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Activation mechanisms of endothelial NF-κB, IKK, and MAP kinase by *tert*-butyl hydroperoxide

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Abstract
Lipid peroxidation plays a major role in vascular dysfunction and age-related cardiovascular diseases. A major product of lipid peroxidation, *tert*-butyl hydroperoxide (*t*-BHP), has been reported to modulate vascular reactivity and cellular signaling. To better understand vascular abnormality, we set out to delineate the activation mechanism of nuclear factor kappa B (NF-κB) by *t*-BHP and the regulation of MAPK in endothelial cells.

The results showed that *t*-BHP induces NF-κB activation by an inhibitor of κB (IκB) phosphorylation through IκB kinase (IKK) activation. Our data from this *t*-BHP study also showed increased p38 MAP kinase and ERK activity; however, interestingly, *t*-BHP showed no influence on JNK. Pretreatment with the p38 MAP kinase inhibitor, SB203580 and the ERK1/2 inhibitor, PD98059, prevented *t*-BHP-induced increases in p65 translocation, NF-κB luciferase activity, and phospho-IKKα/β. Data suggested that *t*-BHP induces NF-κB activation through the IKK pathway, which involves p38 MAPK and ERK activation. This study illustrates a role of *t*-BHP in NF-κB activation and MAPK related-signaling pathways. The *t*-BHP-induced activation of NF-κB and MAPK could be a major player in vascular dysfunctions, as seen in oxidative stressed responses and the vascular inflammatory process.

Keywords: *Tert*-butyl hydroperoxide, IKK, NF-κB, p38 MAPK, ERK, endothelial cells

Introduction

Altered endothelium of the vasculature with advancing age is a major causative factor for the endothelial dysfunctions responsible for chronic vascular diseases [1]. Vascular changes in structure and function due to altered endothelium cells (ECs) are closely related to the redox-sensitive nuclear factor kappa B (NF-κB) activation seen in the pro-inflammatory response [2]. Based on available data on the effects of age-related oxidative stress, we recently proposed that inflammation and related pro-inflammatory processes are major underlying causes of age-related chronic disease processes, included vascular changes [3–5].

Oxidative stress-induced lipid peroxidation in plasma or vascular tissues is a common occurrence in aging and age-related diseases. Increased oxidative stress produces several lipid peroxidation products that are intimately involved in the pathogenesis of cardiovascular diseases like atherosclerosis [6, 7].

A reactive hydroperoxide produced from lipid peroxidation, *tert*-butyl hydroperoxide (*t*-BHP), is known to modulate vascular reactivity by modifying the Ca^{2+} signaling mechanism [8] and cause vasocontraction in rabbit aorta [9]. Recently, *t*-BHP was reported to induce vasoconstriction and the down-regulation of superoxide dismutase (SOD) expression in

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hypertensive and aged rats [10, 11]. *t*-BHP can trigger apoptosis through cytochrome *c* release from mitochondria [12].

Because of its lipophilicity and a long half-life compared to charged free radicals, *t*-BHP can migrate with relative ease through the membrane and cytosol, and therefore, is capable of attacking targets with a greater reactivity [13, 14]. In addition, *t*-BHP can be converted to potent *tert*-butoxyl radicals (*t*-BuO·) [15]. Thus, it seems important to uncover the molecular mechanism underpinning the effect of *t*-BHP on vascular endothelial cells.

One of the most versatile transcription factors in the cellular signal pathways, NF-κB, is exquisitely sensitive to cellular oxidative status [16]. In vascular smooth muscle cells, oxidized low density lipoproteins (LDLs) or bioactive aldehyde, 4-hydroxynonenal (HNE), promote apoptotic process through NF-κB activation [17]. This redox sensitive NF-κB transcription factor also plays a crucial role in the modulation of the expression of a variety of genes intricately involved in the inflammatory response [18, 19]; but until now, the modulation of NF-κB activation by *t*-BHP has not been reported.

The importance of NF-κB in aging was highlighted by the recently proposed "Inflammation Hypothesis of Aging" that provides new molecular insights into the modulation of various redox-sensitive transcription factors. The basic tenet of this hypothesis is based on consistent observations of up-regulated pro-inflammatory gene expressions by NF-κB activation in most aged tissues, thereby showing the aged animal's hypersensitivty to inflammatory stimuli. The significance of the hypothesis is to provide a link between age-related biological changes and increased pathogenesis of disease [20]. For example, the dysregulation of NF-κB activation by inflammation [3] is causally related to inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) [4, 5, 21].

The regulation of NF-κB have focused on the phosphorylation of IκBs [22]. Multiple kinases have been shown to phosphorylate IκB at specific amino-terminal serine residue [23, 24]. The most studied kinases are IκB kinase, IKKα (IKK-1) and IKKβ (IKK-2). Evidence indicates that IKKα and IKKβ are phosphorylated and activated by one or more upstream activating kinases. One such upstream kinase, NF-κB inducing kinase (NIK), was recently identified [25]. Phosphorylation of IκB by the IKK pathway eventually leads to the nuclear translocation of NF-κB, which, in turn, activates the expression of target genes in the nucleus [26]. It is worth pointing out that no information is available at present on the regulation of various kinases by *t*-BHP.

The change in the redox state due to oxidative stress is known to alter many signaling pathways including the activation of mitogen-activated protein kinase (MAPK) [27]. MAPKs, serine/threonine-specific, and proline-directed protein kinases are demonstrated to play a central role in transducing extracellular signals to the nucleus [28]. More relevant to our current study, c-jun NH_2-terminal kinase (JNK) and extracellular signal regulated kinase (ERK) were recently reported to be involved in NF-κB activation related to pro-inflammatory responses [29, 30].

In the present study, we attempted to assess how *t*-BHP molecularly affects NF-κB activation, and whether this activation is accompanied by MAP kinase activation under oxidative stress conditions. Furthermore, we sought molecular clues on the triggering mechanisms on the activation of IKK and the involvement of MAP kinase in NF-κB activation.

