Effect of diltiazem isomers and thiamine on piglet liver microsomal peroxidation using dichlorofluorescein

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ABSTRACT

Purpose. We investigated a potential hepatoprotective role of *d-cis* diltiazem, *l-cis* diltiazem, thiamine and the combination *d-cis* diltiazem and thiamine against lipid peroxidation in a piglet liver microsomal model. A modified *in vitro* dichlorofluorescein assay was developed to assess the extent of peroxidative damage induced by reactive oxygen species in the piglet liver microsomal fraction. **Methods.** Microsomal membrane fraction, obtained from 3 week old female piglets, was treated with either the biologically vasoactive *d-cis* diltiazem or the non-vasoactive stereoisomer *l-cis* diltiazem (5-1000 μM) for 1 hour at $37^{o}C$ followed by one hour incubation with the free radical generator AAPH (2,2'-azobis-(2-amidinopropane) dihydrochloride; 1 mM) to initiate lipid peroxidation. In a separate study, piglet liver microsomes were pre-treated with *d-cis* diltiazem (50 or 500 μM) and thiamine (10-100 μM) to assess the antioxidant activity of the combination. **Results.** A dose dependant inhibition of membrane lipid peroxidation was observed with *d-cis* diltiazem ($p<0.05$) but not with *l-cis* diltiazem, suggesting that diltiazem is stereospecific in protecting against microsomal lipid peroxidation. Combining diltiazem with thiamine further protected microsomes against lipid peroxidation compared to use of individual drugs. **Conclusions.** We conclude that diltiazem and the combination of diltiazem and thiamine offers a hepatoprotective effect against free radicals.

INTRODUCTION

Calcium channel blockers represent a pharmacologically diverse group of compounds that have been widely used as antihypertensive and antiarrythmic agents (*1*). These drugs decrease the vascular contractility and arterial tone by modulating the transmembrane influx of calcium through voltage-gated calcium channels (*2*). *d-cis* Diltiazem (vasoactive isomer of diltiazem) is a typical calcium channel blocker that is widely used in the treatment of hypertension either alone or in combination with other antihypertensives. Diltiazem also has been demonstrated to protect the liver from warm ischemia (*3*), carbon tetrachloride hepatotoxicity (*4*), acetaminophen hepatotoxicity (*5*), microsomal lipid peroxidation (*6*), and hepatocellular oxidative stress (*7*). The hepatoprotective role of diltiazem was initially attributed to its calcium blocking mechanism as most hepatotoxic agents are known to increase intracellular calcium levels. However, diltiazem has been shown to protect rat liver microsomes from lipid peroxidative damage despite the fact that hepatic microsomal preparations lack L-type calcium channels required by the vasoactive *d-cis* diltiazem for its vascular action (*8, 9*). This led us to ask the question whether the non-vasoactive *l-cis* diltiazem (optical isomer of diltiazem) protects hepatic microsomal preparations as well as *d-cis* diltiazem against lipid peroxidation. If true, *l-cis* diltiazem could be used as a hepatoprotective agent that is devoid of any vasoactivity (calcium channel blocking) or its associated adverse effects. Thiamine (vitamin B_1) is a water-soluble vitamin that plays a crucial role in mammalian carbohydrate metabolism. Despite a great body of literature available on thiamine, little is known about its antioxidant potential against membrane lipid peroxidation. A recent study reported that thiamine protects rat liver microsomes from divalent iron induced lipid peroxidation (*10*). Therefore, we investigated the antioxidant role of thiamine in piglet liver microsomal lipid peroxidation and whether additional hepatoprotection is offered by the combination of thiamine and diltiazem.

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MATERIALS AND METHODS

l-cis Diltiazem was purchased from Biomol International (Plymouth Meeting, PA). All other chemicals including *d-cis* diltiazem, thiamine, and dichlorofluorescein diacetate (DCFH$_2$-DA) were purchased from Sigma Chemical (St. Louis, MO). The aqueous buffer used throughout all experiments was phosphate buffered saline (PBS), which had a composition of (in mM) 137 NaCl, 2.68 KCl, 1.65 KH$_2$PO$_4$, and 8.92 Na$_2$HPO$_4$, pH adjusted to 7.4 using 0.1 N NaOH.

