行政院國家科學委員會補助專題研究計畫成果報告

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※一氧化氮合成酶抑制劑對大鼠經內毒素誘發急性肺損※
※傷後其嗾中性白血球和第二型肺泡細胞凋亡現象之影※
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本成果報告包括以下應繳交之附件：

□赴國外出差或研習心得報告一份
□赴大陸地區出差或研習心得報告一份
□出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

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一、中文摘要

關鍵詞：急性肺損傷，嗜中性白血球，第二型肺泡細胞一氧化氮合成酶抑制劑，細胞凋亡

急性肺損傷是臨床上常見的病症，它是因為肺臟暴露於有害的外在和內在物質，而造成氣體交換功能的快速喪失。急性肺損傷的病程先是因為發炎反應使得大量的發炎細胞浸潤於肺泡壁，進而造成組織的破壞，微血管、纖維細胞等結締組織細胞的增生。同時為了修復受傷的肺泡壁，急性肺損傷後第 1 天至 32 天可發現第二型肺泡細胞的增生。

急性發炎反應的消退有賴於發炎細胞(嗜中性白血球)的適時消失，同時在肺臟組織修復(重塑)期增生的第二型肺泡細胞，也要消失以恢復正常的肺泡壁構造。造成嗜中性白血球和第二型肺泡細胞消失的詳細機轉並不是很清楚。在多細胞生物體，經由內在和外在的訊息，可產生細胞凋亡的現象，此現象在組織重塑時扮演重要的角色。經由細胞凋亡的現象，對於增生過多的細胞、發育不正常或有遺傳障礙的細胞可加以去除。我們先前的實驗也證明在修復期時增生的第二型肺泡細胞也是經由細胞凋亡的作用來去除，由此可知，細胞凋亡現象可能是在急性肺損傷後，用以消除過多的發炎細胞和為了修復而增生的細胞的一種普遍機轉。

一氧化氮是一種短暫存在的物質，它可被不同的細胞激素和脂多醣(細菌內毒素)所刺激產生。一氧化氮是由一氧化氮合成酶所製造。一氧化氮在正常和疾病的肺上扮演重要的角色。急性肺損傷後，誘發發炎反應的細胞激素如第一型，第六型介白質和腫瘤壞死因子都有明顯增加，而導致誘發型合成酶在肺泡巨噬細胞，內皮細胞，肺泡上皮細胞(第二型肺泡細胞)大量製造，進而使一氧化氮的合成增加。一氧化氮的增加和細胞內一氧化氮合成酶的增加是同步的。一氧化氮和細胞凋亡有密切的關係，Jassen 發現去除一氧化氮後可增加肺臟細胞的凋亡現象；Blaylock 證明一氧化氮會減少嗜中性白血球的凋亡現象。因此我們打算利用一氧化氮合成酶抑制劑，作用於急性肺損傷的大鼠，觀察急性期之嗜中性白血球和恢復期之第二型肺泡細胞之細胞凋亡的現象，以了解經由抑制一氧化氮的產生而調控細胞凋亡，對於肺損傷後發炎反應的消退和修復作用的影響。

大鼠經由氣管注入大腸桿菌內毒素(4mg/kg)後，誘發急性肺損傷，肺組織在 24 小時後其肺損傷指數和對照組分別為 2.38±0.56 和 1.11±0.44。若大鼠再同時注射一氧化氮合成酶抑制劑 SMT(12.5mg/kg)則肺損傷指數更增加至 2.68±0.18。此現象在肺乾溼重比上也可顯現：即對照組，內毒素，內毒素加 SMT 其肺乾溼重比分別為 0.0037±0.00042，0.0055±0.0007，0.0066±0.00071。血清中一氧化氮代謝物 NOx 分別為 2.65±1.32μM，22.78±12.44μM，5.32±3.15μM。肺組織細胞凋亡染色顯示內毒素誘發肺損傷後嗜中性白血球和第二型肺泡細胞細胞凋亡的比率增加，在同時使用 SMT 的老鼠細胞凋亡的比率更高。經由本實驗，可以證明使用一氧化氮合成酶抑制劑(SMT)可以加重急性肺損傷的嚴重度，間接證明一氧化氮在內毒素誘發肺損傷的大鼠動物模型中具有保護的作用，同時 SMT 也會促進嗜中性白血球和第二型肺泡細胞的凋亡作用。

