TRANSPLANT INTERNATIONAL

Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Silent information regulator 1 protects the liver against ischemia–reperfusion injury: implications in steatotic liver ischemic preconditioning*

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Keywords
ischemic preconditioning, liver ischemia–reperfusion injury, nitric oxide, oxidative stress, Sirtuin 1.

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Conflicts of Interest
The authors have declared no conflict of interests.

*This paper was presented as full oral presentation at the 16th Congress of the European Society for Organ Transplantation (ESOT), 8–11 September, 2013, Vienna, Austria

Received: 5 July 2013
Revision requested: 27 July 2013
Accepted: 23 January 2014
Published online: 5 March 2014

doi:10.1111/tri.12276

Abstract

Ischemia–reperfusion (IR) injury is an important problem in liver surgery especially when steatosis is present. Ischemic preconditioning (PC) is the only surgical strategy that has been applied in patients with steatotic livers undergoing warm ischemia. Silent information regulator 1 (SIRT1) is a histone deacetylase that regulates various cellular processes. This study evaluates the SIRT1 implication in PC in fatty livers. Homozygous (Ob) Zucker rats were subjected to IR and IR + PC. An additional group treated with sirtinol or EX527 (SIRT1 inhibitors) before PC was also realized. Liver injury and oxidative stress were evaluated. SIRT1 protein levels and activity, as well as other parameters involved in PC protective mechanisms (adenosine monophosphate protein kinase, eNOS, HSP70, MAP kinases, apoptosis), were also measured. We demonstrated that the protective effect of PC was due in part to SIRT1 induction, as SIRT1 inhibition resulted in increased liver injury and abolished the beneficial mechanisms of PC. In this study, we report for the first time that SIRT1 is involved in the protective mechanisms induced by hepatic PC in steatotic livers.

Introduction

Ischemia–reperfusion (IR) injury is the main cause of organ damage and initial poor function of liver grafts and is inherent to surgical procedures in liver transplantation. The shortage of organs has led to expand the criteria for the acceptance of marginal donors, including the use of steatotic grafts [1]. However, the use of fatty liver grafts

increases the rates of primary nonfunction and consequently compromises the graft viability after transplantation, exacerbating the organ shortage [2].

The high vulnerability of fatty livers against IR injury is due to the abnormal accumulation of fat within the cytoplasm of hepatocytes, resulting in increased hepatocellular volume and narrowing of sinusoid. As a consequence, hepatic flow is severely obstructed and results in important alterations in liver microcirculation that compromises the suitable graft revascularization and viability after transplantation [3]. Also, another important consequence of fat accumulation in steatotic livers is that hepatocytes are more susceptible to oxidative stress [4].

Therapeutic surgical strategies such as ischemic preconditioning (PC) diminish the high vulnerability of steatotic livers against IR injury [5–8]. The induced hepatoprotection is mediated, in part, through nitric oxide (NO) generation by endothelial nitric oxide synthase (eNOS) which interferes with the mechanisms responsible for IR damage, such as the exacerbated lipoperoxidation in steatotic livers [5]. In addition, PC promotes the activation of adenosine monophosphate protein kinase (AMPK), a fuel energy sensor that contributes to maintain cellular function and integrity [9]. In this line, we have previously demonstrated a direct relationship between AMPK and NO in the protective mechanisms of PC in rat steatotic liver transplantation [10].

Silent information regulator 1 (SIRT1) is a member of the family of class III histone deacetylases involved in stress responses including hypoxic stress, heat shock stress, and inflammation [8,11–13]. SIRT1 deacetylates both histone and nonhistone proteins in a NAD^+-dependent manner, including p53, eNOS, and AMPK. [14,15]. SIRT1 deacetylates p53 in the C-terminal Lys-382 residue and thus reduces its transcriptional activity and its ability to induce apoptosis [15,16]. Furthermore, it has been reported that SIRT1 ameliorates vascular function in endothelial cells after laminar shear stress, as enhancement of SIRT1 activity was associated with eNOS activation [17]. Moreover, various studies in cultured cells and in liver *in vivo* have shown evidence of AMPK activation by SIRT1 [18–20].

Silent information regulator 1 protects the heart from IR injury and decreases oxidative stress [21,22]. Moreover, the fact that SIRT1 downregulation under IR insult in heart was attenuated by PC suggests that SIRT1 may partly mediate the benefits induced by PC [23]. Accumulating data demonstrate the relationship between SIRT1 and AMPK/NO [17,24], both mediators of PC but no data have yet been reported in liver, regarding the involvement of SIRT1 in PC.

The role of SIRT1 in liver IR injury has been poorly investigated. For this reason, the aim of this paper is focused on the study of SIRT1 function in fatty liver IR injury, as well as to explore whether it is involved in the protective mechanisms induced in liver by PC.

Material and methods

Experimental animals

Homozygous obese (Ob) Zucker rats (Charles River, France) aged 12 weeks were used. Ob rats lack the cerebral leptin receptor and showed severe macro- and microvesicular fatty infiltration in hepatocytes (40–60% steatosis). All procedures were performed under isofluorane inhalation anesthesia. This study was performed in accordance with European Union regulations (Directive 86/609 EEC). Animal experiments were approved by the Ethics Committees for Animal Experimentation (CEEA, Directive 396/12), University of Barcelona. Animals were randomly distributed into groups as described below.

Experimental design

Group 1: sham [$n = 6$]. Ob rats were subjected to laparatomy, and hepatic hilum vessels were dissected [25].

Group 2: IR [$n = 6$]. Ob rats were subjected to 60 min of partial (70%) ischemia by applying a microvascular clamp to the hepatic artery and the portal vein, thus blocking the hepatic inflow to the median and left lobes. Then, 24-hour reperfusion was followed [25].

Group 3: PC [$n = 6$]. To induce PC, 5 min of partial ischemia (70%) followed by a reflow for 10 min was applied in ob rats [25]. Livers were then subjected to IR as in group 2.

Group 4: sirtinol + PC [$n = 6$]. As in group 3, but treated with sirtinol (dissolved in DMSO), a SIRT1 inhibitor (0.9 mg/kg i.v.) 5 min before PC [26].

Group 5: EX + PC [$n = 6$]. As in group 3, but treated with EX527 (dissolved in DMSO/saline), a SIRT1 inhibitor (5 mg/Kg i.v.) 30 min before PC [27].

