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Interleukin-6 is a key mediator of the hepatoprotective and pro-proliferative effects of ischaemic preconditioning in mice

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***Background/Aims*: The biological effects of ischaemic preconditioning include NF-κB activation, increased TNF synthesis, stimulation of cell cycle entry and hepatoprotection against ischaemia-reperfusion (IR) injury. Low dose TNF initiates the priming phase of liver regeneration via NF-κB and IL-6. To determine whether (1) IL-6 is released during preconditioning and confers protection against hepatic IR injury, and (2) IL-6 could mediate the biological effects of preconditioning.**

***Methods*: Wildtype (*wt*) and $TNF^{-/-}$ C57BL6 mice were subjected to 90 min partial hepatic ischaemia and 2–44 h reperfusion with or without prior 10 min ischaemic preconditioning. To restitute liver injury, $TNF^{-/-}$ mice were administered murine TNF 5 μg/kg iv 1 min prior to IR. Murine recombinant IL-6 (500 ng/kg iv) was administered 30 min prior to IR, either to *wt* mice or to $TNF^{-/-}$-repleted mice; in the latter case, 1 min before preconditioning.**

***Results*: In *wt* mice, IL-6 attenuated hepatic IR injury and stimulated cell cycle entry. IR injury in TNF-repleted $TNF^{-/-}$ mice was not ameliorated by preconditioning. However, prior IL-6 administration conferred hepatoprotection (IL-6/preconditioned: 349 ± 169 U/L vs vehicle/preconditioned: 1250 ± 608 U/L, $P < 0.01$).**

***Conclusions*: IL-6 is one likely mediator of the hepatoprotective and pro-proliferative effects of ischaemic preconditioning.**

***Keywords*: Interleukin-6; Hepatic ischaemia-reperfusion injury; Ischaemic preconditioning; Nuclear factor-kappa B; Signal transducer and activator of transcription 3; Tumour necrosis factor-α; Cell cycle**

1. Introduction

Hepatic ischaemia reperfusion (IR) injury is an important complication of liver surgery and shock [1,2]. Serum tumour necrosis factor-α (TNF) concentrations rise to very high levels during hepatic ischaemia [3,4], and remain detectable until the late reperfusion phase [5–7]: such TNF mediates hepatic IR injury. In TNF gene-deleted ($^{-/-}$) mice, both early and late phases of hepatic IR injury are attenuated, but injury can be restored by TNF replacement, likewise, TNF-$R1^{-/-}$ mice are refractory to hepatic IR injury, and in rats, anti-TNF strategies reduce the inflammatory phase of hepatic IR injury [5,6,8].

Ischaemic preconditioning is a brief period of hepatic IR that protects against subsequent prolonged IR [9–12]. Preconditioning attenuates the later-onset prolonged release of TNF that mediates hepatic IR injury [7], but, paradoxically, is itself associated with a brief period of TNF release [7]. Further, injection of low dose TNF 30 min prior to hepatic IR confers similar protection as ischaemic preconditioning. Moreover, preconditioning is ineffective against TNF-restituted liver injury in $TNF^{-/-}$ mice subjected to hepatic IR, unless a small dose of TNF is injected (TNF augmentation) at the

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Abbreviations: ALT, alanine aminotransferase; EMSA, electromobility (gel) shift assay; IL-6, interleukin-6; IR, ischaemia-reperfusion; NF-κB, nuclear factor-kappa B; PCNA, proliferating cell nuclear antigen; STAT3, signal transducer and activator of transcription 3; TNF, tumour necrosis factor; $TNF^{-/-}$, tumour necrosis factor-α knockout mice.

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same time [7,8]. We have also previously noted that both ischaemic preconditioning and low dose TNF injection stimulate earlier and more vigorous cell cycle entry during subsequent prolonged IR than occurs in the naïve liver subjected to hepatic IR.

TNF is also known to regulate the priming phase of liver regeneration after partial hepatectomy and toxic liver injury [13–17]. For this function, TNF must activate nuclear factor-kappa B (NF-κB) [18,19] and cause 'downstream' release of interleukin-6 (IL-6); IL-6 then activates signal transducer and activator of transcription-3 (STAT3), a pro-proliferative transcription factor. IL-6 can substitute for TNF in the pro-proliferative response to partial hepatectomy [20,21]. Further, we have previously demonstrated that the hepatoprotective and pro-proliferative effects of TNF against hepatic IR are associated with activation of STAT3 DNA binding [8]. These findings implicate IL-6 as a possible mediator of this effect.