二、 英文摘要

Keywords：Acute lung injury, pneumocyte, apoptosis, neutrophil

Acute lung injury (ALI) results in a rapid disruption of the gas exchange apparatus upon exposure to noxious environmental or endogenous agents. The disease process begins with an explosive inflammatory response in the alveolar wall. This inflammation may cause tissue destruction, extensive cellular reaction, and fibroproliferation of alveolar septa, which consist of capillaries, fibroblasts, and their connective-tissue product. The progression from injury to acute inflammatory response is characterized by an influx of polymorphonuclear neutrophilic leukocytes (PMN). The resolution of acute inflammation depends on the timely clearance of these inflammatory cells. Accompanying the inflammation is a reparative hyperplasia of type II pneumocytes that can be detected from day 1 until day 32. In survivors after ALI, there is an apparent resolution of the granulation tissue in concert with repopulation of the air-lung interface by type II pneumocytes. The cellular mechanisms accounting for the resolution of PMN and type II cell hyperplasia have yet to be elucidated. In multi-cellular organisms, programmed cell death (apoptosis), which can be triggered by a variety of intrinsic and extrinsic signals, is important during tissue remodeling. This process allows for the elimination of cells that have been produced in excess, that have developed improperly, or that have sustained genetic damage.

In our previous study we found that type II pneumocyte proliferation developed after ALI. Extensive apoptosis of type II pneumocytes was detected, and is the main cellular mechanism that accounts for the disappearance of these cells. The local or paracrine factors modulate PMN or type II pneumocyte apoptosis are not clear. Nitric oxide (NO), a transient free radical species, produced by a variety of cell types, can induced diverse target cell response. NO is a mediator of vasodilatation and bronchodilatation and is synthesis from L-arginine by the enzyme, NO synthase (NOS). NO can exert both beneficial and detrimental effects. In this study, we plan to evaluate the effect of NOS inhibitor on PMN and type II pneumocyte apoptosis during the development and resolution of LPS-induced ALI in the rat.

Sprague-Dawley Rats were intratracheal instillation of lipopolysaccharide (4mg/Kg) and received the NOS inhibitor, S-methyl isothiourea (SMT) (12.5 mg/Kg). Inducible NOS (iNOS) induction was assessed by immunohistochemical staining. Measurement of NO metabolite in plasma (nitrite/nitrate, NOx) was performed by chemiluminescence analysis. Lung injury was scored by histology. The effects of NOS inhibitor was observed. LPS treatment strongly induced iNOS in inflammatory cells, macrophages, bronchial epithelial cells & type II pneumocytes. NOx concentration in plasma was elevated 24 h after LPS instillation (22.78±12.44 Vs. 2.654±1.32 □M). SMT reduces NOx concentrations in plasma of LPS treated rats (5.32±3.15 Vs. 22.78± 12.44 □M). Rats treated with LPS show apparent lung injury (2.38± 0.56 Vs. 1.11± 0.44), SMT treatment aggravated lung injury (2.69± 0.18 Vs. 2.38± 0.56). In situ apoptotic assay showed apoptosis of neutrophil and type II pneumocyte increased after LPS induced ALI, administration of SMT further increased the apoptosis of both cells. This study shows that SMT aggravated lung damage induced by LPS. It suggested that iNOS play a major role in modulating LPS induced lung injury and increases the apoptosis of neutrophil and type II pneumocyte. NO has a protective effect in acute lung injury.

