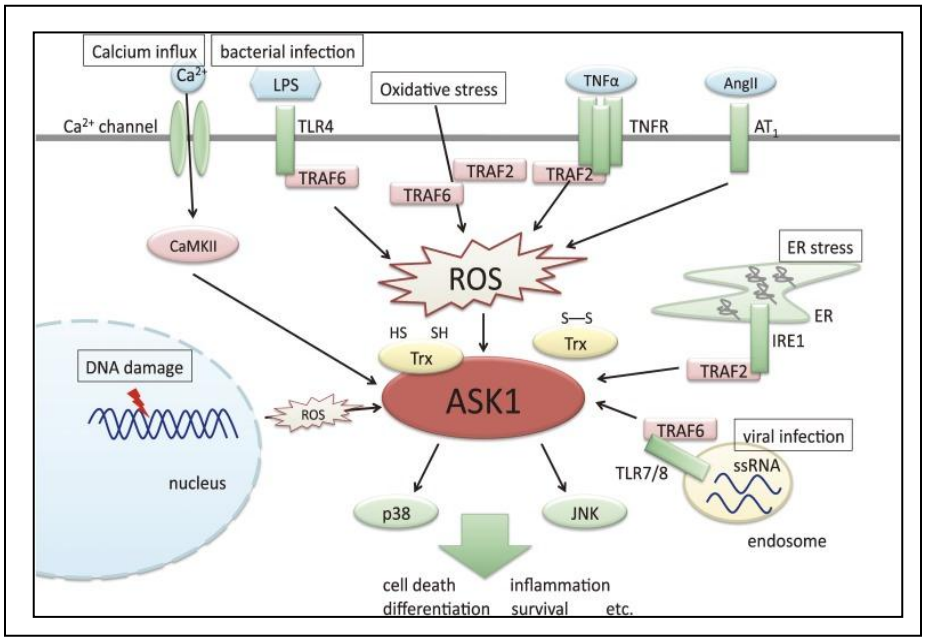

Figure 6*: Overview of the functions of ASK1.*

Unfolded/misfolded/mutated proteins (Hetz et al., 2006), disturbance in cellular redox regulation, endogenous reactive oxygen species (ROS) production (Fedoroff et al., 2006) and hypoxia (Sawada et al., 2008) act as stress signals altering ER homeostasis, if the stress signal is severe and/or prolonged, ER triggers cell death pathways (Szegezdi et al., 2006; Kim et al., 2008; Cheng et al., 2011; Benbrook et al., 2012).

ASK1 is highly conserved among eukaryotes and its activation mechanism appears to be common among the ASK family of proteins, i.e., ASK1, ASK2, NSY-1, and DASK1. ASK1 is a serine/threonine protein kinase activated by phosphorylation of a threonine residue (Thr838 in

human ASK1) within the activation loop. In the past decade, various regulatory mechanisms of ASK1 have been elucidated. It has been reported that ASK1 activity is regulated by many ASK1-interacting proteins, among which thioredoxin (Trx) plays an important role. Trx is a redox protein that changes its structure depending on the cellular redox state. Only the reduced form of Trx binds to the N-terminus of ASK1 and inhibits ASK1 activity by inhibiting homophilic interaction through the N-terminal coiled-coil domains in the pre-existent ASK1 oligomer under unstimulated conditions (Hattori et al., 2009).

Upon ROS stimulation, the ASK1 signalosome unbinds from Trx and forms a fully activated higher-molecular-mass complex, in part by recruitment of tumor necrosis factor receptor-associated factor 2 (TRAF2) and TRAF6. However, the precise mechanisms by which Trx inhibits and TRAF2 and TRAF6 activate ASK1 have not been elucidated fully (Fujino et al., 2007; Hattori et al., 2009). ASK1 activation in response to TNF and LPS signaling has been reported to depend on ROS generation, suggesting that ROS play a key role in the regulation of ASK1 activity (Hattori et al., 2009). Investigations will be necessary to determine whether simple steatosis and progression from isolated fatty liver to NASH be preceded by higher ROS generation (Nassir et al., 2014). Kim et al demonstrated that ASK1 is a substrate for phosphorylation by Akt and that this phosphorylation in a consensus sequence at Ser83 level is associated with a decrease in stimulated ASK1 kinase activity. So, the negative regulation of ASK1 activity and consequent activation of downstream signaling molecules can be negatively regulated by Akt stimulation. This regulatory event has measurable consequences for ASK1 downstream signaling, including apoptosis induced by ASK1. ASK1 may be a physiological target of Akt and raise the intriguing possibility that the ability of Akt to inhibit stress-activated kinases in specific cell contexts is a consequence of this interaction (Kim et al., 2001).

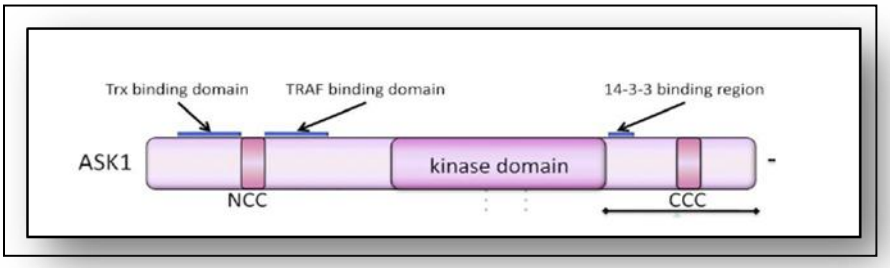

Figure 7: The domain structures of ASK1. *The binding domains of Trx and TRAF exist in N terminus of ASK1. Two coiled coil domains (NCC and CCC) are important for the homomeric interaction and activation of ASK1.*

1.6.4 C-JUN N-TERMINAL KINASES –JNK

C-Jun N-terminal kinases (JNKs), were originally identified as Kinases that bind and phosphorylate c-Jun on Ser 63 and Ser-73 within its transcriptional activation domain. They belong to the mitogen activated protein kinase family, and are responsive to stress stimuli, such as cytokines, ultraviolet irradiation, heat shock, and osmotic shock. They also play a role in T cell differentiation and the cellular apoptosis pathway. Activation occurs through a dual phosphorylation of threonine (Thr) and tyrosine (Tyr) residues within a Thr-Pro-Tyr motif located in kinase subdomain VIII. Activation is carried out by two MAP kinases, MKK4 and MKK4 and JNK can be inactivated by Ser/Thr and Tyr protein phosphatases (Tony et al., 1998).

The c-Jun N-terminal kinases consist of ten isoforms derived from three genes: JNK1 (four isoforms), JNK2 (four isoforms) and JNK3 (two isoforms) (Waetzig et al., 2005). Each gene is expressed as either 46 kDa or 55 kDa protein kinases, depending upon how the 3' coding region of the corresponding mRNA is processed. There have been no functional differences documented between the 46 kDa and the 55 kDa isoform, however, a second form of alternative splicing occurs within transcripts of JNK1 and JNK2, yielding JNK1-α, JNK2-α and JNK1-β and JNK2-β. Differences in interactions with protein substrates arise because of the mutually exclusive utilization of two exons within the kinase domain (Tony et al., 1998). C-Jun N-terminal kinase isoforms have the following tissue distribution:

JNK1 and JNK2 are found in all cells and tissues; JNK3 is found mainly in the brain, but is also found in the heart and the testes (Bode et al., 2007).

Inflammatory signals, changes in levels of reactive oxygen species, ultraviolet radiation, protein synthesis inhibitors, and a variety of stress stimuli can activate JNK. One way this activation may occur is through disruption of the conformation of sensitive protein phosphatase enzymes; specific phosphatases normally inhibit the activity of JNK itself and the activity of proteins linked to JNK activation (Vlahopoulos et al., 2004). JNKs can associate with scaffold proteins JNK interacting proteins as well as their upstream kinases JNKK1 and JNKK2 follows their activation. JNK, by phosphorylation, modifies the activity of numerous proteins that reside at the mitochondria or act in the nucleus. Downstream molecules that are activated by JNK include c-JUN ATF2, ELK1, SMAD4, p53 and HSF1.The downstream molecules that are inhibited by JNK activation include NFAT4, NFATC1 and STAT3. By activating and inhibiting other small molecules in this way,

JNK activity regulates several important cellular functions including cell growth, differentiation, survival and apoptosis.

1.7 CELL SURVIVAL SIGNAL MEDIATOR

1.7.1 PHOSPHOINOSITIDE 3-KINASE (PI3K) AND PROTEIN KINASE B (PKB)-AKT AXIS

Phosphoinositide 3-kinases (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 (Cantley et al., 2002). 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 (Webster et al., 2001; Hateno et al, 2002; Muller et al 2003). Consistently, recent studies have shown that ischemic preconditioning activates PI3K signaling in rodent livers, while the block of this kinase abolishes the protective action of preconditioning both in isolated hepatocytes and in the whole organs (Izuishi et al., 2006; Carini et al., 2004).

By using primary rat hepatocytes preconditioned with a brief hypoxia-reoxygenation in the last years we have begun to analyze the signal network that following A2aR stimulation induces hepatocyte resistance to hypoxia. This network involves Gs protein, adenylate cyclase and protein kinase A (PKA) that phosphorylates A2AR and shifts its coupling to Gi protein and Src kinase and activates PI3K and its downstream effector Akt (Alchera et al., 2010)

The lipid product of PI3K, PIP3, facilitates phosphorylation of Akt, also known as Protein kinase B (PKB) by PDK1. This phosphorylation stimulates the catalytic activity of Akt, resulting in the phosphorylation of a host of other proteins that affect cell growth, cell cycle entry, and cell survival (Cantley et al., 2002).

Akt, is a Serine /threonine- specific protein kinase that. It is one of the key molecules downstream of the phosphoinositide 3-kinase (PI3K) signaling pathway. In mammals, Akt comprises of three highly homologous members, including Akt1 (PKBα), Akt2 (PKBβ) and Akt3 (PKBU), which are encoded by three different genes located on different chromosomes (Dillon et al., 2010; Nicholson et al., 2002; Hanada et al., 2004, Schultze et al., 2011). The Akt kinases control an array of diverse functions including cell growth, survival, proliferation and metabolism (Gonzalez et al., 2009).

Akt1 and Akt2 are widely expressed, whereas Akt3 expression is restricted to brain, testis, lung, fat, mammary glands and pancreatic islets (Schultze et al., 2011). In the liver, only Akt1 and Akt2 are expressed, with Akt2 as the major isoform (accounting for approximately 70% of total Akt protein) (Schultze et al., 2011). Several growth factors and cytokines are known to confer resistance to Fas-induced liver injury by activation of the Akt pathway (Schulze et al., 2004, Moumen et al., 2007). Akt1 is involved in cellular survival pathways, by inhibiting apoptotic processes. Akt1 is also able to induce protein synthesis pathways and is therefore a key signaling protein in the cellular pathways. Since it can block apoptosis, and thereby promote cell survival, Akt1 has been implicated as a major factor in many types of cancer. Akt (now also called Akt1) was originally identified as the oncogene in the transforming retrovirus AKT8 (Staal et al., 1977).

Akt is involved in the PI3K/AKT/mTOR pathway and other signaling pathways. Akt possesses a protein domain known as a PH domain, or pleckstrin homology domain, named after pleckstrin, the protein in which it was first discovered. This domain binds to phosphoinositides with high affinity. In the case of the PH domain of Akt, it binds either PIP3 (phosphotidylinositol (3, 4, 5) - triphosphate, Ptdins (3, 4, 5) P3) or PIP2, phosphotidylinositol (3, 4) bisphosphate, PtdIns (3, 4) P2) (Franke et al., 1997). Once correctly positioned at the membrane via binding of PIP3, Akt can then be phosphorylated by its activating kinases, phosphoinositide dependent kinase 1 (PDPK1 at Thr308) and the mammalian target of rapamycin complex 2 (mTORC2 at Ser473) (Sarbassov et al., 2005).

2. AIMS OF THE PROJECT

The increasing prevalence of NAFLD in the general population translates directly into an increasing graft steatosis, affecting both, the quality and the quantity of donor livers available for transplantation. Ischemia-Reperfusion (IR) injury of liver results in hepatocytes irreversible damage occurring during surgical procedures that include hepatic resections and liver transplantation. In particular, the use of steatotic livers for transplantation is associated with an increased risk for primary nonfunction or dysfunction after surgery respect to a normal liver because steatotic livers tolerate poorly I/R injury. Ischemic Preconditioning (IP) has shown to be an effective method that reduces liver injury induced by I/R, but its application to human surgery has given conflicting results. Thus, a better understanding of the mediators and pathways involved in IP might represent a way to optimize strategies against IR damage in normal and fatty livers. On the base of these considerations, the aims of my project were:

- To understand the phenotypic changes occurring in the liver during I/R or as a response to preconditioning treatments.
- To set up a cellular model able to reproduce “in vitro” the ischemia/reperfusion (I/R) damage using normal and steatotic primary mouse hepatocytes and to characterize the molecular mediators and pathways which could sensitize the steatosis for the exacerbation effect of I/R injury in fatty liver.
- To investigate the protective action of different adenosine receptor agonists during hypoxia reoxygenation damage in normal and fatty liver.

PAPER-1

Mouse hepatocytes and LSEC proteome reveal novel mechanisms of ischemia/reperfusion damage and protection by A2aR stimulation

Summary

Ischemia-reperfusion (IR) of liver results in irreversible damage of hepatocytes (HP) and sinusoidal endothelial cells (LSEC). Ischemia/reperfusion damage causes up to 10% of early organ graft failure, following liver transplantation, and can lead to a high incidence of both acute and chronic rejections. Minimizing the adverse effects of this injury could significantly increase the number of transplantable livers, improving graft outcome. Previous studies have shown that Ischemic Preconditioning (IP) protects IR damage upon stimulation adenosine A2a receptor (A2aR) enhancing cell tolerance against hepatic IR damage. These effects are also mimicked by the A2aR agonist CGS21680. Understanding the phenotypic changes that underlie hepatocellular damage and protection is critical for optimizing strategies against IR. This work describes, for the first time, the proteome alterations of mouse HP and LSEC isolated from murine livers exposed to IR in the presence or absence of A2aR stimulation, elucidating the liver cell contribution to IR damage and hepatoprotection by pharmacological preconditioning. To this aim the proteome of HP and LSEC isolated from sham or IR exposed mice receiving or not the A2aR agonist CGS21680 (0.5 mg/kg b.w) was analysed by 2-D DIGE/MALDI-TOF. Using this procedure we identified 64 proteins involved in cytoprotection, regeneration, energy metabolism and response to oxidative stress; among them, 34 were associated with IR injury and A2aR protection. The main pathways, down regulated by IR and up regulated by CGS21680 in HP and LSEC, were related to carbohydrate, protein and lipid supply and metabolism. In LSEC, IR reduced stress response enzymes that were instead up regulated by CGS21680 treatment. Functional validation experiments confirmed the metabolic involvement and showed that the inhibition of pyruvate kinase, 3-chetoacylCoA thiolase, and arginase affected the protection exerted by CGS21680 on "in vitro" hypoxia-reoxygenation injury, whereas hepatocyte supplementation with the metabolic products of these pathways reduced IR-induced liver cell damage. Moreover, LSEC, but not HP, were sensitive to H_2O_2-induced oxidative damage and CGS21680 protected against this effect. Taken together the results of this study show that IR injury is characterized by specific modifications of HP and LSEC proteomes that are partially reverted by A2aR stimulation providing novel insides in the pathways leading to liver protection by preconditioning treatments.

PAPER-1

Mouse hepatocytes and LSEC proteome reveal novel mechanisms of ischemia/reperfusion damage and protection by A2aR stimulation

Giorgia Mandili[1,2], Elisa Alchera3,, Simone Merlin3,, Chiara Imarisio3,, Bangalore R. Chandrashekar3, Chiara Riganti4, Alberto Bianchi3, Francesco Novelli1,2,, Antonia Follenzi3,, Rita Carini3,

Department of Health Science, University of Piedmont Orientale, Novara, Italy

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ABSTRACT

Background & Aims: Ischemia-reperfusion (IR) of liver results in hepatocytes (HP) and sinusoidal endothelial cells (LSEC) irreversible damage. Ischemic preconditioning protects IR damage upon adenosine A2a receptor (A2aR) stimulation. Understanding the phenotypic changes that underlie hepatocellular damage and protection is critical to optimize strategies against IR.

Methods: The proteome of HP and LSEC isolated from sham or IR exposed mice receiving or not the A2aR agonist CGS21680 (0.5 mg/kg b.w.) was analysed by 2-D DIGE/MALDI-TOF

Key results: 64 proteins were identified involved in cytoprotection, regeneration, energy metabolism and response to oxidative stress; among them, 34 were associated with IR injury and A2aR protection. The main pathways, down regulated by IR and up regulated by CGS21680 in HP and LSEC, were related to carbohydrate, protein and lipid supply and metabolism. In LSEC, IR reduced stress response enzymes that were instead up regulated by CGS21680 treatment. Functional validation experiments confirmed the metabolic involvement and showed that inhibition of pyruvate kinase, 3-chetoacylCoA thiolase, and arginase reduced the protection by CGS21680 of in vitro hypoxia-reoxygenation injury, whereas their metabolic products induced liver cell protection. Moreover, LSEC, but not HP, were sensitive to H_2O_2-induced oxidative damage and CGS21680 protected against this effect.

Conclusions: IR and A2aR stimulation produces pathological and protected liver cells phenotypes respectively characterized by down- and up- regulation of proteins involved in the response to O_2 and nutrients deprivation during ischemia, oxidative stress and reactivation of aerobic energy

synthesis at reperfusion. This provides novel insides in IR hepatocellular damage and protection and suggests additive therapeutic options.

Introduction

Inflow occlusion during liver surgery, with consequent reperfusion, causes liver ischemia-reperfusion (IR) injury. IR causes up to 10% early graft dysfunction or failure during liver transplantation [1]. IR injury is the result of a complex series of alterations that mainly involve hepatocytes (HP) and sinusoidal endothelial cells (LSEC) [2]. Several events contribute to liver damage by IR. The lack of oxygen during the ischemic period is associated with mitochondrial de-energization, ATP depletion that impairs $Ca2^{+}$, H^{+}, and Na^{+} homeostasis, with alteration of the volume regulatory mechanisms, and eventually necrosis. Upon oxygen readmission, the uncoupled mitochondria generate reactive oxygen species (ROS) with oxidative stress, mitochondrial permeability transition, and decreased capacity to synthesize ATP. These events, along with caspase activation, lead to cell death by both necrosis and apoptosis. Concomitantly, activation of the inflammatory reactions is also associated with the onset of IR [3,4]. Minimizing the adverse effects of IR could significantly increase the number of transplantable organs and improve the outcome of the grafts [5]. Preconditioning is a powerful protective phenomenon able to activate endogenous systems that make tissues resistant to a subsequent lethal stress [6]. Liver ischemic preconditioning, defined as brief periods of ischemia and reperfusion before sustained hepatic ischemia, can preserve energy loss, reduce transaminases release, inhibit inflammatory reactions, and promote liver regeneration after IR injury [4,7]. The surgical application of ischemic preconditioning represents a promising approach to protect against hepatic IR in humans. However, its use has the main disadvantage of inducing trauma to major vessels and stress to the target organ [8]; clinical studies have given conflicting results preventing the clinical use of ischemic preconditioning [4,8,9]. These observations show the need to explore alternative approaches to activate ischemic preconditioning in patients. To this respect, pharmacological induction of liver preconditioning could represent a more efficient and reliable technique. In vitro and in vivo studies have established a key role of the adenosine A2a receptor (A2aR) stimulation as an approach for pharmacological induction of liver preconditioning [4,10-12]. In fact, even short periods of hypoxia lead to the enhanced breakdown of adenine nucleotides to adenosine, because of the decreased production of ATP. Adenosine accumulation protects tissues from injury upon signalling through the adenosine receptor A2aR [4,12]. Expression of new synthesized

proteins can also contribute to the production of the protected liver cell phenotypes [13]. The changes of protein expression of preconditioned as well as IR injured HP and LSEC are presently poorly characterized. With the aim of identifying new targets for the development of innovative therapeutic hepatoprotective approaches, the present work analysed the proteomic patterns of primary HP and LSEC isolated from mouse liver following IR, with or without pre-treatment with the A2aR agonist CGS21680.

Materials and Methods

Chemicals and reagents:

Protease inhibitors, nuclease, ammonium persulfate (APS), bromophenol blue, glycerol,N,N,N9,N9-tetramethylethylene-diamine (TEMED), sodium dodecyl sulphate (SDS), TRIZMA, urea, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulphonate (CHAPS), dithiothreitol (DTT), iodoacetamide, Dulbecco's modified Eagle medium culture medium (DMEM), trypan blue, 2p-(2-carboxyethyl)-phenyl- amino-5-N-ethylcarboxya-mido-adenosine (CGS21680), palmitic acid, nonessential amino acid mixture (AA, 100X), suramine (SUR), norvaline (NRV), pyruvate, trimetazidine (TMZ), 2,7-dichlorofluorescin diacetate (DCFH-DA), BCA kit, Enzymatic Assay of Pyruvate Kinase kit and ATP Bioluminescent Assay kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). DC Protein Assay kits, acrylamide, agarose, ready-made immobilized pH gradient (IPG) strip (17-cm IPG strips, pH 3-10NL) were purchased from Bio-Rad (Hercules, CA, USA). Ampholine pH 3.5–10, Western blot detection system, membranes for blotting, anti-rabbit and anti-mouse IgG horseradish-peroxidase-labelled antibodies were obtained from GE Healthcare (MI, Italy). Rabbit antibody against arginase 1 was purchased from Thermo Scientific (Illkirch Cedex, France), rabbit antibody against 3-ketoacyl-CoA thiolase from Aviva System Biology (San Diego, CA, USA). TaqMan gene expression master mix and TaqMan gene expression probes for mouse 3-ketoacyl- CoA thiolase, arginase 1, α-enolase and β-actin or 18S were purchased from Applied Biosystems Italia (Monza, Italy).

Animals:

Male C57BL/6 mice used for this study were purchased from Harlan SRL, Italy. All experiments involving animals were approved by the Italian Ministry of Health and the ethical committee for animal care of the University del Piedmont Orientale ''A. Avogadro''.

Ischemia-reperfusion injury:

Mice were exposed to a non-lethal (70% of the total liver volume) hepatic ischemia for 30 min, followed by 120 min reperfusion, as previously described [14]. Pharmacological A2aR activation was induced by I.P. injection of CGS21680 (0.5 mg/kg of body weight) 20 min before ischemia induction. Liver injury was assessed by measuring the ALT serum transaminase activity, with a commercial kit (Gesan Production, Italy), and the morphological alterations by histological observation. Details are provided in Supplementary Materials.

Liver cells isolation and treatment

Liver cells were isolated by liver perfusion with collagenase digestion, from sham operated mice or mice exposed to IR, pre-treated or not with CGS21680. HP were obtained by differential centrifugation at 50g for 5 min at 4°C and LSEC by immunomagnetic separation, using a negative selection with a mouse anti-CD45, and a positive selection with anti-CD146 antibodies linked to immunomagnetic beads (Miltenyibiotec, Calderana di Reno, BO, Italy), as previously reported [15] and described in details in Supplementary Materials. Isolated HP and LSEC for proteomic analysis were stored at 80°C until solubilisation. For evaluation of hypoxia-reoxygenation injury, primary HP and LSEC were resuspended (106/ml cell density) in Viaspan solution (University of Wisconsin solution without additives), fluxed with 95% N_2/ 5% CO_2 and maintained at 4^0C for 16 hrs in sealed flasks. For reoxygenation, cells were transferred to an oxygenated Krebs-Henseleit buffer containing 20 nmol/L N-(2-hydroxyethyl)-piperazine- N0-(2-ethanesulfonic acid) (pH 7.4 at 37^0C), and the incubation flasks were further fluxed with a 95% air/5% CO2 gas mixture. When indicated, liver cells, suspended in the Viaspan solution, were preincubated 15 min at 37^0C before cold preservation with CGS21680 (5 μmol/L) and/or suramine (SUR, 20 μmol/L), norvaline (NRV, 50 μmol/L), trimetazidine (TMZ, 100 μmol/L), pyruvate (10 μmol/ L), Palmitic acid (PA, 2 μmol/L) or non-essential amino acid mixture (AA, 10%). To evaluate oxidative damage, HP or LSEC in Krebs-Henseleit buffer were treated with H_2O_2 (500 μmol/L) in the presence or absence of CGS21680 (5 μmol/L) and incubated for 30 min at 37^0C under a 95% air/5% CO_2 gas atmosphere.

Determination of cell viability

Cell viability was estimated by the determination of nuclear fluorescence staining with propidium iodide using a FACScan analyser (Becton-Dickinson, San Jose, CA) and Cell Quest software (Becton-Dickinson) [13].

Measurement of reactive oxygen species (ROS)

Intracellular ROS production was measured as reported in [14], by quantifying the DCFH-DA (2,7-dichlorofluorescin diacetate) fluorescence intensity with a Hitachi F-4500 fluorescence spectrophotometer. Details are provided in Supplementary Materials.

Data analysis:

Statistical analysis was performed with In Stat 3 statistical software (Graph Pad Software, Inc., San Diego, CA) by 1-way analysis of variance, testing with Bonferroni correction for multiple comparisons when more than 2 groups were analysed. The distribution normality of all groups was preliminarily verified with the Kolmogorov and Smirnov test. Significance was established at the 5% level.

Proteomic analysis:

Two-dimensional gel electrophoresis (2-DE) on ready-made IPG strip (17-cm IPG strips, pH 3-10NL) was performed as described [16]. For 2-D DIGE analysis, fifty micrograms of each sample (control, CGS21680, IR or CGS21680+IR) was minimally labelled with CyDye DIGE Fluors following manufacturer's instructions (GE Healthcare). For 2DE Coomassie stained gel, 1 mg of total liver protein was loaded. Destaining and in-gel enzymatic digestion of G-stained spots were performed as previously described [16]. All digests were analysed by MALDI-TOF (Tof Spec SE, Micro Mass). Details are provided in Supplementary Materials. To verify the significance of protein expression variations, two-sided Student's t test was used. Experiments were performed in triplicate. Statistical significance was set at p 60.05. Proteins were classified as differentially expressed if ratio in spot intensity was greater than 1.5-fold (protein overexpressed) or lower than 0.5-fold (protein under expressed). The protein and RNA levels of ketoacyl-CoA thiolase, arginase 1, and α-enolase were evaluated by Western blotting and RT-PCR as described in Supplementary materials.

Enzymatic assays:

Aldolase B activity was measured as described in [17], with minor modifications. α-enolase activity was measured accordingly to [18]. The activity of pyruvate kinase was detected with the Enzymatic Assay of Pyruvate Kinase kit, following manufacturer's instructions. Fatty acids b-oxidation was measured as previously reported [19], with minor modifications. The activity of carbamoyl phosphate synthase I was measured on mitochondrial extracts, isolated as previously reported [20]. Arginase activity was measured with a spectrophotometric method [21]. To measure the isocitrate dehydrogenase activity, 25 μg mitochondrial proteins was resuspended in 0.3 ml of Tris-acetate (pH 7.4), containing 5 mM/L DL-isocitrate trisodium salt and 5 mM/L MgCl2. The reaction was started by adding 0.5 mMol/L NAD^+ and the absorbance at 340 nm was followed for 5 min. Results were expressed as nM NADH/mg mitochondrial proteins. The rate of cytochrome c reduction was measured according to [22] with minor modifications. The ATP level in mitochondria extracts was measured with the ATP Bioluminescent Assay Kit. Additional details are provided in Supplementary Materials.

Results

Analysis of liver injury following IR and A2aR stimulation

Mice exposure to 30 min of hepatic ischemia, followed by 120 min reperfusion, caused substantial liver injury as determined by the serum ALT (alanine transaminase) release and hepatic histology (Supplementary Fig. 1). In accordance to previous observations [4, 12], stimulation of adenosine A2 receptors by mouse treatment with CGS21680 (0.5 mg/kg b.w.) before IR, significantly reduced the serum ALT increase and markedly attenuated the signs of hepatocyte necrosis and sinusoidal congestion detected by hematoxylin and eosin staining (Supplementary Fig. 1).

Proteomic analysis following IR and A2aR stimulation

2-D DIGE proteomic analysis was performed, to elucidate the phenotypic changes of HP and LSEC isolated from mice livers exposed to IR, with or without A2aR stimulation (Supplementary Fig. 2, Supplementary Tables 1–3).