Biochemical determinations

Transaminases assay

Hepatic injury was assessed in terms of transaminases levels with commercial kits from RAL (Barcelona, Spain). Briefly, plasma extracts were collected before liver extraction and centrifuged at 4 °C for 10 min at 0.8 *g*. Then, 200 μl of the supernatant were added to the substrate provided by the commercial kit. ALT levels were determined at 365 nm with an UV spectrometer and calculated following the supplier instructions [28].

Lipid peroxidation assay

Lipid peroxidation in liver was used as an indirect measurement of the oxidative injury induced by ROS. Lipid peroxidation was determined by measuring the formation of malondialdehyde (MDA) with the thiobarbiturate reaction [29]. MDA in combination with thiobarbituric acid (TBA)

forms a pink chromogen compound whose absorbance at 540 nm was measured. The result was expressed as nmols/mg protein.

SIRT1 activity assay

Silent information regulator 1 activity was determined according to the method described by Becatti *et al.* [30] with some modifications. Protein extracts were obtained using a mild lysis buffer (50 mM Tris–HCl pH 8, 125 mM NaCl, 1 mM DTT, 5 mM MgCl2, 1 mM EDTA, 10% glycerol, and 0.1% NP40). SIRT1 activity was measured using a deacetylase fluorometric assay kit (CY-1151; CycLex, MBL International Corp.), following the manufacturer's protocol. A total of 25 µl of assay buffer containing the same quantity of protein extracts (5 µl) were added to all wells, and the fluorescence intensity was monitored every 2 min for 1 h using the fluorescence plate reader Spectramax Gemini, applying an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The results are expressed as the rate of reaction for the first 30 min, when there was a linear correlation between the fluorescence and this period of time.

Western blotting analysis

Liver tissue was homogenized in RIPA buffer (Tris–HCl pH = 7.5 50 mM, NaCl 150 mM, SDS 0.1%, $C_{24}H_{39}O_4Na$ 1%, NP-40 1%, EDTA 5 mM, Na_3VO_4 1 mM, NaF 50 mM, DTT 1 mM, 1 complete tablet/100 ml) for SIRT1 immunodetection and in Hepes buffer (NaCl 40 mM, EDTA 1 mM, Triton X 0.1%, glycerol 5%, NaP_2O_7 10 mM, b-glycerophosphate 10 mM, Na_3VO_4 1,5 mM, NaF 50 mM, 1 complete tablet/100 ml, Hepes-KOH pH = 7.4 50 mM) for the rest of proteins. Fifty µg of proteins were electrophoresed on 8–15% SDS-PAGE gels and transblotted on PVDF membranes (Bio-Rad). Membranes were then blocked with 5% (w/v) nonfat milk in TBS containing 0.1% (v/v) Tween 20 and incubated overnight at 4 °C with anti-SIRT1 (#07-131, Merck Millipore, Billerica, MA), anti-ac-p53 (ab37318, abcam, UK), anti-p-AMPK (Thr172, #2535), anti-caspase 3 (#9662), anti-cytochrome C (#4272), anti-p-p38 MAP kinase (Thr180/Tyr182, #9211), anti-p-p44/42 MAPK (Erk1/2; Thr202/Tyr204, #9101; all the above antibodies were purchased from Cell Signaling, Danvers, MA) anti-eNOS (610296), anti-HSP70 (610607; both from Transduction Laboratories, Lexington, KY), and anti-β-actin (A5316, Sigma Chemical, St. Louis, MO, USA). After washing, bound antibody was detected after incubation for 1 h at room temperature with the corresponding secondary antibody linked to horseradish peroxidase. Bound complexes were detected using WesternBright ECL-HRP substrate (Advansta) and were quantified using the Quantity One software for image analysis. Results were expressed as the densitometric ratio between the protein of interest and the loading control (β-actin).

Histology

To estimate the severity of hepatic injury, hematoxylin–eosin-stained sections were evaluated using an ordinal scale from 0 to 4 as follows: grade 0: absence of injury; grade 1: mild injury consisting in cytoplasmic vacuolation and focal nuclear pycknosis; grade 2: moderate injury with focal nuclear pycknosis; grade 3: severe necrosis with extensive nuclear pycknosis and loss of intercellular borders; and grade 4: severe necrosis with disintegration of hepatic cords, hemorrhage, and neutrophil infiltration.

Statistics

Data are expressed as mean ± standard error and were compared statistically by the nonparametric Kruskal–Wallis test. A P value <0.05 was considered significant.

Results

SIRT1 protein expression and activity in PC

To study the implication of SIRT1 in PC, we first evaluated its protein expression pattern. As shown in Fig. 1a, the expression of SIRT1 in fatty livers subjected to IR was significantly augmented when compared with sham group. This increase was exacerbated when PC was carried out and reversed after sirtinol (a SIRT1 inhibitor) treatment. Treatment with EX527, another SIRT1 inhibitor, did not affect SIRT1 protein levels during PC. Furthermore, PC group exhibited an increased deacetylase activity compared with both IR and sham groups (Fig. 1b), and as expected, sirtinol and EX527 treatment groups during PC resulted in decreased SIRT1 activity. However, no significant differences in SIRT1 activity were observed between sham and IR groups or between the inhibitors groups. In addition to this, we analyzed the acetylation (Lys-382) of p53 (ac-p53), a direct substrate of SIRT1 (Fig. 1c). PC group was characterized by a marked decrease in ac-p53, which was reversed by treatment of both inhibitors. The increase of ac-p53 was more significant for sirtinol than EX527. Finally, the ac-p53 levels between sham and IR group were not significantly altered.

Liver injury

We next determined whether SIRT1 plays a role in the prevention of IR injury mediated by PC. As shown in Fig. 2a, IR injury increased ALT levels, which were reversed by PC. The administration of both sirtinol and EX527 resulted in

