Pharmacologic Modulation of Experimental Postischemic Hepatic Function

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The present study evaluated and compared the effects of SRI 63-441, a potent platelet activating factor antagonist, superoxide dismutase (SOD), an oxygen free radical scavenger, and ibuprofen, a cyclooxygenase inhibitor on hepatic function after 90 minutes of warm ischemia. After warm ischemia, livers were harvested and underwent 90 minutes of warm, oxygenated, sanguinous perfusion on an isolated liver perfusion apparatus. Pretreatment of donor animals with 20 mg/kg intravenous (I.V.) SRI 63-441 5 minutes before induction of total hepatic ischemia resulted in significantly increased bile production, a significant decrease in transaminase release, and a higher tissue adenosine triphosphate (ATP) content when compared with ischemic nontreated controls. SOD resulted in improved bile production and decreased transaminase liberation only when present in the perfusate at the time of *in vitro* reperfusion. Ibuprofen did not improve postischemic hepatic function in this model. Electron microscopy revealed patchy hepatocellular vacuolization with an intact sinusoidal endothelium in all ischemic livers. However, the degree of damage was less severe in the livers from those rats pretreated with 20 mg/kg SRI 63-441. This study demonstrates that SRI 63-441 pretreatment significantly reduces hepatic warm ischemic injury, and in the present model, appears superior to two other agents that have been advanced in the treatment of ischemic injury. The use of such agents singly or in combinations have important implications as regards gaining a better understanding of the basic mechanisms in organ ischemia, and moreover, for therapeutic applications in organ ischemia and preservation.

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ORGAN INJURY after ischemia and reperfusion remains one of the most formidable limiting factors in the field of transplantation. The ischemia suffered during the process of transplantation is comprised of both a warm and cold component. There is emerging evidence suggesting that warm and cold ischemia result in two different types of injury.[1] The complexity of the interactions between the various mediators, as well as the redundancy and resiliency of the various cascades, prompted us to investigate potential therapeutic agents for organ ischemia that are thought to exert their effects by different mechanisms. In the present study, we have evaluated the effect of three pharmacologic candidates on hepatic function after warm ischemic injury using the isolated perfused rat liver (IPRL).[2]

The mediators and cascades involved in the inflammatory response to ischemia/reperfusion injury are quite complex and interrelated. They include the generation of oxygen-free radicals, arachidonic acid metabolites, and platelet activating factor (PAF). PAF is a key inflammatory mediator elaborated by a variety of cell types, including platelets, neutrophils, and the endothelium, and is intimately involved in the microcirculatory response to nonimmunologic and immunologic inflammatory stimuli.[3-8] Many of the pathophysiologic responses and the microcirculatory failure that result from PAF release mimic the progression of responses observed after ischemia/reper-

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fusion injury. SRI 63-441 (Sandoz Pharmaceuticals, North Hanover, NJ) is a potent receptor antagonist of PAF that can inhibit all of the known biological responses to PAF.[9] Thus, the availability of such an antagonist could give further insight into the basic mechanisms involved in the ischemic process, and could have significant potential as a therapeutic agent for ischemic organ injury.

Recently there has been much interest in the role of oxygen-free radicals in ischemia and reperfusion injury.[10] Oxygen-free radicals have been implicated in ischemia/reperfusion injury in both the liver and kidney.[11-14] In the present study, three different protocols using superoxide dismutase (SOD) (Pharmacia Co., Uppsala, Sweden), an oxygen-free radical scavenger, were evaluated, as well as a combined protocol using SOD and SRI 63-441.

The nonsteroidal anti-inflammatory agent and cyclooxygenase inhibitor, ibuprofen (Upjohn Company, Kalamazoo, MI),[AQ5] has been reported to have the ability to salvage ischemic myocardium during infarction in the dog.[15] Its protective mechanism may be via stabilization of cellular membranes and prevention of thromboxane release.[16] Ibuprofen has also demonstrated a protective effect on bowel ischemia[17] and improves survival and neurologic outcome after resuscitation from cardiac arrest.[18] This protective effect of ibuprofen was further evaluated in these studies of ischemia/reperfusion injury in the liver.

Of the three agents investigated, SRI 63-441 at a dose of 20 mg/kg administered intravenously produced the most significant improvement in postischemic hepatic function as determined by the parameters measured. Histologic evaluation of the SRI 63-441 pretreated livers was carried out using light and electron microscopy, and has given further insight into the mechanism of warm ischemic liver injury, specifically how it compares with cold ischemic liver injury.

Materials and Methods

Animals

Male Lewis rats (Charles River Breed Laboratories, Wilmington, MA) weighing 225–300 g were used as liver donors and male Lewis rat retired breeders were used as blood donors in these experiments. Animals were acclimatized for 1 week before experimentation and housed in a standard animal facility at the University of Pittsburgh. Animals were fed standard rat chow and water *ad libitum*. Guidelines for the care and use of laboratory animals of the University of Pittsburgh were followed.

Operative Procedure

Inhalational anesthesia was administered and maintained with methoxyflurane. The abdomen was entered through a transverse incision. All animals received 300 units of heparin intravenously 5 minutes before cannulation of the bile duct and induction of total hepatic ischemia. Both the hepatic artery and portal vein were ligated. At the time of harvest, the liver was flushed via the portal vein with 60 cc of cold (4 C) heparinized lactated Ringer's solution from a height of 20 cm immediately after venting the vena cava. The portal vein was cannulated using a blunted 18 gauge needle. After 90 minutes of total *in situ* ischemia, the liver was harvested and placed on the perfusion apparatus.

Isolated perfusion

The perfusion apparatus was a recirculating system consisting of a pulsatile pump, "Hamilton Lung" oxygenator, debubbler/reservoir, effluent collecting basin, blood infusion filter, platelet trap, and in-line thermometer.[2,19,20] The temperature within the apparatus was maintained at 37 C with a circulating water bath. The liver was perfused via the portal vein only from a height of 18 cm.[21] The rate of flow through the liver was regulated by the resistance of the intrahepatic microvasculature and varied between 10 and 20 cc/minute. Oxygenation was maintained using a 95% oxygen/5% carbon dioxide mixture, resulting in a pO_2 of approximately 500 torr.

Blood was obtained by aortic puncture after heparinizing both the blood donor and the syringe. The perfusate consisted of a dilute sanguinous solution prepared from two parts by volume of heparinized fresh whole rat blood and 1 part Krebs bicarbonate buffer.[22] The resulting hematocrit was approximately 25%.[23] The pH of the perfusate was maintained at approximately 7.4 with the addition of sodium bicarbonate, as necessary. Blood gases were checked before placing the liver on the circuit and periodically thereafter. All livers were perfused for a period of 90 minutes.

Experimental Protocol

The experimental groups are described in Table 1. All livers in all groups underwent 90 minutes of warm, sanguinous, oxygenated perfusion.