By comparing HP and LSEC of sham operated mice vs. mice undergoing IR, we observed that 16 proteins were down regulated (Fig. 1, Supplementary Table 1). In particular, in both HP and

LSEC, IR reduced the level of proteins involved in glycid, lipid and mitochondrial metabolism (Krebs cycle and oxidative phosphorylation). Notably, IR decreased, in LSEC specifically, two proteins related to the response to oxidative stress (Fig. 1).

Compared to control, treatment with the A2aR agonist CGS21680 alone affected the expression of metabolic proteins: 6 were up regulated and 1 was down-regulated (Fig.1, Supplementary Table 2).

The treatment with CGS21680 and IR vs. control, with the exception of three proteins that were down regulated in HP, up regulated 10 proteins, mostly metabolic enzymes associated with ATP synthesis, glycolysis, lipid and amino acid catabolism, and cell response to stress (Fig.1, Supplementary Table 3). Notably, the CGS treatment completely rescued the expression of the 16 proteins down regulated by IR, with 14 proteins that recovered control level and two that were up regulated (Fig.1, Supplementary Table 3).

It is noteworthy that a more complex and unexpected scenario was seen when cell extracts obtained from mice receiving CGS21680 with IR were compared to those exposed to IR alone. We found, that further 19 proteins, including metabolic, stress related and folding-related proteins, and were up regulated (Fig. 1, Supplementary Table 3).

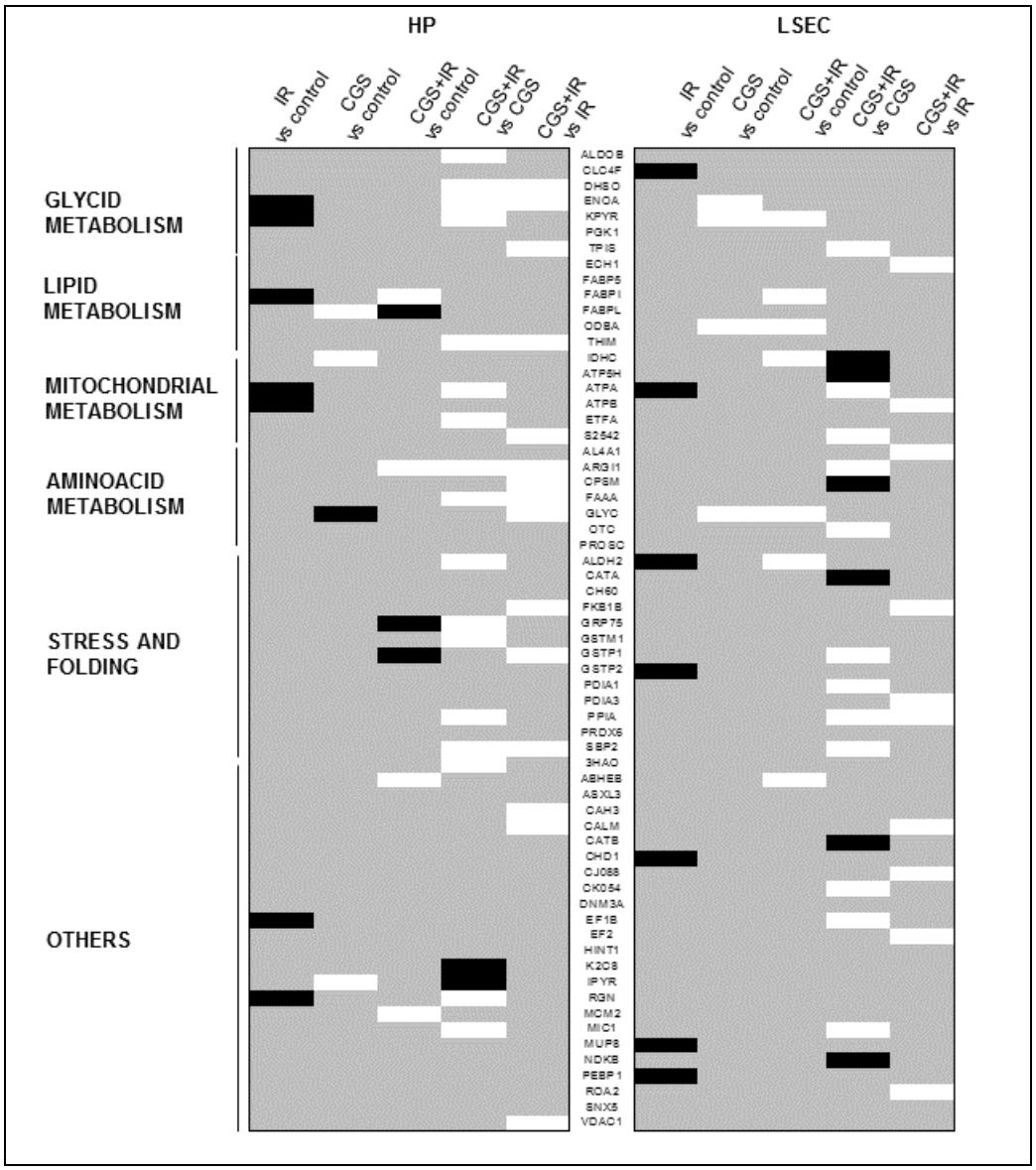

Fig.1 Differentially expressed proteins upon IR, A2aR stimulation or A2aR stimulation plus IR. Down regulated (black) and up regulated (white) identified proteins associated or not (other) to metabolism (glycid, lipid, mitochondrial and amino acid metabolism) or stress-response/folding processes in control conditions or upon A2aR stimulation with the A2aR agonist CGS21680 or IR, in the presence or absence of CGS21680 treatments. All pair conditions were examined.

Also the comparison IR plus CGS21680 vs. CGS21680 did not reproduce the protein profile of IR alone (Fig. 1, Supplementary Tables 1 and 3). We detected the modulation of 41 proteins and, most intriguingly, 34 of them were up regulated, while in IR vs. control, all proteins were down regulated. Among the up regulated proteins, we evidenced metabolic and stress related enzymes. Altogether, in both HP and LSEC, A2aR stimulation by CGS21680 alone and at a greater extent

when followed by IR up regulated proteins associated with DNA synthesis and cytoprotection; intriguingly, the main involved pathways were related to cell response to stress, and more markedly to the carbohydrate, lipid, and amino acids supply and catabolism (Fig. 2). This suggested a possible role of antioxidant and catabolic enzymes in the hepatoprotective effects of A2aR stimulation. Proteomic data have been validated by Western blot and RT PCR analysis on three key metabolic enzymes (ENOA, THIM, and ARGI1) (Supplementary Fig. 3).

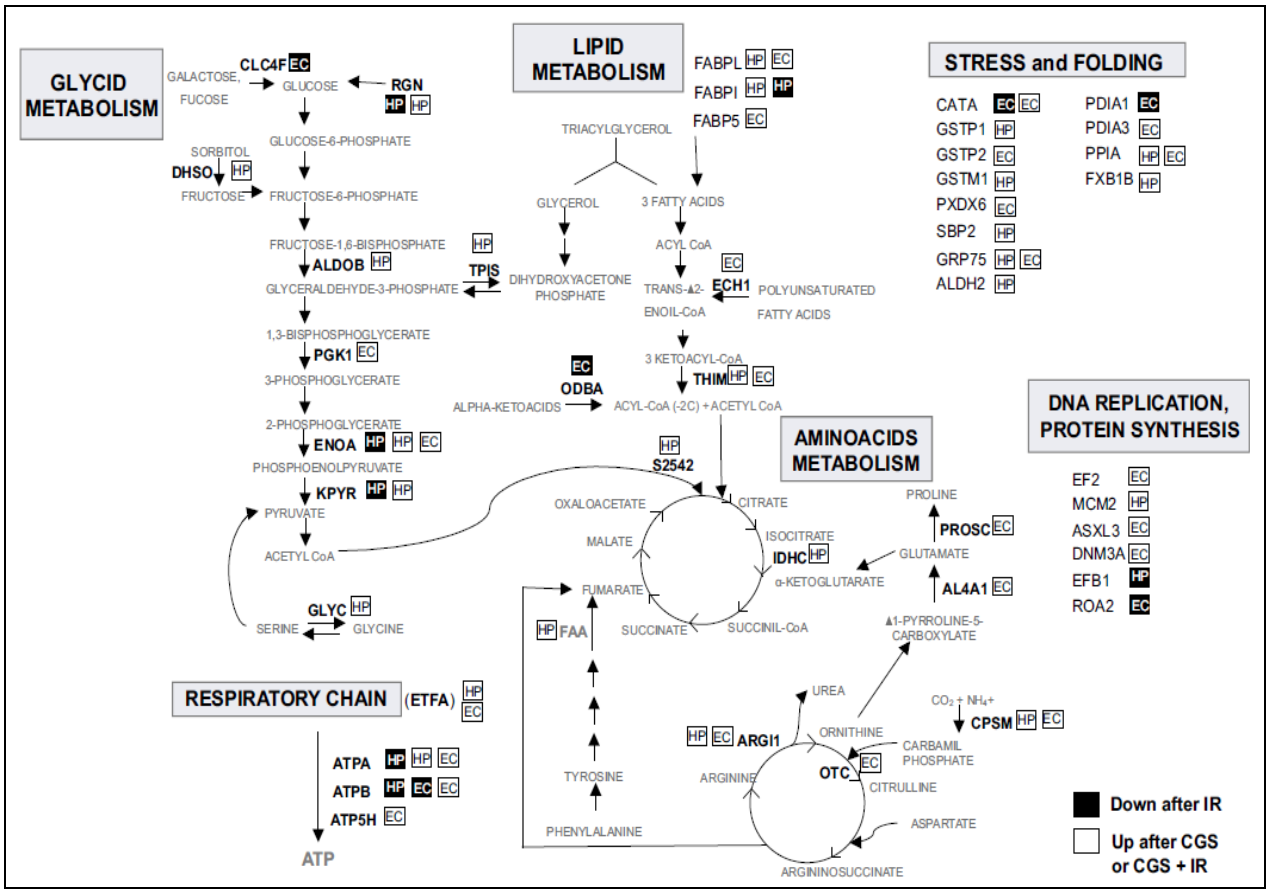

Fig.2 Graphical abstract of the main pathways involved in IR and A2aR stimulation in HP and LSEC. Identified proteins are indicated. Functional validation of the metabolic effect of A2aR stimulation on HP and LSEC

Proteomic data showed that A2aR stimulation increased the expression of several catabolic enzymes that were instead reduced following IR (Fig. 1, Supplementary Tables 1–3). To functionally confirm this observation, the activity of several enzymes referred to glycid, lipid, amino acid and mitochondrial metabolism was assayed.

The activity of glycolytic enzymes aα-enolase (ENOA) and pyruvate kinase (KPYR) was down regulated by IR and up regulated by IR plus CGS21680 in HP and LSEC, while that of fructose-bisphosphate aldolase B (ALDOB) was down regulated by IR and up regulated by IR plus CGS21680 in HP only (Fig. 3).

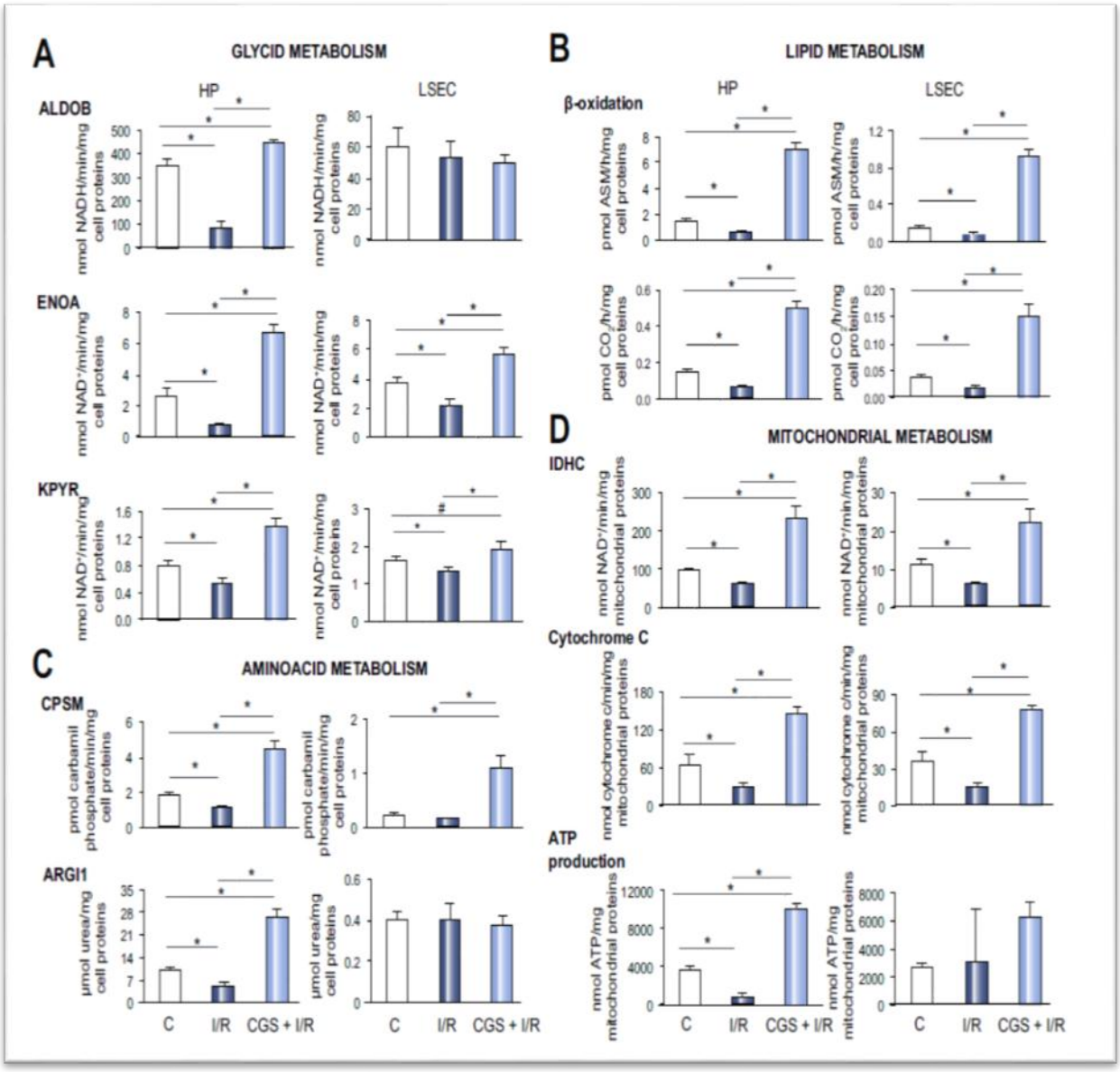

Fig.3 Effects of IR, A2aR stimulation or A2aR stimulation plus IR on metabolic activities. Enzymatic activities of (A) fructose-bisphosphate aldolase B (ALDOB), α-enolase (ENOA), and pyruvate kinase (KPYR), (B) β-oxidation reactions, (C) carbamoyl-phosphate synthase (CPSM) and arginase 1 (ARGI1) and (D) isocitrate dehydrogenase (IDHC), cytochrome C, and ATP production were evaluated in HP and LSEC. The results are means ± SD of four experiments. ⁄p <0.01, #p < 0.05.

For lipid metabolism, we analysed the products of b-oxidation reactions that were down regulated by IR and up regulated by IR plus CGS21680 in HP and LSEC (Fig. 3).

For amino acid catabolism, we evaluated the activity of two enzymes linked to urea cycle, namely carbamoyl-phosphate synthase (CPSM) and arginase 1 (ARGI1). The activity of CPSM was reduced by IR (although not significantly in LSEC) and strongly up regulated by IR plus CGS21680 (Fig. 3). The activity of ARGI1 was significantly down regulated by IR and up regulated by IR plus CGS21680 in HP only.

For mitochondrial metabolism, the activity of isocitrate dehydrogenase (IDHC) and cytochrome C, and ATP production were measured. The activity of IDHC and cytochrome C was significantly down regulated by IR and up regulated by IR plus CGS21680 in both HP and LSEC, while ATP production was the same but only in HP (Fig. 3).

These data clearly indicate that IR strongly reduces the metabolism and that CGS21680 rescues it, in both HP and LSEC, confirming the observations obtained by the proteomic approach.

Functional validation of the cytoprotective role of metabolic enzymes in A2aR-induced resistance to death of HP and LSEC

To evaluate the cytoprotective meaning of the up regulation of the metabolic enzymes in HP and LSEC, obtained from mice treated with CGS21680 before hepatic IR, we applied an in vitro model of IR injury, using primary HP and LSEC preserved in hypoxic conditions in VIASPAN solution, and then reoxygenated in Krebs-Hanslet at 37^0C. As shown in Fig. 4A, chemical inhibition of the 3 key enzymes of carbohydrate, lipid and amino acids catabolism, pyruvate kinase (KPYR), 3-ketoacyl- CoA thiolase (THIM), and arginase 1 (ARGI1) by suramine (SUR, 20 μM/L), trimetazidine (TMZ, 100 μM/L), and norvaline (NRV, 50 μM/L) respectively, significantly reduced the protective effect of CGS21680 against reperfusion damage Similarly, supplementing VIASPAN solution with palmitic acid (2 μM/L), a non-essential amino acid mixture (10%), or Pyruvate (10 μM/L), significantly reduced HP and LSEC mortality induced by 60 min reoxygenation, partially reproducing the cytoprotective action of CGS21680 (5 μM/L) supplementation (Fig. 4B).

Functional validation of the antioxidant effect of A2aR stimulation on LSEC

Proteomic data showed that A2aR stimulation increased the expression of several antioxidant enzymes that were instead reduced following IR, particularly in LSEC (Fig. 1, Supplementary Tables 1–3). These observations were functionally confirmed by evaluating the susceptibility to oxidative stress of primary mouse HP and LSEC upon 30 min exposure to H2O2 (500 mol/L). H2O2 treatment significantly increased ROS and cell damage in LSEC, but not in HP. The stimulation of A2aR with CGS21680 abolished ROS production and prevented loss of LSEC viability induced by H2O2 exposure (Fig. 5).

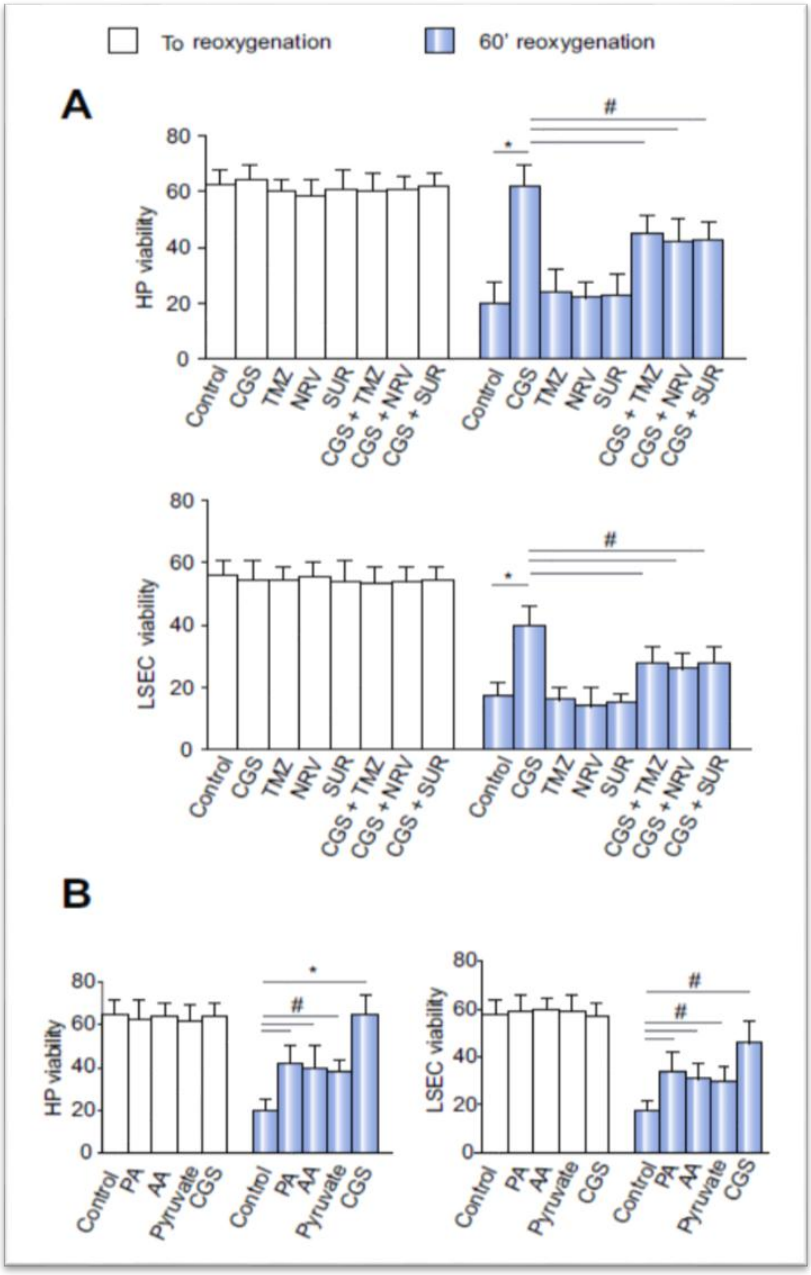

Fig. 4 A2aR stimulation protects HP and LSEC against hypoxia-reoxygenation injury by promoting glycid, lipid and amino acid catabolism. Viability of primary mice HP and LSEC conserved for 16 h in cold hypoxic conditions and exposed to 60 min of warm reoxygenation. HP and LSEC were conserved in VIASPAN solution in the presence or absence of (A) 3-ketoacyl-CoA thiolase inhibitor trimetazidine (TMZ, 100 µmol/L), arginase inhibitor norvaline (NRV, 50 µmol/L) and pyruvate kinase inhibitor suramine (SUR, 20 µmol/L), with or without the A2aR agonist CGS21680 (5 µmol/L) or (B) Palmitic acid (2 µmol/L) (PA), non-essential amino acid mixture (10%) (AA), pyruvate (10 µmol/L) or CGS21680 (5 µmol/L). The results are means ± SD of four experiments. *p <0.001, #p <0.01

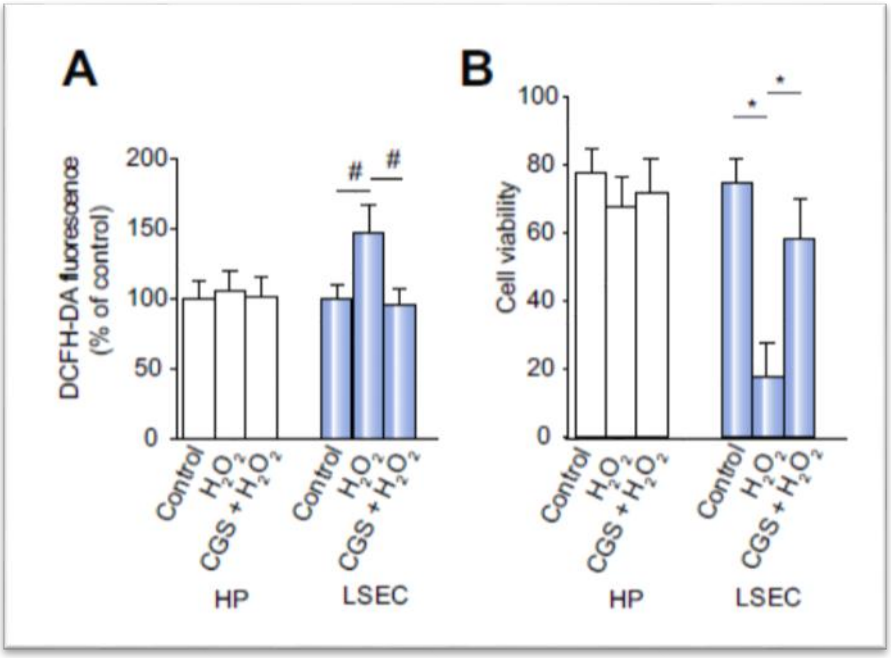

Fig.5 CGS21680 prevents oxidative species production and oxidative damage of LSEC. Intracellular oxidative species production evaluated as (A) DCFH-DA intracellular fluorescence intensity and (B) viability of primary mice HP and LSEC after 30 min exposure to H2O2 (500 µmol/L). The results are means ± SD of four experiments. $p < 0.001$, $\#p < 0.01$.

Discussion

Ischemia/reperfusion damage causes up to 10% of early organ graft failure, following liver transplantation, and can lead to a high incidence of both acute and chronic rejections. Minimizing the adverse effects of this injury could significantly increase the number of transplantable livers, improving graft outcome [5–7]. Ischemic preconditioning demonstrated its efficacy in several models [2–7] and different pharmacological preconditioning approaches have been developed to overcome limitations of surgical preconditioning [2–7,13]. Previous studies have shown that pretreatment with the A2aR agonist CGS21680 enhanced tolerance against hepatic IR damage [4, 11]. This work describes, for the first time, the proteome alterations of mouse HP and LSEC isolated from livers exposed to IR in the presence or absence of A2aR stimulation, elucidating the liver cell contribution to IR damage and hepatoprotection by pharmacological preconditioning.

Our work has shown profound modifications of HP and LSEC proteome and enzymatic activities, highlighting critical processes involved in IR injury and liver preconditioning, and implementing

and dissecting previous observation obtained in the liver [5,23– 28]. Considering all identified proteins, few of the affected proteins were shared between HP and LSEC, highlighting the diversity of these cells and the importance of analysing them separately. However, the pathways involved were almost the same (metabolism, stress response, protein folding and regeneration), showing the existence of a general common response, but with the prevalence of metabolic effects in HP and stress-related effects in LSEC. Notably, the profiling of the enzymatic and functional activities, reduced by IR and rescued by CGS21680, almost completely overlapped with those observed by proteomics.

The severe ATP depletion during ischemic phase in HP has been generally ascribed to the lack of O2 and glycolytic substrate supply, consequent to blood interruption [2–4]. However, such alteration is prevented in the preconditioned ischemic liver, indicating that the block of blood supply is not per se sufficient to justify ATP loss. In addition, one of the most striking alterations of the IR-injured liver is its incapability of recovering aerobic ATP production at blood flow re-establishment with reperfusion. The observations that 1) glycolytic enzymes and ATP synthase subunits were decreased in HP and LSEC derived from a liver exposed to IR, and 2) CGS21680 treatment combined to IR up regulated the glycolytic and mitochondrial pathways, endorses the hypothesis that IR damage is not merely due to a reduction of blood flow requirement, but to a coordinate perturbation of metabolic enzyme expression, which is rescued by preconditioning.

Furthermore, the liver acts as a major organ for lipid metabolism and the hepatic aerobic ATP synthesis is strictly dependent on lipid supply and catabolism. Interestingly, we found that CGS21680 treatment is able to promote lipid transport and β-oxidations, which were instead down modulated by IR. It would be interesting in the future to evaluate the impact of β-oxidation modulation in preventing IR injury.

We observed up regulation of urea cycle enzymes and increase of activity of two key enzymes of this pathway (CPSM and ARGI1) following CGS21680 treatment. This suggests that the improvement of amino acid catabolism could represent a response of HP and LSEC to ATP deprivation caused by IR.

All together, these results indicate that the down regulation of key metabolic enzymes can explain the ATP loss caused by IR. Therefore, A2aR stimulation provides a general metabolic advantage to HP and LSEC, demonstrated by ATP production increase, not only rescuing the metabolic

alteration induced by IR but, in some cases, enhancing the expression of enzymes required for energy production.

The relevance of our observations about the metabolic advantage provided by CGS21680 is also supported by the fact that the cytoprotective action of CGS21680 is reverted by the inhibition of pyruvate kinase (KPYR), 3-ketoacyl-CoA thiolase (THIM), and arginase (ARGI1) – three enzymes of glycolysis, β-oxidation and urea cycle respectively that are impaired by IR. Furthermore, cell supplementation with the glycolic end-product pyruvate, the free fatty acid Palmitic acid or amino acid mixture demonstrated to mimic partly the protective effects of CGS21680 against HP and LSEC hypoxia-reoxygenation damage.

