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Human Umbilical Vein Endothelial Cells (HUVECs) Show Ca^{2+} Mobilization as Well as Ca^{2+} Influx Upon Hypoxia

Yasuhisa Aono,* Hideo Ariyoshi, Masato Sakon, Atsushi Ueda, Yoshihisa Tsuji, Tomio Kawasaki, and Morito Monden

Department of Surgery II, Osaka University Medical School, Suita, Osaka 565-0871, Japan

Abstract Bleb formation is an early event of cellular damage observed in a variety of cell types upon hypoxia. Although we previously found that the $[Ca^{2+}]_i$ rise before bleb formation only at the same loci of HUVECs upon hypoxia (localized $[Ca^{2+}]_i$ rise), the mode of the $[Ca^{2+}]_i$ rise remains ill-defined. In order to clarify the mechanisms causing the localized $[Ca^{2+}]_i$ rise in hypoxia challenged HUVECs, we studied the effects of several Ca^{2+} channel blockers or a Ca^{2+} chelator, EGTA, which reduces extracellular Ca^{2+} concentration on the hypoxia-induced localized $[Ca^{2+}]_i$ rise and bleb formation by employing a confocal laser scanning microscopy (CLSM). After the initiation of hypoxia, $[Ca^{2+}]_i$ rose gradually in a localized fashion up to 15 min, which was associated with bleb formation at the same loci. The maximal $[Ca^{2+}]_i$ rise was 435 ± 84 nM at the loci of bleb formation. Ca^{2+} channel blockers including Ni^{2+} (non-specific, 1 mM), nifedipine (L type, 10 μM), nicardipine (L + T type, 10 μM), and cilnidipine (L + N type, 10 μM) did not inhibit either the localized $[Ca^{2+}]_i$ rise or bleb formation. Although both the localized $[Ca^{2+}]_i$ rise and bleb formation were inhibited by lowering extracellular Ca^{2+} concentration below 100 nM, a diffuse $[Ca^{2+}]_i$ rise through the cytoplasm remained without bleb formation, which was inhibited by a phospholipase C (PLC) inhibitor, U73122. In conclusion, hypoxia causes both the Ca^{2+} mobilization and the Ca^{2+} influx in HUVECs and the Ca^{2+} influx through unknown Ca^{2+} channels is responsible for the localized $[Ca^{2+}]_i$ rise integral to bleb formation. J. Cell. Biochem. 78:458–464, 2000.

Key words: calcium; human umbilical vein endothelial cells; hypoxia; Ca^{2+} mobilization; Ca^{2+} influx

During hypoxic injury, bleb formation, small protrusion of the plasma membrane, is considered as an early evidence of cellular damage in many cell types [Jewell et al., 1982; Jurkowitz-Alexander et al., 1992; Smith et al., 1992; Johnson et al., 1994]. A lot of reports implicated that the cytosolic free calcium ($[Ca^{2+}]_i$) plays an important role to cause cellular damage and cell death. In HUVECs, we have shown that hypoxia causes the $[Ca^{2+}]_i$ rise only in the small area of the cytoplasm (localized $[Ca^{2+}]_i$ rise), which can be clearly identified from the other part of the cytoplasm in a fluo-3 fluorescence image. The "localized $[Ca^{2+}]_i$ rise" was followed by bleb formation at the same loci, suggesting the close relationships between the localized $[Ca^{2+}]_i$ rise and bleb formation [Ikeda et al., 1998]. Several groups reported that the removal of extracellular Ca^{2+} or the Ca^{2+} influx inhibition by Ca^{2+} channel blockers suppresses bleb formation and the rise of $[Ca^{2+}]_i$, which supports the concept that $[Ca^{2+}]_i$ has a pivotal role in this process [Jewell et al., 1982; Greeraerts et al., 1991; Arnould et al., 1992].

However, the exact mechanisms to cause the localized $[Ca^{2+}]_i$ rise is not fully understood. It is important to elucidate the mechanisms, not only because ECs are located at the blood tissue interface and maintain blood flow by secreting a number of vasodilators and substances which inhibit neutrophil adherence and platelet aggregation, but also because the dysfunction of ECs during hypoxia might cause the dysfunction of organs while transplantation or organ ischemia through the deterioration of microcirculation.

