Subacute Mild Hypoxia Increases Histamine-stimulated Calcium Oscillation Frequency in Pulmonary Artery Endothelial Cells*

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Abstract Calcium oscillation may regulate gene transcription in a frequency-decoding manner during agonist stimulation, which provides an indicator of transcription level in cells. To determine whether persistent exposure to hypoxia may sensitize or blunt cell response to histamine, the effects of 24 h subacute mild hypoxia on histamine-stimulated calcium oscillation frequency were examined in pulmonary artery endothelial cells (PAECs). The results are: (1) 24 h subacute mild hypoxia significantly increased the histamine-stimulated calcium oscillation frequency in PAECs. The averaged frequency of calcium oscillation in posthypoxic PAECs was significantly higher than that in normoxic ones. (2) NADPH oxidase inhibitor, diphenylene iodonium chloride (DPI, 10 μmol/L), abolished histamine-stimulated calcium oscillations both in normoxic and posthypoxic PAECs. (3) Xanthine oxidase inhibitor, oxypurinol (100 μmol/L), did not affect the calcium oscillation frequency in normoxic PAECs. However, it significantly decreased the elevation of calcium oscillation frequency in posthypoxic PAECs. These results demonstrated that, during pulmonary disease related to persistent hypoxia, PAECs become more sensitive to histamine. During histamine stimulation, NADPH oxidase plays a critical role in generating calcium oscillations, while xanthine oxidase may contribute to, at least in part, the increase of calcium oscillation frequency in posthypoxic PAECs.

Key words calcium oscillation, histamine, hypoxia, pulmonary artery, NADPH oxidase, xanthine oxidase

Histamine released from mast cells is critically involved in hypoxic pulmonary vasoconstriction and remodeling, it causes relaxation mediated by pulmonary artery endothelial cells (PAECs) and contraction by pulmonary artery smooth muscle cells (PASMCs)[1]. In endothelial cells, histamine stimulates an increase of intracellular $[Ca^{2+}]_i$ concentration due to a rapid mobilization of calcium from intracellular stores [2] followed by a more sustained increase in $[Ca^{2+}]_i$ due to the influx of extracellular Ca^{2+} [3]. Histamine-stimulated expression of target genes as endothelial nitric oxide synthase (eNOS) [4], endothelin-1 [5], vascular endothelium growth factor (VEGF) [6] may ultimately influence the tone or proliferation of pulmonary vessels and contribute to the pathogenesis of pulmonary hypertension. The expression of these genes was dependent on the activation and activity of calcium-activated transcription factors as NF-κB etc[7].

Many inflammatory mediators, including histamine, can induce calcium oscillations in endothelial cells. As calcium oscillation may regulate transcription in a frequency-decoding manner [8~11], a variation in the frequency of $[Ca^{2+}]_i$ oscillations during low-level histamine stimulation resulted in a parallel variation in NF-κB activity. The regulation of nuclear transcriptional activity by the frequency of cytosolic Ca^{2+} oscillations may provide cells with a specific mechanism to control gene expression during agonist stimulation.

During the pathogenesis of many pulmonary diseases such as chronic bronchitis, emphysema, asthma, PAECs were exposed to persistent hypoxia.

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To determine whether this kind of hypoxia may sensitize or blunt their response to histamine, we examined the effects of 24 h subacute mild hypoxia on histamine-stimulated calcium oscillations in PAECs after re-supply of O_2.

1 Materials and methods

1.1 Pulmonary artery endothelial cell culture

Endothelial cells were isolated from main pulmonary arteries of 10- to 12-month-old pigs by trypsin and cultured in medium M-199 (HyClone Laboratories, Inc, Logan, UT) containing 20% fetal bovine serum (Gibco Laboratories, Grand Island, NY), antibiotics (100 U/ml penicillin, 100 mg/L streptomycin) according to methods described previously [12,13] and propagated in monolayer cultures seeded on glass coverslips. Cells were determined to be endothelial in origin by the detection of a typical cobblestone appearance under the inverted microscope. This method was validated in an earlier study by von Willebrand factor detection[14].

