DOI: 10.1111/j.1365-2362.2008.01996.x

ORIGINAL ARTICLE

New evidence for the role of TNF-α in liver ischaemic/reperfusion injury

D. Pevni, I. Frolkis, D. Schwartz, I. Schwartz, T. Chernichovski, A. Kramer, Y. Ben-Gal, G. Uretzky, I. Shapira and A. Weinbroum

Tel-Aviv University, Tel-Aviv, Israel

ABSTRACT

Background Tumour necrosis factor-alpha (TNF-α) plays a key role in causing ischaemia/reperfusion (I/R) injury. I/R also causes activation of xanthine oxidase and dehydrogenase (XDH + XO) system that, via generated free radicals, causes organ damage. We investigated the effect of ischaemia, reperfusion and non-ischaemic prolonged perfusion (NIP) on TNF-α and XDH + XO production in an isolated perfused rat liver model.

Materials and methods Rat livers underwent 150 min NIP (control group) or two hours of ischaemia followed by reperfusion (I/R group). TNF-α (TNF-α mRNA and protein level), XDH + XO production and bile secretion were determined in tissue and effluent at baseline, at 120 min of ischaemia, after 30 min of reperfusion (I/R group) and after 120 and 150 min of prolonged perfusion (control).

Results Unexpectedly, neither ischaemia nor reperfusion had any effect on TNF-α production. TNF-α in effluent was 11 ± 4·8 pg mL^{-1} at baseline, 7 ± 3·2 pg mL^{-1} at the end of ischaemia, and 13 ± 5·3 pg mL^{-1} after 30 min of reperfusion. NIP, however, caused a significant increase of TNF-α synthesis and release. TNF-α effluent level after 120 and 150 min of perfusion was 392 ± 78·7 pg mL^{-1} and 408 ± 64·3 pg mL^{-1}, respectively. TNF-α mRNA in tissue was also significantly elevated compared to baseline levels (1·31 ± 0·2 $P < 0·001$ and 1·38 $P < 0·002$, respectively). Decrease of liver function (expressed by bile secretion) during I/R and NIP was accompanied by significant XDH + XO elevation.

Conclusion This is the first evidence that NIP, and not I/R, is the decisive trigger for TNF-α production. This study leads to a better understanding of pathogenesis of liver I/R and perfusion damage.

Keywords Injury, isolated perfused liver, TNF

Eur J Clin Invest 2008; 38 (9): 649–655

Introduction

Protection of the liver during hepatic surgery is typically achieved by a cooling procedure primarily designed to minimize the oxygen demands of hepatocytes with the aim of avoiding ischaemic injury. While reperfusion is essential for restoring oxygen to an ischaemic organ and to resume delivery of substrates, reperfusion itself may induce tissue damage by activating multiple potentially deleterious oxidative pathways [1–3]. Hepatic ischaemia/reperfusion (I/R) injury is a complication of liver surgery, transplantation and shock, leading to both local and remote cellular damage and organ dysfunction. This complicated and still unclear process results in an acute inflammatory response characterized by the induction of a cascade of proinflammatory mediators leading to parenchymal cell injury [4,5].

The acute phase of hepatic injury characteristically occurs within 1–6 h after reperfusion. It is associated with Kupffer cell activation and the generation of reactive oxygen species (ROS) and pro-inflammatory cytokines, such as tumour necrosis factor alpha (TNF-α) and others [4,5]. The xanthine oxidoreductase system that contains xanthine dehydrogenase and xanthine oxidase (XDH + XO) is one of the major sources of free radicals in biological systems; both XDH and XO cause organ damage via generation of ROS [2,3]. This is followed by a subacute phase response that is characterized by massive neutrophil infiltration that peaks between 9–24 h following reperfusion [6,7].

Recent laboratory studies have shown that several inflammatory cytokines, among them TNF-α and interleukin-6 (IL-6), play key roles in the pathophysiology of hepatic I/R injury [4,8,9]. TNF-α antibodies were found to have a protective effect on rat liver I/R [10], but this could not be reproduced clinically [11]. Mosher *et al*. [9] reported that Kupffer cells are major contributors to cytokine production in hepatic I/R and suggested that their modulation may serve as a potential target for therapeutic

intervention. We had earlier found that ischaemia, but not reperfusion, is the more important signal for TNF-α protein synthesis and release from the isolated I/R rat heart [12]. The aim of the current study is to investigate the influence of ischaemia, reperfusion and non-ischaemic prolonged perfusion (NIP) on TNF-α and XDH + XO production in an isolated perfused rat liver model.

Materials and methods

The present study protocol was approved by the Animal Care Committee of Tel Aviv University, Tel Aviv, Israel (in accordance with the National Institute of Health, Bethesda, MD, USA). Male Wistar rats (mean weight 370 ± 21 g) were selected for experimentation by the institutional veterinarian according to age, weight and general health status. All rats were anesthetized by intraperitoneal injection of phenobarbital sodium (30 mg kg^{-1} body weight).