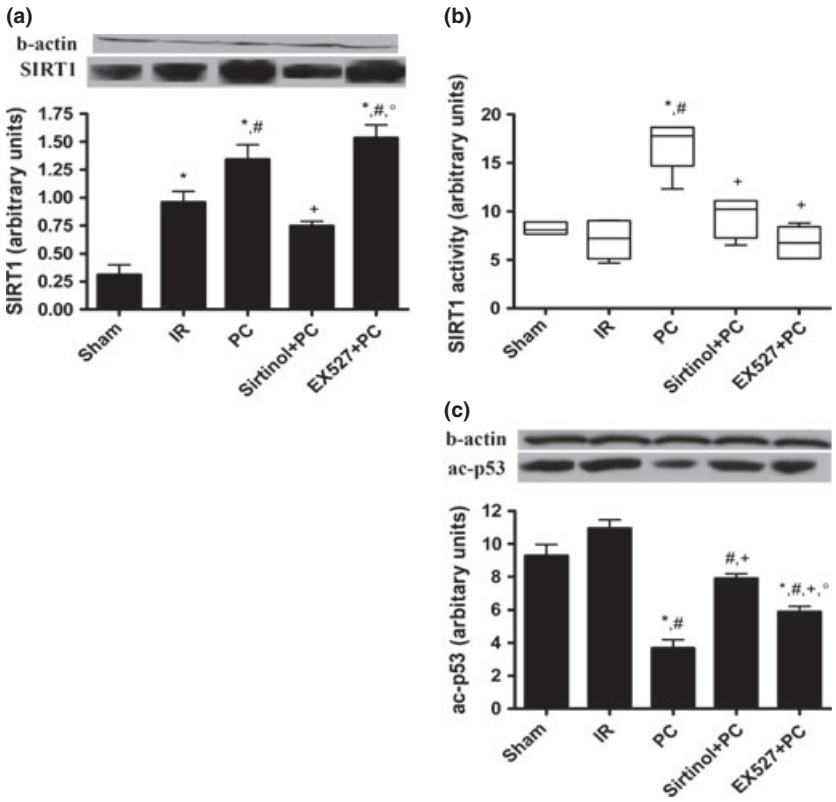

Figure 1 Role of preconditioning (PC) on SIRT1 expression and SIRT1 activity in steatotic livers subjected to ischemia–reperfusion (IR) injury. (a) Western blot and densitometric analysis of SIRT1; (b) SIRT1 enzymatic activity; (c) densitometric analysis of ac-p53. Sham: anesthesia and laparotomy, IR: 60 min partial ischemia and 24 h of reperfusion, PC: IR with previous preconditioning induced by 5 min of ischemia and 10 min of reperfusion, sirtinol + PC: administration of sirtinol 5 min before PC. EX527 + PC: administration of EX527 30 min before PC. PC $^{*}P < 0.05$ versus sham, $^{\#}P < 0.05$ versus IR, $^{\dagger}P < 0.05$ versus PC; $^{\circ}P < 0.05$ versus sirtinol +PC.

increased ALT levels, but sirtinol treatment provoked liver injury to a lesser extent than EX527. This result is consistent with the histological findings shown in Fig. 2b. Steatotic livers subjected to IR exhibited severe and extensive areas of coagulative necrosis with neutrophil infiltration (75%) that were significantly reduced (25%) when PC was performed. Pretreatment with sirtinol and EX527 aggravated tissue lesions as shown by extensive areas of coagulative necrosis (50% and 80% respectively), in comparison with PC group (Fig. 2c).

eNOS and AMPK activation

Given that the benefits of PC are mediated in part by NO, we explored the effects of SIRT1 on eNOS expression and AMPK activation induced by PC. As shown in Fig. 3a,b, PC potentiated IR induced eNOS expression and AMPK phosphorylation, respectively. Furthermore, the increased levels of eNOS expression/AMPK activation induced by PC were completely blocked by sirtinol and EX527 administration.

Oxidative stress and heat shock proteins

We evaluated the relevance of SIRT1 on the prevention of oxidative stress induced by IR. For this reason, we measured MDA levels in liver tissue. As indicated in Fig. 4a, the high MDA levels observed in IR group were reduced when PC was applied. SIRT1 inhibition resulted in increased lipid peroxidation and the highest MDA increase was observed when EX527 was administrated prior to PC. Furthermore, IR induced a significant increase in heat shock protein 70 (HSP70), which was further reinforced during PC. In addition, both sirtinol and EX527 reversed the HSP70 overexpression induced by PC (Fig. 4b).

MAPK kinases

We also explored the effect of SIRT1 on mitogen-activated protein kinases (MAPK) activation. As shown in Fig. 5a, PC increased extracellular signal-regulated kinase (ERK) phosphorylation, when compared with IR and sham groups. Moreover, we observed that PC reversed the

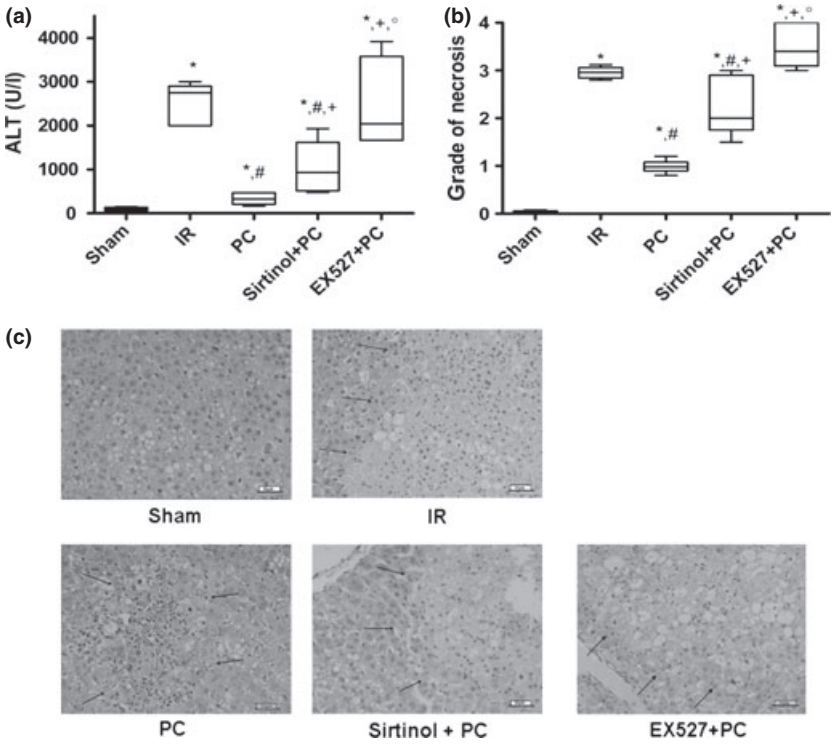

Figure 2 Effect of the inhibition of SIRT1 during preconditioning (PC) in hepatic injury. (a) Photometric analysis of alanine aminotransferase (ALT) levels; (b) Histological lesions in steatotic liver by hematoxylin-eosin-stained sections; (c) Grade of necrosis in the experimental groups. Sham: anesthesia and laparotomy, ischemia–reperfusion (IR) injury: 60 min of partial ischemia and 24 h of reperfusion, PC: IR with previous preconditioning induced by 5 min of ischemia and 10 min of reperfusion, PC + sirtinol: administration of sirtinol 5 min before PC. EX527 + PC: administration of EX527 30 min before PC. $^{*}P < 0.05$ versus sham, $^{\#}P < 0.05$ versus IR, $^{\dagger}P < 0.05$ versus PC, $^{\circ}P < 0.05$ versus sirtinol + PC.