Piglet Liver Microsomes.

All animal studies were performed in accordance with the principles and guidelines of the Canadian Council on Animal Care and the University of Manitoba Fort Garry Campus Protocol Management and Review Committee. Three week old piglets were obtained from the Swine Unit of the University of Manitoba Glenlea Research station. At necropsy, livers were rapidly removed and placed on ice for the preparation of microsomes. Microsomes from hepatocytes were prepared as previously described (*11*). Briefly, livers were minced with scissors in a homogenizing buffer containing 25 mM Tris, 225 mM sucrose, 5 mM glutathione, and 50 mM sodium fluoride and transferred to a large homogenizer. Liver pieces were homogenized twice at 5000 rpm for 1 min using a Polytron homogenizer (Brinkmann, Westbury, NY) at 4°C with 1 min interval between the two runs. The homogenate was centrifuged at 15,000 x g for 20 min (4°C) followed by centrifugation of the supernatant at 100,000 x g for 1 hr (4°C). The resulting microsomal pellet was washed twice with 0.25 M sucrose and resuspended in 0.25 M sucrose at a concentration of 1 mg/ml. Microsomes were frozen at -80°C in 2 ml aliquots until further use. Liver microsomes obtained from 3 individual piglets were pooled for the purpose of the study.

Lipid Peroxidation Studies.

Piglet liver microsomes (1 mg/ml) were pre-incubated with either *l-cis* diltiazem or *d-cis* diltiazem (5, 50, 250, 500, and 1000 μM) or thiamine (10, 50, and 100 μM) for 1 hour at 37°C in a Gyrotory Waterbath Shaker (New Brunswick

Scientific, Canada) maintained at 80 rpm (slow shaking). Control microsomes were treated with 0.25 M sucrose. In combined drug studies, microsomes were co-treated with *d-cis* diltiazem (50 or 500 μM) and thiamine (10, 50, or 100 μM) for 1 hour at 37°C. The microsomes were then incubated for 1 hour with the free radical generator 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH; 1 mM) at 37°C to initiate microsomal lipid peroxidation. At the end of the treatment period, the microsomal mixture was washed and centrifuged several times at 1000 x g using BIOMAX 5000 MWCO ultrafiltration tubes (Millipore, MA) for 10 min with Ca/Mg-PBS to remove excess AAPH, diltiazem, and thiamine. The extent of AAPH-mediated microsomal lipid peroxidation was measured using a modified dichlorofluorescein (DCF) assay procedure.

DCF Assay.

The conversion of the non-fluorescent 2',7'-dichlorodihydrofluorescein (DCFH) to the highly fluorescent dichlorofluorescein (DCF) by reactive oxygen products, such as lipid peroxides, forms the basis of the assay. The active intracellular 2',7'-dichlorodihydrofluorescein (DCFH) form of DCFH$_2$-DA was prepared as described by Tollefson et al. (*12*). Briefly, the diacetate moiety of DCFH$_2$-DA was cleaved by incubating 100 μL stock DCFH$_2$-DA in the presence of 200 μL methanol and 100 μL 2N NaOH at room temperature in the dark. After 2 hours, in the dark, the resulting DCFH solution was diluted with Ca/Mg-PBS to achieve a final probe concentration of 20 μM with the pH adjusted to 7.4 immediately before use.

To measure the extent of lipid peroxidation, 100 μL of the AAPH-treated microsomes was added to 100 μL of the prepared DCFH solution (20 μM) in a 96-well plate at room temperature to give a final DCF concentration of 10 μM. After 15 min of incubation, the fluorescence intensity of emitted DCF fluorescence was measured using a BMG (Durham, NC, USA) Fluostar Galaxy fluorescence plate reader (485 nm excitation wavelength/520 nm emission wavelength). Results were expressed in arbitrary fluorescence units (AFU).

