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The calcium channel blocker nisoldipine minimizes the release of tumor necrosis factor and interleukin-6 following rat liver transplantation

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Abstract. Kupffer cells, when activated, release toxic cytokines such as tumor necrosis factor (TNF), which can cause tissue injury. Takei et al. have reported that nisoldipine, a calcium channel blocker which decreases phagocytotic activity by Kupffer cells, also diminishes liver and lung injury and dramatically improves survival following liver transplantation [27]. Therefore, we studied the effect of nisoldipine on the time course of TNF and interleukin-6 (IL-6) release following cold storage and liver transplantation in the rat. Livers were stored under survival and non-survival conditions in cold Euro-Collins solution in the presence or absence of nisoldipine (1.4 μM). After storage, the effluent was collected for determination of cytokines. The liver was then transplanted orthotopically and serum was collected at various time intervals for up to 5 h. In the effluent, TNF levels were very low in both the control and nisoldipine-treated groups and IL-6 was not measurable. Furthermore, when livers were stored under survival conditions and transplanted (liver stored in the cold for 4 h), serum TNF (2 U/ml) and IL-6 (350 U/ml) values were minimal in both the control and nisoldipine-treated groups. In contrast, when livers were stored under non-survival conditions and transplanted (liver stored in the cold for 10 h), TNF levels increased to 15 ± 2 U/ml, 150 min after graft reperfusion, an increase which was prevented by nisoldipine (6.5 U/ml). Serum IL-6 levels were also elevated 300 min after transplantation in livers stored for 10 h. Nisoldipine also reduced the release of this cytokine. Serum transaminases (SGOT) were elevated to values around 2000 U/l 5 h following transplantation. In the nisoldipine-treated group, values were lower between 60 and 300 min. In the lung, interstitial and alveolar edema and cellular infiltration were detectable 5 h postoperatively and were diminished by nisoldipine. These data confirmed that TNF and IL-6 release were minimal following cold storage and transplantation of livers stored under survival conditions, but were elevated transiently after transplantation under non-survival conditions. Nisoldipine prevented cytokine release, most likely by blocking the activation of Kupffer cells, which may explain how it decreases liver and lung injury very early following liver transplantation.

Key words: Nisoldipine – Tumor necrosis factor – Interleukin-6 – Liver transplantation

Although liver transplantation is now performed routinely in children and adults, the risk of primary non-function still occurs with an unacceptably high frequency [12]. Primary non-function can compromise postoperative recovery and necessitate retransplantation. The mechanism is not well understood, but it is likely that Kupffer cells, the hepatic macrophages, are involved since they are activated following cold storage and reperfusion [28]. When activated, macrophages release toxic mediators including cytokines such as tumor necrosis factor (TNF) as well as eicosanoids [10]. TNF has been implicated in lung injury [7, 16] and in shock states involving multiple organ failure [4], and it is released following warm ischemia [7] and rejection [31]. Nisoldipine, a calcium channel blocker, decreases phagocytosis of Kupffer cells, diminishes liver and lung injury 24 h following liver transplantation and improves survival [27]. We asked, then, whether injury to liver and lung following transplantation involves the release of TNF and if TNF release is affected by nisoldipine. Furthermore, almost no information is available on the release of other cytokines immediately following liver transplantation. Interleukin-6 (IL-6) may also influence postoperative outcome since it is involved in the acute phase response [13] as well as in the regulation of the immune system [2]. Therefore, we investigated the time course of release of TNF and IL-6 following liver transplantation.

Methods

Orthotopic liver transplantation. Inbred Lewis rats (180–220 g, female) were used to exclude immunological rejection and were transplanted according to the procedure described by Zimmermann [33]

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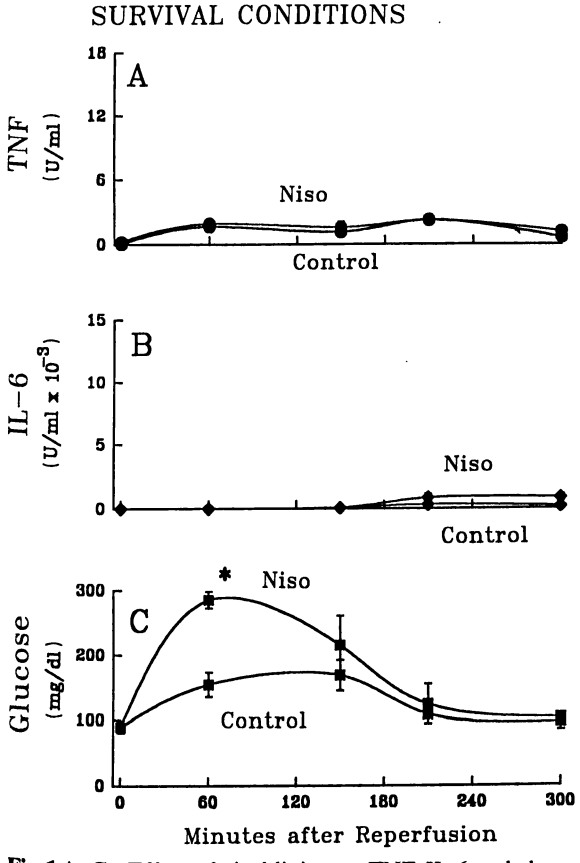