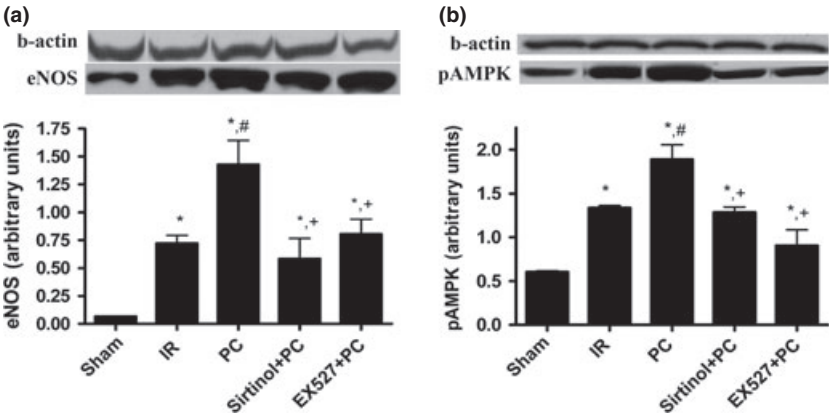

Figure 3 Implication of Silent Information Regulator 1 on eNOS expression and adenosine monophosphate protein kinase (AMPK) activation during preconditioning (PC) in steatotic livers. Western blot and densitometric analysis of eNOS and pAMPK (a and b respectively). Sham: anesthesia and laparotomy, ischemia–reperfusion (IR) injury: 60 min partial ischemia and 24 h of reperfusion, PC: IR with previous preconditioning induced by 5 min of ischemia and 10 min of reperfusion, PC + sirtinol: administration of sirtinol 5 min before PC, EX527 + PC: administration of EX527 30 min before PC. $^{*}P < 0.05$ versus sham, $^{\#}P < 0.05$ versus IR, $^{\dagger}P < 0.05$ versus PC.

increased p-p38 protein levels caused by IR (Fig. 5b). Sirtinol and EX527 administration partially reduced the protective effects of PC on MAP kinases modulation, but no differences between both inhibitors were noted.

Apoptosis

We also evaluated the involvement of SIRT1 activation in PC and its consequences on liver apoptosis by measuring

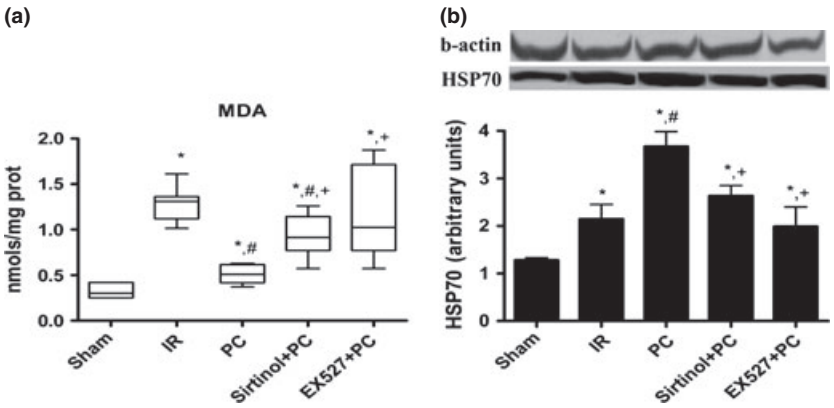

Figure 4 Effect of SIRT1 on oxidative stress and HSP70 expression during preconditioning (PC) in steatotic livers. (a) Photometric analysis of malondialdehyde levels. (b) Western blot and densitometric analysis of HSP70 protein expression. Sham: anesthesia and laparotomy, ischemia–reperfusion (IR) injury: 60 min partial ischemia and 24 h of reperfusion, PC: IR with previous preconditioning induced by 5 min of ischemia and 10 min of reperfusion, PC + sirtinol: administration of sirtinol 5 min before PC. EX527 + PC: administration of EX527 30 min before PC. $^{*}P < 0.05$ versus sham, $^{\#}P < 0.05$ versus IR, $^{\dagger}P < 0.05$ versus PC.

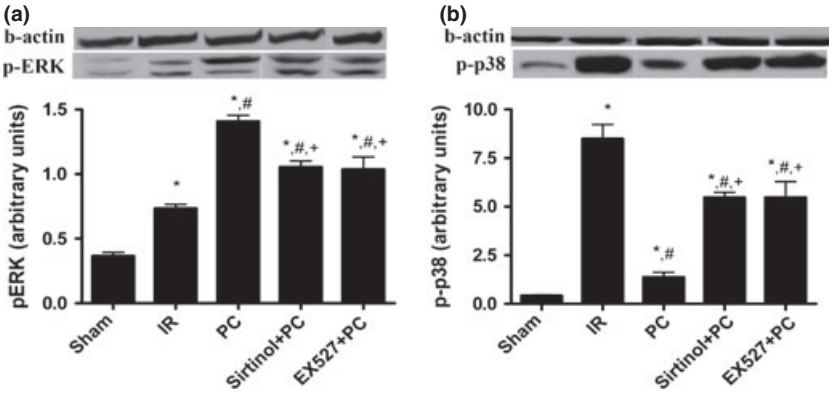

Figure 5 Modulation of MAPK expression and phosphorylation by Silent Information Regulator 1 during preconditioning (PC) in steatotic livers. Western blot and densitometric analysis of pERK (a) and p-p38 (b). Sham: anesthesia and laparotomy, IR: 60 min partial ischemia and 24 h of reperfusion, PC: IR with previous preconditioning induced by 5 min of ischemia and 10 min of reperfusion, PC + sirtinol: administration of sirtinol 5 min before PC. EX527 + PC: administration of EX527 30 min before PC. $^{*}P < 0.05$ versus sham, $^{\#}P < 0.05$ versus IR, $^{\dagger}P < 0.05$ versus PC.

caspase-3, caspase-9 cleavage, and cytochrome c protein levels. Significant increases in the above parameters of apoptosis were seen during IR, which were then reversed when PC was applied (Fig. 6). SIRT1 inhibition by both inhibitors provoked increased liver apoptosis in comparison with PC group.