Isolated perfused rat liver preparation

Liver isolation and perfusion were accomplished as previously described [3,13]. Briefly, perfusion cannulas were placed in the portal vein (in) and suprahepatic inferior vena cava (out). The bile duct cannula was placed for bile secretion monitoring. The liver was then placed in an environmental chamber designed to control temperature at 37 °C. A pressure transducer (Statham Medical P132284, Mennen Medical Inc, Clarence NY., USA), a digital thermometer (NJM-100, Webster Laboratories, Alta Dena, CA., USA) and a bubble trap were set directly in front of the isolated liver, and ports for the collection of aliquots were positioned at the entrance and exit of the liver perfusate. The livers were perfused via the portal vein with freshly prepared haemoglobin-free modified Krebs-Henseleit buffer (KH). The incoming perfusate had a constant temperature of 37 °C, a pH of 7·36–7·42 and was equilibrated with 95%O_2–5%CO_2 to achieve an influent pO_2 of 500 mmHg. The flow rate was set at 3 mL min^{-1} g^{-1} liver ideal weight.

Experimental protocol

Following a stabilization phase (15 min perfusion, baseline) the livers were assigned to the NIP (control) group or to the I/R group. The livers of the NIP group were perfused for a total of 150 min. Their samples of effluent for TNF-α protein and XDH + XO were taken at baseline, after 120 min and after 150 min. We also modified the usual protocol by taking samples of tissue for TNF-α protein (n = 10), TNF-α mRNA (n = 10) and XDH + XO (n = 10) at the same time points.

The livers in the I/R group were exposed after stabilization phase to ischaemic condition for 120 min and followed reperfusion for the 30 min. We modified the usual protocol by taking samples of tissue for TNF-α protein, TNF-α mRNA and total XDH + XO at baseline (n = 10), after 120 min of ischaemia alone (n = 10) and after 30 min of reperfusion (n = 10). Samples for TNF-α protein and XDH + XO in effluent were taken according to the same schedule.

Liver function was determined by bile secretion [13], which was defined as the amount of bile at 1 minute/wet liver weight. At the completion of every protocol, the livers were weighed, maintained at 70 °C for 24 h and reweighed to calculate the wet-to-dry weight (W/D) ratio (n = 5 for each experimental point).

Determination of TNF-α protein concentration in the effluent. TNF-α protein concentration was measured in effluent collected during 1 minute at the following time points: baseline, end of ischaemia (marked by the first minute of reperfusion), after 30 min of reperfusion, and after 120 and 150 min of NIP. All samples were then immediately stored at –70 °C until assay. TNF-α levels were determined using the commercially available high sensitive enzyme linked immunosorbent assay (R & D System Inc, Minneapolis, MN, USA). The limit of detection was 4 pg mL^{-1}.

TNF-α liver tissue protein quantification by Western blotting. TNF-α expression in liver was determined by Western blot analysis. The excised livers were mechanically homogenized in ice cold phosphate buffered saline (PBS) lysis buffer containing 0·01% Triton X-100, 0·1% sodium dodicyl sulphate and protease inhibitors (phenylmethylsulphonyl fluoride 1 mM, leupeptin 10 μg mL^{-1}, aprotinin 10 μg mL^{-1}, ICN Biomedical Inc., Irvine, CA, USA) and left on ice for 30 min. Homogenates were centrifuged (12 000 g, 10 min, 4 °C) and supernatants were stored in aliquots at –80 °C. The total protein content was determined by a protein assay kit (Sigma, St. Louis, MO, USA). Equal amounts of protein (80 μg) were diluted with loading buffer transferred onto nitrocellulose membranes (Hybond-C extra, Amersham Corp, England). Membranes were blocked with PBS-Tween buffer supplied with 5% w/v non-fat dried milk, then incubated for 3 h with mouse monoclonal anti-rat TNF-α antibodies (R&D Systems Inc, Minneapolis, MN, USA), washed three times for 5 min each in PBS-Tween, incubated for 2 h with secondary horseradish peroxidase conjugated goat antimouse IgG (Jackson ImmuniResearch Laboratories Inc, West Grove, PA, USA) and washed as described above. TNF-α was detected by enhanced chemiluminescence, using Pierce chemiluminescent substrate (Pierce Biotechnology, Rockford, IL, USA) and Fuji Super RX film (Fuji Photo Film Co, Ltd, Tokyo, Japan). It was identified as a 21 kDa band and quantified by densitometry (Thermal Imaging System FTJ-500; Fujifilm; Osaka, Japan), a computer-based image capturing software (Image Capture Software, Pharmacia Biotech, Jerusalem, Israel), and a software package (TINA; Raytest Isotope Messgerate, GmbH; Staubenhardt, Germany). The intensities of the bands were expressed in arbitrary densitometry units. Membranes were

re-probed for actin, and all TNF-α band intensities were normalized by respective actin values.

