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Influence of the 15-HETE on Cytosolic $[Ca^{2+}]_i$ of the Rabbit Pulmonary Artery Smooth Muscle Cells

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ABSTRACT

Extracellular Ca^{2+} influx was blocked by L-type Ca^{2+} channel blocker nifedipine to observe the effects of 15-hydroxyeicosatetraenoic acid on the constriction of rabbit pulmonary artery rings and on the changes of Ca^{2+} level in the rabbit pulmonary artery smooth muscle cells, and further to investigate the mechanism of the calcium mobilization induced by the 15-HETE under hypoxic conditions. The effect of extracellular Ca^{2+} on tension of the rabbit PA rings was also studied. Nifedipine (10 μmol/L) had no effect on 1 μmol/L 15-hydroxyeicosatetraenoic acid induced vasoconstriction under normoxic and hypoxic conditions. Intracellular Ca^{2+} increased markedly in the 15-HETE group (cells were exposed to 1 μmol/L 15-HETE for 8 min during culture) compared to the control group ($P < 0.05$). The study demonstrated that the 15-HETE could induce the elevation of Ca^{2+} in the pulmonary artery smooth muscle cells and the elevated calcium came from the release of the intracellular calcium.

Key words: 15-hydroxyeicosatetraenoic acid, hypoxia, pulmonary artery rings, pulmonary artery smooth muscle cells, $[Ca^{2+}]_i$

INTRODUCTION

The mechanism of hypoxia-induced pulmonary vasoconstriction remains unknown. Zhu et al (2003) reported the regulation of the 15-lipoxygenase (15-LO), leading to the elevated levels of the 15- hydroxyeicosatetraenoic acid (15-HETE) under the hypoxic condition. The 15-HETE causes concentration-dependent constriction of the pulmonary artery (PA) rings of the neonatal rabbits from the animals exposed to the hypoxic but not the normoxic environments. The inhibition of the interior 15-HETE results in the phenylephrine-induced concentration-related constriction shifted to the right, suggesting a potential role for the 15-HETE as an initiating factor in modulating the hypoxic pulmonary vasoconstriction (HPV) (Zhu et al, 2003). Though the 15-HETE is closely related to the pulmonary artery vasoconstriction and the constriction is related to cytosolic Ca^{2+} level, there is no report on the effect of the 15-HETE on the cytosolic Ca^{2+} changes. Thus, the aim of this work was to investigate the contribution of the 15-HETE on level of Ca^{2+} in the pulmonary artery smooth muscle cells (PASMCs) under the hypoxic and normoxic conditions, and also examine the source of cytosolic Ca^{2+}.

MATERIALS AND METHODS

Animals

Adult male New Zealand white rabbits (weight 2-3 kg) were housed in the Animal Resource Center of Harbin Medical University (fully accredited by the Institutional Animal Care and Use Committee) and handled in a manner which met all the

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recommendations formulated by the National Society for Medical Research and Guidelines for the Care and Use of Laboratory Animals.

Reagents

The 15-HETE was purchased from the Biomol. Nifedipine, ethylene glycol-bis (beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) were purchased from Sigma. All other chemicals were of analytical grade unless otherwise stated.

Animal model (Zhu et al, 2000)

Neonatal rabbits (kits) were cared in a normoxic environment and immediately after their first feeding, transferred to an environmental chamber in which the fractional inspired oxygen (FIO_2) was either reduced to 0.12 (hypoxia), or 0.21 (normoxia). Six kits from both the groups were returned to their mother (in a normoxic environment) for 20 minutes once a day for the feeding and then replaced in the environmental chambers for 9 days.

Tension studies of rabbit PA rings

These studies were carried out as described (Zhu et al, 1998). PAs 1-1.5 mm in diameter were cut into rings. The rings were mounted on the tungsten wire and immersed in 25 ml pH-adjusted, oxygenated Krebs solution at 37□, the composition of the Krebs solution was (in mmol/L) 118 NaCl, 4.7 KCl, 27 $NaHCO_3$, 10 glucose, 2.5 $CaCl_2$, 0.57 $MgSO_4$, and 1.2 KH_2PO_4, pH 7.2-7.4. The rings were initially loaded with 0.3 *g* tension, which was gradually and incrementally applied over 30 min and then equilibrated for an additional 30 - 40 min at 37□ in Krebs solution before the studies begun. The 15-HETE from a stock solution in ethanol was added at a final concentration of 1 μmol/L to observe the tension changes. Then the 15-HETE was washed out and the baseline tension of the rings was recovered. The rings were incubated with 10 μmol/L nifedipine for 15 min and then with 1 μmol/L 15-HETE again. In a separate experiment, the response of the PA rings to 1 μmol/L 15-HETE in calcium-free Krebs solution was determined. All the tension data were relayed from the pressure transducers to a signal amplifier (600 series eight-channel amplifier, Gould Electronics). Data were acquired and analyzed with the CODAS software (DataQ Instruments, Inc.).

