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PROTECTIVE EFFECTS OF THE CALCIUM ANTAGONISTS DILTIAZEM AND TA3090 AGAINST HEPATIC INJURY DUE TO HYPOXIA

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Abstract—Recent work has shown that dihydropyridine-type calcium channel blockers such as nitrendipine protect against ischemic liver damage in the rat *in vivo* (Thurman RG, Apel E and Lemasters JJ, *J Cardiovasc Pharmacol* **12**: S113–S116, 1988), suggesting that calcium antagonists may have clinical value in preventing ischemic and hypoxic hepatic injury. This study was designed to examine the effects of two benzothiazepine-type calcium channel blockers, diltiazem and TA3090, in the hypoxic perfused rat liver. Livers were isolated and perfused briefly with oxygen-saturated buffer, followed by perfusion for 80 min with nitrogen-saturated buffer with diltiazem or TA3090 (20–200 μM), and concluding with 20 min of perfusion with oxygen-saturated buffer. In control preparations, maximal lactate dehydrogenase (LDH) release into effluent perfusate following hypoxia averaged about 1100 U/L. Diltiazem and TA3090 decreased LDH release at all concentrations studied; both drugs were most effective at the 100 μM concentration (71 and 73% inhibition, respectively). Oxygen uptake by control livers decreased 78% following hypoxia; diltiazem and TA3090 reduced this effect markedly, with maximal effectiveness again observed with 100 μM (O_2 uptake was decreased by 22% with 100 μM diltiazem and by only 9% with 100 μM TA3090). Histological examination for nuclear uptake of the vital dye trypan blue revealed necrosis of parenchymal cells along with cell shrinking and consequent expansion of the sinusoids in control livers. Perfusion with diltiazem markedly reduced parenchymal cell death but did not alter the pattern of cell damage observed. In contrast, livers perfused with TA3090 during hypoxia had virtually no parenchymal cell damage, although moderate damage to nonparenchymal cells in the sinusoids occurred. The difference in mechanisms responsible for the phenomena which occur with diltiazem and TA3090 is not completely understood; however, these and other calcium antagonists clearly have powerful hepatoprotective effects against ischemia and hypoxia.

The calcium ion is widely accepted as a fundamental regulator of intracellular processes in most cell types [1]. The concept of calcium antagonists, agents which inhibit the regulatory action of calcium, was first proposed by Fleckenstein in 1966 for verapamil and by Godfraind in 1968 for cinnarizine [2, 3]. Since then, the number of known calcium antagonists has grown rapidly. Diltiazem, a calcium antagonist of unusual specificity [4, 5], and TA3090 [6] are benzothiazepine-type slow calcium channel blockers. Studies from this laboratory have shown that dihydropyridine-type calcium channel blockers protect against ischemic damage in the perfused liver and *in vivo* [7–9]. The present study was undertaken to evaluate the possible role of diltiazem and TA3090 in protection against hypoxic liver injury. To examine hypoxic liver damage, rat livers were isolated and perfused briefly with an oxygen-saturated buffer, the perfusate was then replaced with nitrogen-saturated buffer for a sustained period, and oxygen-saturated buffer was subsequently reintroduced. The effects of diltiazem or TA3090 were assessed by infusing various concentrations of each drug during the hypoxic stage and examining hepatic damage, as indicated by lactate dehydrogenase (LDH) release, decreased rates of oxygen uptake, and histology.

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METHODS

Liver perfusion. Ninety-five female Sprague–Dawley rats (175–250 g) were fasted for 24 hr prior to experiments. Livers were perfused with Krebs-Henseleit-bicarbonate buffer which contains 1.5 mM calcium (pH 7.4, 37°) saturated with an oxygen–carbon dioxide mixture (95:5) for 10 min in a hemoglobin-free, non-recirculating perfusion system [10], and then with buffer saturated with a nitrogen–carbon dioxide mixture (95:5) for 80 min in the absence or presence of either diltiazem or TA3090. Perfusions were concluded by a 15-min perfusion with oxygen-saturated buffer. Perfusate was pumped via a cannula placed in the vena cava past a Teflon-shielded Clark-type oxygen electrode. Rates of oxygen uptake were calculated from the influent–effluent concentration difference, the flow rate and the liver wet weight. Effluent perfusate was collected for determination of LDH release by standard enzymatic procedures [11]. The addition of diltiazem or TA3090 had no significant effect on LDH release in fully oxygenated livers.