*Correspondence to: Yasuhisa Aono, MD, Department of Surgery II, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan. E-mail: yaono@surg2.med.osaka-u.ac.jp

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The developments of a digital imaging microscopy and fluorescent Ca^{2+} indicators have enabled us to observe $[Ca^{2+}]_i$ in individual cells and the existence of Ca^{2+} gradients inside cells has been reported [Williams et al., 1985; Burnier et al., 1994; Hernandez-Curz et al., 1990; Yelamarty et al., 1990; Geiger et al., 1992; Nakato et al., 1992; Wahl et al., 1992]. Ca^{2+} gradients in cytosol suggest the role of subcellular organelles linked to their localized physiological function [Williams et al., 1985; Yelamarty et al., 1990; Bellomo et al., 1992; Burnier et al., 1994]. We also found that the localized $[Ca^{2+}]_i$ rise proceeding to bleb formation at the same loci [Ikeda et al., 1998]. These observations have suggested the rise in $[Ca^{2+}]_i$ is the initial cellular response in morphological changes including bleb formation induced by hypoxia. Therefore, it is important to understand the mechanisms to cause the $[Ca^{2+}]_i$ rise, while discussing the hypoxia-induced cellular injury. In this study, we analyzed the effects of several reagents to abolish the localized $[Ca^{2+}]_i$ rise and bleb formation and found the existence of the Ca^{2+} mobilization from the Ca^{2+} storage sites in the hypoxia stimulated HUVECs.

MATERIALS AND METHODS

Materials

Penicillin-streptomycin was purchased from Life Technologies Inc. (Grand Island, NY). Fluo-3 acetoxymethyl ester (AM) was purchased from Molecular Probes (Eugene, OR). Cremophor EL, EGTA (ethylene glycol bis[β-aminoethylether]-N,N,N′,N′-tetraacetic acid) and $NiCl_2$ were purchased from Nacalai Tesque (Kyoto, Japan), and digitonin was purchased from Wako Pure Chemical Industries (Osaka, Japan). Nifedipine, nicardipine hydrochloride, and U-73122 were purchased from Research Biochemicals International (Natick, MA). Cilnidipine was purchased from Fuji Rebio (Tokyo, Japan). Other chemicals were of the highest analytical grade available.

Cell Culture and Dye Loading

HUVECs were obtained from human umbilical veins as previously described [Ikeda et al., 1998]. HUVECs were cultured in MCDB 131 containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 10 ng/ml b-FGF in a 37°C humidified atmosphere of 5% CO_2 and 95% air. HUVECs were fed every 1–2 days with a complete change of fresh cultured medium. HUVECs were used between at the passage 5 and 8. The experiments were performed at the time when the confluent monolayer was formed. HUVECs grown on 24 × 24 mm glass coverslips were loaded with fluo-3 AM as described previously [Ikeda et al., 1998]. In brief, after HUVECs were washed twice with modified HEPES Tyrode's buffer (MHTB; 129 mM NaCl, 8.9 mM $NaHCO_3$, 0.8 mM KH_2PO_4, 0.8 mM $MgCl_2$, 5.6 mM dextrose and 10 mM HEPES, pH 7.4), HUVECs were incubated in the presence of 10 μM fluo-3 AM for 45 min at 37°C. All experiments were performed in less than 90 min and no difference was observed in the results with passage numbers.

$[Ca^{2+}]_i$ Imaging

The digital imaging was carried out as described previously [Burnier et al., 1994] with some modifications. A confocal laser scanning microscopy system (CLSM) equipped with an argon-ion laser (Carl Zeiss Microscope Systems LSM 410, Oberkochen, Germany) was employed in this study. A 488 nm laser light was used for excitation; emitted light was collected through a FT510 dichroic beam splitter and finally passed through a 515 nm long pass filter. A 40× oil-immersion objective (Planneofluar, numerical aperture = 1.3) was used. The focal depth of the optical section was adjusted to 0.6 μm by the size of the aperture pinhole in the emission pathway, which is sufficiently small to obtain a fluorescence intensity (F) independently of the thickness of the specimen. The images acquired by a photo multiplier tube (PMT) detector were averaged eight times in real time to reduce noises, and the average was digitalized to 256 gray levels with an analog to digital converter. It takes 8.64 s to acquire one image consisting of 512 × 512 pixels. The sensitivity of the system for $[Ca^{2+}]_i$ was confirmed by in vivo calibration curve [Ikeda et al., 1996b]. In each experiment, the calibration of $[Ca^{2+}]_i$ was carried out by the method described by Gillo et al. [1993]. Briefly, at the end of each experiment, 25 μM digitonin was added to obtain a maximal fluorescence (F_{max}), followed by the addition of 10 mM EGTA to obtain a minimal fluorescence (F_{min}), $[Ca^{2+}]_i$ was calculated using the equation $[Ca^{2+}]_i = Kd \times (F–F_{min})/(F_{max}–F)$, where Kd is the dissociation constant for Ca^{2+}-bound fluo-3 at 320 nM.