Materials and methods

Culture conditions and t-BHP treatments

YPEN-1, rat prostate endothelial cells, was obtained from American type culture collection (ATCC, USA). The cells were grown in Dulbecco's modified eagle medium media (DMEM, Nissui, Tokyo, Japan) containing 2 mM L-glutamine, 100 mg/ml streptomycin, 2.5 mg/l amphotericin B and 5% heat-inactivated fetal bovine serum (FBS). Cells were maintained at 37 °C in a humidified atmosphere containing 5% of CO_2/ 95% of air. Cells were discarded after three months at which time, new cells were obtained from frozen stock. Cells at the exponential phase were used for all experiments, and cell viability (>90%) was assessed by trypan blue exclusion.

The *t*-BHP was obtained from Sigma Inc. Working solutions of *t*-BHP were made in phosphate buffered saline (PBS) immediately before use. For all experiments, cells were seeded at a density of 5×10^5/ml in 100 mm culture dishes with DMEM containing 5% FBS and supplements. Cells were allowed to adhere in dish overnight, and then the culture medium was replaced with fresh DMEM (serum free) with or without *t*-BHP as indicated.

Measurement of intracellular reactive species (RS) generation

The cells were inoculated at a density of 3×10^4cells/well in a Costar 48-well plate, were allowed to adhere overnight, and then were incubated in serum free DMEM with *t*-BHP and 10 μM 2′,7′-dichlorofluorescin diacetate (DCF-DA, Molecular Probes, USA) at 37 °C. The change in fluorescence was measured at excitation wavelength of 485 nm and emission wavelength 530 nm by Microplate Fluorescence Reader FL 500 (Bio-Tek Instruments). A fluorometric assay was performed to determine the relative levels of reactive species including as superoxide radical, hydroxyl radical, and lipid

peroxide [31]. This assay measured the oxidative conversion of stable, nonfluorescent DCFH-DA to the highly fluorescent 2′,7′-dichlorofluorescin (DCF) in the presence of esterases and RS [32].

Analysis of the intracellular RS level by confocal laser microscopy

Intracellular DCF fluorescence was visualized by a confocal laser microscopy [32]. The amount of $ONOO^-$ generated was estimated by assaying the formation of fluorescent rhodamine-1, 2, 3 from the oxidation of non-fluorescent dihydrorhodamine-1,2,3 (DHR 123) according to the modified method of McBride et al. [33]. Briefly, cells grown to sub-confluence on glass cover slips were incubated with *t*-BHP. Following stimulation for 2 h, cells were washed with PBS, and then incubated with 10 μM DCFDA or or DHR 123 for an additional 20 min. The cells were washed with PBS and mounted in the microscope stage. Fluorescence images were recorded using a Zeiss LSM 510 laser scanning confocal microscope with excitation at 488 nm and long-pass detection at 530 nm.

Preparation of cytosolic and nuclear fraction

Nuclear and cytosolic extracts were prepared according to Deng et al. [34]. Briefly, treated cells were washed, and then scraped into 1.0 ml of ice-cold PBS, and pellet at 3000 rpm at 4 °C for 5 min. The pellets were suspended in 10 mM Tris (pH 8.0), with 1.5 mM $MgCl_2$, 1 mM DTT, 0.1% Nonidet P-40 (NP-40), and inhibitors incubated on ice for 15 min. Nuclei were separated from cytosol by centrifugation at 12,000 rpm at 4 °C for 15 min. The cytosolic supernatants were removed, and the precipitated pellets were suspended in 10 mM Tris (pH 8.0), with 50 mM KCl, 100 mM NaCl, and inhibitors, incubated on ice for 30 min, then were centrifuged at 12,000 rpm at 4 °C for another 30 min.

Protein analysis by Western blot

Western blot analysis was performed as described previously [35]. The cells were harvested, washed twice with ice-cold PBS, and lysed in a TNN buffer (50 mM Tris–HCl, pH 8.0, 120 mM sodium chloride, 0.5% NP-40) that was supplemented with protease inhibitors (2 μg/ml aprotinin, 2 μg/ml leupeptin, and 100 μg/ml PMSF, and 5 μg/ml of pepstatin,1 mM DTT) and phosphates inhibitors (20 mM NaF and 2 mM Na_3VO_4) for 1 h on ice, vortexing after every 10 min. Lysates were centrifuged at 12,000 rpm for 30 min to remove insoluble material. The protein concentration was determined by the Lowry method (Sigma, USA) using bovine serum albumin (BSA) as a standard. Equal amounts of protein were separated on 6–15% SDS-PAGE gels. The gels were subsequently transferred onto a nitrocellulose membrane (Hybond C, Amersham Corp.). Monoclonal antibodies to phospho-p38 MAPK and phospho-IKKα/β and polyclonal antibodies to NF-κB (p65), IκBα, phospho-IκBα, phospho-ERK, and phospho-JNK. Monoclonal sheep anti-mouse IgG, or donkey anti-rabbit IgG horseradish peroxidase-conjugated secondary antibodies, was used at 1:1000. Proteins were detected by an enhanced chemiluminescence (ECL) reagent using a commercial kit (Amersham Life Science).

Electrophoretic mobility shift assay (EMSA)

The EMSA method was used to characterize the binding activities of NF-κB in nuclear extracts [36]. NF-κB oligonucleotide was 5′-GAGAGGCAAGGG-GATTCCCTTAGTTAGGA-3′. The protein-DNA binding mixture containing 20 μg of nuclear protein extract was incubated for 20 min at 4 °C in a binding medium containing 5% glycerol, 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, 2 mM DTT, 1% NP-40, 10 mM Tris (pH 7.5), and 1 μg of poly(dI-dC)·poly(dI-dC). Radiolabeled transcription factor consensus oligonucleotide (20,000 cpm of ^{32}P) was added, and the complete mixture was incubated for additional 20 min at room temperature. DNA-binding complexes were resolved by 7% native poly acrylamide gel electrophoresis with 0.5 X TBE (0.045 M Tris–borate/0.001 M EDTA) with 5 mM Tris/38 mM glycine running buffer for 90 min at 200 V. The gel was dried, and complexes were established with excess unlabeled oligonucleotide.

