

## Diltiazem Protects Hepatocytes from Damage Induced by Reactive Oxygen Species Through Actions on Mitochondria

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### ABSTRACT

**AIM:** *d(+)-cis*-Diltiazem, in addition to its ability to inhibit voltage-operated  $\text{Ca}^{2+}$  channels, exhibits antioxidant properties and can protect hepatocytes in the diseased liver. However, the mechanisms involved are not well understood. The aims were to determine whether the protective effects of diltiazem on damaged hepatocytes include actions on the mitochondrial  $\text{Ca}^{2+}$  channels that enable damaged cells to continue to produce energy.

**MATERIALS AND METHODS:** Rates of  $\text{O}_2$  consumption by isolated rat liver mitochondria were measured using an  $\text{O}_2$  electrode. Isolated rat hepatocytes or H4IIE rat liver cells were incubated with  $\text{H}_2\text{O}_2$ . Cell damage was assessed by measuring extracellular lactate dehydrogenase spectrophotometrically.

**RESULTS:** In isolated mitochondria,  $\text{Ca}^{2+}$  (170  $\mu\text{M}$ ) added to partly uncouple oxidative phosphorylation decreased the acceptor control ratio from  $3.5 \pm 0.1$  to  $1.9 \pm 0.1$ . *d(+)-cis*-Diltiazem (410  $\mu\text{M}$ ) pre-incubated for 5 min increased the acceptor control ratio, measured in the presence of  $\text{Ca}^{2+}$ , to  $2.6 \pm 0.1$  ( $n = 18-31$ ,  $p \leq 0.001$ ). Co-addition of thiamine did not affect the actions of diltiazem. About 50% of the added diltiazem was associated with the mitochondrial membranes. Incubation of hepatocytes with  $\text{H}_2\text{O}_2$  for 24 h induced substantial

cell injury. Pre-incubation with *d(+)-cis*-diltiazem (5  $\mu\text{M}$ ) for 2 h reduced this by about 50%. Some additional protection was given by co-addition of thiamine (50  $\mu\text{M}$ ). Protection provided by *d(+)-cis*-diltiazem was comparable with that provided by silymarin.

**CONCLUSION:** Diltiazem protects hepatocytes from damage induced by reactive oxygen species. The mechanism may involve, in part, antioxidant actions of diltiazem on mitochondrial and other intracellular membranes.

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**Key Words:** Diltiazem; Thiamine; Liver; Mitochondria; Reactive Oxygen

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### INTRODUCTION

Many liver diseases, including hepatitis, non-alcoholic fatty liver and alcoholic cirrhosis involve the generation of reactive oxygen species (ROS) and ROS-induced protein modification<sup>[1-3]</sup>. Mitochondria are central to hepatocyte energy metabolism and to hepatocyte function through the provision of ATP in the electron transport and oxidative phosphorylation pathways<sup>[4]</sup>. In addition, under conditions which favour the generation of ROS, mitochondria contribute to the formation of ROS<sup>[5-8]</sup>. While the mechanisms by which ROS cause cellular damage are not yet fully understood, they include direct and indirect actions on the plasma membrane and on intracellular membranes. Changes in the plasma membrane induced by ROS include non-specific increases in membrane permeability to  $\text{Ca}^{2+}$ , other cations, anions and water, and the activation of non-specific cation channels<sup>[9]</sup>. This effect is known to cause cell swelling in

hepatitis and other liver diseases thereby contributing to hypoxia. Increased permeability of mitochondrial membranes initiated by ROS has also been shown to impair mitochondrial function<sup>[10,11]</sup>. Mitochondria are also both direct and indirect targets for ROS action. Ca<sup>2+</sup> entry to hepatocytes across the plasma membrane induced by ROS leads to the uptake of Ca<sup>2+</sup> by mitochondria through the mitochondrial Ca<sup>2+</sup> uniporter<sup>[12]</sup>, and subsequently to Ca<sup>2+</sup>-induced damage to these organelles<sup>[8,9,13-16]</sup>.