Determination of TNF-α mRNA. Total RNA was extracted from the livers using the guanidium thiocyanate method [14]. Pellets of RNA were kept at –20 °C with 75% ethanol until assay. Dried sediments were dissolved in sterile RNAse-free water and quantitated spectrophotometrically at a wavelength of 260 nm. Two μg of total RNA were subjected to reverse transcription reaction in 20 μL using a reverse transcription system (Promega Corporation, Madison, WI, USA). After completion of the reaction, 5 μL of this reaction mixture was used for TNF-α complementary DNA (cDNA) polymerase chain reaction (PCR) amplification, and 5 μL of 1 : 10 diluted reaction mixture was used for glyceraldegide-3-phosphate dehydrogenase (GAPDH) cDNA amplification. Our PCR negative control contained water instead of cDNA, and the cDNA negative control contained water instead of RNA.

For TNF-α complementary DNA amplification, the following primers were used: sense, CACGCTCTTCTGTCTACTGA, and antisense, GGACTCCGTGATGTCTAAGT. These produced a 546-base pair (bp) fragment. An annealing temperature of 57 °C was chosen for this reaction [15].

For GAPDH complementary DNA amplification, the following primers were used: sense, AATGCATCCTGCACCACCAA and antisense, GTAGCCATATTCATTGTCATA. These produced a 515-bp fragment [16]. The annealing temperature in this case was 60 °C. The optimal number of cycles for the TNF-α and GAPDH was 30. A minicycler (MJ Research Inc; Watertown, MA, USA) was used for PCR amplification and reverse transcription reaction. PCR products (10 μL) were separated in a 1·8% agarose gel, which was stained with ethidium-bromide, visualized by ultraviolet irradiation, and photographed (Polaroid, Cambridge, MA, USA) with film that evaluated band densities using the same software as for tissue protein quantification (see above). The intensities of the bands were expressed in arbitrary densitometry units. All TNF-α band intensities were normalized by respective GAPDH values. Each PCR reaction was performed at least twice.

XDH + XO activity. The reduced and oxidized enzyme forms of XDH and XO in the effluent and tissue samples were analysed as described in detail in our previous publications (3). We used the total (sum) XDH + XO activity because XDH may convert to XO with followed ROS formation and XDH itself shows a capacity to produce superoxide anion [2,3,17]

Statistics

All data are expressed as means ± SD. Comparisons between various time points were performed using an analysis of variance (ANOVA). Differences were considered significant when $P < 0{\cdot}05$.

Results

Liver function

The results showed that 120 min of global ischaemia significantly decreased liver function, as expressed by a significant decrease in bile production at the end of ischaemia compared to baseline (0·05 ± 0·04 and 3·32 ± 0·57 μL min^{-1} g^{-1}, respectively, $P < 0{\cdot}0005$). Reperfusion lasting for 30 min failed to improve bile production (it went from 0·05 ± 0·04 to 0·05 ± 0·05 μL min^{-1} g^{-1}). Ischaemia did not significantly change the W/D ratio, however, reperfusion significantly increase the W/D ratio compared to baseline value (from 2·72 ± 0·06 to 3·34 ± 0·14, $P = 0{\cdot}002$). NIP over 120 and 150 min (control group) also caused a less marked (than for the I/R group) but statistically significant time-dependent decrease in bile production compared to baseline (2·3 ± 0·68 μL min^{-1} g^{-1}, $P < 0{\cdot}0005$ and 1·7 ± 0·25 μL min^{-1} g^{-1}, $P < 0{\cdot}0001$, respectively). The 120 minute NIP did not change the W/D ratio, but the 150 minute NIP significantly increased it compared to baseline (3·04 ± 0·58, P = NS and 4·18 ± 0·56, $P < 0{\cdot}001$, respectively). However, bile production under NIP condition was still much higher when compared to the post-ischaemic situation ($P < 0{\cdot}0001$).

Total XDH + XO production

Ischaemia was found to be a strong trigger for total XDH + XO production. Increased total XDH + XO levels in tissue and in effluent at the end of ischaemia were 1·6 and 9·7 times higher, respectively, compared to baseline (Table 1). Reperfusion

Table 1 Total XDH + XO content in tissue and effluent at various measured time points

Time points	Total XDH + XO in tissue (mU g^{-1}) ($n = 10$)	*P*-value (compared to baseline)	Total XDH + XO in effluent (mU mL^{-1}) ($n = 10$)	*P*-value (compared to baseline)
Stabilization (baseline)	4·58 ± 0·56		0·19 ± 0·04	
Ischaemia 120 min	7·47 ± 0·83	< 0·0004	1·84 ± 0·07	< 0·0001
Reperfusion 30 min	3·91 ± 0·85	< 0·02	0·3 ± 0·02	< 0·0001
Perfusion 120 min	5·21 ± 0·51	< 0·02	0·5 ± 0·4	< 0·0003
Perfusion 150 min	5·26 ± 0·66	< 0·02	0·22 ± 0·07	< 0·03