Fig. 1 A–C. Effect of nisoldipine on TNF, IL-6 and glucose release following transplantation under survival conditions. Livers stored for 4 h in cold Euro-Collins solution (i.e., survival conditions) were transplanted as described in Methods. TNF, IL-6 and glucose were measured in the effluent collected after storage (minute 0) and in recipient rat serum collected at various times. Rats were randomized between an untreated control group and a nisoldipine-treated group. Nisoldipine (*Niso;* 1.4 μM) in DMSO was added to Ringer's and Euro-Collins solutions during explantation and storage only. Nisoldipine was a kind gift of Miles, Inc. (West Haven, Conn.). Reperfusion was initiated by opening the portal vascular clamp. Mean ± SEM, * $P < 0.05$ ($n = 3$–6 per group)

and Kamada [17]. Rats were anesthetized lightly with ether, and surgery was performed under clean conditions. The explantation procedure included incision of the skin, sectioning of the hepatic ligaments, insertion of a splint (polyethylene tubing, PE 10) into the bile duct and dissection of the portal vein and inferior vena cava. The portal vein was then clamped and the liver was rinsed with 10 ml of Ringer's solution at 4 °C followed by 15 ml of Euro-Collins solution at 4 °C. Livers were then removed and immersed in Euro-Collins solution at 1 °C and cuffs were placed on the portal vein and inferior hepatic vena cava before storage for 4 h (survival conditions) or 10 h (non-survival conditions). Implantation was performed by connecting the suprahepatic vena cava with a running suture, inserting cuffs into the appropriate vessels, and anastomosing the bile duct with an intraluminal splint. Grafts were rinsed with 5 ml of Ringer's solution at 21 °C before reperfusion, and the effluent was collected. Ischemia of the gut due to clamping the portal vein did not exceed 20 min. Ringer's solution (5 ml) was infused via the tail vein during implantation.

Blood glucose levels were measured in whole blood collected from the superior vena cava with reagent strips (Chemstrip, Boehringer Mannheim) and quantified with an Accu-Check II monitor. Serum glutamate oxaloacetate transaminase (SGOT) activity was measured enzymatically [3].

Blood sample collection and cytokine assays. The rat was anesthetized lightly with ether, the right external jugular vein was exposed and a glass micropipet (Corning) was inserted into the superior vena cava via the jugular vein to collect blood (400 μl) at 60, 150, 210 and 300 min after graft reperfusion. The protease inhibitor aprotinin (3 U/ml) was added to the effluent and blood samples and sera were stored at −70 °C until assayed. The cytotoxicity of TNF was assayed in L-M fibroblasts incubated with serum or effluent in the presence of 1 μg/ml actinomycin D as described elsewhere [19]. IL-6 was measured using the growth stimulation of B-9 cells as described by Aarden [1]. Results from unknown samples were compared with curves prepared from authentic standards.

Lung histology. Rats were sacrificed 5 h postoperatively, and lungs were fixed by immersion in 2% paraformaldehyde in Krebs-Henseleit buffer, emmbedded in paraffin, and processed for light microscopy. Sections were stained with hematoxylin and eosin. Lung injury was indexed on a scale from 0 to 2 for interstitial edema, alveolar hemorrhage and infiltration of inflammatory cells (maximal score = 6).

Statistics. Data are presented as mean ± SEM Statistical analyses were performed using Students' *t*-test [26]. The criterion for significance was $P < 0.05$.

Results

Release of cytokines and glucose following transplantation under survival conditions

Following 4 h of storage in Euro-Collins, all rat recipients survived (4 of 4), – survival conditons. Blood levels of TNF, IL-6 and glucose were measured at various time points during the first five h postoperatively and are depicted in Fig. 1. In the rinse effluent and in sera from livers stored under survival conditions and transplanted, TNF levels were detectable but low (less than 4 U/ml) in samples from both control and nisoldipine-treated groups (Fig. 1 A). IL-6 was not detectable in the effluent, but reached levels between 150 and 1500 U/ml 300 min after graft reperfusion (Fig. 1 B). However, there were no statistical differences between the groups under these conditions.

