· Original Article in English ·

Gene expressions and roles of matrix metalloproteinases-8 and tissue inhibitor of metalloproteinases-1 in hyperoxia-induced pulmonary fibrosis in neonatal rats

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Abstract: **Objective** Extracellular matrix (ECM) deposition is a major reason of pulmonary fibrosis in hyperoxia-induced lung injury. However, the relevant mechanism has not been identified. This study examined the gene expressions of matrix metalloproteinases-8 (MMP-8, a catabolic enzyme of type Ⅰ collagen) and tissue inhibitor of metalloproteinases-1 (TIMP-1) in neonatal rats with hyperoxia-induced pulmonary injury in order to explore the role of MMP-8 and TIMP-1 in pulmonary fibrosis. **Methods** Eighty term newborn rats were randomly exposed to hyperxia (FiO_2 = 0.90, hyperxia group) and to room air (FiO_2 = 0.21, control group) (n = 40 each). Lung injury was induced by hyperxia exposure. The content of type I collagen and the expressions of type I collagen protein and MMP-1 mRNA and TIMP-1 mRNA were assayed with enzyme linked immunoadsorbent (ELISA), immunohistochemistry and reverse transcription polymerase chain reaction (RT-PCR) respectively on days 1, 3, 7, 14 and 21 after exposure. **Results** The content of type I collagen and the expression of type I collagen protein in the hyperxia group were statistically higher than those in the control group at 14 and 21 days post-exposure. The MMP-8 mRNA expression decreased while the TIMP-1 mRNA expression increased significantly in the hyperxia group as compared to the control group at 14 and 21 days post-exposure. **Conclusions** Hyperxia exposure down-regulates MMP-8 mRNA expression and up-regulates TIMP-1 mRNA expression. This results in a reduction of ECM degradation, thereby ECM deposition occurs in lung tissue, which may be an important mechanism of pulmonary fibrosis following hyperoxia-induced lung injury. [**Chin J Contemp Pediatr**, **2007**, **9** (**1**):**1 - 5**]

Key words: Matrix metalloproteinases-8; Tissue inhibitor of metalloproteinases-1; Pulmonary fibrosis; Neonatal rats

基质金属蛋白酶-8 及其组织抑制因子-1 在高氧致新生鼠肺纤维化中的基因表达及其意义

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[**摘　要**] **目的**　近年来研究发现细胞外基质(ECM)的过度沉积与肺纤维化发生密切相关,而 ECM 合成与降解在新生儿慢性肺疾病的肺纤维化发生、发展中作用如何尚不清楚。本文着重研究基质金属蛋白酶-8(MMP-8,Ⅰ型胶原的降解酶)及其组织抑制因子-1(TIMP-1)基因在高氧致新生鼠肺损伤中的表达,并探讨其在纤维化中的作用。**方法**　80 只足月新生大鼠依吸氧浓度(FiO_2)随机分为高氧组(FiO_2 = 0.90)和对照组(FiO_2 = 0.21),每组均为 40 只。采用高浓度氧诱导新生鼠肺损伤模型,应用酶联免疫吸附法(ELISA)、免疫组织化学及反转录聚合酶链反应(RT-PCR)技术,动态研究 MMP-8 及 TIMP-1 mRNA 表达,并同时观察肺组织Ⅰ型胶原蛋白的表达强度及其含量变化。**结果**　实验后 14 d 和 21 d 的高氧组肺组织中,Ⅰ型胶原蛋白表达和Ⅰ型胶原水平均高于对照组,差异有显著性意义。而实验后 14 d 和 21 d,高氧组肺组织 MMP-8 mRNA 表达降低,TIMP-1 mRNA 表达增高,与对照组比较差异有显著性意义。**结论**　高氧通过下调 MMP-8 及上调 TIMP-1 基因表达,导致 ECM 降解减少,过量 ECM 沉积于肺组织,这可能是高氧致肺纤维化的机制之一。[**中国当代儿科杂志,2007,9(1):1 -5**]