Selzner et al. have shown that cold ischaemia during liver preservation impairs liver regeneration after partial liver transplantation in the rat [22]. This impairment is associated with significant *reduction* in hepatic synthesis of both TNF and IL-6, and it can be corrected by pretreatment with IL-6. IL-6 also significantly *improves* recipient survival. Others have shown that high concentrations of IL-6 (500–1000 μg/kg body weight) added to an isolated rat liver perfusion system or administered prior to warm hepatic ischaemia in mice protect against IR injury [23–26]. In the present work, we sought to characterize the role of IL-6 as a candidate cytokine that mediates protection against warm hepatic IR injury compared with naïve mice subject to equivalent IR. We first noted that preconditioning stimulates exaggerated IL-6 release from the liver. We then injected low dose IL-6, 30 min prior to prolonged IR in a well-characterized in vivo murine model to test whether such a manoeuvre reproduces both the hepatoprotective and hepatocellular proliferative responses induced by ischaemic preconditioning. Finally, our TNF-repleted $TNF^{-/-}$ mouse model of hepatic IR injury allowed us to adopt an IL-6 conditioning protocol to distinguish the separate mechanistic contributions of IL-6 and TNF in ischaemic preconditioning.

2. Methods

2.1. Murine models of partial hepatic ischaemia reperfusion injury and ischaemic preconditioning

Female C57BL6 wildtype (*wt*) and TNF gene-deleted ($TNF^{-/-}$) mice weighing 18–25 g were used for all experiments. $TNF^{-/-}$ mice with C57BL6 background (courtesy of Dr Lisa Sedger) were obtained from the Animal Facility at Westmead Hospital, University of Sydney were maintained in a specific pathogen-free facility, fed on a commercial pellet diet and allowed water ad libitum. Experimental protocols and procedures complied with the International Criteria of Animal Experimentation and were approved by the Western Sydney Area Health Service Animal Ethics Committee. The left/median partial IR injury model and collection of liver samples have been described [7,8]. Whole blood (1 mL) was obtained by cardiac puncture and used fresh to assay serum alanine aminotransferase (ALT) levels, or snap frozen for IL-6 assay.

2.2. Experimental protocols for cytokine administration

In *wt* mice, IL-6 was administered (500 ng/kg of murine recombinant IL-6) (Calbiochem, San Diego, CA) intravenously (iv) via the lateral tail vein 30 min prior to 90 min IR (Fig. 1). In $TNF^{-/-}$ mice, 'TNF-repletion' was accomplished by administration of murine TNF (Calbiochem, San Diego, CA) at 5 μg/kg body weight iv, 1 min prior to commencement of prolonged hepatic ischaemia. Preconditioning was performed by 10 min ischaemia followed by clamp release 10 min before IR. To test the hypothesis that IL-6 is an essential mediator of ischaemic preconditioning, we studied the effects of IL-6 (500 ng/kg body weight) compared with saline vehicle control, injected 1 min prior to ischaemic preconditioning against hepatic IR in TNF-repleted $TNF^{-/-}$ mice (Fig. 1). Four mice were studied in each experimental group.

2.3. Assessing severity of liver injury

The severity of liver injury was determined from serum ALT levels and quantitative liver histology [7,8]. Liver sections were also used for proliferating cell nuclear antigen (PCNA) immunohistochemistry (ImmunoCruz Staining System, Santa Cruz Biotechnology, Santa Cruz, CA). PCNA-positive staining nuclei were counted in five high power fields (×400 magnification); results were expressed as a percentage of hepatocyte nuclei.

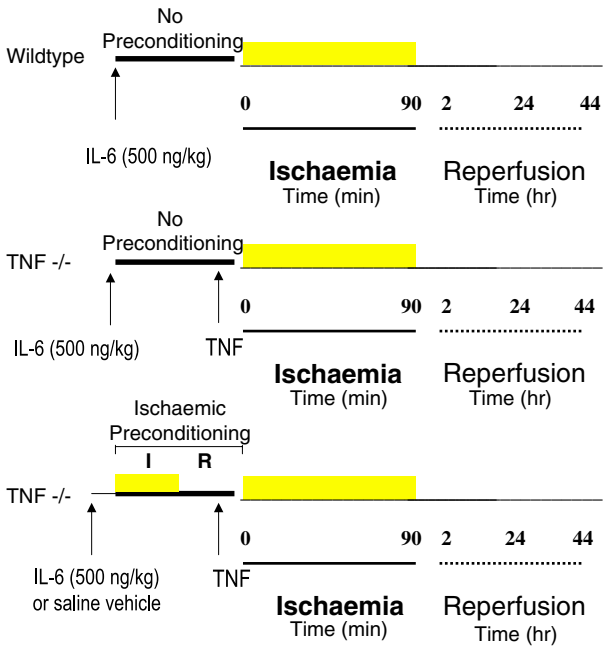

Fig. 1. Experimental protocols used in these studies. All mice were subjected to 90 min ischaemia followed by 2–44 h reperfusion. In some experiments, IL-6 (500 ng/kg body weight) was administered 30 min prior to ischaemia-reperfusion in *wt* or $TNF^{-/-}$ mice. In $TNF^{-/-}$ mice (which are refractory to hepatic IR injury), TNF was administered TNF (5 μg/kg body weight), 1 min prior 90 min hepatic ischaemia so as to cause liver injury. Ischaemic preconditioning was induced by 10 min of ischaemia (I) followed by 10 min of reperfusion (R). In order to test whether IL-6 effects preconditioning in $TNF^{-/-}$ mice, IL-6 (500 ng/kg) was administered 1 min prior to preconditioning.