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SPC-100270, a protein kinase C inhibitor, reduced hypoxic injury due to reperfusion following orthotopic liver transplantation in the rat

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Abstract Recently, we reported that SPC-100270, a sphingosine derivative and inhibitor of protein kinase C (50–90 μM) in mixed micelle assays, reduced reperfusion injury resulting from hypoxia in a low-flow, reflow model of liver perfusion [8]. Here we report that SPC-100270 has similar beneficial effects following liver transplantation in vivo. Rat liver transplantation was performed using nonarterial and rearterial techniques. Livers from syngenic rats were harvested surgically, prepared with vascular cuffs and a splint, and stored for 24 or 48 h in University of Wisconsin (UW) cold storage solution. Just prior to completion of vascular reconstruction, the organ was rinsed with 3 or 10 ml of Ringer's solution, vehicle, or a solution containing SPC-100270 (up to 500 μM). Following implantation surgery, low doses of SPC-100270 were ineffective at reducing both parenchymal and nonparenchymal cell death, yet significant ($P < 0.05$) reductions were observed with 500 μM. Further, nonparechnymal cell viability was improved nearly four fold by the drug. SPC-100270 (500 μM) tended to increase survival following 48 h cold storage in UW solution, but the improvement was not statistically significant. SPC-100270 also did not diminish carbon-centered free radical formation in transplanted livers from alcohol-treated rats. Collectively, these data support the hypothesis that pretreatment of donor livers with an inhibitor of protein kinase C is effective in vivo at reducing reperfusion injury, particularly to nonparenchymal cells, following orthotopic liver transplantation in the rat.

Key words PKC inhibitor
Sphingosine · Reperfusion
Liver transplantation

Introduction

Liver transplantation has become an accepted therapy worldwide for irreversible liver disease, yet primary graft nonfunction still occurs in about 15% of cases, threatening the patients' lives [5]. Studies on the pathophysiological mechanisms of primary nonfunction upon reperfusion have focused on damage to endothelial cells, activation of Kupffer cells [1], adherence of leukocytes [7], and disturbances in the microcirculation [9]. It is likely that activated Kupffer cells and infiltrating neutrophils produce toxic mediators upon reperfusion such as tumor necrosis factor and free radicals [2] which are involved in mechanisms of reperfusion injury to the graft, leading to

the development of primary nonfunction following liver transplantation. By using a low-flow, reflow model of liver perfusion, we demonstrated recently that SPC-100270, a sphingosine analogue and an inhibitor of protein kinase C, was effective in reducing reperfusion injury in the hemoglobin-free perfused rat liver, most likely via inhibition of Kupffer cell activation [8]. However, it is not known whether or not SPC-100270 acts beneficially in transplanted livers following cold ischemic storage and reperfusion with blood. Therefore, these studies were designed to determine if SPC-100270, which minimizes reperfusion injury in vitro, is effective in vivo after cold storage and liver transplantation in the rat.

Materials and methods

Drug preparation

SPC-100270 was dissolved in 3 or 10 ml of lactated Ringer's solution at 37 °C and was used at room temperature for most of this work at various test concentrations (e.g., 1–500 μM).

Transplantation

Orthotopic liver transplantation was performed under ether anesthesia using techniques described by Kamada and Calne [6] and Gao et al [4]. Syngenic female Lewis rats (175–200 g) were used to eliminate rejection. Briefly, 0.5 ml Ringer's solution containing 100 units of heparin was injected into the donor vena cava, and the liver was flushed with 3–5 ml of cold UW solution. Subsequently, cuffs were placed on the portal vein and subhepatic vena cava of the donor liver. Grafts were stored at 0–4 °C for 24 or 48 h in UW solution and were rinsed with 3 ml Ringer's solution, vehicle, or SPC-100270 at various concentrations. Subsequently, livers were implanted by connecting the suprahepatic vena cava with a running suture, inserting cuffs and a splint into appropriate venous vessels and the proper hepatic artery, and anastomosing the bile duct with an intraluminal splint. The explantation required less than 5 min, and the ischemic interval due to clamping the portal vein and inferior vena cava during implantation did not exceed 15 min. Surviving animals were killed after 30 days for histology study.

