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α-Tocopherol Mutually Complementary with Glutathione in Suppression of Cell Membrane bleb Formation under oxidative stress

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Abstract—Multiple bleb formation on cell membrane is a common during cell death. The effects of α-tocopherol and glutathione (GSH) on *tert*-butyl hydroperoxide (TBH)-induced membrane changes in rat hepatocytes were studied. Over 60 min of exposure, TBH (0.5–2.0 mM) caused a dose-dependent membrane blebbing. Cells pretreated with buthionine sulfoximine, a GSH synthesis inhibitor, had significantly greater blebbing and lactate dehydrogenase (LDH) leakage under 0.5 mM TBH treatment as compared to cells without pretreatment. However, the protective effect of GSH disappeared when the TBH concentration was increased to 2.0 mM. In the presence of α-tocopheryl succinate (TS) pretreatment, it was noted that bleb formation, expressed as the percentage of cells bearing blebs, the average bleb size, or the onset of blebbing, was partially suppressed even when TBH concentration was 2.0 mM. TBH-induced thiobarbituric acid reactive substances and LDH leakage were completely abolished by TS pretreatment. Accompanying bleb formation, membrane-insoluble actin was noted to decrease by immunoblot assay . The decrease in actin was also suppressed by TS. These results indicated that intracellular GSH and α-tocopherol status are important to the TBH-induced cell membrane abnormality. Furthermore, TS plays a defensive role against blebbing when GSH is exhausted by TBH. © *2000 Elsevier Science Ltd. All rights reserved*

Keywords: α-tocopherol; glutathione; membrane blebbing; hepatocytes.

Abbreviations: BSA = bovine serum albumin; BSO = L-buthionine sulfoximine; FBS = fetal bovine serum; GSH = glutathione; GSSG = glutathione disulfide; LDH = lactate dehydrogenase; PBS = phosphate buffered saline; PVDF = polyvinylidiene difluoride; SDS–PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBARS = thiobarbituric acid-reactive substances; TBH = *tert*-butyl hydroperoxide; TS = α-tocopheryl succinate.

INTRODUCTION

Antioxidants and prooxidants maintain balance in normal cells. Disruption of this balance in favor of prooxidants result in oxidative damage. The formation of multiple blebs on the plasma membrane is considered to be an early sign of cell oxidative injury (Thor *et al.*, 1984) and is common to cells undergoing necrosis and apoptosis (Trump and Berezesky, 1995). These membrane protrusions gradually enlarge and eventually break and release intracellular components. In addition to dying cells, membrane blebbing has also been found in healthy cells, such as in cells undergoing locomotion during early embryogenesis (Trinkaus, 1973) and in cultured cells during cell flattening (Boss, 1955).

The disturbance of Ca^{2+} homeostasis has been thought to be the major factor responsible for bleb formation (Jewell *et al.*, 1982); however, blebbing also occurs when Ca^{2+} status is not disturbed (Lemasters *et al.*, 1987). Other mechanisms for cell membrane blebbing have been proposed, such as the inhibition of ATP synthesis, damage to cytoskeletal proteins, and interference with the interaction between cytoskeletal proteins and their binding proteins (Lemasters *et al.*,

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1987). Among the cytoskeletal proteins and their roles in bleb formation, most studies have focused on actin and actin binding proteins. Tumor cells lacking actin binding proteins generate extensive blebs (Cummingham *et al.*, 1992). Cleavage of two actin binding proteins, talin and α-actinin by calpain is coexistent with TBH-induced blebbing (Miyoshi *et al.*, 1996). Changes in the thiol redox status in actin correlated well with various oxidant-induced bleb formation in hepatocytes (Mirabelli *et al.*, 1988, 1989). Membrane phospholipid is also susceptible to free radical attack and leads to lipic peroxidation, membrane disruption, and finally cell death (Chen and Stevens, 1991). The formation of blebs in cell membranes may be the result of lipid peroxidation. But evidence indicates that lipid peroxidation is not the determining factor for reoxygeneration- (Caraceni *et al.*, 1994a) and TBH-induced cell death (Liu *et al.*, 1998). Whether membrane lipid peroxidation participates in oxidant-induced membrane blebbing remains to be established.

GSH, the most abundant cellular thiol, plays diverse roles in cells. This tripeptide molecule is important not only in the formation of conjugates with a variety of electrophilic xenobiotics but also in removal of many reactive oxygen species such as hydrogen peroxide and other organic hydroperoxides (Meister, 1989). In the reduction of hydroperoxides, GSH acts as a hydrogen donor and is oxidized to GSSG by GSH peroxidase. Most of the GSSG formed is then reduced to GSH by GSH reductase with the consumption of NADPH. α-tocopherol, a natural lipophilic antioxidant, is also an effective free radical scavenger that prevents membrane lipid peroxidation and maintains membrane integrity (Niki *et al.*, 1995).

To further clarify the actual roles played by GSH and α-tocopherol in TBH-induced membrane abnormality by forming multiple surface blebs, primary rat hepatocytes prepared by collagenase perfusion were used. In this study, hepatocytes in the absence or presence of L-buthionine sulfoximine (a GSH synthesis inhibitor) or α-tocopheryl succinate pretreatment were exposed to various concentrations of TBH for 50 min and the cell shape changes was investigated.

MATERIALS AND METHODS

Materials

tert-Butyl hydroperoxide (TBH), α-tocopheryl succinate (TS), L-buthionine sulfoximine (BSO), HEPES, actin antiserum and type VII rat tail collagen were purchased from Sigma Chemical (St Louis, MO, USA). L-15 medium, bovine serum albumin (BSA), fetal bovine serum (FBS), insulin, transferrin and penicillin–streptomycin solution were obtained from Gibco Laboratory (Grand Island, NY, USA). Collagenase was obtained from Worthington Biochemical (Freehold, NJ, USA).

Cell isolation and culture

8-wk-old Sprague–Dawley rats, with an average body weight of 250–300 g, were purchased from the National Animal Breeding and Research Center (Taipei, Taiwan). Rat hepatocytes were isolated by a two-step collagenase perfusion method as previously described (Wang *et al.*, 1997). After isolation, cell viability was examined by the trypan blue exclusion test and was greater than 90%. Cells were then resuspended in L-15 culture medium (pH 7.6) containing 18 mM HEPES, 5 mg/l each of insulin and transferrin, 1 g/l galactose, 1 μM dexamethasone, 5 μg/l selenium as sodium selenite, 100 mg/l streptomycin, 1×10^5 IU/l penicillin and 2.5% FBS. A 2.5-ml cell suspension with a total of 1.0×10^6 cells was plated on each 30-mm collagen-precoated tissue culture dish (Falcon Labware, Freaklin Lakes, NJ, USA). The cells were then incubated in a humidified incubator at 37°C in an air atmosphere. Cell attachment to the culture dish was completed 4 hr after plating, and the medium was changed to remove dead cells. After attachment, 2 g/l BSA was used to replace FBS in the L-15 culture medium and, thereafter, the culture medium was changed once each day. At 24 hr after plating, hepatocytes were preincubated either in the absence (control) or in the presence of 1 mM BSO (+BSO) or 100 μM TS (+TS). After 24 hr of preincubation, cells were changed to fresh L-15 culture medium but without BSA and TBH treatment was performed according to the designated intervals.

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 and the average bleb size (μm^2/bleb) were determined on pictures that were taken at 0, 15, 30 and 60 min, respectively. At least 150 cells were counted in each analysis. The lag period of blebbing, percentage of cells bearing blebs, and the average bleb size were used to express the extent of membrane blebbing in each group.

Biochemical assays

Cell harvesting was performed by removing the culture medium and washing the hepatocytes twice with cold phosphate buffered saline (PBS), pH 7.0. Cell extracts were prepared by scraping with 20 mM potassium phosphate buffer (pH 7.0). The cell extracts were used immediately for lipid peroxidation determination. Lipid peroxidation was determined by measuring thiobarbituric acid-reactive substances (TBARS) formation (Fraga *et al.*, 1988). TBARS fluorescence was detected at excitation and emission wavelengths of 515 and 555 nm, respectively, in an F4500 fluorescence spectrophotometer (Hitachi,

Tokyo, Japan). 1,1,3,3-Tetramethoxypropane was used as the TBAR standard. Changes in intracellular GSH and GSSG on the TBH exposure were determined by HPLC (Reed *et al.*, 1980). Cell viability was determined by measuring the release of LDH into the extracellular fluid (Moldeus *et al.*, 1978). The α-tocopherol assay was prepared and measured as described by Catignani and Bieri (1983).

SDS–PAGE and immunoblot assay

Modification of membrane-insoluble actin was analyzed by SDS–PAGE as described by Mirabelli *et al.* (1988) with some modifications. Cells were first washed twice with cold PBS and then extracted with 1 m 50 mM Tris–HCl buffer (pH 7.5) containing 40 mM KCl, 5 mM $MgCl_2$ and 1% Triton X-100 for 30 min in an ice-bath. After extraction, cells were scraped and divided into two aliquots, one for reducing and one for non-reducing electrophoresis. Samples for non-reducing electrophoresis were treated with 25 mM *N*-ethylmaleimide for 30 min before centrifugation. The Triton X-100-insoluble fractions were centrifuged at 10,000 *g* for 20 min. The pellets were washed twice with the same extraction buffer and finally suspended in 1.5% SDS and heated in boiling water for 4 min. Samples were subsequently heated at 100°C for an additional 5 min in the presence or absence of β-mercaptoethanol. After heating, samples with equal amount of protein were subjected to 10% SDS–polyacrylamide gels. Following electrophoresis, proteins separated on SDS–polyacrylamide gels were transferred to polyvinylidiene difluoride (PVDF) membranes. Normal goat serum was used to block non-specific binding sites on the membranes. Membranes were incubated with actin antiserum and each of the two Vectastain ABC reagents, biotinylated anti-rabbit IgG and avidin-peroxidase complex, respectively, for 30 min at 37°C. Hydrogen peroxide and 3,3′-diaminobenzidine tetrachloride were used as peroxidase substrates for color development. To estimate the change in the monomeric form of actin following TBH exposure, the density of each band was quantitated by an AlphaImager™ 2000 denistometer (Alpha Innotech Co. San Leandro, CA, USA).

Statistical analysis

Data are expressed as means±SE. Statistical analysis were performed using analysis of variance (SAS Institute, Cary, NC, USA). Duncan's multiple range tests were used to determine significant difference between treatment means ($P < 0.05$).

RESULTS

Changes of membrane morphology

Upon treatment of the hepatocytes with TBH, cell morphological changes such as the formation of multiple blebs on the cell membrane were observed (Plate 1). These spherical surface blebs grew continuously during the exposure period until breakage. To determine the concentration of TBH required for induction of these dramatic morphological changes, a dose–response study was performed (Fig. 1A). Over the 60-min exposure, no blebbing was found in cells treated with 0.5 mM TBH. However, when the TBH concentration was increased to 1.0 mM or 2.0 mM, cell blebbing as well as the percentage of cells bearing blebs were increased. After 60 min of exposure, approximately 15% and 50% of hepatocytes in the two groups had blebbing.

The dynamic changes observed in membrane blebbing were indicative of membrane disintegrity under oxidative stress. To correlate the degree of oxidative stress with membrane blebbing, the extent of LDH release into the extracellular fluid was determined (Fig. 1B). Our results showed that the increase in membrane blebbing preceded the LDH leakage. Membrane blebbing was seen after 15 min of 2.0 mM TBH treatment and then it was increased dramatically; however, the LDH release was found after 30 min of 2.0 mM TBH exposure. The extent of TBH-induced LDH leakage was dose dependent.

In this study, hepatocytes were preincubated with 1 mM BSO to inhibit GSH synthesis before the addition of two different concentrations of TBH. After 24 hr of preincubation, BSO caused a 95% reduction in cellular GSH. Then these cells were exposed to 0.5 mM TBH, 24% of cells had blebbing after 60 min (Table 1). In contrast, cells without BSO preincubation had no blebbing under the same condition. However, when the TBH concentration was increased to 2.0 mM, the cell blebbing was not different, regardless of whether BSO pretreatment was used. Although GSH showed no protective effect against cell blebbing in the presence of 2.0 mM TBH, TS pretreatment suppressed the degree of membrane blebbing. No matter whether the cells were treated

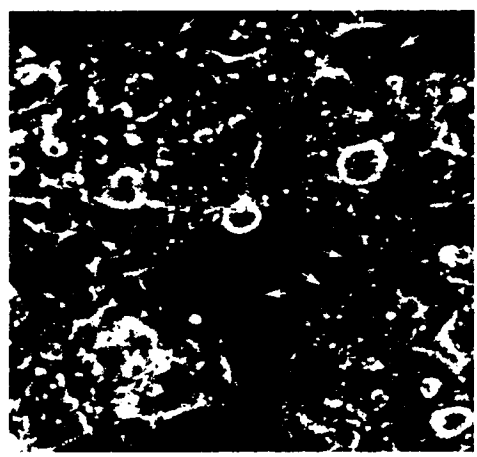

Plate 1. Multiple membrane blebs in hepatocytes. Bleb formation was monitored with a phase constrast inverted microscope after 2.0 mM TBH treatment for 60 min. Multiple surface blebs are indicated by arrows. —,40 μm.

