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The Effects of DTBNP on Intracellular Ca^{2+} Signaling in Cultured Bovine Aortic Endothelial Cells

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The mechanism underlying oxidant-induced intracellular Ca^{2+} ($[Ca^{2+}]_i$) increase was studied in cultured bovine aortic endothelial cells (BAECs) using fura-2 AM. In the presence of 2 mM extracellular Ca^{2+}, the application of DTBNP (20 μM), a membrane-permeable oxidant, caused an increase in $[Ca^{2+}]_i$, and DTT (2 mM) as a reductant completely reversed the effect of DTBNP. The $[Ca^{2+}]_i$ increase induced by DTBNP was also observed in an extracellular Ca^{2+}-free/2 mM EGTA solution, indicating the release of Ca^{2+} from intracellular store(s). After endoplasmic reticulum was depleted by an IP_3-generating agonist, ATP (30 μM) or an ER Ca^{2+} pump inhibitor, thapsigargin (1 μM), DTBNP-stressed BAECs showed an increase of $[Ca^{2+}]_i$ in Ca^{2+}-free/2 mM EGTA solution. Ratio-differences before and after the application of DTBNP after pretreatment with ATP or thapsigargin were 0.42 ± 0.15 and 0.49 ± 0.07, respectively (n=7), which are significantly reduced, compared to the control value of 0.72 ± 0.07 in a Ca^{2+}-free/2 mM EGTA solution. After the protonophore CCCP (10 μM) challenge to release mitochondrial Ca^{2+}, the similar result was obtained. Ratio-difference before and after the application of DTBNP after pretreatment with CCCP was 0.46 ± 0.09 (n=7). Simultaneous application of thapsigargin and CCCP completely abolished the DTBNP-induced $[Ca^{2+}]_i$ increase. The above results together indicate that the increase of $[Ca^{2+}]_i$ by DTBNP resulted from the release of Ca^{2+} from both endoplasmic reticulum and mitochondria.

Key Words: Oxidant, DTBNP, DTT, Bovine aortic endothelial cells, Endoplasmic reticulum, Mitochondria

INTRODUCTION

The vascular endothelial cells, which modulates vascular tone, vasoreactivity, and permeability, are the major target of oxidant stress. Oxidant stress to the vascular endothelial cells causes endothelial dysfunction and the pathophysiology of several vascular diseases, including atherosclerosis, diabetes, neuronal disorders, and ischemia-reperfusion injury (Lum et al, 2001). Oxidant stress can also disrupt normal physiological pathways and cause cell death. Such a switch is largely mediated through intracellular Ca^{2+} signaling. (Ermak et al, 2001).

Ca^{2+} concentration in the cytoplasm is regulated by Ca^{2+} transport into and out of the endoplasmic reticulum (ER), in which calcium can be stored, as well as by Ca^{2+} transport through the plasma membrane between cells and their environments (Berridge et al, 1998; Marin et al, 1999). Mitochondria are active in continuous generation of reactive oxygen species (ROS), thereby favoring a situation of mitochondrial oxidant stress. An increase in the release of calcium from mitochondria by oxidants stimulates calcium-dependent enzymes such as calcium-dependent proteases, nucleases, and phospholipases, which subsequently trigger apoptotic process of the cells (Chakraborti et al, 1999). It has been shown that oxidants cause a rapid increase in Ca^{2+} concentration in the cytoplasm of diverse cell types (Rooney et al, 1991; Roveri et al, 1992; Doan et al, 1994; Wang et al, 2000). The aim of the present study was to investigate the effect of membrane permeable oxidant, DTBNP (2,2'-dithio-bis-5-nitropyridine) on endothelial cell Ca^{2+} signaling.

METHODS

Preparation of cells

Cultured bovine aortic endothelial cells were provided by Dr. Masahiro Oike at Kyushu University in Japan. Endothelial cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco BRL) containing 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin under 5% CO_2 and 95% oxygen at 37°C. Cells of the 4th subculture were used in the following experiments. The grown cells were separated by trypsin (1X), and then single cells were used.

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ABBREVIATIONS: ROS, reactive oxygen species (ROS); DTBNP, 2,2'-dithio-bis-5-nitropyridine; DTT, 1,4-dithiothreitol; CCCP, carbonyl cyanide m-chlorophenylhydrazone; BAEC, bovine aortic endothelial cells.