Histology

Rats were killed 3 h or 30 days postoperatively, depending on the experimental design, and their livers were fixed with 1% paraformaldehyde in Krebs-Henseleit buffer, embedded in paraffin, and processed for light microscopy. Where indicated, the vital dye trypan blue was infused into livers for 5 min at the end of experiments, followed by fixation of the liver with 1% paraformaldehyde in Krebs-Henseleit buffer. Livers were processed for light microscopy, and sections were stained with eosin so that only nuclei of dead cells could be identified [7].

Electron paramagnetic resonance spectroscopy

Grafts were stored in UW solution for 48 h and rinsed with 500 μM SPC-100270 before implantation. After reperfusion with blood containing the spin trap PBN, venous blood samples (4–5 ml) were collected, and the serum was extracted with chloroform. A Varian E-109 electron paramagnetic resonance (EPR) spectrometer equipped with a TM_{110} cavity was used to obtain the spectra. Instrument conditions were 20 mW microwave power, 0.68 G modulation amplitude, and 80 G scan width for all analyses. Spectral data were stored on an IBM compatible computer and were analyzed for EPR hyperfine coupling constants by computer simulation.

Results

Reduction of hepatocellular injury following transplantation by SPC-100270

One goal of this study was to determine whether SPC-100270 would reduce reperfusion injury to transplanted livers in vivo. Since most human livers are stored for less than 24 h before transplantation, the effect of SPC-100270 was studied following 24 h of cold storage. Low doses of SPC-100270 (2–10 μM) were ineffective at reducing both parenchymal and nonparenchymal cell death. However, 500 μM SPC-100270 reduced reperfusion injury to nonparenchymal cells in both periportal and pericentral regions. It also decreased the injury to parenchymal cells in pericentral regions (Fig. 1).

Effect of SPC-100270 on free radical formation in fatty livers produced by alcohol

Since fatty livers from alcoholics transplant poorly and because free radicals formed following orthotopic liver

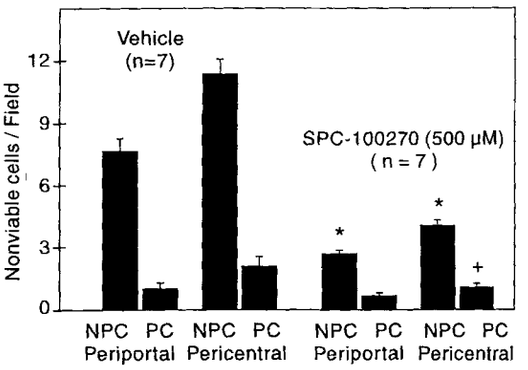

Fig. 1 Reduction of hepatocellular injury by SPC-100270 following transplantation. Livers were stored under survival conditions (24 h in cold UW solution), rinsed with 500 μM SPC-100270, and then implanted using the Kamada method [6]. The vital dye trypan blue was infused into livers for 5 min at the end of experiments as described in Materials and methods. Values are mean ± SEM for seven livers in each group. ($^+$ $P = 0.05$, * $P < 0.05$ compared with vehicle by Student's *t*-test; *NPC* nonparenchymal cells; *PC* parenchymal cells)

