·Original Article in English·

Effects of Tumor Necrosis Factor-α and IκBα on Pulmonary Hemorrhage of Neonatal Rats Induced by Lipopolysaccharide

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Abstract: Objective To investigate the effects of tumor necrosis factor-α (TNF-α) and inhibitor protein of nuclear factor-κB (IκBα) on pulmonary hemorrhage (PH) of neonatal rats induced by lipopolysaccharide (LPS). **Methods** Seven to ten days old Wistar rats were randomly separated into 2 groups: LPS group, the rats of which were injected with LPS intraperitoneally at the dosage of 5 mg/kg, and they were sacrificed 30 min, 1, 2, 4, 8, 16 and 24 hs after injection of LPS, respectively; normal saline (NS) group, where equal amount of NS was injected intraperitoneally. The number of each time point was 6. The lungs of the rats were examined by eyes and microscope. The protein and mRNA of TNF-α in the lung tissues were detected by the method of ELISA and reverse transcription polymerase chain reaction. The expression of IκBα protein was measured by Western Blot. **Results** One hour after administration of LPS, patch-like bleeding was observed in more than two lobes of the lung. From the 1st h after injection of LPS, TNF-α protein increased and was significantly higher than that of NS control group ($P < 0.01$), it peaked at the 2nd h. TNF-α mRNA increased 30 min after injection of LPS and was significantly higher than NS control ($P < 0.01$) and the peak was reached 2 hs after injection of LPS. The expression of IκBα quickly weakened from the 1st h after injection of LPS ($P < 0.05$), it was the lowest at the 8th h. **Conclusions** LPS may lead to lung hemorrhage in the neonatal rats, which may be associated with the decrease of IκBα protein in the lung tissue and activation of NF-κB, up-regulation of the transcription of TNF-α gene.

[Chin J Contemp Pediatr, 2003, 5(4): 301 - 305]

Key words: Tumor necrosis factor-α; Inhibitor protein of nuclear factor-κB; Lipopolysaccharide; Pulmonary hemorrhage; Neonatal rat

TNF-α 和 IκBα 在内毒素致新生大鼠肺出血中的作用研究

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[**摘 要**] **目的** 探讨肿瘤坏死因子-α(TNF-α)和核因子-κB 抑制蛋白-α(IκBα)在内毒素脂多糖(LPS)致新生大鼠肺出血发生机制中的作用。**方法** 将 48 只 7～10 日龄 Wistar 大鼠分为 LPS 组和生理盐水(NS)组。LPS 组腹腔注射 LPS 5 mg/kg,按 LPS 注射后观察的时间分为 30 min,1 h,2 h,4 h,8 h,16 h 和 24 h 组,每组 6 只;NS 组腹腔注射等量生理盐水作为对照。对鼠肺进行大体和光镜观察,分别采用酶联免疫吸附(ELISA)、反转录聚合酶链反应(RT-PCR)和 Western Blot 杂交法动态观察肺组织 TNF-α 蛋白和 mRNA 表达及 IκBα 表达的变化。**结果** LPS 注射后 1 h 可引起新生大鼠明显的肺出血及炎性改变。肺组织 TNF-α 蛋白含量从 LPS 注射后 1 h 增加,2 h 达高峰;TNF-α mRNA 的表达从 LPS 注射后 0.5 h 明显增强($P < 0.01$),2 h 达高峰;而 IκBα 于 LPS 注射后 1 h 起表达很快减弱,明显低于 NS 组($P < 0.05$),8 h 表达最弱($P < 0.01$)。**结论** LPS 可致新生大鼠肺出血。新生大鼠肺出血可能与肺组织 IκBα 蛋白表达减弱,导致 NF-κB 活化、TNF-α 基因转录上调及蛋白合成增加有关。

[**中国当代儿科杂志,2003,5(4):301 - 305**]

[**关 键 词**] 肿瘤坏死因子-α;核因子-κB 抑制蛋白;脂多糖;肺出血;新生大鼠

[Received] October, 25, 2002; [Revised] February 20, 2003

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[中图分类号] R-33;R563.6 [文献标识码] A [文章编号] 1008-8830(2003)04-0301-05