Solutions and drugs

The normal solution contained (mM): 135 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 HEPES (pH=7.4, titrated with NaOH). Ca^{2+} was omitted from normal solution, and 2 mM EGTA was added to make a Ca^{2+}-free solution. As an oxidant and reductant, DTBNP (2,2'-dithio-bis-5-nitropyridine) and DTT (1,4-dithiothreitol) were used. For depletion of endoplasmic reticulum, ATP and thapsigargin were used. For disrupting mitochondrial potential, CCCP (carbonyl cyanide m-chlorophenylhydrazone) was used. All chemicals and drugs used in this study were purchased from Sigma (USA).

Fura-2 loading and $[Ca^{2+}]_i$ measurement

Single cells were loaded with acetoxymethyl ester form of fura-2 (2 μM diluted from 1 mM stock in dimethyl sulfoxide) in Ca^{2+}-free normal solution for 25 min at room temperature. After then, the cell suspension was briefly centrifuged (800 r.p.m., 5 min) and washed twice with Ca^{2+}-free normal solution. Fura-2 loaded cells were stored at 4°C until use. The recording of $[Ca^{2+}]_i$ was performed with a microfluorometric system consisting of an inverted fluorescence microscope (Diaphot 300, Nikon, Japan) with a dry-type fluorescence objective lens (x40, NA0.85), a photomultiplier tube (type R 1527, Hamamatsu, Japan) and PTI-Deltascan illuminator (Photon Technology International Inc. USA). One drop of cell suspension was placed on a superfusion chamber (100 μl), and cells were allowed to settle down and thereafter superfused at a constant flow of 2 ml/min. Light was provided by a 75 W xenon lamp (Ushino, Japan) and, to control excitation frequency, a chopper wheel alternated the light path to monochromators (340 and 380 nm) with a frequency of 5 or 10 Hz. A short-pass dichroic mirror passed emission light of <570 nm onto the photomultiplier tube, and intensity at 510 nm was measured. A mechanical image mask was placed in the emission path, thus limiting measurement to a single cell. Both data acquisition and control of light application were done by using a computer software (Felix v. 1.1, PTI). Because of uncertainties in calibrating the fura-2 signals in intact cells, no attempt was made to calibrate $[Ca^{2+}]_i$, and all results were instead reported as changes in the ratio of fluorescence signals of two different wave lengths (340 nm/380 nm).

Statistics

Pooled data are expressed as means $\pm$ S.E.M. Significance was tested using Student's t test.

RESULTS

Response of the intracellular Ca^{2+} ($[Ca^{2+}]_i$) to oxidant, DTBNP

Application of DTBNP (2,2'-dithio-bis-5-nitropyridine), a hydrophobic SH-group-oxidizing substance, to bovine aortic endothelial cells (BAECs) suspended in 2 mM Ca^{2+}-containing normal extracellular solution increased $[Ca^{2+}]_i$ in a dose-dependent manner (Fig. 1A). The half maximum concentration of DTBNP was 9.7 μM. For all subsequent experiments, DTBNP concentration equal to 20 μM was employed. The extracellular application of 20 μM DTBNP increased $[Ca^{2+}]_i$, which reached a stable plateau after 300~500 seconds and sustained during the application of DTBNP (Fig. 1B). Ratio-difference before and after the application of DTBNP with duration of 300 seconds was 0.71 ± 0.05 (mean $\pm$ S.E.M., n=7). The effect of DTBNP was reversed to the control level after the application of DTT (1,4-dithiothreitol, 2 mM), disulfide bond reducing substance (Fig. 2A).

