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Research Article

The Protection of Hepatocyte Cells from the Effects of Oxidative Stress by Treatment with Vitamin E in Conjunction with DTT

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We investigated the effect of vitamin E on membrane protein thiols under oxidative stress, which we induced by treating hepatocytes with *tert*-butyl hydroperoxide (TBH) for 60 mins. Those cells which we pretreated with vitamin E formed fewer blebs (22.3% compared to 60.0% in nonvitamin E-treated cells) and maintained cytosolic calcium concentration and the number of membrane protein thiols instead of showing the usual symptoms in cells undergoing oxidative stress. Dithiothreitol (DTT) also commonly reduces bleb formation in hepatocytes affected by TBH. However, our experiments clearly demonstrate that DTT does not prevent the changes in cytosolic calcium and membrane protein thiols in the blebbing cells. Consequently, we decided to pretreat cells with both DTT and vitamin E and found that the influence of TBH was entirely prevented. These findings may provide us with a new aspect for investigating the mechanism of bleb formation under oxidative stress.

1. Introduction

Formation of blebs on the surface of hepatocytes is not only an early sign of toxic injury under ischemic conditions or oxidative stress but also has a significant association with apoptosis or necrosis [1]. This morphological abnormality has been attributed to a change in intracellular calcium homeostasis [2, 3]. An increase in the concentration of intracellular calcium may induce a series of calcium-dependent reactions catalyzed by the calcium-dependent proteases, phospholipases, or endonucleases [4]. These enzymes may disrupt the integrity of the cytoskeleton and lead to bleb formation and growth. The course of plasma membrane blebbing on hepatocytes has been divided into three stages: formation, shedding and fusion, and finally rupture [5]. Injuries to hepatocytes in the first two stages are reversible whereas bleb rupture is irreversible and results in cell lysis [6].

In addition to its nutritional importance, vitamin E (α-tocopherol) is also a natural antioxidant which can prevent lipid peroxidation in cellular and subcellular membrane phospholipids under oxidative stress [7]. Lipid peroxidation may cause damage of the plasma membrane and an increase in the number of cytosolic-free calcium ions. This can result in the change in the verapamil and nifedipine-sensitive Ca^{2+} channels [8] or an increased possibility of arachidonic acid-induced toxicity of CYPE1-expressing cells [9]. This increase in cytosolic Ca^{2+} concentration can be prevented by vitamin E; moreover, we have also demonstrated that vitamin E may prevent bleb formation and the loss of protein thiols in *tert*-butyl hydroperoxide-(TBH-) treated hepatocytes [10, 11]. Since vitamin E only protects protein thiols which have been depleted due to interaction with endogenously generated lipid peroxidation products [12], it is possible that the attenuation of plasma membrane protein thiols may be related to the maintenance of intracellular calcium homeostasis.

In order to determine the role of vitamin E in this mechanism, we employed confocal microscopy, high-pressure liquid chromatography (HPLC), and spectrophotometry to investigate the changes in the concentration of intracellular calcium ions and the number of plasma membrane protein thiols of rat hepatocytes under oxidative stress induced by TBH.

2. Materials and Methods

2.1. Isolation and Culture of Hepatocytes. All animal experiments were conducted with approval from Chung Shan Medical University Animal Care and Use Committee. Male Sprague-Dawely rats (8 weeks) were purchased from the National Animal Breeding and Research Center, Taipei, Taiwan. Hepatocytes were isolated from the liver of these animals by collagenase perfusion [10], and >90% were found to be viable by the trypan blue exclusion test. The cells were then plated to collagen-precoated 30-mm plastic tissue culture dishes (Falcon Labware, USA) with a total of 0.7×10^6 cells in L-15 culture medium (pH 7.6) containing 18 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 2.5% fetal bovine serum, 5 mg/L each of insulin and transferrin, 5 μg/L sodium selenite, 1 g/L galactose, 1 μmol/L dexamethasone, 100,000 IU/L penicillin, and 100 mg/L streptomycin. After culturing in a 37°C humidified incubator in ambient air for 4 hours, unattached and dead cells were removed from the culture. The cells were then cultured in the L-15 culture medium with 0.2% bovine serum albumin without fetal bovine serum at 37°C for 4 hours; cells were incubated at 37°C without treatment or treated with 100 μM vitamin E for 20 hours. Cultures with vitamin E treatment were then treated with 5 mM dithiothreitol (DTT) for 15 min or without this treatment. Those without vitamin E treatment were treated with 5 mM DTT and/or 15 mM ethylene glycol tetraacetic acid (EGTA) for 15 min or without any treatment. These cultures were treated with indicated concentrations of TBH, and changes in the cells were detected.