Platelet Activating Factor Antagonist

SRI 63-441, a PAF receptor antagonist, was supplied by Dr. Robert Saunders of Sandoz Pharmaceuticals, North Hanover, New Jersey. The pharmacologic activity and physicochemical properties of this specific compound have been described previously.[9] It was supplied in a powdered form and a 15-mg/ml solution was prepared daily in 0.9% sodium chloride. The solution was warmed to 37 C to ensure that the SRI 63-441 was completely solubilized before injection. The agent was administered intrave-

TABLE 1. *Experimental Groups*

Group	No. of Patients	Ischemia	Agent	Dosage	Route	Time Treated
1	7	no				
2	9	yes				
3	8	no	SRI 63-441	20 mg/kg	I.V.	At time of heparinization
4	9	yes	SRI 63-441	20 mg/kg	I.V.	At time of heparinization
5	3	yes	SRI 63-441	10 mg/kg	I.V.	At time of heparinization
6	4	yes	SOD	12 mg/kg 8 mg/kg	flush	At time of harvest perfusate at 0, 5, 10 min. perfusion*
7	4	yes	SOD	12 mg/kg 12 mg/kg	I.V. flush	At time of heparinization at time of harvest
8	4	yes	SOD	12 mg/kg 12 mg/kg 8 mg/kg	I.V. flush	At time of heparinization at time of harvest perfusate at 0, 5, 10 min. perfusion*
9	6	yes	SRI 63-441 SOD	20 mg/kg 6.5 mg/kg 6.5 mg/kg	I.V. flush	At time of heparinization at time of harvest perfusate at 0 min. perfusion†
10	7	yes	Ibuprofen	12 mg/kg 1.8 mg 12 mg/kg	I.V. flush	1 hour before surgery at time of harvest perfusage at 0 min. perfusion†
11	3	no	Ibuprofen	12 mg/kg 1.8 mg 12 mg/kg	I.V. flush	1 hour before surgery at time of harvest perfusate at 0 min. perfusion†

* 8 mg/kg of SOD was added to the perfusate just before placing the liver on the perfusion apparatus (0 min.), 5 minutes after the liver was placed on the perfusion apparatus, and 10 minutes after the liver was placed on the perfusion apparatus, for a total dose of 24 mg/kg added.

† Zero minutes perfusion is just before placing the liver on the perfusion apparatus.

nously at a dose of 20 mg/kg and 10 mg/kg 5 minutes before the induction of total hepatic ischemia.

Superoxide dismutase

The oxygen-free radical scavenger SOD was supplied by Pharmacia Company, (Upsala, Sweden). The bovine SOD was supplied in a powdered form and a 4-mg/ml solution was prepared daily in 0.9% sodium chloride. SOD was given at the time of heparinization, added to the flush at the time of harvest, or added to the perfusate (Table 1).

Ibuprofen

The cyclooxygenase inhibitor ibuprofen was supplied by the Upjohn Company (Kalamazoo, Michigan). It was supplied as a sterile 50-mg/ml solution and, for experimentation, was diluted to a 10-mg/ml solution with 0.9% sodium chloride daily. The animals were pretreated with 12-mg/kg ibuprofen administered intravenously 1 hour before surgery[17] as well as the addition of ibuprofen to the flush at harvest (1.8 mg), and 12 mg/kg was added to the perfusate (Table 1).

Assessment of Liver Status

Baseline perfusate levels of serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), and glucose were measured using the Technicon RA 500 (Technicon Instruments, Tarrytown, NY) before placing the liver on the perfusion apparatus. Liver function during perfusion was determined by measuring bile production, SGOT, SGPT, and glucose levels in the perfusate every 30 minutes. At the completion of the 90-minute perfusion period, tissue was immediately freeze-clamped in liquid nitrogen and adenosine triphosphate (ATP) content was determined biochemically.[24] Specimens were placed in formalin, embedded in paraffin, cut, and stained with hematoxylin and eosin and PAS for light microscopy. An additional three experiments were repeated per group for experimental Groups 1, 2, and 4, and the livers were processed for electron microscopy.

After 90 minutes of warm sanguinous perfusion, the livers were perfused-fixed via the portal vein with 20 cc of 2.0% glutaraldehyde in 0.125 M cacodylate buffer at a rate of 5–10 cc/minute, minced to 1 × 1 × 1 mm cubes and placed in 2.0% glutaraldehyde for 2 hours.[25] The livers were postfixed in buffered 2.0% osmium tetroxide, dehydrated in ethanol and embedded in Epon 812. Blocks were cut using a Reichert microtome. Semi-thin sections were stained with toluidine blue for light microscopy. Ultra-thin sections were collected on grids, stained with uranyl acetate and lead citrate, and were observed using a Phillips 300 electron microscope (Phillips Electronic Instruments, Mahwah, NJ).[26]

TABLE 2. *Parameters of Liver Function Evaluated*

Group	Bile Production	SGOT	SGPT	Glucose	ATP	Morphology: LM	EM
1	×	×	×	×	×	×	×
2	×	×	×	×	×	×	×
3	×	×	×	×	×	×	
4	×	×	×	×	×	×	×
5	×	×	×	×	×		
6	×	×	×				
7	×	×	×				
8	×	×	×				
9	×	×	×	×	×		
10	×	×	×	×			
11	×	×	×	×			

Not all of the parameters described above were determined for all groups. Experimental groups and the variables measured for each group are listed in Table 2.

Statistics

All values were corrected to baseline by subtracting the values obtained before placing the liver on the perfusion apparatus from the subsequent measurements at 30, 60, and 90 minutes of perfusion. The statistical evaluation was carried out using an analysis of variance. When indicated, the Student's t-test was performed, using the variances generated by the ANOVA.[27] All values reported are expressed as a mean ± the standard error of the mean (SEM); p values of less than 0.05 were considered statistically significant.

Results

Treatment with SRI 63-441

There was a significant increase in bile production ($p < 0.005$) by those livers harvested from rats that were pretreated with 20 mg/kg of SRI 63-441 administered intravenously (Fig. 1). Perfusate transaminase levels were significantly lower from the ischemic livers of animals pretreated with 20 mg/kg SRI 63-441 ($p < 0.001$) (Fig. 2). There was no significant difference in transaminase release by SRI 63-441-treated and untreated livers from animals that had not undergone ischemic injury. ATP levels were significantly higher in the ischemic livers pretreated with 20 mg/kg of SRI 63-441 when compared with untreated livers ($p < 0.05$) (Fig. 3). When compared with untreated livers, ischemic livers from rats that were pretreated with only 10 mg/kg of SRI 63-441 administered intravenously did not demonstrate any improvement in terms of bile production, transaminase release, or ATP content (Figs. 1–3). There was a significant difference in bile production, transaminase release, and ATP levels in untreated livers when comparing the group that underwent 90 minutes of total warm ischemia with the group that did not undergo an ischemic period (Figs. 1–3) ($p < 0.001$).