Preparation of diltiazem and TA3090 solutions. Diltiazem or TA3090 was prepared in Krebs-Henseleit-bicarbonate buffer (35 mL buffer plus

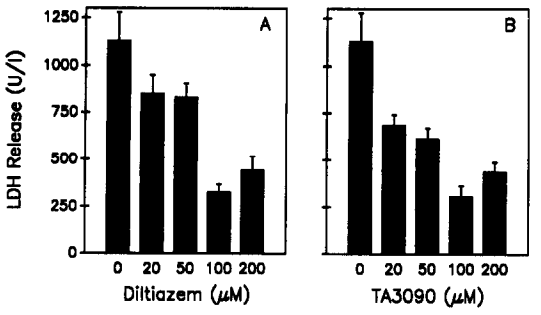

Fig. 1. Effects of various concentrations of diltiazem (A) or TA3090 (B) on maximal rates of LDH release due to hypoxia. Livers were perfused briefly with oxygen-saturated perfusate, then for 80 min with nitrogen-saturated perfusate with diltiazem (A) or TA3090 (B) at the concentrations indicated on the axes, and subsequently with oxygen-saturated perfusate (reflow). LDH in effluent perfusate was determined by standard enzymatic procedures [11]. Data are means ± SEM, N = 10.

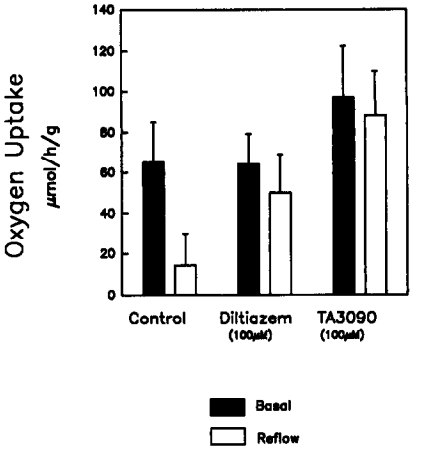

Fig. 2. Effects of diltiazem or TA3090 on rates of oxygen uptake before and after hypoxia. Livers were perfused as in Fig. 1 and oxygen uptake was calculated as described in Methods. Oxygen uptake was calculated initially (basal rate) and when oxygen-saturated perfusate was reintroduced following hypoxia (reflow rate). Data are means ± SEM, N = 10.

2–4 drops acetic acid), and the pH of the solution was adjusted to 7.3 by addition of 2–3 drops 1 N NaOH. Solutions were filtered with a 0.2 μM filter and infused into the livers at final concentrations of diltiazem or TA3090 of 20, 50, 100 and 200 μM. Vehicle control solutions were prepared and infused in an identical manner except for the omission of diltiazem or TA3090.

Histological procedures. At the end of each perfusion, trypan blue (0.2 mM) and paraformaldehyde (1%) in Krebs-Henseleit-bicarbonate buffer were infused for 8 min. Fixed tissues were embedded in paraffin and processed for light microscopy. Sections were stained with eosin, a cytoplasmic stain, so that trypan blue could be identified easily in the nuclei of all nonviable cells. Viability was determined for approximately 1000 cells in each section.

RESULTS

Rates of LDH release due to hypoxia in the presence of various concentrations of diltiazem or TA3090. Control livers perfused for 80 min with nitrogen-saturated buffer released LDH into effluent perfusate at a peak rate of about 1100 U/L (Fig. 1). In contrast, livers perfused with nitrogen-saturated buffer containing 20, 50, 100 or 200 μM diltiazem showed decreased rates of LDH release. Although the decreases in LDH release with 20 and 50 μM diltiazem were small and not significant compared to basal values ($P < 0.1$), the difference at higher concentrations was much larger (about 75% decrease) and was significant ($P < 0.001$ and 0.01 for 100 and 200 μM, respectively).