Notably, among the 28 metabolic proteins identified, only 14 were already connected to IR and preconditioning (FABPL, ATPB, FABPI, ENOA, ATPA, ARGI1, ALDOB, ETFA, THIM, CPSM, TPIS, OTC, HINT, FABP5) [5,24–26,28–32], while the others are completely new (GLYC, IDHC, KPYR, DHSO, FAAA, S2542, PGK1, CLC4F, ODBA, NDKB, ATP5H, PROSC, ECH1, AL4A1).

Another fundamental aspect is the role of antioxidant enzymes in the protection against IR injury by preconditioning. We detected several proteins involved in liver cell response to oxidative stress. Many of these proteins (GRP75, GSTP1, SBP2, PPIA, GSTM1, CATA, PRDX6, CH60, PDIA3) were already known to be involved in IR and preconditioning processes [5,24–28]. Catalase, GSTP1, GSTP2, and GSTM1 are directly linked to detoxification of ROS, and GSH is known as a highly effective antioxidant present in elevated concentrations in HP [33]. PRDX6 is another well-known antioxidant normalizing mitochondrial respiration during IR [26]. Finally, the chaperones GRP75, PDIA1, PDIA3, and CH60 can be involved in protein folding repair mechanism, together with the 2 proline isomerase PPIA and FKB1B, since ROS are known to cause protein misfolding [34]. The majority of stress proteins that we have identified have mitochondrial origin, in agreement with previous observations [28, 35]. We observed that CGS21680 treatment generally increased the antioxidant defences, particularly in LSEC, while IR depressed the antioxidant enzymes content in LSEC exclusively, and that CGS21680 treatment of these cells prevented oxidative damage following in vitro addition of H2O2. These results may explain the high sensitivity of LSEC to cold ischemia and the microcirculatory disturbance induced by IR damage as well as the rescuing action of ischemic preconditioning [2].

An intriguing aspect that may deserve further analysis is that the combined treatment of CGS21680 plus IR often results in more effective production of protective protein modifications than that with CGS21680 alone. This suggests that the genomic changes induced by A2aR stimulation accomplish a full-protected phenotype only in presence of cell stress. Indeed, recent results showed that A2aR stimulation might also effectively prevent pathological conditions different from IR through the activation of noxious-specific mechanisms of protection [36].

In conclusion, this study contributed to the understanding of the molecular bases of IR injury and cytoprotection by A2aR stimulation, showing specific modifications of HP and LSEC proteomes. The great number of new proteins identified demonstrates the strength of our experimental approach. Finally, showing the importance of glycid, lipid, amino acid and antioxidant availability in IR injury and in A2aR-induced liver cell protection, this study suggests the protective potential of supplementing organ preservation solutions with energy-linked metabolites and natural or synthetic antioxidants.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Authors' contributions

Giorgia Mandili, Elisa Alchera, Simone Merlin, Chiara Imarisio, BR Chandrashekar, Chiara Riganti, Alberto Bianchi, and Antonia Follenzi performed the experiments; Francesco Novelli, Antonia Follenzi,and Rita Carini planified the study; Giorgia Mandili and Rita Carini wrote the manuscript, Francesco Novelli and Antonia Follenzi critically revised the manuscript.

Acknowledgements

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Supplementary Materials

Table of content:

SUPPORTING INFORMATION

EXPERIMENTAL PROCEDURES

Ischemia-reperfusion injury

Male C57BL6 mice were anesthetized with isoflurane, the abdominal cavity was opened, the liver vessels were exposed and normothermic partial hepatic ischemia was induced by the clamping of portal structures to the left and median lobes with a micro vascular clip; this yielded approximately 70% of hepatic ischemia. The abdomen was covered with saline-humidified gauze during the ischemic period. After 30 minutes of partial hepatic ischemia, the clip was removed to initiate hepatic reperfusion, the abdominal cavity was closed with a 4-0 silk suture and metal clips were applied to the skin. The temperature was maintained at 37°C during hepatic ischemia and in the post-surgical period with a warming pad. Sham-operated mice underwent the same procedure without clamping of the pedicle of the liver lobes. Mice were randomly assigned to 1 of 4 groups with a sample size of 4 mice per group. CGS21680 (0.5 mg/kg) was administered by intraperitoneal injection 20 min before the ischemia procedure. Mice were killed 120 min after reperfusion or employed for the procedures of liver cells isolation. For the "in vivo" analysis of liver injury, before mice sacrifice, blood was collected for serum ALT transaminase activity determination. Tissues from ischemic lobes were fixed in 4% formaldehyde and then embedded into paraffin. Sections were cut and stained with hematoxylin and eosin for histological analysis.

Liver cells isolation

Liver cells were isolated from sham operated mice or mice exposed to hepatic ischemia/reperfusion and treated or not with the A2aR agonist CGS21680 (0.5 mg/kg), after liver perfusion by collagenase digestion. After liver digestion, cells were dispersed and HP recovered

by differential centrifugation. An initial immunomagnetic separation by a mouse anti-CD45 antibody linked to immunomagnetic beads (Miltenyi biotec.) was used to collect hematopoietic cells. The negative fraction of the CD45+ cells was used to isolate LSEC by positive selection with anti-CD146 antibody linked to immunomagnetic beads. Typically, the yield of LSEC cells was $5x10^6$ per mouse liver and average of $40x10^6$ HP (15).

Cell viability estimated at the beginning of the experiments, ranged from 82% to 90%. Isolated HP and LSEC for proteomic analysis were stored at -80°C until solubilization.

Measurement of Reactive Oxygen Species (ROS)

Cells were incubated for 10 minutes at 37°C with 5µmol/L DCFH-DA in phosphate-buffered saline. After 2 washes with phosphate-buffered saline, cells were transferred to a fluorometer cuvette, and the fluorescence was recorded with a Hitachi F-4500 fluorescence spectrophotometer (490-nm excitation and 530-nm emission). ROS production was calculated as a percentage of the DCFH-DA fluorescence intensity versus untreated control cells.

Proteomic analysis

Samples preparation

Samples were solubilized in a solution containing 9 M urea, 4% w/v CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulphonate), protease inhibitors and nuclease. The sample was incubated O.N. at 4°C and spun down at 13,800 g for 10 min at 4°C. The clear supernatant recovered, quantified with DC Protein assay kit and stored at -20°C until analysis.

Two-dimensional gel electrophoresis (2-DE) coomassie-stained gels

2-DE was performed using ready-made IPG strip (17-cm IPG strips, pH 3-10NL). Each sample (1 mg of total liver protein) was applied onto an IPG gel by in-gel rehydration for 20 h, adding DTT 1% w/v, final concentration and ampholine pH 3.5–10, 2% v/v, final concentration. Isoelectric focusing, strips equilibration and second dimension were performed as previously described (16). Gels were stained with colloidal Coomassie (18% v/v ethanol, 15% w/v ammonium sulfate, 2% v/v phosphoric acid, 0.2% w/v Coomassie G-250) for 48 h.

2D DIGE

Samples were labelled with CyDye DIGE Fluors following the manufacturer's instruction (GE Healthcare). Fifty micrograms of each sample was minimally labelled with 400 pmol of either Cy

2 or Cy3 or Cy5. Cy3 and Cy5 were alternately used for samples, whereas Cy2 was used for the internal standard (a pooled standard containing total liver proteins treated or not with CGS21680). Labelling reactions were performed in the dark for 30 min on ice and then quenched with the addition of 10mM lysine. Three 2D DIGE experiments (containing one gel each) were performed to analyse three biological replicates of control and CGS21680 or IR and CGS21680+IR samples. 2-DE was performed as described above.

Image analysis

Gel images were acquired with Chemi Doc Imaging System (Bio-Rad). Image analysis was performed using PD-Quest software (version 7.2, Bio-Rad) according to the manufacturer's instructions. Normalization of each individual spot was performed according to the total quantity of the valid spots in each gel, after subtraction of the background values. The spot volume was used as the analysis parameter to quantify protein expression.

Protein identification by mass spectrometry and database search

Coomassie G-stained spots were excised from 2-DE preparative gels; distaining and in-gel enzymatic digestion performed as previously described (16). All digests were analysed by MALDI-TOF (TofSpec SE, MicroMass) equipped with a delayed extraction unit. Peptides solution was prepared with equal volumes of saturated a-cyano-4-hydroxycinnamic acid solution in 40% v/v acetonitrile-0.1% v/v trifluoroacetic acid. The MALDI-TOF was calibrated with a mix of PEG (PEG 1000, 2000 and 3000 with the ratio 1:2:2) and mass spectra were acquired in the positive-ion mode. Peak lists were generated with Protein Lynx Data Preparation (Protein Lynx Global Server 2.2.5) using the following parameters: external calibration with lock mass using mass 2465.1989 Da of ACTH, background subtract type adaptive combining all scans, performing de isotoping with a threshold of 1%. The 25 most intense masses were used for database searches against the SWISSPROT database using the free search program MASCOT (http://www.matrixscience.com). The following parameters were used in the searches: taxa Mus musculus, trypsin digest, one missed cleavage by trypsin, carbamido methylation of cysteine as fixed modification, methionine oxidation as variable modifications and maximum error allowed 100 ppm. Were taken on to consideration only protein with a Mascot score≥56.

Western blotting

Lysates containing equal amounts of proteins (30 μg), containing Laemmli buffer, were subjected to SDS/PAGE (12% gel). The separated proteins were transferred to a nitrocellulose membrane. The blot was blocked using 5% w⁄ v dried no fatty milk in PBS containing 0.1% Tween-20, and probed using rabbit antibody against arginase 1 (diluted 1:1000), mouse antibody against α–enolase (diluted 1:5000), rabbit antibody against 3-ketoacyl-CoA thiolase (diluted 1:3000) overnight at 4°C. After washing using PBS containing 0.1% Tween-20 for 30 min, the blot was incubated for 1 h with horseradish-peroxidase labelled antibodies against rabbit or mouse IgG (diluted 1:10000), and immune reactivity was detected using an enhanced chemiluminescence kit.

Real-time quantitative RT-PCR

Total RNA was isolated from frozen isolated HP and LSEC taken from sham liver or liver exposed to ischemia-reperfusion from mice treated or not with CGS21680, using the Charge Switch® Total RNA Cell Kit (Applied Biosystems Italia, Monza, Italy) following manufacturer's instructions. RNA was reverse transcribed for first-strand complementary DNA (cDNA) synthesis using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Italia, Monza, Italy) according to the manufacturer's recommendations. Quantitative real-time polymerase chain reaction (RT-PCR) was performed in the CFX96 Touch™ Real-Time PCR Detection System-Bio-Rad (Bio-Rad Laboratories S.r.l, Milan, Italy) using TaqMan Gene Expression Master Mix and Taqman Gene Expression probes for mouse 3-ketoacyl-CoA thiolase (THIM), arginase1 (ARGI1), α-enolase (ENOA), and β-actin or 18S as control genes (Applied Biosystems Italia, Monza, Italy). All samples were ran in duplicate, and the relative gene expression calculated as $2^{-\Delta Ct}$ is expressed as fold increase over control samples. Values were normalized to those of β-actin for ENOA or to those of 18S for THIM and ARGI1 and expressed by using the comparative $2^{-\Delta Ct}$ method.

Enzymatic assays

Glycid metabolism

Cells were rinsed with PBS, sonicated with 10 bursts of 1 s, centrifuged at 13,000 x g for 5 min, re-suspended in 100 mMol/L Tris (pH 7.4). A 50 μL aliquot was used for the protein

quantification with the BCA Kit (Sigma Chemical Co., St. Louis, MO). 50 µg of whole cell lysates were used in each assay. Aldolase B activity was measured as described in (17), with minor modifications: samples were incubated at 37°C, in the presence of 100 mMol/L K_2HPO_4 (pH 7.2), 1 mMol/L fructose 1,6-biphosphate, 10 mMol/L EDTA, 2 mg/mL α-glycerophosphate dehydrogenase, 2 mg/mL triose phosphate isomerase, 100 µg/mL bovine serum albumin, 0.15 mMol/L NADH, in a final volume of 300 µL. The rate of NADH oxidation was followed for 5 min, monitoring the absorbance at 340 nm with a Packard microplate reader EL340 (Bio-Tek Instruments, Winooski, VT). Results were expressed as mMol NADH produced/min/mg cell proteins. Enolase A activity was measured accordingly to (18). Results were expressed as nMol NAD^+/min/mg cell proteins. The activity of pyruvate kinase was detected with the Enzymatic Assay of Pyruvate Kinase kit, following the manufacturer's instruction. Results were expressed as nMol NAD^+/min/mg cell proteins.

Lipid metabolism

Fatty acids β-oxidation was measured as previously reported (19), with minor modifications. Cells were washed twice with PBS, detached with trypsin/EDTA (0.05/0.02% v/v) and centrifuged at 13,000 x g for 5 min. A 50 µL aliquot was collected, sonicated and used for the intracellular protein quantification. The remaining sample was re-suspended in culture medium containing 0.24 mMol/L fatty acid-free bovine serum albumin, 0.5 mMol/L L-carnitine, 20 mMol/L Hepes, 2 µCi [1-^{14}C]Palmitic acid (3.3 µCi/mMol) and transferred into test tubes tightly sealed with rubber caps. After 2 h incubation at 37°C, 0.3 mL of a 1:1 v/v phenyl ethylamine/methanol solution was added into each sample by a syringe, followed by 0.3 mL of 0.8 N $HClO_4$. Samples were incubated for 1 hr further at room temperature, and then centrifuged at 13,000 x g for 10 min. Supernatants, containing $^{14}CO_2$, and precipitates, containing ^{14}C-acid soluble metabolites (ASM), were collected. The radioactivity of each sample was counted by liquid scintillation. Results were expressed as pmol of [$^{14}CO_2$] or ^{14}C-ASM/h/mg cell proteins.

Amino acid metabolism

The activity of carbamoyl phosphate synthetase I was measured on mitochondrial extracts, isolated as reported previously (20). Samples were sonicated and a 50 µL aliquot was used for protein quantification. 25 µg of mitochondrial proteins were incubated in 0.5 mL of the assay

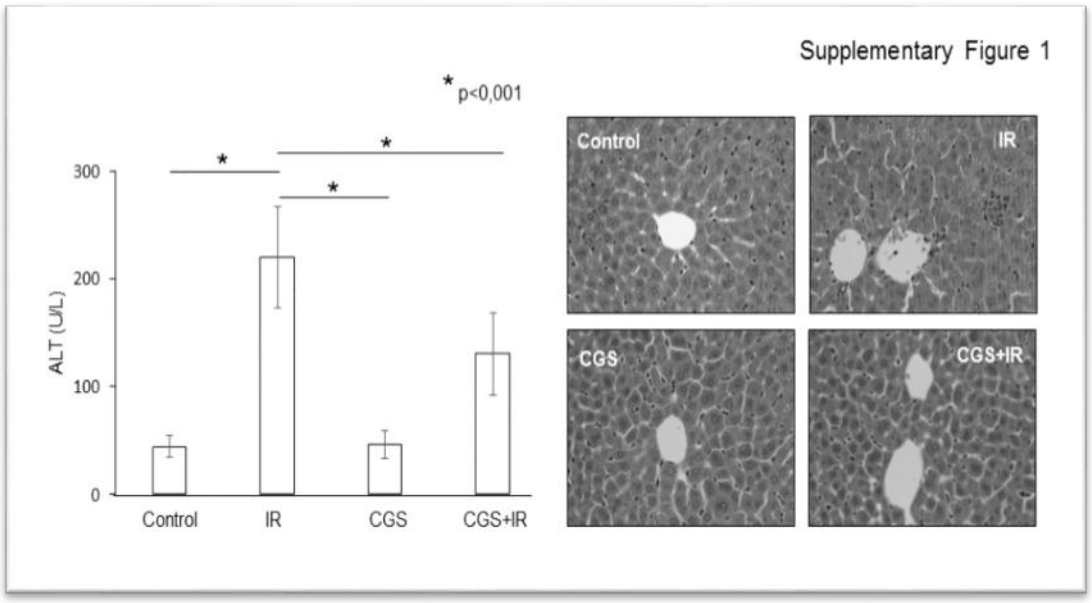
buffer (87 mMol/L Tris/HCl, 87 mMol/L KCl, 25 mMol/l $MgCl_2$, 10 mMol/L ATP, 20 mMol/L NH_4Cl, 0.8 mMol/L dithiothreitol, 6.5% v/v dimethyl sulfoxide, 2.2% v/v glycerol) with 4 µCi [^{14}C]-$NaHCO_3$ (54 µCi/mMol) for 30 minutes at 37°C. The reaction was stopped by adding 0.2 mL of 80% w/v trichloroacetic acid. To remove the unincorporated $^{14}CO_2$, the tubes were heated at 85°C for 3 h; the remaining samples, containing [^{14}C]-carbamoyl phosphate, were analyzed by liquid scintillation counting. Results were expressed as pmol carbamoyl phosphate/min/mg cell proteins. Arginase activity was measured on 50 µg of whole cell lysates by a spectrophotometric method (21). Results were expressed as µMol urea/mg cell proteins.

Mitochondrial metabolism

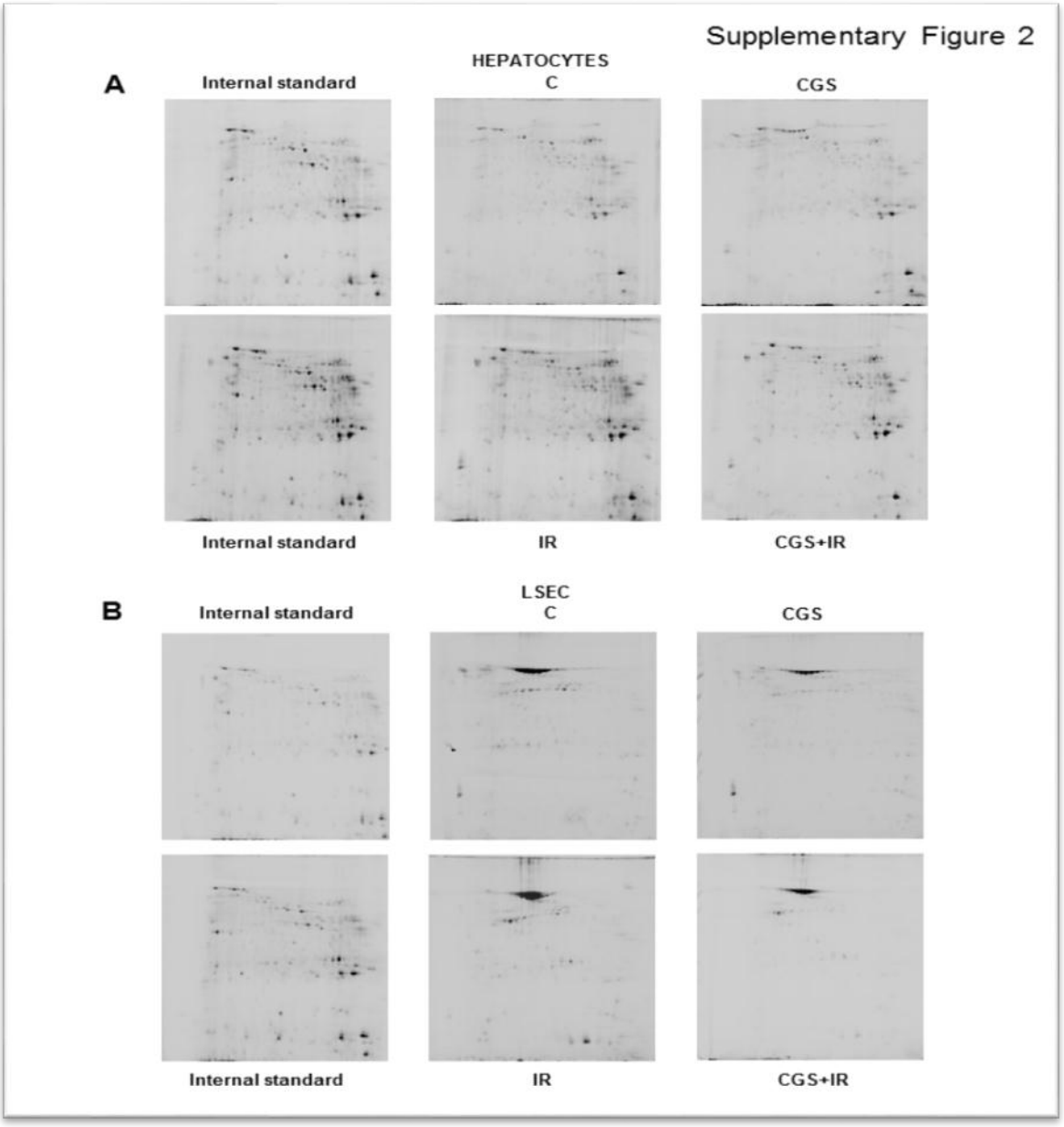
Mitochondria were isolated as reported above. To measure the isocitrate dehydrogenase activity, 25 µg mitochondrial proteins were re-suspended in 0.3 mL of Tris-acetate (pH 7.4), containing 5mMol/L DL-isocitrate trisodium salt and 5 mMol/L $MgCl_2$. The reaction was started by adding 0.5 mMol/L NAD^+ and the absorbance at 340 nm was followed for 5 minute. Results were expressed as nMol NADH/mg mitochondrial proteins.

The rate of cytochrome c reduction was taken as an index of the activity of the electron flux from complex I to complex III, and was measured according to (22) with minor modifications. 50 µg of non-sonicated mitochondrial samples, re-suspended in 0.59 mL buffer A (5 mMol/L KH_2PO_4, 5 mmol/L $MgCl_2$, 5% w/v bovine serum albumin), were transferred into a quartz spectrophotometer cuvette. Then 0.38 mL buffer B (25% w/v saponin, 50 mMol/L KH_2PO_4, 5 mMol/L $MgCl_2$, 5% w/v bovine serum albumin, 0.12 mMol/L cytochrome c-oxidized form, 0.2 mMol/L NaN_3) were added for 5 min at room temperature. The reaction was started with 0.15 mMol/L NADH and was followed for 5 min, reading the absorbance at 550 nm by a Lambda 3 spectrophotometer (PerkinElmer).The ATP level in mitochondria extracts was measured with the ATP Bioluminescent Assay Kit, using a Synergy HT Multi-Mode Microplate Reader (Bio-Tek Instruments). ATP was quantified as arbitrary light units and converted into nMol ATP/mitochondrial proteins, according to the calibration curve previously set.

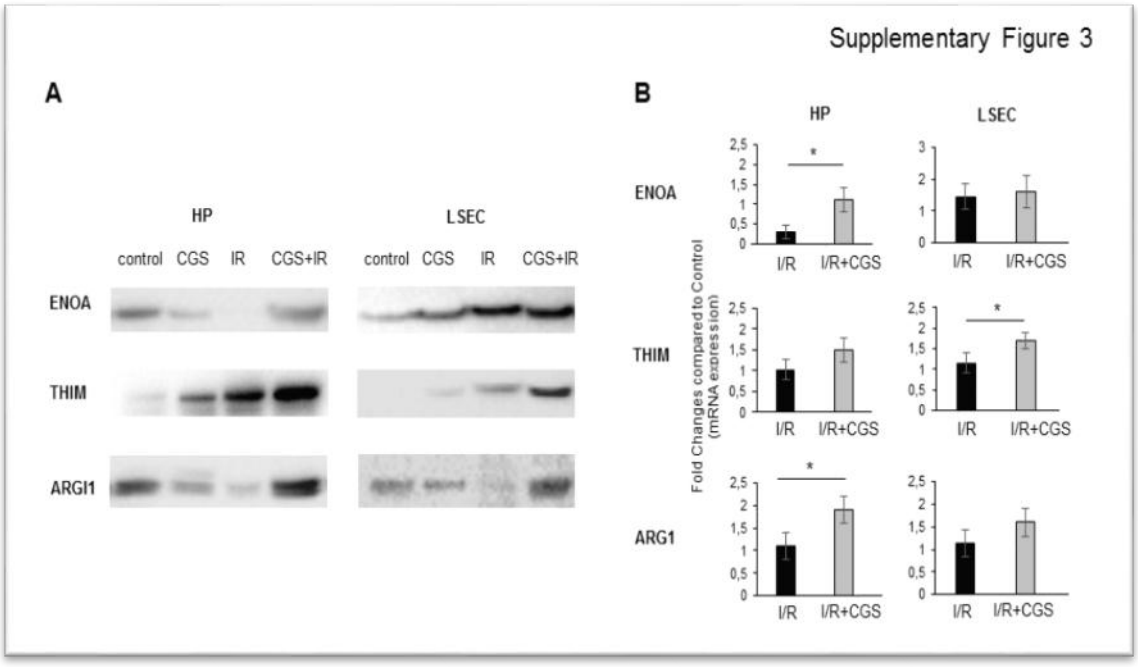
LEGENDS TO SUPPLEMENTARY FIGURES



The administration of CGS21680 ameliorates liver IR injury. Liver IR damage was induced by 30 minutes of warm ischemia followed by 120 minutes of reperfusion. CGS21680 (0.5 mg/kg) was injected intraperitoneal 20 min before liver ischemia. Sham-operated mice were used as controls. Hepatic injury was evaluated by the measurement of serum ALT release or at histology. Results are mean ± SD of 6 experiments. * $p < 0.05$.



2D-DIGE. Representative images (of three independent experiments) of 2DE DIGE gels. HP (A) and LSEC (B) proteins expression were studied in control conditions and upon A2aR stimulation with the A2aR agonist CGS21680 (CGS) or IR in presence (CGS+IR) and in absence (IR) of CGS21680. Internal standard gels are also reported.



Western blot and RT-PCR analysis of ENOA, THIM and ARGI1: (A) Representative western blot (of three independent experiments) with anti-ENOA, anti-THIM and anti-ARGI1 antibodies. HP and LSEC proteins expression were analyzed in control conditions (control) or upon A2aR stimulation with the A2aR agonist CGS21680 (CGS) or IR in presence (CGS+IR) or in absence (IR) of CGS21680. (B) Total RNA was isolated from HP and LSEC from sham mice (control) or mice exposed to IR and pretreated or not with CGS21680 (CGS) and ENOA, THIM and ARG-1 were determined by quantitative RT- PCR. Results are mean ± SD of 3 independent experiments. * $p < 0.05$.

PROTEINS ABBREVIATIONS:

ABHEB (Abhydrolase domain-containing protein 14B); **ALDH2** (Aldehyde dehydrogenase, mitochondrial); **AL4A1** (Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial); **ALDOB** (Fructose-bisphosphate aldolase B); **ATPA** (ATP syntase A), **ATPB** (ATP syntase B); **ATP5H** (ATP synthase subunit d, mitochondrial); **ARGI1** (Arginase-1); **ASXL3** (Putative Polycomb group protein ASXL3); **CAH3** (Carbonic anhydrase 3); **CALM** (Calmodulin); **CATA** (Catalase); **CATB** (Cathepsin B); **CH60** (60 kDa heat shock protein, mitochondrial); **CHD1** (Chromodomain-helicase-DNA-binding protein 1); **CJ088** (Uncharacterized protein C10orf88 homolog); **CK054** (Ester hydrolase C11orf54 homolog); **CLC4F** (C-type lectin domain family 4 member F); **CPSM** (Carbamoyl-phosphate synthase [ammonia], mitochondrial); **DHSO** (Sorbitol dehydrogenase); **DNM3A** (DNA (cytosine-5)-methyltransferase 3); **ECH1** (Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial); **EF1B** (Elongation factor 1-beta); **EF2** (Elongation factor 2); **ENOA** (Alpha-enolase); **ETFA** (Electron transfer flavoprotein subunit alpha, mitochondrial); **FAAA** (Fumarylacetoacetase); **FABP5** (Fatty acid-binding protein, epidermal); **FABPI** (Fatty acid-binding protein, intestinal); **FABPL** (Fatty acid-binding protein, liver); **FKB1B** (Peptidyl-prolyl cis-trans isomerase FKBP1B); **GLYC** (Serine hydroxymethyltransferase, cytosolic);

GRP75 (Stress-70 protein, mitochondrial); **GSTM1** (Glutathione S-transferase Mu 1); **GSTP1** (Glutathione S-transferase P 1); **GSTP2** (Glutathione S-transferase P 2); **3HAO** (3-hydroxyanthranilate 3,4-dioxygenase); **HINT1** (Histidine triad nucleotide-binding protein 1); **IDHC** (Isocitrate dehydrogenase [NADP] cytoplasmic); **IPYR** (Inorganic pyrophosphatase); **K2C8** (Keratin, type II cytoskeletal 8); **KPYR** (Pyruvate kinase isozymes R); **MCM2** (DNA replication licensing factor MCM2); **MIC1** (Uncharacterized protein C18orf8 homolog); **MUP8** (Major urinary proteins 8 (Fragment)); **NDKB** (Nucleoside diphosphate kinase B); **ODBA** (2-oxoisovalerate dehydrogenase subunit alpha, mitochondrial); **OTC** (Ornithine carbamoyltransferase, mitochondrial); **PDIA1** (Protein disulfide-isomerase); **PDIA3** (Protein disulfide-isomerase A3); **PEBP1** (Phosphatidylethanolamine-binding protein 1); **PGK1** (Phosphoglycerate kinase 1); **PPIA** (Peptidyl-prolyl cis-trans isomerase A); **PRDX6** (Peroxiredoxin-6); **PROSC** (Proline synthase co-transcribed bacterial homolog protein); **RGN** (Regucalcin); **ROA2** (Heterogeneous nuclear ribonucleoproteins A2); **S2542** (Solute carrier family 25 member 42); **SBP2** (Selenium-binding protein 2); **SNX5** (Sorting nexin-5); **THIM** (3-ketoacyl-CoA thiolase); **TPIS** (Triosephosphate isomerase); **VDAC1** (Voltage-dependent anion-selective channel protein 1).

