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Effect of Ca^{2+} Channel Blockers, External Ca^{2+} and Phospholipase A_2 Inhibitors on t-butylhydroperoxide-induced Lipid Peroxidation and Toxicity in Rat Liver Slices

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Objectives : *This study was undertaken to examine the effect of oxidant on lipid peroxidation and lethal cell injury in rat liver slices.*

Methods : *t-Butylhydroperoxide (t-BHP) was employed as a model of an oxidant. The lipid peroxidation and lethal cell injury were estimated by measuring the formation of malondialdehyde (MDA) and lactate dehydrogenase (LDH) release, respectively.*

Results : *t-BHP increased lipid peroxidation and LDH release in a dose-dependent manner over concentrations of 0.5-10mM. t-BHP-induced lipid peroxidation was completely prevented by an antioxidant, N,N-diphenyl-p-phenylenediamine (DPPD), but LDH release was partially decreased. Both t-BHP-induced lipid peroxidation and LDH release were significantly protected by iron chelator, deferoxamine, sulfhydryl reducing agent, dithiothreitol and glutathione. Ca^{2+} channel blockers, verapamil, diltiazem and nifedipine exerted a significant protective effect against t-BHP-induced lipid peroxidation and LDH release. By contrast, addition of external Ca^{2+} chelator, ethylene glycol bis(b-aminoethyl ether)-N,N-tetraacetic acid (EGTA) did not alter t-BHP-induced lipid peroxidation, whereas t-BHP-induced lethal cell injury was significantly prevented. Phospholipase A_2 (PLA_2) inhibitors, mepacrine and butacaine produced a partial protective effect.*

Conclusions : *These results suggest that t-BHP induces cell injury by lipid peroxidation-dependent and -independent mechanisms which can be partially prevented by Ca^{2+} channel blockers and PLA_2 inhibitors.*

Key Words : *Oxidant, Lipid peroxidation, Ca^{2+} channel blockers, Phospholipase A_2 inhibitors*

INTRODUCTION

Oxygen free radicals have been considered to be responsible for the pathogenesis of carcinogenesis, aging, ischemia/reperfusion injury and tissue injuries by certain xenobiotics and anticancer drugs[1]. All aerobic cells generate, enzymatically or nonenzymatically, oxygen free radicals such as superoxide, hydrogen peroxide and, probably, hydroxyl radicals during normal and abnormal metabolic processes from the metabolism of exogenous drugs and toxins. At the same time, the abundant antioxidant defenses of most cells prevent oxygen free radical-induced cell injury. Nevertheless, when the rate of oxygen free radical generation is increased and/or the anti-

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oxidant defenses of the cells are weakened, oxidative cell injury would result[2].

Exposure of isolated hepatocytes to oxidants, such as t-butylhydroperoxide (*t*-BHP) or H_2O_2, results in peroxidation of membrane lipids and a rapid loss of cell viability[3-6]. Lipid peroxidation has been recognized to be an important mediator of oxygen free radical-induced cell injury. Nevertherless, the role of lipid peroxidation in hepatocyte injury is controversial. Masaki et al. reported that t-BHP causes cell death by a mechanism that depends on the peroxidation of cellular lipids in cultured hepatocytes[6]. By contrast, Rush et al. reported that lipid peroxidation does not play a critical role in the acute toxicity of t-BHP in isolated hepatocytes[3]. t-BHP can be metabolized to free radicals by iron to result in the formation of the t-butyl alkoxyl radical. This radical can initiate the peroxidation of cellular lipids which is responsible for the loss of cell viability. Alternatively, the t-butyl alkoxyl radical may cause cell injury by lipid peroxidation-independent mechanism. In the latter case, lipid peroxidation could be induced as a consequence rather than as a cause of cell death or as epiphenomenon accompanying lethal attack on the cell[6]. Thus, the role of lipid peroxidation in the underlying mechanism of *t*-BHP-induced cell injury is not clearly defined.

