The Role of Calcium Ions and Calcium Channel Entry Blockers in Experimental Ischemia–Reperfusion-induced Liver Injury

RUSSELL J. NAUTA, M.D., EVANGELOS TSIMOYIANNIS, M.D., MARIO URIBE, M.D., DANIEL B. WALSH, M.D., DEBORAH MILLER, M.D., and ARTHUR BUTTERFIELD, D.V.M., PH.D.

Verapamil administered before treatment, but not after treatment, had a beneficial effect on a 90-minute warm ischemia–reperfusion rat liver injury model. The possible activation of proteases converting the xanthine dehydrogenase to xanthine oxidase, the significant mitochondrial calcium loading during the ischemic period, and the potentiation of calcium and oxygen-derived free radicals to promote injury to mitochondria are mechanisms supported by this study, based on both histologic observations and on the pattern of enzyme leak after the acute ischemic event.

From the Division of General Surgery, Georgetown University Hospital, Washington, D.C.

LIVER ISCHEMIA MAY occur in patients operated on for trauma,[1] cancer,[2] or hepatic transplantation.[3] Hepatic failure unassociated with surgery, but following a period of hemodynamic or cardiogenic shock, is a well-recognized phenomenon.[4,5]

Although such conditions may result in hypoperfusion of liver cells and eventual hepatocyte dysfunction, the mechanisms by which postischemic liver damage occurs are not clear. In the past two decades, many potentially damaging factors, such as adenosine triphosphate (ATP) depletion, activation of autolytic systems, cellular acidosis, superoxide-induced membrane change, and mitochondrial dysfunction have been implicated as playing roles in ischemic injury.[6] Steroids,[7] dopamine,[8] ATP,[9] alpha tocopherol,[10] activated carbon hemoperfusion,[11] allopurinol,[12–14] superoxide dismutase, and catalase[13–15] have been implicated as theoretically protective agents addressing these factors.

Recent interest has been displayed in the calcium-blocking agent verapamil for protection of hearts subjected to global ischemia, and for protecting against postischemic acute tubular necrosis in acute renal failure models.

Previous work in our laboratory defined a pattern of hepatic enzyme leak that is substantially and predictably elevated over sham-operated controls[13] and showed that this enzyme leak corresponds to a pattern of histologic damage most evident at day 2 and resolved by 3 weeks after operation.[14]

Address reprint requests to Russell J. Nauta, M.D., Chief, Division of General Surgery, Georgetown University Hospital, 3800 Reservoir Rd. NW, 4th Floor PHC Bldg., Washington, D.C. 20007.

Accepted for publication January 3, 1990.

The present experiments were designed to study the role of administered verapamil on the histologic and biochemical response to an *in vitro* timed liver ischemic episode of 90 minutes, and, by inference, the role of ionized calcium and calcium channel blockade in the mediation of ischemia–reperfusion liver injury.

Material and Methods

Sprague-Dawley rats weighing 280 to 330 g were fasted for 12 hours before experiments but were allowed water *ad libitum*. The animals were anesthetized with ketamine (10 mg/kg body weight) plus innovar (0.1 mL/kg body weight), and the internal jugular vein was exposed for drug administration. Midline laparotomy was performed and 100 units of heparin were administered. The liver hilus was exposed, and the portal vein, hepatic artery, and bile duct of the left lateral and median lobes of the liver were occluded by a small vascular clamp distal to the origin of the vessels supplying the omental (caudate) and right lobe, as previously described. The blood supply to the omental and right lobes was uninterrupted and the portal blood flow was maintained through them without evidence of vascular congestion of the alimentary tract. The abdominal incision was temporarily closed by towel clips, and the animals were maintained anesthetized in the Research Resources Facility of Georgetown University. Reperfusion was induced by declamping the vessels after 90 minutes of ischemia, the abdomen was closed in two layers with 3-0 silk sutures, and the animals were allowed to recover.

Three groups of 20 rats each were used in this study. All rats had blood determinations of lactic dehydrogenase (LDH), serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase, sorbitol dehydrogenase, alkaline phosphatase, and total and direct bilirubin before operation and at 2, 7, 14, and 21 days after ischemia. Rats were killed on day 21. Liver biopsies were performed at

the time of initial laparotomy before the induction of ischemia, 2 days after operation, at 1 and 2 weeks, and at the time the animals were killed. All of the biopsies were performed by laparotomy under a ketamine-innovar general anesthesia.