Acute lung injury (ALI) is a clinical syndrome that involves rapid disruption of the gas exchange apparatus when the lungs are exposed to noxious environmental or endogenous agents. The disease process begins with an explosive inflammatory response in the alveolar wall. This inflammation may cause tissue destruction, extensive cellular reaction, and fibroproliferation of alveolar septa, which consist of capillaries, fibroblasts, and their connective-tissue product (1). The progression from injury to acute inflammatory response is characterized by an influx of polymorphonuclear neutrophilic leukocytes (PMN). The resolution of acute inflammation depends on the timely clearance of these inflammatory cells. Accompanying the inflammation is a reparative hyperplasia of type II pneumocytes that can be detected from day 1 until day 32 (2). In patients who die from ALI, this airspace granulation persists (3). In survivors after ALI, there is an apparent resolution of the granulation tissue in concert with repopulation of the air-lung interface by type II pneumocytes. The repair is complete when the alveolar epithelium resumes its physiologic conformation, in which type I cells cover 95 percent of the air-lung interface and are present in numbers equal to those of type II cells. The cellular mechanisms accounting for the resolution of PMN and type II cell hyperplasia have yet to be elucidated.

In multi-cellular organisms, programmed cell death (apoptosis), which can be triggered by a variety of intrinsic and extrinsic signals, is important during tissue remodeling (2). This process allows for the elimination of cells that have been produced in excess, that have developed improperly, or that have sustained genetic damage (4). Apoptotic cell death can be distinguished from necrotic cell death by the following unique processes: controlled autodigestion of cells, activation of endogenous protease, cytoskeletal disruption, cell shrinkage and membrane blebbing, nuclear condensation, DNA fragmentation (oligonucleosome formation), and elimination of the dying cell without induction of an inflammatory response (4). The latter process permits orderly cell removal without disruption of adjacent tissue. A hallmark feature of apoptosis is based on the observation that nuclear DNA extracted from apoptotic cells is often degraded in an internucleosomal pattern caused by cytoplasmic proteases signaling endonuclease activation. This DNA fragmentation was observed by agarose gel electrophoresis, which demonstrated a ladder pattern at 200-base-pair intervals (5). Recently, an in situ apoptosis (DNA fragmentation) assay (ISAA) that permits the visualization of apoptosis by conventional immunohistochemical light microscopy of paraffin-embedded tissue section was described (6). This technique is based on the specific binding of terminal deoxynucleotidyl transferase (TdT) to the 3'-OH ends of DNA, and this enzyme is used to incorporate biotinylated deoxyuridine at sites of DNA breaks. A subsequent incubation with fluorescein- or peroxidase-labeled avidin or an avidin-biotin-peroxidase complex (ABC) allows the distinct localization of cells undergoing apoptosis (7,8). This technique is more sensitive than DNA laddering in detecting cells undergoing apoptosis (9).

Cox and coworkers showed that PMN apoptosis in the lipopolysaccharide (LPS) model of ALI occurs between 6 and 72 h after injury, and they demonstrated the ingestion of apoptotic PMNs by macrophages(10). Hussain et al has demonstrated, in oleic acid model of ALI, that 1) PMN influx is followed by PMN apoptosis between 1 and 24 h, 2) onset of PMN apoptosis correlates with histologic signs of resolution between 24 and 72 h, and 3) phagocytosis of apoptotic PMNs by macrophages helps in their clearance (11). Apoptosis has also been shown to be responsible for the remodeling of certain mesenchymal cells during the repair phase