Discussion

In this study, we report for the first time that SIRT1 is implicated on the prevention of fatty liver IR injury by PC. Firstly, we have evidenced a significant up-regulation of SIRT1 protein levels induced by PC, and secondly, we have demonstrated that SIRT1 inhibition reverses the benefits of PC during liver damage. Furthermore, high SIRT1 deacetylase activity was observed in PC group, which was significantly decreased when SIRT1 was inhibited during PC by either sirtinol or EX527. The diminished levels of ac-p53 (a direct substrate of SIRT1) during PC were consistent with the high deacetylase activity, and this effect was reversed by both SIRT1 inhibitors. These results are in accordance with previous reported data in heart [23,31,32] and brain [15,33,34] where SIRT1 confers protection to those tissues against IR injury.

In addition, our findings support the fact that an overexpression of SIRT1 occurs in fatty livers subjected to IR. This observation agrees with previous *in vivo* and *in vitro* investigations in heart, where the SIRT1 levels were up-regulated by certain stresses, including IR injury, suggesting that SIRT1 could act as a self-compensatory mechanism for preventing tissue damage [21,22,30]. However, we observed that SIRT1 activity, as well as ac-p53 protein levels, was not

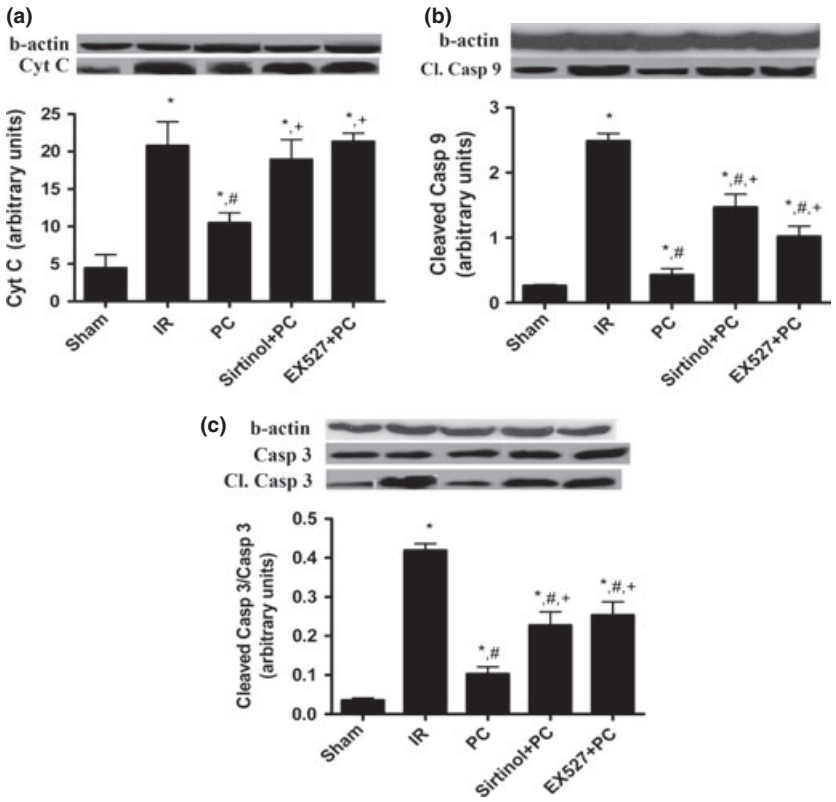

Figure 6 Effect of SIRT1 on liver apoptosis. Western blot and densitometric analysis of cytochrome C (a), cleaved caspase 9 (b) and cleaved caspase3/caspase 3 (c). Sham: anesthesia and laparotomy, ischemia–reperfusion (IR) injury: 60 min partial ischemia and 24 h of reperfusion, preconditioning (PC): IR with previous preconditioning induced by 5 min of ischemia and 10 min of reperfusion, PC + sirtinol: administration of sirtinol 5 min before PC. EX527 + PC: administration of EX527 30 min before PC. $^{*}P < 0.05$ versus sham, $^{\#}P < 0.05$ versus IR, $^{\dagger}P < 0.05$ versus PC.

altered during IR, which implicates that various factors can affect its activity. For example, in a similar study, it was observed that the activity of liver histone deacetylases is decreased only in short times of reperfusion, whereas it remains unchanged after 24 h of reperfusion [35].

Recent investigations in rodent aortic and human endothelial cells reported the relevance of SIRT1 in eNOS activation; SIRT1 interacts with eNOS, resulting in the activation of the enzyme [36–40]. In our study, SIRT1 up-regulation during PC was well correlated with the expression of eNOS which was inhibited after sirtinol or EX527 administration. This result suggests that SIRT1 is involved in PC hepatoprotection that is mediated by NO, counterbalancing the exacerbated microcirculation in fatty livers [5,41].

Protective PC mechanisms are associated with the activation of AMPK, as we have previously reported [9,10]. Once activated, AMPK phosphorylates various substrates to conserve ATP levels and switch on metabolic pathways that generate ATP [9]. The present study demonstrated that SIRT1 inhibition abolished the activation of AMPK during PC, suggesting a potential link between SIRT1 and AMPK signaling in liver PC. Our results are in agreement with reported investigations in hepatic cultured cells and mouse liver *in vivo*, showing that SIRT1 activates AMPK through LKB1 deacetylation [18–20]. In addition, we have previously reported that AMPK and eNOS activation are involved in the benefits of PC in a model of rat steatotic liver transplantation [10]. The fact that SIRT1 inhibition completely abrogated the activation of AMPK and eNOS suggests a potential relationship between SIRT1 and the above factors.

Results reported here also confirm that the overexpression of SIRT1 in PC is responsible for the attenuation of oxidative stress caused by PC. Indeed, SIRT1 inhibition reduced the prevention of lipoperoxidation induced by PC. A similar effect was observed in heart, where the overexpression of SIRT1 also attenuated oxidative stress through the stimulation of FoxO1 transcription factor, thus enhancing antioxidant enzymes like manganese superoxide dismutase [23].

It is well established that stressful conditions such as IR can induce besides ROS, the heat shock transcription response [42]. In this line, we previously provided evidence that HSP70 is activated during PC and protected against IR

injury [43]. Here, we demonstrate that SIRT1 is involved in the regulation of heat shock proteins expression in fatty liver PC, as confirmed by the decrease in HSP70 expression when SIRT1 was inhibited. These data agree with other studies in HeLa cells, demonstrating that SIRT1 enhances HSP70 expression through the regulation of HSF1 transcriptional activity. [44].