Treatment with SOD

Results of bile production and transaminase release for Groups 6–8 are described in Table 3. Of the ischemic livers that were treated with the three SOD protocols, only the group that received SOD, 12 mg/kg in the flush at harvest and 8 mg/kg in the perfusate at 0, 5, and 10 minutes of perfusion (*i.e.*, Group 6), experienced a significant increase in bile production after 90 minutes of perfusion, as well as a significant decrease in transaminase release when compared with ischemic controls ($p < 0.02$). The group of livers that underwent pretreatment with 12 mg/kg of SOD followed by 12 mg/kg of SOD in the flush at harvest, but did not have SOD added to the perfusate (*i.e.*, Group 7), had no significant improvement in bile production after 90 minutes of perfusion and did not differ significantly from ischemic controls in the degree of transaminase release. Those livers that were pretreated with 12 mg/kg SOD, flushed with 12 mg/kg of SOD and had 8mg/kg of SOD added to the reperfusate at 0, 5, and 10 minutes of perfusion (*i.e.*, Group 8), did not demonstrate a significant increase in bile production after 90 minutes of perfusion, but did release significantly less SGOT ($p < 0.05$) and SGPT ($p < 0.001$) into the perfusate when compared with untreated ischemic controls.

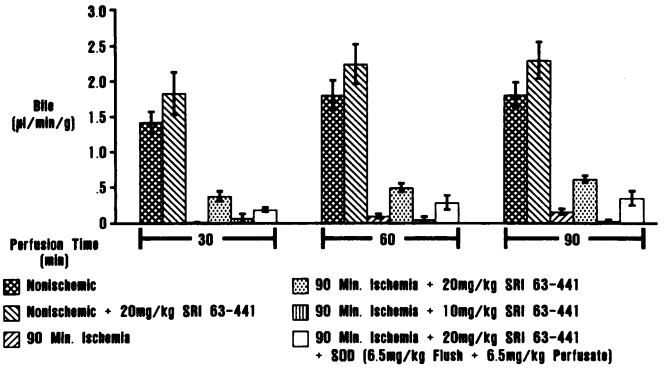


FIG. 1. Bile production (SRI 63-441). Bile production by ischemic livers was essentially nil. Low dose SRI 63-441 (10 mg/kg) did not result in an increase in bile production. SRI 63-441 (20 mg/kg), pretreated livers produced significantly more bile ($p < 0.005$).

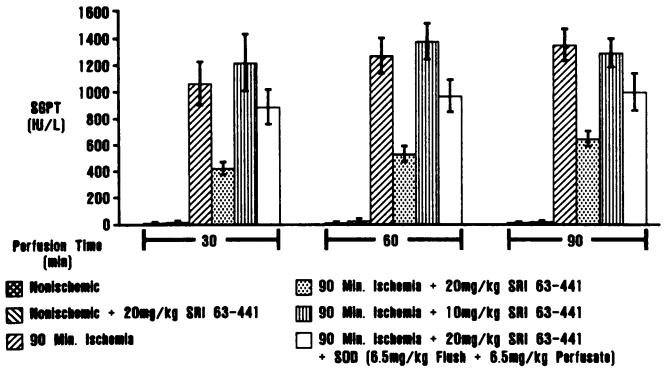


FIG. 2. Perfusate SGPT levels (SRI 63-441). A significant reduction in SGPT liberation was exhibited only by those ischemic livers harvested from donors pretreated with SRI 63-441 (20 mg/kg) ($p < 0.001$). An almost identical pattern was seen with SGOT release (not shown).

Combined Treatment with SRI 63-441 and SOD

Bile production was significantly increased by those livers from the group that underwent a combination of pretreatment with 20 mg/kg of SRI 63-441 administered intravenously and received 6.5 mg/kg SOD in the flush at harvest and 6.5 mg/kg SOD in the perfusate ($p < 0.001$) when compared with ischemic controls (Fig. 1). ATP content was significantly higher ($p < 0.05$) in those livers treated with SOD plus SRI 63-441 than that of ischemic controls (Fig. 3). However, there was no significant difference in the amount of transaminase release between livers treated with a combination of SOD and SRI 63-441 and ischemic controls (Fig. 2).

Treatment with Ibuprofen

The ischemic livers that were pretreated with 12 mg/kg ibuprofen, flushed with 1.8 mg ibuprofen, and received 12 mg/kg ibuprofen in the perfusate did not exhibit a significant difference in bile production or transaminase release when compared with ischemic controls (Figs. 4 and 5). There was also no significant difference in bile production after 90 minutes of perfusion, or in transaminase release for ibuprofen-treated nonischemic livers and nonischemic controls (Figs. 4 and 5).

There was no significant difference in perfusate glucose levels between ischemic controls and any of the ischemic experimentally treated groups, or between nonischemic controls and any of the nonischemic treated groups. Therefore, glucose release does not appear to be a valid predictor of liver function in this model.

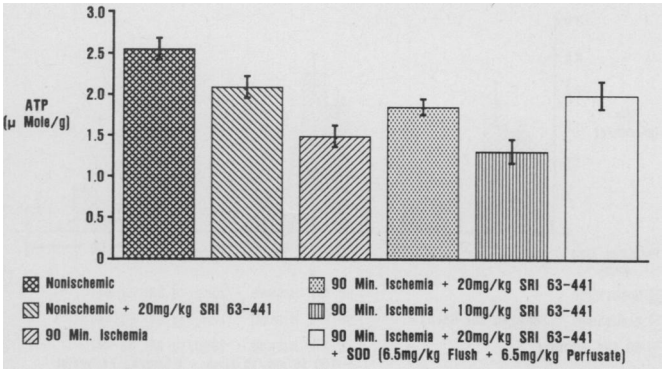


FIG. 3. Tissue ATP content. Ischemic livers manifested a significant reduction in ATP content when compared with nonischemic livers ($p < 0.001$). The liver tissue ATP content was significantly higher in the group treated with SRI 63-441 (20 mg/kg) and the combined SOD/SRI 63-441 treatment group ($p < 0.05$).

Electron Microscopy

All samples for electron microscopy were taken after the 90-minute perfusion period. Electron microscopy was done for Groups 1, 2, and 4 only. Electron microscopy of the nonischemic control livers (Group 1) revealed intact, healthy-appearing hepatocytes as well as intact, well-preserved sinusoidal endothelial cells (Fig. 6A). The ischemic livers (Group 2) exhibited a patchy picture of hepatocellular damage (Fig. 6B). Vacuolated and pale-staining nonviable hepatocytes containing many disrupted mitochondria were found interspersed with viable hepatocytes. The sinusoidal endothelium remained intact when covering viable hepatocytes, but was disrupted in areas adjacent to nonviable hepatocytes (Fig. 7). Sections from ischemic livers that were harvested from donors pretreated with SRI 63-441 (20 mg/kg) (Group 4) demonstrated relatively normal-appearing hepatocytes with few vacuoles and intact sinusoidal endothelium (Figure 6C). The patchy distribution of injury combined with the small cross-sectional area observed with electron microscopy introduces the possibility that the sections viewed were not representative of the true morphologic picture. In fact, sections of the ischemic nontreated livers could be found that manifested relatively healthy-appearing hepatocytes. In order to obtain a better overview, paraffin embedded tissue was cut and stained with hematoxylin and eosin and PAS for light microscopy.