Ca<sup>2+</sup> channel blockers (inhibitors of voltage-operated Ca<sup>2+</sup> channels) are widely used clinically as anti-hypertensive and anti-arrhythmic agents<sup>[17,18]</sup>. One of these, *d(+)-cis*-diltiazem, is used extensively to treat hypertension and angina<sup>[17,18]</sup>. Diltiazem has also been shown to protect the liver from injury induced by warm ischemia and reperfusion, carbon tetrachloride, acetaminophen, other hepatotoxic drugs, lipid peroxidation, and forms of oxidative stress<sup>[19-24]</sup>. While some hepato-protective effects of diltiazem, for example after acute ischaemia-reperfusion injury, are due to effects on the vasculature<sup>[25]</sup>, other effects, particularly in chronic liver disease, are due to direct effects on hepatocytes<sup>[22]</sup>. Experiments with isolated microsomes have shown that *d(+)-cis*-diltiazem at a concentration of about 500 µM protects these organelles from peroxidative damage, suggesting that some of the actions of diltiazem on the liver may be due to its role as an antioxidant acting on intracellular organelles<sup>[26]</sup>. Taken together, results for the actions of diltiazem on liver suggest that diltiazem may protect the liver from injury through two independent mechanisms: one involving inhibition of Ca<sup>2+</sup> channels and/or Ca<sup>2+</sup> transporters, and the other involving antioxidant action.

Two other compounds with hepatoprotective and antioxidant properties are thiamine and silibinin. Thiamine (vitamin B<sub>1</sub>) is a co-factor for mitochondrial pyruvate dehydrogenase, α-ketoglutarate dehydrogenases and several other enzymes, all of which play central roles in intermediary metabolism and energy production. Thiamine also exhibits antioxidant properties<sup>[4]</sup> and can protect cells from carbonyl- and oxidative-induced damage<sup>[27,28]</sup>. Silibinin, the active component of the milk thistle extract silymarin, has a broad range of biological activities, including actions as an antioxidant<sup>[29,30]</sup>. Through its antioxidant actions the active components of silymarin can reduce the onset and extent of liver damage mediated by ROS<sup>[29,31,32]</sup>.

Silymarin and thiamine are both hydrophilic antioxidants and appear to act within the cytoplasmic space. By contrast, diltiazem is lipophilic and is absorbed into the phospholipid of cell membranes. The antioxidant and protective effects of diltiazem and thiamine (and silymarin) appear to additive and complementary<sup>[26]</sup>.

The aims of this study were to determine whether diltiazem can protect mitochondria and hepatocytes from damage induced by the generation of ROS. The experiments have been conducted using isolated mitochondria, in order to assess the direct effects of diltiazem on these organelles, and isolated rat hepatocytes. In the mitochondrial studies, Ca<sup>2+</sup> was employed to induce damage and uncoupling of mitochondria since an increase in cytoplasmic Ca<sup>2+</sup> concentration is a key step in damage induced in hepatocytes by ROS. Increased cytoplasmic Ca<sup>2+</sup> is likely to lead to opening of the mitochondrial transition pore and subsequent loss of the capacity of mitochondria to synthesise ATP<sup>[33-36]</sup>. In the experiments with isolated hepatocytes, H<sub>2</sub>O<sub>2</sub> was used to initiate the generation of ROS<sup>[34]</sup>.

## MATERIALS AND METHODS

### Materials

*d(+)-cis*- and *l(-)-cis*-diltiazem HCl, thiamine, thiamine

pyrophosphate, silymarin, H<sub>2</sub>O<sub>2</sub> (30%) phenylmethanesulfonyl fluoride (PMSF), leupeptin, pepstain A, β-nicotinamide adenine dinucleotide, reduced dipotassium salt (NADH), ethylenediaminetetraacetic acid (EDTA), Triton X-100, D- (+) – glucose, and pyruvic acid were obtained from Sigma-Aldrich. Collagenase (Type IV) was obtained from Worthington Biochemical Corporation; Dulbecco's Modified Eagles Medium (DMEM) from Invitrogen and Gibco; fetal bovine serum (FBS) was from Bovogen; penicillin, streptomycin ovalbumin, and EZQ protein quantification reagent were from Invitrogen; trypsin (2.5% solution) from Gibco; and Nonidet P40 from Roche and methanol from BDH Chemicals Ltd. Silymarin and diltiazem were prepared as stock solutions in water.

### Isolation of liver mitochondria and measurement of O<sub>2</sub> consumption and acceptor control ratio

Liver mitochondria were isolated, and their respiratory activities measured, as described previously<sup>[37]</sup>. Mitochondrial integrity was monitored at 25°C by measuring the ratio (respiratory-control ratio) of the rate of ADP-stimulated respiration to the rate of respiration obtained after the ADP had been depleted, after the addition of limiting amounts of ADP to the incubation medium<sup>[38]</sup>. In the presence of 12.5 mM succinate, respiratory-control ratios of 4-6 were obtained. Mitochondrial protein concentration was determined by a modification of the biuret method<sup>[37]</sup>. The mitochondrial incubation medium contained (mM): sucrose, 100; KCl, 50; Tris buffer, 15; KH<sub>2</sub>PO<sub>4</sub>, 10; MgCl<sub>2</sub>, 2; EDTA, 1, adjusted to pH 7.4 with HCl.