TA3090 also inhibited cell death due to hypoxia in the perfused liver; however, unlike diltiazem, protection was observed with all concentrations studied (Fig. 1). The decrease in peak LDH release was statistically significant at the lower concentrations ($P < 0.02$ at 20 μM and < 0.01 at 50 μM) as well as the higher concentrations studied. As with diltiazem, the 100 μM concentration proved most effective in inhibiting peak rates of LDH release, producing a value 73% lower than control preparations. TA3090 had a much greater effect than diltiazem at influent concentrations of 20 and 50 μM, whereas the two drugs had nearly equal effects at 100 and 200 μM.

Rates of oxygen uptake following hypoxia in the presence of various concentrations of diltiazem or TA3090. Oxygen uptake is a critical indicator of cell viability. The decrease in rates of oxygen uptake by the liver following hypoxia was also diminished by diltiazem and TA3090 (Fig. 2, Table 1). In control livers, oxygen uptake declined by an average of 78% after 80 min of perfusion with nitrogen-saturated buffer, and in some livers oxygen uptake ceased altogether. With the addition of diltiazem in concentrations ranging from 20 to 200 μM, however, oxygen uptake decreased only 22–36%. Once again the 100 μM concentration was most effective in preserving oxygen uptake. The same pattern occurred with TA3090, as influent concentrations between 20 and 200 μM inhibited the decline in oxygen uptake, and rates decreased only 9–38% from basal values.

Trypan blue uptake in the perfused liver following hypoxia in the presence of diltiazem or TA3090. In control livers perfused for 80 min with nitrogen-saturated buffer, 88% of cells showed nuclear uptake of trypan blue, indicating nonviability (Fig. 3). Livers perfused with nitrogen-saturated buffer in the presence of diltiazem or TA3090, however, exhibited markedly reduced cell death. With both drugs the 100 μM concentration was most effective at reducing irreversible cell death due to hypoxia. At the 100 μM concentration, diltiazem reduced trypan blue uptake to 17%, and TA3090 diminished cell death to 15%. In control preparations, cell death occurred mainly

Table 1. Effects of diltiazem and TA3090 on the decrease in oxygen uptake following hypoxia

Treatment	Oxygen uptake before N_2 perfusion—basal rate (μmol/g/hr)	Oxygen uptake after N_2 perfusion—reflow rate (μmol/g/hr)	Percentage decrease in oxygen uptake due to hypoxia
Control	65.0 ± 20.0	14.3 ± 15.6	78.0
Diltiazem			
20 μM	83.6 ± 22.0	53.6 ± 20.9	35.9
50 μM	73.7 ± 17.4	52.3 ± 19.1	29.0
100 μM	64.0 ± 15.1	49.9 ± 22.0	22.1
200 μM	71.1 ± 19.8	50.0 ± 18.2	29.6
TA3090			
20 μM	85.7 ± 31.3	53.1 ± 24.4	38.0
50 μM	60.9 ± 30.5	45.8 ± 22.8	24.9
100 μM	96.9 ± 25.5	87.9 ± 22.2	9.3
200 μM	71.7 ± 17.2	56.1 ± 17.6	21.8

Livers were perfused briefly with oxygen-saturated perfusate, then for 80 min with nitrogen-saturated perfusate containing diltiazem or TA3090 at the concentrations listed above, and subsequently with oxygen-saturated perfusate. Oxygen uptake was calculated as described in Methods. Data are means ± SEM, N = 10.