1.2 Exposure to hypoxia

PAEC monolayers of 70%~80% confluence on glass coverslips were gently put into flasks and were then exposed to a mixed gas of 5% O_2, 5% CO_2, 90% N_2 in the incubator (NUARE,USA) for 24 h.

The partial pressure of oxygen (PO_2) in the medium measured using a blood-gas analyzer (AVL, Switzerland) was (38±5) mmHg after exposure to hypoxia and rapidly increased to normoxic values (150 mmHg) within 10 min.

1.3 Intracellular free Ca^{2+} measurement

To measure $[Ca^{2+}]_i$, PAEC monolayers on glass coverslips were incubated with culture medium containing 2 μmol/L Fura-2 (acetoxymethyl ester form; Calbiochem) in 37℃, 95% air-5% CO_2 atmosphere for 40 min. The coverslips were then gently washed 3 times with an indicator-free Hepes buffered saline (HBS) composed of (in mmol/L) NaCl 140, KCl 4.5, $CaCl_2$ 1.5, $MgSO_4$ 1.0, D-glucose 10, and HEPES 21, pH 7.40, at room temperature to allow deesterification of the indicator. Fura-2 loaded monolayers were first exposed to HBS or HBS with the presence of 10 μmol/L diphenylene iodonium chloride (DPI, Sigma, USA) or 100 μmol/L oxypurinol (Sigma, USA) for at least 5 minutes' equilibration before histamine was added to a final concentration of 1 μmol/L. Fura-2 fluorescence was recorded in a field of 1~7 connected cells of a subconfluent PAEC monolayer on a coverslip in a perfusion chamber mounted on the stage of an inverted epifluorescence microscope (Olympus, I×70, Japan). Fura-2 fluorescence was alternatively excited at 340 nm and 380 nm using a polychrome (photonics, Germany) corresponding to the Ca^{2+}-bound and -free forms of the indicator, respectively. Emitted fluorescence through bandpass interference filters (photonics, Germany) with selected wavelength bands at 510 nm was captured by a computer coupled device (CCD, Imago-QE, photonics, Germany) and transferred to Till-vision software (photonics, Germany). Autofluorescence from unloaded PAECs was subtracted from Fura-2 fluorescence recordings before the calculation of the ratio of the emitted fluorescence intensity excited at 340 nm and 380 nm respectively (F340/F380).

Because the dissociation constant (K_d) of fura-2 for Ca^{2+} in pig artery endothelial cells may be different from that obtained *in vitro* [15] and of the general uncertainties of the calibration techniques of $[Ca^{2+}]_i$ measurement [16], the absolute amount of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was not calculated and therefore F340/F380 was used as a relative indicator of $[Ca^{2+}]_i$ in this study. This will not affect the analysis of calcium oscillation frequency.

1.4 Data analysis and statistics

All data are expressed as ($\bar{x}\pm s$); ANOVA analysis was used to evaluate the significance of differences, with $P<0.05$ as significant.

2 Results

2.1 24 h-hypoxia increased histamine-stimulated calcium oscillation frequency in PAECs

In all monolayers studied (n=6 respectively. Note that n number means cell monolayers on separate coverslips. In each monolayer, calcium was measured from 1~7 cells. An average was first calculated from the 1~7 cells in one monolayer as 1 mean. 6 means from 6 cell monolayers were used to perform a statistical analysis. When n=6, the mean represents an average from 6 separate cell monolayers composed of 18 ~ 40 cells. The n number had the same significance throughout this work.), 1 μmol/L histamine induced repetitive calcium oscillations both in normoxic and posthypoxic PAECs. The averaged frequency of calcium oscillations in posthypoxic PAECs was significantly higher than that of normoxic ones. [(7.598±1.069) vs. (5.365±0.867) mHz respectively,

P<0.01, n=6] (Figure 1 and Figure 4)

2.2 DPI abolished histamine-stimulated calcium oscillations both in normoxic and posthypoxic PAECs

In all monolayers studied (n=6 respectively), 1 μmol/L histamine only induced a single spike and no repetitive oscillations in HBS with 10 μmol/L DPI both in normoxic and posthypoxic PAECs (Figure 2).