### Isolation and culture of rat hepatocytes

Hepatocytes were isolated from rat liver by perfusion with collagenase, as described previously<sup>[39]</sup>. Animals received humane care, and the experimental protocols were conducted according to the criteria outlined in the "Australian Code of Practice for the Care and Use of Animals for Scientific Purposes" (National Health and Medical Research Council of Australia). Hepatocytes were plated onto the surface of 6-well plates [Greiner Bio-One International AG (Austria)] and incubated for 24-72 h in 1.5 ml of DMEM/F12 supplemented with 10% (v/v) fetal calf serum, penicillin (100 U/ml), streptomycin (0.1 mg/ml) and 10 mM HEPES in 5% (v/v) CO<sub>2</sub> (pH 7.4) at 37°C. H<sub>2</sub>O<sub>2</sub>, *d-cis*-diltiazem, *l-cis*-diltiazem, thiamine, thiamine pyrophosphate and silymarin were added at the times and concentrations indicated in the legend to figure 1.

### Assessment of liver cell damage by measurement of lactate dehydrogenase (LDH) leakage

Hepatocyte injury was assessed by measuring the amount of lactate dehydrogenase released to the extracellular medium. Lactate dehydrogenase, which is located in the cytoplasmic space of hepatocytes, is released to the extracellular medium following cell injury and/or cell death<sup>[40]</sup>. For each experimental condition, the amount of lactate dehydrogenase in the extracellular medium was expressed as a percentage of the total (intracellular plus extracellular) after subtraction of the amount of lactate dehydrogenase in the extracellular medium of control cells (no H<sub>2</sub>O<sub>2</sub> present). Total lactate dehydrogenase activity was determined by lysing all the cells using digitonin (100 µmol/L). Lactate dehydrogenase was measured spectrophotometrically (Market, 1984) using a Cobas Fara autoanalyser and standard Cobas Fara protocols.

### Measurement of diltiazem concentrations

Samples of the mitochondrial suspension were centrifuged at 10,000

g for 5 min at room temperature. The concentration of diltiazem present in the extramitochondrial medium was measured by HPLC/MS using a Waters liquid chromatograph (Waters, Milford, USA), consisting of a 2,695 Separation Module and 2,487 dual wavelength UV detector and a Micromass Quattro Micro LCMS triple quadrupole mass spectrometer (Mass Range: 2-2000  $m/z$ ) complete with ESI probe. A SGE Wakosil, 2 mm  $\times$  150 mm, C18 column was used for the separation at a flow rate of 0.2 mL/min and a gradient profile of 98% solvent A (0.1% aqueous formic acid), 1 min hold to 100% solvent B (0.1% acetonitrile) in 10 min with a 5 min hold at 100% B, the gradient was then returned to 98% solvent A and equilibrated for 5 min. An injection volume of 10  $\mu$ L was used. Data were recorded at 254 and 280 nm, the 280 nm trace was used for quantification of the diltiazem. The MS data was used for confirmation.

### Statistical Analysis

Mean values between two groups were compared using independent Student's *t*-test and between multiple groups using the one way ANOVA followed by Fisher's LSD *posthoc* test.

## RESULTS

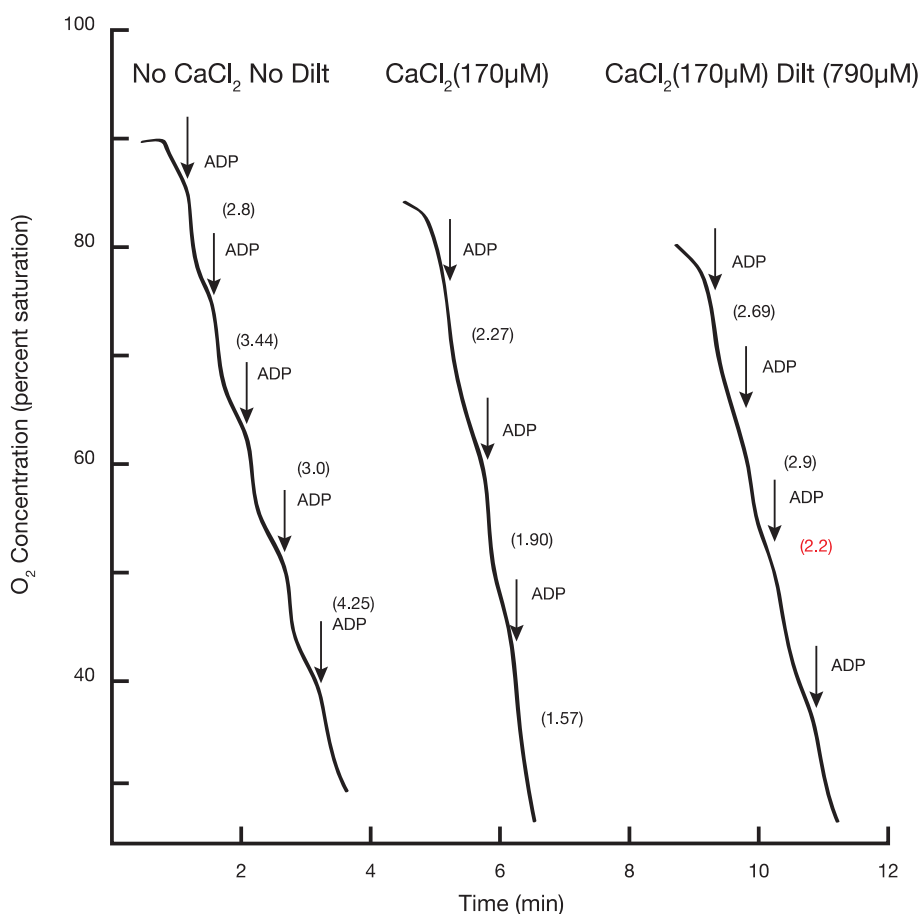
### Effects of *d*(+)-*cis*-diltiazem on $\text{Ca}^{2+}$ -induced damage to mitochondria