Light microscopic review was performed by the pathologist in a blinded fashion and fatty deposition and centrilobular necrosis were rated as absent, moderate, or severe.

The drug treatment regimens (excluding the heparin, ketamine, and innovar, which all animals received) were as follows: group A, none (control group); group B, verapamil before treatment (0.3 mg/kg body weight intravenously as a bolus *via* the internal jugular vein before the induction of the liver ischemia); and group C, verapamil after treatment (0.3 mg/kg body weight intravenously as a bolus *via* the internal jugular vein beginning 1 minute before the liver hilus declamping and continuing intravenous administration for 2 minutes during early reperfusion).

Results of the enzyme determinants are expressed as the mean of these values plus or minus the standard de-

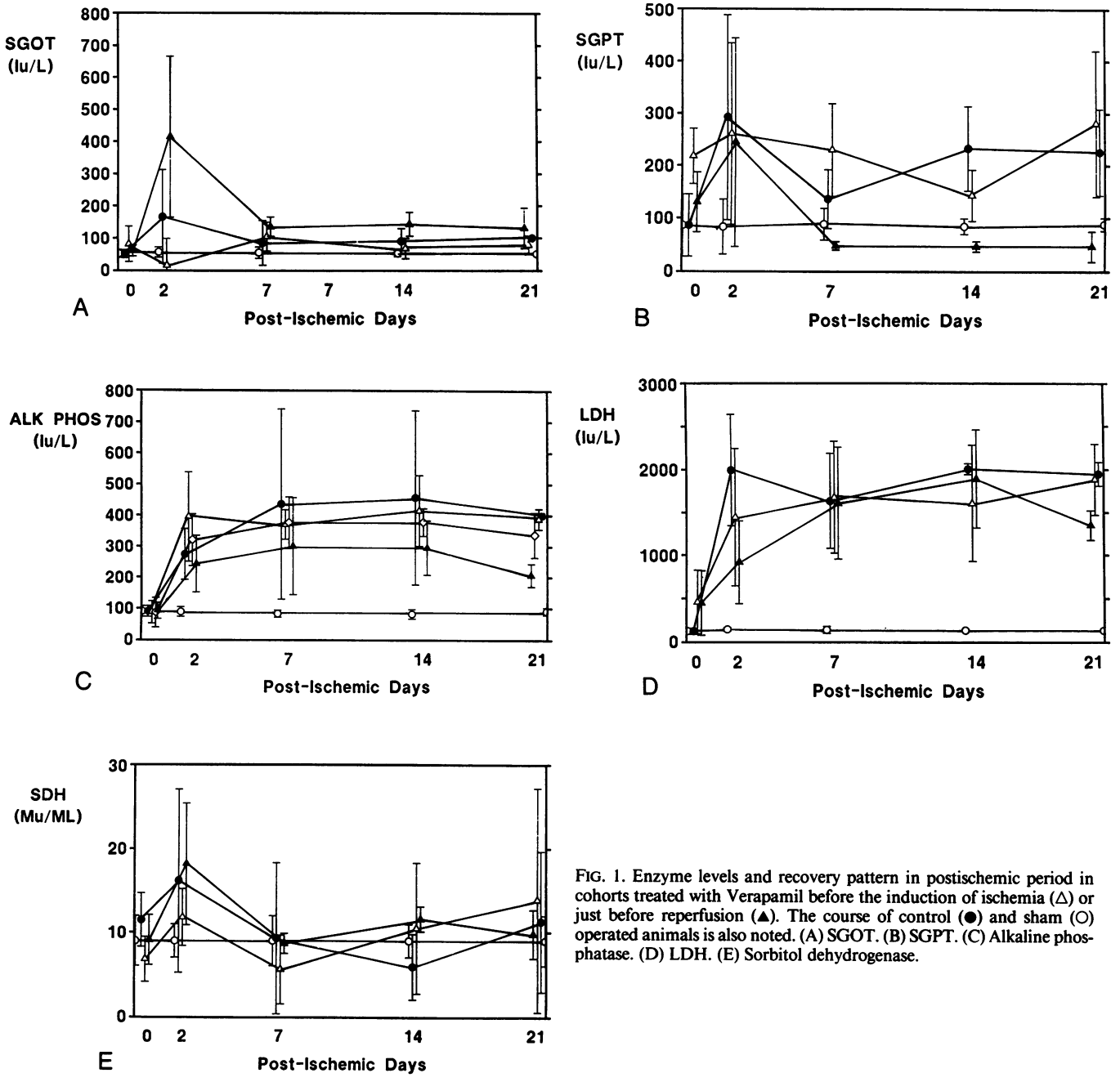


FIG. 1. Enzyme levels and recovery pattern in postischemic period in cohorts treated with Verapamil before the induction of ischemia (△) or just before reperfusion (▲). The course of control (●) and sham (○) operated animals is also noted. (A) SGOT. (B) SGPT. (C) Alkaline phosphatase. (D) LDH. (E) Sorbitol dehydrogenase.

viation. Student's t test and chi square test were used to analyze differences.

Results

During the ischemic period, portal flow was satisfactory through the uninterrupted circulation of the nonischemia liver lobes without evidence of vascular congestion of the alimentary tract.

The mean and the standard deviation of all biochemical assays in each group is presented in Figure 1.

Serum glutamic oxaloacetic transaminase values in group A (control) showed a significant change on days 2, 14, and 21. This was also true in the verapamil post-treatment group, whereas in group B, no significant increase was noted. On the second day, group C had significantly higher levels than the control group ($p < 0.005$) and group B ($p < 0.005$). On the 14th day, group C maintained significantly higher levels than group A ($p < 0.001$). By 21 days after ischemia, no significant differences were observed. Serum glutamic pyruvic transaminase values were increased significantly during the entire postischemic period in the control group. However, in group C, a significantly elevated level was only measured on postoperative day 2 ($p < 0.02$), and no significantly higher ($p < 0.001$ every day) levels in group A were noted each day when compared to group C.

In groups A and C, significantly increased levels of alkaline phosphastase during the entire postischemic period were observed. No significant increase in group B was noted. In group C significantly higher levels were noted than in the control group at days 2 and 21 ($p < 0.001$).

In all groups significantly increased levels of LDH compared to the preischemic baseline were noted. In the control group, higher levels than in group B were noted only on day 14 ($p < 0.005$). In the control group, values were higher than group C only on days 2 ($p < 0.002$) and 21 ($p < 0.001$).

In group A no significant increased levels of sorbitol dehydrogenase were measured. In group B significant increases on days 2 and 21 were observed, whereas in group C significantly increased levels on days 2 and 14 were noted. In group C a significantly higher level than in group A ($p < 0.001$) was observed on day 14.

No significant total and direct bilirubin level differences in either group were noted when compared to baseline levels. When compared, all groups had identical bilirubin levels throughout the experiment period.

The total number of significantly elevated levels of all the biochemical assays during the postischemia–reperfusion period compared to the preischemic baseline levels were tabulated (Table 1). In group B a significantly better result than in group A or in group C was noted ($p < 0.05$).