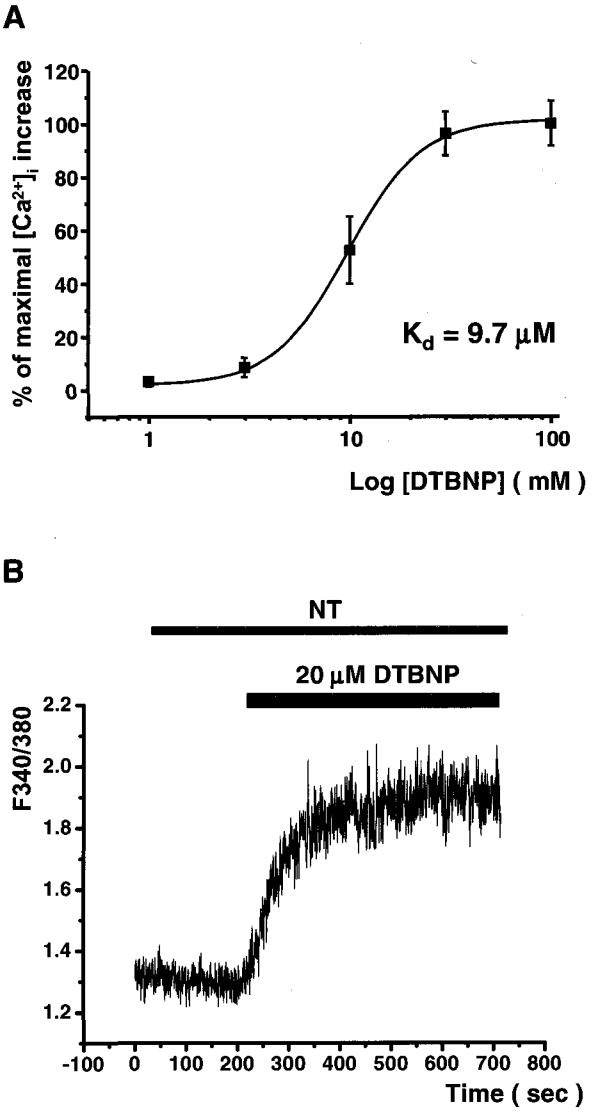

Fig. 1. Responses of intracellular Ca^{2+} to DTBNP in bovine aortic endothelial cell. BAECs were suspended in 2 mM Ca^{2+}-containing normal solution. Increase in $[Ca^{2+}]_i$ was plotted against DTBNP concentrations (A). 20 μM DTBNP caused a sustained increase in $[Ca^{2+}]_i$ (B).

The release of an intracellular stored Ca^{2+} by DTBNP

The dependence of the DTBNP-induced $[Ca^{2+}]_i$ increase on extracellular Ca^{2+} was examined in Fig. 2. When Ca^{2+}-free solution including 2 mM EGTA replaced normal