Measurements of transfection and luciferase reporter assay for NF-κB activity

The activity of NF-κB was examined using a luciferase plasmid DNA, pTAL-NF-κB that contains a specific binding sequence for NF-κB (BD Biosciences Clontech, CA, USA) [37]. Transfection was carried out using FuGENE 6 Reagent (Roche, Indianapolis, IN). Briefly, 5×10^4 cells per each well were seeded in 24-well plates. When cultured cells reached about 50% confluence, cells were treated with 0.2 μg DNA/0.5 μl FuGENE 6 complexes in a total volume of normal media (5% serum contained) with 500 μl for 48 h. Subsequently, the plate was changed with serum-free medium, and 1 μM of *t*-BHP and inhibitors were co-treated. After additional incubation for 6 h, cells were washed with PBS and were added to the plate with Steady-Glo Luciferase Assay System (Promega, Madison, WI, USA). Luciferase activity was measured by a luminometer (GENious, TECAN, Salzburg, Austria). The obtained raw luciferase activities were normalized by protein concentration in each well.

Statistics

The results were presented as means ± SE of three individual experiments and each measurement was performed in triplicate. Statistical significance was tested using one-way ANOVA allowed by the Student-Newman-Keuls test. Statistical significance was set at $P < 0.05$.

Results

Induction of RS generation by t-BHP

Because *t*-BHP is a strong oxidant, we examined the oxidative status of vascular endothelial YPEN cells in serum-free media to minimize the binding to serum albumin. For the kinetics experiments, endothelial cells were incubated with *t*-BHP at various doses. The time course for the generation of RS was followed by observing changes in the intensity of DCF fluorescence. At concentrations of 1, 5, and 10 μM, we observed *t*-BHP to gradually increase RS generation in a dose-dependant manner, as shown in Figure 1A.

To estimate the intracellular RS generation induced by *t*-BHP, we visually analyzed RS production using confocal microscopy. As shown in Figure 1B, RS levels were increased in cells treated with 5 μM *t*-BHP compared to the untreated control. Treatment with 5 μM *t*-BHP also increased the fluorescence intensity in