Light Microscopy

The nonischemic control livers exhibited normal, healthy-appearing hepatocytes. PAS-stained sections were glycogen-laden (Fig. 8A). The ischemic livers contained large patches of pale-staining, nonviable hepatocytes and vacuolization interspersed with normal appearing hepatocytes. PAS-staining revealed large patchy areas of glycogen depletion (Fig. 8B). Ischemic livers from donors pretreated with SRI 163-441 (20 mg/kg) also contained some foci of pale-staining, nonviable hepatocytes and vacuolization. Although quantitative analysis was not carried out, the foci of injury in the SRI 63-441 pretreated

TABLE 3. *The Effect of SOD on Parameters of Hepatic Function*

Group*	No. of Patients	Min. Perfusion	Bile (μl/min/g)	SGOT	SGPT
2	9	30	0.003 ± 0.003	946 ± 107	1066 ± 162
		60	0.09 ± 0.03	1108 ± 143	1269 ± 132
		90	0.15 ± 0.04	1237 ± 142	1349 ± 118
6	4	30	0.14 ± 0.11	366 ± 103†	383 ± 103†
		60	0.35 ± 0.14†	455 ± 122†	448 ± 82†
		90	0.37 ± 0.11†	603 ± 163†	625 ± 187†
7	4	30	0.23 ± 0.12†‡	720 ± 186	668 ± 203
		70	0.19 ± 0.03	901 ± 185	844 ± 202
		90	0.17 ± 0.03	1095 ± 196	1023 ± 175
8	4	30	0.23 ± 0.09†‡	478 ± 129†	377 ± 82†
		60	0.17 ± 0.06	559 ± 130†	459 ± 113†
		90	0.12 ± 0.05	740 ± 170†	646 ± 134†

* See Table 1 for descriptions of groups. All values expressed X ± SEM.

† p of at least <0.05 when compared with corresponding values for untreated ischemic controls (Group 2). There is no significant difference between Groups 6, 7, and 8.

‡ Although there is a significant increase at 30 minutes of perfusion, this is not considered biologically significant because the first 30 minutes of perfusion are considered an equilibration period.[42]

ischemic livers were generally smaller in size and less abundant than in the untreated ischemic livers. The pretreated ischemic livers were largely glycogen depleted, in fact, to a greater extent than even the untreated ischemic livers (Fig. 8C). Nonischemic livers from rats pretreated with 20 mg/kg SRI 63-441 had normal-appearing hepatocytes, but also demonstrated patchy glycogen depletion (not shown).

Discussion

Ischemia/reperfusion injury appears to be integrally tied to the inflammatory response, resulting in microcirculatory failure followed by necrosis and cell death. The pathways implicated as causative of the injury to the microcirculation that occurs in this process are multiple and complex. These highly interrelated and redundant cascades involved in the mediation of ischemia/reperfusion injury make it highly likely that effective pharmacologic modulation of the injury, leading to prevention of both warm and cold ischemic injury and ultimately to prolongation of organ preservation, will depend on a polypharmaceutic approach.

Attempts to prevent or diminish ischemic injury associated with organ procurement and preservation have already involved the use of several pharmacologic agents. Prostacyclin has been used to enhance survival of canine livers preserved by hypothermic storage, and it has been shown to improve the survival of warm ischemically damaged liver allografts.[28,29] Chlorpromazine has been found to prevent much of the calcium-mediated injury seen during reperfusion.[30,31]

There has been much interest recently in the role of oxygen-free radicals in ischemia and reperfusion injury.[10-14] Catalase, SOD, and allopurinol have been shown to protect against reperfusion injury mediated by oxygen-free radicals.[10-14] Other antioxidants and calcium channel blockers have also been used in an attempt to ameliorate ischemia/reperfusion injury.[32-34] Lidoflazine, a calcium channel blocker, has been shown to protect against re-

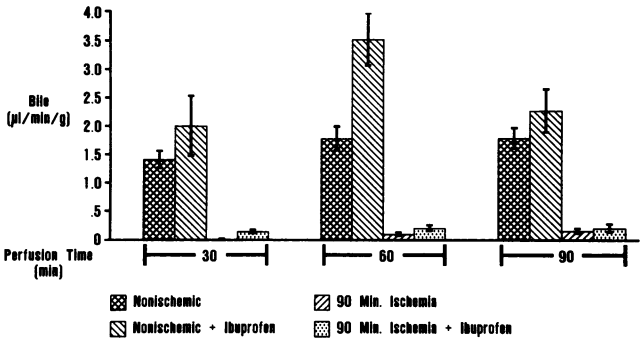


FIG. 4. Bile production (ibuprofen). Bile production by ibuprofen-treated nonischemic livers was no less than that of controls. Ischemic livers produced minimal amounts of bile regardless of ibuprofen treatment.