Histologic Findings

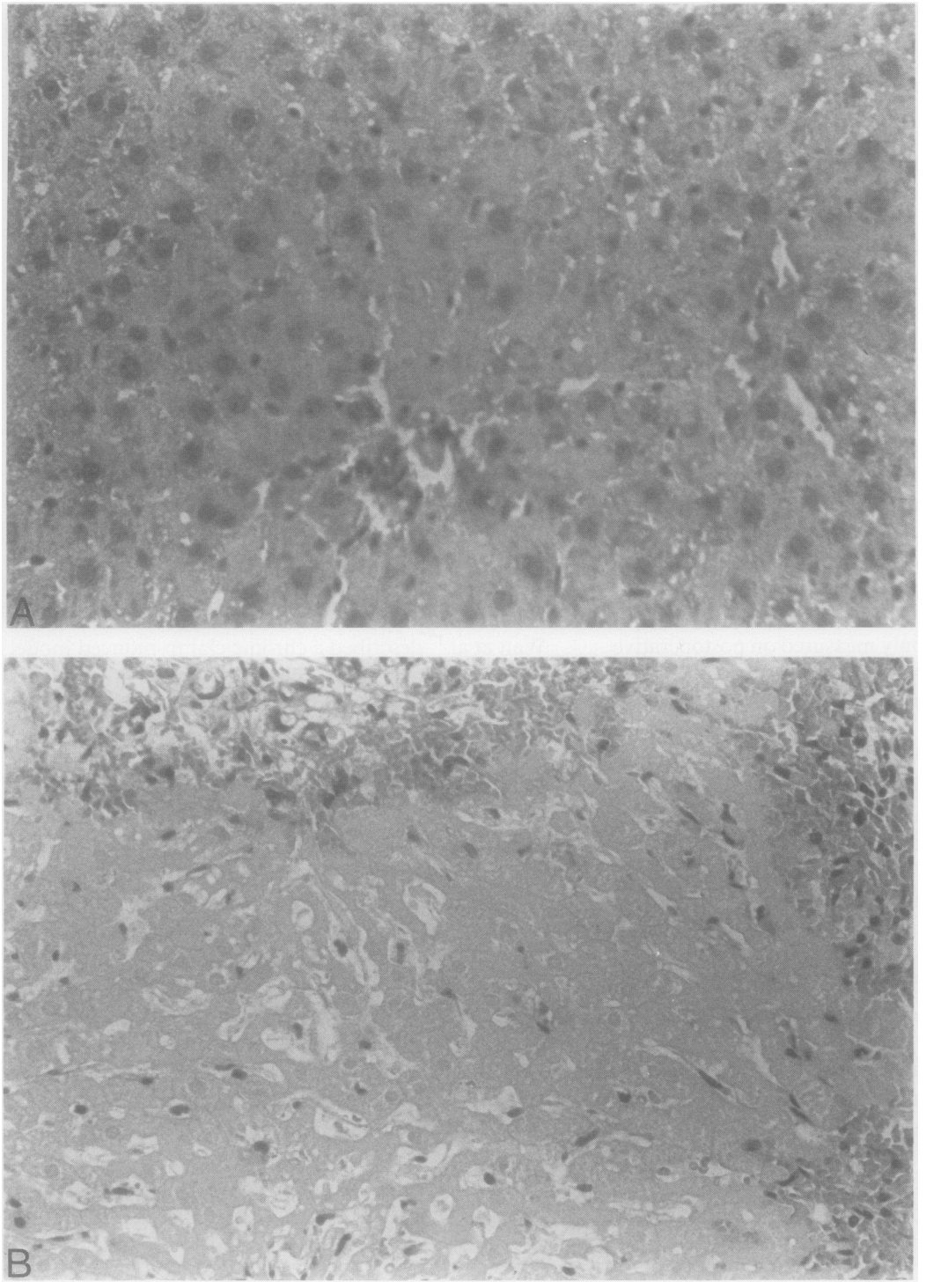
Severe centrilobular liver cell necrosis in groups A and C was observed. In group B the pretreatment use of verapamil had significant protective effect on the ischemia–reperfusion-induced liver injury (Table 1, Fig. 2).

Discussion

Wait et al.[17] described a chronic clamp-induced model of ischemic renal failure, occluding the renal artery of rats for 45 minutes and then performing contralateral nephrectomy. Fifteen-minute infusions of verapamil to 66 mg/kg were noted to offer protection in this model. Previously we demonstrated[13,14] that 90-minute infusion produces a reproducible histologic injury and biochemical leak pattern and we sought, in these experiments, to test whether the same dose of verapamil would be protective against hepatic ischemia–reperfusion injury. Our results show that verapamil, a slow calcium channel entry blocker, administered before an ischemic insult, had a beneficial effect during the 90-minute period of warm liver ischemia in the *in vivo* rat liver followed through the 21-day reperfusion period. When verapamil was given during the end of ischemia and the early reperfusion period, the biochemical and histologic findings were very close to controls, and minimal, if any, protection was afforded.

TABLE 1.

Experimental Group	Extent of Centilobular Necrosis				
	Days Following Ischemic Event				
	0	2	7	14	21
Sham	20 Absent	20 Absent	20 Absent	20 Absent	20 Absent
Group A	20 Absent	12 Severe	12 Severe	17 Moderate	3 Moderate
		8 Moderate	8 Moderate	3 Absent	17 Absent
Group B		8 Moderate	4 Moderate	3 Moderate	2 Moderate
		12 Absent	16 Absent	17 Absent	18 Absent
Group C		13 Severe	13 Severe	16 Moderate	5 Moderate
		7 Moderate	7 Moderate	4 Absent	10 Absent



FIGS. 2A and B. (A) Moderate centrilobular necrosis. Note some preservation of portal architecture. (B) Severe centrilobular necrosis.