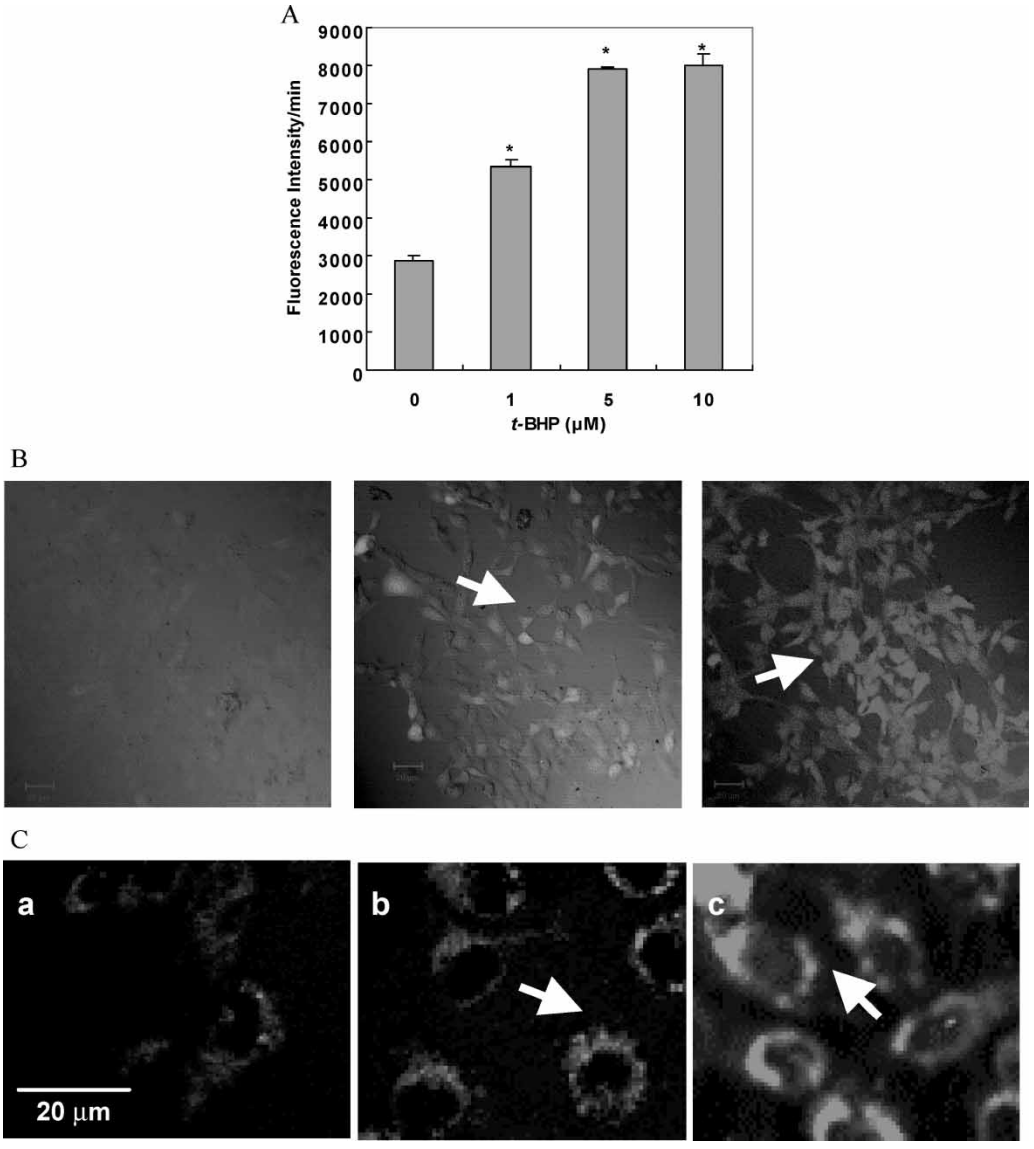

Figure 1. Induction of RS generation by *t*-BHP. A: Dose-dependent intracellular RS generation by *t*-BHP. The cells were incubated in serum-free media with the concentrations of *t*-BHP indicated. The DCF-DA (B) and DHR123 (C) methods were used to determine intracellular RS generation. Analysis of intracellular RS generation induced by *t*-BHP using confocal laser microscopy. The cells were incubated in serum-free media with the 5 μM *t*-BHP for 3 h. In B, panels were merged with images of fluorescence and light microscopy. In C, panels are images of fluorescence microscopy. Panel a, control; Panel b, 1 μM *t*-BHP; Panel c, 5 μM *t*-BHP. The results are presented as means ± SE. of three independent experiment. Statistical significance: $*p < 0.05$ vs. untreated control.

RS levels more than what was observed for treatment with 1 μM *t*-BHP (see the arrow in panel c), indicating the generation of RS and lipid hydroperoxide by *t*-BHP. iNOS under conditions of limiting L-arginine can produce superoxide as well as NO [38, 39]. One of the products of nitrogen-derived free radicals, $ONOO^-$, is formed by the reaction of two ubiquitous free radical species: superoxide (O_2^-) and nitric oxide (NO) [40]. In mitochondria, when fluorescent probe DHR 123 is oxidized by RS, such as peroxynitrite, the fluorescent intensity intensified [41]. As shown in Figure 1C, RS levels were increased in the *t*-BHP treated cell compared to the untreated control cells (see the arrow), indicating the generation of RS by *t*-BHP.

Enhancement of NF-κB (p65) translocation and cytosolic IκBα degradation by t-BHP

The active forms of NF-κB are heterodimers, most often composed of p50 and p65. Under normal conditions, these forms exist in cytosol as an inactive complex by the inhibitory subunit, IκB. Because nuclear translocation is a key step in NF-κB transcriptional activity, the status of p65 in nucleus is crucial. To monitor NF-κB translocation, we performed Western blot analysis. Data shown in Figure 2A demonstrated that treatment of endothelial cells with *t*-BHP resulted in enhanced p65 protein in the nuclear extracts. When stimulated with 5 μM *t*-BHP, an increased expression of p65 was detectable within 1 h, continued to 2 h, and then declined to the level of the untreated control after 3 h. When we examined the disappearance of IκBα in cytoplasm, as shown Figure 2B, IκBα expression was significantly decreased with 1 h exposure to 5 μM *t*-BHP. We then assessed the effect of *t*-BHP on the phosphorylation of IκBα to obtain signaling data for NF-κB activation. Data showed that *t*-BHP markedly increased the Phospho-IκBα at 30 min (Figure 2C).

Up-regulation of NF-κB binding by t-BHP

To determine the extent of *t*-BHP-induced activation of NF-κB, EMSA was carried out on nuclear proteins. Treatment of endothelial cells with *t*-BHP at various times led to activation of the NF-κB transcription

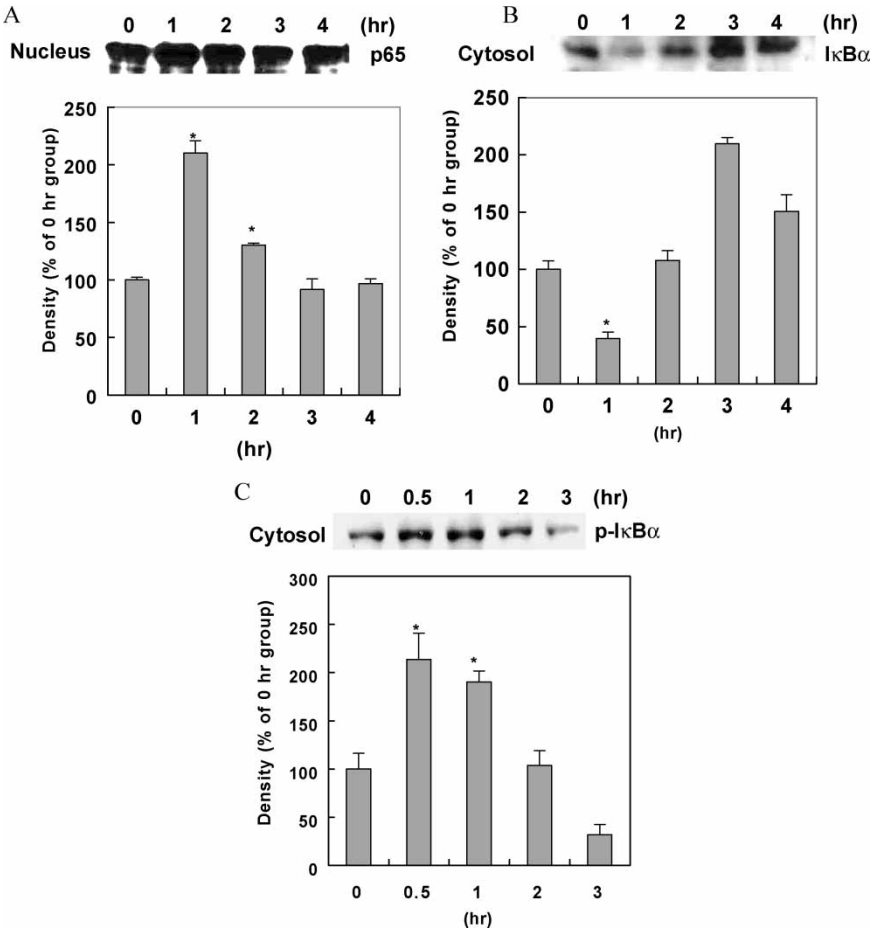

Figure 2. Induction of NF-κB activation through IκBα degradation by *t*-BHP. The cells were incubated in serum-free media with 5 μM *t*-BHP for 0–4 h. A: The levels of p65 protein in nuclear extracts (30 μg/lane) were analyzed by Western blot; levels of IκBα (B) and p-IκBα (C) in cytosolic extracts (40 μg/lane). The data presented is representative of at least three separate experiments. Quantitation of the p65, IκBα, and p-IκBα expression were performed by densitometric analysis. Statistical significance: $*p < 0.05$ vs. untreated control.