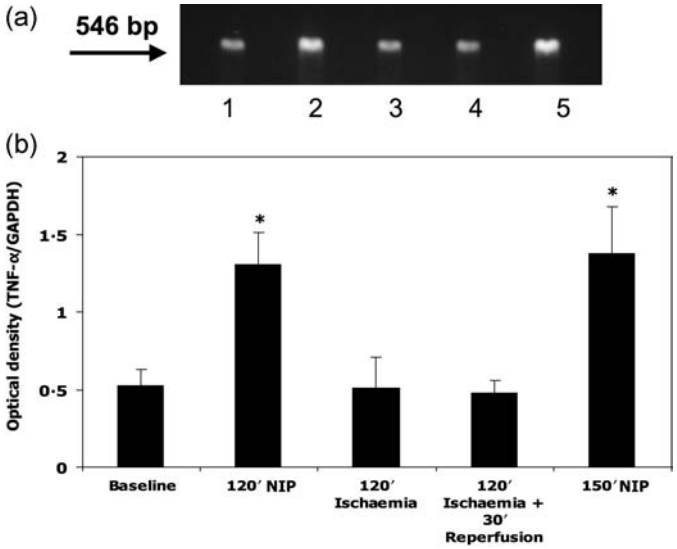

Figure 1 Representative PCR analysis of TNF-α mRNA in the liver samples (a) and relative optical density of TNF-α PCR signal (b). Data were normalized to appropriate GAPDH PCR signals. Samples were drawn at the end of the stabilization period (1 or Baseline), at 120 min of non-ischaemic perfusion -**NIP**- (2 or 120′ **NIP**), at 120 min of global ischaemia (3 or 120′ Ischaemia), at 30 minute of reperfusion (4 or 120′ Ischaemia +30′ Reperfusion), and at the end of 150 min of non-ischaemic perfusion (5 or 150′ **NIP**). Significantly increased TNF-α mRNA expression was observed after 120 ($P < 0{\cdot}001$) and 150 ($P < 0{\cdot}002$) minutes of non-ischaemic perfusion, while no changes were observed after 120 min of global ischaemia, and no changes were observed after 120 min of global ischaemia following 30 min of reperfusion.

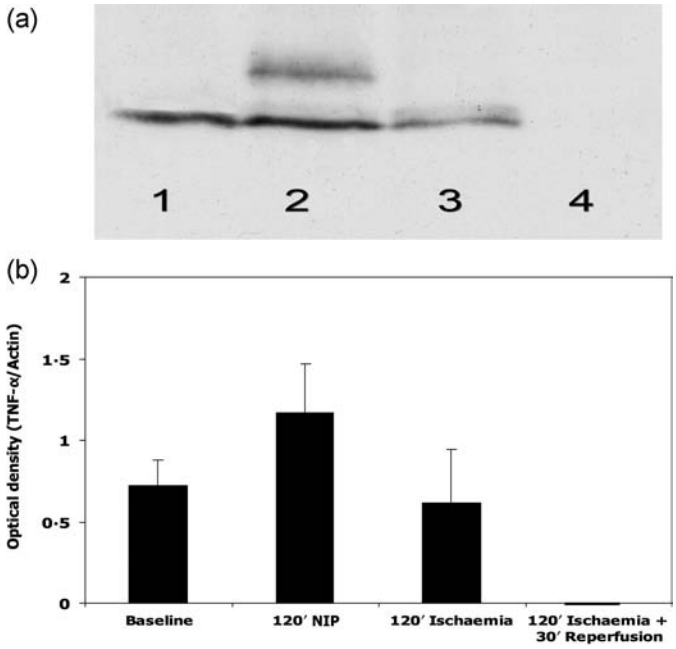

Figure 2 Representative Western blot analysis TNF-α protein in the liver samples (a) and relative optical density of a TNF-α Western blot signal (b). Data were normalized to appropriate actin Western blot signals. Samples were drawn at the end of the stabilization period (1 or Baseline), at 120 min of non-ischaemic perfusion -**NIP**- (2 or 120′ **NIP**), at 120 min of global ischaemia (3 or 120′ Ischaemia) and at the end of 30 min of reperfusion (4 or 120′ Ischaemia +30′ Reperfusion). There was a tendency toward an increase of TNF-α protein after 120 min of perfusion ($P < 0{\cdot}065$), and an additional TNF-α protein band appeared at that time point (upper band 2). No changes were observed after 120 min of global ischaemia. TNF-α protein was below detectable levels after the 120 min of global ischaemia that followed 30 min of reperfusion.

significantly decreased the total XDH + XO level in tissue ($P < 0{\cdot}0001$), probably by a washout effect. Total XDH + XO in effluent after 30 min of reperfusion was significantly lower than after ischaemia, but significantly higher than at baseline (Table 1). NIP for 120 and 150 min was also associated with elevation of total XDH + XO production. This elevation was less marked than at the end of ischaemia, but still significant (Table 1).

TNF-α synthesis and release

Neither ischaemia nor reperfusion increased TNF-α synthesis and release in the liver. The band intensities of TNF-α mRNA in hepatic tissue were 0·53 ± 0·1 at baseline, 0·51 ± 0·2 at the end of ischaemia and 0·48 ± 0·08 at the end of reperfusion (Fig. 1). Band intensities of TNF-α protein in tissue were 0·72 ± 0·16 after stabilization, 0·62 ± 0·32 at the end of ischaemia, and below detectable levels at the end of reperfusion (Western blot quantification, Fig. 2). TNF-α in effluent was 11 ± 4·8 pg mL^{-1} at baseline, 7 ± 3·2 pg mL^{-1} at the end of ischaemia, and 13 ± 5·3 pg mL^{-1} after 30 min of reperfusion. The differences in TNF-α levels at all these points were not significant (Fig. 3).