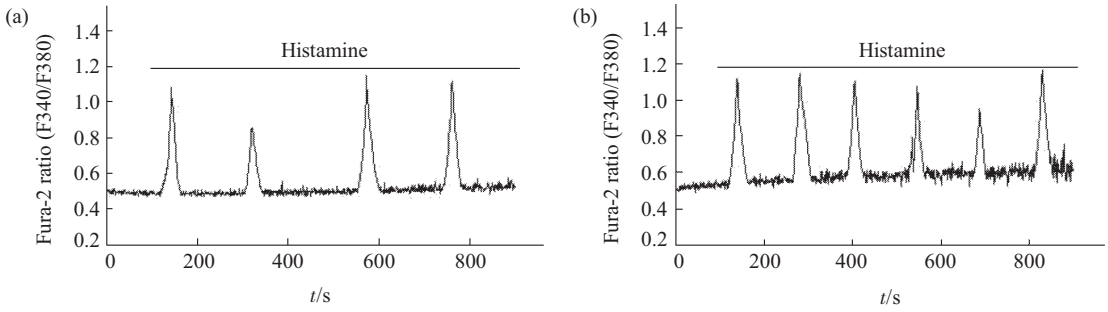

Fig.1 Hypoxia increased histamine-stimulated calcium oscillation frequency in pulmonary artery endothelial cells

(a) Representative Fura-2 fluorescence from a normoxic PAEC monolayer exposed to 1 μmol/L histamine in HBS. Repetitive $[Ca^{2+}]_i$ oscillations were observed in all monolayers studied (n=6). The averaged $[Ca^{2+}]_i$ oscillation frequency was (5.365 ±0.867) mHz. (b) Representative Fura-2 fluorescence from a posthypoxic PAEC monolayer exposed to 1 μmol/L histamine in HBS. Repetitive $[Ca^{2+}]_i$ oscillations were observed in all monolayers studied (n=6). The averaged $[Ca^{2+}]_i$ oscillation frequency was (7.598±1.069) mHz.

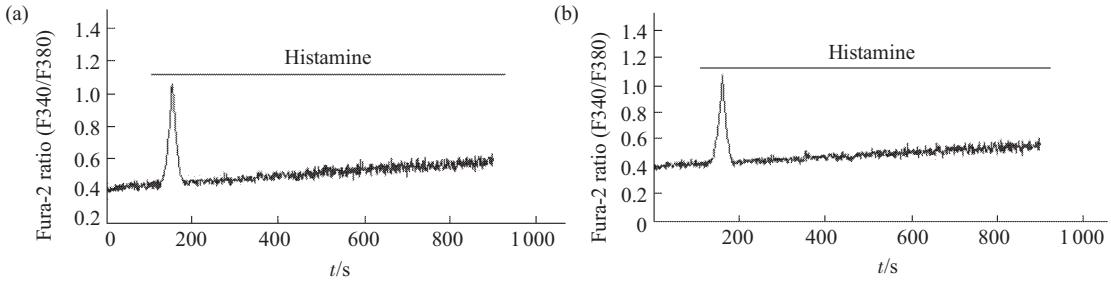

Fig.2 Effect of NADPH oxidase inhibitor, 10 μmol/L DPI, on histamine-induced calcium oscillation in normoxic and posthypoxic PAECs

(a) Representative tracing of six similar experiments from Fura-2-loaded normoxic PAEC stimulated by 1 μmol/L histamine in HBS with 10 μmol/L DPI. Only a single $[Ca^{2+}]_i$ spike and no oscillations were observed.(b) Representative tracing of six similar experiments from Fura-2-loaded posthypoxic PAEC stimulated by 1 μmol/L histamine in HBS with 10 μmol/L DPI. Only a single $[Ca^{2+}]_i$ spike and no oscillations were observed.