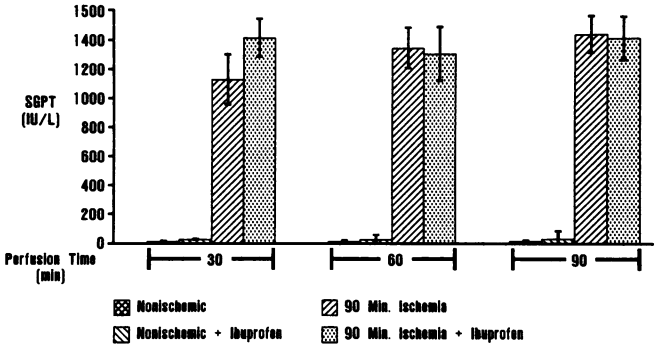
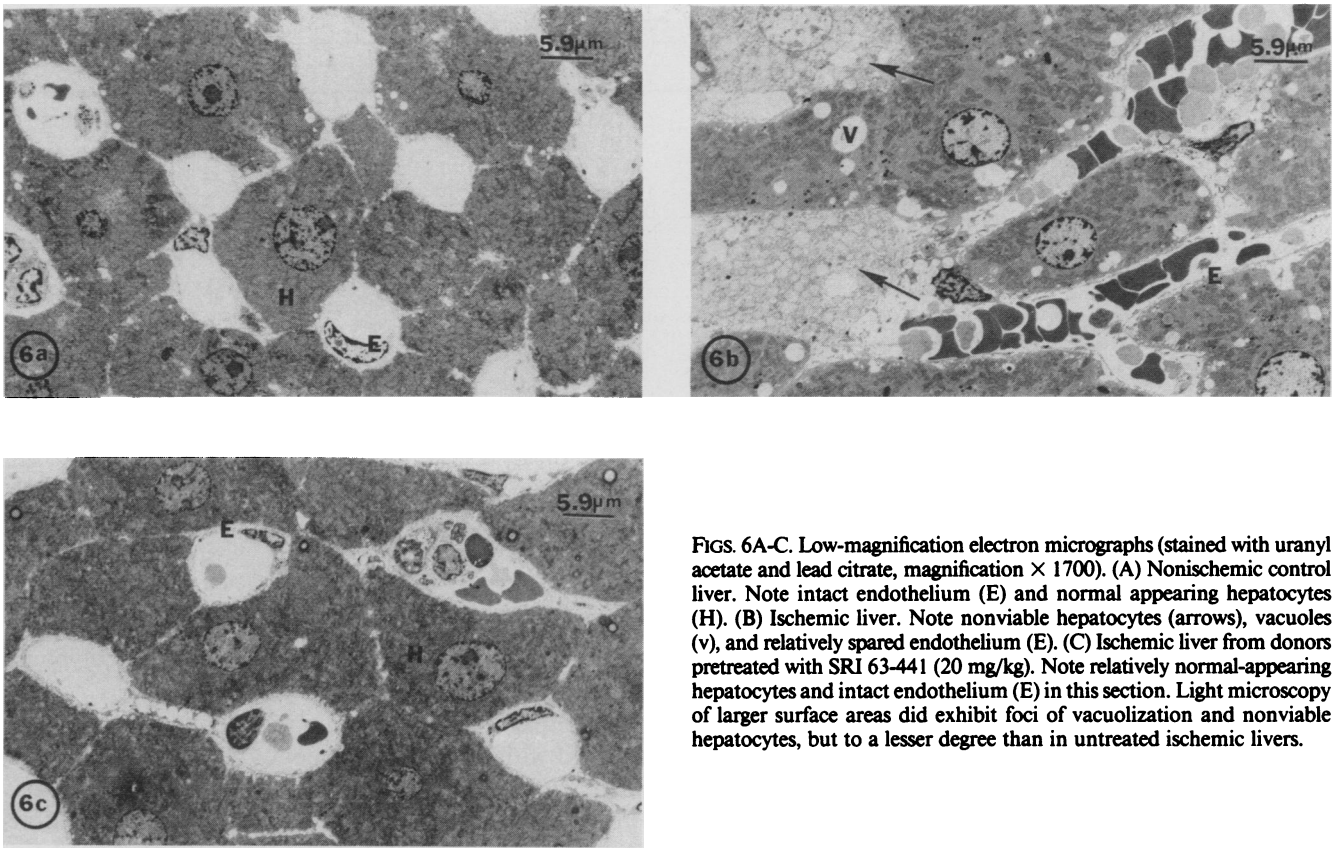


FIG. 5. Perfusate SGPT Levels (ibuprofen). The addition of Ibuprofen did not significantly alter SGPT liberation by ischemic and nonischemic livers. SGOT release followed a similar pattern (not shown).



FIGS. 6A-C. Low-magnification electron micrographs (stained with uranyl acetate and lead citrate, magnification × 1700). (A) Nonischemic control liver. Note intact endothelium (E) and normal appearing hepatocytes (H). (B) Ischemic liver. Note nonviable hepatocytes (arrows), vacuoles (v), and relatively spared endothelium (E). (C) Ischemic liver from donors pretreated with SRI 63-441 (20 mg/kg). Note relatively normal-appearing hepatocytes and intact endothelium (E) in this section. Light microscopy of larger surface areas did exhibit foci of vacuolization and nonviable hepatocytes, but to a lesser degree than in untreated ischemic livers.

perfusion injury during small bowel transplantation.[32] Alpha-tocopherol has been demonstrated to prevent oxygen-free radical damage in ischemic rat liver.[33] Marubayashi et al.[34] have also used co-enzyme ubiquinone (Q10) to prevent ischemic hepatocyte damage, presumably through the protection of membranes from lipid peroxidation, with preservation of calcium homeostasis and restoration of mitochondrial function.

Platelet activating factor (PAF) has been proposed as a potential key inflammatory mediator of the microcirculatory failure that results after ischemic organ injury. PAF is released from several cells, including endothelial cells, platelets and neutrophils, and plays a significant role in almost every aspect of the basic inflammatory response.[8] In addition to its effects on platelets, PAF induces neutrophil aggregation, activation, superoxide anion release, and acts directly on vascular endothelium to increase permeability. It also has smooth muscle contracting properties.[4] SRI 63-441 is one of the most potent PAF receptor antagonists developed to date.[9]

A dose-dependent protective effect against warm ischemic/reperfusion injury was clearly demonstrated after donor pretreatment with SRI 63-441. Bile production, transaminase release, and ATP content all exhibited significant improvement when compared with untreated ischemic controls in those livers from rats pretreated with 20 mg/kg of SRI 63-441 administered intravenously. When a lower dose of 10 mg/kg of SRI 63-441 was administered, no significant improvement in liver function was demonstrated for any parameter measured when compared with ischemic controls. These results substantiate the important role of PAF in ischemic organ injury, and moreover, introduces a potential therapeutic approach to organ ischemia and preservation through the inhibition of PAF by specific receptor antagonism. It is clear from the results reported here that prevention of warm ischemic injury to the liver and improved hepatic function could be achieved by the administration of SRI 63-441 as a pretreatment to liver donors. Because rat platelets are thought to be unresponsive to PAF,[9] PAF-mediated ischemic liver injury may be neutrophil-dependent or, alternatively, may be due to some other PAF-mediated pathway. The improved postischemic hepatic function demonstrated with SRI-63-441 pretreatment has encouraging implications for liver preservation, especially because the same protective effect has been demonstrated for cold ischemic injury.[35]

SOD has been shown to have a protective effect on oxygen-free radical induced damage in kidneys subjected to warm ischemia and reperfusion.[13] During the period of ischemia, the foundation is laid for the reperfusion component of the injury. ATP is utilized and degraded