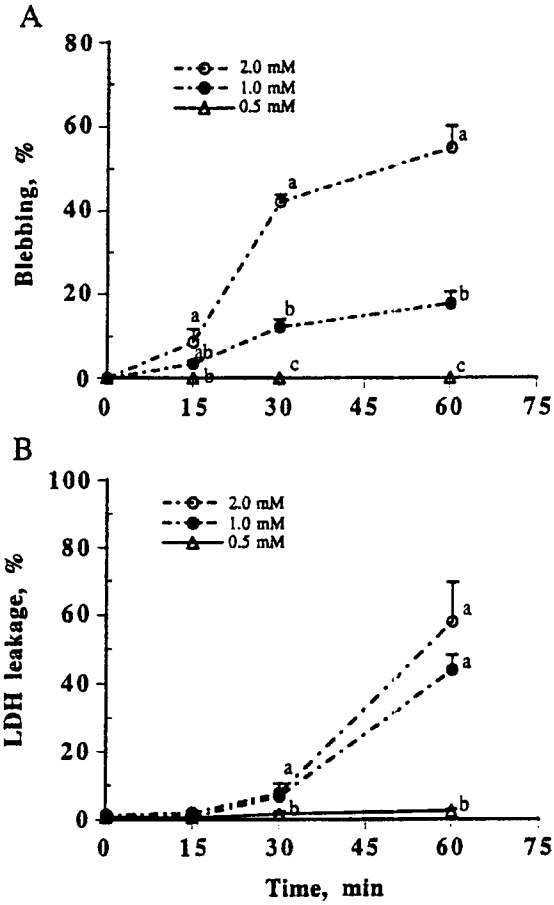

Fig. 1. TBH-induced membrane bleb formation (A) and LDH leakage (B). Values are means±SE, n=4.[a,b,c]Groups at the same time point that do not share the same letter differed significantly ($P<0.05$).

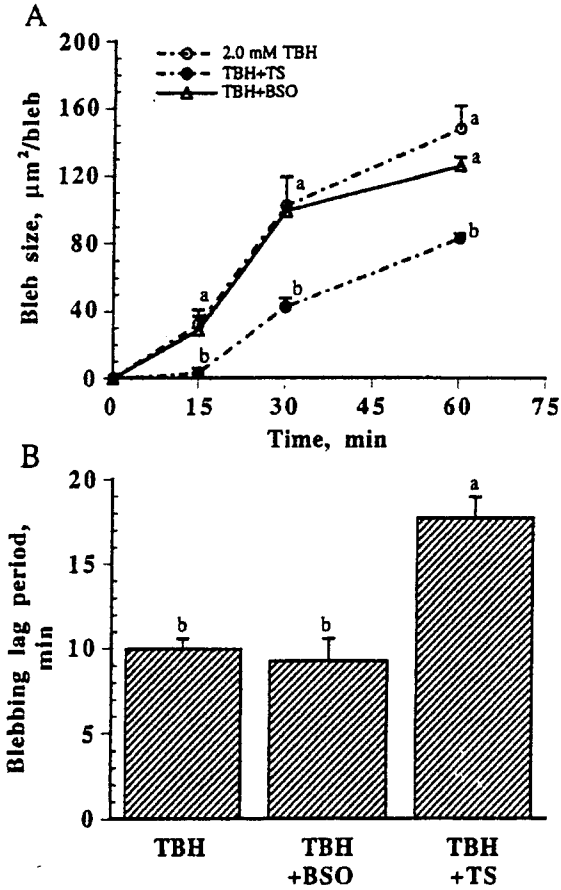

Fig. 2. The effects of TS and BSO on the average size (A) and the lag period of the first observable bleb (B) caused by 2.0 mM TBH. Values are means±SE from 4 separate experiments. [a,b]Groups at the same time point that do not share the same letter differed significantly ($P<0.05$).

Table 1. The effect of BSO and α-tocopheryl succinate on bleb formation and LDH leakage induced by TBH

Treatment	% Blebbing	% LDH leakage
TBH (0.5 mM)	0.0±0.0[c]	2.5±0.8[d]
+BSO	24.1±1.2[b]	31.9±5.5[c]
TBH (2.0 mM)	54.8±5.2[a]	58.0±11.4[b]
+BSO	52.5±12.7[a]	86.3±3.9[a]
+TS	25.1±3.4[b]	4.2±1.3[d]

%Blebbing = number of cells with one or more membrane blebs/total cells counted×100%. In the presence or absence of TS pretreatment for 24 hr, cellular α-tocopherol levels were 33.9±2.7 and 0.04±0.01 μg/mg protein, respectively. Values are means±SE of three or four separate experiments.

[a,b,c,d]Treatment means not sharing the same letter differed significantly ($P<0.05$).

with 0.5 or 2.0 mM TBH, the LDH leakage was closely related to intracellular GSH level (Table 1). LDH leakage was significantly enhanced in the presence of BSO preincubation and TS dramatically suppressed TBH-induced LDH leakage.

When the extent of blebbing was expressed in terms of the average size of blebs (Fig. 2A), the results showed a dynamic growth of these blebs over the 60-min period and breakage of the large bleb in some cells observed. TS preincubation not only suppressed the percentage of cells bearing TBH-induced blebs but also significantly decreased the size of blebs compared to those without TS preincubation. Again, under 2.0 mM TBH treatment, BSO showed no effect on the average size of blebs (Fig. 2A) and the onset of blebbing (Fig. 2B), but TS did delay the onset of blebbing.

GSH and GSSG status

With 2.0 mM TBH treatment, GSH level continuously decreased (Fig. 3A). Accompanying the decrease in GSH, GSSG accumulated within the first 15 min and then decreased thereafter (Fig. 3B). When 0.5 mM TBH was added to the cells, GSH decreased and GSSG increased similar to the 2.0 mM treatment during the first 15 min; however, the later event was totally different. After 15 min, 0.5 mM TBH treatment caused the GSSG level to decrease dramatically, but this reduction was accompanied by the increase in GSH and the GSH content recovered to 80% of the initial level after 60 min. TS pretreatment had no effect on GSH and GSSG concentrations following the addition of 2.0 mM TBH (data not shown).

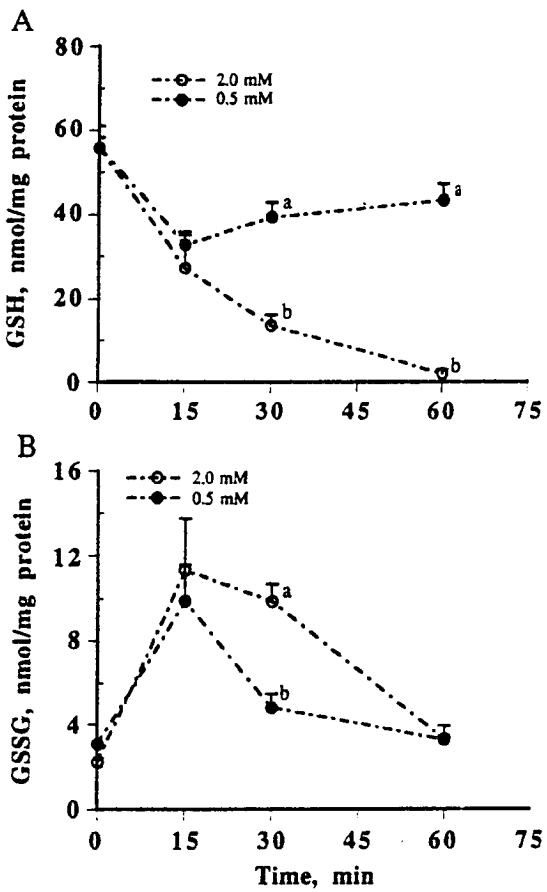

Fig. 3. Changes of intracellular GSH (A) and GSSG (B) levels. Values are means±SE, n=4. [a,b]Groups at the same time point that do not share the same letter differed significantly ($P<0.05$).

Table 2. Lipid peroxidation in rat hepatocytes

Treatment	TBARS nmol/mg protein
Control	1.46±0.14[c]
TBH (0.5 mM)	1.88±0.12[c]
+ BSO	3.36±0.51[c]
TBH (2.0 mM)	9.99±0.51[a]
+ BSO	9.95±0.52[a]
+ TS	0.13 ±0.03[d]

Hepatocytes were preincubated with or without BSO or TS for 24 hr before the addition of 0.5 or 2.0 mM TBH. TBARS level was determined after TBH treatment for 60 min. Values are means ±SE (n=3–4).

[a,b,c,d]Treatment means not sharing the same letter differed significantly ($P<0.05$).

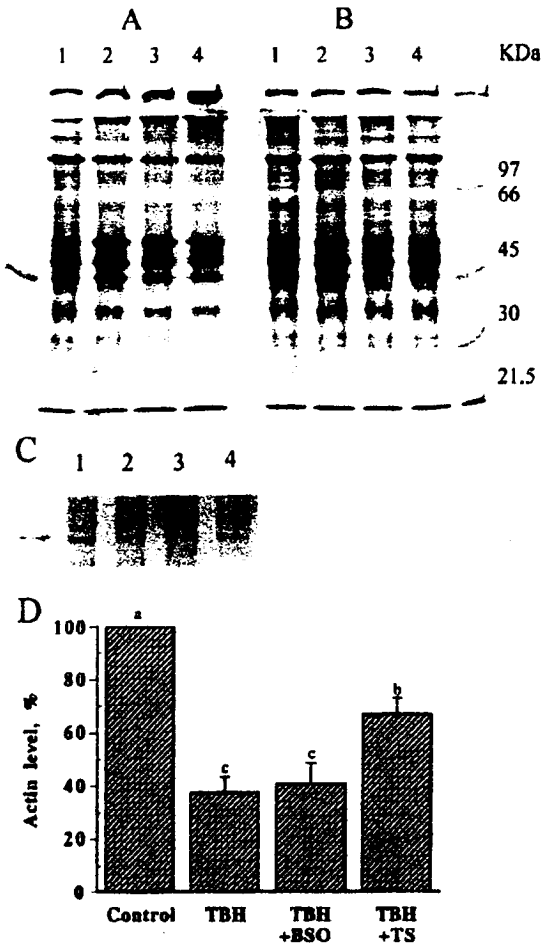

Fig. 4. TBH-induced actin changes. Equal amounts of membrane Triton X-100-insoluble protein were analyzed under non-reducing (A) and reducing (B) SDS-polyacrylamide gels which were stained with Coomassie blue. Proteins separated on gels were transferred to PVDF membranes and were immunostained by actin antiserum and antibody-linked peroxidase (C). Data are shown before (lane 1) and 15 (lane 2), 30 (lane 3), and 60 min (lane 4) after the addition of TBH. The band at the left side of lane 1 shows the actin (45 kDa). The decrease of actin due to TBH treatment at 60 min was measured by densitometry (D). The integrated density value of each group at time 0 was regarded as 100%. Values are means±SE, n=3–4. [a,b,c]Groups at the same time point that do not share the same letter differed significantly ($P<0.05$).

Lipid peroxidation

The TBARS level between 0.5 mM TBH-treated and control cells after 60 min of exposure was not significantly different (Table 2). However, in the presence of BSO preincubation, 0.5 mM TBH caused significantly greater lipid peroxidation. Cells treated with 2.0 mM TBH had significantly greater TBARS than cells treated with 0.5 mM TBH, and BSO preincubation had no effect on the TBARS level of cells exposed to 2.0 mM TBH. Again, TS suppressed lipid peroxidation caused by 2.0 mM TBH treatment.

Actin modification

Results of SDS–PAGE revealed that a protein with similar molecular weight to actin in Triton X-100 insoluble membrane fractions showed a time-dependent decrease following 2.0 mM TBH treatment (Fig. 4A, lanes 2–5). This decrease was accompanied by the formation of high molecular weight molecules which aggregated in the sample wells and at the top of the separating gels. When these same samples were treated with thiol reducing agent, β-mercaptoethanol, the 45 kDa protein returned and the high molecular weight aggregates disappeared (Fig. 4B). The results indicated that such changes are likely related

to thiol modification in the proteins. To confirm whether the 45 kDa protein observed on SDS–PAGE was actin, immunostained with actin antiserum was performed (Fig. 4C). As observed on the Coomassie blue-stained gels, TBH actually caused a time-dependent decrease in the level of actin. Quantitated by densitometry (Fig. 4D), 60% of 45 kDa actin was lost with 2.0 mM TBH treatment after 60 min, and loss of 45 kDa actin was observed in cells pretreated with BSO. However, TS pretreatment suppressed TBH-induced actin loss.