Unbound Diltiazem Concentration:

Piglet liver microsomes (1 mg/ml) were pre-incubated with either l-cis diltiazem or d-cis diltiazem (0, 50, 250, 500 and 1000 μM) for 1 hour at 37°C in water bath and then transferred to microfilters for centrifugation at 2000 rpm for 40 min to separate the unbound from the protein-bound fraction. Analysis of diltiazem was performed as previously described (*13*). Briefly, an iron (III)–1,10–phenanthroline solution was prepared daily (2 g 1,10-phenanthroline + 0.25 concentrated HCl + FeCl$_3$ + 25ml H$_2$O). The mixture of diltiazem and iron (III)–1,10–phenanthroline solution was heated in a water bath for 40 minutes before spectrophotometric analysis. Standard curve of diltiazem was determined using 0, 10, 40, 160, and 640 μM diltiazem with iron (III)–1,10–phenanthroline solution. Finally, the concentrations of diltiazem in the microsome samples were read using a Spectra Max 190 (Molecular Devices) plate reader at 510 nm.

Statistical Analyses.

Data are presented as mean ± SEM. The *n* value refers to the number of replicates performed for each study. Data were analyzed using one-way ANOVA with Student-Newman-Keuls post hoc test taking *p*<0.05 as the level for significance (statistical significance set at *p*<0.05).

RESULTS

Effect of l-cis diltiazem and d-cis diltiazem on piglet liver microsomes.

Microsomal lipid peroxidation studies were conducted to investigate if diltiazem is stereospecific in protecting piglet liver microsomes from lipid peroxidation. Figure 1 shows DCF fluorescence from peroxidized piglet liver microsomes treated with either *l-cis* diltiazem or *d-cis* diltiazem. The vasoactive d-cis diltiazem significantly inhibited AAPH-induced microsomal peroxidation in a dose-dependent manner (*P*<0.05) compared to control microsomes. The DCF fluorescence was significantly lower even at a low *d-cis* diltiazem concentration (*d-cis* diltiazem (0 μM) 53 ± 2; *d-cis* diltiazem (5 μM): 44 ± 0.5, *P*<0.05). The emitted fluorescence from the non-vasoactive *l-cis* diltiazem treated microsomes at the indicated concentrations was not different from control (0 μM) suggesting that *l-cis* diltiazem does not protect liver microsomes from AAPH-induced lipid peroxidation (Figure 1). Therefore, diltiazem appears to be sterospecific in its protective action against microsomal lipid peroxidation.

Effect of thiamine on piglet liver microsomes.

We furthered our work by investigating the effect of thiamine on hepatic microsomal lipid peroxidation. Results indicated that DCF fluorescence readings from microsomes pre-incubated with thiamine (10 – 100 μM) were significantly lower (*P*<0.05) compared to control microsomes (no thiamine) and were dose-dependent (Figure 2), suggesting that thiamine protects piglet liver microsomes from AAPH-induced lipid peroxidation.

Effect of d-cis diltiazem and thiamine combination on piglet liver microsomes.

We conducted studies to investigate whether co-treating liver microsomes with *d-cis* diltiazem and thiamine could further enhance protection against microsomal lipid peroxidation. As shown in Table 1, when microsomes were co-treated with 50 μM *d-cis* diltiazem and thiamine (10, 50, or 100 μM), DCF fluorescence was significantly reduced compared to 50 μM *d-cis* diltiazem alone (*P*<0.05). However, fluorescence values from co-treated microsomes were not different from the respective thiamine concentrations suggesting that combining 50 μM *d-cis* diltiazem and thiamine did not further enhance the protective effect against microsomal lipid peroxidation compared to thiamine alone. On the other hand, combining 500 μM *d-cis* diltiazem and thiamine (10, 50, or 100 μM) significantly reduced DCF fluorescence compared to either 500 μM *d-cis* diltiazem alone or the respective thiamine concentrations (*P*<0.05). Thus, use of thiamine together with 500 μM *d-cis* diltiazem significantly enhanced protection against microsomal lipid peroxidation compared to the use of either drug or thiamine alone.