Studies *in vitro* have shown that oxidants induce an increase in intracellular Ca^{2+} concentration in myocytes[7] and hepatocytes[4, 8]. This rise in intracellular Ca^{2+} mediates the cell injury associated with an acute oxidative stress[5, 9]. Several studies demonstrated that the mobilization of Ca^{2+} from intracellular stores or an inhibition of the Ca^{2+} extrusion pump of the plasma membrane are the major mechanisms responsible for the elevated cytosolic Ca^{2+} concentration[5, 10]. On the other hand, Ca^{2+} fluxes in hepatocytes seem to be, at least in part, regulated by Ca^{2+} channels[11, 12], and the cytoprotective effect of Ca^{2+} channel blockers has been documented by various heptotoxins[13, 14]. However, it has not been known that Ca^{2+} channel blockers exert a protective effect against oxidant-induced liver cell injury.

Elevated intracellular Ca^{2+} by oxidants may initiate a cascade of signaling leading to activation of phospholipase A_2(PLA_2) resulting in cell injury[9]. In fact, previous in vitro studies have also showed that PLA_2 inhibitors attenuated oxidant-induced cell injury in renal cells[15]. However, it is unclear whether similar results could appear in hepatocytes.

This study was undertaken to determine whether Ca^{2+} channel blockers, modulation of external Ca^{2+} and PLA_2 inhibitors affect *t*-BHP-induced cell injury in rat liver slices. The present study demonstrated that LDH release and lipid peroxidation induced by *t*-BHP are significantly prevented by Ca^{2+} channel blockers or PLA_2 inhibitors, and oxidant-induced cell injury does not necessarily result from lipid peroxidation.

MATERIALS AND METHODS

1. Slice preparation

Liver slices were prepared from male Sprague-Dawley rats weighing 150-200g. Livers were rapidly removed and placed in ice-cold isotonic saline solution containing 140mM NaCl, 10mM KCl and 1.5mM $CaCl_2$. Liver slices (approximately 1 cm size and 0.4-0.5mm thick) were prepared using a Stadie-Riggs microtome and were stored in an ice-cold medium containing 130mM NaCl, 10mM KCl, 1.5mM $CaCl_2$, 5mM glucose and 20mM Tris/HCl (pH 7.4). Slices were preincubated for 30 min and treated for 60 min with *t*-BHP in the presence or absence of various drugs at 37℃ under a 100% oxygen atmosphere in a Dubnoff metabolic incubator with slow agitation. After incubation, lactate dehydrogenase (LDH) and lipid peroxidation were measured.

2. Measurement of LDH release

Irreversible cell injury was evaluated by measuring LDH release. Liver slices were homogenized in 2ml of distilled water and centrifuged at 1,000rpm for 5 min. The pellet was discarded and the supernatant was used. LDH activities in the supernatant and incubation medium were determined using LDH measurement kit (Iatron Lab., Japan).

3. Measurement of lipid peroxidation

Lipid peroxidation was estimated by measuring the tissue content of malondialdehyde (MDA)

according to the method of Uchiyama and Mihara[16]. Slices were homogenized in ice-cold 1.15% KCl (5% wt/vol). A 0.5ml of homogenate was added to 3ml of 1% phosphoric acid and 1ml of 0.6% thiobarbituric acid. The mixture was heated for 45 min on a boiling water bath. After addition of 4ml of n-butanol, the contents were vigorously vortexed and centrifuged at 2,000g for 20min. The absorbance of the upper, organic layer was measured at 535 and 520nm with diode array spectrophotometer (Hewelett Packard, 8452A), and was compared to results obtained using freshly prepared malondialdehyde tetraethylacetal standard. MDA values were expressed pmoles per mg protein. Protein was measured by the method of Bradford[17].

4. Chemicals

t-Butylhydroperoxide (*t*-BHP), verapamil, diltiazem, nifedipine, mepacrine, butacaine, glutathione (GSH), dithiothreitol (DTT), ethylene glycol bis (b-aminoethyl ether)-*N,N*-tetraacetic acid (EGTA) and malondialdehyde tetraethylacetal were purchased from Sigma Chemical (St. Louis, MO). N,N-diphenyl-p-phenylenediamine (DPPD) was purchased from Aldrich Chemical (Milwaukee WI). All other chemicals were of the highest commercial grade available.