Following sham-operation (i.e., surgery as described in Methods without graft implantation), levels of TNF and IL-6 were similar to values observed following control transplantation of livers stored for 4 h in Euro-Collins. Highest values of TNF (2.68 ± 0.1 U/ml, $n = 4$) were found 60 min after the operation. IL-6 reached 366 ± 72 U/ml at 150 min, and blood glucose was increased to 164 ± 26 mg/dl.

A transient hyperglycemia occurred 60 min following graft reperfusion which was significantly greater in the nisoldipine group (Figs. 1 C and 2 C). Treatment of the donor rat with gadolinium chloride, a procedure which selectively destroys Kupffer cells [5], prevented the postoperative increase in blood glucose (data not shown). This observation indicated that the transient hyperglycemia observed following transplantation involved rapid release of prostanoids from Kupffer cells [24] which are known to stimulate phosphorylase A, causing glucose release from

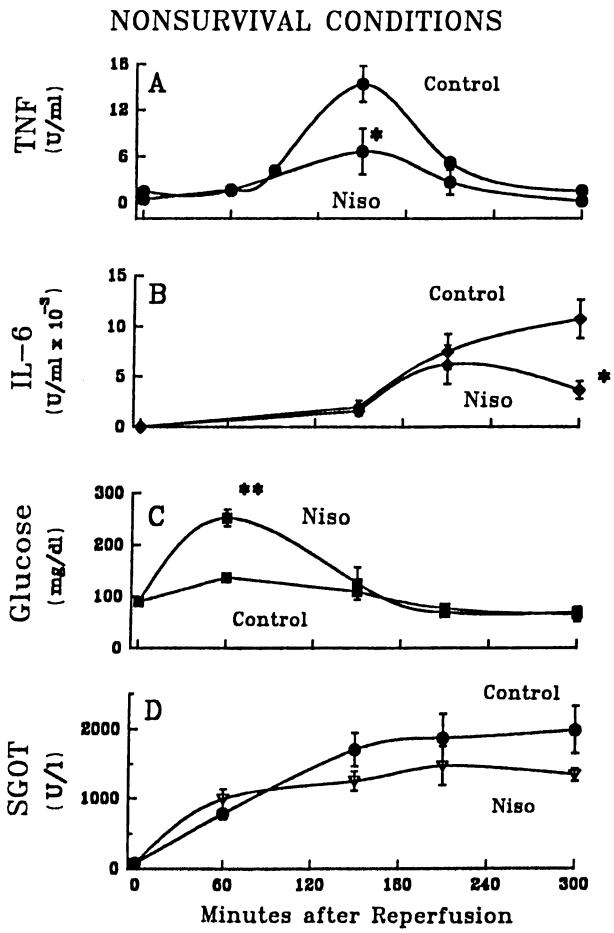

Fig. 2 A–D. Effect of nisoldipine on TNF, IL-6, glucose and SGOT release following transplantation under non-survival conditions. Livers stored for 10 h in cold Euro-Collins solution (i.e., non-survival conditions) were transplanted, and TNF, IL-6, glucose and SGOT were measured postoperatively as described in Methods and in the legend of Fig. 1. Mean ± SEM, * $P < 0.05$; ** $P < 0.01$ ($n = 4$–5 per group)

parenchymal cell glycogen stores [21]. Surprisingly, nisoldipine stimulated glucose release (Fig. 1 C and 2 C) for reasons that remain unclear. A partial agonist effect of the drug may be involved [14].

Nisoldipine prevented postoperative cytokine release in livers stored under non-survival conditions

Following 10 h of storage, survival was poor (3 of 8) – non-survival conditions. In the graft effluent, TNF was minimal in both the control (0.5 U/ml) and the nisoldipine-treated groups (1.5 U/ml). Following transplantation under these conditions, TNF levels in the blood increased slowly, beginning 90 min following graft reperfusion and reaching values of around 15 U/ml at 150 min before declining to basal levels at 210 min (Fig. 2 A). Importantly, this increase was prevented by nisoldipine. IL-6 reached values around 10000 U/ml 5 h following transplantation, a phenomenon which was also minimized by nisoldipine (Fig. 2 B).