This implicates calcium as a primary etiologic agent in ischemic cell injury.

In heart models others have found similarly that to be protective, the drug must be given before the induction of global ischemia.[16] It is believed in this model that calcium channel blockers protect the heart from oxygen and substrate deprivation by decreasing the contractile activity and the energy demand.[19] Although the time of drug administration with respect to the insult appeared less critical in kidney models, verapamil has been reported to protect

kidneys from the acute ischemic renal failure induced by norepinephrine,[20] cold,[21] or warm ischemia.[17,21] In other studies it was demonstrated that verapamil had global beneficial effects when given to dogs during a 2-hour period of hemorrhagic shock.[22] The drug partially or completely prevented the development of ventricular subendocardial necrosis and hemorrhage and of zonal lesions resulting in less intestinal mucosal hemorrhage and effecting a significantly higher survival rate.

In ischemic states elevated levels of calcium in cells or tissues have been observed in *in vitro* models. In rat kidneys that were rendered ischemic for 45 minutes, mitochondria isolated before reperfusion had marked impairment of respiratory function but only slightly increased levels of calcium.[25] During reflow the mitochondrial calcium increased progressively so that a mitochondrial calcium loading occurred despite improvement in respiratory function within 1 to 4 hours of reperfusion.[20,25] Verapamil has been shown to prevent this mitochondrial calcium loading and mitochondrial respiratory dysfunction.[20] In contrast to these studies of warm ischemia and cold ischemia, a significant calcium loading occurs during the ischemic period, and the protective effects of verapamil appear to take place during the ischemia and not during reflow.[21]

In liver ischemia, no direct evidence of the role of slow calcium channel entry blockers has been reported in a chronic model.

Verapamil was protective, if present, during the ischemic period, but not if administered after established ischemia. This suggests that high levels of calcium in cells or tissues might contribute to the deleterious events leading to cellular injury.[19]

Different mechanisms could explain this phenomenon, none of them giving clear evidence of calcium's role in cellular injury. In fact all inferences to date with respect to the role of calcium in liver injury have been indirect. The salutary effect of chlorpromazine,[23] thought to be protective by inhibiting calcium-dependent processes by binding to calmodulin,[24] is an example of such an inference.

One proposed mechanism of the protection afforded by verapamil relates to the conversion of xanthine dehydrogenase to xanthine oxidase, which is the main source of the superoxide radical.[26] The elevated cytosolic calcium concentration during ischemia, it is believed, activates a protease capable of converting the dehydrogenase to the oxidase. The xanthine oxidase can use molecular oxygen during reperfusion, producing superoxide anion and, secondarily, hydrogen peroxide and hydroxyl radical. These oxygen-derived free radicals are responsible for ischemia–reperfusion-induced liver injury.[13,14] The present study is unable to contradict this theory.

The second proposed mechanism, one that relates to cellular calcium loading during reperfusion,[20,25] is not supported by this study because no protective effect of verapamil was noted when the drug was given during the reperfusion period.

The second proposed mechanism, one that relates to cellular calcium loading during the period of cold ischemia, could be supported by our study if it is known that calcium loading during warm ischemia also is present.

Recent evidence[27] indicates that calcium potentiates the damaging effect of oxygen-derived free radicals on the mitochondrial electron transport chain due to impairment of NADH-coenzyme Q-reductase activity. This interesting fourth proposed mechanism is supported by this study.

Acknowledgment

The authors thank Kathleen Blamer for preparation of the manuscript.