NIP, but not ischaemia or reperfusion, caused a significant increase of TNF-α synthesis and release. NIP lasting for 120 and 150 min caused significant increases of TNF-α mRNA in tissue compared to baseline levels (1·31 ± 0·2 $P < 0{\cdot}001$ and 1·38 $P < 0{\cdot}002$, respectively, Fig. 1). The TNF-α protein level in tissue after 120 min of NIP showed a trend towards elevation compared to baseline (1·17 ± 0·3, $P < 0{\cdot}065$, Fig. 2), and this was associated with significantly elevated TNF-α effluent levels after 120 and 150 min of perfusion (392 ± 78·7 pg mL^{-1} $P < 0{\cdot}0001$ and 408 ± 64·3 $P < 0{\cdot}0001$ pg mL^{-1}, respectively, Fig. 3). An additional TNF-α protein band appeared after 120 min of NIP: it was identified as 26 kDa protein (upper band 2, Fig. 2a) and it corresponded to a well known transmembrane TNF-α.

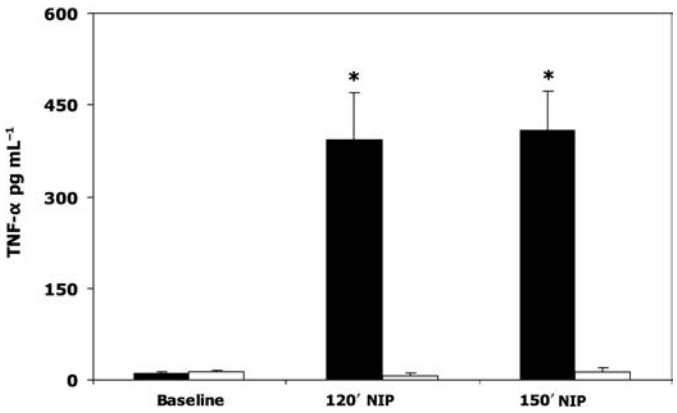

Figure 3 TNF-α protein content in the effluent from an isolated liver during prolonged non-ischaemic perfusion -**NIP**, (black bars) and during ischaemia/reperfusion (white bars). Samples were drawn at the end of a stabilization period (Baseline, $n = 10$), at 120 ($n = 10$) and 150 ($n = 10$) minutes of **NIP**, at 120 min of global ischaemia ($n = 10$) and at the end of 30 min of reperfusion ($n = 10$) following 120 min of global ischaemia. Significantly increased levels of TNF-α protein were observed in the effluent after 120 ($P < 0{\cdot}0001$) and 150 ($P < 0{\cdot}0001$) minutes of **NIP**, no changes were observed after 120 min of global ischaemia and no changes were observed after 120 min of global ischaemia following 30 min of reperfusion.

Discussion

The current study was originally designed to clarify whether TNF-α causes liver damage during the ischaemic or reperfusion part of the I/R process. We followed the usual protocols for isolated liver I/R models and decided to also check the influence of prolonged perfusion on the production of TNF-α and total XDH + XO. Control group addition enabled us to quantitatively monitor TNF-α synthesis and release at three time points in a unique fashion: at baseline, after 120 min (control to ischaemia) and once more after another 30 min of perfusion (control to reperfusion).

The results of the present study demonstrate that global ischaemia and reperfusion cause a decrease of liver function and a significant elevation of total XDH + XO.

Unexpectedly, neither 120 min of ischaemia alone nor 30 min of reperfusion were associated with induction of TNF-α synthesis and release. TNF-α mRNA expression and the tissue TNF-α protein level as well as the TNF-α content in effluent at the end of 120 min of ischaemia did not exceed baseline levels. The 30 min of reperfusion that followed caused no elevation of TNF-α mRNA in tissue or TNF-α protein in effluent, and TNF-α protein in tissue was below detectable level. This decrease of TNF-α in tissue can probably be explained by a washing out and/or a tissue degradation process.

A less marked but significant decrease of liver function was also observed during NIP (120 and 150 min – control group). It was associated with a significant elevation of total XDH + XO, although it was less pronounced than the extent of elevation after ischaemia and reperfusion. Moreover, NIP was unexpectedly associated with a significant elevation of TNF-α synthesis and release. NIP (120 min) increased the TNF-α protein level in effluent as well as the TNF-α mRNA content in tissue. There was a tendency for tissue TNF-α protein to increase or remain unchanged compared to baseline, and an additional transmembrane TNF-α protein band appeared after 120 min of NIP. All these lines of evidence strongly point to the probability that NIP is the trigger for the increase of TNF-α synthesis and release that occurred in the isolated rat liver. Despite the significant elevation of TNF-α during the period of NIP, liver function (bile excretion and liver oedema) was significantly less impaired than at the time of I/R. This is in agreement with Simeonova *et al*. who reported that TNF-α is not responsible for direct hepatocyte damage [18].