[**关　键　词**]　基质金属蛋白酶-8;基质金属蛋白酶组织抑制因子-1;肺纤维化;新生鼠

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Oxygen therapy is the most frequently used in newborn infants with heart and lung diseases. However, prolonged inhalation of high concentration oxygen may induce lung injury. In some serious cases, it will lead to chronic lung disease (CLD) because of pulmonary interstitial fibrosis and the patients must depend on oxygen or repeated mechanical ventilations for several months or even several years. Due to the level of pulmonary fibrosis closely correlates to the prognosis of hyperoxia-induced lung injury, studying the mechanism of pulmonary fibrosis becomes an important topic. Recent research has shown that extracellular matrix (ECM) deposition in lung tissue is a main reason of bleomycin-induced rat pulmonary fibrosis and adult idiopathic pulmonary interstitial fibrosis. Previous studies by the authors also indicate that ECM deposition is a major reason for pulmonary fibrosis in hyperoxia-induced lung injury. However, the precise mechanism has not been fully identified [1,2]. In this study, a model of hyperoxia-induced lung injury of neonatal rats was prepared. The gene expressions of matrix metalloproteinases-8 (MMP-8, a catabolic enzyme of type Ⅰ collagen) and tissue inhibitor of metalloproteinases-1 (TIMP-1), and the type I collagen content and its protein expression in lung tissue were detected so as to study the effects of synthesis and degradation of ECM on pulmonary fibrosis in CLD.

Subjects and methods

The preparation of lung injury model and animal grouping

Eighty newborn rats of both sexes which were spontaneously delivered at gestation of 22-23 days (provided by the Department of Experimental Animal, China Medical University) were randomly exposed to hyperxia (FiO_2 = 0.90, hyperxia group) and to room air (FiO_2 = 0.21, control group) (n = 40 each). The average weights of the hyperxia and control groups were 6.13 ± 0.12 and 6.19 ± 0.14 g respectively. A lung injury model was induced according to the published literature[1,3]. Neonatal rats together with their foster mothers were put into an oxygen chamber immediately after birth. Oxygen was continuously supplied at a FiO_2 of 0.90 and CO_2 concentration < 0.5%. The chamber temperature was maintained at between 25-27℃, and relative humidity between 50%-70%. The rats' foster mothers were exchanged with the control group in order to avoid the decrease of feeding ability caused by oxygen intoxication.

Collection of specimens

At each time interval of 1, 3, 7, 14 and 21 days after experiment, eight rats of both groups were randomly chosen and were sacrificed. The left lung was taken out and fixed in 4% paraformaldehyde for the detection of collagen protein Ⅰ expression. The right lung was removed and frozen in a refrigerator (-80℃) and kept for the detection of the expressions of MMP-8 and TIMP-1 mRNA and the content of type Ⅰ collagen.

Detection of the type I collagen content

Samples were assayed for the content of type Ⅰ collagen in lung tissue by enzyme linked immunosorbent assay (ELISA) in accordance with the manufacturer's instructions (Shanghai Sengxiong Biological Product Company). The lung tissue was washed with cooled normal saline to remove the residual blood. One gram of wet lung tissue was taken out and cut into small pieces. Ten percent tissue homogenate was prepared after ultrasonication. The supernatants were collected after centrifugation at a low temperature. The result was presented as the content of type I collagen per milligram lung tissue.

Detection of collagen protein

The expression of type Ⅰ collagen protein in lung tissue was detected with the method of SABC immunohistochemistry. The rabbit-anti-rat type Ⅰ collagen protein antibody (1∶100) was used as the first antibody (provide by Wuhan Boshide Biological Product Company). After SABC stainting the cells that had brown particles in cytoplasm were defined as positive ones. The expression intensity of type Ⅰ collagen protein was evaluated by the USA Universal Imaging Porporation image analysis system and was expressed with average gray scale value. The lower average gray scale value, the higher the positive expression, which indicated a higher protein content.

Detection of MMP-8 mRNA and TIMP-1 mRNA expressions

The MMP-8 mRNA and TIMP-1 mRNA expressions in lung tissue were detected by the RT-PCR technique. The total RNA was extracted using TRIZOL. cDNA was synthesized by reverse transcription and was then amplified by PCR primer sequences. The conditions of PCR are shown in Table 1. The amplification products were fractionated by 2% agarose electrophoresis, stained with ethidium bromide, and visualized under ultraviolet light. The absorbance ratio between MMP-8 or TIMP-1 and β-actin amplification products represented the relative MMP-8 or TIMP-1 mRNA levels.