2.2. Confocal Microscopy. Alternations in intracellular calcium were determined by confocal microscopy with a calcium-sensitive fluorescent dye (fluo 3-AM) and video microscopic imaging using the method of Burnier et al. [13] with modifications. Fluo 3-AM (5 μM) was added to culture medium, and the hepatocytes were incubated at 37°C for 30 min in the dark. The pluronic acid (2 μL/mL) was added to fluo 3-AM for dispersing the dye. After removing the culture medium, the cells were washed with L-15 culture medium without bovine serum albumin and then cultured with 1 mL of this medium in a 30-mm culture dish.

After labeling with fluo 3-AM, the culture dish was placed into a thermostatic stage maintained at 37°C. Hepatocytes with various treatments or without treatment were scanned under a confocal microscope (LSM 410 invert, Zeiss, Germany). Confocal microscopy was performed according to the procedures as previously described [13].

2.3. Cell Morphology Examination. Tissue culture dishes were placed on a heated microscope stage (37°C). Following the addition of TBH, cell membrane bleb formation was monitored under a phase-contrast inverted microscope (Nikon, Tokyo, Japan) equipped with a CCD camera monitor. The percentage of hepatocytes-bearing blebs was determined on pictures that were taken at 15, 30, 45, and 60 min, respectively. At least 150 cells were counted in each analysis. The percentage of cells-bearing blebs was used to express the extent of membrane blebbing in each group.

2.4. High-Pressure Liquid Chromatography. These cells were allowed to stand for 30 min to dissolve glutathione (GSH) into perchloric acid. To the acid solvent containing GSH (400 μL), 40 μL iodoacetic acid (120 mg/mL) and potassium bicarbonate ($KHCO_3$) were added and placed in the dark for 15 min before adding 440 μL 3% 2, 4-dinitrofluoro benzene in ethanol. The mixture was then vigorously shaken and stored at 4°C for 8 hours. After centrifuged at $6{,}000 \times g$ for 5 min, the supernatant was filtered through a 0.45-μm filter. Concentrations of GSH were determined by HPLC using the method as previously described [14].

2.5. Spectrophotometry. To determine lipid peroxidation, hepatocytes were washed twice with cold phosphate-buffered saline (PBS, pH 7.4) after removal of the culture medium. The cells were extracted with 200 μL of 50 mM potassium phosphate buffer (pH 7.4). Lipid peroxidation was determined as thiobarbituric acid reactive substances (TBARS) [15]. The fluorescence of the samples was detected at an excitation wavelength of 515 nm and an emission wavelength of 555 nm in a F4500 fluorescence spectrophotometer (Hitachi, Japan) and 1, 1, 3, 3-tetramethoxypropane was used as a TBARS standard.

For the determination of membrane protein thiols, the hepatocytes were washed twice with PBS, and 600 μL of 20 mM potassium phosphate buffer (pH 7.4) was added, after removing the culture medium. The cells were then scraped and centrifuged at $800 \times g$ for 10 min. The supernatant was centrifuged again at $105000 \times g$ to obtain the cytosolic fractions (supernatant) and the membrane fractions (pellet). The membrane fractions were then mixed thoroughly with the same buffer (800 μL) containing 5% SDS. The total membrane protein thiols were measured after the incubation with 5,5′-dithio-bis-nitrobenzoic acid as previously described [16], and the total protein concentrations were determined by the method as previously described [17]. To express the cell viability, the lactate dehydrogenase (LDH) leakage was analyzed according to the method as previously described [18].

2.6. Statistical Analysis. Data were expressed as mean ± standard deviation. Significant differences among the groups were analyzed by one-way analysis of variance. Duncan's multiple tests were used to determine the difference among groups, and Student's t-test was used in case of the two group comparison. $P < .05$ was considered to have statistical significance.