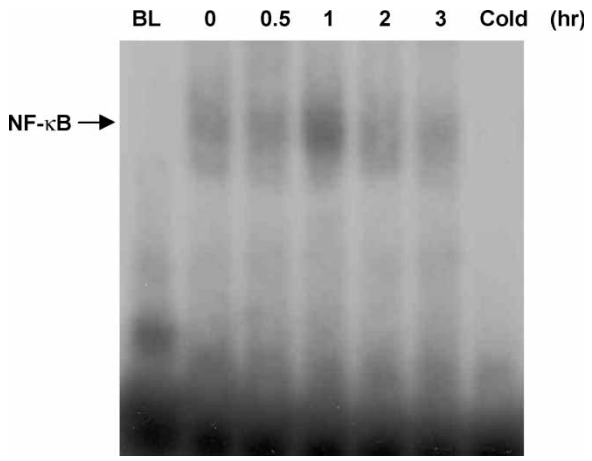

Figure 3. Upregulation of NF-κB binding activity induced by *t*-BHP. The cells were incubated in serum-free media with 5 μM *t*-BHP for the indicated times. Nuclear fractions were incubated with ^{32}P-end labeled probe containing a binding site for NF-κB. Gel shift assays with NF-κB probes were performed as described under "Material and Methods". Statistical significance: $*p < 0.05$ vs. untreated control. BL, negative control without nuclear extract; Cold, specific competition with unlabeled NF-κB oligonucleotide.

factor, as determined by increased DNA binding activity of NF-κB in nuclear fractions. One-hour *t*-BHP stimulation significantly enhanced NF-κB binding activity, as shown in Figure 3. These data strongly indicated that the *t*-BHP enhanced NF-κB activation due to increased oxidative stress.

Modulation of IKK by t-BHP

Because evidence shows that NF-κB activators induce IκB phosphorylation and degradation by the activation of the IKK complex, we wanted to verify whether or not *t*-BHP-induced NF-κB activation through the IKK pathway, as revealed by increased phospho-IκB, which is associated with IKK phosphorylation [26]. Although we used the phospho-IKKα (Ser180)/IKKβ (Ser181) antibody for detection of phospho-IKK, only phospho-IKKα was detected. In Figure 4, we show the results from examining the ability to induce IKKα expression and IKK phosphorylation. Data document that the treatment of endothelial cells with *t*-BHP resulted in enhanced IKKα expression. Increased expression IKKα was detectable within 1 h, continued to 2 h. Data also show that the treatment of endothelial cells with 5 μM *t*-BHP increased phospho-IKKα that was detectable within 10 min and continued to 60 min. Cells exposed to 60 min *t*-BHP showed a greater than 2-fold increase in IKKα phosphorylation than the control cells.

Activation of MAP kinase by t-BHP

The sensitivity of MAP kinase activation to oxidative stress is well established, but the inter-relationship of the activation of MAP and NF-κB, particularly in *t*-BHP-treated cells, has not been well documented. Because ERK activation is dependent on tyrosine and threonine phosphorylation [42], we examined the phosphorylation of ERK proteins in *t*-BHP-treated cells. To do that, protein levels by Western blot analysis was performed using an anti-active ERK antibody that recognizes dually phosphorylated (tyrosine and threonine) ERK1 (44 kDa) and ERK2 (42 kDa). The results shown

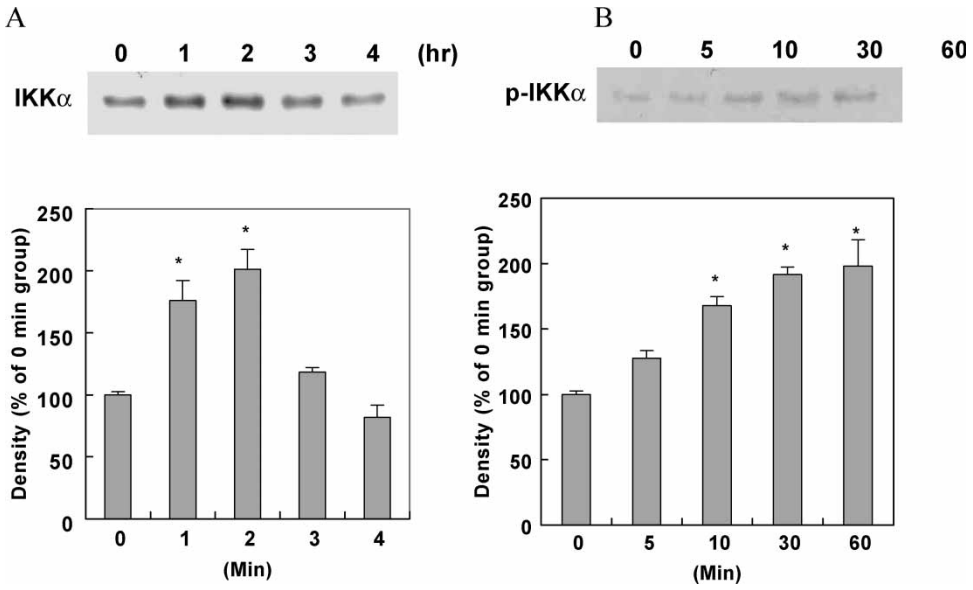

Figure 4. Regulation of IKK activation by *t*-BHP. The cells were incubated in serum-free media with 5 μM *t*-BHP for the indicated times. The IKK and p-IKKproteins in whole-cell lysate were determined by Western blot analysis. The data presented is representative of at least three separate experiments. Quantitation of the IKKα (A) and p-IKK (B) expression were performed by densitometric analysis. Statistical significance: $*p < 0.05$ vs. untreated control.