Table 1　Primer sequences and conditions of PCR

Primers	Application fragments(bp)	Conditions
MMP-8 upstream: 5′-AGT GCC CGA CTC TGG TGA TTT-3′ downstream: 5′-GTT GAT GTC TGC TTC TCC CTC-3′	202	94℃ 2 min→94℃ 45 s→52.3℃ 1 min→72℃ 1 min (35 circles) →72℃ 7 min
TIMP-1 upstream: 5′-CTT CCA CAG GTC CCA CAA-3′ downstream: 5′-CAG CCC TGG CTC CCG AGG-3′	285	94℃ 3 min→94℃ 30 s→63℃ 1 min→72℃ 1.5 min (35 circles) → 72℃ 7 min
β-actin(internal reference) upstream: 5′-GAT TGC CTC AGG ACA TTT CTG-3′ downstream: 5′-GAT TGC TCA GGA CAT TTC TG-3′	690	94℃ 1 min→55℃ 1 min→72℃ 2 min→(35 circles) → 72℃ 7 min

Results

The type I collagen protein expression and type I collagen content

There were no significant differences in the type I collagen protein expression and type I collagen content in lung tissue between the hyperxia and the control groups within 7 days after exposure. The content of type I collagen and the expression of type I collagen protein in lung tissue in the hyperxia group were statistically higher that those in the control group at 14 and 21 days post-exposure. See Table 2 and Figure 1.

Table 2　Type I collagen protein expression and type I collagen content　($n=8$, $\bar{x}\pm s$)

Group	Type I collagen protein					Type I collagen (ng/mg)				
	1 d	3 d	7 d	14 d	21 d	1 d	3 d	7 d	14 d	21 d
Control	80.0±1.2	80.2±1.5	78.2±9.1	82.2±3.2	82.0±2.4	406.7±20.3	491.3±15.2	501.5±18.2	496.0±11.0	511.9±49.6
Hyperxia	78.9±1.1	79.9±2.1	78.2±3.4	73.8±4.7[a]	71.9±5.0[a]	422.1±49.5	470.9±29.4	528.1±43.4	556.6±40.0[b]	874.9±81.3[a]

Compared with the control group, a $P<0.01$, b $P<0.05$.

Table 3　MMP-8 mRNA and TIMP-1 mRNA expressions　($n=8$, $\bar{x}\pm s$)

Group	MMP-8 mRNA expression					TIMP-1 mRNA expression				
	1 d	3 d	7 d	14 d	21 d	1 d	3 d	7 d	14 d	21 d
Control	1.3±0.1	1.3±0.1	1.3±0.2	1.3±0.2	1.2±0.2	1.1±0.5	1.1±0.2	1.0±0.3	0.9±0.2	1.0±0.1
Hyperxia	1.3±0.1	1.2±0.2	1.3±0.2	1.0±0.2[a]	0.8±0.3[a]	1.1±0.3	1.0±0.1	1.0±0.2	1.3±0.3[a]	1.3±0.3[a]

Compared with the control group ,a $P<0.05$.

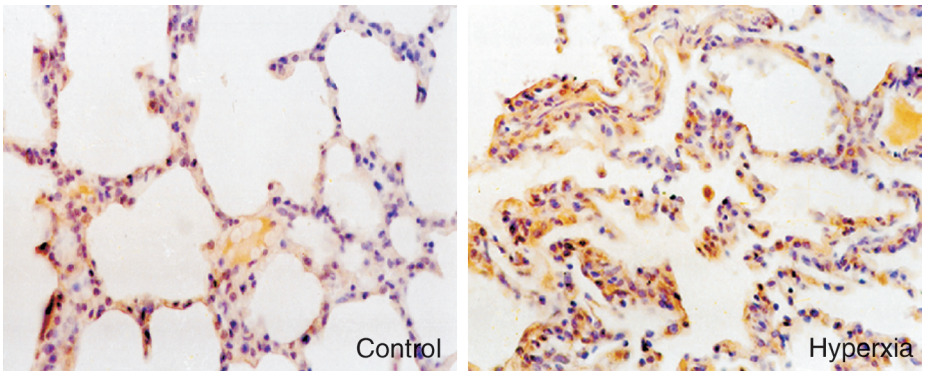

Figure 1　The expression of type I collagen protein at 21 days post-exposure (× 400). Type I collagen protein was weakly expressed in few lung interstitial cells and endochylema was stained as light yellow in the control group. The type I collagen protein in lung tissue in the hyperxia group increased significantly, mainly expressing in fibroblasts of lung interstitium, and endochylema was stained as brown.