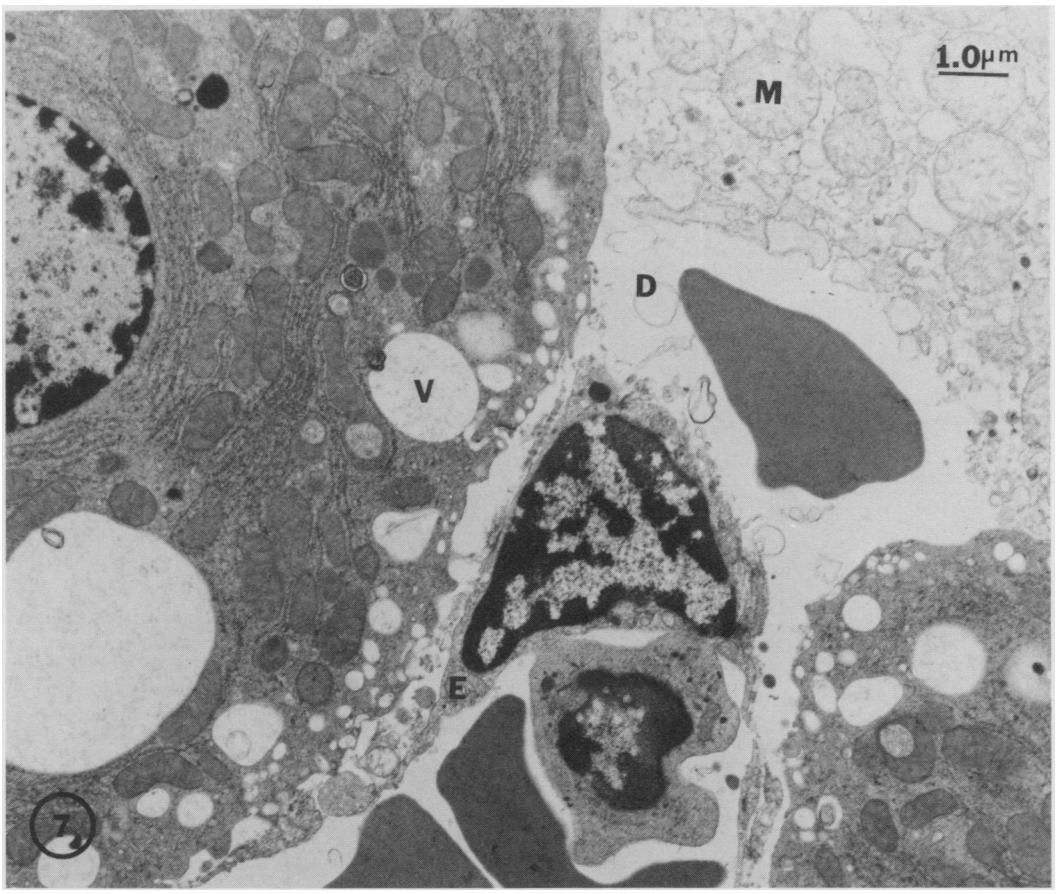
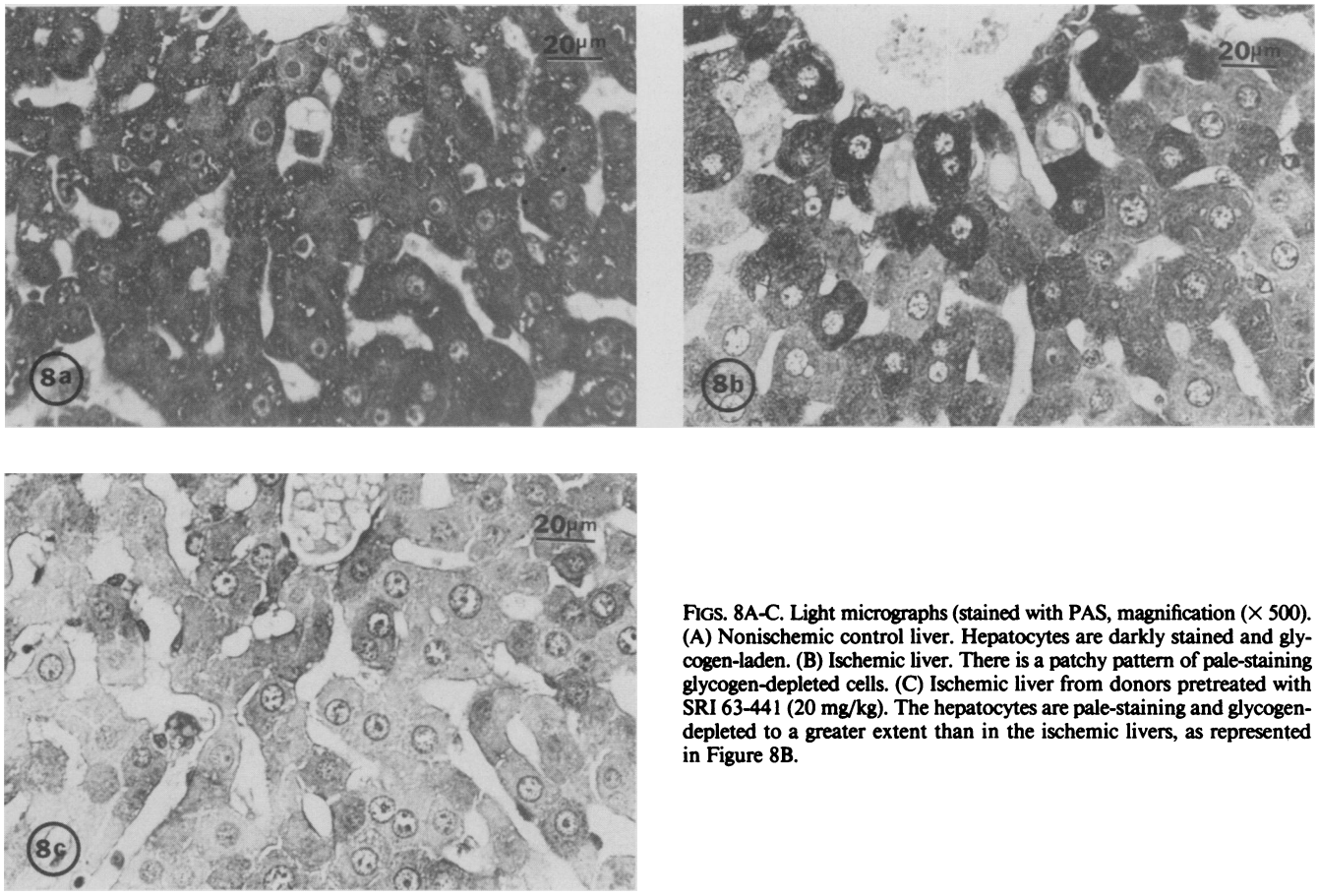


FIG. 7. Higher magnification electron micrograph of ischemic liver (stained with uranyl acetate lead citrate, magnification × 9700). A nonviable hepatocyte with disrupted mitochondria (M) and destruction of space of Disse (D) is seen adjacent to vacuolated (C) but viable hepatocytes. Note that the endothelial cell (E) remains in close contact with the damaged but viable hepatocyte, but has pulled away from the nonviable hepatocyte.

to hypoxanthine, and xanthine dehydrogenase is converted to xanthine oxidase. At the time of reperfusion, oxygen-free radicals are generated.[36] The presence of SOD at the time of reperfusion can reduce the levels of superoxide anions, and hence, can reduce (at least theoretically) oxygen-free radical-mediated injury. The treatment groups in which SOD was added to the perfusate and was present at the time of reperfusion (Groups 6 and 8) exhibited significant improvement in postischemic hepatic function. This was manifested as increased bile production (Group 6) and decreased transaminase liberation into the perfusate (Groups 6 and 8). The group in which SOD was not added to the perfusate during the reperfusion period (Group 8) did not show any significant improvement in hepatic function over ischemic controls, despite donor pretreatment with SOD and the presence of SOD in the flushing solution at the time of harvest (Table 3). The critical moment of action for SOD is at the instant of reperfusion. Therefore, it is necessary that SOD be present in the perfusate at the time of reperfusion, as in Groups 6 and 8. The livers in experimental Group 8 did exhibit decreased transaminase release, although bile production was not significantly improved when compared with nontreated ischemic controls. This lack of improved bile production could possibly be a manifestation of SOD toxicity as the result of an increased total dosage of SOD resulting from the addition of SOD pretreatment to the protocol.