To assess the ability of *d*(+)-*cis*-diltiazem to protect mitochondria from damage, isolated liver mitochondria were partially uncoupled by addition of  $\text{Ca}^{2+}$  (170  $\mu$ M). Rates of  $\text{O}_2$  consumption in the presence

of a limiting amount of ADP (state 3) and after ADP depletion (state 4) were measured, and acceptor control ratios calculated. As shown previously<sup>[41]</sup>,  $\text{Ca}^{2+}$  decreased the acceptor control ratio (Figure 1, Table 1). Figure 1 shows plots of  $\text{O}_2$  concentration as a function of time, obtained by incubating isolated mitochondria in an oxygen electrode chamber, for mitochondria incubated in the absence of  $\text{Ca}^{2+}$  and *d*(+)-*cis*-diltiazem, in the presence of 170  $\mu$ M  $\text{Ca}^{2+}$ , and in the presence of 170  $\mu$ M  $\text{Ca}^{2+}$  and diltiazem.

Since it has previously been shown that concentrations of diltiazem in the range of 500  $\mu$ M protect isolated liver microsomes from peroxidation<sup>[26]</sup>, we tested the ability of diltiazem at 410 and 800  $\mu$ M to protect mitochondria from  $\text{Ca}^{2+}$ -induced damage. At these concentrations *d*(+)-*cis*-diltiazem reduced the rate of  $\text{Ca}^{2+}$ -activated  $\text{O}_2$  utilisation in state 4 and increased the acceptor control ratio (Table 1). Much smaller effects were observed at 210  $\mu$ M diltiazem. Co-addition of thiamine (50-100  $\mu$ M) did not improve protection provided by diltiazem (results not shown). The effects of diltiazem were also tested in the absence of ADP. Diltiazem (410  $\mu$ M) reduced the rate of  $\text{Ca}^{2+}$ -stimulated  $\text{O}_2$  utilisation from  $6.10 \pm 0.5$  to  $4.6 \pm 0.2$  (means  $\pm$  SEM,  $n = 5$ ,  $p < 0.05$ ).

Diltiazem, like other lipophilic drugs, partitions into the lipid phase of cell membranes<sup>[42]</sup>. To determine the free concentration of *d*(+)-*cis*-diltiazem in mitochondrial incubations, the concentration of *d*(+)-*cis*-diltiazem in the extramitochondrial medium was measured after centrifugation of the mitochondrial suspension to precipitate the mitochondria. For incubations containing 210 and 410  $\mu$ M added total *d*(+)-*cis*-diltiazem, the measured concentration of the drug in



**Figure 1** Effect of *d*(+)-*cis*-diltiazem on  $\text{Ca}^{2+}$ -induced oxygen consumption by mitochondria. Plots of  $\text{O}_2$  concentration as a function of time for mitochondria incubated in the absence of  $\text{Ca}^{2+}$  and *d*(+)-*cis*-diltiazem (DiH), in the presence of 170  $\mu$ M  $\text{Ca}^{2+}$ , and in the presence of 170  $\mu$ M  $\text{Ca}^{2+}$  and diltiazem. Each plot is representative of those obtained in 18-31 measurements made with 6-11 mitochondrial preparations.

**Table 1** Effects of *d(+)-cis-diltiazem* on the rates of  $\text{Ca}^{2+}$ -stimulated  $\text{O}_2$  utilisation and acceptor control ratio of rat liver mitochondria.