References

1. Numes G, Blaisdell F, Margaretten N. Mechanism of hepatic dysfunction following shock and trauma. Arch Surg 1970, 100:546–556.
2. Starzl T, Bell R, Beart R, Putnam R. Hepatic trisegementectomy and other liver resections. Surg Gynecol Obstet 1975, 141:429–437.
3. Toledo-Pereyra L. Improvement of liver transplant survival: new avenues of research. Transplant Proc 1985, 17:298–301.
4. Champion R, Jones R, Trump B, et al. A clinicopathologic study of hepatic dysfunction following shock. Surg Gynecol Obstet 1976, 142:657–663.
5. Bulkley G, Oshima A, Bailey R. Pathophysiology of hepaic ischemia in cardiogenic shock. Am J Surg 1986, 151:87–97.
6. Leaf A, Cheung J, Mills J, Bonventre J. Nature of the cellular insult in acute renal failure. *In* Brenner B, Lazarus J, eds. Acute Renal Failure. Philadelphia: WB Saunders, 1983; pp 2–20.
7. Fornander J, Hellman A, Hasselgren P. Effects of methylprednisone on protein synthesis and blood flow in the post-ischemic liver. Circ Shock 1984, 12:287–295.
8. Hasselgren P, Biber B, Fornander J. Improved blood flow and protein synthesis in the post-ischemic liver following infusion of dopamine. J Surg Res 1983, 34:44–52.
9. Clemens M, McDonagh P, Chaudry I, Bave A. Hepatic microcirculatory failure after ischemia and reperfusion: improvement with ATP-$MgCL_2$ treatment. Am J Physiol 1985, 248:H804–H811.
10. Marubayashi S, Dohi K, Ochi K, Kawasaki T. Role of free radicals in ischemic rat liver cell injury: prevention of damage by a-tocopherol administration. Surgery 1986, 99:184–191.
11. Toledo-Pereyra L. Role of activated carbon hemoperfusion in the recovery of livers exposed to ischemic damage. Arch Surg 1985, 120:462–465.
12. Nordstrom G, Seeman T, Hasselgren P. Beneficial effect of allopurinol in liver ischemia. Surgery 1985, 97:679–683.
13. Nauta R, Uribe M, Walsh D, et al. Histopathologic correlates of murine hepatic ischemic reperfusion injury: evidence from a chronic in vivo model. J Inv Surg 1988, 1:155–162.
14. Nauta R, Uribe M, Tsimoyiannis E, et al. Description of a chronic in-vivo murine model for the study of warm hepatic ischemia/reperfusion injury. Surg Res Comm 1989 (In press).

15. Granger D, Adkinson D, Hollwarth M, et al. Role of oxygen free radicals in ischemia-reperfusion injury to the liver. Gastroenterology 1985, 88:1662(Abstr).
16. Watts J, Koch C, Lanoue K. Effects of Ca^{2+} antagonism on energy metabolism: Ca^{2+} and heart function after ischemia. Am J Physiol 1980; 232:H909–H916.
17. Wait R, White G, Davis T. Beneficial effects of verapamil on post-ischemic renal failure. Surgery 1983, 94:276–282.
18. Baker H de C. Ischemic necrosis in the rat liver. J Pathol Bact 1956, 71:135–143.
19. Cheung T, Bonventre J, Malis C, Leaf A. Calcium and ischemic injury. N Eng J Med 1986, 314:1670–1676.
20. Burke T, Arnold P, Gordon J, et al. Protective effect of intrarenal calcium membrane blockers before or after renal ischemia: functional, morphological, and mitrochondrial studies. J Clin Invest 1984, 74:1830–1841.
21. Shapiro J, Cheung C, Itabashi A, et al. The effect of verapamil on renal function after warm and cold ischemia in the isolated perfused rat kidney. Transplantation 1985, 40:596–600.
22. Hackel D, Mikat E, Whalen G, et al. Treatment of hemorrhagic shock in dogs with verapamil: effects on survival and on cardiovascular lesions. Lab Invest 1979, 41:356–359.
23. Mittnacht S, Farbet J. Reversal of ischemic mitochondrial dysfunction. J Biol Chem 1981, 256:3199–3206.
24. Godfraind T. Calcium entry and calcium entry blockade. *In* Godfraind T, et al., eds. Calcium Entry Blockers and Tissue Protection. New York: Raven Press, 1985; pp 1–19.
25. Wilson D, Arnold P, Burke T, Schrier R. Sequential changes in mitochondrial function in ischemic acute renal failure in the rat. Kidney Int 1983, 23:209(Abstr).
26. McCord J. Oxygen-derived free radicals in post-ischemic tissue injury. N Eng J Med 1985, 312:159–163.
27. Malis C, Bonventre J. Mechanism of Ca and oxygen free radical injury to the mitochondrial electron transport chain. Kidney Inst 1986, 29:305(Abstr).