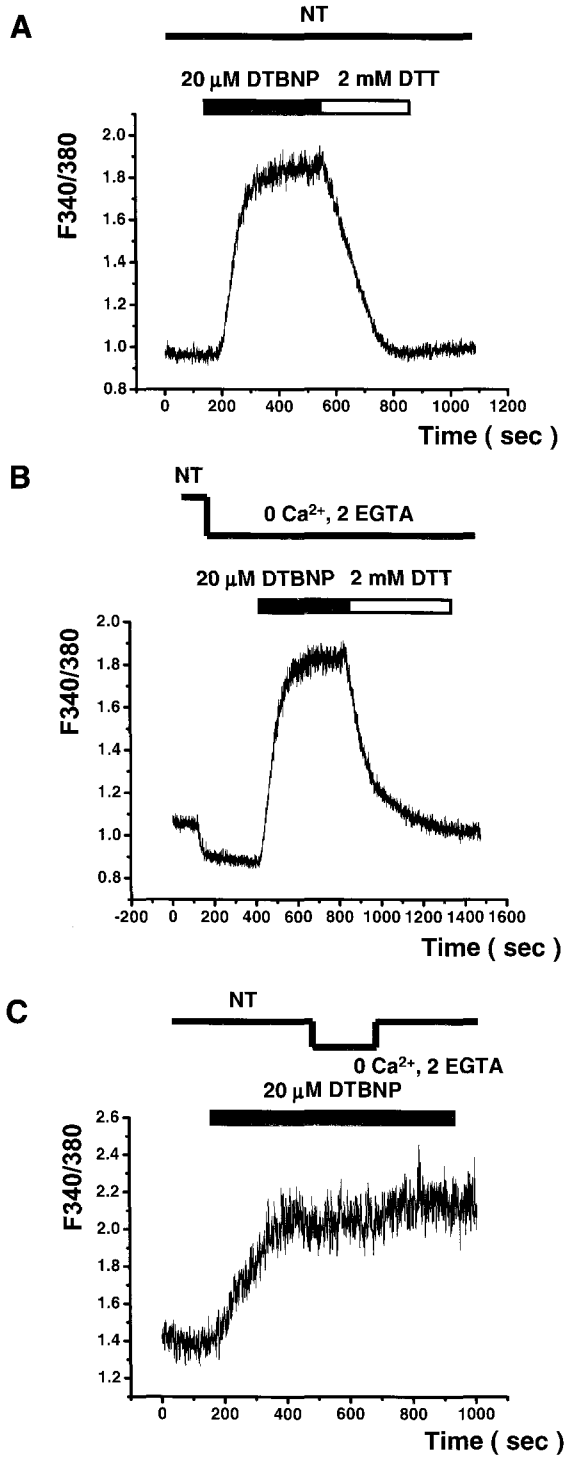

Fig. 2. Mobilization of an intracellular Ca^{2+} increase in response to DTBNP. BAECs were perfused with 20 μM DTBNP followed by 2 mM DTT in 2 mM Ca^{2+} -containing normal (A) and Ca^{2+}-free/2 mM EGTA solution (B). Changes of solutions from normal to Ca^{2+}-free/2 mM EGTA solution and again to normal solution had no effect on $[Ca^{2+}]_i$ during the application of DTBNP (C).