2.3 Oxypurinol decreased the elevated calcium oscillation frequency in posthypoxic PAECs

In normoxic PAECs, 1 μmol/L histamine induced repetitive calcium oscillations in HBS with 100 μmol/L oxypurinol, the frequency of which had no significant difference with that in HBS without oxypurinol [(5.145 ±0.81) vs. (5.365 ±0.867) mHz, P>0.05, n=6]. In posthypoxic PAECs, 1 μmol/L histamine induced repetitive calcium oscillations in HBS with 100 μmol/L oxypurinol, the frequency of which was significantly higher than that of normoxic PAECs [(6.523±0.906) vs. (5.145±0.81) mHz, P<0.05, n=6], but was significantly lower than that of posthypoxic PAECs in HBS without oxypurinol. [(6.523±0.906) vs. (7.598±1.069) mHz, P<0.05, n=6] (Figure 3 and Figure 4).

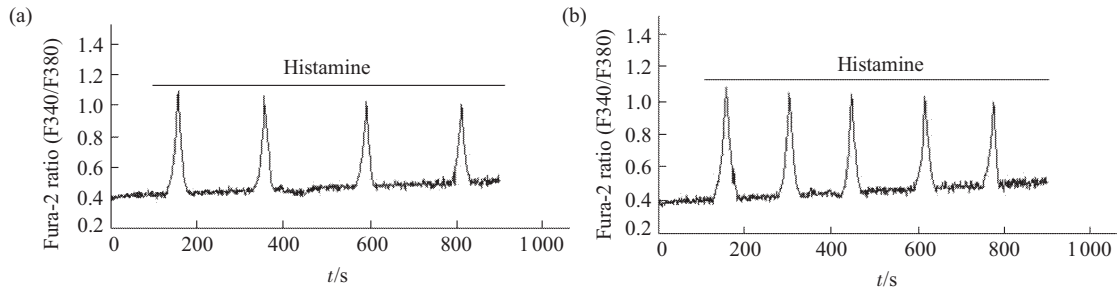

Fig.3 Effect of xathine oxidase inhibitor, 100 μmol/L oxypurinol, on histamine-induced calcium oscillation in normoxic and posthypoxic PAECs

(a) Representative Fura-2 fluorescence from a normoxic PAEC monolayer stimulated by 1 μmol/L histamine in HBS with 100 μmol/L oxypurinol. Repetitive $[Ca^{2+}]_i$ oscillations were observed in all monolayers studied (n=6). The averaged $[Ca^{2+}]_i$ oscillation frequency was (5.145±0.81) mHz.(b) Representative Fura-2 fluorescence from a posthypoxic PAEC monolayer stimulated by 1 μmol/L histamine in HBS with 100 μmol/L oxypurinol. Repetitive $[Ca^{2+}]_i$ oscillations were observed in all monolayers studied (n=6). The averaged $[Ca^{2+}]_i$ oscillation frequency was (6.523±0.906) mHz.

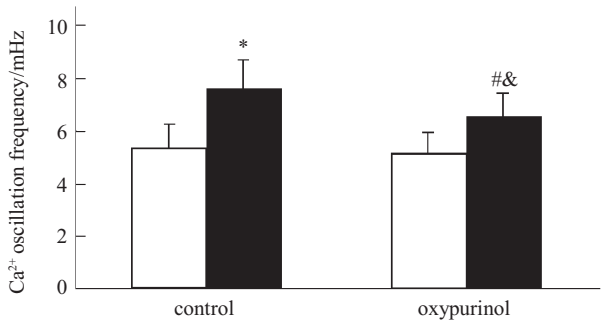

Fig.4 Comparison of the calcium oscillation frequency between different cell groups