Table1.

	SSP		AC	name	IR/control	p value	function	Match. pept./ 25	coverage %	Mascot score
HP	1506	EF1B	O70251	Elongation factor 1-beta	0,11	0,002	protein biosynthesis	5	24	56
	2503	Mixture			0,34	0,038				108
		RGN	Q64374	Regucalcin			calcium binding protein; vitamine C biosynthesis	8	31	88
		KPYR	P53657	Pyruvate kinase isozymes R/L			metabolism, glycolysis	7	18	60
	2602	ATPB	Q3U774	ATP synthase subunit beta, mitochondrial	0,25	0,009	metabolism, oxidative phosphorylation	15	44	192
	4303	FABPI	P55050	Fatty acid-binding protein, intestinal	0,32	0,013	metabolism, lipid binding protein	8	54	106
	4703	ENOA	P17182	Alpha-enolase	0,21	0,032	metabolism, glycolysis	9	36	116
	6402	ATPA	Q03265	ATP synthase subunit alpha, mitochondrial	0,39	0,015	metabolism, oxidative phosphorylation	8	21	61
LSEC	701	PDIA1	P09103	Protein disulfide-isomerase	0,18	0,054	stress protein	12	28	126
	1604	ATPB	Q3U774	ATP synthase subunit beta, mitochondrial	0,14	0,029	metabolism, oxidative phosphorylation	16	45	222
	3505	CLC4F	P70194	C-type lectin domain family 4 member F	0,20	0,039	metabolism, receptor with an affinity for galactose and fucose	7	15	56
	5512	CATA	P24270	Catalase	0,05	0,040	stress protein	9	24	93
	5515	ODBA	P50136	2-oxoisovalerate dehydrogenase subunit alpha, mitochondrial	0,26	0,015	metabolism, conversion of alpha-keto acids to acyl-CoA and CO2	13	38	138
	5908	CJ088	Q9D2Q3	Uncharacterized protein C10orf88 homolog	0,12	0,016	unknown	6	18	57
	7406	NDKB	Q01768	Nucleoside diphosphate kinase B	0,26	0,035	metabolism, synthesis of nucleoside triphosphates other than ATP	11	69	132
	8409	ROA2	O88569	Heterogeneous nuclear ribonucleoproteins A2/B1	0,25	0,052	pre-mRNA processing	6	20	57

IR modulated spots : Spot number (SSP), accession number on Swiss Prot database (AC), name, densitometry ratio between IR and control sample, p value, biological function, number of

matched mass values (match. pept.) on number of total mass values searched (25), coverage percentage and Mascot score are indicated.

Table 2.

	SSP		AC	name	CGS/control	p value	function	match. pept./ 25	coverage %	Mascot score
HP	1604	IPYR	Q9D819	Inorganic pyrophosphatase	3,22	0,018	pyrophosphatase	10	42	122
	4901	GLYC	P50431	Serine hydroxymethyltransferase, cytosolic	0,48	0,025	metabolism, aminoacids	9	25	87
	5609	IDHC	O88844	Isocitrate dehydrogenase [NADP] cytoplasmic	4,74	0,009	metabolism, Krebs cycle	7	25	64
	9949	FABPL	P12710	Fatty acid-binding protein, liver	5,58	0,031	metabolism, lipids binding protein	6	61	82
LSEC	3410	Mixture			4,41	0,001				75
		OTC	P11725	Ornithine carbamoyltransferase, mitochondrial			metabolism, urea cycle	6	22	59
		PGK1	P09411	Phosphoglycerate kinase 1			metabolism, glycolisis	6	26	57
	5602	ENOA	P17182	Alpha-enolase	1,67	0,051	metabolism, glycolisis	13	44	154
	8604	THIM	Q8BWT1	3-ketoacyl-CoA thiolase, mitochondrial	2,84	0,052	metabolism, fatty acids beta oxidation	13	51	166

CGS modulated spots: Spot number (SSP), accession number on Swiss Prot database (AC), name, densitometry ratio between CGS and control sample, p value, biological function number of matched mass values (match. pept.) on number of total mass values searched (25), coverage percentage and Mascot score are indicate

Table:3

	SSP		AC	name	CGS+IR/ control	p value	function	match. pept./ 25	coverage %	Mascot score
HP	804	GRP75	P38647	Stress-70 protein, mitochondrial	0,47	0,042	stress protein	7	14	61
	2202	FABPI	P55050	Fatty acid-binding protein, intestinal	5,51	0,039	metabolism, lipids binding protein	8	54	106
	2203	ABHEB	Q8VCR7	Abhydrolase domain-containing protein 14B	4,19	0,051	hydrolase activity towards p-nitrophenyl butyrate	5	37	64
	4202	GSTP1	P19157	Glutathione S-transferase P 1	0,46	0,007	stress protein	7	40	84
	4803	ARGI1	Q61176	Arginase-1	2,99	0,029	metabolism, urea cycle	10	40	105
	8201	MCM2	P97310	DNA replication licensing factor MCM2	13,09	0,020	DNA replication	10	12	62
	9105	FABPL	P12710	Fatty acid-binding protein, liver	0,26	0,030	metabolism, lipids binding protein	7	60	78
LSEC	3301	ATP5H	Q9DCX2	ATP synthase subunit d, mitochondrial	5,79	0,029	metabolism, oxidative phosphorylation	5	39	58
	4401	OTC	P11725	Ornithine carbamoyltransferase, mitochondrial	22,01	0,012	metabolism, urea cycle	6	19	57
	4605	Mixture			6,20	0,014				75
		OTC	P11725	Ornithine carbamoyltransferase, mitochondrial			metabolism, urea cycle	6	22	59
		PGK1	P09411	Phosphoglycerate kinase 1			metabolism, glycolisis	6	26	57
	4702	CATA	P24270	Catalase	15,27	0,019	stress protein	9	24	93
	6210	FABPL	P12710	Fatty acid-binding protein, liver	7,38	0,049	metabolism, lipids binding protein	7	51	60
	6212	ASXL3	Q8C4A5	Putative Polycomb group protein ASXL3	110,23	0,002	transcriptional control	12	8	56
	7702	THIM	Q8BWT1	3-ketoacyl-CoA thiolase, mitochondrial	3,15	0,044	metabolism, fatty acids beta oxidation	8	28	82
	SSP		AC	name	CGS+IR/ CGS	p value	function	match. pept./ 25	coverage %	Mascot score
HP	1502	K2C8	P11679	Keratin, type II cytoskeletal 8	0,27	0,035	structural	11	24	106
	2504	Mixture			3,70	0,042				108
		RGN	Q64374	Regucalcin			calcium binding protein; vitamine C biosynthesis	8	31	88
		KPYR	P53657	Pyruvate kinase isozymes R/L			metabolism, glycolysis	7	18	60
	3502	IPYR	Q9D819	Inorganic	0,35	0,027	pyrophosphatase	10	42	122

				pyrophosphatase						
	3601	ENOA	P17182	Alpha-enolase	2,47	0,033	metabolism, glycolysis	9	27	92
	3702	SBP2	Q63836	Selenium-binding protein 2	3,69	0,018	stress protein	10	33	121
	3705	SBP2	Q63836	Selenium-binding protein 2	2,77	0,029	stress protein	11	29	134
	3708	GRP75	P38647	Stress-70 protein, mitochondrial	5,09	0,010	stress, folding of proteins	9	16	82
	4401	3HAO	Q78JT3	3-hydroxyanthranilate 3,4-dioxygenase	2,98	0,045	cofactor biosynthesis	6	31	61
	4501	ARGI1	Q61176	Arginase-1	3,59	0,004	metabolism, urea cycle	9	40	103
	4706	ENOA	P17182	Alpha-enolase	0,25	0,021	metabolism, glycolysis	9	36	116
	4707	ALDH2	P47738	Aldehyde dehydrogenase, mitochondrial	4,52	0,011	stress protein	14	33	181
	5203	PPIA	P17742	Peptidyl-prolyl cis-trans isomerase A	3,25	0,027	folding of proteins	5	24	74
	5302	GSTM1	P10649	Glutathione S-transferase Mu 1	3,63	0,043	stress, folding of proteins	7	39	75
	5502	ARGI1	Q61176	Arginase-1	6,34	0,055	metabolism, urea cycle	10	36	112
	5504	DHSO	Q64442	Sorbitol dehydrogenase	3,08	0,026	metabolism, sorbitol to fructose conversion	7	32	71
	5505	Mixture			4,89	0,004				106
		DHSO	Q64442	Sorbitol dehydrogenase			metabolism, sorbitol to fructose conversion	8	36	86
		ARGI1	Q61176	Arginase-1			metabolism, urea cycle	6	25	58
	5506	ARGI1	Q61176	Arginase-1	5,21	0,015	metabolism, urea cycle	10	40	105
	5508	ALDOB	Q91Y97	Fructose-bisphosphate aldolase B	3,59	0,026	metabolism, glycolysis	6	20	56
	5603	FAAA	P35505	Fumarylacetoacetase	3,81	0,051	metabolism, aa degradation	10	39	108
	5701	ENOA	P17182	Alpha-enolase	2,37	0,050	metabolism, glycolysis	13	44	154
	6203	MIC1	Q8VC42	Uncharacterized protein C18orf8 homolog	2,52	0,021	unknown	7	20	70
	6302	ATPA	Q03265	ATP synthase subunit alpha, mitochondrial	1,85	0,029	metabolism, oxidative phosphorylation	8	21	61
	6402	ETFA	Q99LC5	Electron transfer flavoprotein subunit alpha, mitochondrial	4,14	0,038	metabolism, oxidative phosphorylation	8	39	92
	7401	CAH3	P16015	Carbonic anhydrase 3	2,58	0,036	reversible hydration of carbon dioxide	12	51	168
	9402	VDAC1	Q60932	Voltage-dependent anion-selective channel protein 1	2,41	0,042	cell volume regulation and apoptosis	7	44	89

	9601	THIM	Q8BWT1	3-ketoacyl-CoA thiolase, mitochondrial	3,37	0,020	metabolism, fatty acids beta oxidation	11	39	136
LSEC	2105	DNM3A	O88508	DNA (cytosine-5)-methyltransferase 3A	27,48	0,043	DNA methylation	9	11	66
	2201	PROSC	Q9Z2Y8	Proline synthase co-transcribed bacterial homolog protein	8,96	0,013	metabolism, aminoacids	5	20	57
	2203	MUP8	P04938	Major urinary proteins 11 and 8 (Fragment)	2,71	0,053	pheromones binding	10	78	130
	2308	PEBP1	P70296	Phosphatidylethanolamine-binding protein 1	0,24	0,053	ATP, opioids and phosphatidylethanolamine binding	8	60	124
	3201	ATP5H	Q9DCX2	ATP synthase subunit d, mitochondrial	0,48	0,036	metabolism, oxidative phosphorylation	5	39	58
	3304	PRDX6	O08709	Peroxiredoxin-6	92,13	0,002	stress protein	8	32	98
	3601	ATPB	Q3U774	ATP synthase subunit beta, mitochondrial	9,79	0,003	metabolism, oxidative phosphorylation	15	44	184
	3701	CH60	P63038	60 kDa heat shock protein, mitochondrial	0,32	0,021	stress protein	10	32	98
	5304	PRDX6	O08709	Peroxiredoxin-6	11,16	0,010	stress protein	8	51	111
	5401	PDIA3	P27773	Protein disulfide-isomerase A3	4,37	0,021	stress protein	12	25	129
	5501	Mixture			5,04	0,010				213
		3HAO	Q78JT3	3-hydroxyanthranilate 3,4-dioxygenase OS=Mus musculus			catalyzes the oxidative ring opening of 3-hydroxyanthranilate to 2-amino-3-carboxymuconate semialdehyde, which spontaneously cyclizes to quinolinate	11	52	131
		ECH1	O35459	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial			metabolism, fatty acids beta oxidation	11	48	114
	6302	EF2	P58252	Elongation factor 2	18,04	0,006	protein synthesis	14	18	115
	7302	CPSM	Q8C196	Carbamoyl-phosphate synthase [ammonia], mitochondrial	15,04	0,001	metabolism, urea cycle	11	9	64
	7606	FAAA	P35505	Fumarylacetoacetase	0,10	0,012	metabolism, aminoacids degradation	6	24	69
	9105	GSTP2	P46425	Glutathione S-transferase P 2	16,76	0,052	stress protein	5	39	60
	9701	AL4A1	Q8CHT0	Delta-1-pyrroline-5-carboxylate	8,06	0,035	metabolism, aminoacids degradation	7	23	78

				dehydrogenase, mitochondrial						
	9702	Mixture			0,33	0,056				84
		CHD1	P40201	Chromodomain-helicase-DNA-binding protein 1			DNA replication	12	9	70
		ATPA	Q03265	ATP synthase subunit alpha, mitochondrial			metabolism, oxidative phosphorylation	8	18	61
	SSP		**AC**	**name**	**CGS+IR/ IR**	**p value**	**function**	**match. pept./ 25**	**coverage %**	**Mascot score**
HP	202	CALM	Q498A3	Calmodulin	2,51	0,020	control of a large number of enzymes, ion channels and other proteins by Ca^{2+}	5	50	57
	3701	SBP2	Q63836	Selenium-binding protein 2	2,82	0,017	stress protein	10	30	114
	3705	SBP2	Q63836	Selenium-binding protein 2	3,55	0,040	stress protein	11	29	134
	4501	ARGI1	Q61176	Arginase-1	2,21	0,022	metabolism, urea cycle	9	40	103
	5504	DHSO	Q64442	Sorbitol dehydrogenase	2,44	0,044	metabolism, sorbitol to fructose conversion	7	32	71
	5505	Mixture			2,36	0,021				106
		DHSO	Q64442	Sorbitol dehydrogenase			metabolism, sorbitol to fructose conversion	8	36	86
		ARGI1	Q61176	Arginase-1			metabolism, urea cycle	6	25	58
	5506	ARGI1	Q61176	Arginase-1	2,62	0,036	metabolism, urea cycle	10	40	105
	5602	GLYC	P50431	Serine hydroxymethyltransferase, cytosolic	2,35	0,013	metabolism, aminoacids	9	25	87
	5603	FAAA	P35505	Fumarylacetoacetase	2,99	0,051	metabolism, aminoacids degradation	10	39	108
	5701	ENOA	P17182	Alpha-enolase	2,95	0,037	metabolism, glycolysis	13	44	154
	6401	CPSM	Q8C196	Carbamoyl-phosphate synthase [ammonia], mitochondrial	3,04	0,002	metabolism, urea cycle	10	8	65
	7301	Mixture			2,94	0,045				98
		TPIS	P17751	Triosephosphate isomerase			metabolism, glycolysis	7	28	77
		S2542	Q8R0Y8	Solute carrier family 25 member 42			metabolism, transport of coenzyme A (CoA) in mitochondria	7	23	56
	7604	THIM	Q8BWT1	3-ketoacyl-CoA thiolase, mitochondrial	3,46	0,041	metabolism, fatty acids beta oxidation	8	28	82
	8302	GSTP1	P19157	Glutathione S-transferase P 1	2,88	0,031	stress protein	5	37	61

	8602	THIM	Q8BWT1	3-ketoacyl-CoA thiolase, mitochondrial	3,84	0,033	metabolism, fatty acids beta oxidation	13	51	166
	8605	THIM	Q8BWT1	3-ketoacyl-CoA thiolase, mitochondrial	5,35	0,045	metabolism, fatty acids beta oxidation	14	39	173
	8607	THIM	Q8BWT1	3-ketoacyl-CoA thiolase, mitochondrial	5,34	0,030	metabolism, fatty acids beta oxidation	8	28	82
	9502	FKB1B	Q9Z2I2	Peptidyl-prolyl cis-trans isomerase FKBP1B	3,30	0,004	folding of proteins	5	38	66
	9601	THIM	Q8BWT1	3-ketoacyl-CoA thiolase, mitochondrial	2,51	0,034	metabolism, fatty acids beta oxidation	11	34	124
LSEC	1303	SNX5	Q9D8U8	Sorting nexin-5	27,99	0,028	intracellular trafficking	6	24	57
	1405	CATB	P10605	Cathepsin B	10,43	0,004	intracellular degradation and turnover of proteins	6	23	66
	2909	GRP75	P38647	Stress-70 protein, mitochondrial	6,15	0,040	stress protein	9	17	72
	2912	GRP75	P38647	Stress-70 protein, mitochondrial	11,47	0,017	stress protein	8	15	57
	3101	FABP5	Q05816	Fatty acid-binding protein, epidermal	4,13	0,002	metabolism, lipids binding protein	5	31	59
	3303	PRDX6	O08709	Peroxiredoxin-6	8,23	0,052	stress protein	8	51	111
	3404	CK054	Q91V76	Ester hydrolase C11orf54 homolog	7,03	0,003	ester hydrolase activity on the substrate p-nitrophenyl acetate	8	30	104
	4103	HINT1	P70349	Histidine triad nucleotide-binding protein 1	3,70	0,006	Hydrolyzes purine nucleotide phosphoramidates with a single phosphate group	7	71	97
	5301	ETFA	Q99LC5	Electron transfer flavoprotein subunit alpha, mitochondrial	3,69	0,035	metabolism, oxidative phosphorylation	6	31	63
	5502	ARGI1	Q61176	Arginase-1	5,69	0,020	metabolism, urea cycle	11	49	136
	7104	PPIA	P17742	Peptidyl-prolyl cis-trans isomerase A	3,52	0,052	folding of proteins	5	34	59

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PAPER- 2

ISCHEMIA/REPERFUSION INJURY ON MICE STEATOTIC HEPATOCYTES AND DIFFERENTIAL EFFECTS OF ADENOSINE A2A AND A1 RECEPTORS STIMULATION

Summary

Ischemia re-perfusion injury is regarded as a major cause of liver dysfunction or failure, after tissue resection and transplantation and these problems are particularly evident in patients with fatty livers. The lack of available organs has forced the use of steatotic liver for transplantation despite their higher susceptibility to ischemia-reperfusion injury. In order to elucidate the variability of the protective action of Ischemic Preconditioning (IP), we investigated the effect of separate adenosine receptors stimulation on JNK-dependent lipotoxicity and on hypoxia/ reoxygenation (H/R) injury of steatotic mice hepatocytes.

In this study mice hepatocytes were exposed to Palmitic acid (50 μM) to induce steatosis and then incubated in presence or absence of the adenosine A2 receptor agonist CGS21680b [2-p-(2-carboxyethyl)phenethylamino-5-Nethyl carboxyamidoadenosine] (5μM) or the A1 receptor agonist CCPA [2-Chloro-N6-cyclopentyl adenosine] (100μM) and stored at 4°C for hypoxia induction. After cold ischemic preservation, steatotic hepatocytes were exposed to reoxygenation at 37°C. For "in vivo" experiments, mice were fed with HFD diet for 9 weeks and treated with or without CCPA (1.5 mg/kg) and CGS21680 (0.5 mg/kg) and ASK1 inhibitor NQDI-1 (50μM) and subjected to liver ischemia/reperfusion.

The results obtained indicated that damage of steatotic hepatocytes exposed to H/R is mediated by the activation of the ASK1-JNK axis and increased sensitivity of steatotic hepatocytes to H/R injury is related to an augmented and ROS-dependent stimulation of this cytotoxic pathway. CGS21680 A2aR stimulation protected steatotic hepatocytes exposed to H/R by activating the PI3K-Akt axis, which blocks ASK-1/JNK axis through inhibitory phosphorylation of Ask-1 in Ser83. On the contrary, CCPA A1R stimulation was unable to exert hepato-protection being unable to induce the Akt dependent negative regulation of Ask-1. In HFD fed steatotic mice subjected to I/R, NQDI-1 or CGS21680 treatment showed to prevent liver damage induced by steatosis and I/R and inhibit ASK1 and JNK activation, while CCPA treatment further increases liver damage due to an augmented JNK and Ask1 activation

Altogether these observations suggest A2aR activation and Ask1 inhibition as effective protective conditions against I/R injury of steatotic liver and suggest that pharmacological interventions aimed to directly stimulate A2aR or block ASK1 can represent a novel and efficient therapeutic approach to prevent the injurious consequences of I/R application in fatty livers.

ISCHEMIA/REPERFUSION INJURY ON MICE STEATOTIC HEPATOCYTES AND DIFFERENTIAL EFFECTS OF ADENOSINE A2A AND A1 RECEPTORS STIMULATION

Bangalore R Chandrashekar, Chiara Imarisio, Elisa Alchera, and Rita Carini*

Department of Health Science, University of Piedmont Orientale, Novara, Italy

A B S T R A C T

Hepatic steatosis is a major risk factor after liver surgery because steatotic livers poorly tolerate ischemia/reperfusion (I/R) injury and is the main cause of both initial poor function and primary non function of liver allograft. In the light of the growing need for new therapeutic options specific for protective strategies for the I/R injury of steatotic livers during liver transplantation, we investigated the pharmacological preconditioning action of different adenosine receptors against IR injury on steatotic liver. The effects of the A2aR agonist CGS21680b[2-p-(2-carboxyethyl)phenethylamino-5-Nethyl carboxyamidoadenosine] and A1R agonist CCPA-2-Chloro-N6-cyclopentyl adenosine were evaluated 'in vitro' in liver cells exposed to PA (Palmitic acid) and 'in vivo' in mice with steatosis induced by 9 weeks of feeding with an HFD diet (high fat diet). In primary mice steatotic hepatocytes, CGS21680- A2aR stimulation protected fatty hepatocytes exposed to I/R by activating the PI3K-Akt axis which blocks ASK-1/JNK axis through inhibitory phosphorylation of Ask-1 in Ser83. On the contrary, CCPA -A1R stimulation was unable to exert hepato-protection being unable to induce the Akt dependent negative regulation of Ask-1. In mice receiving the HFD diet, the development of steatosis was associated with JNK-1/2 activation. CGS21680 (0.5 mg/kg of body weight, intraperitoneal) but not CCPA (1.5 mg/kg of body weight, intraperitoneal) administration to HFD-fed mice, effectively reduced HFD-associated ALT (alanine aminotransferase) release and prevented JNK-1/2 and Ask1 activation. Taken together, these results indicate that pharmacological interventions aimed to directly stimulate A2aR or block ASK1 can represent a novel and efficient therapeutic approach to prevent the injurious consequences of I/R application in fatty livers.

Keywords: Ischemia reperfusion injury; Steatosis; Adenosine receptors; steatotic liver;

*Corresponding author. Address: Department of Health Sciences, University of Piedmont Orientale 'A. Avogadro', via Solaroli, 17, Novara 28100, Italy. Tel.: +39 0321660685; fax: +39 0321620421.

E-mail address: carini@med.unipmn.it (R. Carini).

Abbreviations: HP, hepatocytes; SHp, steatotic hepatocytes; A2aR, adenosine 2a receptor; IR, ischemia-reperfusion;CGS21680,2p-(2-carboxyethyl)-phenyl-amino-50-Nethylcarboxyamido-adenosine; ROS-reactive oxygen species; CCPA-2-Chloro-N6-cyclopenty–l adenosine, HFD-high fat diet, NQDI-1-2,7-Dihydro-2,7-dioxo-3H-naphth-o[1,2,3dequin- oline-1-carboxylic acid ethyl ester.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Authors' contributions

Bangalore.R.Chandrashekar, Chiara Imarisio, Elisa Alchera performed the experiments; Chiara Imarisio, Elisa Alchera and Rita Carini planified the study; B.R Chandrashekar, Chiara Imarisio, Elisa Alchera and Rita Carini wrote the manuscript, Rita Carini critically revised the manuscript.

1. INTRODUCTION

Hepatic ischemia/reperfusion (I/R) injury is defined as the phenomenon during which cellular damage in an organ, caused by hypoxia, is paradoxically exacerbated after the restoration of oxygen delivery [1]. It is a dynamic process which involves the two interrelated phases of local ischemic insult and inflammation-mediated reperfusion injury [2]. Hepatic I/R injury is a frequent and major complication in clinical practice, which compromises liver function and increases postoperative morbidity, mortality, recovery and overall outcome and is the main cause of both initial poor function and primary non function of liver allograft [3]. Liver, being an organ with high energy requirements, is highly dependent on oxygen supply and susceptible to hypoxic or anoxic conditions [4]. The shortage of organs has led to expand the criteria for the acceptance of marginal donors, including the use of steatotic grafts [5].

Moreover hepatic steatosis is a major risk factor after liver surgery because steatotic livers tolerate poorly I/R injury. After major liver resection, steatosis is associated with mortality higher than 14% respect to the 2% using normal liver. The tolerance of the liver to I/R injury is reduced dramatically due to the presence of fatty infiltration. Several hypotheses have been suggested to explain the decreased tolerance of steatotic liver to I/R injury compared with normal livers. These include increased lipid peroxidation, neutrophil infiltration, and release of pro-inflammatory mediators and the alteration of micro circulation [6]. The latter process is the major event of re-perfusion injury in steatotic livers, and is due to the abnormal accumulation of fat within the cytoplasm of hepatocytes, resulting in increased hepatocellular volume and narrowing of sinusoid, compromising the suitable graft revascularization and viability after transplantation. Moreover, several evidences indicated that an increased sensitivity of fatty hepatocytes to the harmful effects of reactive oxygen species (ROS) plays a pathogenic role in this event. All together, these studies suggested different therapeutic strategies to prevent loss of liver functions and failed to focus more on the search of specific protective strategies for the steatotic livers.

A short period of ischemia with subsequent re-perfusion triggers natural defense mechanism against future ischemic insults and protects the organ against the IR damage (IR). This phenomenon is regarded as ischemic preconditioning (IP) [8]. IP can be applied intermittently or as a single short period of 5-10 ten minutes of ischemia followed by 10-15 minutes re-perfusion [9].

“In vivo” and “in vitro” studies have clearly established that the onset of IP is triggered by the release of adenosine and by the subsequent stimulation of adenosine A2a receptor [10-14]. This was confirmed in our laboratory with experiments using primary rat hepatocytes. In this model, the released adenosine to extra-cellular space induced hepatocyte protection by the autocrine stimulation of A2a receptors [15]. Ischemic preconditioning has also shown to be effective in reducing re-perfusion damage during hepatic resection in humans, as well as to improve the outcome of hepatic transplants in experimental animals. These beneficial effects are particularly evident in fatty livers where preconditioning reduced by about 50%, the transaminase release and histological evidence of necrosis [6]. Surgical ischemic preconditioning raised hopes that it could

be applied to patients to prevent the side-effect of major liver surgery, but the application of IP in clinical trials have given conflicting results as in some cases IP did not afford protection and in other its protective action was variable [16-21].

Thus the mechanism by which steatosis increases the sensitivity of the liver to ischemia-re-perfusion injury is poorly understood. More specifically, the primary event responsible for the exacerbated response has not been identified, at the same time the surgical ischemic preconditioning gave promising results in animal models but when applied on human, often failed to induce reliable graft protection. This gave a rationale to investigate the effects of adenosine receptor agonist on steatotic mice hepatocytes in order to evaluate the possible use of these agents for liver pharmacological preconditioning.