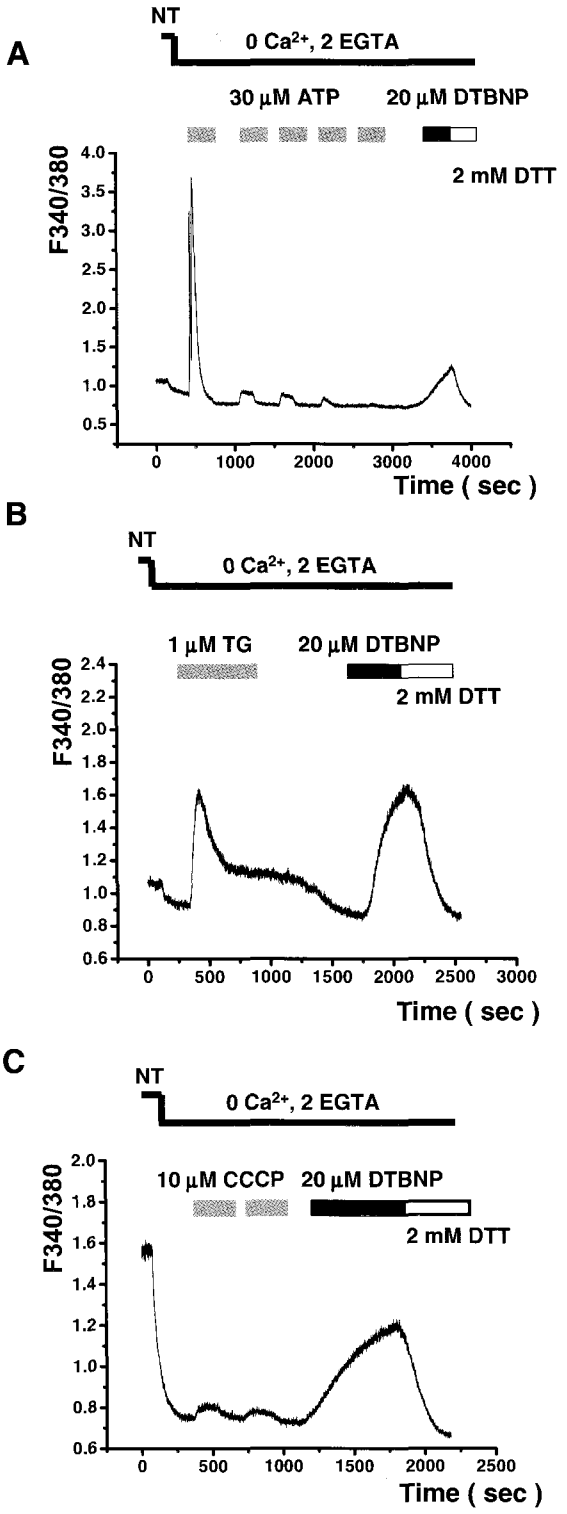

Fig. 3. Effect of pretreatment with ATP, TG, or CCCP on DTBNP-induced $[Ca^{2+}]_i$ increase. BAECs were perfused intermittently 5 times with 30 μM ATP in Ca^{2+}-free/2 mM EGTA buffer solution, followed by 20 μM DTBNP (A). BAECs were perfused with 1 μM TG for 10 min in Ca^{2+}-free/2 mM EGTA solution, followed by 20 μM DTBNP (B). BAECs were perfused intermittently 2 times with 10 μ M CCCP in Ca^{2+}-free/2 mM EGTA solution, followed by 20 mM DTBNP (C).

extracellular solution, the application of DTBNP induced a rise in $[Ca^{2+}]_i$, indicating that DTBNP-induced $[Ca^{2+}]_i$ increase resulted from the mobilization of intracellular stored Ca^{2+}. Ratio-difference before and after the application of DTBNP for 300 seconds was 0.72 ± 0.07 (mean $\pm$ S.E.M., n=7) in Ca^{2+}-free/2 mM EGTA solution, which was not significantly different from the value of 0.71 ± 0.05 in normal solution (Fig. 2B). To confirm that the $[Ca^{2+}]_i$ increase by DTBNP resulted from the release of Ca^{2+} from internal Ca^{2+} store(s), we changed the extracellular normal solution to Ca^{2+}-free/2 mM EGTA, and again to normal solution (Fig. 2C). The $[Ca^{2+}]_i$ increase was not changed during the application of DTBNP, thus providing evidence that DTBNP released Ca^{2+} from internal Ca^{2+} store(s). Therefore, we next tried to find out the Ca^{2+} store(s) that are affected by membrane-permeable oxidant, DTBNP.

DTBNP-induced $[Ca^{2+}]_i$ signaling after IP_3 or thapsigarginsensitive Ca^{2+} and mitochondrial Ca^{2+} store depletion

After ER (endoplasmic reticulum) was depleted by an IP_3 (inositol 1,4,5-trisphosphate)-generating agonist, ATP (30 μM) or an ER Ca^{2+} pump inhibitor, thapsigargin (1 μM), the perfusion of cells with DTBNP showed an increase in $[Ca^{2+}]_i$ in Ca^{2+}-free/2 mM EGTA solution (Fig. 3A, B). However, ratio-differences before and after the application of DTBNP after pretreatment with ATP or thapsigargin were 0.42 ± 0.15 and 0.49 ± 0.07, respectively (n=7), which were significantly reduced, compared to the control value of 0.72 ± 0.07. Time to plateau was 519 ± 20 seconds (n=5) after ER was depleted by ATP, and 427 ± 11 seconds after ER was depleted by thapsigargin. After mitochondrial Ca^{2+} depletion by CCCP (10 μM), which is a mitochondrial uncoupler that collapses the mitochondrial membrane potential and Ca^{2+} uptake, the effect of DTBNP was tested. As shown in Fig. 3C, mitochondrial Ca^{2+} store was also affected by DTBNP. Ratio-difference before and after the application of DTBNP after pretreatment with CCCP was 0.46 ± 0.06 (n=7). Time to plateau was 618 ± 34 seconds, which was significantly different from the value of 442 ± 39 seconds in Ca^{2+}-free/2 mM EGTA buffer. Subsequent application of ATP, TG, or CCCP during the $[Ca^{2+}]_i$ increase induced by DTBNP failed to elicit any detectable response, respectively (Fig. 4A, B, C). Ratio-differences and time to plateau are depicted as bar in Fig. 5. These findings suggest that DTBNP-sensitive Ca^{2+} stores include IP_3/thapsigarginsensitive Ca^{2+} store and mitochondrial Ca^{2+} stores. Thus, we explored simultaneously both IP_3/ thapsigarginsensitive Ca^{2+} store and mitochondrial Ca^{2+} store in the next experiment.