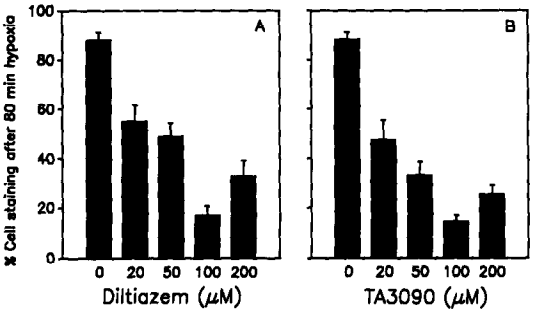

Fig. 3. Effects of various concentrations of diltiazem (A) or TA3090 (B) on cell death due to hypoxia. Livers were perfused as in Fig. 1, and perfusions were concluded by infusion of the vital dye trypan blue. Sections were stained and prepared for histology, and the percentage of cells which took up trypan blue was calculated as described in Methods. Data are means ± SEM, N = 8–13 per group.

in parenchymal cells and was accompanied by cell shrinking and consequent expansion of the sinusoidal space (Fig. 4A). Diltiazem (100 μM) significantly reduced cell death and shrinking (Fig. 4C). TA3090 also reduced the total levels of cell death and appeared to shift the site of damage from parenchymal to nonparenchymal cells in the sinusoids (Fig. 4B).

DISCUSSION

Protective effects of diltiazem and TA3090 against hypoxic injury in the liver. Recently, the hepatoprotective effect of the calcium antagonist nitrendipine against hypoxic injury was applied to the development of a new rinse solution, Carolina Rinse, to diminish injury to organs stored for transplantation [12, 13]. This solution, which contains a calcium channel blocker, dramatically reduced postoperative injury to liver grafts [13]. Although the exact mechanism of this phenomenon has not been established, a delay of the activation of Kupffer cells is clearly involved. Further, we recently described a voltage-dependent calcium channel in hepatic Kupffer cells [14].

These findings with a dihydropyridine-type calcium channel blocker led to an investigation of other types of calcium channel blockers. Experiments described above were designed to examine the effect of two benzothiazepine-type calcium channel blockers, diltiazem and TA3090, on hepatic injury due to hypoxia. This study utilized three indicators of hepatic viability following sustained hypoxia: LDH release, oxygen uptake, and vital dye uptake. In control preparations, 80 min of hypoxia caused extensive cell death, as indicated by a massive release of LDH into the effluent perfusate and by a significant decrease in the amount of oxygen taken up by the liver compared to the amount taken up before the hypoxic phase (Figs. 1–3). The addition of diltiazem or TA3090 to perfusate during the hypoxic phase reduced hepatic damage as assessed by all three methods.

Nuclear uptake of the vital dye trypan blue was used in these experiments to assess the extent and location of cell death due to hypoxia. In control preparations, histological examination revealed extensive parenchymal cell death and shrinking, with corresponding expansion of the sinusoids (Fig. 4A). When maximum protection of hepatocytes was achieved by inclusion of diltiazem (100 μM), a much smaller number of parenchymal cells were damaged, with little or no cell shrinking (Fig. 4C). Interestingly, TA3090 (100 μM) afforded slightly greater hepatic protection as assessed by the number of stained nuclei recorded, LDH release, and oxygen uptake but resulted in a markedly different pattern of cell