from ALI (12). In our previous study, we used a rat model of ALI to evaluate the role of apoptosis of type II pneumocytes in alveolar remodeling during the resolution phase (13). Sprague-Dawley rats had *E. coli* lipopolysaccharide (LPS) instilled transtracheally for induction of ALI. Animals were sacrificed on various days after LPS instillation. Lung specimens from all animals were examined for the presence of apoptosis in type II pneumocytes by an *in situ* apoptosis assay (ISAA), and for proliferative nuclear antigen (PCNA) and cytokeratin-18 with an immunohistochemical stain. Histologic examination by light microscopy revealed that the lungs of ALI rats showed thickened alveolar septa, intra-alveolar hemorrhage, infiltration of numerous inflammatory cells in the intra-alveolar and/or interstitial space, and hyperplasia of type II pneumocytes. The inflammatory response reached its maximum on day 3 and subsided around day 14. Type II pneumocyte proliferation, detected by PCNA staining, developed maximally around day 3 after ALI (Fig. 1A). In the ISAA, positive signals in type II pneumocytes were obvious and were distributed diffusely in the lung parenchyma from day 1 after ALI, became maximal around day 7, then declined until day 21 (Fig. 1B). DNA fragmentation analysis revealed that a DNA ladder pattern was detectable from day 3, persisted until day 10, and disappeared after day 14. Our results confirm that type II pneumocyte proliferation in response to ALI is mainly a reparative phenomenon. During the resolution phase of ALI, extensive apoptosis of type II pneumocytes is the main cellular mechanism that accounts for the disappearance of these cells. It is therefore reasonable to suggest that the timing of apoptosis and the resolution response are not related to specific injurious agent or route of delivery but are more general responses during resolution of ALI. The local or paracrine factors modulate PMN or type II pneumocyte apoptosis are not clear. Identification of these factors would lead to an understanding of the triggers that determine whether an acute inflammatory response would lead to repair or progress to chronic inflammation and fibrosis.

Nitric oxide (NO), a transient free radical species produced by a variety of cell types, can induced diverse target cell response. NO is a mediator of vasodilatation and bronchodilatation and is synthesized from L-arginine by enzyme, NO synthase (NOS). The expanding family of NOS isoforms falls into two catergories: 1) a constitutive from (cNOS), regulated by calcium and calmodulin, and 2) a cytokine-inducible form (iNOS) which is transcriptionally regulated (14,15). NO plays a critical role in many aspects of pulmonary function in health and disease. There are reports concerning the NO and its role in apoptosis: Blaylock showed NO can decrease the apoptosis of PMN (16); Janssen et al demonstrated that depletion of nitric oxide causes cell cycle alterations and increase apoptosis in pulmonary cells (17). In this study, we plan to evaluate the effect of NOS inhibitor on PMN and type II pneumocyte apoptosis during the development and resolution of LPS-induced ALI in the rats.

Sprague-Dawley Rats were intratracheal instillation of lipopolysaccharide (4mg/Kg) and received the NOS inhibitor, S-methyl isothiourea (SMT) (12.5 mg/Kg). Inducible NOS (iNOS) induction was assessed by immunohistochemical staining. Measurement of NO metabolite in plasma (nitrite/nitrate, NOx) was performed by chemiluminescence analysis. Lung injury was scored by histology. The effects of NOS inhibitor was observed. LPS treatment strongly induced iNOS in inflammatory cells, macrophages, bronchial epithelial cells & type II pneumocytes. NOx concentration in plasma was elevated 24 h after LPS instillation (22.78±12.44 Vs. 2.654±1.32 □M). SMT reduces NOx concentrations in plasma of LPS treated rats (5.32±3.15 Vs. 22.78± 12.44 □M). Rats treated with LPS show apparent lung injury

(2.38± 0.56 Vs. 1.11± 0.44), SMT treatment aggravated lung injury (2.69± 0.18 Vs. 2.38± 0.56). In situ apoptotic assay showed apoptosis of neutrophil and type II pneumocyte increased after LPS induced ALI, administration of SMT further increased the apoptosis of both cells. This study shows that SMT aggravated lung damage induced by LPS. It suggested that iNOS play a major role in modulating LPS induced lung injury and increases the apoptosis of neutrophil and type II pneumocyte. NO has a protective effect in acute lung injury.

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