Pulmonary hemorrhage (PH) is a serious complication causing acute respiratory distress in the newborns and it is associated with high mortality and morbidity. The most common causes of PH include severe infection, asphyxia or hypoxia, cold injury or hypothermia, premature birth and low birth weight[1]. The pathogenesis is not clearly known yet. It has been previously reported that inflammatory injury in the lung was associated with PH of newborn[2]. Recently, Baier[3] reported that concentrations of interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) in tracheal aspirates increased significantly in premature infants with PH. Cytokine is the mediator in the inflammatory reaction. Tumor necrosis factor (TNF) is one of the important inflammatory cytokines, which stimulates the production of proinflammation factors and mediums. Nuclear factor-kappaB (NF-κB) plays an important role in the transcription of cytokine. The activation of NF-κB is mediated by an upstream kinase that regulates the phosphorylation and subsequent degradation of inhibitory-kappaB (IκB)-alpha, which is the major cytosolic inhibitor of NF-κB[4]. In the present study, we investigated the changes of tumor necrosis factor-alpha (TNF-α) and IκBα after intraperitoneal administration of bacterial endotoxin lipopolysaccharide (LPS) in neonatal rats to explore the pathogenesis of PH of the newborns and offer new clues to treat the disease.

Materials and methods

Animal models

Seven to ten days old Wistar rats (provided by the Experinental Animal Center of the Second Affiliated Hospital) were divided into 2 groups randomly. The rats in the LPS group were injected with Escherichia coli LPS (LPS) intraperitoneally at the dosage of 5 mg/kg and they were sacrificed 30 min, 1, 2, 4, 8, 16 and 24 h after injection of LPS respectively. The number of each time point was 6. The rats in the normal saline (NS) group were injected with equal amount of NS intraperitoneally (n = 6).

Lung histopathology

The rats were anesthetized with 5 % soluble pentobarbitone at the dosage of 20 mg/kg. Then they were killed and the lower lobe of the right lung was removed and fixed in 4 % formaldehydum polymerisatum. After the fixed tissue was dehydrated, it was embedded in wax and sectioned. Stained with HE, it was then observed under the microscope. The other part of lung tissues was put in the RNase-free Eppendorf tubes. After freezing in liquid nitrogen rapidly, it was stored in a freezer at -80 ℃.

Detection of the TNF-α level in rat lungs

The lung was homogenized in 1 ml of ice-colded PBS and then centrifuged at 1 500 rpm/min for 10 min at 4 ℃. The supernatant was collected and stored at -20 ℃. TNF-α in lung homogenates was detected by a sandwich ELISA method (kit from R&D Systems).

Expression of TNF-α mRNA in lungs

The expression of TNF-α mRNA in lungs was determined by RT-PCR. Total cellular RNA was extracted by TRIZOL reagent (Promega, USA), and the concentration of total RNA was determined by spectrophotometry. The RNA was reversely transcribed using oligo (dT) 12-18 primers (TaKaRa). The cDNA was amplified by PCR according to protocols. The primers of TNF-α were 5'TAC TGA CAT TCG GGG TGA TTG GTC C3' and 5'CAG CCT TGT CCC TTG AAG AGA ACC3' (Aoke Biotechnology, Beijing). The reversely-transcribed cDNA (3 μl) was added into the 25 μl mixtures of TNF-α primer and Taq DNA polymerase (TaKaRa). The PCR was carried out for 3 min at 94 ℃, for 30 s at 94 ℃, for 45 s at 64 ℃, for 1.5 min at 72 ℃. After 30 cycles, it was reacted at 72 ℃ for 7 min. The PCR products were identified by electrophoresis on a 2 % agarose gel containing ethidium bromide. The results of electrophoresis was observed under UV light and analysed by an automated gel-imaging system (Kodak-1D). The amount of TNF-α mRNA was expressed as the ratio of TNF-α absorption value to that of β-actin.

Expression of IκBα protein in lungs

The IκBα protein in lungs was determined by Western Blot method. The lung tissues were finely minced and homogenized in 10 volumes of ice-colded

protein-extracting buffer. The homogenate was centrifuged at 36 000 g/min at 4 ℃ for 1.5 h, then the supernatant was collected. The protein concentration was determined by the improved Lowry method. Equal amount of protein (40 μg/lane) was loaded and separated on 15 % SDS-polyacrylamide slab gel under denatured conditions. Broad range protein molecular weight marker was used as reference. Proteins were electroblotted to nitrocellulose membrane. After incubation in blocking solution [5 % milk in Tris buffered saline with Tween 20 (TBST)] at room temperature for 1 h, the monoclonal antibody of IκBα (Santa Cruz, CA) was added and they were reacted at 4 ℃ overnight. The second antibody alkaline phosphatase-conjugated goat anti-rabbit IgG was added the next day. The results of Western Blot was analysed by an automated gel-imaging system (GIS-700D).