Dispersion and culture of rabbit PASMCs

The PASMCs were collected according to Gebremedhin et al (1998). The PAs were cut into small pieces and dispersed using a constantly stirred solution containing collagenase (10 units/ml). The dispersed PASMCs were incubated for 8 - 9 min at 25□ and then centrifuged for 10 min to get the cell pellet. The pellet was resuspended in RPMI-1640 culture medium with 20% serum and 1% penicillin/streptomycin and plated into T75 culture flasks for culturing. The cell viability (usually greater than 98%) was determined by the Trypan Blue exclusion (Huang, 1997).

Intracellular Ca^{2+} measurement

Ca^{2+} transients in the individual cells were determined using the fluorescence video microscope by the method of Gerboth et al (1993). The cells (PASMCs, which adhered to glass cover slips for < 48 h) were loaded with 5 μmol/L of Ca^{2+} indicator Fura-2 for 30 min at 37□, followed by 1 μmol/L 15-HETE for 8 min. The cells were washed several times to remove the non-hydrolyzed dye and then visualized using inverted microscope equipped for the epifluorescence. Alternating measurements of fluorescence excited at 340 and 380 nm with an emission wavelength of 510 nm were obtained using a dual wavelength fluorescence imaging and photometric system (InCyt IM2; Cincinnati Ohio). Ca^{2+} concentrations were calculated using the ratio method according to the equation $[Ca^{2+}]_i$ = Kd (Sf2/Sb2) [(R□Rmin)/Rmax□R]] where R was the fluorescence ratio at 340/380, Sf2 and Sb2 are the ratios of free and bound forms of the dye. Rmin and Rmax were the 340/380 ratios of full free (in the presence of 20 mmol/L EGTA) and full bound (in the presence of 60 μmol/L digitonin). Kd was assumed to be 224 nm.

Statistical Analysis

Data were presented as means ± SE. The significances in mean values among the groups with the different treatments were measured using the two-tailed analyses of the variance (ANOVA), followed by the Dunnett's test when significant differences were identified. $P \leq 0.05$ using a two-tailed test was considered significant.

RESULTS

Effect of L-type Ca^{2+} channel blocker nifedipine on tension of rabbit PA rings

After 1 μmol/L 15-HETE was added to pH 7.4 oxygenated Krebs solution at 37□, the tension of the rabbit PA rings that were incubated in the solution were increased greatly in both normoxic (161.0 ± 13.3, % basal tension) and hypoxic groups (219.0 ± 29.0, % basal tension). When the rings were pretreated with 10 μmol/l nifedipine for 15 min before conducting the same experiment as above, the tension values were 169.8 ± 8.0 (% basal tension, normoxic group) and 233.7 ± 20.4 (% basal tension, hypoxic group), respectively. Compared with the treatment of giving 1 μmol/L of 15-HETE only, the results had no statistical significance ($P > 0.05$, Fig. 1).

Effect of extracellular Ca^{2+} on the tension of rabbit PA rings

The rings from the hypoxic and normoxic groups were treated with 1 μmol/L 15-HETE in pH 7.4, oxygenated Krebs solution (with calcium) at 37□. Tension values of the rings from the hypoxic and normoxic groups were 138.75 ± 4.24 and 173.0 ±5.62 (% basal tension), respectively. After washing, the rings were treated with 1 μmol/L 15-HETE again in pH 7.4 oxygenated Krebs solution without calcium. Tension values of the rings from the hypoxic and normoxic groups were almost the same as above, 140.7 ± 4.42 and 174.3 ± 5.98 (% basal tension), respectively (Fig. 2).