MATERIALS AND METHODS

Hepatocytes isolation, preparation and treatments:

Male mice C57BL/6 weighing 20-30g were anesthetized i.p. with Zoletil/Xilazine mixture (Zoletil 43mg/Kg; Xilazine 17,2mg/Kg) were used for isolating primary murine hepatocytes by perfusing liver with collagenase. The liver was washed via the portal vein at first with T1 buffer at 37°C and later with T2 buffer containing collagenase for liver digestion. The hepatocytes were purified from the other cells by centrifugation at 500 rpm for 5 minutes followed by a further 3 minutes centrifugation at 1050 rpm through a layer of Percoll. Cell purity was assessed according to Benten [22] Cell viability, estimated at the beginning of experiments, ranged between 82% and 90%. **Details provided in Supplementary Materials**. After counting, isolated hepatocytes were centrifuged and resuspended in Viaspan solution (University of Wisconsin Solution without additives) at $1x10^6$ cells/ml. Hepatocytes suspended in Via Span solution were kept for 16hrs in hypoxic atmosphere (95%N_2 and 5%C_{O2}) at 4°C (H). Palmitic Acid (50µM) was added in Viaspan solution to induce steatosis in hepatocytes. Isolated hepatocytes, where indicated, were treated with the following drugs: CGS21680 (5µM) and CCPA (100µM), the A2a and A1a receptors agonist respectively, JNK inhibitor - SP600125 (10µM), ASK1 inhibitor-NQDI1 (500nM), ER stress inhibitor- APY29 (285nM), DPPD (5µM), PI3K inhibitor-Wortmannin (250nM). After 16hrs of cold storage, the hepatocytes were reoxygenated by fluxing (95%air and 5%CO_2) gas mixture in DMEM medium on the heater at 37°C (R) for 0, 15 and 30 minutes time course analysis (H/R T0', T15', T30').

Determination of cell viability

Cell viability was estimated by microscope-counting of the hepatocyte by Trypan blue excluding test and viability was also estimated by the determination of nuclear fluorescence staining with propidium iodide using a FACScan analyser (Becton-Dickinson, San Jose, CA) and Cell Quest software (Becton-Dickinson). **Details provided in the Supplementary Materials.**

Steatosis colorimetric assay:

Intracellular lipid accumulation in the mouse hepatocytes was evaluated using the Steatosis Colorimetric Assay Kit (Cayman Chemical), according to the manufacturer's instructions. To evaluate the intracellular lipid distribution in mouse hepatocytes steatosis slides were prepared using ORO staining. **Details provided in the Supplementary Materials.**

Measurement of Reactive Oxygen Species (ROS):

Intracellular ROS quantity was measured by the method of Jakubowski and Bartosz [23], with minor modifications, by quantifying the DCFH-DA (2,7-dichlorofluorescin diacetate) fluorescence intensity with Victor X4 2030 multi reader (Perkin Elmer). **Details provided in the Supplementary Materials.**

C1C7: Transfection and Treatment:

The C1C7 mice Hepatocarcinoma cell line was obtained from the European Collection of Cell Cultures and cultured on modified DMEM medium containing 10% FBS (fetal bovine serum), 1% penicillin/streptomycin. Murine ASK1 SiRNAs (small interfering RNA) were purchased from Sigma-Mission (Milan, Italy). Sequences are as follows: SiRNA2 sense: CAGAUAGUCCACCGGGAUAdTdT and antisense: UAUCCCGGUGGACUAUCUGdTdT. Control siRNA was used as negative control of transfection. C1C7 cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer recommendations. Transfection efficacy was analyzed after 48 hrs using BLOCK-iT Fluorescent Oligo (Invitrogen) and the transfection efficiency of C1C7 cells was more than 75%. **Details provided in the Supplementary Materials.**

Data analysis:

Statistical analysis was performed with InStat 3 statistical software (Graph Pad Software, Inc., San Diego, CA) by 1-way analysis of variance, testing with Bonferroni correction for multiple comparisons when more than 2 groups were analysed. The distribution normality of all groups was preliminarily verified with the Kolmogorov and Smirnov test. Significance was established at the 5% level.

In Vivo Studies:

Male C57BL/6 mice used for this study were purchased from Harlan SRL, Italy. All experiments involving animals were approved by the Italian Ministry of Health and the ethical committee for animal care of the University del Piedmont Orientale ''A. Avogadro''. Mice were fed with an isocaloric control diet or an high fat diet to induce steatosis (HFD: 58% of energy derived from fat, 18% from protein, and 24% from carbohydrates; 5.6 kcal/g) (Laboratorio Dottori Piccioni,

Gessate, Milan, Italy) for 9 weeks. From the second week control or steatotic mice were treated by intraperitoneal injection twice a week either with sterile saline or CGS21680 (0.5 mg/kg of body weight in sterile saline) or CCPA (1.5 mg/kg of body weight in sterile saline) or NQDI-1 (50μM). At the end of the dietary and pharmacological treatments, mice were subjected to ischemia/reperfusion injury. At the end of the treatments mice were exposed to a non-lethal (70% of the total liver volume) hepatic ischemia for 30 min, followed by 120 min reperfusion as previously described (Mandili et al., 2015). Immediately after the reperfusion period, blood was collected to assess liver injury by measuring the ALT serum transaminase activity, with a commercial kit (Gesan Production, Italy), and then mice were sacrificed and the liver fragments immediately frozen in liquid nitrogen for WB analysis and triglyceride content determination. **Details provided in the Supplementary Materials.**
Liver triglyceride accumulation and hepatocyte injury as serum transaminase activity were assessed by measuring with a commercial kit (Gesan Production, Italy) according to the manufacture instructions.

Analysis of the phosphorylation state of AKT, JNK, TRAF2 and ASK1:

Akt, JNK and TRAF2 and ASK1 Protein extracts were electrophoresed by SDS/PAGE (10% gel) and, after blotting on to nitrocellulose membranes, the membranes were probed with antibodies against phospho-Akt (Ser473), Akt, phospho-JNK (Thr183/Tyr185), JNK, TRAF2 (Cell Signaling Technology). Phospho-ASK1 (Thr845) (Biorbyt), inhibitory phospho-ASK1 (Ser83) (Sigma-Aldrich) and ASK-1 (Santa Cruz Technology).The β-actin monoclonal antibody (Sigma-Aldrich) was used to assess equal protein loading. The antigens were detected by Western Lightning Chemiluminescence Reagent plus (ECL) (PerkinElmer) and VersaDoc 3000 quantitative imaging system (BioRad Laboratories). The results were expressed as ratios. **Details provided in the Supplementary Materials.**

RESULTS

Effect of Palmitic Acid supplementation on hypoxia/reoxygenation injury of primary mouse hepatocytes

To investigate the mechanisms of ischemia/reperfusion (I/R) damage in the presence of steatosis we developed "in vitro" models of I/R by employing primary mouse hepatocytes. The hepatocytes were isolated by mouse liver perfusion with collagenase and were suspended in the University of Wisconsin Solution (UWS), the most commonly used graft preservation solution in clinical practice, and to reproduce the phase of ischemic liver graft preservation stored at 4°C for 16 hours in hypoxic conditions using flasks fluxed with $N_2$95 CO_2 5% (H). To evaluate the effect of I/R injury on steatotic hepatocytes, Palmitic acid (C16:0) (PA; 50uM) one of the most abundant circulating fatty acids (FFAs) in patients with nonalcoholic fatty liver disease (NAFLD) was added to the UWS solution. The reperfusion phase was then mimicked, exposing the hepatocytes,

previously preserved in the UWS, to an oxygenated atmosphere (O_2 95% and CO_2 5%) at 37°C in DMEM (R).

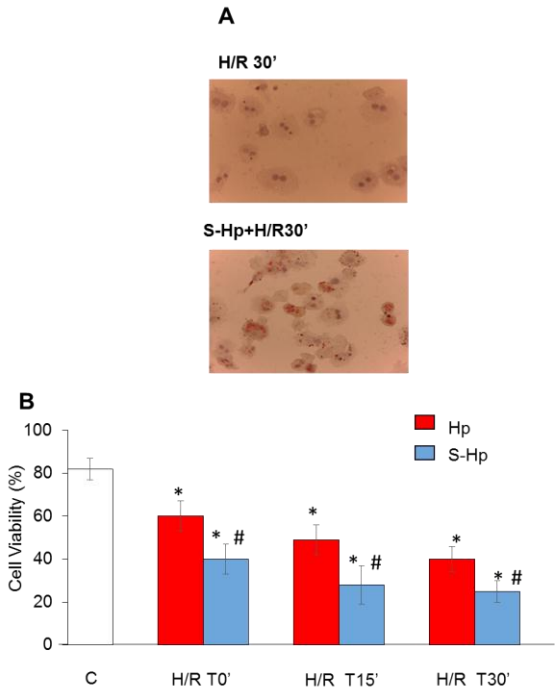

Figure 1: Effect of Palmitic Acid supplementation on hypoxia/reoxygenation (H/R) injury of primary mouse hepatocytes

A) *Steatosis was detected by Oil Red O staining (ORO) on murine primary hepatocytes exposed to 16hrs of hypoxia and to 30' of reoxygenation at 37°C and incubated with or without Palmitic acid (PA; 50μM). The images (20X) represent the two conditions after 30 min of reoxygenation.* ***B)*** *Steatotic hepatocytes (treated with PA 50μM: S-Hp) and non steatotic hepatocytes (Hp) were stored at 4°C in hypoxic conditions for 16hrs and reoxygenated at 37°C for 15 and 30 min. Cells viability was evaluated before hypoxic storage or after hypoxia (H/R T0') and after 15 and 30 min of reoxygenation (H/R T15' and T30'). The results are expressed as mean of 3 experiments ± SD.*p<0.05 vs Control; #p<0.05 vs Hp.*

As shown in the figure 1A PA (50 μM) treatment of primary hepatocytes exposed to H/R increased the intracellular lipid content, measured by Oil Red O staining, as compared to control primary hepatocytes exposed to H/R without PA treatment. Upon cold hypoxic storage cell viability was reduced by 20% and further decreased by another 20% following 30 min of warm reoxygenation (**Fig 1B**). In presence of PA cell damage was further increased by an additional 15%-20% after both hypoxia and reoxygenation.

Role of endoplasmic reticulum stress and JNK activation on hypoxia/reoxygenation damage of steatotic and non steatotic hepatocytes

Recent studies show that both ischemia/reperfusion (I/R) injury and hepatic steatosis can induce endoplasmic reticulum stress (ER). The adaptor protein tumour necrosis factor-receptor associated factor 2 (TRAF2) plays a central role in regulating cellular responses to death stimuli from the ER stress activating among other the JNK/SAPK pathway.

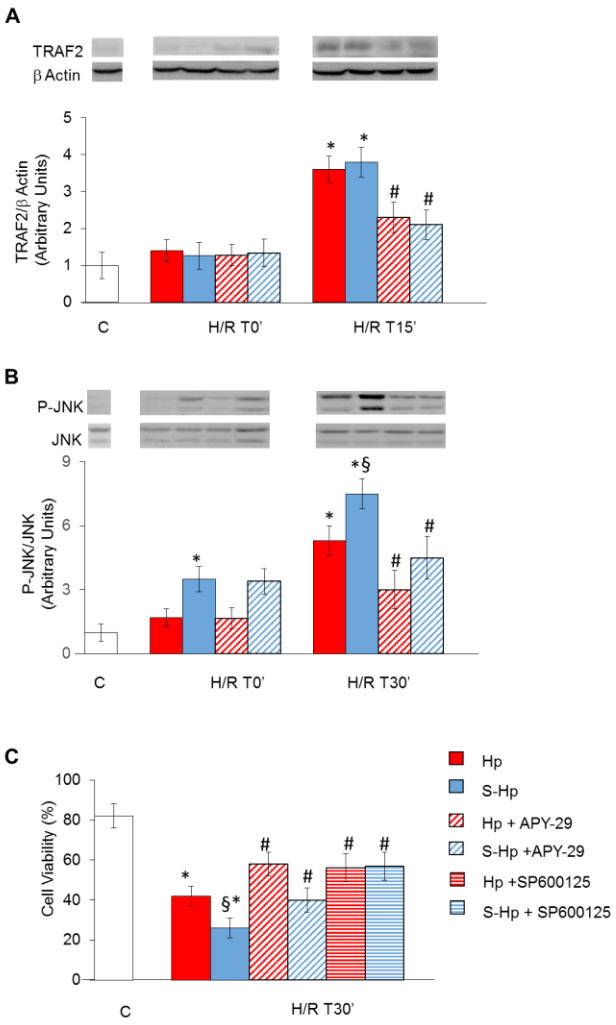

Figure 2: Role of TRAF2 and JNK in H/R damage of control and steatotic hepatocytes

Steatotic hepatocytes (PA 50μM) and non steatotic hepatocytes were stored at 4°C in hypoxic conditions for 16hrs and then reoxygenated at 37°C for 15'-30 min in presence or absence of APY29(285nM) ER stress inhibitor or SP600125(10μM) JNK inhibitor.

***A)** TRAF2 expression was evaluated before hypoxic storage (C) and after 15 min of reoxygenation at 37°C (H/R T15'). The results are expressed as ratio TRAF2/β-actin, and represent the mean of 3 experiments ± SD.*p<0.001 vs Control; # p<0.05 vs Hp+H/R T15' or S-Hp+H/R T15'*

***B)** JNK activation was evaluated before hypoxic storage (C) and after* 30 min of reoxygenation (H/R T30'). *JNK activation was evaluated as phosphorylation on Thr183/Tyr185. The results represent the mean of 3 experiments ± SD.*p<0.001 vs Control §p<0.05 vs Hp+H/R T30'; #p<0.05 vs Hp+H/R T30' or vs S-Hp +H/R T30'*

***C)** Cells viability was evaluated before hypoxic storage (C) and after 16 hrs of hypoxia and 30 min of reoxygenation (H/R T30'). The results are expressed as mean of 3 experiments ± SD. *p<0.001 vs Control; §p<0.05 vs Hp+H/R T30' #p<0.05 vs Hp+H/R T30' or S-Hp+H/R T30'.*

Accordingly, we observed an increase of TRAF2 expression in both control and steatotic hepatocytes exposed to hypoxia and subsequent 15 min of reoxygenation (**Fig 2A**), that was inhibited by the treatment with the ER stress inhibitor APY29 (285nM).

Furthermore, the measurement of JNK activation expressed as increased phosphorylation on Thr183/Tyr185 showed that subjecting hepatocytes to hypoxia/reoxygenation (H/R), induced a significant activation of JNK after 30' min of reoxygenation **(Fig 2B).** Interestingly we found that steatosis further augmented JNK activation and such process was independent from ER stress, since APY29, an ER stress inhibitor, abolished JNK activation only induced by H/R but did not affect the further increase of JNK activation induced by PA.

The role of ER stress and JNK in the development of H/R injury in steatotic and not steatotic hepatocytes was then investigated. As shown in figure 2C**,** APY29 significantly reduced the damage induced by H/R but did not decrease the further increase of toxicity induced by PA. On the contrary JNK inhibition by SP600125 protected the H/R damage both in control and in steatotic hepatocytes **(Fig 2C).** This indicated that H/R damage depended on ER stress related-

JNK activation both in control and in steatotic hepatocytes, but that the exacerbation of H/R damage in steatotic hepatocytes was still dependent by JNK but not induced by ER stress.

ROS-dependent and independent Ask1 and JNK activation mediates the hypoxia/reoxygenation injury of steatotic hepatocytes

Upon ER stress, ASK1 is the upstream mediator responsible for TRAF2-mediated JNK activation. Different studies also show that ASK1 activation can be induced by a mechanism related to an increased production of reactive oxygen species (ROS) [33]. These observations prompted us to evaluate the activation of ASK1 and its role in the development of H/R damage in our models of steatotic and non steatotic hepatocytes. As shown in the (**Fig 3A**), hypoxia followed by 15 min of reoxygenation induces the stimulation of ASK1, evaluated as increased phosphorylation of Thr845, in both control and steatotic hepatocytes. In steatotic hepatocytes, however, ASK1 stimulation is significantly greater than non steatotic cells. Moreover, the exposure to H/R in presence of APY29 entirely inhibited ASK1 activation in control hepatocytes, but only reduced ASK1 activation in steatotic hepatocytes. On the contrary, the treatment with the antioxidant DPPD decreased ASK1 activation but only in steatotic hepatocytes. This suggested that steatosis could induce an increase of oxidative stress that was involved in exacerbating the stimulation of ASK1. Indeed, the DCFH-DA fluorimetric determination of reactive oxygen species (ROS) **(Fig 3B**), evidenced that in steatotic hepatocytes exposed to H/R there was an increase of ROS production and as shown in figure 3B, ROS production was entirely prevented by DPPD addition. Additionally, we observed that PA 50μM exposure of mouse hepatocytes cultured 16 hours in normoxic conditions in DMEM at 37°C induced a significant increase of ROS production that was inhibited in presence of DPPD. This indicated that, independently from H/R exposure, steatosis is a condition "per se" sufficient to induce oxidative stress.

We next evaluated the role of the ROS-mediated ASK1 stimulation, on the JNK activation over the increased damage of steatotic hepatocytes exposed to H/R. As shown in figure 3C-a, DPPD prevented both the enhanced JNK activation **(Fig 3C-a)** and the increased toxicity induced by PA **(Fig 3C-b).** On the other hand, ASK1 inhibition prevented JNK activation and the H/R damage in both control and steatotic hepatocytes. All together these data indicated that ASK1 activation has a central role in the development of the H/R injury in steatotic hepatocytes and that the exacerbation of H/R damage in steatotic hepatocytes is due to a further increase of ASK1 stimulation.

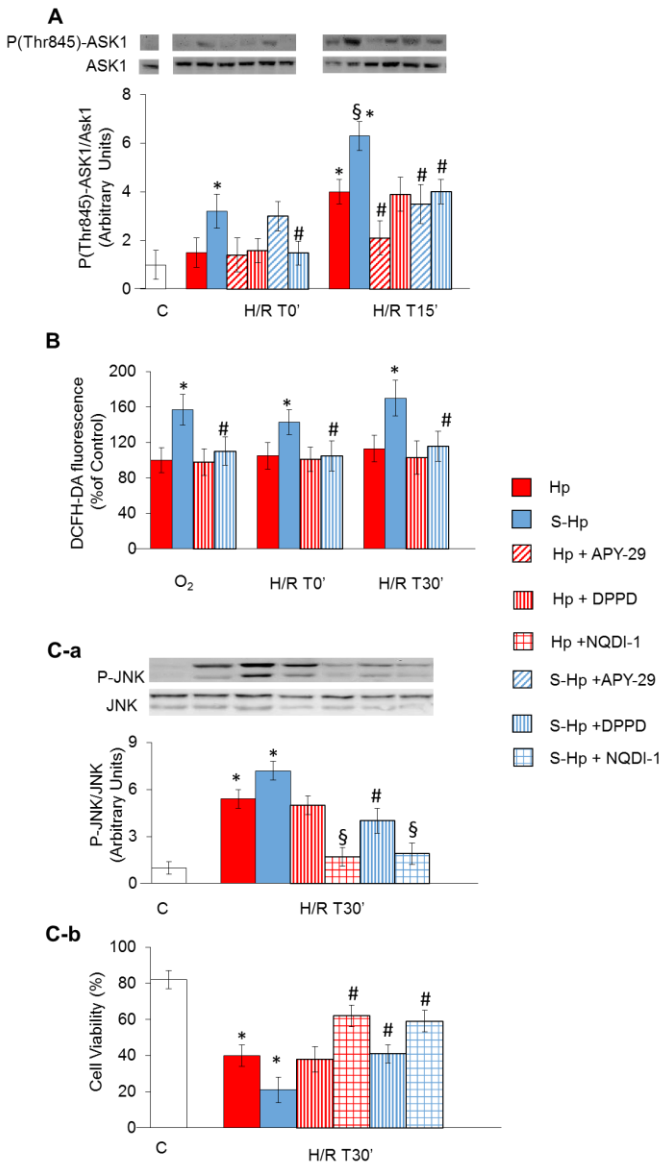

Figure 3: **Effect of ROS production on ASK1 and JNK activation and H/R damage of steatotic and non steatotic hepatocytes**

A) *Steatotic hepatocytes (PA50μM) and non steatotic hepatocytes were stored at 4°C for 16 hrs in hypoxic conditions and reoxygenated at 37°C, in presence or absence of the antioxidant DPPD (5μM) or of the ER stress inhibitor APY29(285nM). The stimulation of ASK1 was evaluated as phosphorylation in Thr845 before hypoxic storage (C) and after hypoxia (H/R T0') and after 15' of reoxygenation (H/R T15'). The results represent the mean of 3 experiments ± SD. *p<0.01 vs Control; §p<0.01 vs Hp+H/R T15'; #p<0.05 vs Hp+H/R T15' or S-Hp+H/R T0' and T15'.*

B) *Steatotic hepatocytes (PA 50μM) and non steatotic hepatocytes were stored at 4°C in hypoxic conditions for 16 hrs (H/R T0') and reoxygenated at 37°C for 30 min (H/R T30'), in presence or in absence of the antioxidant DPPD. In other experiments steatotic hepatocytes (PA 50μM) and non steatotic hepatocytes were maintained for 16 hrs in normoxic condition (O_2) in presence or in absence of the antioxidant DPPD. ROS production was evaluated as DCFH-DA fluorescence. The results represent the mean of 3 experiments ± SD. *p<0.05 vs Control; #p<0.05 vs S-Hp+ O_2 or S-Hp+H/R T0' or S-Hp+H/R T30'.*

C) *Steatotic hepatocytes (PA 50μM) and non steatotic hepatocytes were stored at 4°C in hypoxic conditions for 16 hrs and reoxygenated at 37°C for 30 min (H/R T30'), in presence or in absence of the antioxidant DPPD (5μM) or of the ASK1 inhibitor NQDI-1(500nM). a)JNK activation evaluated as phosphorylation on Thr183/Tyr185 and (b)Cell viability. The results represent the mean of 3 experiments ± SD. *p<0.001 vs Control; #p<0.05 vs S-Hp+H/R T30'; §p<0.001 vs Hp+H/R T30' or S-Hp+H/R T30'.*

Critical role of ASK 1 in hypoxia/reoxygenation injury of steatotic and non steatotic mice hepatoma cells

To confirm the critical role of ASK1 in the H/R injury of steatotic hepatocytes, we carried out experiment of silencing of ASK1. We decreased ASK1 expression with specific SiRNA in C1C7 hepatoma cells, treated with PA (700μM) for 16 hrs and exposed them to H/R. As shown in figure 4 the down regulation of ASK1 expression significantly reduced the damage of steatotic C1C7 cells exposed to H/R.

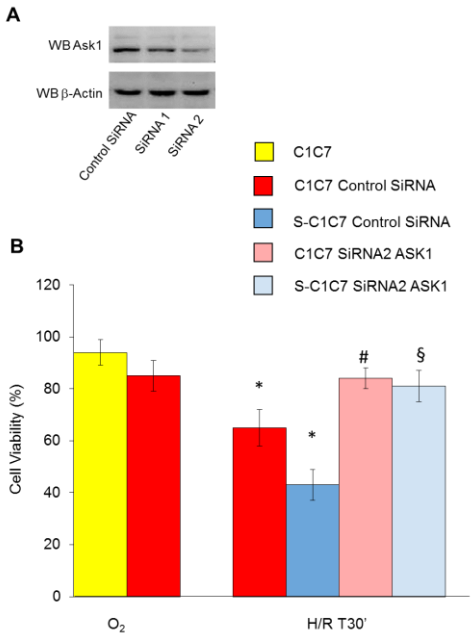

Figure 4: ASK1 down regulation in C1C7 reduces the hypoxia/reoxygenation injury of steatotic C1C7 cells.

*A) WB analysis of Ask1 expression after C1C7 transfection with Control SiRNA or two different ASK1 SiRNAs (SiRNA1 and SiRNA2). B) Steatotic C1C7 cells (PA 700μM) (S-C1C7) and non steatotic C1C7 cells, transfected with control SiRNA or SiRNA 2 stored at 4°C for 16 hrs in hypoxic conditions and reoxygenated at 37°C for 30 min (H/R T30'). The results represent the mean of 3 experiments ± SD. *p<0.01 vs C1C7 control SiRNA; #p<0.05vs C1C7 control SiRNA+H/R T30'; §p<0.01 vs S-C1C7 control SiRNA+H/R T30'.*

Differential effects of A1 and A2a receptor activation in hypoxia/reoxygenation injury of steatotic hepatocytes

The effect of A1 and A2a adenosine receptors stimulation on cell damage induced by subjecting steatotic hepatocytes to cold hypoxic storage and subsequent reoxygenation, were analyzed using specific pharmacological agonists of the two adenosine receptors, CCPA and CGS21680 respectively. Specifically, isolated hepatocytes were incubated in Wisconsin Solution at 4°C for 16 hrs with PA 50μM in presence or absence of CGS21680 (5μM) and CCPA (100μM) for hypoxic state and then exposed to reoxygenation at 37°C. As illustrated in Figure 5A, in steatotic hepatocytes exposed to hypoxia/reoxygenation (H/R), CGS21680 significantly reduced cell damage (25%), whereas CCPA increases it of 10%.

Although an increase of TRAF2 expression and JNK activation were associated to the development of the H/R injury of steatotic hepatocytes neither the treatment with A1 nor A2a adenosine receptors agonists, CCPA and CGS21680 was able to modulate TRAF2 expression induced by H/R (**Fig 5B).** On the contrary, the measurement of JNK activation showed that the activation of JNK induced by subjecting steatotic hepatocytes to H/R damage was significantly prevented by CGS21680 treatment and further increased by CCPA treatment **(Fig 5C).**

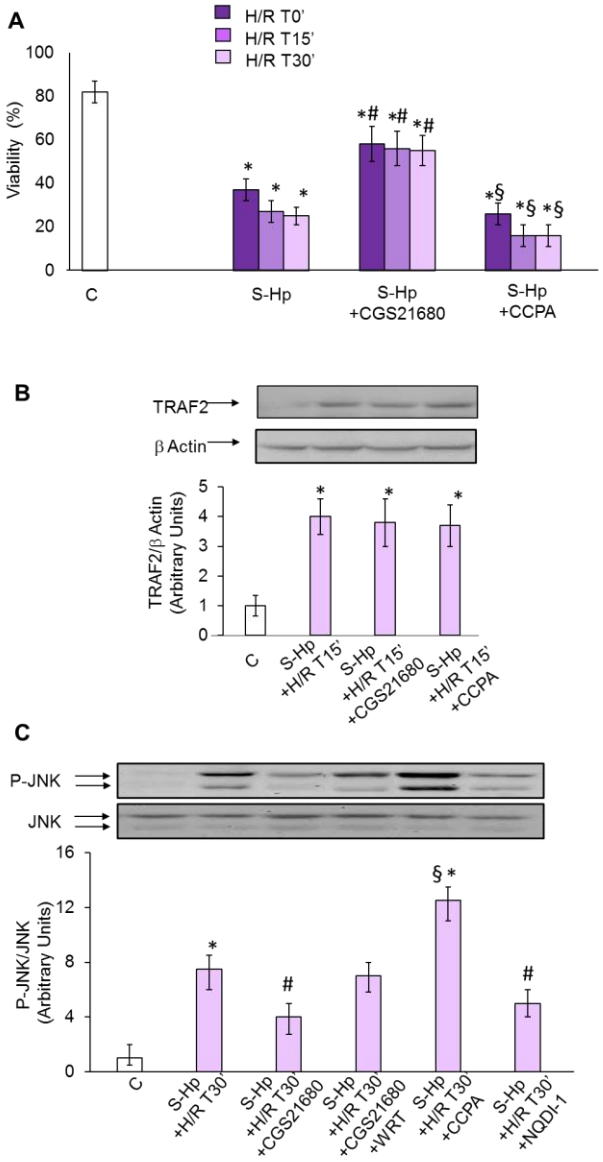

Figure 5: Effects of CGS21680 and CCPA treatment on cell viability, TRAF2 expression and JNK activation in steatotic hepatocytes exposed to hypoxia/reoxygenation.