5. Statistical analysis

The data are expressed as the mean±SE and evaluated for significance using Student's t-test. A probability level of 0.05 was used to establish significance.

RESULTS

The exposure of *t*-BHP to liver slices resulted in an increase of lipid peroxidation in a dose-dependent manner (Fig. 1A). Similar results were observed in LDH release (Fig. 1B). Thus, there is close correlation between t-BHP-induced lipid peroxidation and LDH release (Fig. 2).

In order to determine whether antioxidant could prevent lipid peroxidation as well as *t*-BHP-induced cell injury as estimated by LDH release, the effect of a phenolic antioxidant, DPPD, was examined. As shown in Fig. 3, 20mM DPPD

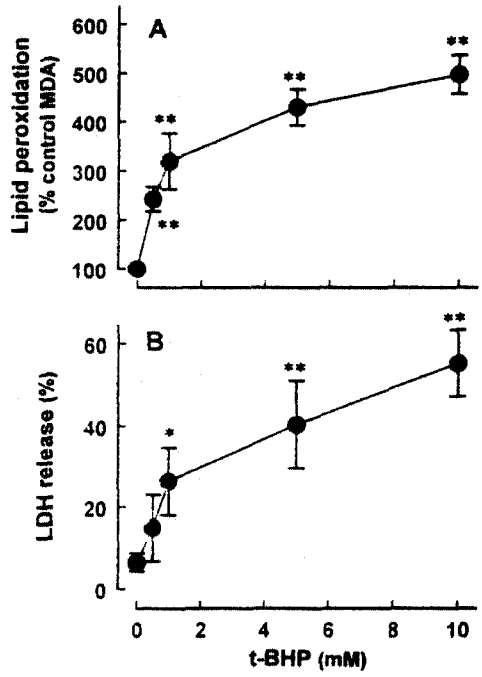

Fig. 1. Effect of various concentrations of *t*-BHP on lipid peroxidation (A) and LDH release (B) in rat liver slices. Data are mean±SE of four experiments. *p<0.05, **p<0.01 compared with the control in the absence of *t*-BHP.

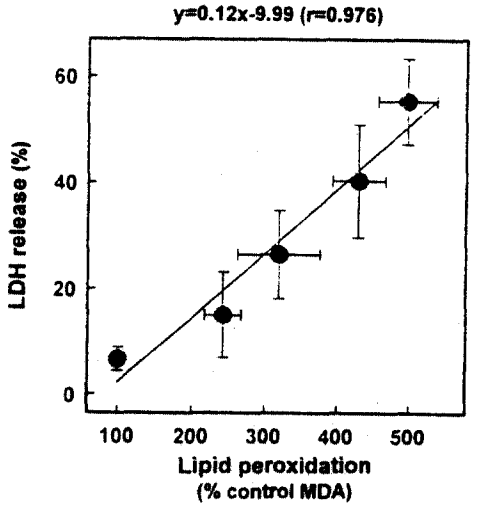

Fig. 2. Relationship between *t*-BHP-induced lipid peroxidation and LDH release. Data are obtained from Fig. 1.

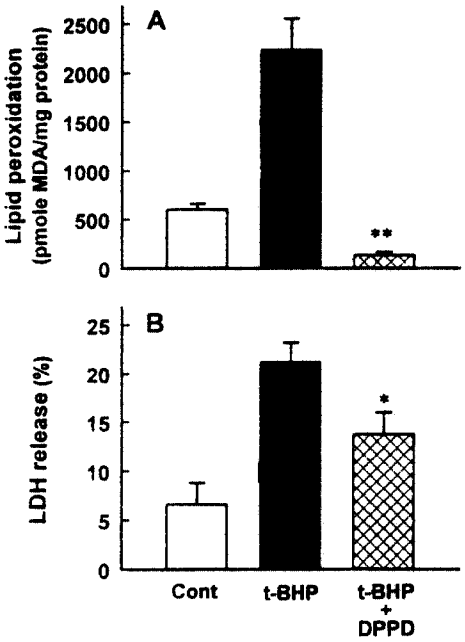

Fig. 3. Effect of DPPD on *t*-BHP-induced lipid peroxidation (A) and LDH release (B). Liver slices were treated with 1 mM *t*-BHP for 60 min at 37℃ in the presence or absence of 20 mM DPPD. Data are mean±SE of four experiments. *p<0.05, **p<0.01 compared with *t*-BHP alone.