Moreover, the oxidative stress can activate MAPK by dual phosphorylation on tyrosine and threonine residues [45,46]. Given that PC affects the MAPK pathways [43,47,48], we examined whether SIRT1 regulated these kinases. We observed that SIRT1 inhibition decreased the expression of p-ERK and augmented p-p38 protein levels. ERK activation during PC protects against IR injury, by inhibiting apoptosis [49], whereas treatment with a p38 activator resulted in increased liver injury when PC was performed on steatotic livers [43]. It has also been reported that SIRT1 modulated MAPK pathways in an experimental model of IR using cardiomyocytes [30].

A variety of stressors, such as DNA damage and ROS, can activate a cascade of mediators, leading to increased apoptosis during IR injury [50]. This is accompanied by the release of cytochrome c, which promotes caspase 9 activation, which in turn activates caspase 3 and the final steps of apoptosis [51]. In our study, decreased levels of apoptotic parameters (caspase 3, caspase 9, and cytochrome c) were observed in the PC group when compared with the IR group, whereas inhibition of SIRT1 during PC promoted the increase in fatty liver apoptosis and would be in accordance with the concomitant p-ERK expression diminution, as previously commented. In addition, as it occurs with MAPK kinases, both inhibitors, sirtinol and EX527, partially reversed the protective effect of PC on apoptosis, suggesting that additional mechanisms can be involved in the beneficial effects of fatty liver PC.

Sirtinol and EX527 are both inhibitors of SIRT1 activity. However, it has been reported that sirtinol can also inhibit human SIRT2 activity *in vitro* [52], whereas EX527 has been described as a more specific inhibitor of SIRT1 and with a lower efficiency for SIRT2 inhibition [53]. In our model, treatment with either sirtinol or EX527 during PC resulted in increased liver injury. The fact that treatment with EX527 dramatically reduced the protective effect of PC, confirm our hypothesis that SIRT1 is involved in the beneficial effects of PC against IR. On the other hand, sirtinol is less potent to prevent the protection provided by PC. This fact may be attributed to its additional inhibitory effect on SIRT2; given that in recent studies, inhibition of SIRT2 has been found to be protective [54,55], the results obtained after sirtinol treatment might be the consequence of the inhibition of both SIRT2 (possible protective effect) and SIRT1 (detrimental effect).

Moreover, it has been shown that inhibition of SIRT1 by sirtinol contributes to the expression of inflammatory cytokines, through the acetylation of NF-κB [56]. However, more recent studies provided data showing that administration of sirtinol in rats subjected to trauma-hemorrhage decreased hepatic/lung injury and production of pro-inflammatory mediators [26,57]. As in our study, the PC+sirtinol group resulted in increased hepatic injury compared with PC group and the administration of sirtinol and EX527 in a sham group provoked no significant changes in the parameters studied (data not shown), a possible protective role of sirtinol should be ruled out. Furthermore, we observed that treatment with sirtinol diminished SIRT1 levels, and a similar effect has been observed in other experimental model, but the underlying mechanisms are to be investigated [58].

In summary, our study demonstrates that SIRT1 is involved in the protective effects of PC against IR injury in fatty livers. More concretely, SIRT1 is associated with the activation of eNOS and AMPK, the attenuation of oxidative stress, and apoptosis (Fig. 7). Therefore, the application of SIRT1 activators, such as resveratrol, could be a potential pharmacological treatment of patients with steatotic livers submitted to liver transplantation. Indeed, it has already been shown that resveratrol prolongs allograft survival after liver transplantation in rats [59].

In conclusion, the data reported here provide new insights into the liver protection, suggesting that SIRT1 is a

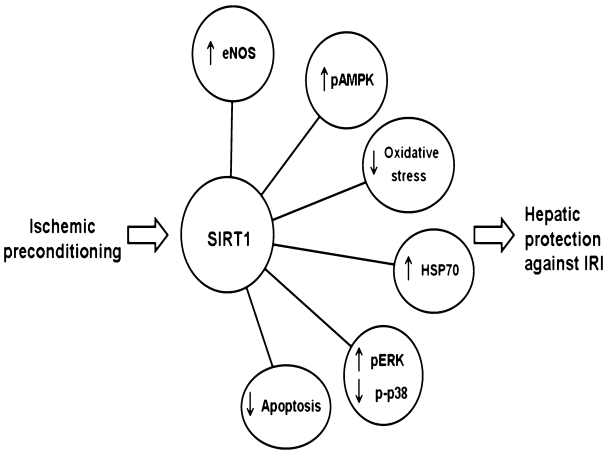

Figure 7 Effects of Silent Information Regulator 1 (SIRT1) in liver ischemic preconditioning. Ischemic preconditioning in fatty livers induces SIRT1 upregulation and enhancement of its deacetylase activity that leads from one hand, to the enhancement of cytoprotective pathways, including eNOS, HSP70, pERK expression and adenosine monophosphate protein kinase activation and from the other hand to downregulation of p-p38 and apoptosis and to decreased oxidative stress. The final result is an enhanced protection of fatty liver against ischemia–reperfusion (IR) injury.

promising pharmacological target to increase the fatty liver tolerance against IR injury.

Authorship

EP and MB: carried out the experimental work. EP, MAZ, and EF-P: provided protocols and analyzed data. MAZ and MB: established the animal experimental model. AS: carried out the histological study. VP, NDeV, EF-P, HBA, and AR: contributed to the critical analyses of the data. EP, MAZ, and JR-C: designed the study, coordinate the experiments, and wrote the paper.

Funding

Eirini Pantazi is fellowship-holder of AGAUR (2012FI_B 00382), Generalitat de Catalunya, Barcelona, Catalonia, Spain. Mohamed Bejaoui is a recipient from CSIC for the development program (I-COOP0005). This work was supported by the Fondo de Investigaciones Sanitarias (FIS PI12/00519) and CiberEHD.

Acknowledgments

This work was supported by the Fondo de Investigaciones Sanitarias (FIS PI12/00519) and CiberEHD. The authors thank Generalitat de Catalunya and CSIC for the economical support for Eirini Pantazi and Mohamed Bejaoui, respectively.