Control: The averaged frequency of 1 μmol/L histamine-stimulated calcium oscillation in posthypoxic PAECs was significantly higher than that of normoxic ones.[*,(7.598±1.069) vs. (5.365±0.867) mHz, P<0.01, n=6]. Oxypurinol: In normoxic PAECs, the frequency of calcium oscillations induced by 1 μmol/L histamine in HBS with 100 μmol/L oxypurinol had no significant difference with that in HBS without oxypurinol [(5.145±0.81) vs. (5.365±0.867) mHz, P>0.05, n=6]. In posthypoxic PAECs, the frequency of calcium oscillations induced by 1 μmol/L histamine in HBS with 100 μmol/L oxypurinol was significantly higher than that of normoxic PAECs [#, (6.523±0.906) vs. (5.145±0.81) mHz, P<0.05, n=6], but was significantly lower than that of posthypoxic PAECs in HBS without oxypurinol [&, (6.523±0.906) vs. (7.598±1.069) mHz, P<0.05, n=6]. □: normoxic, ■: hypoxic.

3 Discussion

24h-hypoxia of P_{O_2} below (38±5) mmHg was considered as a kind of subacute mild hypoxia as previously described[17,18].

In this study, histamine of physiological concentration induced repetitive calcium oscillations in PAECs. Subacute mild hypoxia increased the histamine-stimulated calcium oscillation frequency. As gene expression may be regulated by calcium oscillation frequency as previously studied [8~11], the increased calcium oscillation frequency in posthypoxic PAECs may imply an elevated level of gene expression after persistent exposure to hypoxia. These results demonstrated that, during pulmonary disease related to persistent hypoxia, PAECs became more sensitive to histamine.

NADPH oxidase and xanthine oxidase were two important enzymes involved in reactive oxygen species (ROS) generation in endothelial cells[19]. ROS plays a pivotal role in amplifying calcium signaling [20~22]. 10 μmol/L DPI[23~26] and 100 μmol/L oxypurinol[26~28] had been shown to effectively inhibit the activity of NADPH oxidase and xanthine oxidase respectively. In this study, when NADPH oxidase was inhibited, PAECs failed to generate repetitive calcium oscillations, which implies a critical role of NADPH oxidase in generating calcium oscillations in PAECs during histamine stimulation. This was in accordance with the observation in human aortic endothelial cells. As ROS may increase the sensitivity of calcium release through IP_3R from endoplastic reticulum, ROS generated from NADPH oxidase pathway plays an important role in maintaining repetitive calcium oscillations[29]. Xanthine oxidase inhibitor, 100 μmol/L oxypurinol, did not abolish the repetitive calcium oscillations induced by histamine in PAECs, however, it decreased the elevated calcium oscillation frequency in posthypoxic PAECs. This implies that xanthine

oxidase may not be involved in generating calcium oscillations in normoxic PAECs during histamine stimulation, but it may be contribute to, at least in part, the elevation of calcium oscillation frequency caused by hypoxia. This maybe explained by the shortage of xanthine oxidase in normoxic PAECs, but it was largely formed from xanthine dehydrogenase after exposure to hypoxia, which had been observed in human umbilical vein endothelial cells[30]. As for why the ROS generated from NADPH oxidase seems to play a more important role in generating calcium oscillations than that generated from xanthine oxidase, we speculated that, in this case, the former was more abundant than the latter.

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References

1 Satoh H, Inui J. Endothelial cell-dependent relaxation and contraction induced by histamine in the isolated guinea-pig pulmonary artery. Eur J Pharmacol, 1984, **97** (3-4): 321~324

2 Ryan U S, Avdonin P V, Posin E Y, *et al*. Influence of vasoactive agents on cytoplasmic free calcium in vascular endothelial cells. J Appl Physiol, 1988, **65** (5): 2221-2227

3 Hallam T J, Jacob R, Merritt J E. Evidence that agonists stimulate bivalent-cation influx into human endothelial cells. Biochem J, 1988, **255** (1): 79~184