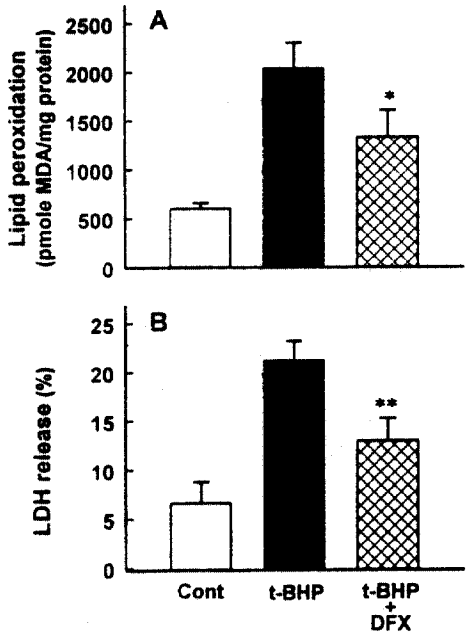

Fig. 4. Effect of iron chelator on *t*-BHP-induced lipid peroxidation (A) and LDH release (B). Liver slices were treated with 1 mM t-BHP for 60 min at 37℃ in the presence or absence of 2 mM deferoxamine. Data are mean±SE of four experiments. *p<0.05, **p<0.01 compared with *t*-BHP alone.

exerted a siginficant protective effect against lipid peroxidation and LDH release caused by 1mM *t*-BHP. However, the extent of protective effect on LDH release was less than that on lipid peroxidation. *t*-BHP-induced lipid peroxidation was completely prevented by DPPD, whereas *t*-BHP-induced LDH release was partially (although significantly) reduced.

t-BHP reacts with ferrous iron to produce a more potent oxidant, the t-butyl alkoxyl radical[6, 8]. Thus, the effect of iron chelator was examined to ascertain if iron chelator could prevent both lipid peroxidation and LDH release by t-BHP. Slices were pretreated for 10min with 2mM deferoxamine before treatment of *t*-BHP. The results depicted in Fig. 4 indicated that the lipid peroxidation and LDH release induced by *t*-BHP were siginficantly decreased by pretreatment of deferoxamine.

Fig. 5 shows the effect of a sulfhydryl reducing agent, DTT, and GSH on *t*-BHP-induced lipid peroxidation and LDH release. Addition of 2mM DTT completely protected against the lipid peroxidation and LDH release caused by 1 mM *t*-BHP. Likewise, both *t*-BHP-induced lipid peroxidation and LDH release were markedly prevented by 2mM GSH.

Sippel et al. reported in liver cells that Ca^{2+} channel blockers exert a protective effect against cell death by 98/202 which causes cell injury through a disturbance of intracellular calcium homeostasis[14]. Therefore, effects of Ca^{2+} channel blockers on *t*-BHP-induced lipid peroxidation and LDH release were examined. As shown in Fig. 6A, *t*-BHP- induced lipid peroxidation was partially but significantly reduced by addition of diltiazem, nifedipine or verapamil.