References

1. Busuttil RW, Tanaka K. The utility of marginal donors in liver transplantation. *Liver Transpl* 2003; **9**: 651.
2. Selzner M, Clavien PA. Fatty liver in liver transplantation and surgery. *Semin Liver Dis* 2001; **21**: 105.
3. Ramalho FS, Fernandez-Monteiro I, Rosello-Catafau J, Peralta C. Hepatic microcirculatory failure. *Acta Cir Bras* 2006; **21**(Suppl. 1): 48.
4. Czubkowski P, Socha P, Pawlowska J. Oxidative stress in liver transplant recipients. *Ann Transplant* 2011; **16**: 99.
5. Serafin A, Rosello-Catafau J, Prats N, Xaus C, Gelpi E, Peralta C. Ischemic preconditioning increases the tolerance of fatty liver to hepatic ischemia-reperfusion injury in the rat. *Am J Pathol* 2002; **161**: 587.
6. Massip-Salcedo M, Zaouali MA, Padrissa-Altes S, *et al.* Activation of peroxisome proliferator-activated receptor-alpha inhibits the injurious effects of adiponectin in rat steatotic liver undergoing ischemia-reperfusion. *Hepatology* 2008; **47**: 461.
7. Clavien PA, Selzner M, Rudiger HA, *et al.* A prospective randomized study in 100 consecutive patients undergoing major liver resection with versus without ischemic preconditioning. *Ann Surg* 2003; **238**: 843, discussion 51-2.
8. Brunet A, Sweeney LB, Sturgill JF, *et al.* Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. *Science* 2004; **303**: 2011.
9. Peralta C, Bartrons R, Serafin A, *et al.* Adenosine monophosphate-activated protein kinase mediates the protective effects of ischemic preconditioning on hepatic ischemia-reperfusion injury in the rat. *Hepatology* 2001; **34**: 1164.
10. Carrasco-Chaumel E, Rosello-Catafau J, Bartrons R, *et al.* Adenosine monophosphate-activated protein kinase and nitric oxide in rat steatotic liver transplantation. *J Hepatol* 2005; **43**: 997.
11. Finkel T, Deng CX, Mostoslavsky R. Recent progress in the biology and physiology of sirtuins. *Nature* 2009; **460**: 587.
12. Yeung F, Hoberg JE, Ramsey CS, *et al.* Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. *EMBO J* 2004; **23**: 2369.
13. Majmundar AJ, Wong WJ, Simon MC. Hypoxia-inducible factors and the response to hypoxic stress. *Mol Cell* 2010; **40**: 294.
14. Pillarisetti S. A review of Sirt1 and Sirt1 modulators in cardiovascular and metabolic diseases. *Recent Pat Cardiovasc Drug Discov* 2008; **3**: 156.
15. Hernandez-Jimenez M, Hurtado O, Cuartero MI, *et al.* Silent information regulator 1 protects the brain against cerebral ischemic damage. *Stroke* 2013; **44**: 2333.
16. Luo J, Nikolaev AY, Imai S, *et al.* Negative control of p53 by Sir2alpha promotes cell survival under stress. *Cell* 2001; **107**: 137.
17. Chen Z, Peng IC, Cui X, Li YS, Chien S, Shyy JY. Shear stress, SIRT1, and vascular homeostasis. *Proc Natl Acad Sci U S A* 2010; **107**: 10268.
18. Hou X, Xu S, Maitland-Toolan KA, *et al.* SIRT1 regulates hepatocyte lipid metabolism through activating AMP-activated protein kinase. *J Biol Chem* 2008; **283**: 20015.
19. Lan F, Cacicedo JM, Ruderman N, Ido Y. SIRT1 modulation of the acetylation status, cytosolic localization, and activity of LKB1. Possible role in AMP-activated protein kinase activation. *J Biol Chem* 2008; **283**: 27628.
20. Suchankova G, Nelson LE, Gerhart-Hines Z, *et al.* Concurrent regulation of AMP-activated protein kinase and SIRT1 in mammalian cells. *Biochem Biophys Res Commun* 2009; **378**: 836.
21. Alcendor RR, Kirshenbaum LA, Imai S, Vatner SF, Sadoshima J. Silent information regulator 2alpha, a longevity factor and class III histone deacetylase, is an essential endogenous apoptosis inhibitor in cardiac myocytes. *Circ Res* 2004; **95**: 971.
22. Alcendor RR, Gao S, Zhai P, *et al.* Sirt1 regulates aging and resistance to oxidative stress in the heart. *Circ Res* 2007; **100**: 1512.
23. Hsu CP, Zhai P, Yamamoto T, *et al.* Silent information regulator 1 protects the heart from ischemia/reperfusion. *Circulation* 2010; **122**: 2170.