A) *Steatotic hepatocytes (PA 50μM) were stored at 4°C for 16 hrs in hypoxic conditions and reoxygenated at 37°C, in presence of CGS21680 (5μM) or CCPA (100μM). Control cells viability was evaluated before hypoxic storage. In the other samples cell viability was evaluated after 16hrs of hypoxia and after 15' and 30' of reoxygenation time course (H/R T0'-15'-30'). The results are expressed as mean of 3 experiments ± SD. *p<0.001 vs Control; #p<0.05 vs S-Hp + H/R T0', 15' and 30'; § p<0.01 vs S-Hp + H/R T0', 15' and 30'*

B) *TRAF2 expression was evaluated in control hepatocytes (C) before storage in hypoxic conditions, or stored at 4°C per 16 hrs with PA (50μM) in hypoxic conditions in presence or absence of CGS21680 (5μM) or CCPA (100μM) and then exposed to 15' of reoxygenation at 37°C. The results are expressed as ratio TRAF2/β-actin, and represent the mean of 3 experiments ± SD. $^{\#}p<0.001$ vs Control.*

C) *JNK activation was evaluated as phosphorylation on Thr183/Tyr185 in control hepatocytes (C) before storage in hypoxic conditions or stored at 4°C per 16 hrs with PA (50μM) in hypoxic conditions in presence of CGS21680 (5μM), CGS21680 + Wortmannin (250nM), CCPA(100μM) or NQDI-1 (500nM) and then reoxygenated at 37°C for 30 min.. The results represent the mean of 3 experiments ± SD *p<0.001 vs Control; #p<0.005 S-Hp+H/R T30'or S-Hp+H/R T30'+CGS21680+WRT; $^{\S}p<0.01$ vs S-Hp+H/R T30'.*

Role of PI3K/AKT and ASK1 in the production of the cytoprotective effects of A2aR activation and effect of A1 and A2a adenosine receptors agonists on steatosis.

Previous studies from our and other laboratories have evidenced that PI3K/Akt axis plays a key role in the production of hepatoprotection induced by preconditioning and in the cytoprotection of hepatocytes after A2a adenosine receptors stimulation. Hence by measuring the phosphorylation of Ser 473 on Akt as a marker of PI3K stimulation, we evidenced that in steatotic hepatocytes exposed to hypoxia followed by 15 minutes of reoxygenation, A2a receptors stimulation with CGS21680 induced a significant increase of Akt phosphorylation **(Fig 6A),** while A1 receptors stimulation with CCPA did not produce any effect **(Fig 6A).**

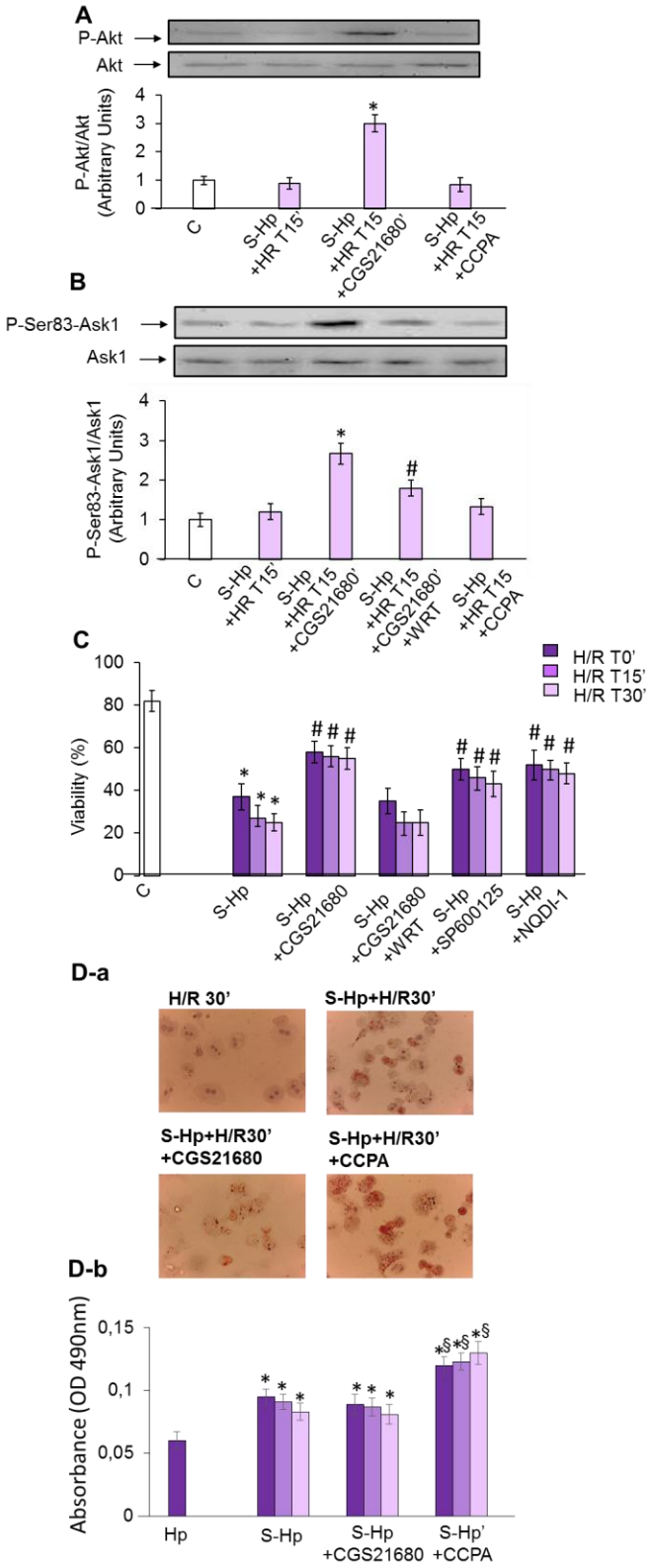

Figure 6: Different effects of A1R and A2aR stimulation on Akt activation, Ask1 inhibition, intracellular steatosis and hepatocytes viability.

A) *Akt activation in Control hepatocytes (C) or hepatocytes treated with PA (50μM), PA+CGS21680 (5μM) or PA+CCPA (100μM) in hypoxic conditions at 4°C for 16 hrs and then exposed to 15' of reoxygenation at 37°C, measured as ratio Phospho Akt (Ser473)/Akt. The results represent the mean of 3 experiments ± SD.*p<0.001 vs Control and vs S-Hp+H/R T15'.*

B) *Inhibitory phosphorylation of ASK1 in hepatocytes treated with PA (50μM), PA+CGS21680 (5μM) in presence or not of Wortmannin (250nM) and PA+CCPA (100μM) exposed to 15' of reoxygenation, evaluated as phosphorylation of Ser83 on ASK1. The results represent the mean of 3 experiments ± SD. *p<0.001 vs Control and S-Hp+H/R T15'; #p<0.005 vs S-Hp+H/R T15'+CGS21680.*

C) *Cell viability of Steatotic hepatocytes (PA 50μM) stored at 4°Cfor 16 hrs in hypoxic conditions and reoxygenated at 37°C, in presence or in absence of NQDI-1 (500nM), SP600125 (10μM) or CGS21680 (5μM) or CGS21680 + Wortmannin (250nM). The results represent the mean of 3 experiments ± SD. *p<0.001 vs Control; #p<0.05 vs S-Hp+H/R or S-Hp+H/R+WRT.*

D) *a)Steatosis detected by Oil Red O staining (ORO) in murine primary hepatocytes exposed to 16hrs of hypoxia and to 15'-30' of reoxygenation at 37°C and incubated with or without Palmitic acid (PA; 50μM), PA+CGS21680(5μM), PA+CCPA(100μM). The images (20X) represent each condition after 30' of reoxygenation. b) Steatosis quantified spectrophotometrically by the measurement of absorbance from dye ORO at 490nm. The results are expressed as mean of 3 different experiments ± SD. *p<0.01 vs Hp; §p<0.01 vs S-Hp.+H/R.*

We have shown that a TRAF2-dependent and a ROS-dependent ASK1 activation is responsible for JNK stimulation and for the production of H/R damage of steatotic hepatocytes. In this regard, it is interesting to note that Kim et al [34] reported that the mediators of cytoprotection, PI3K/Akt, can produce an inhibitory phosphorylation on Ser 83 of ASK1, avoiding its activation. Starting from these observations, we evaluated the levels of the phosphorylation of Ser 83 on ASK1 in steatotic hepatocytes treated with or without CGS21680 or CCPA and subjected to hypoxia/reoxygenation. As shown in figure 6B, the treatment with hypoxia/reoxygenation in presence or absence of CCPA did not modify the levels of ASK1 phosphorylation on Ser 83. On the contrary, hepatocyte incubation with CGS21680 induced a significant increase of the inhibitory phosphorylation of ASK1 that was prevented by the concomitant inhibition of PI3K with Wortmannin (250nM). To confirm the involvement of PI3K in ASK1 and JNK inhibition in the production of the cytoprotective effects of CGS21680, we investigated the effect of PI3K inhibitors on cellular damage and compared the capacity of ASK1 and JNK inhibitors to reproduce CGS21680 cytoprotection. As shown in Figure 6C, pharmacological inhibition of ASK1 with NQDI-1 (500nM) simulated the protective effects of A2a receptor stimulation with CGS21680 and at the same time, inhibited JNK activation **(Fig 5B).** Similarly, the treatment with the JNK inhibitor SP600125 (10μM) prevented cell death induced by hypoxia/reoxygenation in steatotic hepatocytes, reproducing the effect of NQDI-1 and CGS21680. By contrast, the treatment with the PI3K inhibitor Wortmannin (250nM) abolished the protective effects of CGS21680 on cell damage (**Fig 6B,**) and on JNK activation **(Fig 5B).**

In previous studies about alcoholic steatohepatitis [24] mice KO for A1 adenosine receptors have been shown to be protected from hepatic steatosis. On the light of these data, we investigated, in our "in vitro" model of hepatocytes treated with PA and exposed to hypoxic/reoxygenation damage, the effect of A1 and A2a receptors stimulation on lipid intracellular accumulation.

Our results indicated that the treatment of steatotic hepatocytes exposed to 16 hrs of hypoxia and 15'-30'of reoxygenation, with A2aR agonist, CGS21680 5μM, did not modify the lipid intracellular content induced by PA alone. On the contrary, the treatment with A1R agonist, CCPA 100μM, increased hepatocyte steatosis induced by PA **(figure 6D-a)**. Intracellular lipid accumulation in steatotic hepatocytes exposed to hypoxia/reoxygenation was also quantified with spectrophotometer by measurement of absorbance at 490 nm and the results **(figure 6D-b)**. The data obtained confirmed the observations shown by the ORO visualization showing the capacity of CCPA to increase the lipid content of about 30%. Thus we investigated whether the increased lipid content of CCPA treated hepatocytes was associated to the increase of ROS production, cytotoxicity, ASK1 and JNK activation. As shown in figure 7, steatotic hepatocytes exposed to CCPA and H/R showed an increased production of ROS **(Fig 7A)** and an increased ASK1 **(Fig 7C)** and JNK phosphorylation **(Fig 7D)** that were inhibited by DPPD treatment. Consistently DPPD also prevented the stimulation of cell death induced by CCPA treatment **(Fig 7B)**.

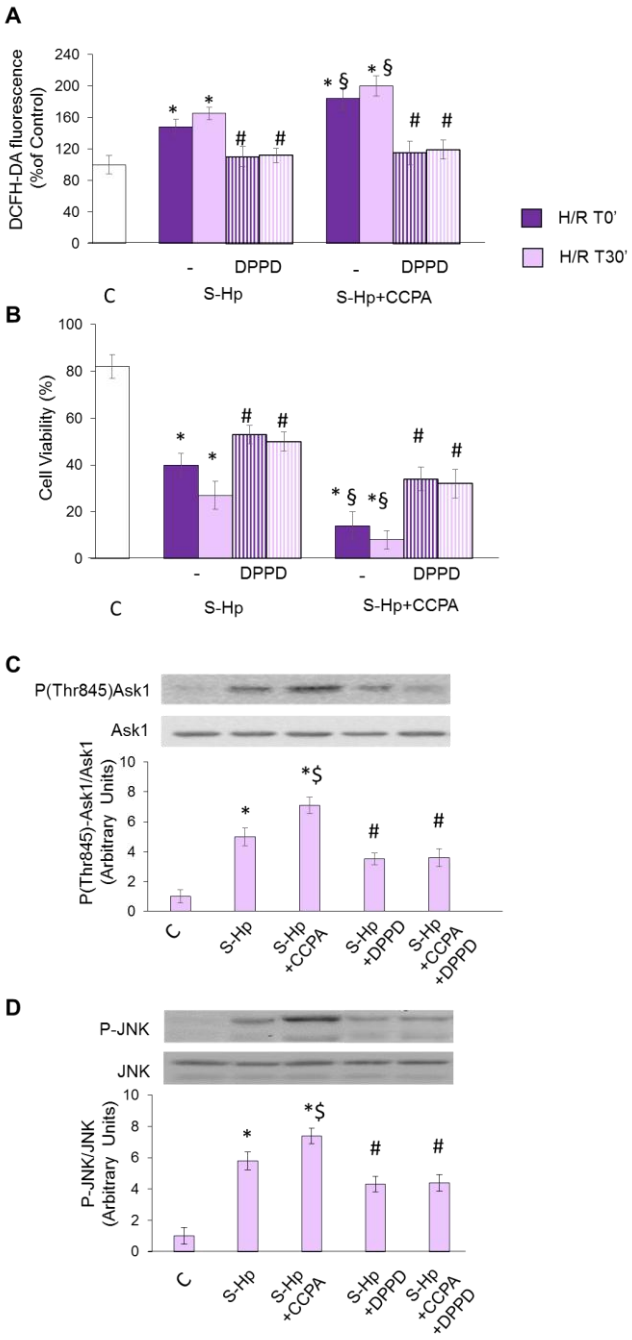

Figure 7: Effect of CCPA treatment on ROS production, cytotoxicity, ASK1 and JNK activation in steatotic hepatocytes exposed to hypoxia/reoxygenation .

Steatotic hepatocytes (PA 50μM) were stored at 4°C for 16 hrs in hypoxic conditions and reoxygenated at 37°C until 30' min, in presence of CCPA (100μM) with or without DPPD (5μM). ***A)*** *ROS production was evaluated as DCFH-DA fluorescence after 16hrs of hypoxia and after 30' of reoxygenation.* ***B)*** *Viability evaluated in Control cells before hypoxic storage and in the other samples after 16hrs of hypoxia and after 30' of reoxygenation.* ***C)*** *Stimulation of ASK1 evaluated as phosphorylation on Thr845 after 15' of reoxygenation.* ***D)*** *JNK activation evaluated as phosphorylation on Thr183/Tyr185 after 30' of reoxygenation. The results represent the mean of 3 experiments ± SD.*p<0,001 vs Control §p<0.05 vs S-Hp; $p< 0.05 vs S-Hp; #p<0.05 Vs S-Hp or S-Hp+CCPA.*

In Vivo Studies

To confirm *in vivo* the observations obtained in the cellular systems, we employed a model liver ischemia/reperfusion injury on mice fed with a standard diet or high fat diet (HFD) for 9 weeks. The effect of the stimulation of A1R or A2aR or the inhibition of ASK1 was evaluated in mice

treated respectively with CCPA (i.p. 1.5mg/Kg), CGS21680 (i.p. 0.5mg/kg) or NQDI-1 (i.p. 50mM). Hepatic steatosis induced by feeding HFD, as evaluated as liver triglyceride content, was not significantly affected neither by I/R or NQDI-1 and CGS21680 treatment **(Figure 8B).** By contrast, CCPA treatment further increased the lipid content of steatotic livers **(figure 8B),** confirming the *in vitro* observations obtained with steatotic hepatocytes treated with CCPA. In HFD-fed mice, both NQDI-1 and CGS21680 treatment showed to prevent liver damage induced I/R, as evaluated as serum ALT releases **(Fig 8A).** Moreover, both NQDI-1 and CGS21680 treatments inhibited Ask1 and JNK activation **(Fig 8 C&D).** On the contrary, CCPA treatment further increased ALT release and such effect was associated to an augmented JNK and Ask1 activation. On the other hand, liver damage protection induced by CGS21680 was associated to an increased phosphorylation of Akt **(Fig 8F)** and of Ask1 in Ser 83 **(Fig 8 E).**

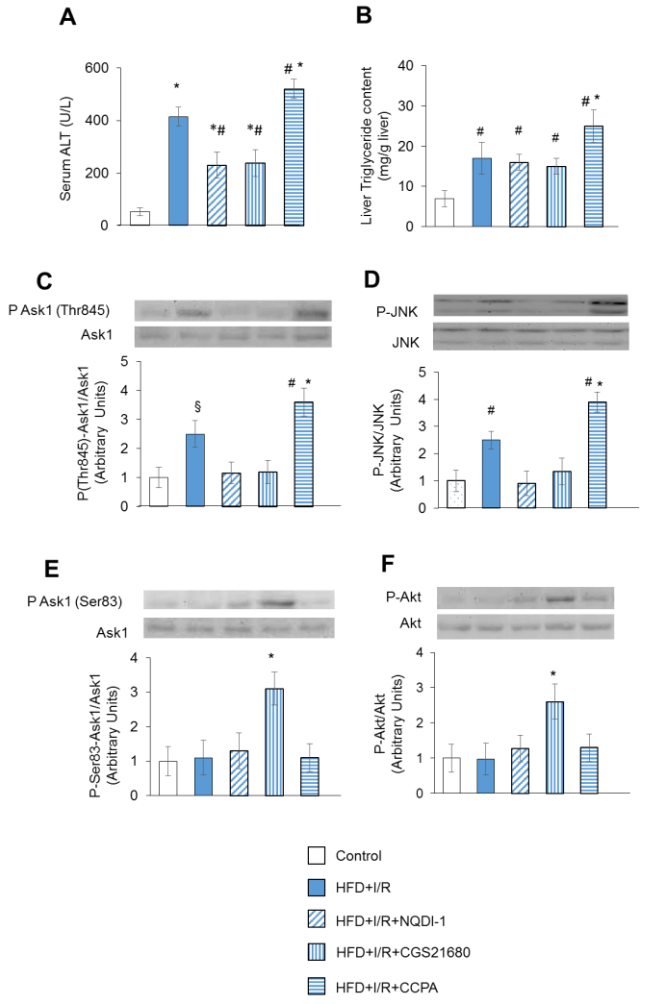

Figure 8: Effect of ASK1 inhibition or A2aR or A1R activation on hepatic I/R injury of steatotic mice.

Mice fed with normal or high fat diet for 9 weeks, treated with I.P. injection of CGS21680 (0.5mg/Kg), CCPA(1.5mg/Kg), NQDI-1 (50μM) were subjected to 30' of ischemia followed by 120'(I/R) of reperfusion and ***A)****Hepatic damage evaluated by serum ALT release after I/R.* ***B)****Hepatic steatosis evaluated as triglyceride content after mice sacrifice.* ***C)*** *Stimulation of ASK1 evaluated as phosphorylation on Thr845 after I/R.* ***D)****JNK activation evaluated as phosphorylation on Thr183/Tyr185 after I/R.* ***E)*** *inhibitory phosphorylation of ASK1 on Ser83 evaluated after I/R.* ***F)****Akt activation evaluated as phosphorylation of Akt after I/R. The results represent the mean of 3 experiments ± SD; A: *p<0,001 vs Control; #p<0.05 vs HFD+I/R; B: *p<0,001 vs Control; #p<0.05 vs Control or HFD+I/R; C: *p<0,001 vs Control ; §p<0,01 vs Control; #p<0.05 vs HFD+I/R; D: *p<0,001 vs Control ; #p<0.05 vs Control or HFD+I/R; E and F: *p<0,001 vs Control.*

DISCUSSION

This study investigated the molecular mechanisms involved in I/R injury of fatty livers showing a central role of ROS-dependent and -independent ASK1 activation in promoting hepatocyte injury. We observed, in fact, that in the cellular model of steatotic hepatocytes (S-Hp) exposed to cold hypoxia/warm reoxygenation (H/R) as well as "in vivo" in mice with fatty liver undergoing I/R, hepatocellular injury was associated to an increased activation of ASK1. We additionally observed that genetic or pharmacological inhibition of ASK1 reduced H/R or I/R injury. In vitro data also demonstrated that ASK1 activation was induced by two different mechanisms: one was dependent from the endoplasmic reticulum (ER) stress and associated to TRAF2 expression and the other related to an increased production of reactive oxygen species (ROS). The critical role of the TRAF2-dependent signaling in I/R hepatic damage is in agreement with the recent findings of Zhang and colleagues [25]. These Authors by employing TRAF-1 KO mice demonstrated a significant prevention of I/R injury and an inhibition of the ASK1-JNK axis. Accordingly, our data showed that H/R increases TRAF2/ASK1/JNK pathway and that its inhibition prevented I/R injury. However, we additionally demonstrated that such effect was evident only during reoxygenation, and this indicated the TRAF2/ASK1/JNK cytotoxic pathway was specifically implicated in the damaging effects of reperfusion, independently from the alterations produced during the ischemic phase. Our data also enlightened that steatosis induced an additional increase of ASK1 activation that was associated to an exacerbation of hepatocellular and hepatic damage. On this later, we showed that such steatosis-dependent increase of ASK1 was unrelated to ER stress and TRAF2 expression, but was instead associated to ROS production. We found, in fact, that the antioxidant DPPD entirely prevented the increased activation of ASK1 as well as the increased cytotoxicity induced by steatosis. Moreover, the activation of ASK1 induced by steatosis was associated to an increased oxidative stress. We also showed that H/R was not "per se" able to increase the ROS content of control hepatocytes and that on the contrary, the increased lipid content in hepatocytes incubates with Palmitic acid promoted ROS production. Such observation is in agreement with previous researches that correlated the increase of intracellular fatty acids and the promotion of oxidative stress as consequence of their increased catabolism. Hepatocytes have been, in fact, shown to respond to the excess of intracellular fatty acids by storing them as triglycerides and by boosting their catabolism through mitochondrial and peroxysomal β-oxidation [26]. These processes are instrumental to the removal of fat but also augment the generation of ROS and specifically of superoxide anion [27].

Also the pathophysiology of IR is, however, characterized by conditions that encompass ROS production. The most relevant of them in term of entity of oxidative stress, are related to the ROS production by activated Kupffer cells and by infiltrating leucocytes. However, in the early phase of reperfusion, liver cells too, can produce ROS by a process independent from inflammation. Such process is, in fact, due to the increased superoxide anion formation by the uncoupled mitochondrial as consequence of the oxygen re-admissions [28]. In this regard, we recently observed that, by comparing the sensitivity of liver sinusoidal cells (LSECs) and hepatocytes to oxidative stress and H/R, only LSECs were sensitive to oxidative stress [29]. These observations,

along with the present results indicate that intracellular production of ROS is not relevant for hepatocyte damage during I/R of control liver. This study, however, enlighten that steatotic hepatocytes produce ROS that are, in turn responsible for ASK1 activation and for the consequent stimulation of JNK. Thus, the intracellular production of ROS appears responsible for the detrimental effects associated to I/R in fatty liver.

JNK is an established mediator of tissue and hepatic injury and it has been found involved in both I/R injury [30] and lipotoxicity [31,15], JNK is activated by phosphorylation, translocate to the nucleus and activates target gene like c-Jun that, by inducing AP-1, is involved in the transcription of several apoptotic proteins [32]. Consistently to such cytotoxic role of JNK, we here showed that JNK inhibition significantly reduced S-Hp death induced by H/R and that the hepatoprotective effects of ASK1 inhibition were associated, both in the *cellular* and in the *in vivo* models, to the prevention of JNK stimulation.

This study also elucidated the effect of the A1 adenosine receptor (A1R) activation on H/R injury in I/R damage of fatty liver. We found, that in contrast to the A2aR agonist CGS21680, the A1R agonist CCPA failed to protect the lethal I/R injury of steatotic liver and hepatocytes. Such effect was associated to the incapability of CCPA to activate Akt and to inhibit JNK stimulation**.** Notably, A1R stimulation by CCPA increased hepatotoxicity and JNK activation. Such an effect was associated intracellular lipid accumulation and to an increased ROS production, with consequent further stimulation of the ASK1/JNK cytotoxic pathway.

In conclusion, the results illustrated in the present study strongly indicate A2aR activation and ASK1 inhibition as effective protective conditions against I/R injury of fatty liver and suggest that pharmacological interventions aimed to directly stimulate A2aR or block ASK1 can represent a novel and efficient therapeutic approach to prevent the injurious consequences of I/R application in fatty livers.

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ISCHEMIA/REPERFUSION INJURY ON MICE STEATOTIC HEPATOCYTES AND DIFFERENTIAL EFFECTS OF ADENOSINE A2A AND A1 RECEPTORS STIMULATION

Supplementary materials

Supplementary Experimental Procedures

Hepatocytes isolation, preparation and treatments

Male mice C57BL/6 weighing 20-30g (Harlan Italy S. Pietro al Natisone (UD), Italy) anesthetized i.p. with Zoletil/Xilazine mixture (Zoletil 43mg/Kg; Xilazine 17,2mg/Kg) were used for isolating primary murine hepatocytes by perfusing liver with collagenase. The Italian Ministry of health approved the use and care of the animals. The liver was washed via the portal vein at first with T1 buffer at 37°C containing NaCl 143mM, KCl 7mM, Hepes 10mM at pH 7. 4. After T1, the organ was perfused with the T2 buffer containing NaCl 100 mM, KCl 40 mM, $CaCl_2$ 5 mM, Hepes–NaOH 50 mM, and EGTA, pH 7.4 with collagenase type IV 0.05% for liver digestion Finally, the liver was removed from the animal and resuspended in the medium and scrapped. The dissociated liver cells suspended in the medium were passed through a filter. Later, the hepatocytes were purified from the other cells by centrifugation at 500 rpm for 5 minutes followed by a further 3 minutes centrifugation at 1050 rpm through a layer of Percoll. Cell purity was assessed according to Benten (Benten et al., 2005). Cell viability, estimated at the beginning of experiments, ranged between 82% and 90%.

After counting, isolated hepatocytes were centrifuged and resuspended in Viaspan solution (University of Wisconsin Solution without additives) at $1x10^6$cells/ml. Hepatocytes suspended in Via Span solution were kept for 16hrs in hypoxic atmosphere (95%N2 and 5%CO_2) at 4°C (H). Palmitic Acid (50µM) was added in Viaspan solution to induce steatosis in hepatocytes. Isolated hepatocytes, where indicated, were treated with the following drugs: CGS21680 (5µM) and CCPA (100µM), the A2a and A1a receptors agonist respectively, JNK inhibitor - SP600125 (10µM), ASK1 inhibitor-NQDI1 (500nM), ER stress inhibitor- APY29 (285nM), DPPD (5µM), PI3K inhibitor-Wortmannin (250nM). After 16hrs of cold storage, the hepatocytes were reoxygenated by fluxing (95% air and 5% CO_2) gas mixture in DMEM medium on the heater at 37°C (R) for 0, 15 and 30 minutes time course analysis (H/R T0', T15', T30').

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.

Trypan Blue

Trypan Blue test is routinely used cell stain to assess cell viability using the dye exclusion test. This test is often performed while counting cells with the Burker slide during routine sub-culturing. The results were confirmed by flow Cytometry evaluation of the cells Propidium iodide (PI). Briefly, aliquot of 1 x 10^6 cells/100 µL are harvested into FACS tubes and washed by adding 2 mL of PBS, centrifuged at 1000rpm for 5 minutes, and then the buffer decanted from the pelleted cells. The cells are resuspended in 100 µL of Flow Cytometry Staining Buffer. 5 - 10 µL

of PI staining solution was added to each sample just prior to analysis. The stop count was setted on the viable cells from a dot-plot of forward scatter versus PI. Cell viability was estimated by the determination of nuclear fluorescence staining with propidium iodide using a FACScan analyzer (Becton-Dickinson, San Jose, CA) and Cell Quest software (Becton-Dickinson).

Steatosis Colorimetric Assay:

Intracellular lipid accumulation in the in mouse hepatocytes treated or not with PA in presence or absence of CGS21680 or CCPA was evaluated using the Steatosis Colorimetric Assay Kit (Cayman Chemical), according to manufacturer's instructions of the kit. This assay provides a convenient tool for evaluating steatosis, where the neutral lipids are stained using oil red O (ORO) stain and quantified the lipid accumulation with the dye extraction solution. The dye extracted from the lipid droplets was quantified in spectrophotometer at 490 nm.