Based on the improved postischemic function attained with SRI 63-441 (20 mg/kg) pretreatment and with the addition of SOD to the perfusate, a combined treatment protocol was evaluated for any synergistic effect. It was anticipated that the combination of two agents that acted via different mechanistic pathways might further improve postischemic hepatic function. A lesser dose of SOD was added in the combination treatment study because initial experiments at the higher dosage did not show an increase in bile production and also because there was some concern of toxicity. The addition of SOD to SRI 63-441 pretreatment did not result in an augmentation of the improvement already observed with SRI 63-441 pretreatment alone. In fact, the results of the combined treatment group (Group 9) are somewhat equivocal. Although there was a significant increase in bile production and ATP content in the combined treatment group as compared to ischemic controls, there was no significant difference in transaminase release between the two groups. A polypharmaceutic approach may indeed be required to achieve maximal pharmacologic modulation of ischemic/reperfusion injury, because of the multiplicity and complexity of the cascades involved, although such an ap-



FIGS. 8A-C. Light micrographs (stained with PAS, magnification (× 500). (A) Nonischemic control liver. Hepatocytes are darkly stained and glycogen-laden. (B) Ischemic liver. There is a patchy pattern of pale-staining glycogen-depleted cells. (C) Ischemic liver from donors pretreated with SRI 63-441 (20 mg/kg). The hepatocytes are pale-staining and glycogen-depleted to a greater extent than in the ischemic livers, as represented in Figure 8B.

proach may, in fact, "backfire" on occasion because of this very same complexity and incompletely understood interaction of these multiple pathways. The ultimate "cocktail" would contain several agents that act at different critical points along the multiple pathways. Therefore it should also not be unexpected that, as with the combined SRI 63-441 SOD treatment group, agents anticipated to behave synergistically could upset the fine balance in the cell's homeostatic mechanisms and invoke a detrimental response.

The cyclooxygenase inhibitor, ibuprofen, has been implicated as a beneficial agent in reducing ischemia/reperfusion injury to the brain, myocardium, and bowel.[15-18] The ischemic livers treated with ibuprofen (Group 10) did not exhibit any significant improvement in postischemic hepatic function by any of the parameters measured in this study. Cyclooxygenase is involved in the production of certain prostaglandins that would be beneficial in reducing ischemia/reperfusion injury, as well as in the production of thromboxanes and other prostaglandins that would be detrimental in ischemia/reperfusion injury.[37,38] An overall inhibition of the activity of cyclooxygenase may, therefore, be too overwhelming a block in the cascade and cancel out the beneficial effect of reducing thromboxane production. It may be that the addition of prostacyclin to ibuprofen treatment may act synergistically and result in improved postischemic hepatic function.

Because the group of animals pretreated with SRI 63-41 (20 mg/kg) exhibited the most marked improvement in postischemic hepatic function, electron microscopy was performed on these livers in order to ascertain any histologic improvement in organ status. Ischemic pretreated livers (Group 4) were compared with ischemic (Group 2) and nonischemic (Group 1) controls, which were processed in an identical fashion.

The morphologic appearance of the nonischemic control livers was that of normal liver without any evidence of hepatocellular or endothelial injury, indicating that changes in the experimental groups are not artifactual. The general pattern of injury observed in the ischemic livers and the ischemic livers harvested from donors pretreated with SRI 63-441 (20m g/kg) was almost identical in nature and consisted of patchy hepatocellular vacuolization and nonviable hepatocytes interspersed with viable cells. However, the degree of injury appeared less severe in the group pretreated with SRI 63-441 (Group 4); the foci of damage were smaller and less abundant than in the untreated ischemic group (Group 2). The vacuolization observed is characteristic of hypoxic injury.[39,40]

The sinusoidal endothelium was relatively spared even

in the most severely damaged warm ischemic livers. This is of singular importance because the primary abnormality seen in livers after cold ischemic or preservation injury is endothelial disruption.[1,35] Sinusoidal endothelial injury is apparent in livers injured by cold ischemia while still manifesting normal hepatocellular architecture. Hepatocellular injury manifested by vacuolization does not occur until prolongation of cold ischemic injury well past the point where endothelial injury becomes apparent.[35] This difference in morphology after warm and cold ischemia has only recently been appreciated and lends further credence to the belief that warm and cold ischemia represent two different and distinct types of injury.[1] Moreover, it should become possible to use these different and specific pathologic changes as predictors of organ function.

The glycogen depletion observed in those livers pretreated with SRI 63-441 (Groups 3 and 4) was at first puzzling because it would be expected that the more "healthy," better-functioning livers would retain their glycogen. However, in addition to its role in the inflammatory response, PAF has a potent agonist effect on the glycogenolytic system in the rat liver.[41] It is possible that SRI 63-441, the structurally similar PAF receptor antagonist, may have the same action as PAF itself on the glycogenolytic system activate glycogenolysis.

In summary, the platelet activating factor antagonist, SRI 63-441, significantly reduced hepatic warm ischemic injury in a dose-dependent fashion, thereby substantiating the role of PAF in ischemic organ injury and introducing a novel therapeutic approach. The oxygen-free radical scavenger, SOD, exerted a protective effect on postischemic hepatic function only if present at the exact time of reperfusion (and of generation of free radicals). The cyclooxygenase inhibitor, ibuprofen, was ineffective in protecting the liver from warm ischemic injury under the conditions represented by these experiments. The morphologic findings in this study reveal a pattern of hepatic injury involving primarily parenchymal cells after warm ischemia that differs significantly from the primarily endothelial injury seen subsequent to cold ischemia.[1,35] Liver allografts undergo periods of both warm and cold ischemia during the transplantation process. Therefore, pharmacologic amelioration of ischemic injury with prolongation of organ preservation will involve the administration of agents, singly or in well-defined combinations, which will not only protect against parenchymal cellular injury and disruption, but will also maintain the endothelial integrity of the organ.