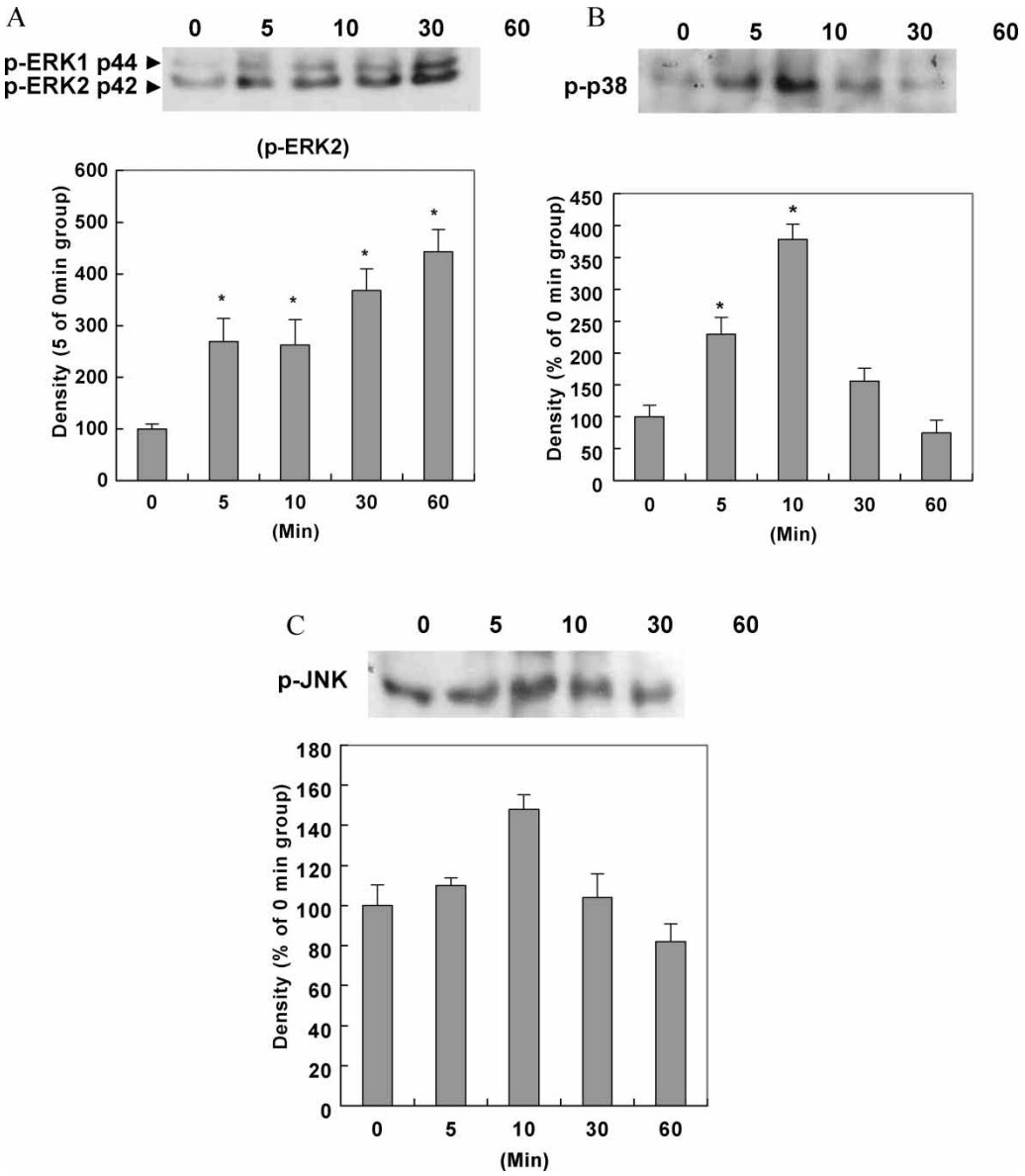

Figure 5. Induction of MAP kinase phosphorylation by *t*-BHP. The cells were incubated in serum-free media with 5 μM *t*-BHP for the indicated times. The MAP kinase proteins were determined by Western blot analysis; levels of p-ERK (A), p-p38 (B), and p-JNK (C) in whole-cell lysate. The data presented is representative of at least three separate experiments. Quantitation of the phosphoproteins was performed by densitometric analysis. Statistical significance: $*p < 0.05$ vs. untreated control.

in Figure 5A indicate that both ERK1 and ERK2 activities were markedly induced in the 5 μM *t*-BHP treated cells compared to untreated control cells. Using 5 μM *t*-BHP stimulation, increased phospho-ERK1/2 was detectable within 5 min and continued to 60 min, with a 4-fold increase compared to controls.

Similar to ERK, p38 MAPK also can be activated by cellular oxidative stresses. To assess the activation of p38 MAPK during exposure to *t*-BHP in endothelial cells, Western blotting was carried out with an antiserum that is reactive with the protein doubly phosphorylated on Thr-180 and Tyr-182 [43]. Our result found that with 5 μM *t*-BHP stimulation, p38 MAPK phosphorylation was enhanced at 5 min, continued to 10 min, and then declined after 30 min. (Figure 5B).

Because JNK, also known as stress-activated protein kinase (SAPK), is an important constituent of the MAPK signaling cascade, we studied the effect of *t*-BHP on JNK activity. Figure 5C shows a representative Western blot using an anti-phospho-JNK antibody. Although ERK and p38 MAPK phosphorylation were enhanced by *t*-BHP, little change occurred in JNK phosphorylation with 10 min exposure to *t*-BHP. The change of phospho-JNK showed no statistical difference from the control. These results suggeste that *t*-BHP does not elicit to JNK activity, but modulates ERK1/2 and p38 MAP kinase activity.