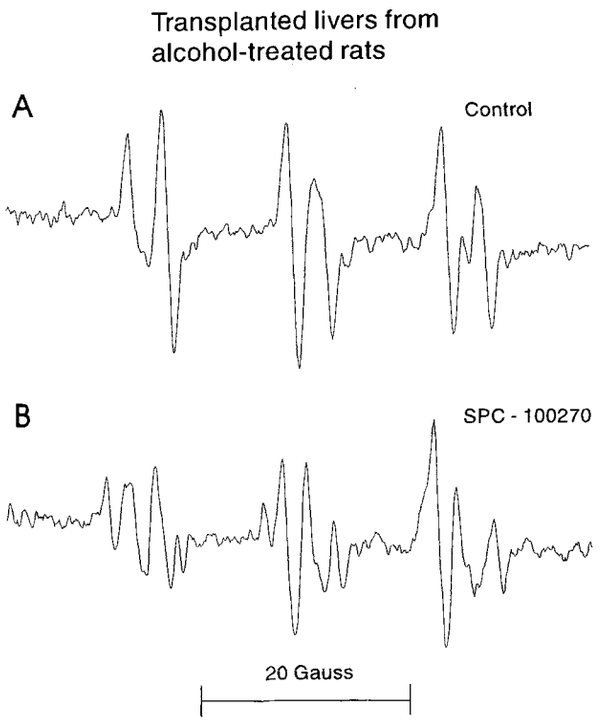

Fig. 2A, B Effect of SPC-100270 on free radical formation in fatty livers produced by alcohol. Livers were stored under nonsurvival conditions (48 h in cold UW solution), rinsed with 500 μM SPC-100270, and then implanted using the rearterial techniques described in the Methods. **A** EPR spectra of livers from alcohol-treated rats (3–5 weeks on Lieber-DeCarli protocol) [3] after cold storage in UW solution for 48 h; **B** conditions as in **A** except livers were rinsed with SPC-100270 (500 μM) during implantation. Representative spectra

transplantation in the rat are involved in graft failure, these experiments utilizing EPR spectroscopy were designed to determine whether or not free radicals formed during transplantation could be reduced by SPC-100270. In livers from alcohol-treated rats, a robust six-line EPR signal was detected following liver transplantation (Fig. 2). The hydrophobic behavior of these species in extraction and sample preparation suggested that they were lipid-derived radicals. SPC-100270 did not reduce free radical formation significantly.

Effect of SPC-100270 on survival

Following 48 h in UW solution, which is much longer than clinical storage time (nonsurvival conditions), the 30-day survival rate was around 25% in the vehicle-treated group. Low doses of SPC-100270 (1–5 μM) did not improve survival, and most of the rats died of primary graft nonfunction (data not shown). When grafts were rinsed with 3 ml of cold 500 μM SPC-100270, the survival rate increased from25% to 50% compared with vehicle, but the changes were not significant (Table 1).

Table 1 Effect of SPC-100270 on survival following liver transplantation in the rat. Livers were stored in cold UW solution for 48 h (nonsurvival conditions) and rinsed with 500 μM SPC-100270 as described in Methods. Values are mean ± SEM for 4–6 livers in each group

Groups	Three-day survival	Thirty-day survival	Percentage
Ringer's	3/6	3/6	50
Vehicle	1/4	1/4	25
SPC-100270	2/4	2/4	50

Discussion

Selective injury to nonparenchymal cells occurs following liver perfusion [8] as well as cold storage and liver transplantation (Fig. 1). Upon reperfusion, free radicals were indeed formed in minutes [2], followed rapidly by endothelial cell injury and disturbed microcirculation [9]. Activated Kupffer cells and leukocytes are both involved in free radical formation, which could cause graft failure following transplantation [1, 5]. For these reasons, nonparenchymal cells seem more vulnerable upon reperfusion and play a key role in the mechanism of primary graft nonfunction. Interestingly, the results presented above showed that SPC-100270 minimized reperfusion injury primarily to nonparenchymal cells following transplantation surgery in vivo. The concentration of SPC-100270 needed in these studies to protect against reperfusion injury was high (500 μM); however, the organ was only rinsed for minutes. SPC-100270 also reduced hepatocellular injury in the isolated perfused liver [8]. Even though SPC-100270 reduced reperfusion injury to liver cells, it did not improve survival or reduce free radical formation significantly. This may be due to negative effects on the coagulation system or simply due to the small size of the groups compared. For this reason, use of an emulsion of SPC-100270 to maximize drug delivery may be helpful in future experiments.

Taken together, these data indicate that SPC-100270 is effective in reducing reperfusion injury to nonparenchymal cells following orthotopic liver transplantation, suggesting that it may be beneficial in inflammatory liver diseases which involve macrophages and neutrophils in their mechanisms of action.

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