In order to determine whether depletion of external Ca^{2+} affects *t*-BHP-induced cell injury, effects of Ca^{2+}-free medium and the external Ca^{2+} chelator EGTA on *t*-BHP-induced lipid peroxi-

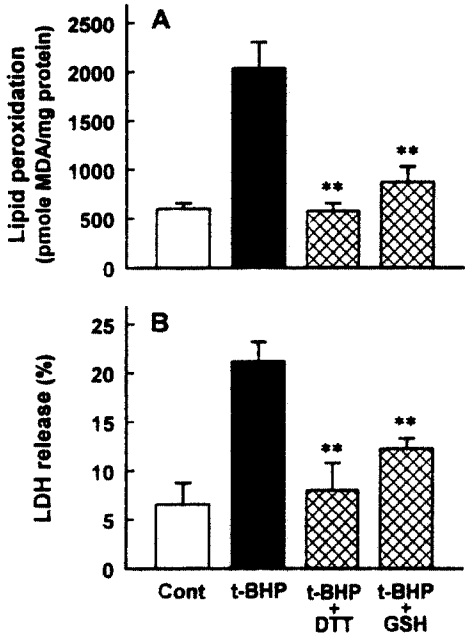

Fig. 5. Effect of DTT and GSH on *t*-BHP-induced lipid peroxidation (A) and LDH release (B). Liver slices were treated with 1 mM *t*-BHP for 60 min at 37℃ in the presence or absence of 2 mM DTT or GSH. Data are mean±SE of four experiments. **p<0.01 compared with t-HP alone.

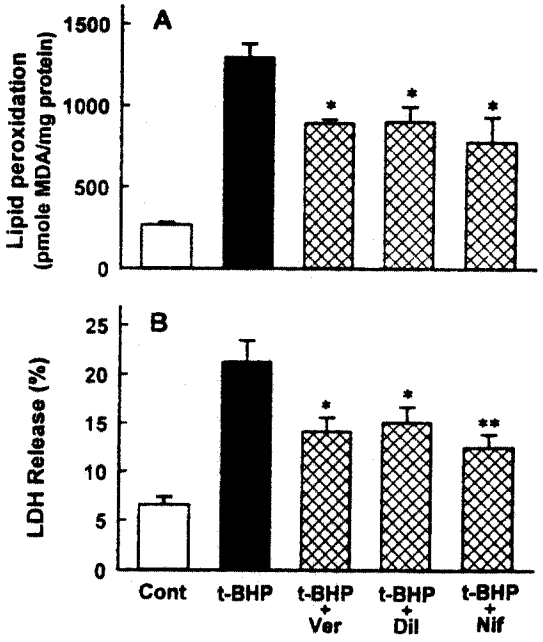

Fig. 6. Effect of Ca^{2+} channel blockers on *t*-BHP-induced lipid peroxidation (A) and LDH release (B). Liver slices were treated with 1 mM *t*-BHP for 60 min at 37℃ in the presence or absence of Ca^{2+} channel blockers (0.1 mM), verapamil(Ver), diltiazem (Dil) or nifedipine (Nif). Data are mean±SE of five experiments. *p< 0.05 compared with *t*-BHP alone.

dation and LDH release was examined. As shown in Fig. 7, when slices were exposed to Ca^{2+}-free medium in the absence of *t*-BHP, there was a siginficant increase in LDH release and a partial but nonsignificant increase in lipid peroxidation. However, both lipid peroxidation and LDH release induced by *t*-BHP rather decreased in the Ca^{2+}-free medium as compared with those in the normal medium, although the difference was nonsignificant. The results depicted in Fig. 8 indicated that *t*-BHP-induced lipid peroxidation was not significantly altered by the addition of 2mM EGTA, whereas *t*-BHP-induced LDH release was significantly reduced by EGTA.

Since previous in vitro studies have showed that PLA_2 activation plays a role in the pathogenesis of cell injury by oxidants or ischemia in various cell types[15, 18-20], it was examined whether if *t*-BHP-induced lipid peroxidation and LDH release could be protected by PLA_2 inhibitors. The results are depicted in Fig. 9. Mepacrine and butacaine at 0.25mM concentration exerted a significant protective effect against both *t*-BHP-induced lipid peroxidation and LDH release. The treatment of liver slices with PLA_2 inhibitors in the absence of t-BHP did not induce liver cell toxicity (data not shown).