Involvement of MAP kinase in NF-κB transactivation by t-BHP

To evaluate the interrelationship between NF-κB transactivation and MAP kinase, the alteration of NF-κB activity induced by *t*-BHP was examined using MAP kinase inhibitors (Figure 6). Pretreatment with SB203580, a p38 MAP kinase inhibitor, prevented the increase of NF-κB translocation and IκB degradation by *t*-BHP (Figure 6A). Although *t*-BHP induced NF-κB luciferase activity in a dose-dependent manner, SB203580 blocked the increase of NF-κB luciferase activity induced by *t*-BHP (Figure 6B). At 5 μM SB203580, NF-κB luciferase activity was inhibited almost to the basal level.

In addition, *t*-BHP led to an increase in NF-κB activation that was effectively inhibited with treatment of 5 μM PD98059, a specific inhibitor of MEK1/2. However, SP600125, a JNK inhibitor, did not affect NF-κB luciferase activity. Thus, present data verified that p38 MAPK and ERK were regulators of NF-κB transactivity induced by *t*-BHP and that JNK was not involved in *t*-BHP-induced NF-κB transactivation.

Effects of MAP kinase on IKK phosphoylation in NF-κB activation

To verify the possibility that p38 MAP kinase and ERK activation by *t*-BHP caused the increased IKK activation that led to NF-κB activation, the extent of blockage of IKK phosphorylation was examined using the MAP kinase inhibitors, SB203580 and PD98059 (Figure 7). Activation of IKK depends on phosphorylation at serines, 177 and 181 whose phosphorylation causes a conformational change that results in kinase activation [44]. SB203580 and PD98059 blocked the phospho-IKKα/β induced by *t*-BHP. These results strongly indicated that NF-κB activation required IκBα phosphorylation by *t*-BHP in IKK activation. In turn, p38 MAP kinase and ERK activation are involved in this IKK activation.

Discussion

Shvedova et al. showed that *t*-BHP induces oxidative stress and damage to vascular smooth muscle cells [45]. Awe et al. reported that in rat, *t*-BHP enhanced vasoconstriction during DOCA-salt hypertension and

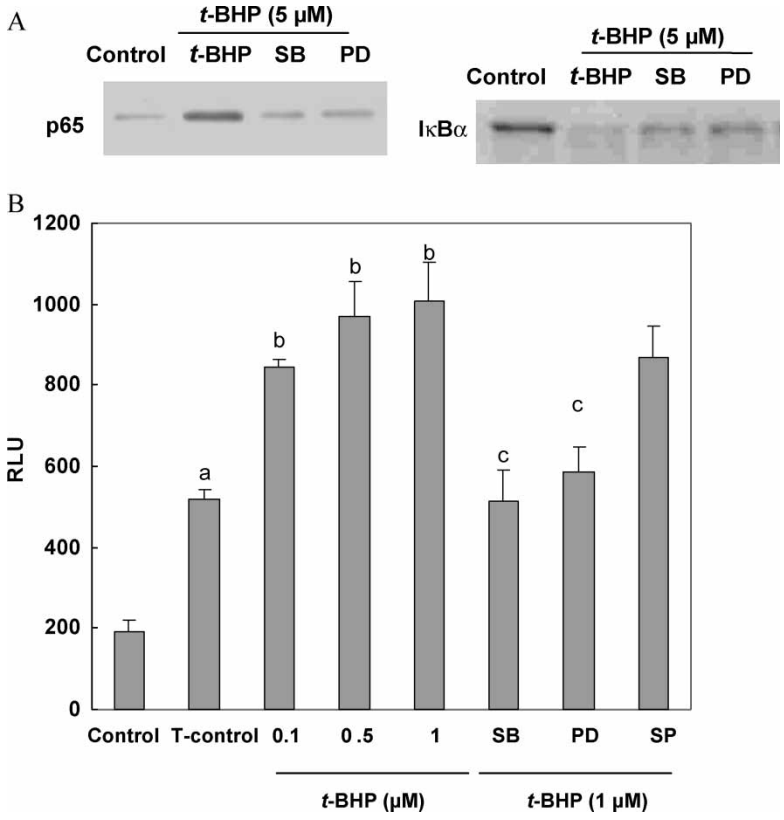

Figure 6. Requirement of p38 MAP kinase and ERK in the NF-κB transactivation by *t*-BHP. A: the level of p65 protein in nucleus and IκB protein in cytosol. The cells were incubated with *t*-BHP (5 μM) for 1 h after pre-treatment for 30 min with SB203580 (10 μM) or PD98059 (5 μM). B: luciferase activity of NF-κB. Cells were grown in 80–90% confluences after transfecting a reporter plasmid containing pTAL-NFκB. Cells were treated for 6 h with *t*-BHP after pre-treatment with inhibitor for 30 min, and lysed for determination of luciferase. Control, untransfected cells; T-control, transfected and untreated cells; *t*-BHP, transfected cells with *t*-BHP (0.1–1 μM); SB, transfected cell with *t*-BHP (1 μM) and SB203580 (10 μM); PD, transfected cells with *t*-BHP (1 μM) and PD98059 (10 μM); SP, transfected cells with *t*-BHP (1 μM) and SP600125 (10 μM); RLU, reactive light units. Statistical significance: $^{a}p < 0.05$ vs. untreated control; $^{b}p < 0.05$ vs. T-control; $^{c}p < 0.05$ vs. 1 μM *t*-BHP.