The Hypoxic Chamber and the Perfusion System

We originally produced the parallel-plate flow chamber and the flow circuit of hypoxic perfusion system [Ikeda et al., 1998]. Briefly, MHTB containing 1 mM $CaCl_2$ was immersed in 38°C water bath and equilibrated with humidified N_2 (95%) and CO_2 (5%) mixed gas and perfused at 1.4 ml/min by a peristaltic pump. The pH was adjusted by sodium bicarbonate to maintain a pH of 7.4, and the osmolarity of the buffer after pH adjustment was 309.1 m-osmol/l which is comparable to physiological osmolarity. The O_2 concentration was assessed by using a 288 Blood Gas System (Ciba-Corning Diagnostics Cooperation, Medfield) and that in buffer was about 40 mmHg with this system [Ikeda et al., 1998]. The effect of shear stress in this system was negligible, because it was less than 1 dyne/cm^2 [Ikeda et al., 1996a].

RESULTS

As shown in Figure 1, HUVECs showed smooth spindle like appearance and basal $[Ca^{2+}]_i$ was homogeneous through the cytosol as previously reported [Ikeda et al., 1996b]. Twenty-five min after initiation of hypoxia, $[Ca^{2+}]_i$ locally rose to 321 ± 46 nM in the peripheral region of HUVECs, which was associated with bleb formations at the same loci. The localized $[Ca^{2+}]_i$ rise is not due either to the artifacts from dye compartmentalization or to the loss in membrane integrity, because both the localized $[Ca^{2+}]_i$ rise and bleb formation are reversible phenomena in hypoxia-induced cellular damages (data not shown). The concentration of nuclear $[Ca^{2+}]$ ($[Ca^{2+}]_n$) also elevated from 145 ± 42 nM to 888 ± 32 nM. In contrast, neither remarkable changes in $[Ca^{2+}]_i$ nor bleb formation was noted for at least 30 min during perfusion of the control medium (data not shown). In order to clarify the origin of Ca^{2+} causing the localized $[Ca^{2+}]_i$ rise induced by hypoxia, we first tested the effect of various Ca^{2+} channel blockers. L type Ca^{2+} channel blocker (10 μM nifedipine), L + T type Ca^{2+} channel blocker (10 μM nicardipine), L + N type Ca^{2+} channel blocker (10 μM cilnidipine), and a non-specific plasma membrane Ca^{2+} channel blocker (1 mM Ni^{2+}) failed to inhibit the localized $[Ca^{2+}]_i$ rise and bleb formation. The peak values of the $[Ca^{2+}]_i$ rise were 435 ± 84 nM, 507 ± 94 nM, 503 ± 74 nM, 432 ± 65 nM, and 481 ± 149 nM in control, nifedipine-treated, nicardipine-treated, cilnidipine-treated, and Ni^{2+}-treated HUVECs, respectively.

In order to abolish the Ca^{2+} influx from extracellular space to cytosol, we employed Ca^{2+}-EGTA buffer to inhibit the Ca^{2+} influx by lowering extracellular Ca^{2+} concentration to 100 nM so as to abolish the Ca^{2+} gradient between the extracellular space and cytosol. The concentration of ionized Ca^{2+} in Ca^{2+}-EGTA buffer was calculated by the method described by Harafuji et al. [1980]. We did not completely chelate extracellular Ca^{2+} by EGTA to inhibit the Ca^{2+} influx, because the complete chelation of extracellular Ca^{2+} may lead to the cellular damage and detachment under our assay conditions. Hypoxia were started 10 min after the adjustment of extracellular Ca^{2+}. As shown in Figure 2, both the localized $[Ca^{2+}]_i$ rise and bleb formation were completely inhibited by lowering the extracellular Ca^{2+} concentration, suggesting that the Ca^{2+} influx through an unknown type of Ca^{2+} channel other than L type, T type, N type, Ni^{2+}-inhibitable Ca^{2+} channels, is responsible for the hypoxia-induced localized $[Ca^{2+}]_i$ rise. Although inhibiting the Ca^{2+} influx abolished the localized $[Ca^{2+}]_i$ rise and bleb formation, rather a homogeneous rise of $[Ca^{2+}]_i$ to 130 ± 22 nM was observed in the hypoxia stimulated HUVECs, which was inhibited by incubating cells with a PI specific PLC inhibitor, U73122 [Yule et al., 1992; Chung et al., 1998], as shown in Figure 2. These findings might suggest that hypoxia causes the Ca^{2+} mobilization through the activation of PLC. The diffuse $[Ca^{2+}]_i$ rise observed was not due to the Ca^{2+} influx, because it could be observed with the lower Ca^{2+} concentration in the extracellular medium upon hypoxia (data not shown).