DISCUSSION

Oxidative stress causes diverse cell damage (Halliwell and Gutteridge, 1999) and membrane blebbing is one of its consequences (Jewell *et al.*, 1982; Kuo *et al.*, 1997; Mirabelli *et al.*, 1989). The mechanisms responsible for this phenomenon are not yet clear, but multiple factors are believed to be involved in (Lemasters *et al.*, 1988; Trump and Beerezesky, 1995). Membrane lipid peroxidation and damage to cytoskeletal proteins may be the most important ones (Malorni *et al.*, 1991; Miyoshi *et al.*, 1996; Torgerson and McNiven, 1998). To minimize the damage caused by reactive oxygen species, an effective antioxidant defense system is absolutely necessary. TBH is an organic hydroperoxidant which is metabolized by GSH peroxidase accompanying the oxidation of GSH to its disulfide (GSSG). The resultant GSSG is then reduced to GSH by GSH reductase with the consumption of NADPH. If this defense system is overwhelmed, TBH yields free radicals and initiates free radical chain reactions (Chen and Stevens, 1991; Shertzer *et al.*, 1994; Sies, 1985). TBH-derived radicals and GSSG accumulation could disturb the cellular Ca^{2+} buffering system and allow the intracellular Ca^{2+} to increase as found in previous studies (Hoyal *et al.*, 1996; Liu *et al.*, 1998). The increase in intracellular Ca^{2+} activates a series of Ca^{2+}-dependent degradative responses which are also involved in bleb formation (Miyoshi *et al.*, 1996; Trump and Berezesky, 1995). Thus, TBH is an ideally suitable prooxidant for use in studying the cellular function of GSH and/or α-tocopherol under oxidative stress. In this study, the rapid decrease in cellular GSH level accompanied by the increase in GSSG indicated that GSH peroxidase acts on TBH. To avoid the accumulation of GSSG and damage to the cells, GSSG needs to be removed. The regeneration of GSH was found in cells treated with 0.5 mM TBH and the damage was minimized. However, the cells pretreated with BSO had significantly greater percentage of blebbing than the control cells. Although we demonstrated that GSH may prevent morphological changes in 0.5 mM TBH-treated hepatocytes, this protection disappeared when 2.0 mM TBH was used. Cellular GSH and GSSG changes were determined after 15 min; however, the effect of TBH on GSH regeneration and TBH-derived radical formation may occur earlier. This explains why the hepatocytes treated with 0.5 and 2.0 mM TBH have the same level of GSH at 15 min but differ in bleb formation.

α-Tocopherol, a natural and lipophilic antioxidant, is the most important radical scavenger that protects membrane phospholipids and proteins from oxidative damage (Niki *et al.*, 1995; Takenaka *et al.*, 1991). These functions account for its protective effect against membrane morphological changes. It was noteworthy that TS effectively suppressed membrane blebbing even when cells treated with 2.0 mM TBH, and this suggested that α-tocopherol is a secondary defense system against TBH-induced toxicity.

The membrane phospholipid is susceptible to free radical attack and leads to lipid peroxidation, membrane disruption, and cell death (Chen and Stevens, 1991). However, evidence also indicates that lipid peroxidation may not be the determining factor for oxidant-induced cell death. For instance, lipid peroxidation is not correlated with reperfusion- or reoxygenation-caused cell death (Caraceni *et al.*, 1994a,b). TBH-caused cell death was suppressed by blocking the increase in intracellular Ca^{2+} even when lipid peroxidation was high (Liu *et al.*, 1998). Although there is an association between lipid peroxidation and cell death in this model system, membrane blebbing can also be induced through other mechanisms, since blebs were found in 25% of the cells preincubated with TS which completely blocked TBARS generation. In addition to phospholipid, membrane proteins are susceptible to damage under oxidative stress (Stadtman, 1992). It has been found that the menadione- or TBH-induced thiol-related abnormal actin polymerization is related to bleb formation in hepatocytes (Kuo *et al.*, 1997; Mirabelli *et al.*, 1988). Moreover, GSSG at high level may react with actin to form the actin-GSH mixed disulfide (Chai *et al.*, 1994; Rokutan *et al.*, 1994). This reaction not only weakens the actin filament but also causes the disassociation of proteins (Hinshaw *et al.*, 1991; Stournaras *et al.*, 1990). Both of which can possibly cause cell shape changes.

It was found that bleb formation proceeds the LDH leakage but correlates well with LDH leakage in cells treated with 0.5 or 2.0 mM TBH. This suggests that bleb formation is an early sign of cell damage (Thor *et al.*, 1984). Blebs gradually enlarge and release cellular components on breakage. The exception was 25% TS-pretreated cells bearing blebs but with low LDH leakage. This can be due to the long lag time for initial bleb formation and slower bleb enlargement, so, the release of cellular components did not occur during the 60-min incubation. However, the extent of release of intracellular enzyme cannot be simply explained by membrane blebbing, factors such as membrane phospholipid oxidation, membrane protein oxidation, and cellular GSH status may also contribute to LDH leakage.

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Title

The roles of Cytoskeleton and Thiol Redox Status in Oxidant-induced membrane blebbing

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Running Tile

Cytoskeletons and Cellular Thiols

Key Word

Actin, ß-tubulin, Glutathione, Protein Thiol, Membrane Blebbing

Footnotes

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The abbreviations used are: DTT, DTT; GSH, glutathione; GSSG, glutathione disulfide; LDH, lactate dehydrogenase; TBARS, thiobarbituric acid-reactive substances;

INTRODUCTION

In the process of cell death, several membrane changes are noticed. Formation of multiple blebs on cell membranes is one of these changes and is common in cells undergoing necrosis and apoptosis (Trump and Berezesky, 1995). Blebs gradually enlarge and finally release intracellular components upon breakage. The disturbance of Ca^{2+} homeostasis, which triggers so called "Ca^{2+}-dependent toxic cell death", has been considered to play a crucial role upon this morphology aberration (Jewell et al., 1982; Murata et al. 1994). Increased intracellular Ca^{2+} concentration activates Ca^{2+}-dependent nonlysomal proteinase and causes the proteolysis of membrane proteins and cytoskeletal proteins (Mellgren, 1987; Mirabelli et al., 1989) , which in turn damage the membrane integrity. However, others reported the blebbing can be dissociated from changes in intracellular Ca^{2+} homeostasis (Lemaster et al., 1987; Sakaidi et al., 1991), and conflicts arose about the role of Ca^{2+} in cell injury. Mechanisms attributed to membrane morphology aberration is thought to be a multiple biochemical consequences.

Cytoskelatal proteins play a crucial role on determining cell shape. Damage to these proteins interferes membrane integrity and functions (Alberts et al., 1989). Among cytoskeletal proteins, most of studies of membrane blebbing have focused on the role of actin and actin binding proteins. Actin polymerization (F actin level) (Cunningham, 1995) and thiol-related abnormal actin polymerization (Kuo et al., 1997; Mirabelli et al., 1988) are correlated well with bleb formation. Extensive blebs are observed in tumor cells lacking actin binding protein (Cummingham et al., 1992). Cleavage of two actin binding proteins, talin and α-actinini, by calpain is coexisted with tert-butyl hydroperoxdie-induced blebbing (Miyoshi et al., 1996). Recently, bleb formation in cholecystokinin-suprastimulated pancreatic acinar cells has been demonstrated to require the reorganization of actin and myosin cytoskeletons (Torgerson and McNiven, 1998).

In addition to actin, microtubule cytoskeleton is also cell shape determinant, but the the actual role of tubulin on the formation of membrane blebs is limited. Even so, it is known both tubulin and tau protein, a microtubule-associated protein that stabilizes of microtubules, are good substrates of calpain (Billger et al., 1988; Litersky et al., 1993).

Increase of calpain activity under oxidative stress may therefore destabilizes microtubules and results in bleb formation. Blebbing caused by nocodazole, a microtubule destabilizer, further suggests the involvement of microtubules on this membrane change (Mills et al., 1998).

The role of intracellular thiol redox status and lipid peroxidation in the process of cell injury has attracted a lot of attention. Membrane phospholipid is susceptible to free radical attack and leads to lipid peroxidation, membrane disruption, and finally cell death (10). The formation of blebs in cell membranes may be the result of lipid peroxidation. But evidence indicates that lipid peroxidation is not the determining factor for reoxygeneration- (11) and TBH-induced cell death (12). Whether membrane lipid perioxidation participates in TBH-induced membrane blebbing remains to be established. Instead, the maintenance of cellular thiols as the cytoprotective mechanism against oxidative damge has been reported (Marchetti et al., 1997; Pascoe et al., 1987; Pascoe and Reed, 1989). Recently, Sato et al. (1995) even reported that the redox status of cellular protein thiols play more important role than GSH in oxidant-induced cell death.

To investigate whether the cytoskeletal changes and cellular thiol redox status associate with membrane bleb formation under oxidative stress and the role of cellular thiol redox, hepatocytes were treated with either a free radical-generating oxidant, tert-butyl hydroperoxide, or a thiol depletion oxidant, diamide, in the presence or absence of DTT. Decrease of monomeric form of actin and α- and β-tubulin and changes of cytoskeletal network, especially the microtubules structure, accompanied with the dynamic blebbing. tert-Butyl hydroperoxide- or diamide-initiated changes of microtubule network was not affected by taxol and nocodazole. Dynamic blebbing and decrease of actin and tubulin was significantly suppressed DTT pretreatment. This report demonstrates that changes of cytoskeletal structures, especially the microtubule network, are related to bleb formation and it may be controlled by the cellular thiol redox status.

MATERIALS AND METHODS

Materials tert-Butyl hydroperoxide, diamide, rat tail collagen type VII, DTT, 2,4-dinitrofluoro benzene (FDNB), 5,5'-dithio-bis-nitrobenzoic acid (DTNB), taxol, nocodazole, polyclonal actin antibody, Triton X-100, and formaldehyde were purchased from Sigma Chemical Co. (St. Louis, MO). Collagenase was ordered from Worthington Biomedical Co. (Freehold, NJ). Chemicals used for culture medium were purchased from Gibco Laboratory (Grand Island, NY). Monoclonal anti-β-tubulin and secondary fluorescein-conjugated goat anti-mouse antibodies were purchased from Chemicon International Inc. (Temecula, CA). NBD-phallacidin was ordered from Molecular Probes (Eugene, OR).

Cell Culture Hepatocytes were isolated from Sprague Dawley rats by a two-step collagenase perfusion as previously described (Wang et al., 1997). Cell viability was examined by trypan exclusion test and was greater than 90%. Hepatocytes were finally resuspended in L-15 culture medium (pH 7.6) containing 18 mM HEPES, 5 mg/L each of insulin and transferrin, 1 g/L galactose, 1 μM dexamethasone, 5 μg/L selenium as sodium selenite, 100 mg/L streptomycin, 1 X 10^5 IU/L penicillin and 2.5% fetal bovine serum. A 2.5 mL cell suspension with a total of 1.0 X 10^6 cells were plated on each 30-mm tissue culture dish precoated with type VII rat tail collagen . Cell attachment was completed 4 h after plating, and the medium was changed to remove dead cells. Two g/L BSA was used to replace fetal bovine serum in the L-15 culture medium and, thereafter, the culture medium was changed once each day. At 48 h after plating, hepatocytes were changed to fresh L-15 culture medium but without BSA and treated with tert-butyl hydroperoxide or diamide for the designated intervals. DTT was added to cells 30 min before tert-butyl hydroperoxide or dimaide. Cells pretreated with taxol or nacodazole was performed 60 min before adding of tert-butyl hydroperoxide. Part of cells were preincubated with a lipophilic antioxidant, α-tocopherol succinate (100 μM), to determine the role of membrane lipid peroxidation in bleb formation under oxidative stress.

For observing the dynamic bleb formation in living hepatocytes, cells were examined under a phase contrast inverted microscope (Diaphot-TMD, Nikon, Tokyo, Japan). Following the addition of tert-butyl hydroeproxide or diamide, cell culture dishes were put

on a 37°C thermostatic plate and left on the microscopic stage. Blebbing was monitored in the same field before addition or at 15, 30, and 60 min after tert-butyl hydroperoxide or diamide treatment, In each cultured dish, a total of 150-200 cells was counted and the extent of blebbing was expressed as the percentage of cells bearing blebs.

Immunofluorescence of Cytoskeletal Structure To examine the distribution of filamentous actin and tubulin under bleb formation, 10^5 hepatocytes were cultured on each well of 4-well ChamberSlide (Nunc, Denmark). The procedure used was adapted from that described by Li et al., (1987) with some modifications. To stop tert-butyl hydroperoxide or diamide reaction, cells were washed twice with cold phosphate-buffered saline (PBS, pH 7.4) and then rinsed once with 100 mM Pipes buffer (pH 6.9), containing 1 mM MgSO4, 2 mM glycerol, and 2 mM EGTA. Cells were fixed with 3.7% formaldehyde in Pipes buffer for 10 min. Then, cells were permeabilized in 2% Triton X-100 in Pipes buffer for additional 10 min and rinsed with PBS again. For determining filamentous actin, cells were incubated with NBD-phallinidin (0.1 unit/well) for 45 min and then washed with twice with PBS. For studies designed to test tubulin filament distribution, cells were incubated in blocking buffer for 30 min and then incubated in monoclonal anti-β-tubulin for 30 min. Fluorescein-conjugated secondary antibody was added and incubated for additional 60 min. Cell nuclei were then stained in the presence of 2 μM actinomycin for 10 min. All wells were rinsed extensively with PBS before mounting on coverslips with mounting medium (Sigma, St. Louis, MO). Cytoskeleton structure of cells bearing blebs was examined with a Zeiss confocal laser microscope.

Electrophoresis and Western Blotting Cells were first washed twice with cold PBS and then extracted in a 30-min ice bath with 1 mL of 50 mM Tris-HCl buffer (pH 7.5), which contained 40 mM KCl, 5 mM $MgCl_2$ and 1% Triton X-100 (Mirabelli et al., 1988). After extraction, cells were scraped and divided into two aliquots, one for reducing and one for nonreducing gel electrophoresis. The one for nonreducing analysis was treated with 25 mM N-ethylmaleimide for 30 min before centrifugation. The Triton X-100-insoluble fractions were centrifuged at 10,000 X g for 20 min. The pellets were rinsed twice with

the same extracting buffer and finally suspended and sonicated in a solution containing 1.0% SDS and 8 M urea. Following protein determination (Lowry et al., 1956), samples were heated at 100°C for 5 min in the Laemmli buffer with or without ß-mercaptoethanol. After electrophoresis, the SDS-polyacrylamide gels were stained with Coomassie blue or were used for Western blot. Protein separated on gels were transferred to polyvinlidiene difluoride (PVDF) membranes. Normal goat serum was used to block nonspecific binding sites on membranes. Each incubation with primary actin antiserum, monoclonal β-tubulin antibody, and secondary peroxidase-conjugated antibodies were performed at 37°C for 30 min. Hydrogen peroxide and 3,3'-diaminobenzidine tetrachloride were used as peroxidase substrates for color development.

