The protection of hepatocyte cells from the effects of oxidative stress by treatment with vitamin E in conjunction with DTT

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Abstract

We investigated the role of vitamin E on those membrane protein thiols under oxidative stress. In hepatocytes treated with TBH for 60 min, 60.6% bore blebs whereas only 22.3% of cells which had also been pre-treated with vitamin E formed blebs. Moreover, both the increase in the concentration of cytosolic calcium and the decrease in membrane protein thiols which are affected by TBH were completely prevented in cells pre-treated with vitamin E. As is widely known, DTT also reduces bleb formation in hepatocytes affected by TBH. However, our experiments clearly demonstrate that DTT is powerless to prevent the changes in cytosolic calcium and membrane protein thiols. Consequently, we decided to pre-treat cells with both DTT and vitamin E and found that in these cells, 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.

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 have a significant association with apoptosis or necrosis [1]. This morphological abnormality has been attributed to the damage of intracellular calcium homeostasis [2, 3]. Increase in the intracellular calcium concentration 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 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 rupture [5]. The injuries to the 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 in the prevention of lipid peroxidation in the cellular and subcellular membrane phospholipids under oxidative stress [7]. Lipid peroxidation may cause the damage of plasma membrane and the increase in the cytosolic free calcium ion resulting in the change of verapamil-and nifedipine-sensitive Ca^{2+} channels [8] or arachidonic acid-induced toxicity to CYPE1-expressing cells [9], which 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 the protein thiol which is depleted by interacting to endogenously generated lipid peroxidation products [12], it is possible that the attenuation of plasma membrane protein thiols modification by vitamin E may be related to the maintenance of intracellular calcium homeostasis. In order to determine

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

Materials and methods

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 h, 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 h, cells were incubated at 37°C without treatment or treated with 100 μM vitamin E for 20 h. 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.

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).

Cell morphology examination

Tissue culture dishes were placed on a heated microscope stage (37ºC). 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.

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 h. After centrifuged at 6,000× *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].

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 thiobarituric 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× *g* for 10 min. The supernatant was centrifuged again at 105000× *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).

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. $P < 0.05$ was considered to have statistical significance.

Results

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 (Fig. 1A, C, E, G, and I). The fluorescence intensity from the hepatocytes treated with 2.0 mM TBH increased with time (Fig. 1B, D, F, H, and J), such as cell a, b, c, d and e of Fig. 1B whose concentration of intracellular calcium rapidly increased from 12 min, and reached the maximum at 18 min (Fig. 2). A significant increase in fluorescence intensity and a bleb in a hepatocyte were observed at 18 min after TBH treatment, as arrow indicated (Fig. 1E and F). These changes became more severe at 30 min, as arrow indicated (Fig. 1G and H). The fluorescence intensity disappeared at 60 min because of bleb rupture (Fig. 1I and J).

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 pre-treatment with vitamin E ($P < 0.05$). Moreover, pre-treatment 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 pre-treatment with vitamin E and EGTA ($P > 0.05$).

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 pre-treated with vitamin E, the response was in a steady level and significantly lowers than control in the middle period. Whereas, pre-treated 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 (Fig. 3A).

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. Pre-treatment with DTT significantly increased the percentage of blebbing from 25.0 ± 2.2% in the hepatocytes only treated with 2 mM TBH for 60 min to 62.2 ± 1.2% ($P < 0.05$). 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 pre-treatment of DTT increased with time in the blebbing cells but no significant difference was found in the prior period (Fig. 3B).

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. Pre-treatment 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 or 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 pre-treatment 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 pre-treatment of vitamin E or vitamin E plus DTT (Table 1).

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. Pretreatment with DTT in 2.0 mM TBH-treated hepatocytes, although the levels of protein thiols of the membrane fraction were significantly lower than that 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 pre-treated 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 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.

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 demonstrated visually the important role of intracellular 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 hepatocyte were observed and the intensity of fluorescence was proportional to the size of blebs. By pre-treating hepatocytes with EGTA to remove the extracellular calcium, we found that no fluorescence intensity was observed and the percentage of blebbing significantly decreased from 61% in the control group to 27%. Moreover, treatment with EGTA after bleb formation may also reduce the percentage of blebbing (data not shown). These data confirmed that bleb formation may be associated with the increase concentration of intracellular calcium. However, the prevention of blebbing may be due to the combination of EGTA with the intracellular iron which is required for lipid peroxidation [21], since lipid peroxidation may cause the damage of plasma membrane resulting in the increase in the cytosolic free calcium ion [8]. In order to rule out this possibility, we have 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 may protect TBH-treated cells from death [22, 23]. These findings confirm that the intracellular calcium increase by TBH is exclusively due to calcium influx from the extracellular site (22, 24), and signify 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 has reported 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 lipid peroxidation-induced calcium accumulation is completely prevented by vitamin E [8]. In this study, we demonstrated that vitamin E blocks not only the elevation of intracellular calcium concentration but also the loss of protein thiols in the membrane fraction in TBH-treated hepatocytes. These findings indicated that the integrity of cell membrane conserved by vitamin E may be important to the maintenance of homeostasis of intracellular calcium.

Although there is an association between membrane blebbing and intracellular calcium concentration, it may also induce through other mechanisms, since blebs were found in 22% of the hepatocytes pre-treated with vitamin E after TBH treatment. It has been reported that the alteration of cytosolic free calcium may be not required for bleb formation (28, 29). Moreover, Hg^{2+}-treated hepatocytes may 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 reduce the loss of cytosolic protein thiols and decrease bleb formation at TBH-treated hepatocytes, it can not prevent the increase in the intracellular calcium at the remaining blebbing cells. However, pretreatment with ether vitamin E or DTT could entirely block bleb formation and prevent the loss of the consumption of GSH, total protein thiol loss, lipid peroxidation, and homeostasis of intracellular calcium, indicating that plasma membrane blebbing is considered to be relatively complex and may be due to many factors.

Whereas, it has been reported that DTT is effective in preserving the homeostasis of intracellular calcium and the integrity of cell membrane [31, 32]. The controversial results may be due to different cell conditions and various treatment doses. Similar data to our study have been reported that pretreatment of neutrophils with DTT effectively inhibited the phenylarsine oxide-induced intracellular calcium elevation by 75% [33]. However, based on the observations of this study, vitamin E protects specifically the loss of protein thiols in the plasma membrane, and DTT does specifically 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 a new aspect for investigating the mechanism of bleb formation under oxidative stress, and for developing a new preventive strategy.

Competing interests

All authors have no competing interests to declare.

Authors' contributions

JH Tsai wrote the manuscript, HW Chen and YS Hsieh performed the statistical analysis and helped to draft the manuscript, YW Chen carried out the determination of confocal microscopy and spectrophotometry, JY Liu and CK Lii participated in the design of the study. All authors read and approved the final manuscript.

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Legends

Fig. 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.

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

Fig. 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), or 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 ($P<0.05$).

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<0.05).

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

	Protein thiol level (%)	
Treatment	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<0.05).