Table 1. DCF fluorescence from AAPH-induced lipid peroxidized hepatic microsomes pre-treated with both thiamine and *d-cis* diltiazem.

Thiamine (μM)	Arbitrary Fluorescence Index (AFU)		
	d-cis diltiazem (0 μM)	*d-cis* diltiazem (50 μM)	*d-cis* diltiazem (500 μM)
0	39260 ± 993	36497 ± 235	32271 ± 453
10	31112 ± 414	29219 ± 689 *	28891 ± 457 *Δ
50	27265 ± 736	26318 ± 594 *	23468 ± 590 *Δ
100	23477 ± 448	22014 ± 204 *	21200 ± 180 *Δ

* Significantly different from the respective *d-cis* diltiazem concentration ($P<0.05$). Δ Significantly different from the respective thiamine concentration ($P<0.05$). Piglet liver microsomes (1 mg/ml) were pre-incubated with *d-cis* diltiazem (50 or 500 μM) and thiamine (10, 50, or 100 μM) for 1 hour at 37°C followed by 1 hour incubation with the free radical generator 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH; 1 mM) at 37°C to initiate microsomal lipid peroxidation. Control microsomes were pre-incubated with 0.25 M sucrose. The extent of AAPH-mediated microsomal lipid peroxidation was measured using a modified dichlorofluorescein (DCF) assay procedure. Data represent mean±SEM (n=8).

Unbound Diltiazem Concentration.

Total concentrations of *d-cis* and *l-cis* diltiazem used in this study ranged from 0 to 1000 μM. Since diltiazem has limited water solubility it is bound to proteins in the circulation and intracellular proteins within the cell. The pharmacologically effective concentration is thus much lower than that reconstituted. We determined the unbound concentration in our microsome studies to elucidate the concentration that is associated with the antioxidant effect. Figure 3 shows the unbound concentrations of *d-cis* and *l-cis* diltiazem in the presence of microsomes. In both cases the unbound concentration and unbound fraction was not statistically different from each other. Overall the unbound fraction was 30.0 ± 5.5% for d-cis diltiazem and 28.1 ± 5.8% for *l-cis* diltiazem.

DISCUSSION

Calcium antagonists such as diltiazem have been shown to retard the progression of atherosclerosis in various experimental and clinical models (*14-16*). The antiatherogenic property has been primarily attributed to its vasodilatory effect. Recent experimental evidence, however, suggests that calcium antagonists inhibit membrane lipid peroxidation, an early step in the development of atherosclerosis, by mechanisms independent of its vascular effect (*17, 18*). Also, a number of calcium antagonists have been shown to inhibit LDL oxidation *in vitro* and to exhibit antioxidant activity in various membrane preparations (*19-21*).

Studies with hepatic microsomal fractions have shown that diltiazem protects microsomal membranes from lipid peroxidation by a direct mechanism independent of its calcium flux modulation (*8, 9*). Diltiazem is thought to modulate physicochemical properties of membrane lipid bilayers by directly interacting with membranes (*9*). Our finding that *d-cis* diltiazem confers a dose-dependent protective action against AAPH-induced lipid peroxidation in piglet liver microsomes is in agreement with those studies. A novel finding from the present study is the inability of non-vasoactive *l-cis* diltiazem to confer a similar effect in hepatic microsomal preparations. This was surprising given the fact that the antioxidant property of diltiazem was thought to be independent of its calcium channel modulation. These results would seem to suggest that the effect of diltiazem is stereospecific in interacting with a specific binding site on membrane lipid bilayers, such as microsomes. To our knowledge, this is the first study to report on the stereospecificity of diltiazem in protecting hepatic microsomal fraction against lipid peroxidation.