Statistical analysis

All data were expressed as the mean ± standard deviation ($\bar{x} \pm SD$). Dunnet *t* test was used to analyze the difference of muti-means.

Results

The pathological changes in the lung

Fifteen minutes after administration of LPS, the rats manifested with tachypnea, cyanosis, few movement and drowsiness of various degree; 30 min after injection of LPS, bleeding was seen in the lung of neonatal rats; 1 h after injection of LPS, the lung swelled, with patch-like bleeding in more than two lobes which spread out from the hilus of the lung. See Figures 1 and 2. Two hours after injection of LPS, the diffuse bleeding was seen in every lobe of the lung. See Figure 3.

One hour after injection of LPS, it was observed that alveoli became narrowed, the capillaries of alveoli were dilated and that there was a lot of red blood cells in the lung interstitial tissue and alveoli. See Figure 4. All these became severe with time. Twenty-four hs after injection of LPS, interstitial tissue of the lung became widened, with a lot of endothelial, erythrocytes and neutrophils in alveoli. See Figure 5.

The changes of TNF-α protein

From the 1st h after injection of LPS, the TNF-α protein in the lung of neonatal rats increased and was significantly higher than that of NS group ($P < 0.01$), and peaked at the 2nd h. Then it decreased gradually, but was higher than that of NS group till the 8th h ($P < 0.01$). See Table 1.

The changes of TNF-α mRNA in the lung of neonatal rats

The expression of TNF-α mRNA in NS group was weak. Thirty minutes after injection of LPS, TNF-α mRNA level increased and was higher than that of NS group ($P < 0.01$). It peaked at the 2nd h after injection of LPS, then decreased gradually, but still remained at a relatively high level and until 24 h it was significantly higher than that of NS control ($P < 0.01$). See Figure 6 and Table 1.

The changes of IκBα in the lung

It was shown that the expression of IκBα was obvious in the lung tissue of NS group. From the 1st h after injection of LPS, the expression of IκBα quickly weakened, and was significantly lower than that of NS group ($P < 0.05$). It was the lowest at the 8th h ($P < 0.01$). Afterwards, there was an increase, but until the 24th h, it was still significantly lower than that of NS group ($P < 0.05$). See Figure 7 and Table 1 (All figures were on the Cover Ⅱ).

Table 1 The changes of TNF-α protein, TNF-α mRNA and IκBα in the lung of the neonatal rats ($n = 6$, $\bar{x} \pm s$)

Groups	TNF-α protein (pg/mg)	TNF-α mRNA	IκBα
NS	8.56 ± 4.76	0.28 ± 0.08	182.62 ± 6.80
LPS 0.5 h	9.06 ± 4.22	0.95 ± 0.08[b]	175.38 ± 7.66
1 h	29.40 ± 12.20[b]	1.07 ± 0.15[b]	171.53 ± 7.60[a]
2 h	148.33 ± 62.75[b]	1.23 ± 0.22[b]	168.88 ± 9.84[a]
4 h	116.37 ± 44.36[b]	1.07 ± 0.08[b]	163.63 ± 11.40[a]
8 h	36.32 ± 9.42[b]	0.96 ± 0.09[b]	162.29 ± 9.53[b]
16 h		0.99 ± 0.14[b]	164.95 ± 7.23[b]
24 h		0.93 ± 0.14[b]	170.37 ± 7.44[a]

Note: a vs NS group $P < 0.05$; b vs NS group $P < 0.01$

Discussion

Severe infection and septicemia are the most common causes of PH in newborns. According to Tillema's method of acute lung injury models of adult animal[6], the neonatal rats were injected with LPS (5 mg/kg). We found that 1 h after injection of LPS, the lungs of neonatal rats swelled and bled. Under the optic microscope, it was observed that the the alveoli became narrowed and the alveolar capillaries were dilated, with polymorphonuclear leukocytes and erythrocytes in the lung interstitial tissue and alveoli. All these became severe with time. Two hours after injection of LPS, diffuse bleeding was seen in each lobe of the lung, but no bleeding was seen in other organs. These changes were corresponded with the pathologic changes of neonatal PH[7].