Steatosis Assay: At the end of the treatments, medium was removed and the cells were washed with the PBS 1X twice. Later the cells are fixed to the plate with 1X fixative agent and the wells are subjected to washing with wash solution for 5 minutes on the basculant. The air dried wells are treated with the oil red solution (60% in water) and incubated for 20 minutes. Later the oil red solution is removed and the wells are washed with tap water until the wash solution contains no visible pink color. Then wells are washed twice for 5 minutes each with wash solution and completely air dried for 20 minutes. Lipid accumulation inside the cells are quantified by adding a 100 µl/well lipid droplets assay dye extraction solution and incubated for 30 minutes on the basculant and the final absorbance is read in spectrophotometer Victor X4 2030 multi reader (Perkin Elmer) at 490nm. To evaluate the intracellular lipid distribution in mouse hepatocytes treated or not with PA and in presence or absence of CGS and CCPA the steatosis slides prepared with ORO staining as indicated in the data sheet kit. After the staining procedure done, the lipid accumulation and distribution inside the cells were analyzed.

At the end of the treatments, from wells medium is removed and the cells were washed with the PBS 1X twice. Later the cells are fixed to the cover glass with 1X fixative agent and the wells are subjected to washing with wash solution for 5 minutes on the basculant. The air dried wells are treated with the oil red solution (60% in water) and incubated for 20 minutes. Later the oil red solution is removed and the wells are washed with distilled water until the wash solution contains no visible pink color. Then wells are washed twice for 5 minutes each with wash solution and completely air dried for 20 minutes. Then intracellular distribution of lipid droplets were examined by staining with 100µL of hematoxylin to each well and immediately washed with tap water. Later the cells were allowed to develop nuclear staining under tap water for ten minutes. The slides were prepared by taking out the glass dish with plated cells and gently placed on glass slide. Later slides are used to capture the pictures at microscope of intracellular lipid distribution.

Measurement of Reactive Oxygen Species (ROS)

Intracellular ROS quantity was measured by the method of Jakubowski and Bartosz (Jakubowski et al., 2000) with minor modifications. In primary normal or steatotic hepatocytes exposed to I/R injury in the presence or absence of DPPD, DCFH-DA (10µM) was added and incubated for 30 minutes at 37°C. Then, the samples were washed with PBS 1X. The fluorescence was measured at excitation and emission wavelengths of 495 and 525 nM respectively in the Victor X4 2030 multi reader (Perkin Elmer). DCFH-DA is a non-polar compound that is converted into non-fluorescent

polar derivative (H_2DCFH) by cellular esterases after incorporation into cells. H_2DCFH is rapidly oxidized to the highly fluorescent DCF in presence of intracellular hydrogen peroxide and other peroxides (Bass et al., 1983). ROS production was calculated as a percentage of the DCFH-DA fluorescence intensity versus untreated control cells.

C1C7: Transfection and Treatment

The HEPA-1 wild type C1C7 Hepatocarcinoma cell line was obtained from the European Collection of Cell Cultures and cultured on modified DMEM medium containing 10% FBS (fetal bovine serum), 5% penicillin/streptomycin, non-essential amino acid, 1% vitamin solution and 1% sodium pyruvate. Later the cells were collected, centrifuged and re-suspended in the medium and cell count performed. $1x10^5$cells (70% covering approximately) were plated in six multiwells plate and allowed 24hrs for the attachment of the cells in the incubator.
Later the cells were used for small interference RNA (SiRNA) transfection procedure as described below.
Murine ASK1 SiRNAs were purchased from Sigma-Mission (Milan, Italy). Sequences are as follows:
SiRNA1 sense: GUACUUCCGGGAAUCCAUAdTdT
SiRNA1 antisense: UAUGGAUUCCCGGAAGUACdTdT
SiRNA2 sense: CAGAUAGUCCACCGGGAUAdTdT
SiRNA2 antisense: UAUCCCGGUGGACUAUCUGdTdT.
Control siRNA was used as negative control of transfection. C1C7 cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer recommendations. Transfection efficacy was analyzed after 48 hrs using BLOCK-iT Fluorescent Oligo (Invitrogen) and the transfection efficiency of C1C7 cells was more than 75%. After 48hrs of SiRNA transfection we treated the C1C7 cells with PA at 700μM as final concentration in Viaspan solution and we incubated the six multiwells at 4°C for 16hrs. At the end of the cold hypoxia we started the reoxygenation until 30 minutes at 37°C (H/R). At the end of the treatments we performed the cell viability assay.

Ischemia-reperfusion injury

Mice were anesthetized and the abdomen opened with a mid-line incision. The Branches of hepatic artery and portal vein to the left lateral and median lobes were occluded with a non-traumatic micro vascular clip for 30 mins of ischemic state resulting in deprivation of blood flow to approximately 70% of the liver. The abdomen was covered with saline-humidified gauze during the ischemic period. After 30 minutes of partial hepatic ischemia, clip will was removed, to initiate hepatic reperfusion, later the abdominal cavity was closed with a 4-0 silk suture and metal clips were applied to the skin. And the mice was allowed to recover spontaneously and fed the standard chow diet and water ad libitum for 120 minutes of reperfusion time.The temperature was maintained at 37°C during hepatic ischemia and in the post-surgical period with a warming pad. Immediately after the reperfusion period, blood was collected to evaluate the liver injury, as ALT release, and then mice were sacrificed and the liver fragments immediately frozen in liquid nitrogen for WB analysis and triglyceride content determination.

Analysis of the phosphorylation state of AKT, JNK, TRAF2 and ASK1

Western blot is often used in research to separate and identify proteins. In this technique a mixture of proteins is separated based on molecular weight through gel electrophoresis. At the end of the treatments, hepatocytes were collected by centrifugation, washed twice with PBS 1X and treated with lysing buffer. For "in vivo" experiments at the end of the treatment mice were sacrificed and liver pieces were collected and homogenized in the specific buffer. Protein concentration was determined by modified Lowry method (Lowry et al., 1951) and equal amounts (40-60 μg) of protein were subjected to gel electrophoresis.
The samples were electrophoretically transferred to a nitrocellulose membrane and later incubated with blocking buffer (5% non-fat milk) for 1hr at room temperature and then washed with TBS Tween (0.1% Tween-20) 1X buffer and then incubated with the primary antibody of interest overnight at 4°C. In particular, we used as primary antibody: phospho-Akt (Ser473), Akt, phospho-JNK (Thr183/Tyr185), JNK and TRAF2 (Cell Signaling Technology). Phospho-ASK1 (Thr845) (Biorbyt), inhibitory phospho-ASK1 (Ser83) (Sigma-Aldrich) and ASK-1 (Santa Cruz Technology). The β-actin monoclonal antibody (Sigma-Aldrich) was used to assess equal protein loading. The bound primary antibodies are then detected by using secondary antibodies, anti-rabbit or anti-mouse, incubated at room temperature for 1hr. developing the blot after washing the membrane 3 times with TBS-T (Tris, NaCl and 0.1% Tween-20) of 5 minutes each. The proteins of interest were detected using an chemiluminescence-based immunodetection of alkaline phosphatase (AP) or horse radish peroxidase (HRP) on western blots or dot blots detection kit (ECL) (Invitrogen) at Versa Doc 3000 quantitative imaging system (BioRad Laboratories) and analyzed with the Quantity-One software (Bio Rad, Hercules, CA). The results are expressed as ratio of phosphorylated protein on the correspondent basal protein or, for TRAF2 expression, as ratio of total TRAF2 and β-Actin

5 GENERAL DISCUSSIONS

Ischemia/reperfusion (IR) injury is one of the most critical complications commonly associated with liver surgery. I/R can induce liver dysfunction or failure following major liver surgery. Hepatic I/R injury is a complex, multifaceted process that occurs during the ischemic period as well as during the reperfusion phase. Compared with healthy livers, steatotic livers are vulnerable to IR injury. Hepatic I/R injury is a frequent and major complication in clinical practice, which compromise liver function and increases postoperative morbidity, mortality, recovery and overall outcome (Serracino-Inglott et al., 2001). Hepatic ischemia/reperfusion (I/R) injury can be defined as the phenomenon during which cellular damage in an organ, caused by hypoxia, is paradoxically exacerbated after the restoration of oxygen delivery (Peralta et al., 2010). This concept occurs in several organs such as brain, liver, heart, lung, intestine, skeletal muscle and kidney (Eltzschig et al., 2004). Ischemia/reperfusion (IR) injury is a dynamic process which involves the two interrelated phases of local ischemic insult and inflammation-mediated reperfusion injury (Zhai et al., 2013). Liver, being an organ with high energy requirements, is highly dependent on oxygen supply and susceptible to hypoxic or anoxic conditions (Teoh et al., 2011). Liver steatosis is a frequent condition in western countries and fatty livers poorly tolerate cold and warm ischemia/reperfusion (I/R) injury; thereby steatosis is associated with an higher mortality after major liver surgery and including liver transplantation (Feng et al., 2006).

Early researches from our Group analyzed the molecular mechanisms involved in I/R injury employing an "in vitro" model of primary rat hepatocytes exposed to hypoxic damage. Hepatocytes death upon ATP depletion during the lack of oxygen is precipitated by the deregulation of Na^+ homeostasis. Na^+ alterations that follow ATP depletion are the result of a combined block of the ATP-dependent Na^+ efflux through the Na^+/K^+ ATPase and of the activation of Na^+/H^+ exchanger and $Na^+/HCO3^-$ co-transporter in response to cytosolic acidification. In the phase that precedes death, hepatocytes respond to the progressive increase of intracellular Na^+ with the stimulation of the volume regulatory decrease mechanisms, that is, activation of the K^+ channels and K^+ efflux. The decrease of intracellular K^+ under a critical threshold definitively impairs the volume regulatory systems and leads to a sudden increase of hepatocytes volume, with osmotic lysis and death of hepatocytes (see for review: Alchera et al., 2010). Upon oxygen readmission, ROS production by uncoupled mitochondria promotes oxidative

stress and mitochondrial permeability transition and is associated with a decreased capacity to synthesize ATP (Jaeschke et al., 2003).

As reported in paper1, we analyzed the proteome alterations of murine cell isolated from liver exposed to I/R elucidating their contribution to I/R damage. Our results showed deep modifications in the proteomic pattern of hepatocytes and LSEC and in the enzymatic activities. In particular we demonstrated that I/R reduced the expression of enzymes involved in the carbohydrate, lipids and mitochondrial metabolism (Krebs cycle and oxidative phosphorylation) in hepatocytes and in LSEC and decreased proteins related to oxidative stress that is particularly evident in LSECs. On this respect the severe ATP depletion occurring in hepatocytes during ischemic phase has been generally ascribed to the lack of oxygen and glycolytic substrates, consequent to blood interruption (Peralta et al., 2013; Jaeschke et al., 2003; Alchera et al 2010). However, ATP loss was prevented in the preconditioned liver, indicating that the block of blood was not per se sufficient to justify ATP loss. In addition, one of the most striking alterations of the IR-injured liver is the incapability of recovering aerobic ATP production when blood flow is re-establishment with reperfusion. Our observations that glycolytic enzymes and ATP synthase subunits were decreased in HP and LSEC obtained from liver exposed to IR, and that CGS21680 treatment up regulated the glycolytic and mitochondrial pathways, endorses the hypothesis that IR damage is not merely due to a reduction of blood flow, but also involves a coordinate perturbation of metabolic enzymes, which can be rescued by preconditioning. Beside to the effect on glucose metabolism, we found that CGS21680 treatment is able to promote lipid transport and beta-oxidation, as well as an up regulation of urea cycle. This suggests that the improvement of amino acid and lipid catabolism could represent a response of HP and LSEC to ATP deprivation caused by IR. Therefore, A2aR stimulation provides a general metabolic advantage to HP and LSEC, demonstrated by ATP production increase, not only rescuing the metabolic alteration induced by IR but, in some cases, enhancing the expression of enzymes required for energy production. The relevance of these observations is also supported by the fact that the cytoprotective action of CGS21680 is reverted by the inhibition of pyruvate kinase (KPYR), 3-ketoacyl-CoA thiolase (THIM), and arginase (ARGI1), three enzymes involved in respectively, glycolysis, β-oxidation and urea cycle. Furthermore, cell supplementation with the glycolic end-product pyruvate, the free fatty acid Palmitic acid or amino acid mixture demonstrated to mimic partly the protective effects of CGS21680 against HP and LSEC hypoxia-reoxygenation damage.

Beside the effect of A2aR activation on energy rescue and mantainance, a further critical aspect enlightened by our studies was the capacity of A2aR stimulation to improve the antioxidants defence of liver cells. Interestingly we additionally demonstrated that I/R itself also affected hepatic antioxidant defences and that such effect was specific for LSEC. Accordingly we demonstrated that CGS21680 treatment of LSEC prevented oxidative damage following in vitro addition of H_2O_2. These results are particularly interesting since they may represent the molecular explanation of the high sensitivity of LSEC to cold ischemia, the microcirculatory disturbance induced by IR damage and finally the rescuing action of ischemic preconditioning (Peralta et al., 2013).

I/R are a still a significant clinical problem after transplantation surgery. The dramatic organ shortage for transplantation forces consideration of steatotic grafts, which have a higher susceptibility to I/R. Hepatic steatosis is usually an asymptomatic condition but is a significant risk factor in liver transplantation; in fact, greater than 30% steatosis constitutes one of the single greatest risk factors, similar to the risk associated with donors after cardiac death for liver transplantation (Busuttil et al., 2003). Transplanted steatotic livers exhibit increased rates of primary nonfunction and initial poor graft function and the current consensus is that donors with greater than 60% fat content should not be used (Adam et al., 1991; Todo et al., 1989; Chui et al., 2001).

Based on steatotic animal models, fatty livers tend to have enlarged hepatocytes due to cytoplasmic lipid droplets with a concomitant reduction in sinusoidal space, as well as reduced total hepatic blood flow and microvascular perfusion (Takeda et al., 1991; Saefalien et al., 1999). Furthermore, experimental animal data suggest that steatotic livers are much more sensitive to ischemia-reperfusion than normal “lean” livers. For example, hepatic warm ischemia in obese Zucker rats had a much more pronounced effect on animal survival and led to more hepatocyte necrosis, microvascular disruption, and oxidative damage than in lean animals (Koneru et al., 1995). Rats fed a choline- and methionine deficient diet (CMDD) exhibited more functional impairment after ischemia-reperfusion in situ, and increased oxidative stress compared to animals fed a normal diet (Koneru et al., 1995., Nakana et al 1997). Studies using the same animal model on the effect of cold storage of liver followed by rewarming and perfusion also show more extensive damage in fatty livers than in lean livers, and a reduced “safe” preservation time before transplantation (Nakana et al 1997., Hayashi et al., 1993). The mechanism whereby steatosis increases the sensitivity of the liver to ischemia-reperfusion injury is poorly understood. More

specifically, the primary event responsible for the exacerbated response has not been identified, and it is unclear whether the enhanced inflammatory response is the result of or a causative factor in this response. Previous studies have shown that fatty livers exhibit serious microcirculatory disturbances after ischemia-reperfusion (Hasegawa et al., 1997; Ijaz et al 1995; Sun et al, 2001). Furthermore, there is evidence of a greater inflammatory response in fatty livers after transplantation (Mokuno et al., 2004), while treatments that reduce the inflammatory response, such as heat shock (Mokuno et al., 2006) and endotoxin antibodies (Fiorini et al., 2004), improve survival of fatty livers. None of these studies has, however, identified the primary or triggering event leading to hepatic failure.

We employed a cellular model to investigate the molecular mechanisms involved in I/R injury of steatotic hepatocytes. The results of our study (paper2) indicate that the damage of fatty hepatocytes exposed to I/R is mediated by the activation of the ASK1-JNK axis and that increased sensitivity of fatty hepatocytes to I/R injury is related to an augmented and ROS-dependent stimulation of this cytotoxic pathway. **(Fig 8)**

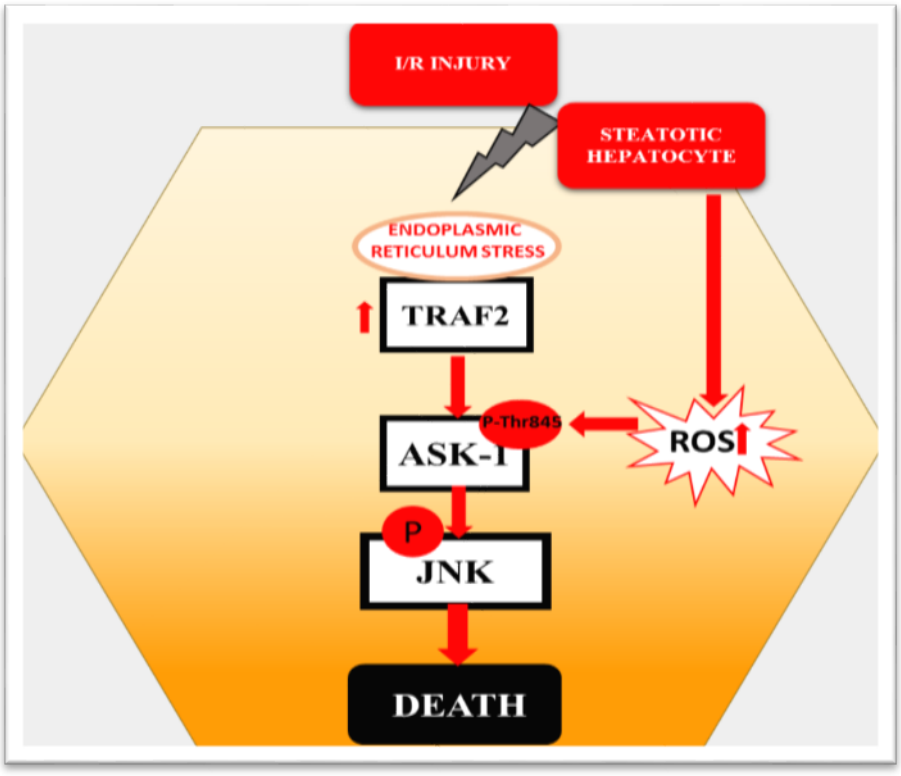

Figure 8: *hepatic damage of fatty hepatocytes exposed to I/R is related to an augmented and ROS-dependent stimulation of the cytotoxic ASK1-JNK axis.*

Preclinical studies have shown several strategies able to reduce hepatic damage by individually targeting the different alterations that contribute to I/R injury (Jaeschke et al, 2003; Alchera et al, 2010; Selzner et al., 2003).

The discovery of ischemic preconditioning (IP) has shown the existence of intrinsic systems of cytoprotection whose activation can stave off the progression of irreversible tissue damage. Besides its conceptual interest, deciphering the molecular mediators that underlie the cytoprotective, pro-regenerative and anti-inflammatory effects of preconditioning can open important therapeutic possibilities. Pharmacological activation of critical mediators of IP emulate or even to intensify its salubrious effects. Thus pharmacological preconditioning could become a novel therapeutic procedure to be applied when surgical IP is not clinically applicable.

Studies "in vitro" and "in vivo" have clearly demonstrated a key role of the adenosine A2a receptor (A2aR) as main trigger of liver IP. In previous studies we illustrated some molecular mechanisms involved in the prevention of hypoxic hepatocyte injury by A2aR. Such mechanisms were responsible for the maintenance of intracellular pH by a p38 MAPK and a PI3K-dependent activation of the vacuolar ATPase that acting as Na^+-independent alternative pH buffering system avoided a toxic Na^+ accumulation. Also the delayed protective effects of A2aR were mediated by PI3K and were dependent by an HIF-1 induced prevention of the hypoxic pH and Na^+ alterations. HIF-1, in fact, by inducing the expression of CAIX maintained the physiological cytosolic pH and prevented Na^+ accumulation (see for review: Carini et al., 2003; Alchera et al., 2010 and 2015).

The use of proteomic analysis allowed us to evidence profound changes of hepatocytes and LSECs proteome, providing new insights into some critical aspects of I/R injury and IP-induced hepatoprotection. Our results showed that hepatic cells isolated from liver exposed to I/R develop a “pathological phenotype” characterized by a decrease of the metabolic enzymes involved in the aerobic and anaerobic ATP production and, in the specific case of LSECs, an additional decrement of antioxidant defenses. On the contrary, A2aR stimulation induces the expression of a “protected phenotype” characterized by an enhancement of enzymes necessary for energy production and ROS detoxification. This gives a sort of metabolic and antioxidant advantage to precondition compared to non-preconditioned cells and can account for the increased resistance to death of preconditioned hepatic tissue during I/R exposure.

Previous studies demonstrated an interesting effect of the application of IP to fatty livers. IP, in fact, almost halved transaminase release and the histological evidence of liver cell death showing a

greater efficacy of IP in steatotic liver compared to normal liver (Serafin et al., 2002). In the past we demonstrated the capacity of A2aR stimulation to prevent lipoapoptosis of primary rat hepatocytes and to inhibit the development of nonalcoholic steatohepatitis in rat fed with MCD (methionine choline-deficient) diet preventing JNK-1/2 activation by a PI3K/Akt-mediated block (Imarisio et al., 2012).

In the present study, we investigated the effects of the pharmacological preconditioning with adenosine A2aR on I/R injury of steatotic hepatocytes. A2aR agonist CGS21680 prevented hepatocellular injury in both S-HP and fatty liver upon H/R and I/R. The hepatoprotective activity of CGS21680 depended on the inhibition of Ask1 and induced, as consequence, the prevention of JNK activation.

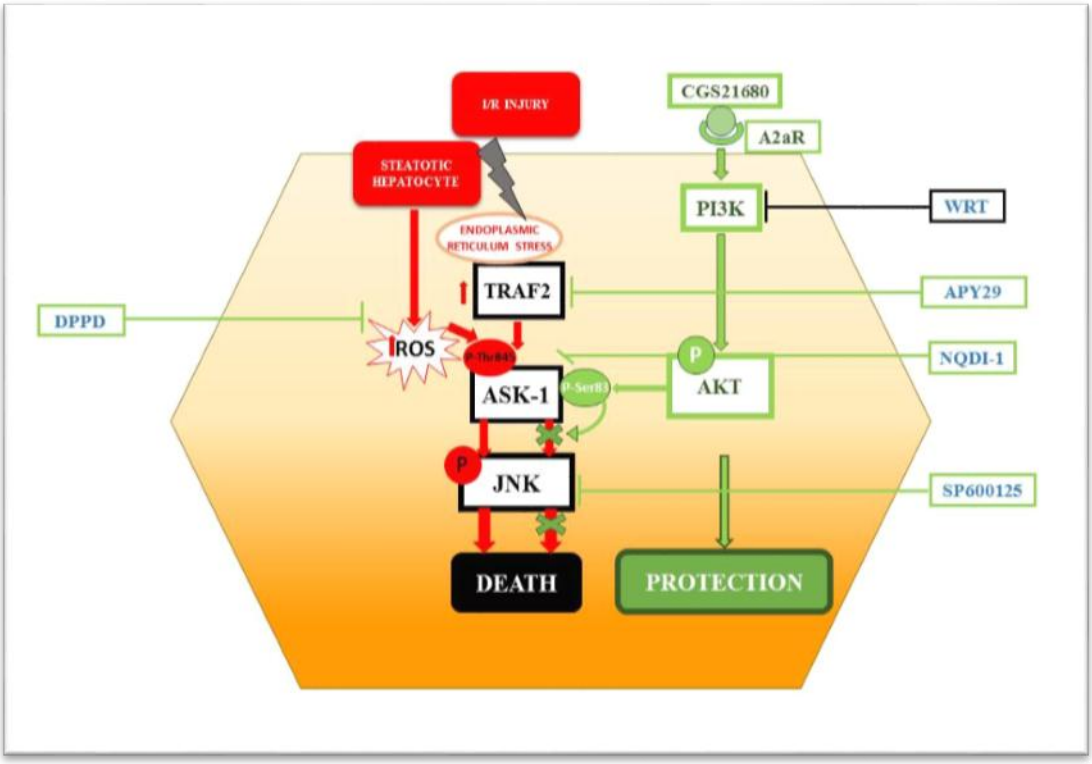

Figure 9: *CGS21680 A2aR stimulation protects fatty hepatocytes exposed to I/R by the PI3K-Akt axis activation able to block ASK-1/JNK axis through inhibitory phosphorylation of Ask-1 in Ser83.*

These results show for the first time, a novel mechanism of protection of the main inductor of IP, the activated A2aR. The mechanism involves a PI3K/Akt mediated block of the cytotoxic axis ASK1 vs. JNK. CGS21680 in fact prevents ASK1 activation without interfering with the toxic mechanisms upstream ASK1 activation, i.e. ER stress with increased TRAF2 expression or ROS

production, and is the result of the Akt- mediated inhibitory phosphorylation of ASK1 on Ser83 **(Fig 9).**

In conclusion the data emerging from the molecular analysis of the effects induced by A2aR stimulation suggest novel potential pharmacological strategies to be applied human hepatic surgery, as transplantation. First, the findings of the multiple mechanisms of liver cell protection induced by A2aR activation strongly enforce the idea to translate A2aR agonists to the clinical practice as hepatoprotective tool.

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Review Article

Pharmacological Preconditioning by Adenosine A2a Receptor Stimulation: Features of the Protected Liver Cell Phenotype

Elisa Alchera,[1] Chiara Imarisio,[1] Giorgia Mandili,[2,3] Simone Merlin,[1] Bangalore R. Chandrashekar,[1] Francesco Novelli,[2,3] Antonia Follenzi,[1] and Rita Carini[1]

[1]*Department of Health Science, University of Piemonte Orientale, 28100 Novara, Italy*
[2]*Centre for Experimental and Clinical Studies (CERMS), Azienda Universitaria Ospedaliera Città della Salute e della Scienza Città di Torino, 10100 Turin, Italy*
[3]*Department of Molecular Biotechnology and Heath Sciences, University of Turin, 10100 Turin, Italy*

Correspondence should be addressed to Rita Carini; rita.carini@med.unipmn.it

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Ischemic preconditioning (IP) of the liver by a brief interruption of the blood flow protects the damage induced by a subsequent ischemia/reperfusion (I/R) preventing parenchymal and nonparenchymal liver cell damage. The discovery of IP has shown the existence of intrinsic systems of cytoprotection whose activation can stave off the progression of irreversible tissue damage. Deciphering the molecular mediators that underlie the cytoprotective effects of preconditioning can pave the way to important therapeutic possibilities. Pharmacological activation of critical mediators of IP would be expected to emulate or even to intensify its salubrious effects. *In vitro* and *in vivo* studies have demonstrated the role of the adenosine A2a receptor (A2aR) as a trigger of liver IP. This review will provide insight into the phenotypic changes that underline the resistance to death of liver cells preconditioned by pharmacological activation of A2aR and their implications to develop innovative strategies against liver IR damage.

1. Ischemia/Reperfusion Injury of the Liver

Inflow occlusion during liver surgery with consequent reperfusion causes ischemia/reperfusion (I/R) injury of the liver. I/R injury is recognised as a main risk factor after major hepatic surgery and liver transplantation since it may affect patients recovery and carries a risk of poor postoperative outcome [1].

Hepatic I/R injury is a complex, multifaceted process that occurs during the ischemic period as well as during the reperfusion phase. During ischemia, mitochondrial deenergization, ATP depletion, and ionic and volume alterations lead to liver cell necrosis. Upon oxygen readmission, reactive oxygen species (ROS) production by uncoupled mitochondria promotes oxidative stress and mitochondrial permeability transition and is associated with a decreased capacity to synthesize ATP. Caspase activation, necrosis, and apoptosis of liver cells and activation of the inflammatory reactions follow these events. Resident Kupffer cells and infiltrating neutrophils and lymphocytes release ROS, proteases, and cytokines and further contribute to the progression of hepatic injury [2–4]. Preclinical studies have shown several strategies able to reduce hepatic damage by individually targeting the different alterations that contribute to I/R injury [2–6]. Their potential adverse effects and their focused approach have inhibited, however, their translation to patients and, to date, no definitive methods have become part of the clinical practise [1, 2].

2. Hepatic Ischemic Preconditioning

The term ischemic preconditioning (IP) refers to the increase in tissue tolerance to ischemia/reperfusion (I/R) damage that can be induced by the preexposure to brief periods of ischemia followed by reperfusion [7]. This phenomenon was first described by Murry et al. in the myocardium [8], but it was subsequently observed in many other tissues [7]. In liver, studies in rodents have shown that 10 min interruption

of blood flow followed by 10 min reperfusion reduces hepatic injury, oxidative stress, microvascular disturbances, and inflammation during a subsequent extended period of I/R [1–8].