Other biochemical Assays The cell viability was determined by measuring the release of lactate dehydrogenase (LDH) into the extracellular medium (Moldeus et al., 1978). Cellular reduced glutathione (GSH) and oxidized GSH (GSSG) were measured by HPLC method (Reed et al., 1980). Briefly, cells were washed twice with cold PBS and acid-soluble GSH and GSSG were obtained by adding 1 ml of 5% perchloric acid solution into each dish. Samples were then derivatized with iodoacetic acid, neutrized with potassium biocarbonate, and finally incubated with FDNB. Lipid peroxidation was determined by measuring the thiobarbituric acid-reactive substances (TBARS) production (Fraga et al., 1988). TBARS fluorescence was detected at an excitation and emission wavelength of 515 and 555 nm in a F4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). For membrane protein thiol determination, cells extracts were prepared by scraping with 20 mM potassium phosphate buffer (pH 7.4) and centrifuged at 4,000 X g for 10 min. The resulting membrane fractions were extensively rinsed with the same buffer and resuspended again in a 20 mM potassium phosphate buffer (pH 7.4) containing 5% SDS. Total membrane protein thiol contents were measured after incubation with DTNB as reported by Boyne and Ellman (1972).

Statistical Analysis Data are expressed as means ± SE. Statistical analysis were performed using analysis of variance (SAS Institute, Cary, NC). Duncan's multiple range tests were used to determine significant differences among treatment means ($P<0.05$).

RESULTS

Rapid bleb Induction Both tert-butyl hydroperoxide (Fig. 1A-C) and diamide ((Fig. 1D-F) caused a dramatic and dynamic changes on the plasma membranes in hepatocytes. Membrane blebbing was noted on some cells at 15 min, and then the numbers and sizes of bleb increased following the treatment. Finally, bleb breakage occurred. As the extent of blebbing expressed by the percentage of cells bearing blebs, 5-10% of cells beared blebs at 15 min, thereafter, the number of blebbing cells significantly increased up to 60 min (Fig. 2). At 60 min, half of the cells examined bearing blebs when treated with 2 mM TBH and was 25% with 2 mM diamide.

To examine whether oxidant-induced blebbing was related to cellular thiol redox status, DTT effect on cell shape change was examined. Results showed that DTT suppresses tert-butyl hydroperoxide- and diamide-induced blebbing but somehow in a different manner. In case of TBH treatment, 52% of cells bearing blebs decreased to 33% in the presence of DTT preincubation (Fig. 2A). However, DTT protection in diamide-treated cells was much more effective (Fig. 2B). In the presence of DTT, diamide caused blebbing at 60 min was decreased from 27% to 2%. Due to tert-butyl hydroperoxide metabolism can gengerate free radical species, which affect not only cellular thiol redox status but also attack other cellular macromolecules. In the next study, we tested a radical scavenger to examide whether mechanisms other than thiol status is involved in tert-butyl hydroperoxide caused membrane blebbing. As results showed, α-tocopheryl succinate (TS) significantly suppressed tert-butyl hydroperoxide-induced blebbing and had greater protection than DTT over this 60 min experiment (Fig. 2A). Only 6% of cells beared blebs in TS-pretreated group as compared to 33% of cells pretreated with DTT and 52% with tert-butyl hydroperoxde alone.

Cell toxicity by TBH and Diamide Cell death was determined by measuring the leakage of intracellular LDH into culture medium (Fig. 3). With either TBH (Fig. 3A) or diamide (Fig. 3B) treatment, enzyme leakage remained unchanged at the first 30 min and then increased. Similar to those observed in membrane bleb formation, DTT protected cell death from TBH or diamide treatment. TS again significantly suppressed LDH leakage

caused by TBH. With regard to membrane blebbing, the extent of enzyme leakage was correspondent to the percentage of cells bearing blebs. It was also noted that blebbing preceded cell death, indicating blebs dynamically grow and finally release intracellular components upon breakage.

TS protection of tert-butyl hydroperoxide-induced cytotoxicity suggested that lipid peroxidation may be an important in causing membrane blebbing. As shown, TBARS was significantly increased after 60 min of 2 mM of tert-butyl hydroperoxide exposure (Fig. 4). DTT pretreatment reduced 30% of TBARS production. When cells were preincubated with TS, lipid peroxidation by tert-butyl hydroperoxide was completely suppressed over this 60 min period. A slight increase of lipid peroxidation was also obtained in diamide-treated cells as compared to control cells. DTT abolished diamide-induced TBARS increase.

GSH and Protein Thiols For cellular thiol status, we determined both GSH, the most abundant nonprotein thiol, and membrane protein thiol levels (Table 1). After adding tert-butyl hydroperoxide and diamide to cells, reduced GSH level was rapidly decreased and less than 1% of GSH left after 60 min exposure. Accompanying the decrease of GSH, oxidized GSH are generated (data not shown). The decrease and oxidation of GSH, however, were prevented by pretreating cells with DTT before adding tert-butyl hydroperoxide and diamide. Similar results were also noted in the loss of membrane protein thiols. A total of 40-50% of protein thiols were lost after 60 min of tert-butyl hydroperoxide and diamide treatment.. Again, the loss of protein thiols was significantly prevented by DTT.

Filamentous Actin and Tubulin Distribution Filamentous actin and microtubule distribution in hepatocytes companied with tert-butyl hydroperoxide- and diamide-induced blebbing were examined using confocal laser microscopy. To clearly identifiy the site of blebs, we align pictures taken under laser confocal (Fig. 5E-L) and transmission conditions (Fig. 5A-D). In normal hepatocytes, microtubule network extended from near nucleus to the peripheral area of cells (Fig. 5E) When cells were treated with tert-butyl hydroperoxide, microtubule network was apparently changed in cells bearing blebs. A

much greater fluorescence density was noted in blebs as compared to the cell body, suggested large amounts of microtubules were translocated into the blebs during blebbing (Fig. 5F). When blebs grew bigger higher fluorescence density still existed in large blebs (Fig. 5G). Similar changes were also observed in the diamide treatment (Fig. 5H). Moreover, microtubules were noted to intensify close to plasma membranes where no bleb formation or in cells before blebbing under oxidative stress (Fig, 6B). This transitional stage supported that microtubule translocation occurs and probably relates to the membrane bleb formation.

Whether the microtubule dynamic instability is related to the blebbing, two microtubule toxins, nacodazole and taxol, were applied to the next experiment. We proposed that changes noted during blebbing are likely due to microtubule instability, and the existence of nacodazole and taxol should affect oxidant-induced blebbing. As results indicated, however, 60 min preincubation with nacodazole and taxol did not enhance or suppress tert-butyl hydroperoxide caused blebbing. These findings suggested tert-butyl hydroperoxide-induced microtubule network changes as stated above were unlike the microtubule dynamic instability.

The actin cytoskeleton is also a cell shape determinant. Changes of actin network in hepatocytes following the addition of tert-butyl hydroperoxide or diamide were examined using confocal laser microscopy (Fig. 7). As seen, actin cytoskeleton changes during blebbing apparently differed from those observed for microtubules. After the adding of tert-butyl hydroperoxide (Fig. 7A,C) or diamide (Fig. 7B,D) into cells, actin extended into the surface blebs, but the fluorescence intensity in blebs were much less relative to the cell body.

SDS-PAGE and Western Blotting The changes in amounts of insoluble actin and ß-tubulin with blebbing were also determined by immunoblotting. As indicated, Triton X-100 insoluble β-tubulin decreased rapidly following the addition of tert-butyl hydroperoxide (Fig. 8B). Most of the β-tubulin lost at the first 15 min then the decrease remained constant. In the presence of DTT preincubation, β-tubulin decrease was largely prevented.

In contrast to the rapid decrease noted in β-tubulin, actin was also decreased following the exposure of tert-butyl hydroperoxide but in a slower and time-dependent manner (Fig. 8A). DTT again prevented the loss of actin. In case of diamide, similar results as observed with tert-butyl hydroperoxide treatment were also obtained (data not shown).

DISCUSSION

In this study, both tert-butyl hydroperoxide and diamide caused apparent membrane morphology changes suggesting the cellular macromoleucle oxidation, i.e. the membrane phospholipids and cellular thiols, plays important roles in the bleb formation on plasma membranes. Under such a circumstance, cytoskeleton networks, especially the microtubule cytoskeleton, were dramatically disturbed by accompanying with the decrease of insoluble actin and ß-tubulin. The coincidence of microtubule changes with blebbing further indicates the crucial role of microtubule in determining bleb formation under oxidative stress and is worth investigating in the future study.

Membrane blebbing is a common phenomena of cell undergoing necrosis and apoptosis under various conditions (Trump and Berezesky, 1995). Recently, this cell shape change was also demonstrated under a normal physiological condition in pancreatic acinar cells, so called cholecystokinin-suprastimulation (Torgenson and McNiven, 1998). The molecular mechanism of blebbing attracts a lot of interesting and cellular Ca^{2+} homeostasis disturbance is generally regarded as a key factor (Jewell et al., 1982), although Ca^{2+}-independent blebbing is also obtained under certain circumstances (Lemaster et al., 1987). In this study, although Ca^{2+} change was not determined, evidence demonstrated that both lipid peroxidation and cellular thiol status contributed to this membrane abnormality under oxidative stress.

Diamide and tert-butyl hydroperoxide are two widely used prooxidants in the study of oxidative stress in intact cells (Sies, 1985). tert-Butyl hydroperoxide is an organic hydroperoxide whose metabolism causes GSH oxidation to its disulfide (GSSG) with the

action of GSH peroxidase. GSSG is then reduced to GSH by GSH reductase with the consumption of NADPH. The depletion of NADPH causes cell damage (Reed, 1990). Otherwise, when this defense is overwhelmed, tert-butyl hydroperoxide generates tert-butyl alkoxyl radicals in the presence of transient metals or upon the reaction of cytochrome P450 (Hogberg et al., 1975). Both tert-butyl hydroperoxide metabolism to free radicals and/or GSSG accumulation damage cellular phospholipids and proteins (Liu et al., 1998; Sakaida et al., 1991; Sies, 1985). For instance, membrane Ca^{2+}-ATPase is one the targets and therefore disrupts the major cellular buffering system and interferes the intracellular Ca^{2+} homeostasis (Henschke and Elliot, 1995; Hoyal et al., 1996). Consequently, such a condition activates Ca^{2+}-sensitivity protein-digesting enzymes (Berridge et al., 1998). Increase of calpain activity accelerates the cleavage of talin and α-actinin and is associated with tert-butyl hydroperoxdie-induced blebbing (Miyoshi et al., 1996). Diamide, although not generating radical per se, results in oxidation of cellular thiols, including both GSH, the most abundant cellular nonprotein thiol, and protein thiols. Therefore, tert-butyl hydroperoxide and diamide are suited for studying the effects of free radical-derived damage and thiol depletion on oxidant-induced blebbing.

The importance of membrane phospholipids on the membrane integrity under oxidative stress can be supported by tert-butyl hydroperoxide caused TBARS generation is correlated well to the extent of blebbing and LDH leakage. Even so, 8% of cell beared bleb even when lipid peroxidation was completely inhibited in cells pretreated with TS indicating factors other than lipid peroxidation participate in this cell shape change. Twenty-five % of blebbing cells in diamide-treated group, which did not increase TBARS production, and DTT inhibition on tert-butyl hydroperoxide- and diamide-induced blebbing demonstrate the cruicial roles of cellular thiols on membrane morphology (Mirabelli et al., 1988). DTT maintains both GSH and protein thiols in reduced state. GSH, thus, helps to detoxify tert-butyl hydroperoxide and diamide caused damage. Cellular thiols as an alternative factor that might be important in cells against oxidative stress has been proposed (Caraceni et al., 1994; Marchetti et al., 1997; Mirkovic, et al., 1997; Pascoe and Reed, 1989; Sato et al., 1995).