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References

1. McKeown CMB, Edwards V, Phillips MJ, et al. The critical injury in cold preservation of liver allografts in the rat is sinusoidal lining cell damage. Transplantation (in press).
2. Miller LL, Bly CG, Watson ML, et al. The dominant role of the liver in plasma protein synthesis. J Exp Med 1951; 94:431–453.
3. Snyder F. Chemical and biochemical aspects of platelet activating factor: a novel class of acetylated ether-linked choline-phospholipids. Med Res Rev 1985; 5:107–140.
4. Camussi G, Brentjens JR. The role of platelet-activating factor in inflammation. *In* F. Snyder, ed. Platelet Activating Factor and Related Lipid Mediators. New York: Plenum Press, 1987; 299–322.
5. Varfaftig BB, Chignand M, Benveniste J, et al. Background and present status of research on platelet-activating factor (PAF-acether). Ann NY Acad Sci 1981; 370:119–137.
6. Vargaftig BB, Chignand M, Lefort J, Venveniste J. Platelet-tissue interaction: role of platelet-activating factor (PAF-acether). Cellular Secretion and Tissue Breakdown 1980; 10:502–506.
7. Bussolino F, Biffignandi P, Arese P. Platelet activating factor: a powerful lipid autacoid possibly involved in microangiopathy. Acta Haematol 1986; 75:129–140.
8. Pinckard RN. Platelet-activating factor. Hosp Pract 1983; 18:67–76.
9. Saunders RN, Handley DA. Platelet-activating factor antagonists. Ann Rev Pharmacol Toxicol 1987; 27:237–255.
10. Das DK, Engleman RM, Dobbs WA, et al. The role of oxygen-derived free radicals in pathogenesis of reperfusion injury. Ann NY Acad Sci 1984; 463:274–277.
11. Arthur MJP, Bently IS, Tanner AR, et al. Oxygen derived free radicals promote hepatic injury in the rat. Gastroenterology 1985; 89: 1114–1122.
12. Atalla SL, Toledo-Pereyra LH, Mackenzie GH, Cederna JP. Influence of oxygen derived free radical scavengers on ischemic livers. Transplantation 1985; 40:584–589.
13. Baker GL, Corry RJ, Autor AP. Oxygen free radical induced damage in kidneys subjected to warm ischemia and reperfusion: protective effect of superoxide dismutase. Ann Surg 1985; 202:628–641.
14. Koyama I, Bulkley GP, Williams GM, Im MJ. The role of oxygen free radicals in mediating the reperfusion injury of cold-preserved ischemic kidneys. Transplantation 1985; 40:590–595.
15. Jugdutt BI, Hutchins GM, Bulkley BH, Becker LC. Salvage of ischemic myocardium by ibuprofen during infarction in the conscious dog. Am J Cardiol 1980; 46:74–82.
16. Lefer AM, Polansky EW. Beneficial effects of ibuprofen in acute myocardial ischemia. Cardiology 1979; 64:265–279.
17. Grosfeld JL, Kamman K, Gross K, et al. Comparative effects of indomethacin, prostaglandin E and ibuprofen on bowel ischemia. J Pediatric Surg 1983; 18:738–742.
18. Kuhn JE, Steimle CN, Zellnock GB, D'Alecy LG. Ibuprofen improves survival and neurologic outcome after resuscitation from cardiac arrest. Resuscitation 1986; 14:199–212.
19. Hamilton RL, Berry MN, Williams MC. A simple and inexpensive membrane lung for small organ perfusion. J Lipid Res 1974; 15: 182–186.
20. Ontell SJ, Colella MS, Horowitz J, et al. Applications of the isolated perfused rat liver in transplantation research. J Invest Surgery 1988; 1:25–27.
21. Levine RA, Pesh LA, Klatskin G, Giarman NJ. Effect of serotonin on glycogen metabolism in isolated rat liver. J Clin Invest 1964; 43:797–809.
22. Krebs HA, Henseleit K. Untersuchungen uber die hrarnstoffbildung in tierkorper. Hoppe-seyler's Z Physiol Chem 1932; 210:33–66.
23. Riedel GL, Scholle JL, Shepherd AP, Ward WF. Effects of hematocrit on oxygenation of the isolated perfused rat liver. Am Physiol Soc 1983; 245:G769–G774.
24. Lamprecht W, Trautschold I. Adenosine-5-triphosphate determination with hexokinase and glucose-6-phosphate dehydrogenase. *In* HU Bergmeyer, ed. Methods of Enzymatic Analysis, 2nd ed. New York: Academic Press, Inc., 1974; 2101–2110.
25. Fahimi HD. Perfusion and immersion fixation of rat liver with glutaraldehyde. Lab Invest 1967; 16:736–751.

26. Reynolds ES. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J Cell Biol 1963; 17:208–212.
27. Sokal RR, Rohlf FJ. Biometry: The Principles and Practice of Statistics in Biological Research, 2nd ed. San Francisco: W.H. Freeman, 1981.
28. Monden M, Fortner JG. 24- and 48-hour canine liver preservation by simple hypothermia with prostacyclin. Ann Surg 1982; 196: 38–42.
29. Toledo-Pereyra LH. Role of prostaglandins (PGI_2) in improving the survival of ischemically damaged liver allografts. Trans Am Soc Artif Intern Organs 1984; 30:390–394.
30. Farber JL, Chien KR, Mittnacht SM. The pathogenesis of irreversible cell injury in ischemia. Am J Path 1981; 102:271–281.
31. Chien KR, Abrams J, Pfau RG, et al. Prevention by chlorpromazine of ischemic liver cell death. Am J Path 1977; 88:539–558.
32. Croitoru DP, Sybicki M, Grand RJ, Cho SI. Lidoflazine protects against reperfusion injury during small bowel transplantation, abstracted. 20th Annual Meeting of the Association for Academic Surgery, November 1986; 125.
33. Marubayashi S, Dohi K, Ochi K, Kawasaki T. Role of free radicals in ischemic rat liver cell injury: prevention of damage by alpha-tocopherol administration. Surgery 1986; 99:184–192.
34. Marubayashi S, Dohi K, Yamada K, Kawasaki T. Changes in the levels of endogenous coenzyme Q homologs, alphatocopherol, and glutathione in rat liver after hepatic ischemia and reperfusion, and the effect of pretreatment with coenzyme Q10. Biochim Biophys Acta 1984; 797:1–9.
35. Ontell SJ, Makowka L, Ove P, Starzl TE. Improved hepatic function in the 24 hour preserved rat liver with UW-lactobionate solution and SRI-63–441. Gastroenterology 1988; 1:25–27.
36. McCord JM. Oxygen-derived free radicals in post-ischemic tissue injury. N Engl J Med 1985; 312:159–163.
37. Parks WM, Hoak JC, Czervionke RL. Comparative effect of ibuprofen on endothelial and platelet prostaglandin synthesis. J Pharmacol and Experimental Therapeutics 1981; 219:415–419.
38. Lelcuk S, Alexander F, Kobzik L, et al. Prostacyclin and thromboxane A2 moderate postischemic renal failure. Surgery 1985; 98:207–212.
39. Ashford TP, Burdette WJ. Response of the isolated perfused hepatic parenchyma to hypoxia. Ann Surg 1965; 162:191–207.
40. Nakata K, Fukumoto O, Fujimoto K, Fujikawa Y. Development of hypoxic change of the liver cells as revealed by the isolated perfused rat liver. Acta Pathol Jap 1971; 21:313–328.
41. Buxton DB, Shukla SD, Hanahan DJ, Olson MS. Stimulation of hepatic glycogenolysis by acetylglyceryl ether phosphorylcholine. J Biol Chem 1984; 259:1468–1471.
42. Ritchie HD and Hardcastle RJ. Isolated organ perfusion. University Park Press, 1973.