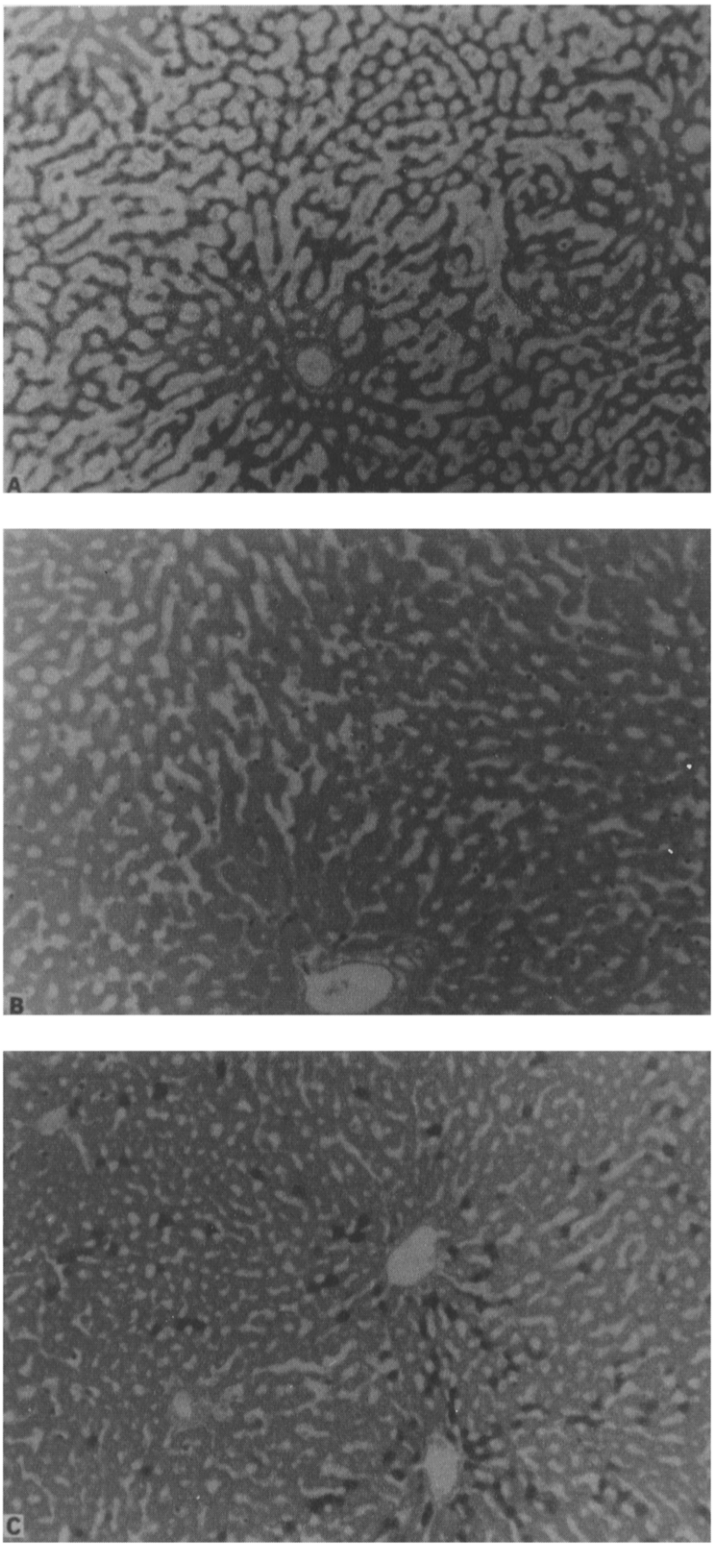

Fig. 4. Photomicrographs depicting the effects of TA3090 and diltiazem on hepatic ultrastructure. Livers were perfused and then fixed as described in Methods. Magnification: 100×. (A) Control preparation. (B) TA3090 (100 μM) infused during hypoxia. (C) Diltiazem (100 μM) infused during hypoxia.

damage (Fig. 4B). Stained nuclei were found almost exclusively in the sinusoids (i.e. they are nonparenchymal cells).

Possible mechanisms for the protective effects of diltiazem and TA3090. Previous studies have indicated that dihydropyridine-type calcium channel blockers protect against ischemic damage in isolated hepatocytes [7–9], but the mechanism responsible for this effect had not been clarified. Recently, however, Hijioka *et al.* [14] demonstrated that Kupffer cells contain L-type voltage-dependent calcium channels. When activated, Kupffer cells release biologically active compounds such as proteases, toxic radicals, eicosanoids and cytokines [15], and the resulting inflammatory, cytotoxic, and vasoactive responses have been reported to lead to hepatic damage and graft failure following liver transplantation [16]. Further, calcium is necessary for the activation of Kupffer cells [17, 18], and calcium channel blockers improve survival following cold storage and liver transplantation in the rat [19]. Therefore, we hypothesize that diltiazem and TA3090 most likely act on voltage-dependent calcium channels, thereby preventing the release of toxic cytokines by hepatic Kupffer cells. On the other hand, alternative mechanisms are possible.

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