As demonstrated by immunoblotting and confocal laser microscopy examination in this study, both membrane insoluble actin and tubulin are the target under oxidative stress. Decrease of actin and ß-tubulin caused was noted to accompany with bleb formation, and this change was inhibited in the presence of DTT. The decrease of actin accompanied with menadione-induced blebbing was attributed to the abnormal actin polymerization (Mirabelli et al., 1988). This formation of high molecular weight molecules disappeared when analyzed by reducing electrophoresis indicates it is thiol-related. There are a total of five thiols groups in the actin molecule exhibiting variable reactivity to a number of thiol reactive agents and, G-actin is more reactive than F-actin (Lusty and Fasoil, 1969). When cysteine at the 374th residue in actin is modified with GSSG, it weakens the mechanical stability of actin filaments (Stournaras et al., 1990). Similarly, ß-tubulin contains 8 cysteines (Krauhs et al., 1981) and blocking of certain of them with thiol modifying agents, such as N-ethylmaleimide, prevents microtubule assembly (Kuriyama and Sakai, 1974). GSSG accumulation in cells exposed to tert-butyl hydroperoxide and diamide could be possible to react with ß-tubulin thiol groups, therefore, to disturb the assembly of microtubules.

tert-Butyl hydroperoxide-caused membrane bleb formation has been attributed to talin and α-actinin degradation which results in plasma membrane detaching from submamebrane microfilament cytoskeleton (Miyoshi et al. 1996). Under such a circumstance, the cytoskeleton structure seemed to be well preserved under transmission electron microscopy. In this study, however, it was noted that both microtubule and actin structure extent into blebs under confocal laser microscopy, indicating the cytoskeleton structure was damaged, Meanwhile, the microtubule network rather than actin filaments had a dramatic change with tert-butyl hydroeproxide or diamide treatment. Accompanying with bleb formation, normal microtubules structure appears to be disrupted by translocating microtubules as evidenced by the condensed intensity of microtubules closed to plasma membranes. Microtubules then dumped into blebs. As we known, we are the first to report such a finding in microtubule structure in the oxidant-induced blebbing, however, the molecular mechanism of microtubule that undergoing translocation is unknown. At least in this

point, the characteristics of microtubules dynamic instability seems to be not the cause of tert-butyl hydroperoxide- or diamide-induced membrane bleb formation. Several microtubular-targeted drugs, such as vinblastin, nocodazole, colchicine and taxol, have been widely applied to study the microtubule assembly dynamics in living cells (Jordan and Wilson, 1998), and demonstrated that the dynamic microtubule in allowing microtubule rearrangement is necessary for cell locomotion in various cell types (Gotlieb et al., 1983; Liao et al., 1995; Zakhireh and Malech, 1980) and growth cone motility and axonal growth in neural cells (Bamburg et al., 1986; Tanaka et al., 1995). However, the lack of effect of taxol and nacodazole in tert-butyl hydroperoxide-induced blebbing suggests the existence of other mechanisms. To clarify this puzzle, further experiment is worth investigating and is necessary.

In conclusion, membrane blebbing was induced in hepatocytes by either a radical-generating or a thiol-modifying oxidant. Although membrane lipid peroxidation related to bleb formation, cellular thiol redox status also plays an important role in oxidant-induced blebbing. Among cellular thiols, the maintenance of the thiol redox of two cytoskeletal proteins, actin and ß-tubulin, could be an important determining factor on cell shape. Furthermore, the changes of microtubular network accompanied with blebbing, which was not seen in microfilament structure, indicated the role of microtubules can be crucial, and the molecular mechanism is worth further investigating.

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Table 1. The effect of DTT on the cellular thiol content.

Treatment	Reduced GSH (nmol/mg protein)	Membrane protein thiols (% of control)
Control	47.8 ± 7.8^{a}	100^{a}
TBH	0.5 ± 0.5^{b}	52.7 ± 8.4^{b}
TBH+DTT	45.8 ± 9.5^{a}	96.3 ± 9.5^{a}
Diamide	ND	61.2 ± 8.7^{b}
Diamide+DTT	47.9 ± 5.8^{a}	89.3 ± 1.5^{a}

Cells were preincubated with 5 mM DTT for 30 min before the addition of TBH or diamide at a final concentration of 2 mM. Samples were collected after 60 min of TBH treatment. Reduced GSH and membrane protein thiols were measured as described under Materials and Methods. The data are means ± standard deviation from three seperate experiments. Means not sharing the same letter are siginificantly different ($P<0.05$). ND = not detectable

Figure 1 The dynamic change of bleb formation in plasma membranes. Hepatocytes were treated with 2.0 mM tert-butyl hydroperoxide (A, B, C) or diamide, respectively, and membrane blebbing was monitored before (A, D) or after 15 (B, E) or 45 min (C, F) of oxidants exposure.

Figure 2 The percentage of membrane bleb formation over 60 min of tert-butyl hyfroperoxide (TBH) or diamide exposure in the absence or presence of dithiothreitol (DTT) preincubation.

Figure 3 Cytotoxicity by tert-butyl hydroperoxide (A) or diamide (B) as determined by lactate dehydrogenase leakage.

Figure 4 Lipid peroxidation in hepatocytes treated with either tert-butyl hydroperoxide or diamide.

Figure 5 The changes of microtubule network examined by confocal laser microscope. Hepatocytes were treated without (A, E, I) or with either 2.0 mM tert-butylhydroperoxide (B, C, F, G, J, K) or 2.0 mM diamide (D, H, L) to induce membrane bleb formation. Microtubule network was immunostained with anti-β-tubulin antibody and FITC-conjugated secondary antibody (E-H). Neclei were stained with actinomycin D (I-L). Cells noted above were also monitored under transmission condition (A-D). _, 25 μm.

Figure 6 The intermediate stage of microtubule translocation to plasma membranes in the presence of tert-butyl hydroperoxided. _, 25 μm.

Figure 7 The changes of actin network examined by confocal laser microscope. Afollowing the exposure to either 2.0 mM tert-butylhydroperoxide (A,C) or 2.0 mM diamide (B, D), actin was labeled with NBD-phallinidin and the microfilamentous

network was examined by confocal laser microscope under laser confocal (C, D) or transmission (A, B) condition. _, 25 μm.

Figure 8 Immunoblot analysis of the changes of α–actin (A) and β–tubulin (B) levels. Hepatocytes were exposed to 2.0 mM tert-butyl hydroperoxide for 0 (lane 1), 15 (lane 2), 30, or 60 min, respectively, in the absence or presence of dithiothreitol pretreatment.

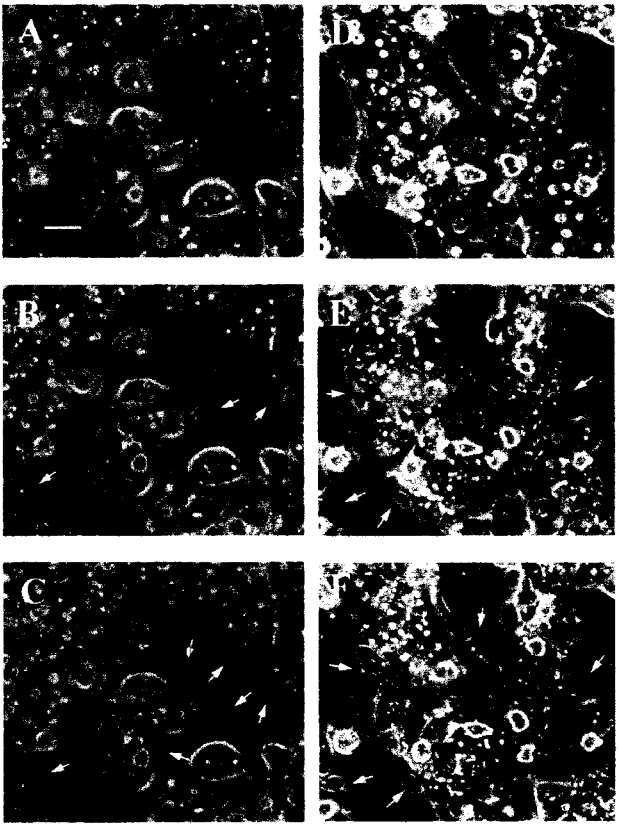

Fig. 1

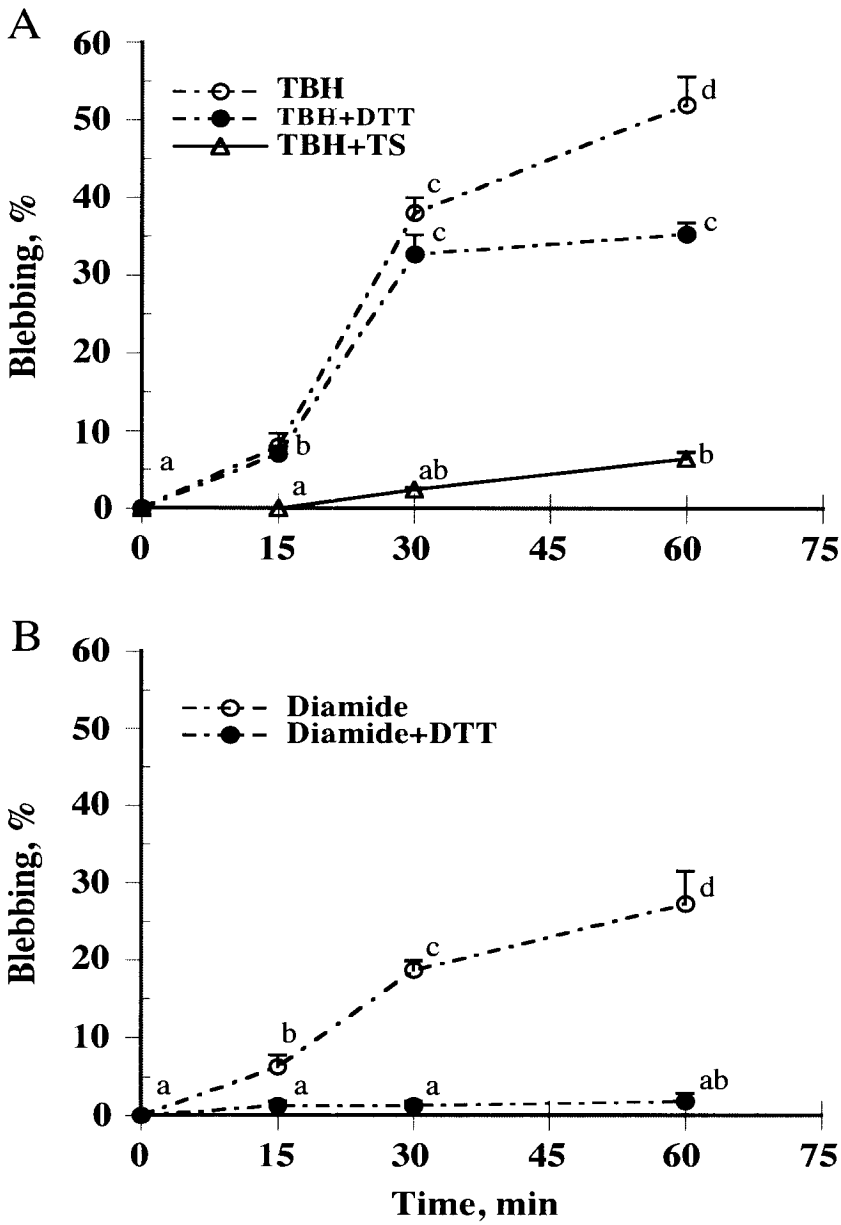
A
Blebbing, %
TBH
TBH+DTT
TBH+TS
0
10
20
30
40
50
60
0
15
30
45
60
75
a
b
a
ab
b
c
c
d
c
B
Blebbing, %
Diamide
Diamide+DTT
0
10
20
30
40
50
60
0
15
30
45
60
75
Time, min
a
b
a
a
ab
c
d

Fig. 2

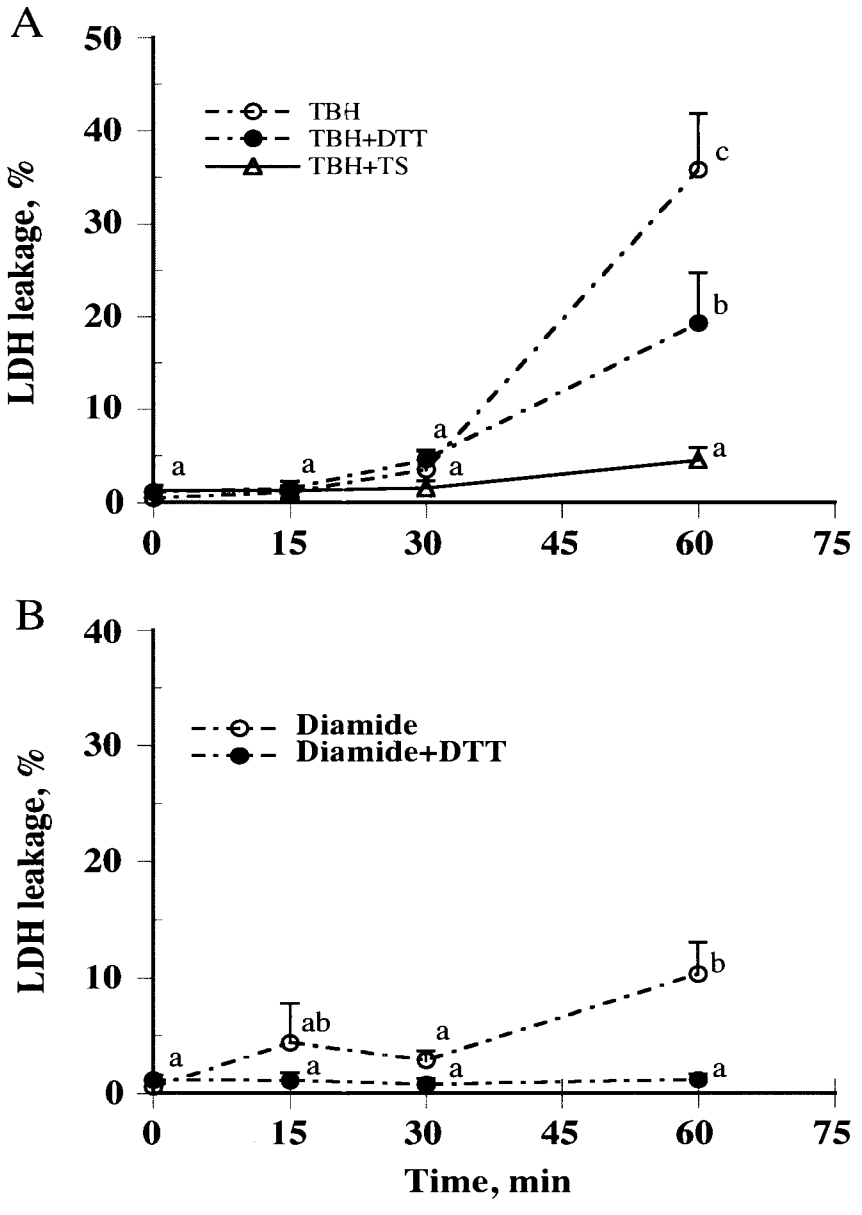
A
LDH leakage, %
TBH
TBH+DTT
TBH+TS
a
a
a
a
b
c
a
0
10
20
30
40
50
0
15
30
45
60
75
B
LDH leakage, %
Diamide
Diamide+DTT
a
ab
a
a
a
b
a
0
10
20
30
40
0
15
30
45
60
75
Time, min