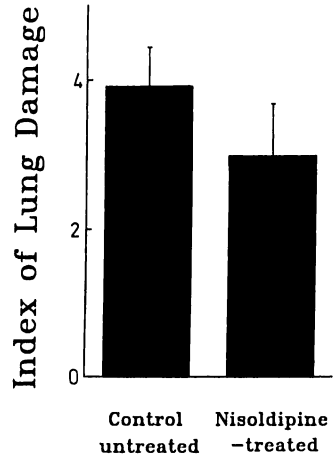

Fig. 3. Effect of nisoldipine on early postoperative lung injury. Five hours following liver transplantation under non-survival conditions (10 h storage in Euro-Collins), rats were sacrificed and their lungs were fixed and evaluated histologically as described in Methods. Nisoldipine was added to the storage solution as described in the legend of Fig. 1. Mean ± SEM, ($n = 5$–6 per group)

Nisoldipine decreased transaminase release and lung injury

Following 10 h of storage and reperfusion, serum glutamate oxaloacetate transaminase (SGOT) increased gradually in both the control and nisoldipine groups. Between 60 and 300 min postoperatively, SGOT values were higher (ca. 2000 U/l) in the control than in the nisoldipine-treated group (1300 U/l; Fig. 2 D). Under non-survival conditions, lung edema and cellular infiltration were prominent 5 h postoperatively. Nisoldipine reduced this injury by about 25% (Fig. 3).

Influence of explantation time on TNF release

Between two series of transplantations under survival conditions performed after 12 and 18 months of surgical training, explantation time decreased from 26 to 15 min. When the data were compared, a significant positive correlation was found between the explantation time and TNF levels measured 60 min after graft reperfusion (Fig. 4). Thus, surgical conditions during explantation of the liver in the donor rat influenced TNF release in the recipient.

Discussion

Tumor necrosis factor release following graft reperfusion

TNF is a toxic mediator present in the blood during pathological states [4]. We observed that TNF was released a few hours (Fig. 2 A) following liver transplantation under non-survival conditions raising the question: what factors influence TNF release after reperfusion of the graft? Two factors, explantation time in the donor (Fig. 4) and duration of cold storage (compare Figs. 1 A and 2 A), appeared important in this study.

One hypothesis is that during explantation of the liver, manipulation of the gut may increase the release of bacteria and endotoxin (LPS) into the portal blood, which is known to activate TNF production [4]. Therefore, a

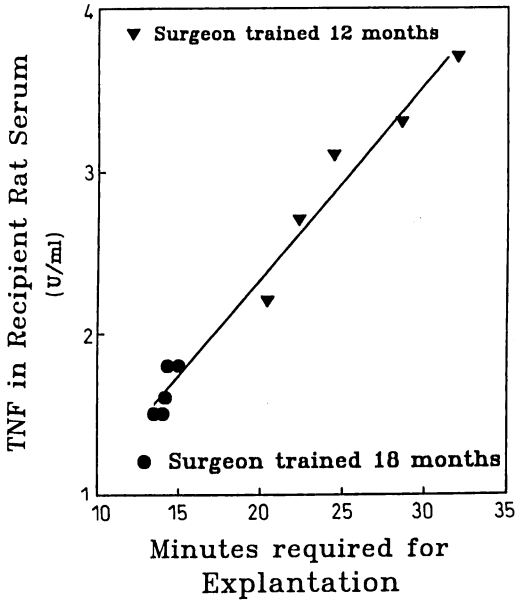

Fig. 4. Effect of explantation time on TNF release. Livers stored for 4 h in cold Euro-Collins solution (i.e., survival conditions) were transplanted orthotopically as described in Methods. TNF was measured in sera of recipient rats 60 min after graft reperfusion as described in Methods. The explantation time was the time from incision of the skin to immersion of liver in cold Euro-Collins. *Triangles:* surgeon trained 12 months (250 transplantations). *Circles:* surgeon trained 18 months (400 transplantations). Coefficient of the linear regression, $r = 0.99$, $P < 0.01$

longer explantation time would be expected to increase hepatic exposure to LPS in the transplant model, leading to greater TNF release after storage and reperfusion (Fig. 2 A). In support of this hypothesis, a dose-dependent release of TNF has been observed in vivo following the injection of LPS [22]. Therefore, it is likely that LPS release into the circulation during explantation contributes to TNF release following transplantation. Thus, by measuring LPS in the donor, it may be possible to predict the clinical outcome in the recipient.