TNF is a key role of cytokine net. It can activate leucocyte, endothelial cells and initiate inflammatory reaction by autocrine or paracrine. TNF has fierce toxicity to lungs. It can make neutrophils adhere vascular endothelial cells and induce apoptosis; it can activate neutrophils to release elastase, collagenase, myeloperoxidase, active oxygen and other inflammatory medium; it can activate extrinsic coagulation to result in microthrombus in the lung and even disseminated intravascular coagulation; it can also increase the permeability of capillary of alveoli. Several studies have found that TNF-α in bronchoalveolar lavage (BAL) or serum and TNF-α mRNA in lungs of adult rats increased after administration of LPS either intravenously or intraperitonealy or by inhaling, which was associated with the development of lung injury. And the inhibition of TNF-α release could attenuate pulmonary edema and vascular endothelial cells injury[8]. In this study, we found that TNF-α protein and TNF-α mRNA in the lung of the neonatal rats increased after the administration of LPS and that the rise of TNF-α mRNA was earlier than that of TNF-α protein. In addition, the increase of TNF-α mRNA and protein was earlier than the peak of inflammatory response and lung injury. Our findings suggest that TNF-α, a proinflammatory cytokine, may play an important role in initiating inflammatory response of the neonatal rats with PH and that the increase of TNF-α in the lungs occured at the transcription level.

NF-κB is one of the transcription factors which bind to the promoter region of proinflammatory cytokine genes and stimulates their transcription. In most cells, NF-κB is sequstered in the cytoplasm by interaction with IκB family, including IκBα. Once stimulated by LPS, IκBα is phosphorylated and degraded. So, free NF-κB dimers translocate into nucleus and bind with the κB site of cytokine genes[9,10]. Therefore, the activation of NF-κB is closely associated with phosphorylation and degradation of IκB. It has been shown that the serine protease inhibitors can inhibit the activation of NF-κB by inhibiting degradation of IκB, and protect macrophages from LPS-mediated injury[11]. Liu found that[12] pyrrolidine dithiocarbamate can prevent activation of NF-κB and inhibited the expression of the cytokine-inducible neutrophil chemoattractant (CINC), intercellular adhesion molecule-1 (ICAM-1) by inhibiting degradation of IκB. In the present study, we found that the expression of IκB in the lung of neonatal rats quickly weakened and the decrease of IκB was associated with the increase of TNF-α mRNA and protein. It indicated that degradation of IκB-α may be a key role of PH.

In summary, LPS can make IκB degrade and initiate the transcription of TNF-α, which promotes adhesiveness and activation of PMN, thus the membranes of alveolar capillary are injured and PH is the result. It indicates that inflammatory reaction mediated by NF-κB may be an important reason of neonatal PH. So inhibition of phosphorylation, degradation of IκB-α and production of TNF-α or antagonize its biological activity may be an important strategy for the treatment of PH of the newborns.

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(Edited by Yan YU)

·消息·

《中国医学文摘·儿科学》2003 年论文集征文通知

《中国医学文摘·儿科学》系中华人民共和国卫生部主管的国家级科技检索期刊,国内外公开发行(刊号:CN21 - 1160/R,邮发代号:6 - 182,大 16 开本)。是一部集权威性、学术性、指导性为一体,以注重现代儿科研究、临床应用为主,兼顾中西医、药结合的临床研究。融实用性、规范性、可读性于一炉的优秀儿科论著。现为解决基层儿科中西医、药医护人员(相关学科)"发表学术论文难"、参加学术会议的长途劳顾、经费负担过重之苦衷,提供推广交流儿科领域医护人员的治疗、护理及中西医、药结合临床应用、研究成果传递、交叉学科的研究成果的快捷载体。应临床儿科医、护、药、教、研人员的迫切要求,经报请卫生部及主管出版部门批准,本社决定编辑出版《中国医学文摘·儿科学》2003 年论文集,特向广大医务人员征文。

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4. 中、西医结合临床儿科的研究进展及发展趋势的探讨;

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1. 来稿应文字简练,内容翔实,观点明确。请用原稿纸工整书写或打印,2 000～3 000 字为宜。中、西药名称请使用《中华人民共和国药典》(2000 年版)的规范名称。现代医学名词术语,应以中国科学院审定的各学科名词为准。使用规范的简化汉字,统计表请使用医学统计学的三线表。

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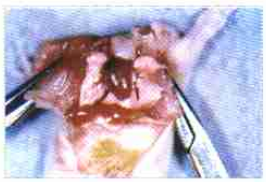
《中国医学文摘·儿科学》杂志社

2003 年 6 月 15 日

Effects of Tumor Necrosis Factor- α and I κ B α on Pulmonary Hemorrhage of Neonatal Rats Induced by Lipopolysaccharide

（正文见第 301 页）



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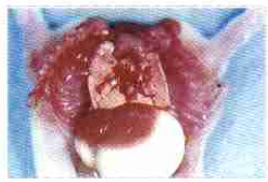