DISCUSSION

Although there have been several reports published discussing the relationships between the $[Ca^{2+}]_i$ rise and bleb formation integral to cellular death [Orrenius et al., 1992], the involvement of the $[Ca^{2+}]_i$ rise in hypoxic injury is still controversial. Although several groups reported the association between the $[Ca^{2+}]_i$ rise and bleb formation [Smith et al., 1992; Friedman et al., 1993], one group found no $[Ca^{2+}]_i$ rise during bleb formation [Lemasters et al., 1987], and other groups have demon-

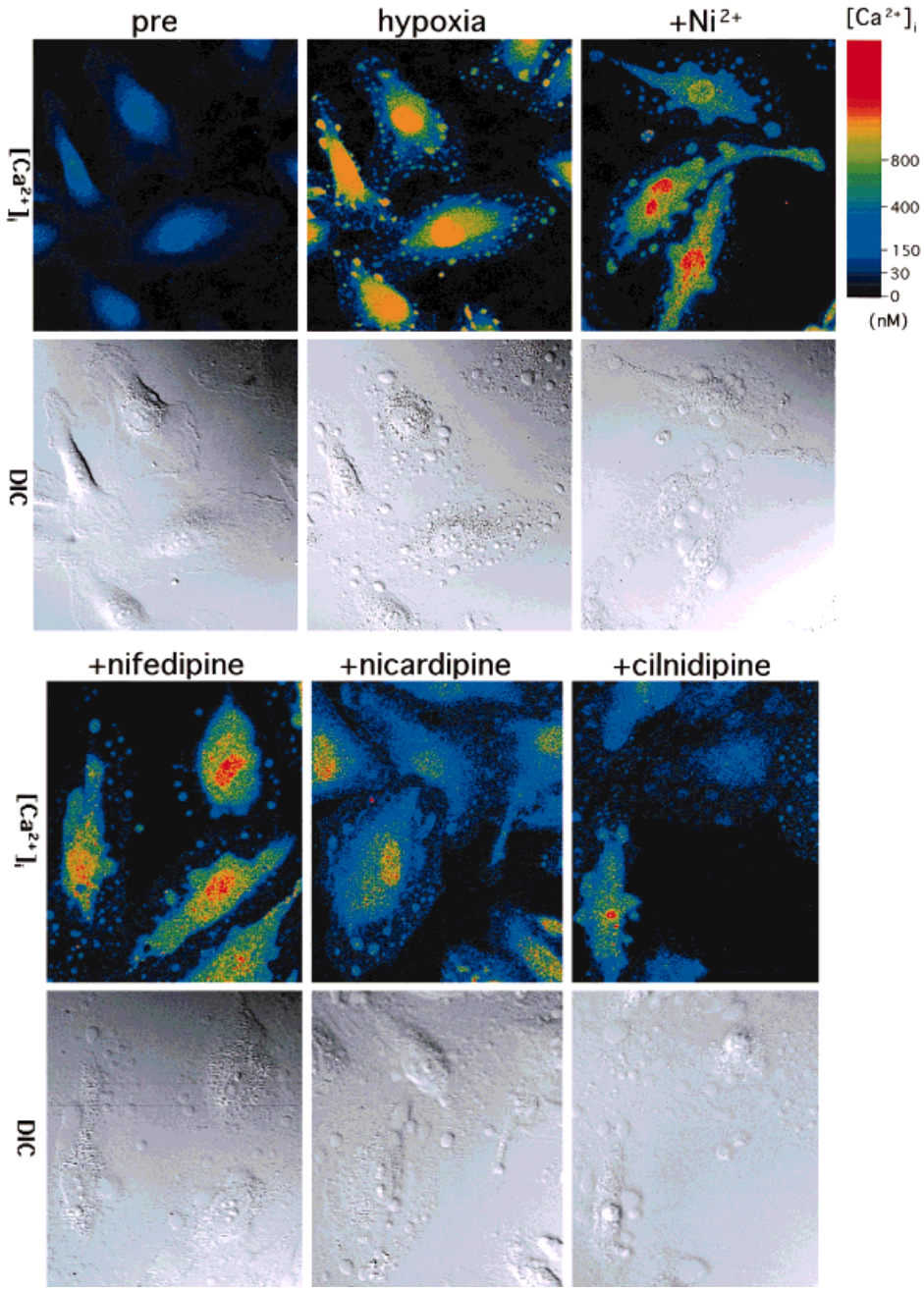

Fig. 1. The effects of various Ca^{2+} channel blockers on the hypoxia-induced localized $[Ca^{2+}]_i$ rise and bleb formation. HUVECs were perfused with hypoxic medium after the incubation with control saline, 1 mM Ni^{2+}, 10 μM nifedipine, 10 μM nicardipine, or 10 μM cilnidipine for 10 min. The $[Ca^{2+}]_i$ images and differential interference contrast (DIC) images before the perfusion and those of 25 min later were shown. The condition of image acquisition was described in Materials and Methods. The results presented were from one representative of four different experiments.

strated bleb formation prior to the $[Ca^{2+}]_i$ rise [Allshire et al., 1987; Geeraerts et al., 1991; Jurkowitz-Alexander et al., 1992; Johnson et al., 1994; Van Winkle et al., 1994].

In previous reports, we confirmed that the localized $[Ca^{2+}]_i$ rise at the same loci of bleb formation precedes these processes in HUVECs [Ikeda et al., 1998]. These temporal and spatial relationship strongly suggested that the localized $[Ca^{2+}]_i$ rise might cause bleb formation. Although it is possible that there may be a Ca^{2+} independent pathway which leads to bleb