4 Li H, Burkhardt C, Heinrich U R, *et al*. Histamine upregulates gene expression of endothelial nitric oxide synthase in human vascular endothelial cells. Circulation, 2003, **107** (18): 2348~2354

5 Ackerman V, Carpi S, Bellini A, *et al*. Constitutive expression of endothelin in bronchial epithelial cells of patients with symptomatic and asymptomatic asthma and modulation by histamine and interleukin-1. J Allergy Clin Immunol, 1995, **96** (5 Pt 1): 618~627

6 Norrby K. Mast cells and angiogenesis. APMIS, 2002, **110** (5): 355~371

7 Tian B, Brasier A R. Identification of a nuclear factor kappa B-dependent gene network. Recent Prog Horm Res, 2003, **58**: 95~130

8 Dolmetsch R E, Xu K, Lewis R S. Calcium oscillations increase the efficiency and specificity of gene expression. Nature, 1998, **392** (6679): 933~936

9 Li W, Llopis J, Whitney M, *et al*. Cell-permeant caged InsP3 ester shows that Ca^{2+} spike frequency can optimize gene expression. Nature, 1998, **392** (6679): 936~941

10 Meldolesi J. Calcium signaling. Oscillation, activation, expression. Nature, 1998, **392** (6679): 863, 865~866

11 Hu Q, Deshpande S, Irani K, *et al*. $[Ca^{2+}]_i$ oscillation frequency regulates agonist-stimulated NF-kappaB transcriptional activity. J Biol Chem, 1999, **274** (48): 33995~33998

12 陆德琴, 李会革, 叶仕桥, 等. 肺动脉内皮细胞缺氧时内皮型一氧化氮合酶基因表达的变化及蛋白激酶 C 亚型的作用. 中华医学杂志, 2004, **84** (2):146~151
Lu D Q, Li H G, Ye S Q, *et al*. National Medical Journal of China, 2004, **84** (2): 146~151

13 陆德琴, 李会革, 叶 红, 等. 组胺对肺动脉内皮细胞一氧化氮合酶基因表达的影响, 生理学报, 2004, **56** (3): 288~294
Lu D Q, Li H G, Ye H, *et al*. Acta Physiologica Sinica, 2004, **56** (3): 288~294

14 Hakim F J, Pasquier C. Reactive oxygen species rapidly increase endothelial ICAM-1 ability to bind neutrophils without detectable upregulation. Blood, 1994, **83** (9): 2669~2677

15 Konishi M, Olson A, Hollingworth S, *et al*. Myoplasmic binding of fura-2 investigated by steady-state fluorescence and absorbance measurements. Biophys J, 1988, **54** (6): 1089~1104

16 Paltauf-Doburzynska J, Frieden M, Spitaler M, *et al*. Histamine-induced Ca^{2+} oscillations in a human endothelial cell line depend on transmembrane ion flux, ryanodine receptors and endoplasmic reticulum Ca^{2+}-ATPase. The Journal of Physiology, 2000, **524** (3): 701~713

17 Hong Z, Weir E K, Nelson D P, *et al*. Subacute hypoxia decreases voltage-activated potassium channel expression and function in pulmonary artery myocytes. Am J Respir Cell Mol Biol, 2004, **31** (3): 337~343

18 Sohn H Y, Krotz F, Gloe T, *et al*. Differential regulation of xanthine and NAD(P)H oxidase by hypoxia in human umbilical vein endothelial cells: Role of nitric oxide and adenosine. Cardiovasc Res, 2003, **58** (3): 638~646

19 Li J M, Shah A M. Endothelial cell superoxide generation: regulation and relevance for cardiovascular pathophysiology. Am J Physiol Regul Integr Comp Physiol, 2004, **287** (5): R1014~1030

20 Brookes P S, Yoon Y, Robotham J L, *et al*. Calcium, ATP, and ROS: a mitochondrial love-hate triangle. Am J Physiol Cell Physiol, 2004, **287** (4): C817~833