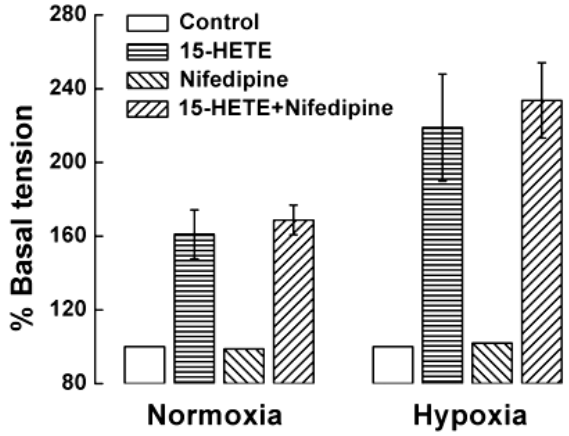

Figure 1 - Mean (± SE) represents effect of L-type Ca^{2+} channel blocker nifedipine (10 μmol/L, incubated for 10 min) on PA vasoconstriction induced by 15-HETE (1 μmol/L) under both normoxic ($FIO_2 = 0.21$) and hypoxic ($FIO_2 = 0.12$) conditions. The vasoconstriction induced by 15-HETE was not affected by nifedipine, ($n = 4$)

Influence of the 15-HETE on cytosolic Ca^{2+} in the cultured rabbit PASMCs

The increase of Ca^{2+} were shown in the 15-HETE group after the cells were treated with 1 μmol/L 15-HETE. The changes happened only a few seconds after the 15-HETE was loaded and reached maximum value after about 3 min (Fig. 3A). Compared with the control group, the cytosolic free $[Ca^{2+}]i$ increased from 113.5 ± 8.7 nmol/L (control group) to 141.7 ± 12.7 nmol/L (the 15-HETE group), and the difference reached statistical significance ($P < 0.05$, Fig. 3B).

DISCUSSION

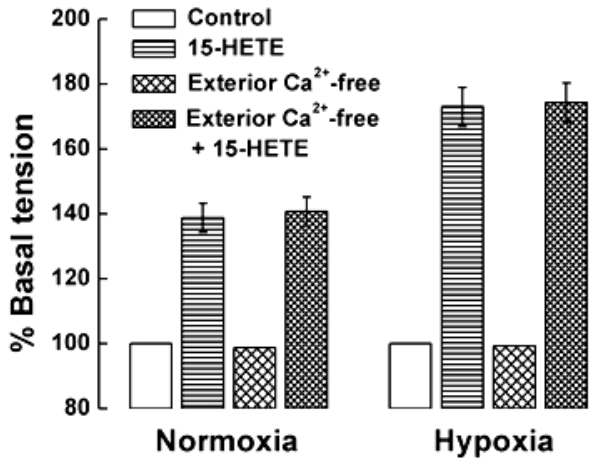

Figure 2 - Mean (± SE) represents effect of exterior Ca^{2+} on rabbit PA vasoconstriction induced by 15-HETE (1 μmol/L) under both normoxic (FIO_2 = 0.12) and hypoxic (FIO_2 = 0.21) conditions. PA vasoconstriction induced by 15-HETE was not affected by exterior Ca^{2+}, (*n* = 4).

The elevations in the global cytoplasmic Ca^{2+} resulting in the contraction are accomplished by the extracellular Ca^{2+} entry through transmembrane channels and the release from the intracellular stores (mainly endoplasmic/sarcoplasmic reticulum) (Sanders, 2001). There are two opinions about the influence of the hypoxia on Ca^{2+} changes. Simonneau et al (1981) and Tolins et al (1986) reported that Ca^{2+} entered smooth muscle cells (SMCs) through the L-type calcium channel leading to the elevation of the $[Ca^{2+}]_i$, and, therefore the HPV. Salvaterra et al (1993) and Gelband et al (1997) found that an initial event in HPV was the cause of the release of Ca^{2+} from the intracellular stores. However, the exact mechanisms of each process as well as the agent(s) causing the elevation of $[Ca^{2+}]_i$ remains an enigma.

In this study, it was observed that the 15-HETE could markedly increase the [Ca2+]i in the cultured rabbit PASMCs, which mean that the 15-HETE was involved in the pulmonary vasoconstriction, and, the constriction was not affected by the L-type Ca2+ channel blocker nifedipine, indicating that the elevation of [Ca2+]i induced by the 15-HETE was not caused by the influx of the extracellular Ca2+ through the L-type Ca2+ channel. As extracellular Ca2+ might enter into cells through other pathways, a different experiment was carried out (Fig. 2).