3. Results

3.1. Initiation of Hepatocyte Blebbing by TBH and Changes in the Intracellular Calcium. Under the confocal microscope, the locations of blebs observed under the transmission mode corresponded to their intensities (Figures 1(a), 1(c), 1(e), 1(g), and 1(i)). The fluorescence intensity from the hepatocytes treated with 2.0 mM TBH increased with time (Figures 1(b), 1(d), 1(f), 1(h), and 1(j)), such as cell a, b, c, d, and e of Figure 1(b) whose concentration of intracellular calcium rapidly increased from 12 min and reached the maximum at 18 min (Figure 2). A significant increase in fluorescence intensity and a bleb in a hepatocyte were observed at 18 min after TBH treatment, as arrow indicated (Figures 1(e) and 1(f)). These changes became more severe at 30 min, as arrow indicated (Figures 1(g) and 1(h)). The fluorescence intensity disappeared at 60 min because of bleb rupture (Figures 1(i) and 1(j)).

3.2. Effects of Vitamin E on the Intracellular Calcium in TBH-Treated Hepatocytes. In hepatocytes treated with 1.0 or 2.0 mM TBH for 60 min under a phase-contrast inverted microscope, 18% ± 4.2% ($n = 3$) or 60.6% ± 1.1% ($n = 4$), respectively, formed blebs on the cell membrane. These phenomenons were similar to the observation of bleb formation from confocal microscope. Significantly lower percentage of 22.3% ± 4.2% ($n = 4$) in 2.0 mM TBH-treated hepatocytes was obtained by the pretreatment with vitamin E ($P < .05$). Moreover, pretreatment with EGTA in 2.0 mM TBH-treated hepatocytes also yielded a significantly lower of 27.4 ± 5.8 ($n = 4$). However, no significant differences were found between the pretreatment with vitamin E and EGTA ($P > .05$).

Although the fluorescence response in 1.0 mM TBH-treated hepatocytes was not observed (data not shown), the positive response was detected at 12 min after treatment with 2.0 mM TBH (control) and increased to 2 folds at 18 min and gradually decreased from 40 min. In 2.0 mM TBH-treated hepatocytes pretreated with vitamin E, the response was in a steady level and significantly lower than control in the middle period. Whereas, pretreated with EGTA in 2.0 mM TBH-treated hepatocytes, the concentration of intracellular calcium was gradually decreased from 15 min, and to zero at 30 min (Figure 3(a)).

3.3. Effects of Vitamin E and DTT on the Intracellular Calcium in TBH-Treated Hepatocytes. In addition to vitamin E, DTT is also an important member of the antioxidative agent. Pretreatment with DTT significantly decreased the percentage of blebbing from 62.2% ± 1.2% in the hepatocytes only treated with 2 mM TBH for 60 min to 25.0% ± 2.2% ($P < .05$). However, after adding vitamin E with DTT to the TBH-treated cells, the blebbing percentage was significantly reduced to zero.

The concentration of intracellular calcium response from the 2.0 mM TBH-treated cells with pretreatment of DTT increased with time in the blebbing cells but no significant difference was found in the prior period (Figure 3(b)).

3.4. Effects of Vitamin E and DTT on Total Glutathione (GSH), LDH Leakage, and Lipid Peroxidation in TBH-Treated Hepatocytes. Intracellular total GSH concentration significantly decreased after treating the hepatocytes with 1.0 or 2.0 mM TBH for 60 min, although the GSH concentration in 2.0 mM TBH-treated cells was significantly lower than that of the 1.0 mM TBH-treated ones. Pretreatment with vitamin E or DTT maintained GSH in 2.0 mM TBH-treated hepatocytes; the levels of GSH were significantly lower than those of the untreated group. However, there was no significant difference in the GSH level between the vitamin E plus DTT-treated group and the untreated group (Table 1).

The levels of LDH leakage in hepatocytes treated with 1.0 or 2.0 mM TBH, EGTA and 2.0 mM TBH, or DTT and 2.0 mM TBH were significantly higher than the untreated group. However, there was no significant difference in the leakage between the untreated group and 2.0 mM TBH-treated cells with pretreatment of vitamin E or vitamin E plus DTT (Table 1).