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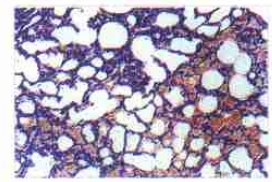

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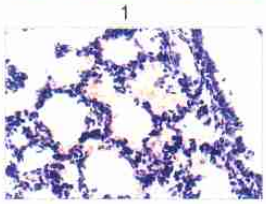

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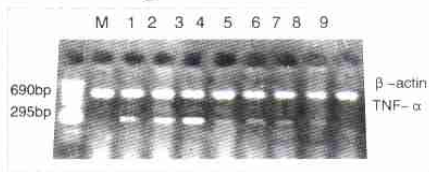

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Figure 1 The lung of normal neonatal rat

Figure 2 The lung of neonatal rats 1 h after administration of LPS

Patch-like bleeding which spread out from the hilus of the lung was observed in more than two lobes

Figure 3 The lung of neonatal rats 2 hs after administration of LPS

The diffuse bleeding was seen in every lobe in the lung

Figure 4 The lung of neonatal rats 1 h after administration of LPS

A lot of red blood cells were seen in the lung interstitial tissue and alveolar cavity (HE × 200)

Figure 5 The lung of neonatal rats 24 hs after administration of LPS

A lot of red blood cells and PMN were seen in the lung interstitial tissue and alveolar cavity (HE × 400)

Figure 6 The expression of TNF-α in the lungs of NS group and LPS groups at various time points

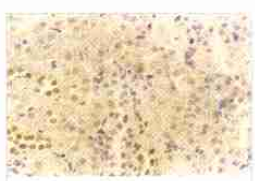
From left to right: M: Marker; 1. NS; 2. 0.5 h; 3. 1 h; 4. 2 h; 5. 4 h; 6. 8 h; 7. 16 h; 8. 24 h; 9. Vacuity

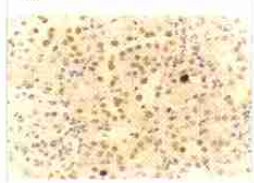
Figure 7 The expression of I κ B α in the lungs of NS group and LPS groups at various time points

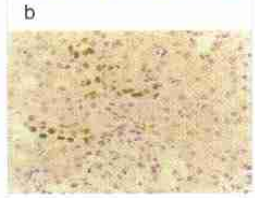
From left to right: 1. NS; 2. 0.5 h; 3. 1 h; 4. 2 h; 5. 4 h; 6. 8 h; 7. 16 h; 8. 24 h

Effects of Valsartan on Apoptosis and Expression of Fas and EasL in the Kidney of Rats with Nephrotic Glomerulosclerosis Induced by Adriamycin

（正文见第 306 页）







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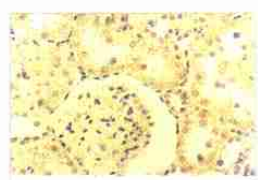

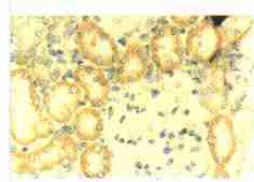

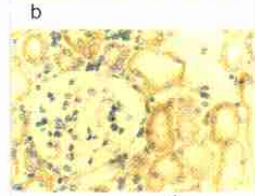

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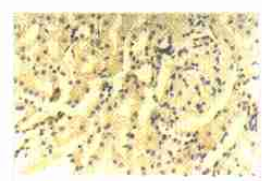

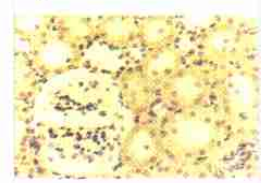

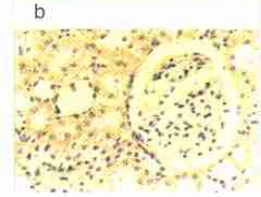

3

Figure 1 Results of TUNEL (× 400)

Compared with control group, the number of apoptotic cells in model group increased significantly, and the number of apoptotic cells in treatment group was significantly lower than that in model group.

Figure 2 Immunohistochemical results of Fas (× 400)

Within the tubular and glomerular regions, expression of Fas in model group was higher than that in control group, and the expression of Fas in treatment group was weaker than that in model group.

Figure 3 Immunohistochemical results of FasL (× 400)

Within renal cortex, the expression of FasL in model group was higher than that in control group, and the expression of FasL in treatment group was weaker than that in model group.