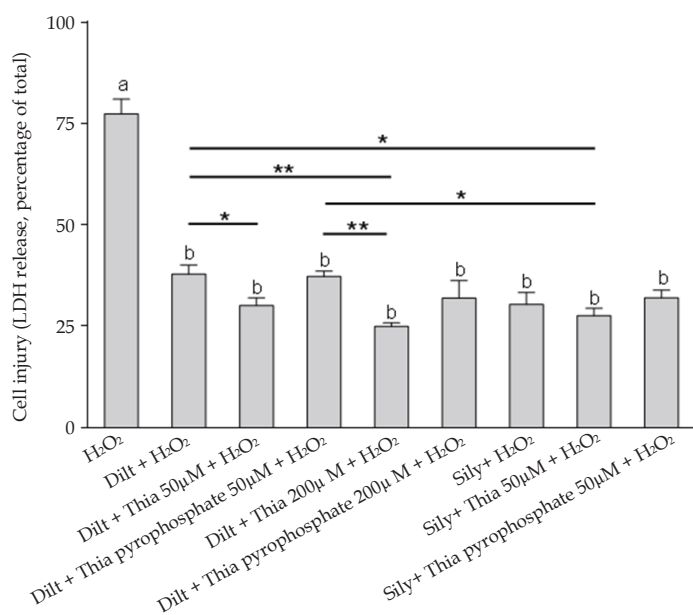
$\text{Ca}^{2+}$ concentration ( $\mu\text{M}$ )	<i>d(+)-cis-Diltiazem</i> concentration ( $\mu\text{M}$ )	Rate of $\text{O}_2$ consumption (% per min) State 3 (ADP present)	State 4 (ADP depleted)	Acceptor control ratio
0	0	22.1 ± 1.2	6.4 ± 0.4	3.5 ± 0.1
170	0	21.2 ± 1.4	11.4 ± 0.7	1.9 ± 0.1
170	210	20.3 ± 1.5	10.0 ± 0.7	2.1 ± 0.1
170	410	19.6 ± 1.1	7.5 ± 0.4***	2.6 ± 0.1***
170	800	21.9 ± 1.6	8.2 ± 0.6**	2.7 ± 0.1***

The values are the means ± SEM of 18-31 measurements made with 6-11 experiments. The degrees of significance, determined using the independent Student's t-test for unpaired samples for comparison of  $\text{Ca}^{2+}$  plus diltiazem with  $\text{Ca}^{2+}$  alone are: \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . The degree of significance for comparison of the values of the acceptor control ratio measured in the absence of diltiazem in the presence and absence of  $\text{Ca}^{2+}$  is  $P < 0.05$ .

**Table 2** Incubation of rat liver mitochondria with *d(+)-cis-diltiazem* leads to the uptake of diltiazem by mitochondria as indicated by a reduction of the concentration of diltiazem in the extramitochondrial medium.

Total concentration of <i>d(+)-cis-diltiazem</i> added to incubation vessel ( $\mu\text{M}$ )	Observed concentration of <i>d(+)-cis-diltiazem</i> in aqueous phase ( $\mu\text{M}$ )
210	142 ± 14 (5)
410	275 ± 10 (6)
790	553 ± 38 (3)

Mitochondria were incubated for 5 min with *d(+)-cis-diltiazem* and the concentration of *d(+)-cis-diltiazem* in the extramitochondrial medium (supernatant) was measured after the centrifugation of the mitochondrial suspension in order to precipitate the mitochondria. The values are the means ± SEM ( $n = 3-6$ ).