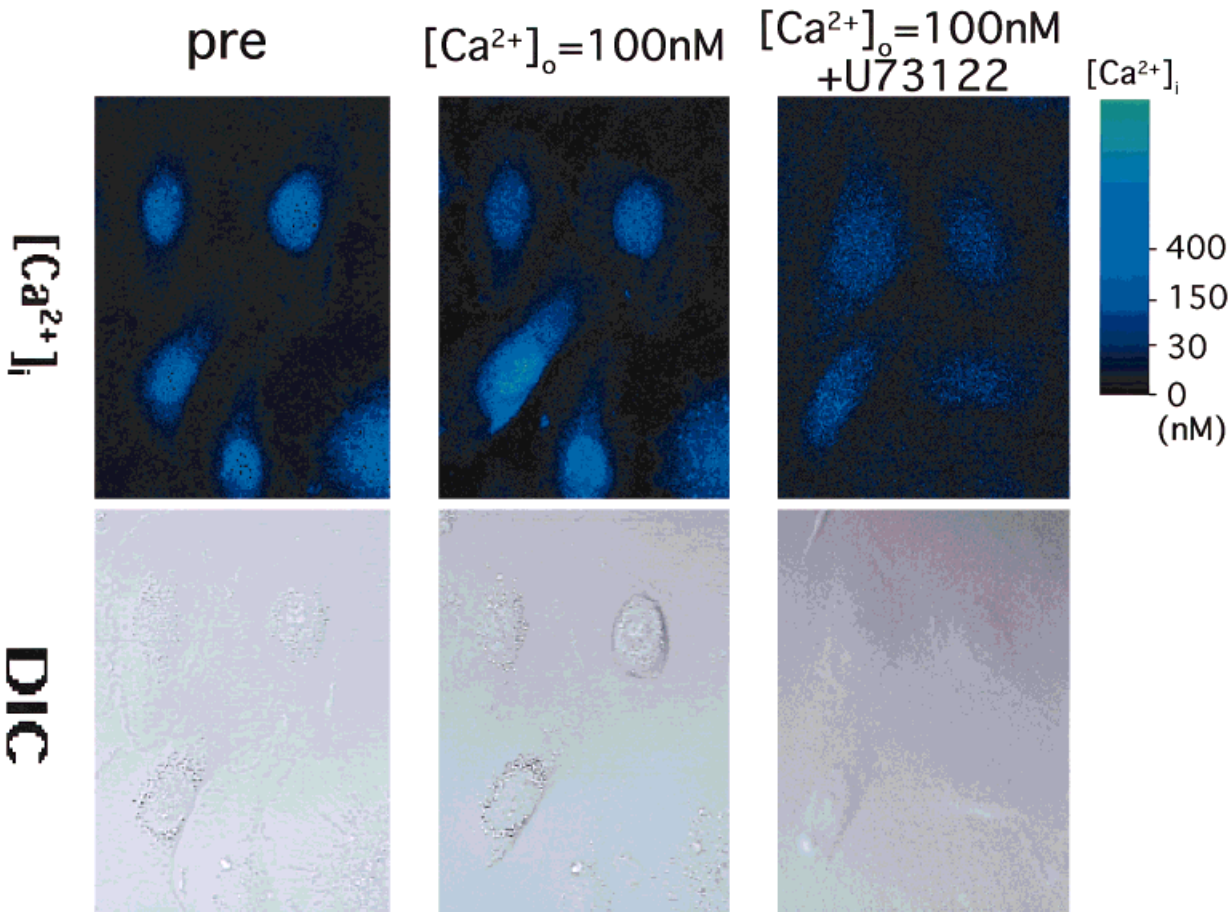

Fig. 2. The $[Ca^{2+}]_i$ gradients in HUVECs perfused with hypoxic medium containing 100 nM Ca^{2+} and the effect of a phospholipase C inhibitor, U73122. HUVECs were perfused with hypoxic medium containing 100 nM Ca^{2+} adjusted by Ca^{2+}-EGTA buffer in the presence or the absence of a phospholipase C inhibitor, 10 μM U73122. The $[Ca^{2+}]_i$ images and DIC images before the perfusion and those of 25 min later were presented. The condition of image acquisition was described in Materials and Methods. The results presented were from one representative of three different experiments.

formation, it is likely that the differences may be due to the differences in cell types or the mode of hypoxia. In brief, many investigators have developed hypoxic injury models using the "chemical hypoxia" induced such agents as cyanide, iodoacetate, or xanthine [Jewell et al., 1982; Allshire et al., 1987; Lemasters et al., 1987; Phelps et al., 1989; Hayashi et al., 1990; Greeraerts et al., 1991; Jurkowitz-Alexander et al., 1992; Smith et al., 1992; Johnson et al., 1994; Zahrebelski et al., 1995], however, it is impossible to exclude possible artifacts due to the nature of these chemicals. Our observations were free from such artifacts, because we utilized a parallel-plate "hypoxic" flow chamber for cultured cells, which allows us to monitor cellular morphology and fluorescence images of fluo-3 under lowered O_2 tension [Ikeda et al., 1998].

However, we failed to confirm the mechanisms or the mode causing the localized $[Ca^{2+}]_i$ rise prior to bleb formation. Therefore, in this study, we studied the origin of the localized $[Ca^{2+}]_i$ rise to elucidate our hypothesis that the $[Ca^{2+}]_i$ rise is the trigger event in hypoxia-induced cellular injury, which works in a localized fashion. In general, it has been believed that the $[Ca^{2+}]_i$ rise during hypoxic injury is produced as a result of a breakdown in the intracellular Ca^{2+} homeostasis due to the energy (ATP) depletion, which cause the Ca^{2+} overload inside cells [Cheung et al., 1986; Orrenius et al., 1992], however, the exact mode of the $[Ca^{2+}]_i$ rise remains unclear. Especially

this theory could not explain the "localized $[Ca^{2+}]_i$ rise" observed in our assay condition. In addition, several groups [Herman et al., 1990] reported the relationship between energy depletion and bleb formation and the role of the Ca^{2+} influx in hypoxia-induced cell death by employing Ca^{2+} channel blockers, Ca^{2+} chelators, or several reagents inhibiting ATP generation, however, they failed to examine the role of the Ca^{2+} mobilization in the hypoxia-induced $[Ca^{2+}]_i$ rise.