Based on these data, we believe that it is reasonable to propose that elevation of total XDH + XO – but not of TNF-α – is responsible for decrease of liver function. XDH-XO system is one of the major sources of free radicals in the biological system [1–3,17]. Previous publications have shown that XDH + XO via generated ROS could directly cause organ damage [2,3].

We realize that the pathophysiological evolution of I/R and long term non-ischaemic perfusion damage may well involve a more complex process consisting of intermediate steps and feedback loops, features that we did not explore within the context of the current study.

The two forms of TNF-α, soluble and membrane-bound, were detected at the end of prolonged perfusion. Most of the members of the TNF-α family are present in both a membrane-bound and a soluble form, which can possess differential bioactivities. TNF-α is primarily produced as a transmembrane protein, but it may be released in soluble trimeric form via proteolytic cleavage by the metalloprotease TNF-converting enzyme [19]. TNF-α exerts a variety of effects that are mediated by TNF-receptor 1 and 2 (TNF-R1 and TNF-R2). Whereas TNF-R1 is efficiently activated by soluble TNF-α, TNF-R2 activation requires binding of membrane-bound TNF-α [18]. Membrane-bound TNF-α has been suggested to have a more localized effect than soluble TNF-α and has been implicated in several other pathological conditions, such as acute hepatitis, rheumatoid arthritis and neurological disorders [20,21].

Despite the multitude of investigations, the true role of TNF-α in liver I/R injury remains unclear. Most of those studies claim that TNF-α plays a key role in mediating I/R injury after liver, kidney, intestine, heart, lung and pancreas transplantation [22]. A recent study by Teoh *et al*. reported that TNF-α has a dual role in I/R

injury [23]. A protective effect of TNF-α in liver I/R injury and an important role in the initiation of liver regeneration after partial resection were also reported [24,25].

Numerous earlier publications demonstrated an elevation of TNF-α production at the time of liver I/R: we found different results in our current study. This discrepancy can be explained by differences in methodology: different time points for sampling TNF-α in tissue and effluent, different models used for investigating TNF-α production during I/R liver injury, and different interpretations of the results. Specifically, the two main models used to imitate liver I/R injury are an isolated non-blood perfused liver in-vitro model or a partial I/R model in which there is temporary closure of the vascular pedicle to the left lateral and median liver lobe *in vivo*. Most experimental animal models for studying hepatic I/R injury involve partial or segmental ischaemia of the liver [26,27]. Those models do not, however, reflect the global ischaemia that occurs during liver transplantation. Importantly, TNF-α was mostly determined *in vivo* or *in vitro* only at different time points of reperfusion, and not during or at the end of ischaemia. Conclusions about the important role of TNF-α during ischaemia in these studies were actually based on the observed elevation of TNF-α during prolonged reperfusion and were not confirmed by direct measurements immediately after ischaemia [28–31]. None of the earlier studies used a control group that had been submitted to non-ischaemic prolonged perfusion (NIP) as we did in our current study. Thus, based on our findings, we propose that the TNF-α elevation evidenced in those studies and ours can be explained by prolonged perfusion but not by I/R injury.

Teoh *et al*. were among the first to document tissue TNF-α elevation at the time of ischaemia using a partial liver ischaemic model [23]. The elevation of tissue TNF-α in their study can be explained by several methodological features: first, the rats were killed by exsanguination which itself can cause elevation of TNF-α production [32]; second, TNF-α levels were determined from whole liver homogenates that contained both ischaemic and non-ischaemic liver tissue. Partial liver ischaemia may cause portal congestion and sinusoidal shear stress by increasing the blood supply to a *non-ischaemic* right lobe, with subsequent elevation of TNF-α production, probably by non-ischaemic tissue such as occurs after partial hepatectomy [26,27].

In our current study, we used an isolated non-blood perfused liver and global liver ischaemia model which we believe more faithfully imitates the state of global ischaemia at the time of liver transplantation, or shock and provides the possibility of investigating endogenous liver TNF-α production. Thus, our model also imitates isolated non-blood perfusion of liver used for treatment of liver cancer [33]. Previous studies that had used this model demonstrated TNF-α elevation at the time of liver reperfusion but not at the time of ischaemia [34–37]. These results can be explained by the timing of TNF-α sampling, i.e. after more than one hour of reperfusion [34,35]. The lack of control groups with long non-ischemic perfusion periods influenced the interpretation of the results in those studies. The elevation of TNF-α during the early reperfusion period that was reported in some other studies may be explained by there having been prolonged liver perfusion (i.e. more than 40 min) before ischaemia [36,37]. Ben-Ari *et al*. reported that they selected a longer (90 min) period of stabilization perfusion before ischaemia because shorter periods did not cause a significant elevation of TNF-α after liver ischaemia [37]. We found that TNF-α elevation began after 30 min of non-ischaemic perfusion and that it reached the maximum level after 60 min where it stayed for up to 150 min (our pilot studies).

Based on our current results and the data in the literature, we hypothesize that the increased release in endogenous liver TNF-α is most likely caused by changes in flow via a hepatic vascular bed such as in the following clinical situations: partial liver resection, portal hypertension, haemorrhagic shock, liver transplantation, and perfusion during treatment of liver cancer. This hypothesis warrants validation by additional experiments.