**Figure 2** *d(+)-cis-Diltiazem* protects hepatocytes against injury induced by  $\text{H}_2\text{O}_2$ . Hepatocytes plated in multiwell plates were incubated in the presence and absence of  $\text{H}_2\text{O}_2$  (0.5 mM), added at the beginning of the incubation period for 24 h. When present, *d(+)-cis-diltiazem* (Dilt) (5  $\mu\text{M}$ ) or silymarin (Sily) and thiamine (Thia) or thiamine pyrophosphate (Thia pyrophosphate) were added at the concentrations indicated, 2 h before  $\text{H}_2\text{O}_2$ . At the end of the 24 h incubation period, the amount of lactate dehydrogenase present in the extracellular medium and the total lactate dehydrogenase (medium plus cell lysate) were measured. For each condition, the amount of extracellular lactate dehydrogenase activity is expressed as a percentage of the total. The values are the means ± SEM of 3 experiments. The degrees of significance, determined using the one way ANOVA followed by Fisher's LSD posthoc test are as follows: For comparison of each condition with  $\text{H}_2\text{O}_2$  alone (a vs b), \*\*\*\* $P < 0.0001$ . For comparison of Dilt plus Thia (50  $\mu\text{M}$ ) plus  $\text{H}_2\text{O}_2$  with Dilt plus  $\text{H}_2\text{O}_2$  alone, \* $P < 0.05$ ; for comparison of Dilt plus Thia (200  $\mu\text{M}$ ) plus  $\text{H}_2\text{O}_2$  with Dilt plus  $\text{H}_2\text{O}_2$  alone, \*\* $P < 0.01$ ; for comparison of Dilt plus Thia pyrophosphate (50  $\mu\text{M}$ ) plus  $\text{H}_2\text{O}_2$  with Dilt plus Thia (200  $\mu\text{M}$ ) plus  $\text{H}_2\text{O}_2$ , \*\* $P < 0.01$ . Additionally, for comparison of Sily plus Thia (50  $\mu\text{M}$ ) plus  $\text{H}_2\text{O}_2$  with Dilt plus  $\text{H}_2\text{O}_2$  alone, \* $P < 0.05$ ; and finally, for comparison Sily plus Thia (50  $\mu\text{M}$ ) plus  $\text{H}_2\text{O}_2$  with Dilt plus Thia pyrophosphate (50  $\mu\text{M}$ ) plus  $\text{H}_2\text{O}_2$ , \* $P < 0.05$ .

the extramitochondrial medium (supernatant) was about 50% of the added total (Table 2). At 790  $\mu\text{M}$  added total *d(+)-cis-diltiazem*, the *d(+)-cis-diltiazem* concentration in the extramitochondrial medium was about 70% of the total concentration. These results indicate that a substantial amount of *d(+)-cis-diltiazem* partitions into the mitochondria under the experimental conditions employed here.

**Effects of *d(+)-cis-diltiazem* on  $\text{H}_2\text{O}_2$ -induced damage to hepatocytes**

In order to assess the ability of diltiazem to protect hepatocytes from ROS-induced damage, we used  $\text{H}_2\text{O}_2$  to initiate the generation of ROS in experiments with isolated hepatocytes<sup>[34]</sup>. Since diltiazem is known to be lipophilic<sup>[43-46]</sup>, and our results above with mitochondria indicate that substantial amounts of diltiazem are taken up by mitochondrial membranes, we employed much lower concentrations of diltiazem for these experiments with isolated hepatocytes. Moreover, it was also considered important to conduct experiments with isolated hepatocytes using concentrations of diltiazem comparable to the blood concentrations achieved clinically<sup>[47,48]</sup>.

Treatment of rat hepatocytes in culture with 0.5 mM  $\text{H}_2\text{O}_2$ , a generator of ROS<sup>[34,49]</sup>, for 24 h caused cell damage, determined by the release of lactate dehydrogenase into the extracellular medium, in a large proportion of the cells (Figure 2). Addition of *d(+)-cis-diltiazem* (5  $\mu\text{M}$ ) 2 h before  $\text{H}_2\text{O}_2$  addition provided substantial protection (\*\*\*\* $P < 0.0001$ ) against  $\text{H}_2\text{O}_2$ -induced cell injury. Co-addition of thiamine with *d(+)-cis-diltiazem* provided a further additional protection (\* $P < 0.05$  and \*\* $P < 0.01$  for 50  $\mu\text{M}$  and 200  $\mu\text{M}$  respectively). Thiamine pyrophosphate (50  $\mu\text{M}$  and 200  $\mu\text{M}$ ) had no additional protective effect (Figure 1). The degree of protection offered by 50  $\mu\text{M}$  *d(+)-cis-diltiazem* was comparable to that provided by 500 ng/mL silymarin (Figure 2). Neither thiamine nor thiamine pyrophosphate enhanced the protection offered by silymarin (Figure 2).

**DISCUSSION**

**Summary of key results**

The results have shown that *d(+)-cis-diltiazem* reduces  $\text{Ca}^{2+}$ -induced damage to isolated mitochondria while pre-incubation of isolated hepatocytes with *d(+)-cis-diltiazem* substantially reduces  $\text{H}_2\text{O}_2$ -induced damage. The effects of diltiazem on mitochondria were associated with the accumulation of a substantial amount of the diltiazem in the mitochondria. The magnitude of the effect of diltiazem in reducing  $\text{H}_2\text{O}_2$ -induced damage to hepatocytes was comparable to that of the herbal antioxidant silybinin, the active component of silymarin<sup>[30]</sup>. The mechanisms by which diltiazem protects hepatocytes from ROS-induced damage may involve actions on both  $\text{Ca}^{2+}$  channel proteins and on intracellular membranes. Thus diltiazem may inhibit  $\text{Ca}^{2+}$  entry across the plasma and mitochondrial membranes and/or opening of the mitochondrial transition pore. In addition, diltiazem may act as an antioxidant in a lipophilic environment, particularly within the phospholipid of cell membranes. These events, in turn, may inhibit the subsequent disruption by elevated intramitochondrial  $\text{Ca}^{2+}$  of the synthesis of ATP.