DISCUSSION

Although there is an increasing recognition of the importance of oxygen free radicals in cell injury, the exact mechanisms or sequence of events by which cells sustain such injury are not clearly defined. Although lipid peroxidation has been considered to be an important mediator of certain deleterious effects of oxygen free radicals in cells, it is not clear whether the cell injury with acute oxidative stress resulted totally from lipid

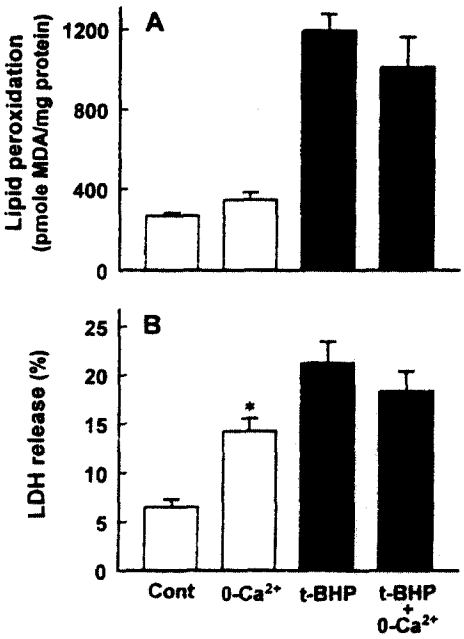

Fig. 7. Effect of external Ca^{2+} depletion on *t*-BHP-induced lipid peroxidation (A) and LDH release (B). Liver slices were treated with 1 mM *t*-BHP for 60 min at 37℃ in the normal or Ca^{2+}-free medium. Data are mean±SE of four experiments. *p<0.05 compared with the control of normal Ca^{2+} concentration.

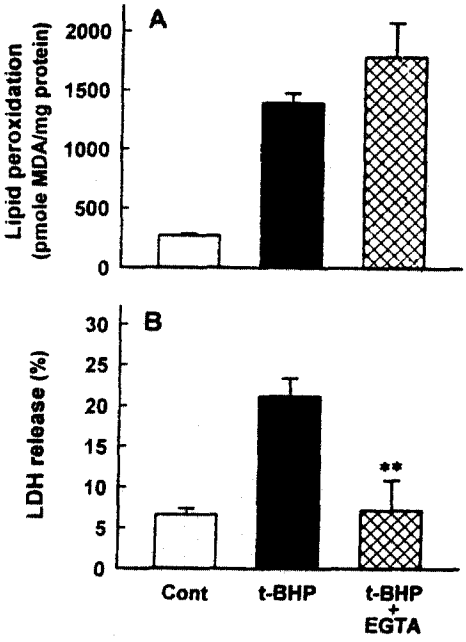

Fig. 8. Effect of external Ca^{2+} chelator on t-BHP-induced lipid peroxidation (A) and LDH release (B). Liver slices were treated with 1 mM *t*-BHP for 60 min at 37℃ in the presence or absence of 2 mM EGTA. Data are mean±SE of four experiments. *p<0.05 compared with *t*-BHP alone.

peroxidation. In liver cells, Masaki et al. proposed that the lipid peroxidation plays a critical role in *t*-BHP-induced cell injury[6]. However, Rush et al. observed that the antioxidant completely blocked the formation of MDA in hepatocytes exposed to t-BHP but had no effect on cell injury or the morphological changes, suggesting that lipid peroxidation does not play an important role in the toxicity of *t*-BHP[3]. As shown in Fig. 10, lipid peroxidation could appear as a consequence of cell injury rather than a cause of cell injury.

In the present study, DPPD completely prevented *t*-BHP-induced lipid peroxidation, whereas *t*-BHP-induced LDH release was partially reduced by the same concentration of DPPD(Fig. 3). *t*-BHP-induced lipid peroxidation was not altered by the addition of an external Ca^{2+} chelator EGTA, but LDH release was significantly reduced(Fig. 8). These results may indicate that lipid peroxidation is not a primary mediator for *t*-BHP-induced cell injury in hepatocytes. This supports the reports of Farber et al. that oxidant-induced cell injury can develop in the absence of detectable lipid peroxidation[21].

Since the cytotoxicity by oxidants is associated with oxidation of the sulfhydryl group, the sulfhydryl reducing agents protect against oxidant-induced cell injury[21]. GSH has been also known to provide a marked protection against oxidant-induced cell injury[22]. As expected, in the present study, DTT and GSH significantly decreased *t*-BHP-induced lipid peroxidation as well as LDH release (Fig. 5).