We conclude that neither liver ischaemia nor reperfusion are causes of TNF-α synthesis and release in isolated perfused livers. Impairment of liver function during I/R is probably associated with an elevation in ROS production. NIP, and not ischaemia or reperfusion, is the decisive trigger for TNF-α synthesis and release. We speculate that TNF-α release during prolonged non-ischaemic liver perfusion can cause cytotoxic effects in different organs. The present study is a new step in the understanding of the mechanism of liver I/R and perfusion injury and sheds new light on TNF-α related and unrelated mechanisms in the evolution of these types of liver injury. The present controversy over the interpretation of data could lead to a better understanding of pathogenesis of I/R and perfusion damage, and potentially reveal new, more specific therapeutic agents.

Acknowledgements

The authors thank Esther Eshkol for editorial assistance.
IS and AW contributed equally to this work.

Address

Departments of Cardiac Surgery (D. Pevni, I. Frolkis, A. Kramer, Y. Ben-Gal, G. Uretzky, I. Shapira) and Nephrology (D. Schwartz, I. Schwartz, T. Chernichovski) and the Animal Research Laboratory (A. Weinbroum), Tel-Aviv Sourasky Medical Centre, Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv, Israel
Correspondence to: Inna Frolkis, MD, PhD, Department of Cardiothoracic Surgery, Tel Aviv Sourasky Medical Centre, 6 Weizmann Street, Tel Aviv 64239, Israel. Tel.: +972-3-6973322; fax: +972-3-6974439, E-mail: frolkisi@tasmc.health.gov.il