**Effective concentration range for the action of diltiazem**

The effect of diltiazem on  $\text{H}_2\text{O}_2$ -induced damage to hepatocytes was observed at lower concentrations of the drug than those which gave protection to  $\text{Ca}^{2+}$ -induced damage to isolated mitochondria. This difference in effective concentrations of diltiazem may be due, in part, to the shorter pre-incubation time employed in the mitochondria

experiments. However, the difference is most likely to be due to the lipophilic properties of diltiazem, which lead to the accumulation of diltiazem in membranes, including the plasma membrane, mitochondrial membranes and the membranes of other intracellular organelles. Thus it was shown in experiments with isolated mitochondria that a substantial amount of diltiazem accumulates in the mitochondria. This is most likely located in the mitochondrial inner and outer membranes since diltiazem is lipophilic<sup>[43-46]</sup>, but some may also accumulate in the mitochondrial matrix. It is likely that diltiazem accumulates in mitochondrial and other intracellular membranes and the plasma membrane during the pre-incubation of hepatocytes with diltiazem. Since diltiazem is readily dissolved in liposomes, the location of diltiazem in cellular membranes may be the hydrophobic lipids of the lipid bilayer<sup>[45]</sup>. Moreover, the experiments with isolated mitochondria were conducted at 25°C and with a relatively short time of exposure to diltiazem. It might be anticipated that at 37°C and at longer incubation times the concentration of diltiazem in the mitochondrial membranes would be greater than that observed at 25°C<sup>[45]</sup>.

Similar differences between the concentrations of diltiazem which affect hepatocytes on the one hand and isolated organelles on the other have been reported for the effects of diltiazem on liver microsomes<sup>[26]</sup>. Moreover, the concentration of diltiazem which protected hepatocytes from ROS-induced damage is comparable with those reported to be achieved in patients receiving oral diltiazem<sup>[50]</sup>. In addition, other studies have shown that oral diltiazem at 25-50 mg/day improved liver function in Hepatitis C patients, whereas higher doses (50 mg/day) were needed to improve propranolol clearance in patients with cirrhosis of the liver<sup>[47,48]</sup>. Taken together, all these observations suggest that while relatively high concentrations of diltiazem were needed to protect isolated mitochondria from Ca<sup>2+</sup>-induced damage, this protective effect might well be achieved at the normal clinical doses of diltiazem under conditions where the time of exposure of the liver, and hence liver mitochondria, to diltiazem is longer than the relatively short times employed here.

#### **Mechanism of diltiazem action likely involves both reduction in ROS-induced membrane damage and inhibition of membrane Ca<sup>2+</sup> channels and transporters**

Since H<sub>2</sub>O<sub>2</sub> principally affects cells by forming ROS and the actions of ROS on cells are known to induce Ca<sup>2+</sup> entry<sup>[34]</sup> leading to Ca<sup>2+</sup>-mediated damage to mitochondria<sup>[8,9,13-16]</sup>, it is likely that the mechanism by which H<sub>2</sub>O<sub>2</sub> induces damage to hepatocytes involves Ca<sup>2+</sup>-induced damage to mitochondria *in situ*. Moreover, it can be concluded that the protective effects of diltiazem on isolated hepatocytes are due, in part, to protection against Ca<sup>2+</sup>-induced damage to mitochondria. The protective effects of diltiazem on ROS-induced damage to hepatocytes are not likely to be due to the inhibition of voltage-operated Ca<sup>2+</sup> channels in the hepatocyte plasma membrane. While *d(+)-cis*-diltiazem is a potent inhibitor of voltage-operated Ca<sup>2+</sup> channels<sup>[17,18]</sup>, these are not thought to be expressed in rat hepatocytes<sup>[51-53]</sup> (but see Park *et al*<sup>[54]</sup>). However, some effects of diltiazem on intact hepatocytes may be due to the inhibition of Ca<sup>2+</sup> entry through store-operated and transient receptor potential (TRP) Ca<sup>2+</sup> entry channels (c.f.<sup>[55]</sup>). Ca<sup>2+</sup> channel blockers such as diltiazem are likely to inhibit mitochondrial Ca<sup>2+</sup> overload and the consequences of this overload by at least three mechanisms. These are: direct inhibition of the mitochondrial Ca<sup>2+</sup> uniporter; indirect inhibition of Ca<sup>2+</sup> entry based on their antioxidant effect which preserves non-specific membrane permeability in both cell and mitochondrial membranes (c.f.<sup>[11,56]</sup>); and inhibition of the opening of

the permeability transition pore in the inner mitochondrial membrane. The relative contribution of each of these three mechanisms is not presently clear.