l-cis and *d-cis* diltiazem have been reported to have anti-ischemic action in the isolated, perfused working rat heart suggesting that
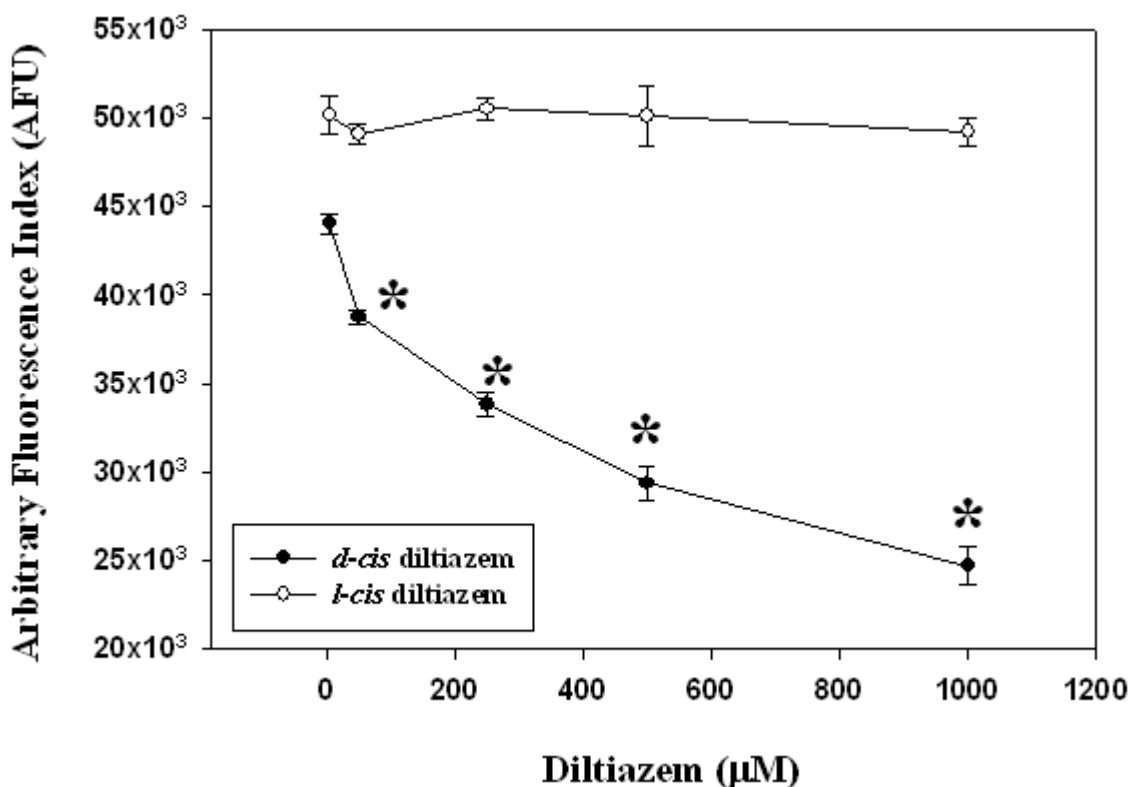
Figure 1. Piglet liver microsomes (1 mg/ml) were pre-incubated with either *l-cis* diltiazem or *d-cis* diltiazem (5, 50, 250, 500, and 1000 µM) for 1 hour at 37°C followed by 1 hour incubation with the free radical generator 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH; 1 mM) at 37°C to initiate microsomal lipid peroxidation. Control microsomes were pre-incubated with 0.25 M sucrose. The extent of AAPH-mediated microsomal lipid peroxidation was measured using a modified dichlorofluorescein (DCF) assay procedure. Data represent mean±SEM (n=8). * Significantly different from the control group (*P*<0.05).

mechanisms other than calcium channel modulation are responsible for diltiazem's effect on ischemic myocardium (*22*). Both diltiazem isomers attenuate hydrogen-peroxide induced derangements in rat hearts partly through an increase in the intracellular myocardial concentration of Na$^+$ (*23*). Moreover, pre-treatment with *l-cis* diltiazem reduces the myocardial infarct size in the ischemic-reperfused rabbit heart without affecting any hemodynamic parameters (*24*). Those studies would seem to suggest a cardioprotective role for *l-cis* diltiazem in conditions involving oxidative damage. However, *l-cis* diltiazem failed to protect hepatic microsomal membrane fraction from ROS-mediated lipid peroxidation in the present study. It is not presently clear how the non-vasoactive *l-cis* diltiazem selectively exhibits cardioprotective properties whereas the vasoactive *d-cis* diltiazem exhibits both cardioprotective and hepatoprotective properties.