Fig. 3

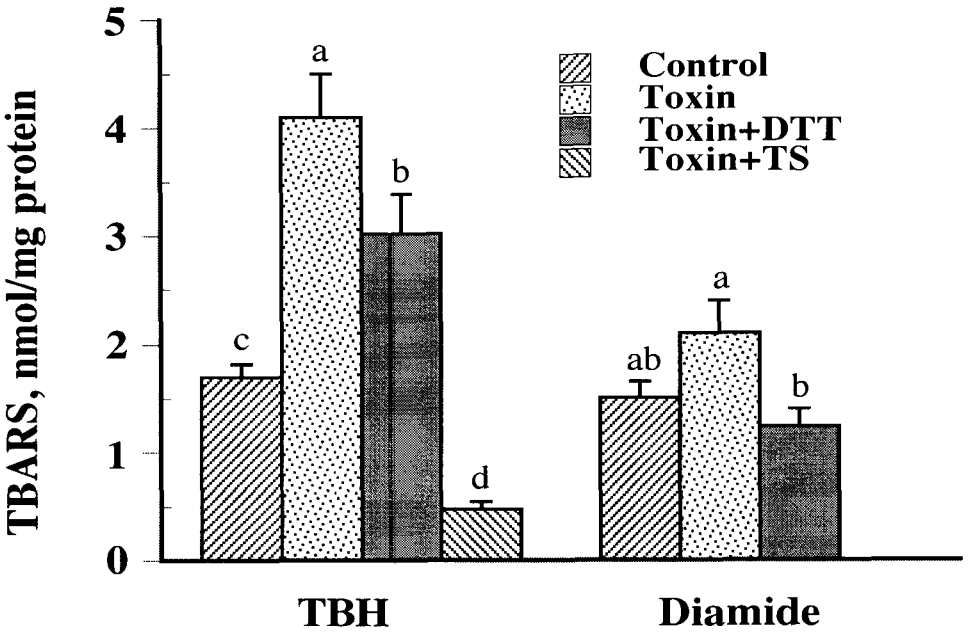
TBARS, nmol/mg protein
5
4
3
2
1
0
a
b
c
d
ab
a
b
Control
Toxin
Toxin+DTT
Toxin+TS
TBH
Diamide