#### **Thiamine may protect by exerting an anti-oxidant action**

Thiamine gave some additional protection, over and above diltiazem, against H<sub>2</sub>O<sub>2</sub>-induced damage to hepatocytes, but did not alter the Ca<sup>2+</sup>-induced changes in mitochondria. Thiamine principally acts as a co-factor in the reactions catalysed by pyruvate- and  $\alpha$ -ketoglutarate-dehydrogenase in mitochondria, but thiamine also possesses antioxidant properties<sup>[4,57]</sup>. Moreover, relatively high daily doses of thiamine were found to provide some reduction in liver damage in patients with chronic hepatitis B<sup>[58]</sup>. Since, in the present study, the hepatocytes and mitochondria were isolated from the livers of normal healthy rats, it is unlikely that the absence of an effect of thiamine on mitochondria is due to the binding of added thiamine to mitochondrial dehydrogenases. By contrast, in thiamine deficiency, the supply of low doses of additional thiamine would likely increase the concentrations of active mitochondrial dehydrogenases. The protective effect of thiamine on isolated hepatocytes may be due to its antioxidant action. Since thiamine is a hydrophilic molecule these antioxidant effects are likely to be exerted in the cytoplasmic space rather than in intracellular membranes and are likely to require a higher dose of thiamine. Since mitochondria are a source of ROS<sup>[5-8]</sup>, the lipophilic properties of diltiazem offer a number of advantages in the action of this molecule as an antioxidant since it can cross both the plasma and mitochondrial membranes.

#### **The hepatoprotective effects of diltiazem and thiamine are likely only manifest in damaged hepatocytes**

The protective effects of diltiazem on ROS-induced damage to hepatocytes and Ca<sup>2+</sup>-induced damage to mitochondria should be almost totally absent in normal cells because these exhibit low levels of ROS and normal low cytoplasmic Ca<sup>2+</sup> concentrations. Similarly, added thiamine should have no detectable effect in normal cells from thiamine replete animals. Therefore the hepatoprotective effects of both diltiazem and thiamine can be profiled as being disease-selective. Since most pharmacological studies examine the effects of drugs on normal tissues, these hepatoprotective effects of diltiazem, diltiazem plus thiamine, or silymarin can be regarded as pathopharmacological actions.

#### **Clinical implications of the results**

There are two important clinical implications which can be drawn from the results of the studies presented in this paper, which were designed to examine the mechanisms of the protective actions of diltiazem and thiamine. The membrane-protective effect produced by diltiazem as a lipophilic antioxidant is consistent with the clinical findings of the study undertaken in patients with chronic hepatitis C in which the drug at doses of 25 mg and 50 mg per day administered as a slow-release capsule lowered lactate dehydrogenase and SGOT enzymes towards normal<sup>[48]</sup>. *In vitro* studies indicate that the combination of diltiazem (lipophilic antioxidant) plus thiamine (hydrophilic antioxidant) will enhance the membrane-protective effect in patients with chronic non-malignant liver disease. The energy protective effect produced by diltiazem by its effect on the mitochondria, and probably by inhibition of the mitochondrial calcium channel is consistent with the findings of the study in patients with cirrhosis of the liver, in which the drug restored the hepatic clearance of propranolol towards normal<sup>[47]</sup>. Thiamine is also well known to have an energy-protective effect, but this appears only

to be active when thiamine is administered to patients with thiamine deficiency.

A key feature of the nature of diltiazem is its lipophilic properties. This makes it very different from most other Ca<sup>2+</sup> channel blockers and accounts for (1) its ability to accumulate within the phospholipid achieving concentrations that have antioxidant actions; (2) its ability to enter cells and inhibit the mitochondrial Ca<sup>2+</sup> importer; and (3) be metabolised by the liver and have a short half-life. This third effect requires that the drugs be administered as a sustained release formulation to maintain the chemical presence of the drug within the liver, but it also means that low doses administered in sustained release formulations are relatively liver-selective thereby minimising the peripheral vasodilator effects of the drug that are best avoid in patients with liver disease who frequently have low-normal blood pressure.

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## CONFLICT OF INTERESTS

Joel Castro, Rachael Hughes, and Greg Barritt each have no conflicts of interest in regard to the present study. Howard Smith is the owner of intellectual property claiming the use of diltiazem and thiamine for the treatment of liver disease.

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