Received 29 February 2008; accepted 2 July 2008

References

1 Buja LM. Myocardial ischemia and reperfusion injury. *Cardiovasc Pathol* 2005;**14**:170–5.

2 Harada H, Hines IN, Flores S, Gao B, McCord J, Scheerens H *et al*. Role of NADPH oxidase-derived superoxide in reduced size liver ischemia and reperfusion injury. *Arch Biochem Biophys* 2004;**423**:103–8.
3 Weinbroum A, Nielsen VG, Tan S, Gelman S, Matalon S, Skinner KA *et al*. Liver ischemia-reperfusion increases pulmonary permeability in rat: role of circulating xanthine oxidase. *Am J Physiol* 1995;**268**:G988–96.
4 Colletti LM, Kunkel SL, Walz A, Burdick MD, Kunkel RG, Wilke CA *et al*. The role of cytokine networks in the local liver injury following hepatic ischemia/reperfusion in the rat. *Hepatology* 1996;**23**:506–14.
5 Jaeschke H, Farhood A. Neutrophil and Kupffer cell-induced oxidant stress and ischemia-reperfusion injury in rat liver. *Am J Physiol* 1991;**260**:G355–G62.
6 Jaeschke H, Hasegawa T. Role of neutrophils in acute inflammatory liver injury. *Liver Int* 2006;**26**:912–9.
7 Crockett-Torabi E, Sullenbarger B, Smith CW, Fantone JC. Neutrophil activation through L selectin and Mac-1. *J Immunol* 1995;**154**:2291–302.
8 Lentsch. AB, Yoshidome H, Cheadle WG, Miller FN, Edwards MJ. Chemokine involvement in hepatic ischemia/reperfusion injury in mice: roles for macrophage inflammatory protein-2 and KC. *Hepatology* 1998;**27**:563–8.
9 Mosher BD, Dean RE, Harkema J, Remick D, Palma J, Crockett E. Inhibition of chemokines production by kupffer cells decreased hepatic ischemia/reperfusion injury in mice. *J Surg Res* 2001;**99**:201–10.
10 Yang YL, Peng Li JI, Xiao Ping XU, Dou KF, Yue SQ, Li KZ. Protective effect of tumor necrosis factor alfa antibody and ulinastin on liver ischemic reperfusion in rats. *World J Gastroenterol* 2004;**100**:3161–4.
11 Abraham E. Anti-TNF therapies. *Sepsis* 1999;**3**:47–50.
12 Pevni D, Frolkis I, Shapira I, Schwartz D, Schwartz I, Chernichovski T *et al*. Cardioplegic ischemia or reperfusion: which is a main trigger for tumor necrosis factor production? *Int J Cardiol* 2008;**127**:186–91.
13 Bessems M, Hart N, Tolba R, Dorschadt B, Leuvenik HGD, Ploeg RJ *et al*. The isolated perfused rat liver: standardisation of time-honoured model. *Lab Anim* 2006;**40**:236–46.
14 Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory;1989,pp. 19–23.
15 Herskowitz A, Choi S, Ansari AA, Wesselingh S. Cytokine mRNA expression in postischemic/reperfused myocardium. *Am J Pathol* 1995;**146**:419–28.
16 Morrissey JJ, McCracken R, Kaneto H, Vehaskari M, Montani D, Klahr S. Location of an inducible nitric oxide synthase mRNA in the normal kidney. *Kidney Int* 1994;**45**:998–1005.
17 Sanders SA, Eisenthal R, Harrison R. NADH oxidase activity of human xanthine oxidoreductase – generation of superoxide anion. *Eur J Biochem* 1997;**245**:541–8.
18 Simeonova PP, Gallucci RM, Hulderman T, Wilson R, Kommineni C, Rao M *et al*. The role of tumor necrosis factor-alpha in liver toxicity, inflammation, and fibrosis induced by carbon tetrachloride. *Toxicol Appl Pharmacol* 2001;**177**:112–20.
19 Wajant H, Pfizenmaier K, Scheurich P. Tumor necrosis factor signaling. *Cell Death Differ* 2003;**10**:45–65.
20 Akassoglou K, Probert L, Kontogeorgos G, Kollias G. Astrocyte-specific but not neuron-specific transmembrane TNF triggers inflammation and degeneration in the central nervous system of transgenic mice. *J Immunol* 1997;**158**:438–45.
21 Küsters S, Tiegs G, Alexopoulou L, Pasparakis M, Douni E, Künstle G *et al*. In vivo evidence for a functional role of both tumor necrosis factor (TNF) receptors and transmembrane TNF in experimental hepatitis. *Eur J Immunol* 1997;**27**:2870–5.
22 Pascher A, Klupp J. Biologics in the treatment of transplant rejection and ischemia/reperfusion injury: new applications for TNF alpha inhibitors? *Biodrugs* 2005;**19**:211–31.
23 Teoh N, Field J, Sutton J, Farrel G. Dual role of tumour necrosis factor-α in hepatic ischemia-reperfusion injury: studies in tumour necrosis factor-α gene knockout mice. *Hepatology* 2004;**39**:412–21.
24 Trautwein C, Rakemann T, Niehof M, Rose-John S, Manns MP. Acute-phase response factor, increased binding, and target gene transcription during liver regeneration. *Gastroenterology* 1996;**110**:1854–62.
25 Yamada Y, Kirillova I, Peschon JJ, Fausto N. Initiation of liver growth by tumor necrosis factor: deficient liver regeneration in mice lacking type I tumor necrosis factor receptor. *Proc Nat Acad Sc USA* 1997;**94**:1441–6.
26 Spiegel HU, Bahde R. Experimental models of temporary normothermic liver ischaemia. *J Invest Surg* 2006;**19**:113–23.
27 Niiya T, Murakami M, Aoki T, Murai N, Shimizu Y, Kusano M. Immediate increase of portal pressure, reflecting sinusoidal shear stress, induced liver regeneration after partial hepatectomy. *J Hepatobiliary Pancreat Surg* 1999;**6**:275–80.
28 Yamada T, Hisanaga M, Nakajima Y, Kanehiro H, Aomatsu Y, Ko S *et al*. The serum interleukin 8 level reflects hepatic mitochondrial redox state in hyperthermochemohypoxic isolated liver perfusion with use of a venovenous bypass. *Surgery* 1999;**125**:304–14.
29 Shibuya H, Ohkohchi N, Tsukamoto S, Satomi S. Tumour necrosis factor-induced, superoxide-mediated neutrophil accumulation in cold ischaemic/reperfused rat liver. *Hepatology* 1997;**26**:113–20.
30 Lopez-Neblina F, Toledo-Pereyra LH. Anti-ischemic effect of selectin blocker through modulation of tumor necrosis factor-alpha and interleukin-10. *J Surg Res* 2007;**138**:275–83.
31 Harada N, Okajima K, Kohmura H, Uchiba M, Tomita T. Danaparoid sodium reduces ischemia/reperfusion-induced liver injury in rats by attenuating inflammatory responses. *Thromb Haemost* 2007;**97**:81–7.
32 Matsutani T, Kang SC, Miyashita M, Sasajima K, Choudhry MA, Bland KI *et al*. Liver cytokine production and ICAM-1 expression following bone fracture, tissue trauma, and hemorrhage in middle-aged mice. *Am J Physiol Gastrointest Liver Physiol* 2007;**292**:G268–74.
33 Grover A, Alexander HR. The past decade of experience with isolated hepatic perfusion. *Oncologist* 2004;**9**:653–64.
34 Le Moine O, Louis H, Stordeur P, Collet JM, Goldman M, Devière J. Role of reactive oxygen intermediates in interleukin 10 release after cold liver ischemia and reperfusion in mice. *Gastroenterology* 1997;**113**:1701–6.
35 Urata K, Nguyen B, Brault A, Lavoie J, Rocheleau B, Huet PM. Decreased survival in rat liver transplantation with extended cold preservation: role of portal vein clamping time. *Hepatology* 1998;**28**:366–73.
36 Schuster H, Blanc MC, Neveux N, Bonnefont-Rousselot D, Le Tourneau A, De Bandt JP *et al*. Protective effects of regulatory amino acids on ischemia-reperfusion injury in the isolated perfused rat liver. *Scand J Gastroenterol* 2006;**1**:342–9.
37 Ben-Ari Z, Hochhauser E, Burstein I, Papo O, Kaganovsky E, Krasnov T *et al*. Role of anti-tumor necrosis factor-alpha in ischemia/reperfusion injury in isolated rat liver in a blood-free environment. *Transplantation* 2002;**3**:1875–80.