Fig. 4

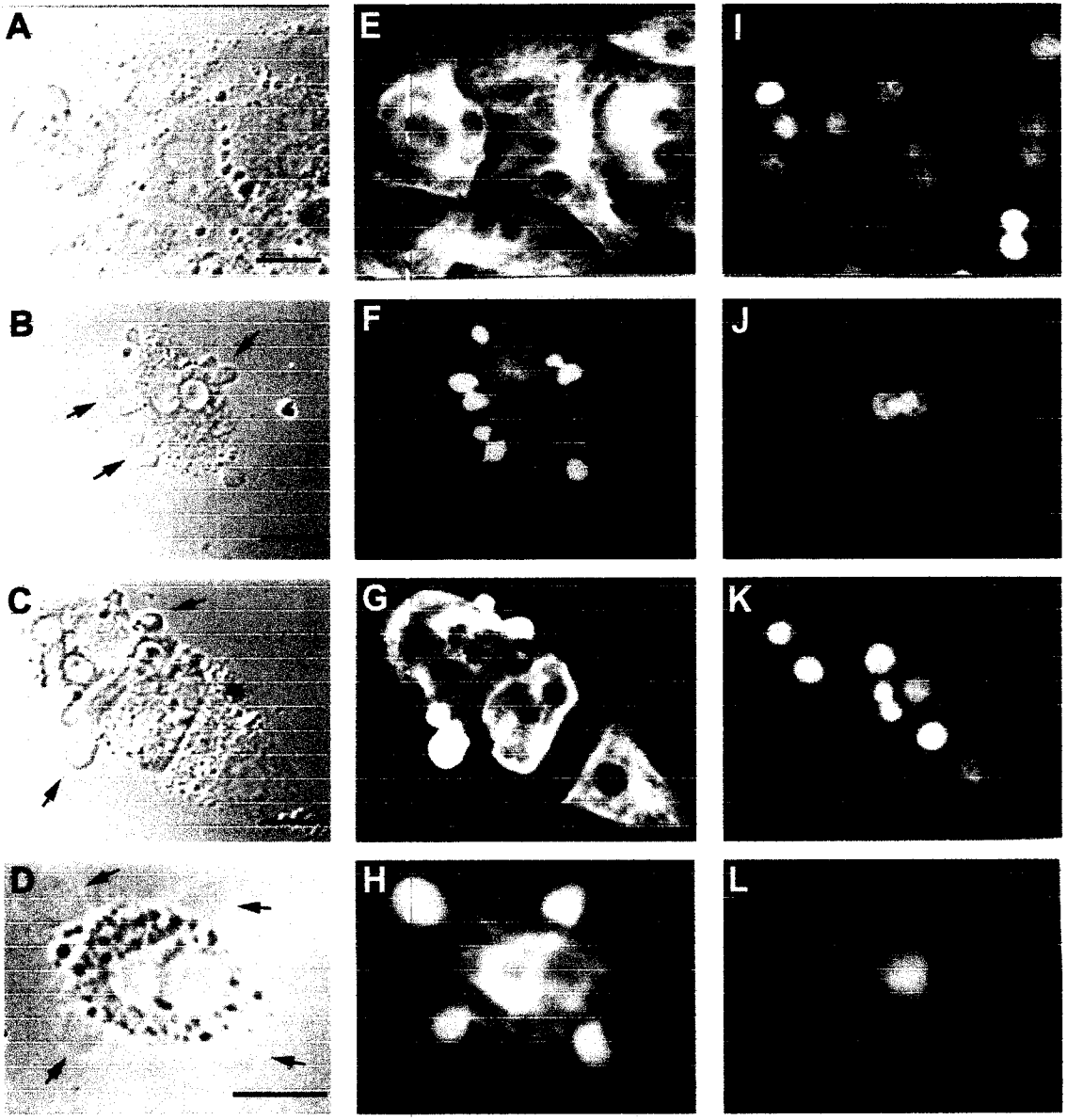

Fig 5

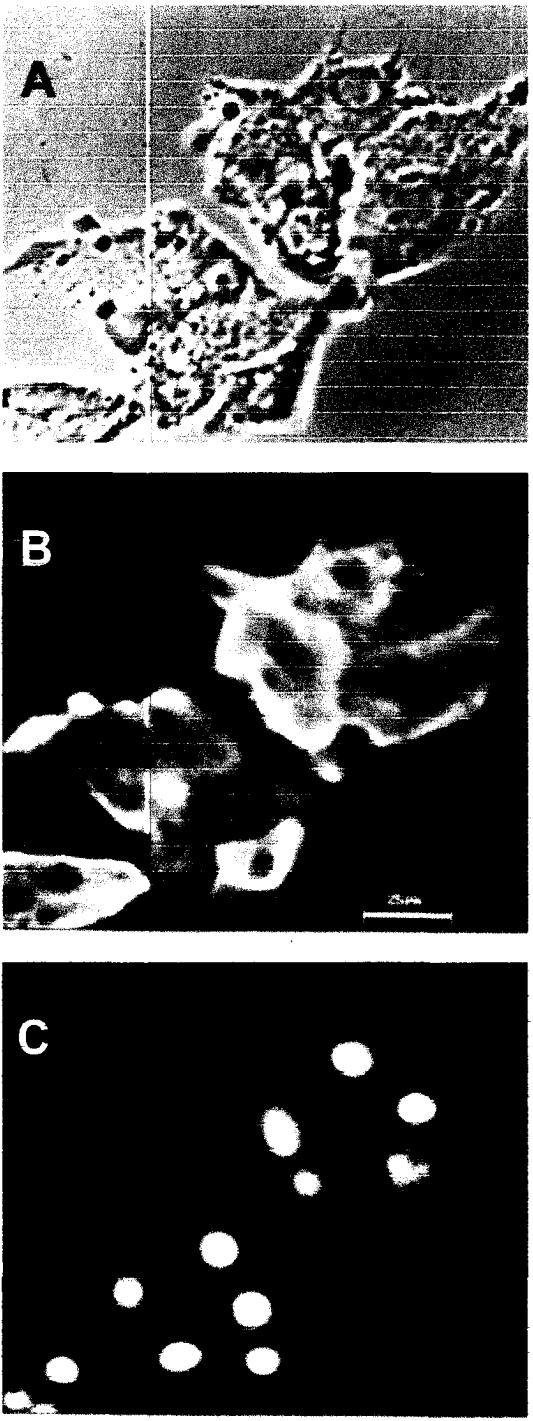

Fig 6

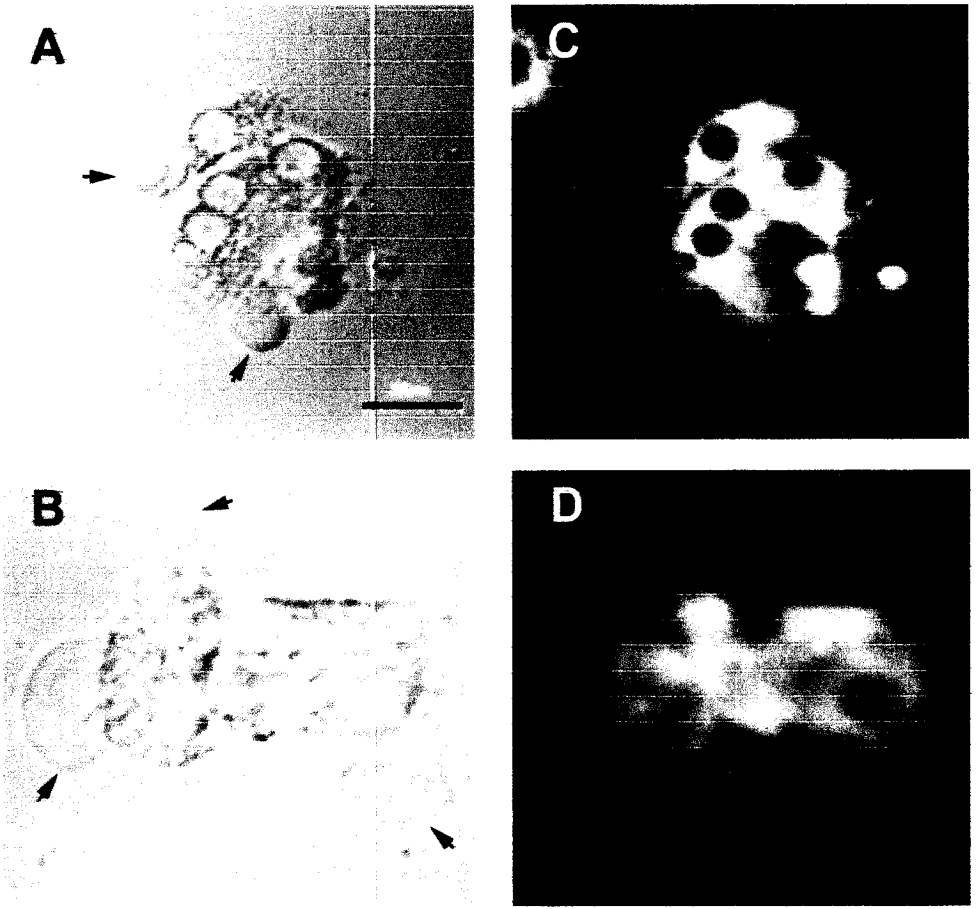

Fig 7

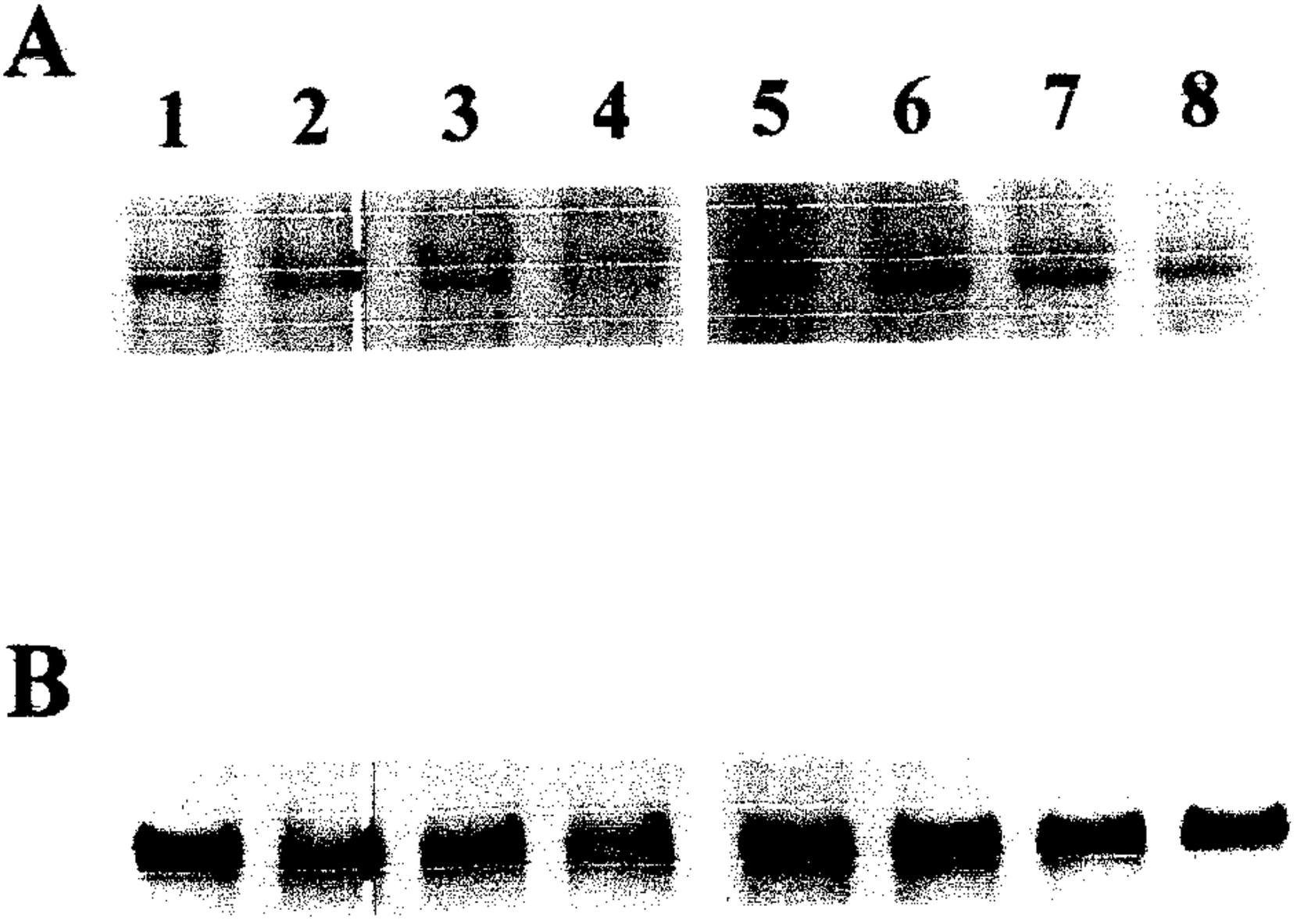

Fig 8

Title

The Effect of Vitamin E on the Intracellular Calcium Ion Using a Model of Plasma Membrane Blebbing of Rat Hepatocytes under an Oxidative Stress Induced by *tert*-butyl hydroperoxide

Authors and Their Affiliations.

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Runnig Title

intracellular Ca^{2+} homeostasis and vitamin E

Key Word

Hepatocyte, glutathione, lipid peroxidation, calcium homeostasis, membrane protein thiol.

Footnotes

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The abbreviations used are: TBH, *tert*-butyl hydroperoxide; BSO, L-buthionine[S,R]-sulfoximine; EGTA, ethylene glycol bis(β-aminoethyl ether) *N*,*N*'-tetraacetic acid; GSH, glutathione; GSSG, glutathione disulfide;DMSO, dimethyl sulfoxide; HPLC, high perfermance liquid chromatography; TBARS, thiobarbituric acid-reactive substances; LDH, lactate dehydrogenase.

ABSTRACT

The effect of vitamin E on the ntracellular calcium under oxidative stress was investigated using a model of plasma membrane blebbing of rat hepatocytes induced by *tert*-butyl hydroperoxide (THB) using confocal microscopy, high pressure liquid chromatography, and spectrophotomety. A percentage of 60.6 ± 1.1% in hepatocytes treated with 2.0 mM TBH and 40.0 ± 8.2% in cells pre-treated with BSO and treated with 1.0 mM TBH beared blebs whereas only a percentage of 22.3 ± 4.2% formed blebs in the cells pre-treated with vitamin E. Moreover, vitamin E maintained intracellular calcium concentration in TBH-treated hepatocytes. Depletion of intracellular and extracellular calcium with EGTA and/or A23187 also caused a significant reduction in the percentage of blebbing. Vitamin E treatment prevented total GSH consumption, LDH leakage, and lipid peroxidation after treatment of TBH. Loss of total membrane protein thiols in TBH-treated hepatocytes became significant lower under the influence vitamin E. These findings indicate that vitamin E may preserve the integrity of the cell membrane by the inhibition of lipid peroxidation and the protection of membrane protein thiols and hence maintain intracellular calcium homeostasis and reduce the level of bleb formation on the cell membrane of hepatocytes 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]. Increase in the intracellular calcium concentration may induce a series of calcium-dependent reactions catalyzed by the calcium-dependent proteases, phospholipases, or endonucleases[3]. These enzymes may disrupt the integrity of cytoskeleton and lead to bled formation and growth. The course of plasma membrane blebbing on hepatocytes has been divided into three stages: formation, shedding and fusion, and rupture[4]. The injuries to the hepatocytes in the first two stages are reversible whereas bled rupture is irreversible and results in cell lysis[5].

In addition to its nutritional importance, vitamin E (α-tocopherol) is also a natural antioxidant in the prevention of peroxidation of polyunsaturated fatty acids in the cellular and subcellular membrane phospholipids under oxidative stress[6]. Moreover, we have demonstrated that vitamin E may prevent plasma membrane blebbing and the loss of protein thiols in *tert*-butyl hydroperoxide (THB)-treated hepatocytes[7]. Although blebs on the cell membrane may be produced under anoxia without changes in the cytosolic free calcium[8], disturbance in the intracellular calcium homeostasis remains the principal explanation for the mechanism of plasma membrane blebbing[5]. It is possible that the protection of cell morphology by vitamin E may be related to the maintenance of intracellular

calcium. In this study, we emp oyed the plasma membrane blebbing of rat hepatocytes as a model to determine the effect of vitamin E on the intracellular calcium under the oxidative stress induced by TBH.

MATERIALS AND METHODS

Isolation and Culture of Hepatocytes 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[7]. After isolation, the cells were 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, and100 mg/L streptomycin. The cells were then cultured in a 37°C humidified incubator in ambient air for 4 h. Unattached and dead cells were removed and the culture medium was changed to L-15 culture medium with 0.2% bovine serum albumin but without 2.5% fetal bovine serum. Cells without treatment or treated with 100 μM vitamin E (α-tocopherol succinate) or 1 mM BSO were incubated at 37°C for 20 h before treating with different concentrations of TBH and detecting changes in the cells.

Confocal Microscopy Alternations in intracellular calcium were determined by confocal microscopy with a calcium-sensitive fluorescent dye (Fluo 3-AM) and videomicroscopic imaging using the method of Burnier et al.[9] with some modifications. Fluo 3-AM (5 μM) was added to culture medium and the hepatocytes was 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 atage 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 described by Burnier et al.[9].

High Pressure Liquid Chromatography (HPLC) Cells pre-treated with BSO (1 mM) were washed twice with PBS and 1 ml 5% perchloric acid was added. These cells were allowed to stand for 30 min to dissolve GSH and GSSG into perchloric acid. To the acid solvent containing GSH and GSSG (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 and GSSG were determined by HPLC using the method described by Reed et al.[10].

Spectrophotomety To determine lipid peroxidation, hepatocytes were washed twice with cold phosphate-buffered saline (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)[11]. 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 TBAR 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 twice at 4,000× *g* for 10 min to obtain the membrane fractions. The membrane fractions were then mixed thoroughly with the same buffer (800 µl). The total membrane protein thiols were measured after the incubation with 5,5'-dithio-bis-nitrobenzoic acid as described by Boyne and Ellman[12] and the total protein concentrations were determined by the method of Lowry et al.[13]. To express the cell viability, the lactate dehydrogenase (LDH) leakage was analyzed according to the method of Moldeus et al.[14].

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 test was used to determine the difference among groups and Student's *t*-testwas used in case of the two group comparison. $P < 0.05$ were considered to have statistical significance.

RESULTS

Initiation of Hepatocyte Blebbing by TBH and Changes in the Intracellular calcium Under the confocal microscope, the intensity of fluorescence from the hepatocytes treated with 2.0 mM TBH increased with time (Fig. 1A, C, E, G, and I). The locations of blebs observed under the transmission mode corresponded to these intensities (Fig. 1B, D, F, H, and J). A significant increase in fluorescence intensity and a bleb in a hepatocyte were observed at 24 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 intensity of fluorescence was proportional to the size of blebs. The fluorescence disappeared at 45 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 2.0 mM TBH for 60 min (control), 60.6 ± 1.1% formed blebs on the cell membrane. Significantly lower percentages of 27.4 ± 5.8% (n = 4) and 19.0 ± 5.1% (n = 4) were obtained by the pre-treatment with EGTA (15 mM) and EGTA (15 mM) with A23187 (10 μM), respectively ($P < 0.05$). The same concentration of EGTA and A23187 were used in the other experiments. Moreover, a significantly lower percentage of 27.5 ± 2.2% was also observed in the cells treated with 2.0 mM TBH for 9 min and then with EGTA with A23187 ($P < 0.05$). Treatment with vitamin E also yielded a significantly lower of 22.3 ± 4.2%. However, no significant differences were found among the experimental groups ($P > 0.05$).

Figure 2 shows the elevation of the fluorescence density in TBH-treated hepatocytes. The fluorescence density of the intracellular calcium rapidly increased since 12 min after TBH, and reached the maximum at 36 min.

The fluorescence response in the control was detected at 12 min after treatment with 2.0 mM TBH and increased to 4 folds at 45 min. In the cells pre-treated with vitamin E, the response was in a steady level and significant lower than the control. After adding EGTA and A23187 to the cells treated with TBH at the first appearance of blebs (9 min), the fluorescence intensity increased at 12 min and then decreased significantly at 18 min (Fig. 3A).

Effects of BSO on the Intracellular calcium in TBH-Treated Hepatocytes To determine the role of GSH in the homeostasis of intracellular calcium, hepatocytes were pre-treated with BSO before the treatment with 1.0 mM TBH. Pre-treatment with BSO significantly increased the percentage of blebbing from 18 ± 4.2% in the hepatocytes only treated with TBH for 60 min to 40 ± 8.2% ($P < 0.05$). However, after adding EGTA with A23187 to the TBH-treated cells, the blebbing percentage was significantly reduced to 3.4 ± 1.4% ($P < 0.05$).

The fluorescence response from the 1.0 mM TBH-treated cells with pre-treatment of BSO increased with time and significant higher than that of the control. After adding EGTA and A23187 at the first appearance of blebs (12 min), the fluorescence intensity in these cells the significantly increased to 21 min followed by gradually decreased (Fig. 3B).

Alternation of GSH in Hepatocyte by TBH GSH concentration in hepatocytes decreased rapidly after treatment with different concentrations of TBH (0.5 mM, 1.0 mM, and 2.0 mM) and the concentration of GSSG increased significantly within 15 min after the treatment. The concentration of GSH gradually increased 15 min after treating with 0.5 mM TBH whereas the concentration remained decrease in the remaining two TBH treatments. However, the concentration of GSSG decreased 15 min after the TBH treatments (Fig. 4A and 4B).

Effects of Vitamin E and BSO on Total 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, 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 BSO completely depleted intracellular GSH in 1.0 mM TBH-treated cells. However, vitamin E maintained GSH in 2.0 mM TBH-treated hepatocytes, although the level of GSH was significantly lower than that of the control (Table 1).

The levels of LDH leakage in hepatocytes treated with 1.0 or 2.0 mM TBH or BSO and 1.0 mM TBH were significantly higher than the control. However, there was no significant difference in the leakage between 2.0 mM TBH-treated cells with pre-treatment of vitamin E and the control (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,

BSO and 1.0 mM TBH or EGTA with 2.0 mM TBH than the control. However, there was no significant difference in the production between 2.0 mM TBH-treated cells with pre-treatment of vitamin E and the control (Table 1).

Effects of Vitamin E to the Loss of Membrane Protein Thiols Induced by TBH Fig. 5 shows the loss of membrane protein thiols after TBH treatment with and without the influence of vitamin E. 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 56% 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 33% at 60 min.

DISCUSSION

The mechanism of plasma membrane blebbing is considered to be relatively complex and may be due to many factors. Damage to the homeostasis of intracellular calcium have been considered to be a crucial factor for this morphological abnormality[15,16]. Under the oxidative stress induced by hypoxanthine and xanthine oxidase, intracellular calcium have been found to play a role in the bleb formation. The formation of blebs in TBH-treated hepatocytes has also been attributed to the elevation of intracellular calcium concentration[17].

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. By pre-treating hepatocytes with EGTA or EGTA with A23187 to remove the intracellular and extracellular calcium, we found that the percentage of blebbing significantly decreased from 61% in the control group to 27% or 19%. These findings signify the importance of intracellular calcium in the formation of plasma membrane blebbing. Moreover, treatment with EGTA and A23187 after bleb formation may also reduce the percentage of blebbing. However, EGTA may also react with iron and the prevention of blebbing may be due to the combination of EGTA with the intracellular iron which may be required for lipic peroxidation[18]. 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[19,20].