In this report, we clearly demonstrated the existence of the Ca^{2+} mobilization from the internal Ca^{2+} storage sites as well as the Ca^{2+} influx from the extracellular medium. In HUVECs, the existence of L type or Ni^{2+}-inhibitable Ca^{2+} channel has been reported [Yoshikawa et al., 1997]. As was reported in the observation by some group [Harrison et al., 1991], we also failed to inhibit the Ca^{2+} influx by a non-specific Ca^{2+} channel blocker, Ni^{2+}, or L type, L + T type and L + N type Ca^{2+} channel blockers under our assay conditions, suggesting that the Ca^{2+} influx observed in our assay condition may be through an "unknown" Ca^{2+} channel. The difference might suggest the difference in the Ca^{2+} pathway between the "chemical" hypoxia and the "real" hypoxia. These findings might explain the failure of Ca^{2+} channel blockers in preventing a hypoxic organ damage in clinical situations [de Broin et al., 1997]. In this study, we first demonstrated the existence of the Ca^{2+} mobilization, inhibitable by a PLC inhibitor, U73122, in the hypoxia stimulated HUVECs, suggesting the existence of an O_2 tension-sensitive signal transduction system in HUVECs, which may explain the mechanisms of the several responses of HUVECs exposed to hypoxia, such as generation of superoxides and free radicals, down-regulation of ATP, or up-regulation of proteases, phospholipases, and endonucleases [Kristian et al., 1998]. Although the Ca^{2+} mobilization we observed was insufficient for bleb formation, it might play an initial and pivotal role in the hypoxia-induced cellular injury, because there have been several reports describing the role of the "cross-talk" in $[Ca^{2+}]_i$ regulating system, such as the "Ca^{2+} induced Ca^{2+} influx" [Arnould et al., 1992]. Our findings might be a clue to understand the nature of the hypoxia-induced $[Ca^{2+}]_i$ rise, which might be integral to the "rescue" of the cells from hypoxic injury.

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REFERENCES

Allshire A, Piper HM, Cuthbertson KSR, Cobbold PH. 1987. Cytosolic free Ca^{2+} in single rat heart cells during anoxia and reoxygenation. Biochem J 244:381–385.

Arnould T, Michiels C, Alexandre I, Remacle J. 1992. Effect of hypoxia upon intracellular calcium concentration of human endothelial cells. J Cell Physiol 152:215–221.

Bellomo G, Perotti M, Taddei F, Mirabelli F, Finardi G, Nicotera P. 1992. Tumor necrosis factor α induces apoptosis in mammary adenocarcinoma cells by an increase in intranuclear free Ca^{2+} concentration and DNA fragmentation. Cancer Res 52:1342–1346.

Burnier M, Centeno G, Burki E, Brunner HR. 1994. Confocal microscopy to analyze cytosolic and nuclear calcium in cultured vascular cells. Am J Physiol 266:C1118–1127.

Cheung JY, Bonventre JV, Malis CD, Leaf A. 1986. Calcium and ischemic injury. N Engl J Med 314:1670–1676.

Chung RJ, Chin MH, Sheng NW, Ching JT. 1998. The phospholipase C inhibitor U73122 increases cytosolic calcium in MDCK cells by activating calcium influx and releasing stored calcium. Life Sci 63:895–908.

de Broin E, Urata K, Giroux L, Lepage R, Huet PM. 1997. Effect of calcium antagonists on rat liver during extended cold preservation-reperfusion. Transplantation 63:1547–1554.

Friedman JE, Haddad GG. 1993. Major differences in Ca^{2+} response to anoxia between neonatal and adult rat CA1 neurons: role of Ca_o^{2+} and Na_o^{+}. J Neurosci 13:63–72.

Geiger RV, Berk BC, Alexander RW, Nerem RM. 1992. Flow-induced calcium transients in single endothelial cells: Spatial and temporal analysis. Am J Physiol 262: C1411–1417.

Gillo B, Ma YS, Marks AR. 1992. Calcium influx in induced differentiation of murine erythroleukemia cells. Blood 81:783–792.

Greeraerts MD, Ronveaux-Dupal M-F, Lemasters JJ, Herman B. 1991. Cytosolic free Ca^{2+} and proteolysis in lethal oxidative injury in endothelial cells. Am J Physiol 261:C889–896.

Harafuji H, Ogawa Y. 1980. Re-examination of the apparent binding constant of ethylene glycol bis (beta-N,N,N′,N′-tetraacetic acid with calcium around neutral pH. J Biochem (Tokyo) 87:1305–1312.

Harrison DG, Treasure CB, Mügge A, Dellsperger KC, Lamping KG. 1991. Hypertension and the coronary circulation with special attention to endothelial regulation. Am J Hypertens 4:454S–459S.

Hayashi H, Miyata H, Kobayashi A, Yamasaki N. 1990. Heterogeneity in cellular response and intracellular distribution of Ca^{2+} concentration during and after metabolic inhibition. Cardiovasc Res 24:605–608.

Herman B, Gores GJ, Nieminen AL, Kawahishi T, Harman A, Lemasters JJ. 1990. Calcium and pH in anoxic and toxic injury. Crit Rev Toxicol 21:127–148.

Hernandez-Curz A, Sala F, Adams PR. 1990. Subcellular calcium transients visualized by confocal microscopy in a voltage-clamped vertebrate neuron. Science 247:858–862.