The demonstration of the pleiotropic protective effects of IP in the experimental models has raised hopes that it could be a useful and easy technique to reduce I/R injury in human liver surgery. IP surgical application, however, has the disadvantage of inducing trauma to major vessels and stress to the target organ [9]. Moreover the contrasting outcomes of the first clinical studies, the different protocols of IP application in humans, and the variable clinical settings have not allowed a definitive demonstration of the benefit of the clinical application of IP [9–13].

This observation has inhibited, by now, the routine use of IP in human liver surgery and has indicated the need of more efficient approaches to activate IP in patients. In this regard, the pharmacological induction of liver preconditioning by targeted activation of one or more of the critical molecular mediators of IP may represent a more reliable technique to activate the intrinsic system of hepatoprotection in patients.

3. Adenosine A2a Receptor Activation: A Main Trigger of Hepatic Preconditioning

The nearly 25 years' research on liver IP has demonstrated that its applications induce deep modifications of liver tissue that make liver cells resistant to damage. The knowledge of the molecular changes responsible for the production of such protected liver cell phenotypes is however still incomplete. To date one of the established notions is the role of the adenosine A2a receptor (A2aR) activation as an inductor of liver preconditioning. Adenosine mainly originates by the breakdown of adenine nucleotides and even a transient damage of cell membranes, like that induced by the brief ischemic stress of IP, leads to massive ATP increase in extracellular space with rapid formation of adenosine [14]. Since the early reports of Peralta et al. [15, 16], *in vivo* and *in vitro* studies have shown that IP increases extracellular adenosine levels that in turn triggers IP protective effects upon stimulation of A2aR of liver cells. Consistently pretreatment with adenosine A2 receptor agonists enhances liver tolerance against hypoxia and I/R damage, while pharmacological or genetic inhibition of A2aR activation prevents the beneficial effects of IP [15–25].

The mechanisms responsible for A2aR-mediated hepatoprotection during IP are both indirect and direct. The indirect mechanisms depend on the maintenance of nitric oxide (NO) synthesis [15, 16] induced by preventing the downregulation of NO synthase of liver endothelial cells induced by I/R [26]. The direct effects are due to the activation of intracellular survival pathways as a consequence of the stimulation of the A2aR expressed on liver cells.

4. Adenosine A2a Receptor Activation Protects Hepatocyte Hypoxic Damage

In the past years, we have employed the *in vitro* model of primary rat hepatocytes preconditioned with a brief hypoxia-reoxygenation period to investigate the intracellular mechanisms responsible for the direct hepatoprotective action of A2aR stimulation. These studies have shown that A2aR stimulation activates a complex array of protective signals that contribute to the induction of hepatocytes resistance to hypoxic damage (Figure 1). Upon A2aR stimulation, with adenosine or pharmacological agonists, the activation of Gs protein and consequently of adenylate cyclase and protein kinase A (PKA) occurs [19, 20, 27]. PKA phosphorylates A2aR and shifts its coupling to Gi protein and Src kinase thus activating the surviving mediator phosphatidylinositol-3-kinase (PI3K) and its downstream effector Akt [21]. This allows the stimulation of phospholipase C, the recruitment of the specific isoforms δ and ε of protein kinase C (PKC), and the activation of p38 MAPK [19, 20, 27]. Full activation of preconditioning responses also needs downmodulation of inhibitory enzymes of PKC and PI3K. Hypoxic preconditioning as well as A2aR stimulation induces, in fact, a RhoA-GTPase-dependent inhibition of the diacylglycerol kinases θ, thus increasing diacylglycerol (DAG) and sustaining activation of the DAG-dependent PKC δ and ε [28]. Consistently recent "*in vivo*" studies with specific PKC δ inhibitors confirmed the critical role of PKC and, particularly, of PKC δ in mediating the protective effect of IP [25]. A2aR stimulation also induces the degradation of the PI3K inhibitor, phosphatase and tensin homologue deleted from chromosome 10 (PTEN), through a NADPH oxidase-dependent mechanism, thus allowing the maintenance of the PI3K-dependent signals [29]. The above observations indicate a key role played by PI3K and p38 MAPK in hepatocyte preconditioning as also confirmed by *in vivo* studies that reported a marked increase in the dual phosphorylation of hepatic p38 MAPK [30] and demonstrated the implication of PI3K in mediating hepatoprotection in preconditioned liver [31].

Biochemical studies shed light on mechanisms by which these protective signal networks induce the increased resistance of preconditioned hepatocytes to hypoxic injury.

As illustrated in Figure 1, hepatocytes death upon ATP depletion is precipitated by the deregulation of Na^+ homeostasis [32]. An irreversible increase of intracellular Na^+ promotes, in fact, hepatocytes killing induced by several insults including oxidative stress, mitochondrial toxins, and warm and cold hypoxia and at the first phases of reoxygenation [32–35]. Na^+ alterations that follow ATP depletion are the result of a combined block of the ATP-dependent Na^+ efflux through the Na^+/K^+ ATPase and of the activation of Na^+/H^+ exchanger and Na^+/HCO_3^- cotransporter in response to cytosolic acidification [32]. In the metastable phase that precedes death, hepatocytes respond to the progressive increase of intracellular Na^+ with the stimulation of the volume regulatory decrease mechanisms, that is, activation of the K^+ channels and K^+ efflux. The decrease of intracellular K^+ under a critical threshold definitively impairs the volume regulatory systems and leads to a sudden increase of hepatocytes volume, with osmotic lysis and death of hepatocytes [35].

Interestingly hypoxic preconditioning and A2aR activation prevent the irreversible Na^+ increase that precedes hypoxic hepatocytes damage. As shown in Figure 1, A2aR stimulation allows the maintenance of intracellular pH and

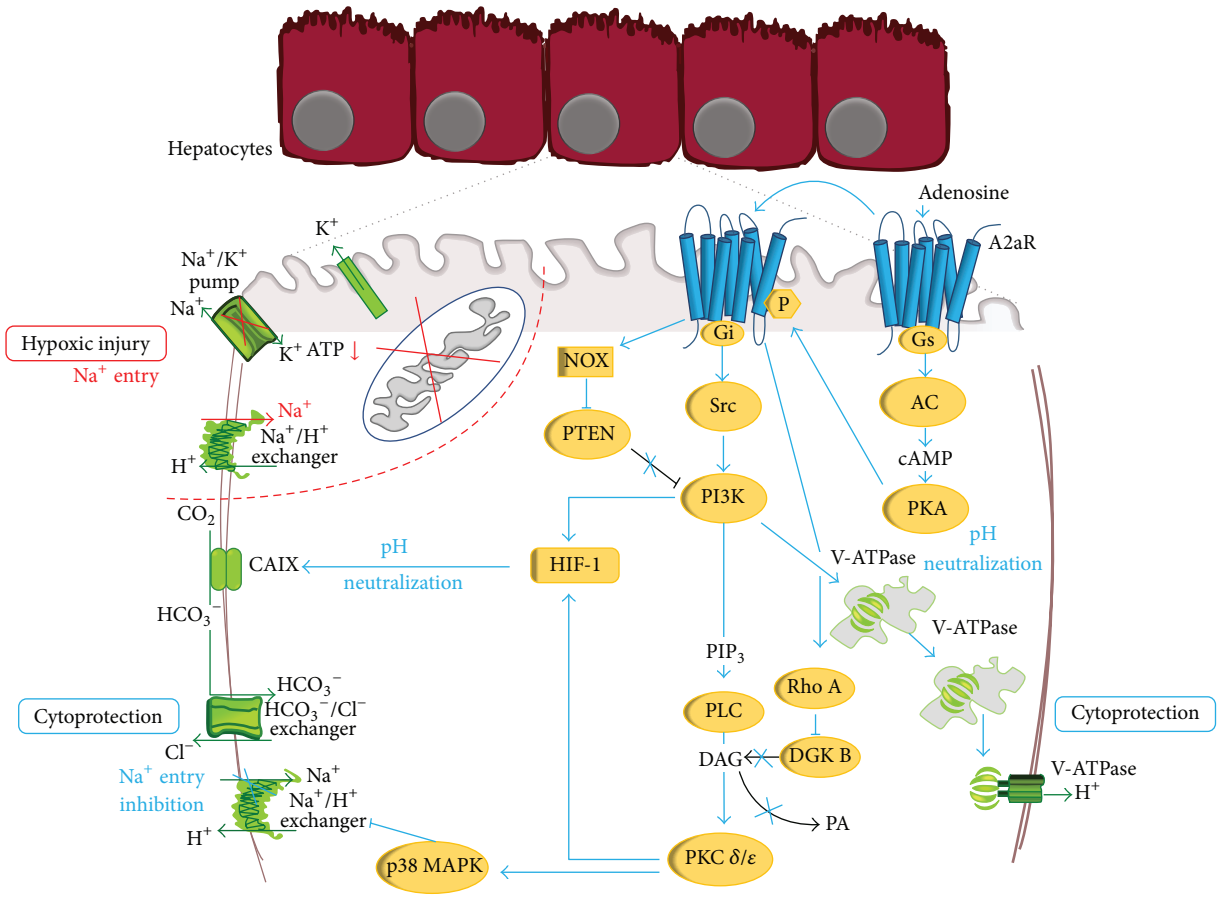

Figure 1: Molecular mechanisms involved in hypoxic injury of primary rat hepatocytes and in their protection upon A2aR stimulation. Hypoxic damage: ATP depletion causes intracellular acidosis, inhibition of the Na^+/K^+ ATPase, and activation of the Na^+/H^+ exchanger with an increase in intracellular Na^+ content and activation of the K^+ channel. A2aR protection: A2aR stimulation induces the sequential activation of PKA, Gs and Gi protein, Src, PI3K, PLC, PKC δ, and ε and p38 MAPK. A2aR also inhibits the negative regulators of PKC and PI3K, DGK, and PTEN. PI3K activates V-ATPase that maintains intracellular pH avoiding the activation of the Na^+/H^+ exchanger and Na^+ overload. PI3K and PKC δ and ε activate HIF with production of CAIX. CAIX converts CO_2 into bicarbonate that enters into hepatocyte through the Cl^-/HCO_3^- exchanger. This neutralizes intracellular pH without activation of the Na^+/H^+ exchanger and the consequent Na^+ increase. (See also text and [19, 20, 27, 28, 36, 37, 40].)

prevents the activation of the Na^+-dependent systems of pH regulation [19, 36]. Such effect is p38 MAPK- and PI3K-dependent and is due to the activation and translocation to the plasma membrane of the vacuolar ATPase (V-ATPase). V-ATPase acts as alternative pH buffering system and allows proton extrusion avoiding the irreversible Na^+ accumulation that precipitates hypoxic hepatocytes death [36, 37].

The protective effects of A2aR stimulation can be either immediate (early preconditioning) or delayed (late preconditioning). Early preconditioning allows hepatocytes to respond to a pathogenic stress that immediately follows the preconditioning stimulus and involves the activation of constitutive molecular systems. Late preconditioning is, instead, able to increase hepatocytes resistance to hypoxia up to 24 hours after the preconditioning stimulus and involves DNA transcription and *de novo* protein synthesis. Hypoxia-inducible factor 1 (HIF-1) is the main regulator of tissue adaptation to oxygen deprivation [38] and it is found to be increased in human transplanted livers exposed to IP [39]. Consistently we found that late hypoxic preconditioning of primary cultured hepatocytes is mediated by an A2aR-dependent nonhypoxic HIF-1 activation and the consequent production of its target protein carbonic anhydrase IX (CAIX) [34]. As shown in Figure 1, A2aR induces a PI3K- and PKC-dependent nuclear translocation, DNA binding, and activation of the nuclear transcription factor HIF-1. In turn, HIF-1 induces the expression of CAIX that converts CO_2 into bicarbonate in the extracellular milieu. Bicarbonate then is transported into the hepatocytes through the Cl^-/CO_3 exchanger and neutralizes the intracellular acids, thus maintaining the physiological cytosolic pH and preventing Na^+ accumulation [40].

5. Adenosine A2a Receptor Activation Protects Hepatocytes Lipotoxicity

The shortage of organs for liver transplantation has led to expansion of the criteria for the acceptance of marginal

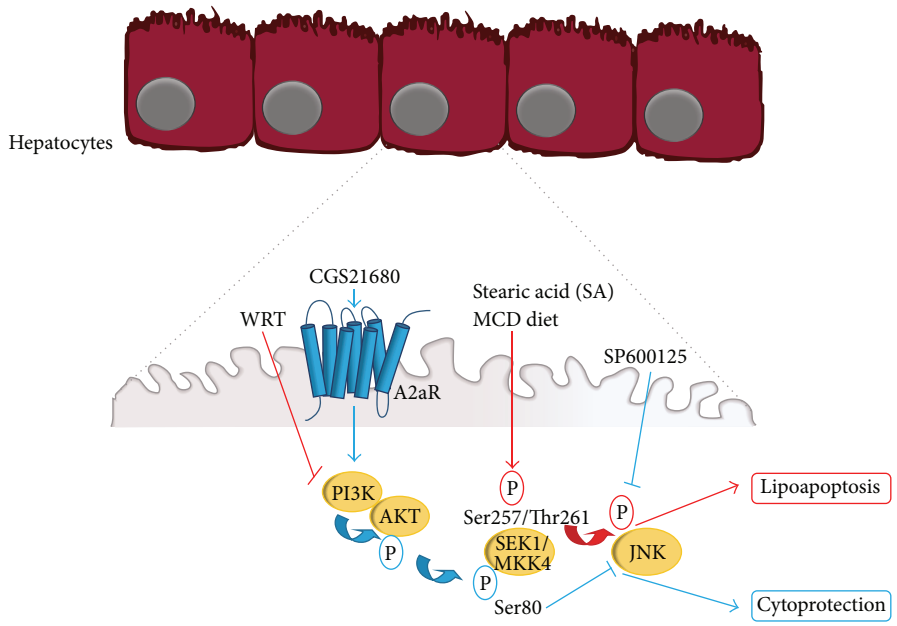

Figure 2: Molecular mechanisms involved in the lipotoxic effects of free fatty acids and in their protection upon A2aR stimulation. Stearic acid (SA) induces primary rat hepatocyte apoptosis by activating JNK-1/2 through the stimulation of MKK4/SEK1. A2aR activation prevents apoptosis by a PI3K/Akt-dependent inhibition of MKK4/SEK1. (See also text and [51, 67].)

donors, including the use of steatotic grafts [41]. Steatosis is characterized by accumulation of excess fat, that is, when the lipid content in cell exceeds 5% of lipid of total liver weight. Steatosis is the most frequent hepatic lesion in western countries with prevalence in the general population ranging from 3% to 15% but reaching up to 70% among overweight individuals [41, 42]. Importantly the presence of fatty infiltration dramatically reduces the tolerance of the liver to I/R injury in experimental models [43] increasing pathological consequences of I/R upon human liver surgery. Indeed clinical meta-analysis shows that patients with steatosis have an up to twofold increased risk of postoperative complications, and those with excessive steatosis have an almost threefold increased risk of death [44]. Several factors such as an increase of oxidative stress, mitochondrial alterations, and ATP depletion can participate in the decreased tolerance of steatotic liver to I/R injury compared with normal livers [45–48]. The accumulating lines of evidence on the phenomenon known as lipotoxicity [49] indicate that the hepatotoxic effects of free fatty acids may represent further attractive mediators of this process. The pathophysiological picture of steatosis is, in fact, characterized by an increase of circulating nonesterified free fatty acids and their metabolites [50] which have been shown to induce hepatic cell apoptosis through JNK activation [45].

The application of IP to fatty livers has demonstrated interesting results. IP, in fact, almost halved transaminase release and the histological evidence of liver cell death showing a greater efficacy of IP in steatotic liver compared to normal liver [48]. The mechanisms responsible for these beneficial effects are, however, unclear.

In recent studies, we evaluated the capacity of A2aR stimulation to prevent lipoapoptosis of primary rat hepatocytes and to inhibit the development of nonalcoholic steatohepatitis in rat fed with MCD (methionine choline-deficient) diet [51]. The treatment of primary rat hepatocytes with the free fatty acid, stearic acid (SA), promoted apoptosis by inducing MKK4 (mitogen activated protein kinase kinase-4)/SEK1 (stress-activated protein kinase/extracellular-signal regulated kinase kinase-1) and JNK-1/2 (c-Jun N-terminal kinase-1/2) activation (Figure 2). The pharmacological A2aR stimulation prevented JNK-1/2 activation by a PI3K/Akt-mediated block of MKK4/SEK1 and also protected lipoapoptosis *in vitro* (Figure 2) and the progression of steatosis to steatohepatitis *in vivo* [45]. These findings may have multiple implications. First, A2aR activation is able to exert separate protective effects against lipotoxicity associated steatosis and against I/R. This may account for additive protective action of A2aR activation and for the increased efficacy of IP in preventing I/R injury in fatty liver (researches are in progress to investigate this point). In addition, the capacity of a molecular inductor of IP to protect against hepatic insults also different from I/R injury potentially broadens the field of clinical application of IP. The activation of IP by pharmacological stimulation of one or some of its mediators would allow, in fact, its employment in all the clinical settings where the chirurgical IP is not applicable.

6. Proteome Reveals Protection Mechanisms in Preconditioned Hepatocytes and LSECs

An important approach to identify new protein mediators of liver preconditioning is the use of the proteomic analysis.

In a recent research we evaluated the proteomic patterns of primary hepatocytes and sinusoidal endothelial cells (LSECs) isolated from mice liver following I/R with or without pretreatment with the A2aR agonist CGS21680 [52]. Hepatocytes and LSECs are the main targets of I/R injury and of the beneficial effects of IP. In comparison to hepatocytes, the knowledge of the molecular mechanisms responsible for the effects of I/R and IP on LSECs is very limited [53]. LSECs, however, have been demonstrated to be largely sensitive to ischemic preservation and I/R [54]. Early studies showed that cultured LSECs exposed to hypoxia-reoxygenation produce high level of oxidative species that can lead to LSECs damage [54]. More recently, ischemic preservation of LSECs demonstrates the downregulation of the transcription factor Kruppel-like factor 2 (KLF2) [55] that is involved in the induction of a number of protective genes including the transcription factor Nrf2 that controls the expression of several antioxidant enzymes such as NAD(P)H dehydrogenase, quinone 1 (NQO1), glutathione peroxidase (GPX), or heme-oxygenase 1 (HO-1) [56]. Consistently recent reports show that remote or intestinal preconditioning prevents hepatic I/R injury via HO-1 mediated mechanisms [57, 58]. In addition the microcirculatory disturbances are a hallmark of hepatic I/R injury [59] and IP application was demonstrated to prevent both LSECs damage and microcirculatory alteration [60, 61].

The employment of proteomic analysis allowed us to evidence profound changes of hepatocytes and LSECs proteome, providing new insights into some critical aspects of I/R injury and IP-induced hepatoprotection. In particular, we observed the modulation of several proteins involved in response to apoptosis and in regeneration and cell signalling and, more importantly, we found major modifications in enzymes involved in oxidative stress protection and energy production, two fundamental processes affected by I/R and IP.

Previous studies clearly showed an increased production of oxidative species during hepatic I/R as well as the capacity of IP to prevent such damaging process [1, 4, 5, 56, 62]. Consistently we evidenced the modulation of several proteins involved in cell response to oxidative stress such as catalase, glutathione transferases GSTP1, GSTP2, and GSTM1, and peroxiredoxin 6. Notably we observed that I/R depressed the antioxidant enzymes content in LSECs exclusively, while A2aR stimulation generally increased the antioxidant defences in both LSECs and hepatocytes. These findings provide a rational base to the greater susceptibility of LSECs to oxidative stress [54] and are consistent with the possible downmodulation of Nrf2 [56]. Our observations indicate, moreover, that the ability of preconditioning to protect against I/R-induced oxidative stress can be explained by an increased antioxidant enzymes expression.

Another critical process is the decrease of ATP content in liver exposed to I/R and its prevention upon preexposure to IP [1–5]. Consistently the proteomic analysis shed light on large modification of enzymes involved in the transport and catabolism of metabolites necessary for energy production. We have observed that I/R induces in hepatocytes and LSECs a decrease of enzymes involved in carbohydrate and lipid catabolism. On the contrary, A2aR stimulation not only rescued the enzymes downregulated by I/R, but even increased enzymes associated with carbohydrate and aminoacids and lipid supply and catabolism. In the specific case of the glycolytic metabolism we found that almost the entire pathway was upregulated in both hepatocytes and LSECs.

The severe ATP depletion of liver tissue exposed to I/R is generally ascribed to the lack of O_2 and glycolytic substrates supply consequent to blood interruption during ischemia [1–3]. Our results indicate that the decrease in the efficiency of the pathways involved in the anaerobic ATP production can significantly exacerbate this process. On the other hand, the rescue or increase of the same pathways by A2aR stimulation can explain the maintenance of the ATP content of preconditioned liver. Another critical aspect is the inability of I/R-injured liver to recover aerobic ATP production at blood flow reestablishment during reperfusion and, on the other hand, the ability of IP to prevent such alteration [1–3]. We observed that I/R inhibited in both hepatocytes and LSECs ATP synthesis downmodulating the regulatory subunit B of ATP synthase and also affecting the catalytic subunit A that is essential for completion of the synthase activity. On the other hand, CGS21680 upregulated in hepatocytes and LSECs both ATPA and ATPB and also, in LSECs, the additional catalytic subunit D (ATPH5). Furthermore, in both cells, CGS21680 increased the electron transfer flavoprotein subunit alpha (ETFA), active in oxidative phosphorylation, and, in hepatocytes, S2542, a carrier mediating the transport of CoA in mitochondria that will then enter in the Krebs cycle to produce ATP. This indicated that I/R, by decreasing the enzymes of the mitochondrial metabolism, affects the capacity to synthesize ATP also in presence of O_2 and that A2aR activation restores this process by rescuing or even increasing these enzymes.

Altogether, these results showed that hepatic cells isolated from liver exposed to I/R develop a "pathological phenotype" characterized by a decrease of the metabolic enzymes involved in the aerobic and anaerobic ATP production and, in the specific case of LSECs, an additional decrement of antioxidant defences (Figure 3). On the contrary, A2aR stimulation induces the expression of a "protected phenotype" characterized by an enhancement of enzymes necessary for energy production and ROS detoxification (Figure 3). This gives a sort of metabolic and antioxidant advantage to preconditioned compared to nonpreconditioned cells and can account for the increased resistance to death of preconditioned hepatic tissue during I/R exposure.

7. Clues for Novel Pharmacological Approaches to Minimize Ischemia/Reperfusion in Patients

The analysis of the molecular changes induced by A2aR stimulation suggests novel potential pharmacological strategies to be applied in human hepatic surgery. First, the findings of the multiple mechanisms of liver cell protection induced by A2aR activation strongly enforce the idea to translate A2aR agonists

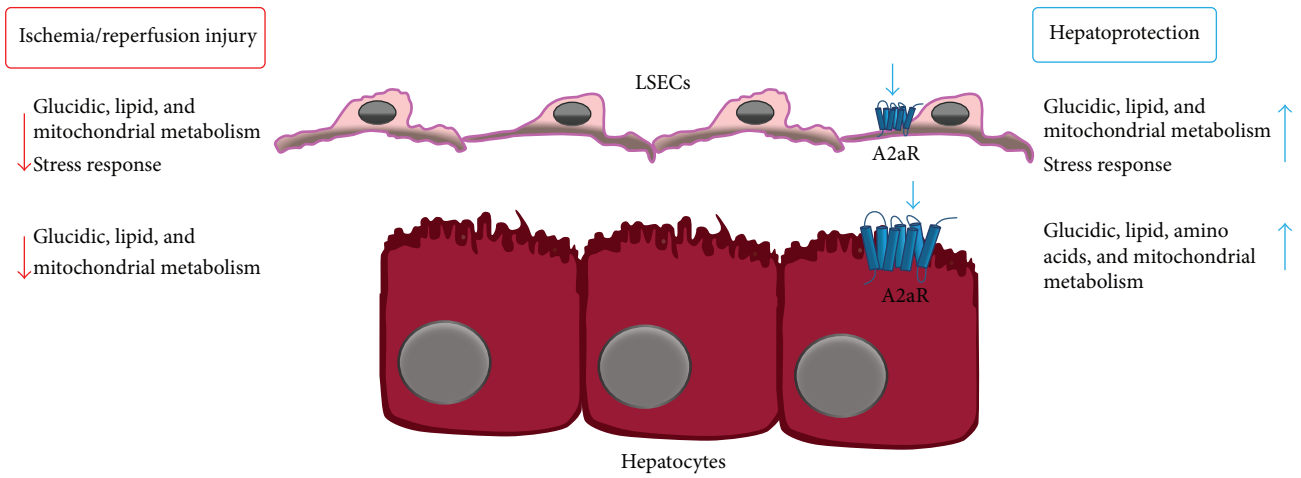

Figure 3: Main proteomic changes of hepatocytes and LSECs upon A2aR stimulation and/or hepatic ischemia/reperfusion. Mice liver exposure to ischemia/reperfusion (I/R) downmodulates proteins associated with glucidic, lipid, and mitochondrial metabolism in hepatocytes and LSECs and, specifically in LSECs, with stress response. These proteins or proteins associated with the same processes are restored or upregulated in both hepatocytes and LSECs, upon A2aR stimulation after mice *in vivo* treatment with the A2aR agonist CGS21680. (See also text and [52].)

to the clinical practise as hepatoprotective tool. In addition to the chemical A2aR agonists such as CGS21680, apadenoson (ALT-146), and ATL-313 largely employed in the preclinical models (see [63] for review), pharmacological agents leading to A2aR activation are already available for clinical purpose in humans. For example, the compound known as regadenoson (CVT-3146) is already approved by the U.S. Food and Drug Administration and it is in use as coronary vasodilator [64, 65].

Additionally, the molecular identification of pleiotropic effects of A2aR stimulation implicates the possibility to intensify these beneficial effects by a concomitant stimulation of their mediators. Moreover, in relation to the needed clinical setting, it might be of interest to achieve a focused stimulation of specific protective signals. For example, in case of short surgical hepatic interventions, it might be favourable to stimulate the protective network of early preconditioning. The choice could be then a simultaneous treatment with A2aR agonists and DGK and PTEN inhibitors in order to sustain the A2aR-induced repression of the negative regulators of PKC and PI3K that are activated within the first hour after stimulation of A2aR. In case of prolonged interventions, like those necessary for major liver surgery and transplantation, the cocktail treatment could additionally include items able to sustain HIF activation such prolyl hydroxylase inhibitors that appear to be well tolerated in patients [66]. Critical would be also the exploitation of antioxidant and metabolic advantages of preconditioned liver cells. In particular, the increased antioxidant enzymatic efficiency of A2aR preconditioned liver cells could be improved by the inclusion in liver graft conservation solutions of natural or synthetic antioxidants [67]. On the other hand, the increased metabolic activities of preconditioned liver cells can take a further advantage by the supplementation with energy-linked metabolites to sustain the glucidic, aminoacids, and lipid catabolism and thus anaerobic and aerobic ATP production.

Abbreviations

IP: Ischemic preconditioning
I/R: Ischemia/reperfusion
A2aR: Adenosine A2a receptor
ROS: Reactive oxygen species
PKA: Protein kinase A
PI3K: Phosphatidylinositol-3-kinase
PKC: Protein kinase C
DGK: Diacylglycerol kinases
DAG: Diacylglycerol
PTEN: Phosphatase and tensin homologue deleted from chromosome 10
V-ATPase: Vacuolar ATPase
HIF-1: Hypoxia-inducible factor 1
CAIX: Carbonic anhydrase IX
SA: Stearic acid
MKK4: Mitogen activated protein kinase kinase-4
SEK1: Stress-activated protein kinase/extracellular-signal regulated kinase kinase-1
JNK 1/2: c-Jun N-terminal kinase-1/2
LSECs: Sinusoidal endothelial cells
KLF2: Kruppel-like factor 2
GST: Glutathione transferase
NQO1: NAD(P)H dehydrogenase quinone 1
GPX: Glutathione peroxidase
HO-1: Heme-oxygenase 1
ETFA: Electron transfer flavoprotein subunit alpha.

Conflict of Interests

The authors declare no conflict of interests.

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