Calcium-free Krebs solution was employed in the experiment, but the constriction of the rings remained the same, which mean that the Ca2+ that caused the constriction of the rings might come from the release of the intracellular stores. Salvaterra et al (1993) reported that the hypoxia could induce a reversible 100-200% increase in the [Ca2+]i that was characterized by the two components: an early rise in [Ca2+]i that was dependent on the rate, as well as the magnitude, of decline in PO2 and later a steady-state increase that was independent of the rate at which PO2 changed. The caffeine lowered [Ca2+]i during the normoxia and blocked the early component of the response to the hypoxia, whereas the steady-state hypoxic response was only partially inhibited (Salvaterra et al, 1993). At the steady state, the hypoxic responses were completely reversed by the removal of the extracellular Ca2+, whereas, on average, verapamil and nifedipine attenuated the hypoxia-induced increases in the [Ca2+]i by only 44 and 35%, respectively. These results suggested that the hypoxia-induced elevation of the [Ca2+]i in the PASMCs consisted of an early release of the Ca2+ from the sarcoplasmic reticulum and a

later influx of the extracellular Ca2+, in part, through the nifedipine- and verapamil-insensitive Ca2+ channels.

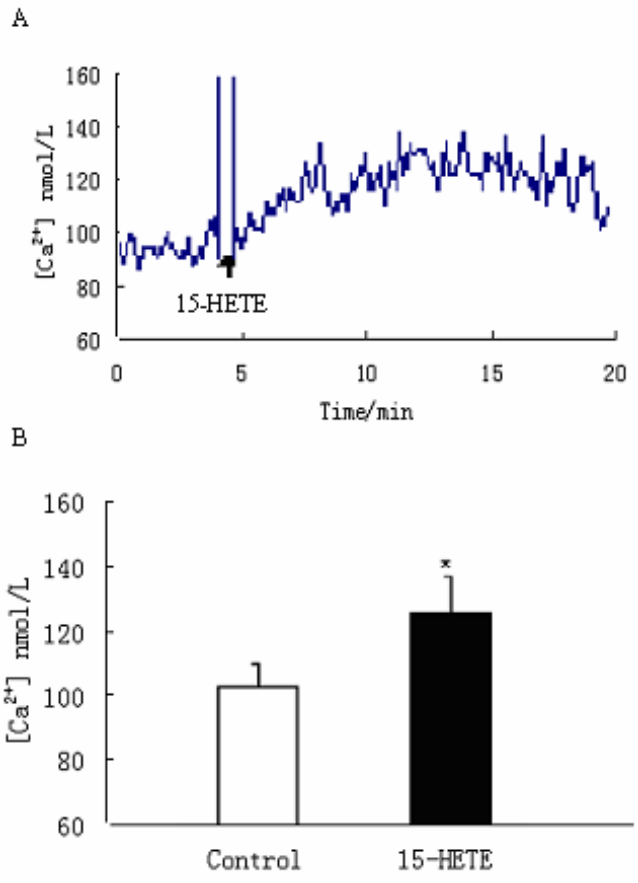

Figure 3 - Effect of 15-HETE on cytosolic free $[Ca^{2+}]_i$ in rabbit PASMCs. A: Representative recording of $[Ca^{2+}]_i$ from rabbit PASMCs treated by 15-HETE (1 μmol/L). B: Summarized data showing changes in $[Ca^{2+}]_I$ induced by 15-HETE. Data are means ± SE, *P<0.05 vs basal $[Ca^{2+}]_i$ levels, n = 38 cells

These results were consistent with Salvaterra's (1993), indicating that the 15-HETE might be involved in the HPV.

The present study demonstrated that the 15-HETE could induce calcium release from the intracellular stores, but the exact sources and the mechanism of the calcium released were unknown. Therefore, further studies should be carried out on the intracellular calcium release induced by the 15-HETE and its mechanism. As there are two calcium releasing channels (Qi et al, 2002; Ma et al, 2003) in the endoplasmic reticulum/sarcoplasmic reticulum membranes, the inositol 1,4,5-trisphosphate receptor (IP3R) and the ryanodine receptor (RyR), the sources of the $[Ca^{2+}]_i$ elevation by using heparin and caffeine to exhaust and block the intracellular calcium release regulated by the two channels should be looked into.

This report is the first to describe a mechanism for the 15-HETE induced calcium elevation in the PASMCs. Apparently, the 15-HETE might play an important role in the elevation of the intracellular $[Ca^{2+}]_i$ leading to the HPV.

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