Vitamin E may prevent the death of cultured hepatocytes treated with TBH[21]. In addition to vitamin E, antioxidants such as *N*,*N*'-diphenyl-p-phenylenediamine, butylated hydroxytoluene, butylated hydroxyanisole, *n*-propyl galate and *t*-butylhydroquinone have been found to be effective in reducing the lipid peroxidation in TBH-treated hepatocytes[22]. In this study, we demonstrated that vitamin E blocks not only lipid peroxidation but also the elevation of intracellular calcium concentration in TBH-treated hepatocytes. It has also been reported that lipid peroxidation-induced calcium accumulation is completely prevented by vitamin E[23]. This suggested that the preservation of intracellular calcium homeostasis by vitamin E may be also an significant factor in preventing the bleb formation.

Our previous study has reported that protection of cell morphology by vitamin E is associated with protein thiols[7]. In this study, we demonstrated that vitamin E partially prevents the loss of membrane protein thiols under oxidative stress. There is a positive correlation between lipid peroxidation in the membrane and the loss of membrane protein thiols[24]. The antioxidative action of vitamin E on the protection of membrane protein thiols is mainly occurring in the cell membrane[25]. These findings suggest that protection of membrane protein thiols by vitamin E should be occurred in the cell membrane. The integrity of cell membrane may be important to the maintenance of homeostasis of intracellular calcium. For instance, regucalcin, a calcium-binding protein, activates (Ca^{2+}-

Mg^{2+})-ATPase by the binding to the plasma membrane lipids[26]. The (Ca^{2+}-Mg^{2+})-ATPase pump is believed to play a role in maintaining the homeostasis of intracellular calcium[27,28]. Under oxidative stress, thiols of regucalcin may be oxidized. This change in regucalcin leads to the inactivation of the (Ca^{2+}-Mg^{2+})-ATPase pump and damage to the homeostasis of intracellular calcium[26,29,30]. These indicate that change in the Ca^{2+}-ATPase activity is associated with the loss of protein thiols in the cells, although the activity of Ca^{2+}-ATPase has been reported to related to a decrease in the membrane lipid fluidity caused by lipid peroxidation[31].

In addition to vitamin E, GSH, an important member of the intracellular anitoxidative system, has also been demonstrated to be effective in preserving the homeostasis of intracellular calcium and the integrity of cell membrane[32]. Inhibition of GSH synthesis may lead to a significant oxidative stress[33]. In the present study, cells pre-treated with BSO, a GSH synthesis inhibitor, had a significantly higher blebbing percentage than the control group. Although we have demonstrated that GSH may prevent morphological changes in TBH-treated hepatocytes, this protection may become ineffective in the treatment with high concentrations of TBH.

Treatment of hepatocytes with TBH may rapidly oxidize the intracellular GSH and enhance lipid peroxidation[34]. These intracellular changes may in turn lead to a significant decrease in the viability of the cells. To prevent the damages to cells from the oxidative stress, the exogenous TBH is converted to t-butyl alcohol by the GSH peroxidase and GSSG is produced[34]. Similarly, results of

this study revealed that oxidat ve stress also leads to an increase in the concentration of intracellular GSSG by decreasing the GSH level. However, GSSG at high concentrations may react with actin to form actin-GSH mixed disulfide. This reaction not only weakens the intensity of actin fibrils but also cause the dissociation of the protein[35]. The morphological alternations observed in cultured gastric mucosal cells may be attributed to the mixed disulfide exchange in actin[36]. Therefore, we speculated that elevation of intracellular GSSG may lead the formation of protein-GSH mixed disulfides and the loss in membrane protein thiols. These alternations in the membrane protein thiols may also change the permeability of the cell membrane to calcium and lead to influx of extracellular calcium to the ntracellular environment.

It has been reported that the alteration of cytosolic free calcium may be not required for bleb formation[8,37]. 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[38]. Although there is an association between vitamin E and intracellular calcium concentration, membrane blebbing may also induced through other mechanisms, since blebs were found in 22% of the hepatocytes pre-treated with vitamin E after TBH treatment. Nevertheless, based on the observations on this study, vitamin E decreases the consumption of GSH, the protein thiol loss and lipid peroxidation under oxidative stress induced by TBH. These mechanisms may contribute to maintain intracellular calcium homeostasis and reduce the level of bleb formation on the cell membrane of hepatocytes under oxidative stress.

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LEGENDS

Fig. 1. Changes in the fluorescence density of intracellular calcium in TBH-treated hepatocytes. Using confocal microscopy, the changes of fluorescence intensity of intracelluar calcium were photographed before (A), 18 (C), 24 (E), 30 (G) and 45 min (I) after 2.0 mM TBH treatment. At the same time, the changes of cell morphology were also photographed before (B), 18 (D), 24 (F), 30 (H) and 45 min (J) after 2.0 mM TBH treatment. Pseudodensity scale indicates fluorescence intensity in arbitrary units. Bar, 20 μm.

Fig. 2. kinetics of changes in the fluorescence density of intracellular calcium in the up (●) and down (○) cells at the image of figure 1 before and after treated with 2.0 mM TBH.

Fig. 3. The effect of vitamin E and BSO on the fluorescence density of intracellular calcium in TBH-treated hepatocytes. (A) Changes in fluorescence intensity 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 2.0 mM TBH for 9 min and then with 15 mM EGTA plus 10 μM A23187. (B) Changes in fluorescence intensity were determined in the cells treated with 1.0 mM TBH (control), or with 1.0 mM TBH by the pretreatment with 1.0 mM BSO for 20 h, or by the pretreatment with 1.0 mM BSO for 20 h and with 1.0 mM TBH for 12 min and then with 15 mM EGTA plus 10 μM A23187. Values are expressed as means±SD. Treatment means in the same time not sharing the same superscripts differ significantly ($P<0.05$).

Fig. 4. Glutathione status in TBH-treated hepatocytes. Changes of the GSH (A) and the GSSG (B) concentration in hepatocytes were measured before and after treatment with different concentration of TBH (0.5 mM, 1.0 mM and 2.0 mM). Values are expressed as means±SD (n=3-4). Treatment means in the same time not sharing the same superscripts differ significantly ($P<0.05$).

Fig. 5. The effect of vitamin E on the loss of membrane protein thiols in TBH-treated hepatocytes. Cells were pre-incubated in the presence (●) or absence (O) of 100 μM vitamin E for 20 h before cells treated with 2.0 mM TBH. Data are expressed as the percentage of total protein thiols left relative to thiol contents at time 0. Values are expressed as means±SD (n=4). Treatment means in the same time not sharing the same superscripts differ significantly ($P<0.05$).

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

Treatments	Total GSH (nmol/mg protein)	LDH leakage (%)	TBARS (nmol/mg protein)
Control	63.0 ± 8.5^{a}	1.2 ± 0.6^{c}	0.66 ± 0.09^{de}
TBH (1.0 mM)	19.5 ± 8.5^{c}	43.8 ± 7.6^{b}	1.31 ± 0.41^{cd}
BSO + TBH (1.0 mM)	0.0^{d}	54.9 ± 5.8^{b}	1.99 ± 0.34^{bc}
TBH (2.0 mM)	4.1 ± 3.2^{d}	76.2 ± 13.8^{a}	3.90 ± 0.31^{a}
Vitamin E + TBH (2.0 mM)	40.7 ± 5.2^{b}	3.0 ± 1.5^{c}	0.41 ± 0.11^{c}
EGTA + TBH (2.0 mM)	ND	ND	2.73 ± 0.51^{b}

Values are expressed as means±SD (n=3-4). Treatment means in the same column not sharing the same superscripts differ significantly (P<0.05). ND = not determined.

Fig. 1.

Fig. 2. (First Author: Jer-Yuh Liu)

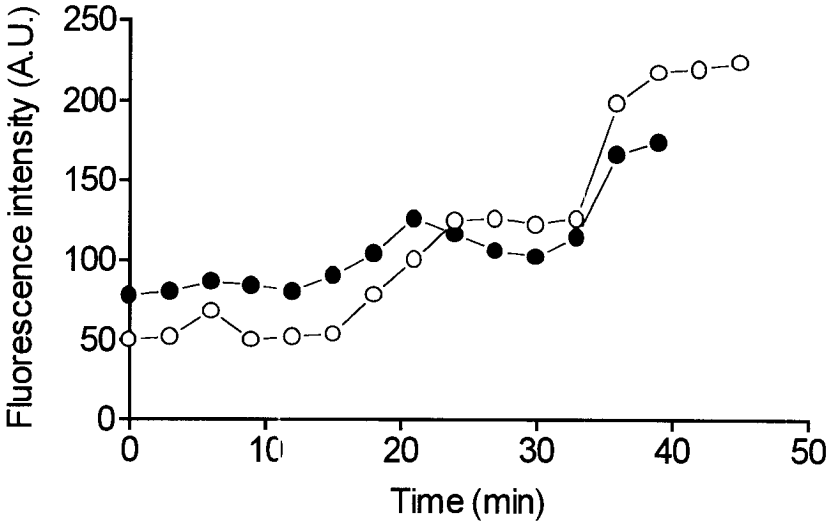

Fig. 3. (First Author: Jer-Yuh Liu)

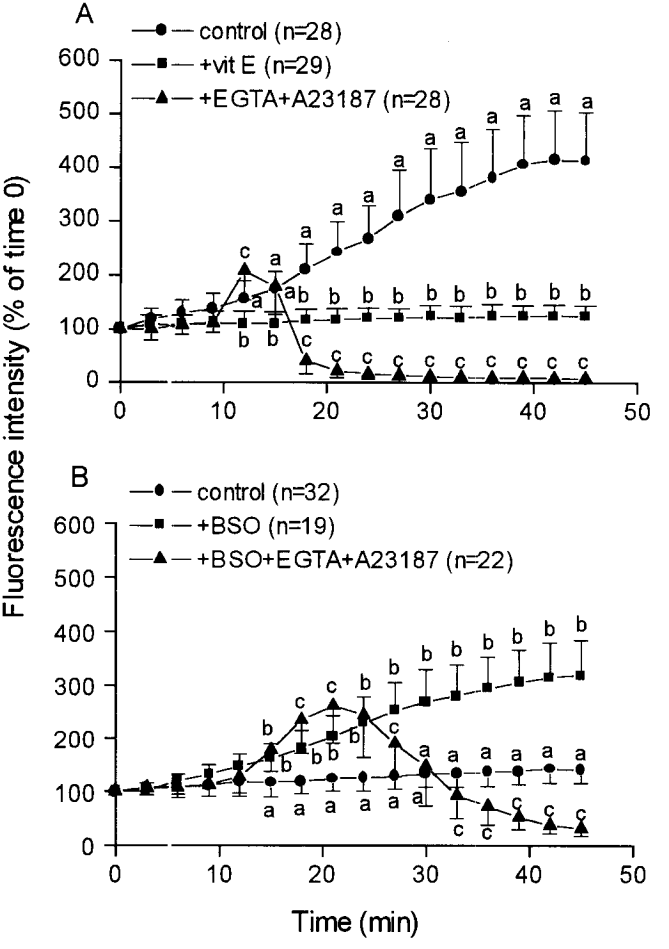

Fig. 4. (First Author: Jer-Yuh Liu)

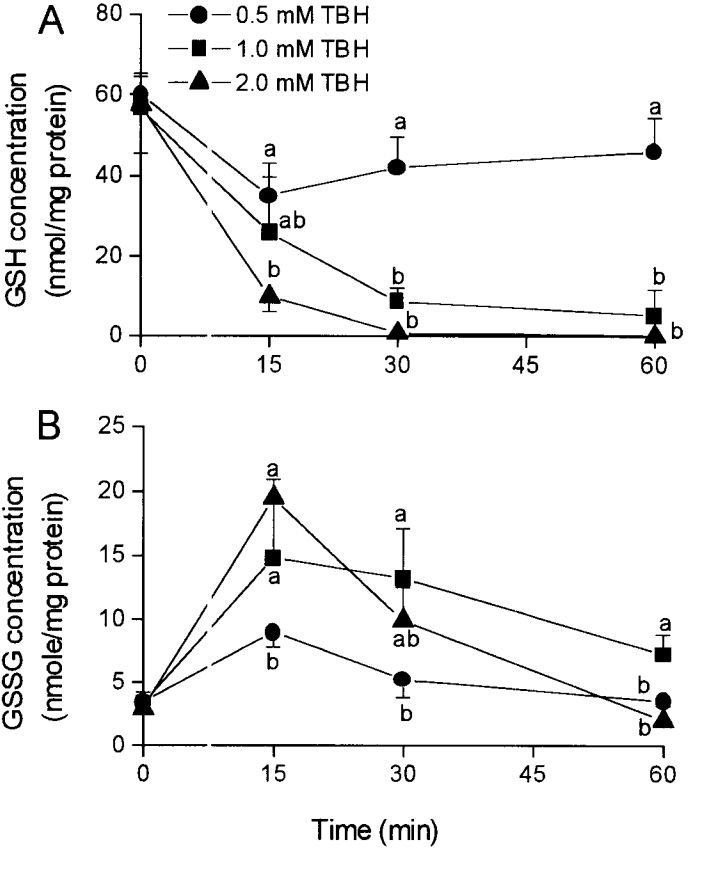

Fig. 5. (First Author: Jer-Yuh Liu)