21 Ward J P, Snetkov V A, Aaronson P I. Calcium, mitochondria and oxygen sensing in the pulmonary circulation. Cell Calcium, 2004, **36** (3~4): 209~220

22 Weir E K, Hong Z, Porter V A, *et al*. Redox signaling in oxygen sensing by vessels. Respir Physiol Neurobiol, 2002, **132** (1): 121~130

23 Reistad T, Mariussen E, Fonnum F. The effect of a brominated flame retardant, tetrabromobisphenol-A, on free radical formation in human neutrophil granulocytes: The Involvement of the MAPKinase pathway and protein kinase C. Toxicol Sci, 2005, **83**(1): 89~100

24 Park S Y, Song C Y, Kim B C, *et al*. Angiotensin Ⅱ mediates LDL-induced superoxide generation in mesangial cells. Am J Physiol Renal Physiol, 2003, **285** (5): F909~915

25 Zhang X, Zhang L, Dong F, *et al*. Hydrogen peroxide is involved in abscisic acid-induced stomatal closure in *Vicia faba*. Plant Physiol, 2001, **126** (4): 1438~1448

26 Hu Q, Ziegelstein R C. Hypoxia/reoxygenation stimulates intracellular calcium oscillations in human aortic endothelial cells. Circulation, 2000, **102** (20): 2541~2547

27 Rieger J M, Shah A R, Gidday J M. Ischemia-reperfusion injury of retinal endothelium by cyclooxygenase- and xanthine oxidase-derived superoxide. Exp Eye Res, 2002, **74** (4): 493~501

28 Arai T, Mori H, Ishii H, *et al*. Oxypurinol, a xanthine oxidase inhibitor and a superoxide scavenger, did not attenuate ischemic neuronal damage in gerbils. Life Sci, 1998, **63** (7): PL 107~112

29 Hu Q, Yu Z X, Ferrans V J, *et al*. Critical role of NADPH oxidase-derived reactive oxygen species in generating Ca^{2+} oscillations in human aortic endothelial cells stimulated by histamine. J Biol Chem, 2002, **277** (36): 32546~32551

30 Sohn H Y, Krotz F, Gloe T, *et al*. Differential regulation of xanthine and NAD(P)H oxidase by hypoxia in human umbilical vein endothelial cells. Role of nitric oxide and adenosine. Cardiovasc Res, 2003, **58** (3): 638~646

亚急性轻度缺氧增加组胺刺激的肺动脉内皮细胞钙振荡频率 *

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摘要 钙振荡 (calcium oscillation) 能以频率解码的形式调节基因转录，钙振荡的频率可反应基因转录的水平. 为探索持续缺氧是强化还是钝化肺动脉内皮细胞对组胺的反应，研究了 24 h 亚急性轻度缺氧对组胺刺激的肺动脉内皮细胞钙振荡频率的影响，并探索了其机制. 结果是：a. 24 h 亚急性轻度缺氧可显著增加组胺刺激的肺动脉内皮细胞钙振荡频率；b.NADPH 氧化酶抑制剂，diphenylene iodonium chloride (DPI，10 μmol/L) 消除了组胺刺激的常氧和缺氧后肺动脉内皮细胞钙振荡；c. 黄嘌呤氧化酶抑制剂，别嘌呤醇 (oxypurinol，100 μmol/L) 能显著降低组胺刺激的缺氧后肺动脉内皮细胞升高的钙振荡频率，但降低后的钙振荡频率仍高于常氧组，别嘌呤醇对组胺刺激的常氧组肺动脉内皮细胞钙振荡频率无显著影响. 以上结果表明，在持续缺氧相关的肺疾患中，肺动脉内皮细胞对组胺反应的敏感性增加. NADPH 氧化酶在组胺刺激的钙振荡的发生中发挥重要作用；黄嘌呤氧化酶的激活是缺氧引起组胺刺激的钙振荡频率增加的重要原因.

关键词 钙振荡，组胺，缺氧，肺动脉，NADPH 氧化酶，黄嘌呤氧化酶

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