One explanation is that the interacting surface of the hepatic microsomal membrane fraction is stereoselective with respect to diltiazem. For example, *d-cis* diltiazem could be favored in binding with microsomal membrane fraction over *l-cis* diltiazem resulting in modulation of membrane physicochemical properties as suggested by Mason et al. (*9*). Future studies will be required to address these issues and other possibilities.

Another important result from the present studies was that thiamine protected hepatic microsomes from lipid peroxidation. Most of the available literature has focused on the role of thiamine as an essential vitamin (i.e., effects of thiamine deficiency and supplementation) rather than on thiamine as an antioxidant itself. A recent study reported that addition of thiamine (1 – 100 µM) was able to inhibit oxygen-induced lipid peroxidation in mouse liver microsomes and that
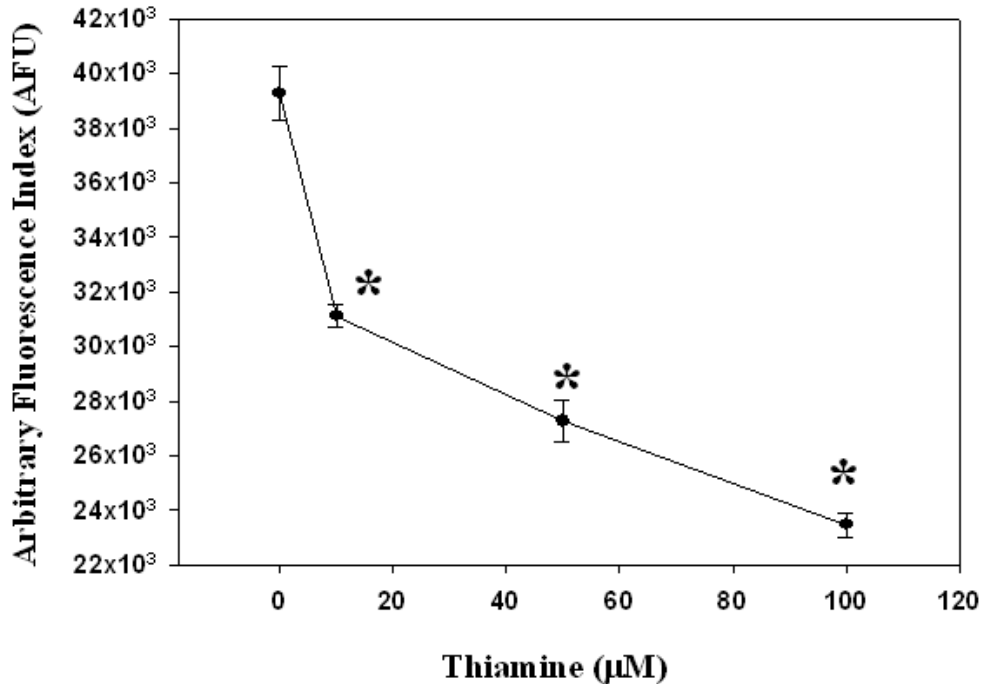
Figure 2. Piglet liver microsomes (1 mg/ml) were pre-incubated with thiamine (10, 50, and 100 µM) for 1 hour at 37°C followed by 1 hour incubation with the free radical generator 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH; 1 mM) at 37°C to initiate microsomal lipid peroxidation. Control microsomes were pre-incubated with 0.25 M sucrose. The extent of AAPH-mediated microsomal lipid peroxidation was measured using a modified dichlorofluorescein (DCF) assay procedure. Data represent mean±SEM (n=6). * Significantly different from the control group ($P<0.05$).
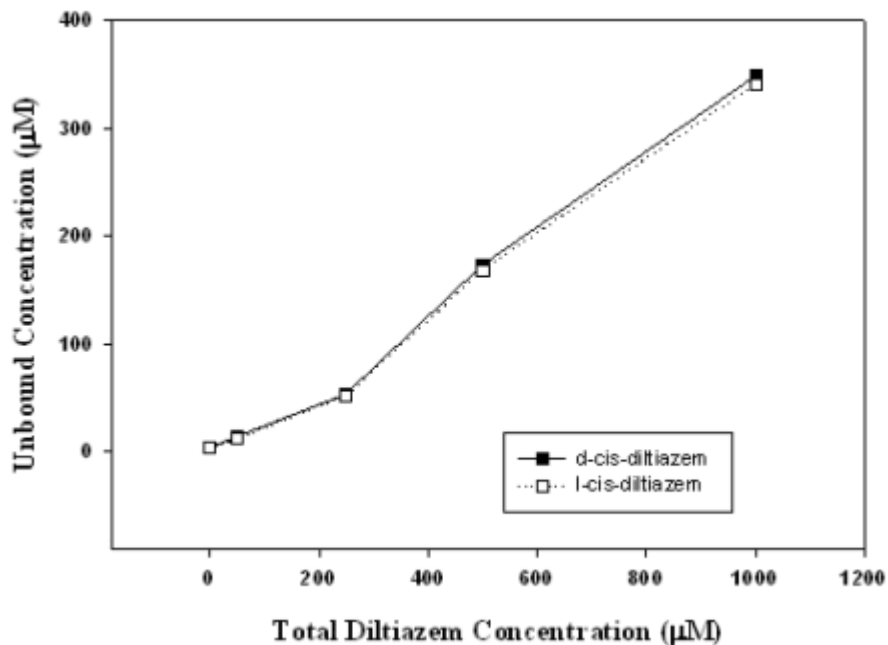