Lipid peroxidation was measured by TBARS production in hepatocytes. TBARS production was significantly higher in the cells treated with 1.0 or 2.0 mM TBH, DTT or EGTA with 2.0 mM TBH than the untreated group. However, there was no significant difference in the production between 2.0 mM TBH-treated cells with the untreated group and pretreatment of vitamin E or vitamin E plus DTT (Table 1).

3.5. Effects of Vitamin E and DTT on the Loss of Membrane Protein Thiols Induced by TBH. In both membrane and cytosol, the levels of membrane protein thiols in hepatocytes treated with 1.0 or 2.0 mM TBH or EGTA and 2.0 mM TBH for 60 min were significantly lower than the untreated group. In the presence of vitamin E, there was no significant difference in the level of protein thiols of the membrane fraction, whereas this level remained significantly lower than the untreated group in the cytosolic fraction. In the pretreatment with DTT in 2.0 mM TBH-treated hepatocytes, although the levels of protein thiols of the membrane fraction were significantly lower than these of the control, there was no significant difference in the cytosolic fraction. However, no significant difference was found in the level of protein thiols of both the membrane and cytosolic fractions in the vitamin E plus DTT pretreated cells (Table 2).

In the cells without the supplement of vitamin E, treatment of 2.0 mM TBH caused a rapid loss of the membrane protein thiols and 37% of the thiols were lost within 15 min. The percentage of loss then became less severe after 15 min, and a total loss of 41% was observed at 60 min after TBH treatment. In the presence of vitamin E, the percentage of loss was also more severe in the first 15 min. However, the total loss of thiols was only 15% at 60 min.

4. Discussion

The formation of blebs in TBH-treated hepatocytes has been attributed to the elevation of intracellular calcium concentration [19, 20]. Using confocal microscopy, we visually demonstrated the important role of intracellular