Effects of simultaneous depletion of mitochondria and ER on DTBNP-induced $[Ca^{2+}]_i$ signaling

To elucidate the mechanisms of DTBNP-induced Ca^{2+} releases, we simultaneously depleted both mitochondrial and endoplasmic reticulum Ca^{2+} stores, and then applied DTBNP in Ca^{2+}-free solution. Fig. 6A shows that simultaneous application of CCCP and thapsigargin clearly abolished the DTBNP-induced $[Ca^{2+}]_i$ increase in 6 out of 10 cells, indicating that DTBNP releases Ca^{2+} from both mitochondrial Ca^{2+} store and endoplasmic reticulum. 10 μM CCCP and 1 μM thapsigargin were sequentially applied to the BAECs during perfusion with 20 μM DTBNP in Ca^{2+}-free solution. Pretreatment of cells with DTBNP abolished the Ca^{2+} release from both endoplasmic reticulum and mitochondrial Ca^{2+} stores in all 4 cells examined. 1 μM Ionomycin was added after the application of CCCP and

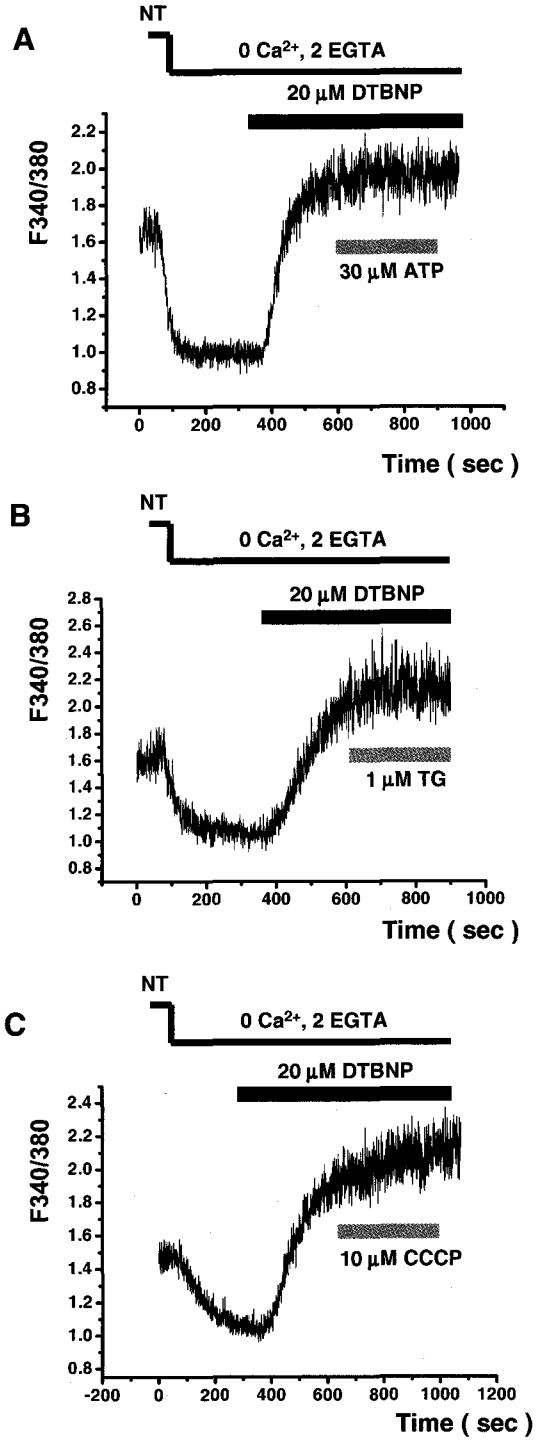

Fig. 4. The application of ATP, TG, or CCCP during the $[Ca^{2+}]_i$ increase induced by DTBNP. The extracellular application of 30 μM ATP (A), 1 μM TG (B), and 10 μM CCCP (C) for 300 seconds caused no further $[Ca^{2+}]_i$ increase during the $[Ca^{2+}]_i$ increase induced by 20 μM DTBNP.