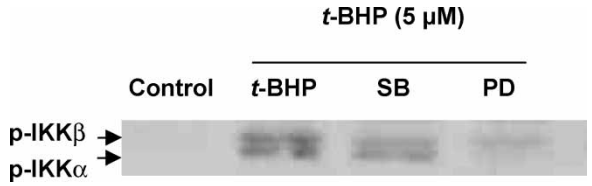

Figure 7. Inhibitory effect of SB203580 and PD98059 on the IKK phosphorylation in NF-κB activation by *t*-BHP. The cells were treated for 30 min with *t*-BHP after pre-treatment with inhibitor for 20 min. The phosphorylated IKK proteins were determined by Western blot analysis. The data presented is representative of at least three separate experiments.

involved reactive radical species and decreases in the expression of endogenous antioxidant enzymes [12].

Recent data revealed that *t*-BHP causes vasoconstriction [12] unlike other well-known membrane-permeant oxidants, such as hydrogen peroxide and cumen hydroperoxide. Biochemical evidence indicates that *t*-BHP-mediated vasoconstriction is modulated through the activation and expression of COX-2, a key enzyme in aortic prostaglandin synthesis [11]. Although vascular reactivity may be altered by *t*-BHP through signaling transduction pathways, at present it is not well known how *t*-BHP induced NF-κB activation is modulated by the IKK pathway.

In our current study, we found evidence showing in YPEN-1 cells that *t*-BHP-induced oxidative stress and enhanced NF-κB activation that likely leads to eliciting the constitutive expression of pro-inflammatory genes such as COX-2 and iNOS. Our findings are in line with the previous study of Cortez-Pinto et al., who reported a *t*-BHP-induced translocation of p65 subunit of NF-κB in hepatocytes [46], although evidence showing NF-κB activation was not reported. Our present study provided clear evidence on NF-κB activation and IκB degradation by phosphorylation in NF-κB binding activity.

The inhibition of IκB and the phosphorylation of IKK are essential steps in the NF-κB activation process. Western blot data on anti-IKK and phospho-IKK antibody showed that both IKK and p-IKK were increased (Figure 4), indicating that the phosphorylation of IKK may be involved in NF-κB activation by *t*-BHP.

The major revelation made in present study is the ability of *t*-BHP to activate p38 MAP kinase and ERK. As showed in Figure 8, a specific inhibitor of MEK, PD98059, (an upstream activator of EERK1 and ERK2) or a specific inhibitor of p38, both SB203580, suppressed the *t*-BHP-induced NF-κB activation and IKK phosphorylation. Thus, our data show for the first time that *t*-BHP is associated with the activation of ERK and p38 MAPK signaling pathways in NF-κB transactivation via IKK in endothelial cells. This interaction can have a wide implication, because the activation of MAP kinase influences the regulation of the inflammatory process as revealed by the ability of pro-inflammatory TNFα to induce rapid activation of ERK, JNK, and p38 MAP kinase [47]. This simultaneous activation of NF-κB and MAP kinase by *t*-BHP may be the reason why *t*-BHP elicits its broader action compared to other hydroperoxides.

One other interesting observation made in the present study is the selective action of *t*-BHP on NF-κB activation without influence in JNK activation. Our data indicated that JNK, and p38 MAP kinase and ERK are preferentially affected by *t*-BHP. In general, p38 MAP kinase and JNK share a similar activation made under environmental stress and pro-inflammatory cytokines. However, as found here, these protein kinases reacted independently when subjected to *t*-BHP induction in endothelial cells. Although it is remained to be determined, this differential effect may be related to that the modulation of p38 MAP kinase and JNK in YPEN cells is controlled by the different upstream activating MAP kinase kinases (MKKs) [48].

Regarding MAP kinase-induced NF-κB activation, *t*-BHP can induce p38 MAP kinase and/or ERK may induce IKK or IκB activation, or *t*-BHP-induced RS may participate in the activation of NF-κB through p38 MAP kinase activation. For instance, in the nucleus, NF-κB transcriptional activity can be modulated by several *t*-BHP responsive protein

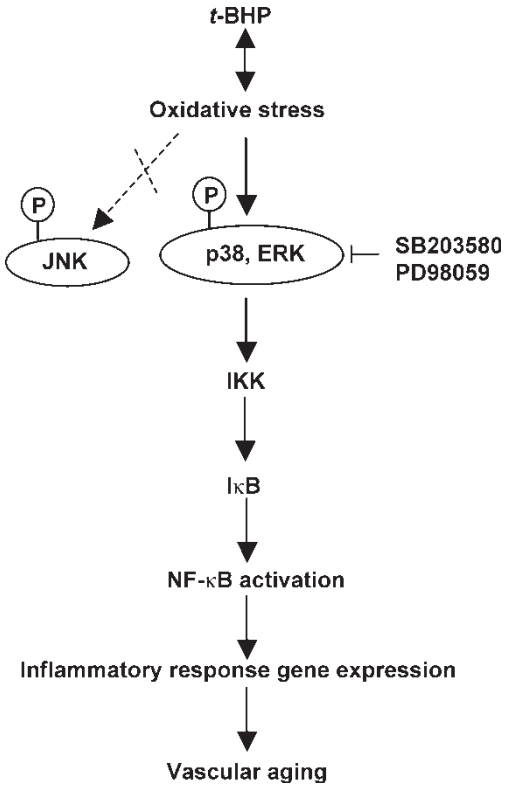

Figure 8. Schematic presentation of *t*-BHP on p38 MAP kinase and ERK in NF-κB transactivation. *t*-BHP induces the NF-κB transactivation by activating p38 MAP kinase and ERK and dependent on IKK. Dotted line denotes the absence of *t*-BHP effect on JNK activation.

kinase, such as the p38 sub-group of MAP kinases. Thus, this study provides a dual role for *t*-BHP on NF-κB activation and MAPK related-signaling pathways, as shown Figure 8.

In summary, *t*-BHP was shown to be a major player in the activation of the redox-sensitive transcription factors responsible for age-related vascular dysfunctions. Our current study provided evidence to delineate an underlying molecular course by which in endothelial cells, *t*-BHP-mediated the activation of NF-κB that elicits p38 MAP kinase and ERK activation and IKK phosphorylation without influencing the JNK pathway.

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