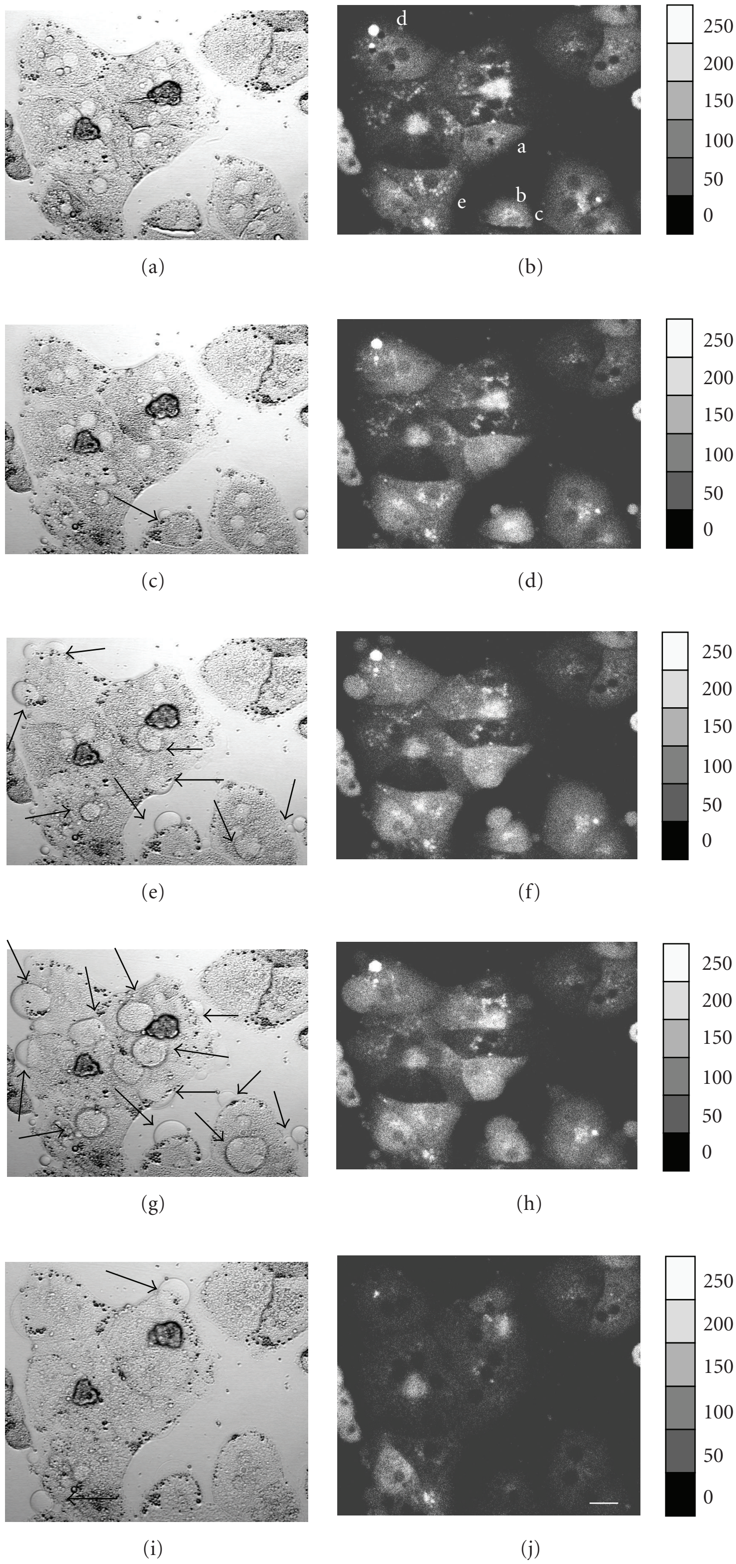

FIGURE 1: Changes in the fluorescence intensity of intracellular calcium in TBH-treated hepatocytes. Using confocal microscopy, the changes of cell morphology were also photographed before (a), 12 (c), 18 (e), 30 (g), and 60 min (i) after 2.0 mM TBH treatment. At the same time, the changes of fluorescence intensity of intracellular calcium were photographed before (b), 12 (d), 18 (f), 30 (h) and 60 min (j) after 2.0 mM TBH treatment. Pseudodensity scale indicates fluorescence intensity in arbitrary units. Arrows indicate the cells with bleb. Bar, 20 μm.

Table 1: Effect of vitamin E and DTT on total GSH content, LDH leakage, and TBARS production in rat hepatocytes with TBH treatment.

Treatment	Total GSH (nmol/mg protein)	LDH leakage (%)	TBARS (nmol/mg protein)
Untreated	47.7 ± 4.5^{a}	1.2 ± 0.6^{a}	0.66 ± 0.09^{ab}
TBH (1.0 mM)	19.5 ± 8.5^{bc}	43.8 ± 7.6^{b}	1.31 ± 0.41^{ac}
TBH (2.0 mM)	4.1 ± 1.2^{d}	76.2 ± 13.8^{c}	3.90 ± 0.31^{d}
Vitamin E (100 μM) + TBH (2.0 mM)	9.1 ± 0.2^{b}	7.6 ± 2.2^{a}	0.41 ± 0.11^{b}
EGTA (15 mM) + TBH (2.0 mM)	2.1 ± 0.1^{d}	62.8 ± 2.2^{c}	2.73 ± 0.51^{e}
DTT (5 mM) + TBH (2.0 mM)	29.7 ± 3.5^{ce}	26.6 ± 1.7^{e}	1.75 ± 0.20^{c}
Vitamin E (100 μM) + DTT (5 mM) + TBH (2.0 mM)	36.9 ± 4.2^{ae}	2.8 ± 1.1^{a}	0.55 ± 0.06^{b}

Values are expressed as means ± SD (n = 3-4). Means in the same column not sharing the same superscripts differ significantly ($P < .05$).

Table 2: Effect of vitamin E and DTT on the loss of membrane protein thiols in TBH-treated hepatocytes 60 min after treatment.