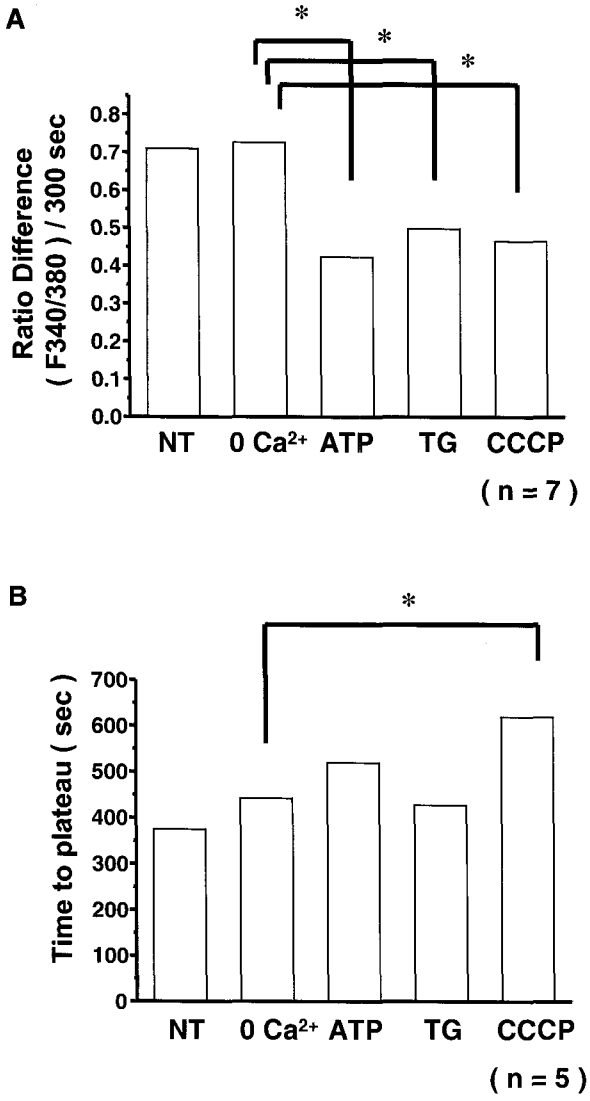

Fig. 5. Ratio difference (F340/380) and time to plateau phase before and after the application of DTBNP. Ratio-differences before and after the application of DTBNP were calculated for 300 seconds. Time to plateau phase is the duration for intracellular Ca^{2+} concentration to reach the plateau under the usage of DTBNP. *Represents p value is less than 0.05.