The expressions of MMP-8 mRNA and TIMP mRNA

There were no significant differences in the expressions of MMP-8 mRNA and TIMP mRNA in lung tissue between the hyperxia and the control groups within 7 days after exposure. The MMP-8 mRNA expression decreased while the TIMP-1 mRNA expression increased

significantly in the hyperxia group when comparing with the control group at 14 and 21 days post-exposure. See Table 3 and Figures 3 and 4.

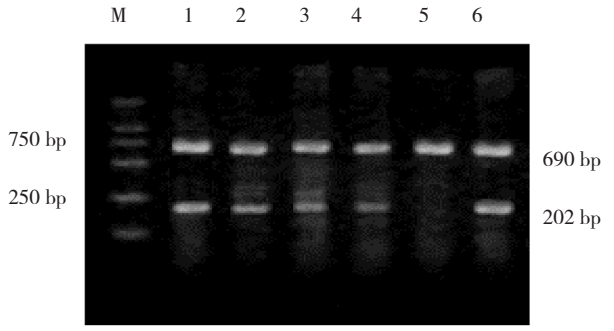

Figure 3 MMP-8 mRNA amplification products of RT-PCR. (M: DNA Marker DL2000; 1-5: 1, 3, 7, 14 and 21 days after hyperxia exposure; 6: control group)

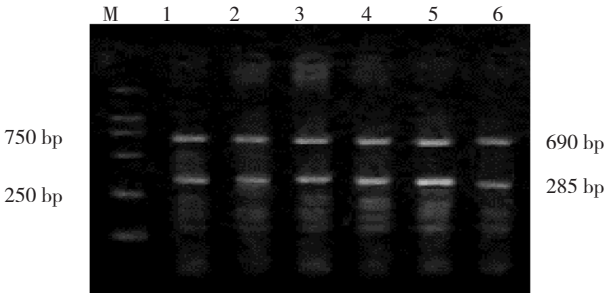

Figure 4 TIMP-1 mRNA amplification products of RT-PCR. (M: DNA Marker DL2000; 1-5: 1, 3, 7, 14 and 21 days after hyperxia exposure; 6: control group)

Discussion

Although the etiology of CLD is complex and the pathogenesis is not clear, the pathological process and pathological changes of this disorder have nearly been identified, i. e, early alveoli pulmonis inflammatory reaction and late pulmonary interstitial fibrosis cause gas exchange function disturbance of alveoli pulmonis, ultimately resulting in respiratory failure. Some seriously affected patients will die within only one year and the survivors must depend on oxygen or repeated mechanical ventilations for a very long time. The level of pulmonary fibrosis closely correlates to the prognosis of CLD patients. It is thus important to understand the mechanism of pulmonary fibrosis following hyperoxia-induced lung injury in neonates.

ECM is a living macromolecule that has complex functions and constructions. It exists among the cells and is composed by collagen, glycosidoprotein and proteoglycans. Adequate ECM not only has influences on the differentiation of the lung cell and on normal array of micrangiums, but also is necessary for the reconstitution of endepidermis structure after lung injury. Much research has shown that collagen deposition in lungs, especially type I collagen protein deposition, is the most important element of pulmonary interstitial fibrosis. This study showed that the expression intensity of type I collagen protein and the content of type I collagen increased obviously in the newborn rats with hyperoxia-induced lung injury at 14 days post-exposure. Furthermore both of them increased with the exposure time. Previous research has proved that the level of type I collagen is coincident with the level of lung fibrosis [1]. The result of this study suggested that excessive deposition of type I collagen in lung tissue is an important reason for pulmonary fibrosis following lung injury in neonatal rats. However, the relevant mechanism remains unclear. Some researchers reported that pulmonary fibrosis might associate with an increased synthesis of ECM induced by over-expression of transforming growth factor-β_1 and protein [2]. Besides the increased synthesis of ECM, whether the reduction of ECM degradation associates with pulmonary fibrosis following lung injury has not identified.