Although the *in vivo* and *in vitro* studies have reported that Ca^{2+} channel blockers attenuate the hepatocellular damage by various hepatotoxins[13, 14, 23-25], it has not been known that Ca^{2+} chennal blockers are benefical on oxidant-induced liver cell injury. In the present study, verapamil, diltiazem

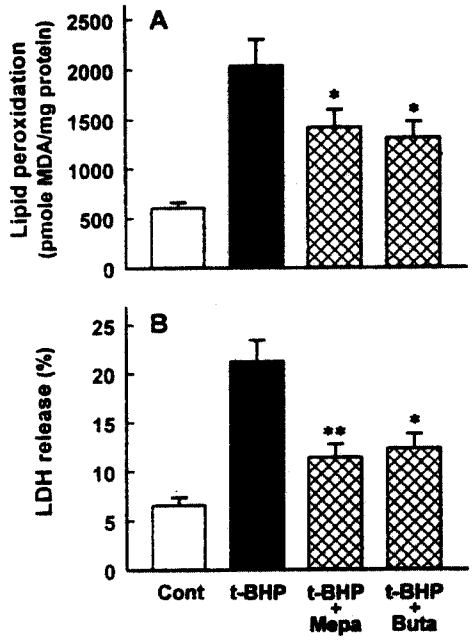

Fig. 9. Effect of PLA2 inhibitor on *t*-BHP-induced lipid peroxidation (A) and LDH release (B). Liver slices were treated with 1 mM *t*-BHP for 60 min at 37℃ in the presence or absence of 0.25 mM mepacrine (Mepa) or butacaine (Buta). Data are mean±SE of four experiments. *$p<0.05$ compared to *t*-BHP alone.

and nifedipine exerted significant protective effect against t-BHP-induced lipid peroxidation and LDH release (Fig. 6). However, it is unclear that such effects are associated with reduction in the influx of extracellular Ca^{2+} and changes in intracellular Ca^{2+} concentration were not determined in the present study. Since nonspecific action of Ca^{2+} channel blockers have been suggested to involve membrane stabilizing effect[26, 27], these agents could exert protective effect without inducing alterations in Ca^{2+} influx. Thus, the precise mechanisms of protective effect by Ca^{2+} channel blockers remain to be determined.

Although the oxidative stress has been reported to be associated with the mobilization of Ca^{2+} from intracellular stores[5, 10], several studies have proposed that increased Ca^{2+} influx across the plasma membrane is essential for the pathogenesis of cell injury and death induced by various chemical agents (Schanne et al., 1979; Kane et al., 1980). In the present study, it was examined whether modulation of external Ca^{2+} affects *t*-BHP-induced liver cell injury. When control slices untreated with *t*-BHP were incubated in the Ca^{2+}-free medium for 60min, LDH release significantly increased (Fig. 7). If oxidant-induced cell injury was not affected by Ca^{2+} depletion, cell injury would be increased by both *t*-BHP and Ca^{2+} depletion as compared with *t*-BHP alone. However, the present study indicated that *t*-BHP-induced lipid peroxidation and LDH release did not more increase in the Ca^{2+}-free medium than those in the normal Ca^{2+} medium. When slices were treated with *t*-BHP in the presence of EGTA, LDH release but not lipid peroxidation induced by *t*-BHP significantly decreased (Fig. 8). These results indicate that the influx of external Ca^{2+} across the plasma membrane may play a role in oxidant-induced liver cell injury. The induction of cell injury by Ca^{2+} depletion was demonstrated in other previous studies[28].

Several *in vitro* studies have also reported that oxidant-induced cell injury is prevented by PLA_2 inhibitors in liver cells[29, 30]. The present study showed that t-BHP-induced lipid peroxidation and LDH release also decreased by mepacrine and butacaine(Fig. 9). These results suggest that oxidant-induced toxicity of liver cells may be, at least in part, associated with PLA_2 activation.

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