24. Ruderman NB, Xu XJ, Nelson L, *et al.* AMPK and SIRT1: a long-standing partnership? *Am J Physiol Endocrinol Metab* 2010; **298**: E751.
25. Serafin A, Rosello-Catafau J, Prats N, Gelpi E, Rodes J, Peralta C. Ischemic preconditioning affects interleukin release in fatty livers of rats undergoing ischemia/reperfusion. *Hepatology* 2004; **39**: 688.
26. Liu FC, Day YJ, Liou JT, Lau YT, Yu HP. Sirtinol attenuates hepatic injury and pro-inflammatory cytokine production following trauma-hemorrhage in male Sprague-Dawley rats. *Acta Anaesthesiol Scand* 2008; **52**: 635.
27. Yan W, Fang Z, Yang Q, *et al.* SirT1 mediates hyperbaric oxygen preconditioning-induced ischemic tolerance in rat brain. *J Cereb Blood Flow Metab* 2013; **33**: 396.
28. Zaouali MA, Padrissa-Altes S, Ben Mosbah I, *et al.* Insulin like growth factor-1 increases fatty liver preservation in IGL-1 solution. *World J Gastroenterol* 2010; **16**: 5693.
29. Zaouali MA, Reiter RJ, Padrissa-Altes S, *et al.* Melatonin protects steatotic and nonsteatotic liver grafts against cold ischemia and reperfusion injury. *J Pineal Res* 2011; **50**: 213.
30. Becatti M, Taddei N, Cecchi C, Nassi N, Nassi PA, Fiorillo C. SIRT1 modulates MAPK pathways in ischemic-reperfused cardiomyocytes. *Cell Mol Life Sci* 2012; **69**: 2245.
31. Rane S, He M, Sayed D, *et al.* Downregulation of miR-199a derepresses hypoxia-inducible factor-1alpha and Sirtuin 1 and recapitulates hypoxia preconditioning in cardiac myocytes. *Circ Res* 2009; **104**: 879.
32. Nadtochiy SM, Redman E, Rahman I, Brookes PS. Lysine deacetylation in ischaemic preconditioning: the role of SIRT1. *Cardiovasc Res* 2010; **89**: 643.
33. Raval AP, Dave KR, Perez-Pinzon MA. Resveratrol mimics ischemic preconditioning in the brain. *J Cereb Blood Flow Metab* 2006; **26**: 1141.
34. Della-Morte D, Dave KR, DeFazio RA, Bao YC, Raval AP, Perez-Pinzon MA. Resveratrol pretreatment protects rat brain from cerebral ischemic damage via a sirtuin 1-uncoupling protein 2 pathway. *Neuroscience* 2009; **159**: 993.
35. Evankovich J, Cho SW, Zhang R, *et al.* High mobility group box 1 release from hepatocytes during ischemia and reperfusion injury is mediated by decreased histone deacetylase activity. *J Biol Chem* 2010; **285**: 39888.
36. Ota H, Akishita M, Eto M, Iijima K, Kaneki M, Ouchi Y. Sirt1 modulates premature senescence-like phenotype in human endothelial cells. *J Mol Cell Cardiol* 2007; **43**: 571.
37. Mattagajasingh I, Kim CS, Naqvi A, *et al.* SIRT1 promotes endothelium-dependent vascular relaxation by activating endothelial nitric oxide synthase. *Proc Natl Acad Sci U S A* 2007; **104**: 14855.
38. Ota H, Eto M, Ogawa S, Iijima K, Akishita M, Ouchi Y. SIRT1/eNOS axis as a potential target against vascular senescence, dysfunction and atherosclerosis. *J Atheroscler Thromb* 2010; **17**: 431.
39. Tajbakhsh N, Sokoya EM. Regulation of cerebral vascular function by sirtuin 1. *Microcirculation* 2012; **19**: 336.
40. Donato AJ, Magerko KA, Lawson BR, Durrant JR, Lesniewski LA, Seals DR. SIRT-1 and vascular endothelial dysfunction with ageing in mice and humans. *J Physiol* 2011; **589**: 4545.
41. Casillas-Ramirez A, Amine-Zaouali M, Massip-Salcedo M, *et al.* Inhibition of angiotensin II action protects rat steatotic livers against ischemia-reperfusion injury. *Crit Care Med* 2008; **36**: 1256.
42. Kume M, Yamamoto Y, Saad S, *et al.* Ischemic preconditioning of the liver in rats: implications of heat shock protein induction to increase tolerance of ischemia-reperfusion injury. *J Lab Clin Med* 1996; **128**: 251.
43. Massip-Salcedo M, Casillas-Ramirez A, Franco-Gou R, *et al.* Heat shock proteins and mitogen-activated protein kinases in steatotic livers undergoing ischemia-reperfusion: some answers. *Am J Pathol* 2006; **168**: 1474.
44. Westerheide SD, Anckar J, Stevens SM Jr, Sistonen L, Morimoto RI. Stress-inducible regulation of heat shock factor 1 by the deacetylase SIRT1. *Science* 2009; **323**: 1063.
45. Kobayashi M, Takeyoshi I, Yoshinari D, Matsumoto K, Morishita Y. P38 mitogen-activated protein kinase inhibition attenuates ischemia-reperfusion injury of the rat liver. *Surgery* 2002; **131**: 344.
46. Kyriakis JM, Avruch J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol Rev* 2001; **81**: 807.
47. Nakano A, Baines CP, Kim SO, *et al.* Ischemic preconditioning activates MAPKAPK2 in the isolated rabbit heart: evidence for involvement of p38 MAPK. *Circ Res* 2000; **86**: 144.
48. Fryer RM, Patel HH, Hsu AK, Gross GJ. Stress-activated protein kinase phosphorylation during cardioprotection in the ischemic myocardium. *Am J Physiol Heart Circ Physiol* 2001; **281**: H1184.
49. Terada K, Kaziro Y, Satoh T. Analysis of Ras-dependent signals that prevent caspase-3 activation and apoptosis induced by cytokine deprivation in hematopoietic cells. *Biochem Biophys Res Commun* 2000; **267**: 449.
50. Datta G, Fuller BJ, Davidson BR. Molecular mechanisms of liver ischemia reperfusion injury: insights from transgenic knockout models. *World J Gastroenterol* 2013; **19**: 1683.
51. Casillas-Ramirez A, Mosbah IB, Ramalho F, Rosello-Catafau J, Peralta C. Past and future approaches to ischemia-reperfusion lesion associated with liver transplantation. *Life Sci* 2006; **79**: 1881.
52. Villalba JM, Alcain FJ. Sirtuin activators and inhibitors. *BioFactors* 2012; **38**: 349.
53. Schlicker C, Boanca G, Lakshminarasimhan M, Steegborn C. Structure-based development of novel sirtuin inhibitors. *Aging (Albany NY)* 2011; **3**: 852.
54. Narayan N, Lee IH, Borenstein R, *et al.* The NAD-dependent deacetylase SIRT2 is required for programmed necrosis. *Nature* 2012; **492**: 199.

55. Donmez G, Outeiro TF. SIRT1 and SIRT2: emerging targets in neurodegeneration. *EMBO Mol Med* 2013; **5**: 344.
56. Yang SR, Wright J, Bauter M, Seweryniak K, Kode A, Rahman I. Sirtuin regulates cigarette smoke-induced proinflammatory mediator release via RelA/p65 NF-kappaB in macrophages *in vitro* and in rat lungs *in vivo*: implications for chronic inflammation and aging. *Am J Physiol Lung Cell Mol Physiol* 2007; **292**: L567.
57. Liu FC, Day YJ, Liao CH, Liou JT, Mao CC, Yu HP. Hemeoxygenase-1 upregulation is critical for sirtinol-mediated attenuation of lung injury after trauma-hemorrhage in a rodent model. *Anesth Analg* 2009; **108**: 1855.
58. Jung-Hynes B, Nihal M, Zhong W, Ahmad N. Role of sirtuin histone deacetylase SIRT1 in prostate cancer. A target for prostate cancer management via its inhibition? *J Biol Chem* 2009; **284**: 3823.
59. Wu SL, Yu L, Meng KW, Ma ZH, Pan CE. Resveratrol prolongs allograft survival after liver transplantation in rats. *World J Gastroenterol* 2005; **11**: 4745.