Treatment	Protein thiol level (%)	
	Membrane	Cytosol
Untreated	100^{a}	100^{a}
TBH (1.0 mM)	78.7 ± 4.7^{b}	83.6 ± 6.9^{b}
TBH (2.0 mM)	59.0 ± 8.3^{c}	71.1 ± 7.9^{c}
Vitamin E (100 μM) + TBH (2.0 mM)	85.4 ± 13.2^{a}	76.8 ± 2.9^{c}
EGTA (15 mM) + TBH (2.0 mM)	76.1 ± 3.2^{b}	83.6 ± 2.1^{b}
DTT (5 mM) + TBH (2.0 mM)	75.7 ± 3.1^{b}	96.4 ± 5.5^{a}
Vitamin E (100 μM) + DTT (5 mM) + TBH (2.0 mM)	114.2 ± 8.8^{a}	92.2 ± 10.0^{a}

Values are expressed as mean ± SD (n = 3-4). Means in the same column not sharing the same superscripts differ significantly ($P < .05$).

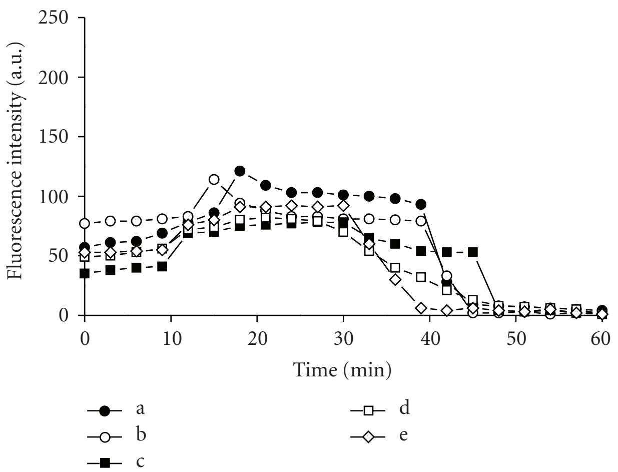

Figure 2: Kinetics of changes in the concentration of intracellular calcium in cell a, b, c, d, and e of Figure 1 before and after treated with 2.0 mM TBH.

calcium in the formation of blebs on the cell membrane of hepatocytes treated with TBH. A significant increase in fluorescence intensity and multiple bleb formation in a single hepatocyte were observed and the intensity of fluorescence was proportional to the size of blebs. By pretreating hepatocytes with EGTA to remove the extracellular calcium, we found that no fluorescence intensity was observed and that the percentage of blebbing significantly decreased from 61% in the control group to 27%. Moreover, treatment with EGTA after bleb formation also reduces the percentage of blebbing. These data confirmed that bleb formation is associated with the increase in concentration of intracellular calcium. However, since lipid peroxidation causes damage of the plasma membrane which results in an increase in the number of cytosolic free calcium ions [8], the prevention of blebbing may be due to the combination of EGTA with the intracellular iron which is required for lipid peroxidation [21]. In order to rule out this possibility, we analyzed the effect of EGTA on the lipid peroxidation caused by TBH and found that EGTA did not decrease lipid peroxidation under oxidative stress. Although EGTA does not affect lipid peroxidation under oxidative stress, treatment with this compound protects TBH-treated cells from death by preventing the increase in concentration of intracellular calcium [22, 23]. These findings confirm that the intracellular calcium increase caused by TBH is exclusively due to calcium influx from the extracellular site [22, 24] and indicate the importance of intracellular calcium in the formation of plasma membrane blebbing.

There is a positive correlation between lipid peroxidation in the membrane and the loss of membrane protein thiols [25]. Our previous study demonstrated that protection of cell morphology by vitamin E is associated with protein thiols [10, 11]. Vitamin E prevents the death of cultured hepatocytes treated with TBH [26, 27]. It has also been reported that calcium accumulation caused by lipid peroxidation is completely prevented by vitamin E [8]. In this study, we demonstrated that vitamin E not only blocks the elevation of intracellular calcium concentration but also prevents the loss of protein thiols in the membrane of TBH-treated hepatocytes. These findings indicate that the integrity of cell membrane conserved by vitamin E may be important in the maintenance of intracellular calcium homeostasis.