In recent years, many researchers have focused on the roles of MMPs and TIMPs (inhibitor of MMPs) in the degradation of ECM. MMPs is a family of calcium and zincum ion dependent proteolytic enzyme that ECM is taken as a substrate. It has been found that the MMPs family has more than twenty members, including collagenase such as MMP-8, MMP-1, types I and III collagen, gelatinase such as MMP-9, MMP-2, type IV collagen, base material hydrolyase and membrane-type MMPs. TIMPs (TIMP-1 - TIMP-4) are specific tissue inhibitors of MMPs. Their biological effects mainly occur in the activation stage of MMPs enzyme precursor. They can form a steady complex with pro-MMPs and block the activation of enzyme precursor. They can also form MMP-TTMP complex with active MMPs at 1∶1 rate and block the combination with their substrates. So, the disturbance of TIMPs and MMPs expressions can lead to degradation abnormities of ECM [4].

Chang [5] reported that the expressions of MMP-2 and MMP-9 obviously increased in lung tissue of pre-

mature rats exposed to hyperoxia at early stage and gradually increased with prolonged exposure time. Sweet [6] also reported that the level of MMP-9 in bronchoalveolar lavage fluid (BALF) of premature infants whom CLD was developed was significantly higher than those whom CLD was not developed. Because the hydrolysis substrate of MMP-2 and MMP-9 is type Ⅳ collagen which is the essential component of alveolar basement membrane of epithelium, it is believed that the increased levels of MMP-2 and MMP-9 at early stage of disease play an important role in acute inflammation stage of hyperxia-induced lung injury by degrading the type Ⅳ collagen to damage the basement membrane [7]. It is known that type Ⅰ collagen is a main component of lung fibrous tissue and that MMP-8 is a catabolic enzyme of type Ⅰ collagen. However the role of MMP-8 in pulmonary fibrosis following hyperxia-induced lung injury remains unknown. This study examined the changes of gene expressions of MMP-8 and its inhibitor TIMP-1 in hyperxia-induced lung injury, and found that the level of MMP-8 mRNA decreased, in contrast, the level of TIMP-1 mRNA increased at 14 and 21 days after hyperxia exposure while type Ⅰ collagen deposition in lung tissue was observed following hyperxia-induced lung injury. So, it is believed that hyperxia exposure down-regulates the MMP-8 mRNA expression and up-regulates the TIMP-1 mRNA expression, resulting in a disequilibrium between MMP-8 and TIMP-1 mRNA expressions at the gene transcription level. This leads to a reduction of ECM degradation, thereby ECM deposition occurs in lung tissue, which may be an important mechanism of pulmonary fibrosis following hyperoxia-induced lung injury.

[References]

[1] Fu JH , Xue XD. Dynamic changes of extracellular matrix in newborn rats with hyperoxia-induced pulmonary injury (in Chinese) [J]. Chin J Perinat Med, 2005,8(3):202-203.

[2] Fu JH, Xue XD. Transforming growth factor-β1 and chronic lung disease of the prematurity (in Chinese) [J]. Chin J Pediatr, 2003, 41(4):313-315.

[3] Qian LL, Chang LW, Zhang QS, Rong ZH. Lung injury of preterm rats induced by prolonged exposure to high oxygen concentration of 85% (in Chinese) [J]. Chin J Contemp Peditr, 2003,5(2):95-98.

[4] Nelson AR, Fingleton B, Rothenberg ML, Matrisian LM. Matrix metalloproteinases: biologic activity and clinical implications[J]. J Clin Oncol, 2000,18(10):1135-1149.

[5] Chang LM, Qian LL, Rong ZH, Zhang QS. Pathogentic role of matrix metalloproteinase and its tissue inhibitors in preterm rat lungs exposed to hyperoxia[J]. Acta Pharmacol Sin, 2002, 23(1):59-63.

[6] Sweet DG, Pizzoti J, Wilbourn M, Chesshyre E, Halliday HL. Matrix metalloproteinase-9 in the airways of infant at risk of developing chronic lung disease[J]. Eur Respir J, 1999, 14(2):248-252.

[7] Buckley S, Driscoll B, Shi W, Anderson K, Warburton D. Migration and gelatinases in cultured fetal, adult, and hyperoxia alveolar epithelial cells[J]. Am J Physiol Lung Cell Mol Physiol, 2001, 281(2):L427-L434.

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