Ikeda M, Ariyoshi H, Kambayashi J, Sakon M, Kawasaki T, Monden M. 1996. Simultaneous digital imaging analysis of cytosolic calcium and morphological change in platelets activated by surface contact. J Cell Biochem 61:292–300.

Ikeda M, Ariyoshi H, Sakon M, Kambayashi J, Fujitani K, Shinoki N, Sakon M, Kawasaki T, Monden M. 1996. Separate analysis of nuclear and cytosolic free Ca^{2+} concentration in human umbilical vein endothelial cells. J Cell Biochem 63:23–36.

Ikeda M, Ariyoshi H, Sakon M, Kambayashi J, Yoshikawa N, Shinoki N, Kawasaki T, Monden M. 1998. A role for local calcium gradients upon hypoxic injury in human umbilical vein endothelial cells (HUVEC). Cell Calcium 24(1):49–57.

Jewell SA, Bellomo G, Thor H, Orrenius S. 1982. Bleb formation in hepatocytes during drug metabolism is caused by disturbances in thiol and calcium ion homeostasis. Science 217:1257–1259.

Johnson ME, Gores GJ, Uhl CB, Sill JC. 1994. Cytosolic free calcium and cell death during metabolic inhibition in a neuronal cell line. J Neurosci 14:4040–4049.

Jurkowitz-Alexander MS, Altschuld RA, Hohl CM, Johnson JD, McDonald JS, Simmons TD, Horrocks LA. 1992. Cell swelling, blebbing, and death are dependent on ATP depletion and independent of calcium during chemical hypoxia during chemical hypoxia in a glial cell line (ROC-1). J Neurochem 59:344–352.

Kristian T, Siesjo. 1998. Calcium in ischemic cell death. Stroke 29:705–718.

Lemasters JJ, DiGuiseppi J, Nieminen A, Herman B. 1987. Blebbing, free Ca^{2+} and mitochondorial membrane potentioal preceeding cell death in hepatocytes. Nature 325:78–81.

Nakato K, Furuno T, Inagaki K, Teshima R, Terao T, Nakanishi M. 1992. Cytosolic and intranuclear calcium singles in rat basophilic leukemia cells as revealed by a confocal Orrenius S, Burkitt MJ, Kass GEN, Dypbukt JM, Nicotera P. 1992. Calcium ions and oxidative cell injury. Ann Neurol 32(Suppl):S33–42.

Phelps PC, Smith MW, Trump BF. 1989. Cytosolic ionized calcium and bleb formation after acute cell injury of cultured rabbit renal tubule cells. Lab Invest 60:630–642.

Smith MW, Phelps PC, Trump BF. 1992. Injury-induced changes in cytosolic Ca^{2+} in individual rabbit proximal tubule cells. Am J Physiol 262:F647–655.

Van Winkle WB, Snuggs M, Miller JC, Buja LM. 1994. Cytoskeletal alterations in cultured cardiomyocytes following exposure to the lipid peroxidation product, 4-hydroxynonenal. Cell Motil Cytoskeleton 28:119–134.

Wahl M. Sleight RG, Gruenstein E. 1992. Association of cytoplasmic free Ca^{2+} gradients with subcellular organelles. J Cell Physiol 150:593–609.

Williams DA, Fogarty KE, Tsien RY, Fay FS. 1985. Calcium gradients in single smooth muscle cells revealed by the digital imaging microscope using fura-2. Nature 318: 558–560.

Yelamarty RV, Miller BA, Scaduto RC, Yu FTS, Tillotson DL, Cheung JY. 1990. Three-dimensional intracellular calcium gradients in single human burst-forming units-erythroblasts induced by erythropoietin. J Clin Invest 85:1779–1809.

Yoshikawa N, Ariyoshi H, Ikeda M, Sakon M, Kawasaki T, Monden M. 1997. Shear-stress causes polarized change in cytoplasmic calcium concentration in human umbilical vein endothelial cells (HUVECs). Cell Calcium 22: 189–194.

Yule DI, Williams JA. 1992. U73122 inhibits Ca^{2+} oscillations on response to cholecystokinin and carbachol but not to JMV-180 in rat pancreatic acinar cells. J Biol Chem 267:13830–13835.

Zahrebelski G, Nieminen A, Al-Ghoul K, Qian T, Herman B, Lemaster JJ. 1995. Progression of subcellular changes during chemical hypoxia to cultured rat hepatocytes: a laser scanning confocal microscopic study. Hepatology 21:1361–1372.