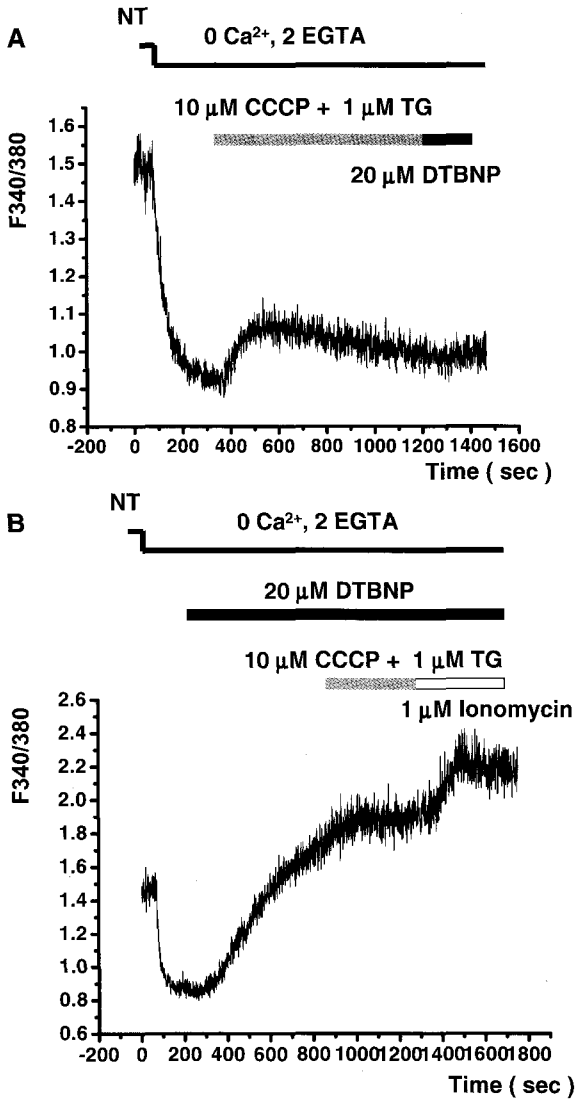

Fig. 6. Effect of pretreatment with both thapsigargin and CCCP on DTBNP-induced $[Ca^{2+}]_i$ increase. BAECs were co-incubated with 1 μM thapsigargin plus 10 μM CCCP followed by 20 μM DTBNP in Ca^{2+}-free solution (A). BAECs were pretreated with 20 μM DTBNP followed sequentially by 10 μM CCCP, 1 μM thapsigargin, and 1 μM ionomycin (B).

thapsigargin during perfusion with 20 μM DTBNP in Ca^{2+}-free solution (Fig. 6B). : Ionomycin, which can induce Ca^{2+} release from IP_3 or thapsigargin insensitive Ca^{2+} store after the discharge of the IP_3 or thapsigargin sensitive Ca^{2+} store (Paola et al, 1997), still evoked the Ca^{2+} release. Therefore, it is quite likely that ionomycin-sensitive Ca^{2+} stores are not involved in DTBNP-induced endothelial Ca^{2+} signaling.

DISCUSSION

This study was designed to identify the cellular mechanisms of oxidant-induced $[Ca^{2+}]_i$ increase in bovine aortic endothelial cells, and showed that the oxidant DTBNP induces a $[Ca^{2+}]_i$ increase in bovine aortic endothelial cells by Ca^{2+} release from both endoplasmic reticulum and mitochondrial Ca^{2+} stores. DTBNP failed to increase $[Ca^{2+}]_i$ in BAECs whose Ca^{2+} stores in both mitochondria and endoplasmic reticulum had been previously depleted by the simultaneous application of CCCP and thapsigargin in Ca^{2+}-free solution.

Changes in intracellular Ca^{2+} homeostasis are thought to play an important role in the endothelial cell response to oxidants (Hoek et al, 1992; Schilling et al, 1992). Abundant evidences indicate that oxidant stress on endothelial cells increases $[Ca^{2+}]_i$, which is due to either an increased Ca^{2+} influx or Ca^{2+} release from intracellular Ca^{2+} stores (Dreher et al, 1995; Volk et al, 1997; Pariente et al, 2001). The increasing effect of DTBNP on resting $[Ca^{2+}]_i$ could be due to a specific effect on the Ca^{2+} release mechanisms and not

related with plasma membrane Ca^{2+} influx mechanisms. Our results demonstrated that DTBNP releases Ca^{2+} from the intracellular Ca^{2+} store in both mitochondria and endoplasmic reticulum. It is important that two major intracellular Ca^{2+} stores were stimulated by DTBNP, reflecting oxidant stress can deplete two major intracellular Ca^{2+} stores. Under oxidant stress conditions, rising Ca^{2+} concentration can disrupt normal metabolism leading to cell death. Since oxidant stress is related to pathophysiology and many diseases, the elucidation of the cellular mechanism of how oxidant stress is related to Ca^{2+} signaling can be helpful to understand process of diseases.

In endothelial cells, the endoplasmic reticulum accounts for approximately 75% of the total intracellular Ca^{2+} stores (Tran et al, 2000) and is in virtually immediate exposure to any intracellular Ca^{2+} signals or Ca^{2+} releasing factors including oxidant. Mitochondria are also important stores of intracellular Ca^{2+} in endothelial cells, accounting for the remaining 25% of the cell's Ca^{2+} storage. The release of Ca^{2+} from mitochondria can occur by oxidation of thiol groups in membrane proteins (Chakraborti et al, 1999).

The effects of DTBNP on Ca^{2+} mobilization were completely blocked by the presence of the sulfhydryl reducing agent such as DTT, suggesting that the effects of DTBNP on Ca^{2+} mobilization are mediated by a mechanism that involves sulfhydryl group oxidation.

From a physiological point of view, our study helps us to understand the complex mechanism of Ca^{2+} signaling under oxidant stress in endothelial cells. Ca^{2+} signaling in the oxidant-stressed endothelial cells may have critical effects on the endothelial dysfunction process.

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