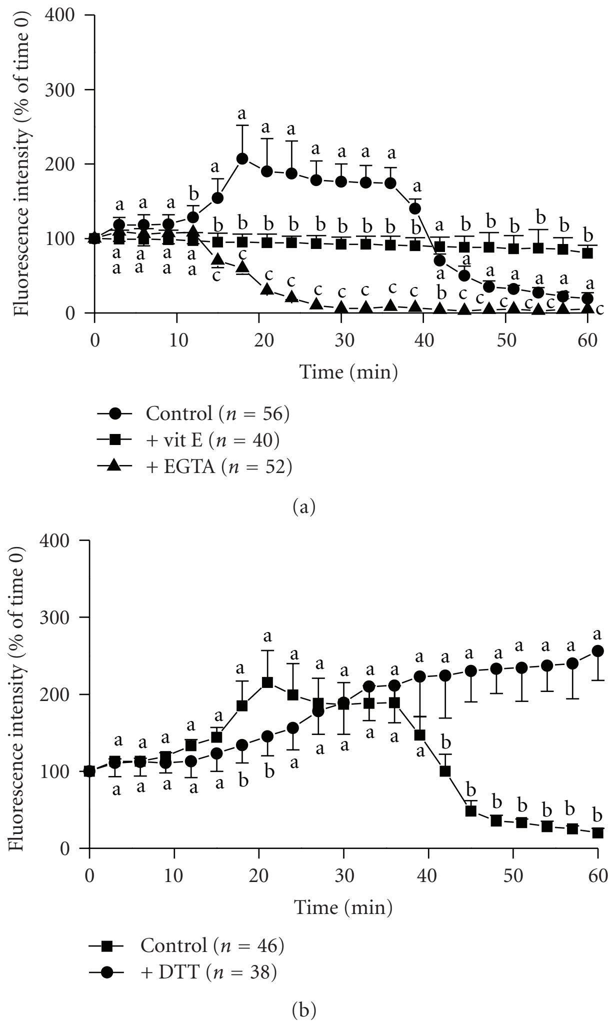

Figure 3: The effect of vitamin E and DTT on the concentration of intracellular calcium in TBH-treated hepatocytes. (a) Changes in concentration of intracellular calcium were determined in the cells treated with 2.0 mM TBH (control), with 2.0 mM TBH by the pretreatment with 100 μM vitamin E for 20 h, or with 15 mM EGTA for 15 min. (b) Changes in concentration of intracellular calcium were determined in the cells treated with 2.0 mM TBH (control) or with 2.0 mM TBH by the pretreatment with 5.0 mM DTT for 15 min. The results were based on three separate experiments, and the values are expressed as mean ± SD. Treatment means in the same time not sharing the same superscripts differ significantly ($P < .05$).

Although there is an association between membrane blebbing and intracellular calcium concentration, blebbing may also be induced by other mechanisms, since blebs were found in 22% of the hepatocytes pretreated with vitamin E, after TBH treatment. It has been reported that the alteration of cytosolic free calcium may not be required for bleb formation [28, 29]. Moreover, Hg^{2+}-treated hepatocytes also form blebs on the cell membrane, and the level of blebbing is independent of the concentrations of intracellular calcium [30]. In this study, we found that although DTT reduces the loss of cytosolic protein thiols and decreases bleb formation in TBH-treated hepatocytes, it can not prevent an increase in the concentration of intracellular calcium in cells which do form blebs. However, pretreatment with both vitamin E and DTT entirely blocks bleb formation, maintains intracellular calcium homeostasis, and prevents total protein thiol loss, lipid peroxidation, and the consumption of GSH. This indicates that plasma membrane blebbing is relatively complex and may be due to many factors.

Although it has been reported that DTT is effective in preserving the homeostasis of intracellular calcium and the integrity of the cell membrane [31, 32], the controversial results may be due to different cell conditions, different time courses and varying treatment doses. Based on the observations in this study, vitamin E specifically prevents the loss of protein thiols in the plasma membrane, while DTT specifically prevents the loss of protein thiols in the intracellular site. Thus, these data indicate that vitamin E may preserve the integrity of the cell membrane by the protection of membrane protein thiols and hence maintain intracellular calcium homeostasis of hepatocytes under oxidative stress. These findings suggest that the different effects of vitamin E and DTT may provide us with a new aspect for investigating the mechanism of bleb formation under oxidative stress and for developing a new preventative strategy.

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