When explantation time was kept constant and duration of cold storage was changed from 4 to 10 h, TNF release increased from minimal to high values (Figs. 1 A and 2 A). Endotoxemia produced by clamping the portal vein [23] cannot explain the high levels of TNF under non-survival conditions since surgical time was identical in the survival and non-survival groups. One possibility is that cold storage influences cells responsible for TNF release. Many studies have reported that cold storage and reperfusion activate Kupffer cells [20, 27, 28], and it is known that macrophages (e.g. Kupffer cells) release TNF when activated [4, 10]. Therefore, it seems reasonable to propose that TNF release following transplantation involves activation of Kupffer cells. Activation may sensitize Kupffer cells to LPS. Another possibility for explaining the release of TNF only under non-survival conditions may be an alteration in regulation of TNF synthesis. Endothelial cells release PGE_2 [20], which suppresses TNF synthesis [18]. Cold storage followed by reperfusion injures endothelial cells substantially [6]; therefore, loss of endothelial cell viability would be expected to decrease PGE_2 and allow uncontrolled synthesis of TNF.

Nisoldipine prevented cytokine release and minimized postoperative injury

Following transplantation under survival conditions, TNF release and liver and lung injury were minimal (data not shown). In contrast, under non-survival conditions, TNF was elevated and liver (Fig. 2 D) and lung injury occurred (Fig. 3), raising the question of whether TNF produces liver and lung injury directly. SGOT was released before the peak of TNF levels occurred (Fig. 2 A and D); therefore, at least part of the hepatic injury observed was TNF-independent and most likely involved other mechanisms such as the production of lipid radicals on reperfusion [8, 29]. However, nisoldipine treatment decreased TNF (Fig. 2 A) and also lowered SGOT release with a similar time course (after 60 min). Moreover, nisoldipine tended to reduce lung injury after 300 min; however, values were only decreased significantly after 24 h (Figs. 2 D and 3; [27]). Thus, it is possible to hypothesize that protection by nisoldipine involves TNF. The beneficial effect of nisoldipine following transplantation is not totally understood, but several arguments support the hypothesis that it prevents Kupffer cell activation (see Fig. 5). Kupffer cells have calcium channels [15] and nisoldipine blocks TNF release in isolated Kupffer cells [9]. Moreover, nisoldipine prevents stimulation of phagocytosis by Kupffer cells in the perfused liver following cold storage [27]. White cells also release TNF [4]; however, because nisoldipine is very lipophilic and since the liver was rinsed before reperfusion, the concentration of the drug in the recipient blood

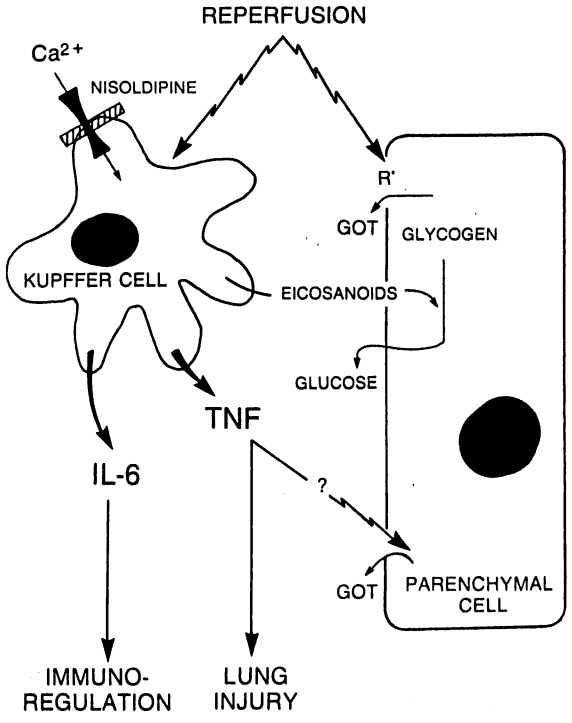

Fig. 5. Scheme depicting cytokine release on reperfusion. *R'*, lipid free radicals; *GOT*, serum enzymes

should be too low to affect TNF release by leukocytes directly. Therefore, it is likely that the major site of action of nisoldipine is the Kupffer cell. Prevention of TNF release by Kupffer cells may decrease postoperative injury, as shown in this study (Figs. 2 D and 3). In support of this hypothesis, TNF has been implicated in other models of liver [30, 32] and lung injury [7, 16]; however, TNF does not appear to cause hepatic injury directly [30, 32]. Thus, the effect of nisoldipine may be complex and involve factors in addition to TNF. To illustrate this point, nisoldipine also decreased IL-6 (Fig. 2 B). It is not known whether the IL-6 detected in this study was derived from donor liver nonparenchymal cells or from recipient blood cells. High levels of IL-6 observed under non-survival conditions may be increased either my TNF or other cytokines [25] or result from decreased clearance. IL-6 may affect transplant outcome directly since it regulates synthesis of most of the hepatic acute phase proteins (i.e., fibrinogen) and can stimulate lymphocyte differentiation [2, 11]. More studies are necessary to determine the consequences of cytokine release on liver function and rejection following transplantation.

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