Figure 3. Unbound diltiazem concentration vs total diltiazem concentration in the presence of microsomes. Data represent mean±SEM (n=6).

thiamine oxidation products, thiochrome and thiamine disulfide, were detected on incubating thiamine with oxidized oleic acid (*10*). Similarly, thiamine was reported to reverse the effects of arsenic-induced oxidative stress (*25*). This would seem to suggest that thiamine inactivates reactive oxygen species (ROS) by directly interacting with the oxidizing free radicals similar to that of vitamin E. Detailed studies are necessary to systematically investigate the antioxidant nature of thiamine.

As the putative antioxidant action of diltiazem and thiamine are thought to involve different mechanisms, we investigated whether combining diltiazem and thiamine could further enhance protection against lipid peroxidation. Diltiazem is a lipophilic drug and as such could protect membrane lipid bilayers against lipid peroxidation by directly interacting with membrane components. Thiamine, on the other hand, is hydrophilic and as such inactivates ROS by being oxidized within the hydrophilic environment. The combination would be expected to further enhance the protective effects. Our results showed that the combination treatment indeed further protected hepatic microsomes but only using 500 µM *d-cis* diltiazem concentration (AFU values were at control thiamine levels when 50 µM d-cis diltiazem was used; Table 1). It appears, therefore, that the use of *d-cis* diltiazem together with thiamine offers further protection compared to use of individual drugs only at a high diltiazem concentration. Whether these observations apply to animal models or clinical scenarios is presently not known and needs to be investigated.

In summary, our work shows that: 1) *d-cis* diltiazem and not *l-cis* diltiazem protects hepatic microsomes from lipid peroxidation; 2) thiamine inhibits microsomal lipid peroxidation; and 3) combining thiamine and diltiazem at high doses further enhances protection against lipid peroxidation compared to the use of either compound alone.

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