計畫名稱: 利用氣喘動物模式研究Adenosine 與其受體在過敏元引發早期與晚期支氣管收縮反應中之角色
The Role of Adenosine and Its Receptors in the Pathophysiology of Antigen-Induced Early and Late Phase Bronchoconstriction in an Animal Model of Asthma

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一、　摘要

關鍵詞：腺核甘，腺核甘受體，氣喘，過敏元，早期與晚期支氣管收縮反應
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吸入性 Adenosine 可於氣喘病患產生支氣管收縮作用，一般認為其乃經由其受體促進發炎細胞釋出介質而引起支氣管收縮。此為期兩年的計劃目的在於建立一氣喘動物模式，並研究 Adenosine 及其受體在過敏元引起之早期與晚期呼吸道收縮中所扮演之角色。在過去兩年的實驗中，我們已成功地利用 OVA 誘導小鼠建立一氣喘動物模式，且可用非侵襲性的方式連續偵測呼吸道阻力之變化，能較以往更精準地界定過敏元誘導產生之早期及晚期支氣管收縮反應。AMP 支氣管激發試驗發現過敏小鼠明顯有支氣管高反應性，而控制組則不明顯。利用 HPLC 的方式我們發現在氣喘小鼠之肺泡沖洗液中 Adenosine 的濃度在早期和晚期收縮期分別為 12.2±0.79 和 14.93±1.84 μM (p = NS)，相對於控制組則為 0.52±0.08 μM. (both P < 0.05)。使用 Adenosine A2a 受體選擇性抑制劑無法減輕或抑制過敏元誘導之早期及晚期支氣管收縮。A1 受體與 A2 非選擇性抑制劑則可稍減少早期收縮反應，但無法抑制晚期收縮反應，且後者抑制早期收縮之能力較前者為強。Adenosine A3 受體抑制劑則無法抑制過敏元誘導之早期及晚期支氣管收縮。顯示 Adenosine 引發過敏小鼠支氣管收縮可能經由 A1 與 A2b 受體而引發收縮介質之釋放。我們利用即時(real-time) PCR 方法定量氣管及肺組織特異性 AdenosineA2b 受體基因於過敏元產生之早期及晚期收縮期之表現與基礎值之差異。蛋白方面之表現則利用 A2b 受體之特殊抗體進行 Western blot 定量。我們發現 A2b 受體 mRNA 表現於早期收縮反應並無增加，但於晚期支氣管收增加 3.2 倍左右。A2b 受體蛋白於早期收縮反應並無增加，但於晚期支氣管收增加。免疫組織染色方面我們發現在正常小鼠呼吸道上皮及肺組織中少有 A2b 受體表現，而在氣喘小鼠則有增加之現象。*in situ* hybridization 之分析則因技術問題尚未成功。這些結果可對 Adenosine 及其受體在氣喘的致病機轉得到進一步的確認，但 adenosine 受體抑制劑對氣喘之治療就此一動物模式之成效而言似乎並不理想。

二、　緣由與目的

Allergen provocation of allergic asthmatics characteristically leads to an early asthmatic response, or early-phase reaction (EPR) within 15–30 minutes. Approximately 60% of subjects also develop a second, late asthmatic or late-phase response (LPR) that is maximal at 6–12 hours, and may persist for up to 24 hours (Cockcroft et al, 1977). Although the EPR appears to depend largely on the release of mediators from airway mast cells, leading to bronchoconstriction and airway edema, the development of the LPR and the concomitant increases in airway reactivity are associated with an influx and activation of inflammatory cells, particularly lymphocytes and eosinophils in the bronchial mucosa (Metzger, 1987)

Adenosine is an endogenous nucleoside that modulates many physiological processes by interaction with specific cell membrane receptors. Four subtypes of

adenosine receptors have been cloned: A_1, A_{2A}, A_{2B}, and A_3. Adenosine has been implicated in the pathophysiology of asthma (Church and Holgate, 1986). Inhaled adenosine, or its precursor AMP, provokes bronchoconstriction in asthmatic patients (Cushley, 1984). The mechanism by which adenosine produces bronchoconstriction has been the focus of recent interest. Whether modulation of the adenosine receptors in allergic subjects or animals can affect allergen-induced reactions remains unclear.

The aim of this study is to investigate the role of adenosine and its receptors in the antigen-induced EPR and LPR using a murine model of asthma.

三、材料及方法:

Sensitization protocol:
BALB/c mice, 8-9 weeks of age, were sensitized on days 1 and 14 by intraperitoneal injection of 20 µg OVA (Grade V; Sigma) emulsified in 2.5 mg aluminum hydroxide in a total volume of 100 µL. Mice were challenged daily with OVA (1% in PBS) for 20 minutes via the airways for 3 days (days 28, 29, and 30), using ultrasonic nebulization (DeVilbiss). Mice were provoked with OVA (5% in PBS) (day 32) 48 hours after the last OVA challenge for 20 minutes to elicit an antigen-induced EPR (5-30 min) and LPR (3-10 hr).

Groups of animals
Group 1 (N = 12): Sensitization to PBS + airway challenge with PBS
Group 2 (N = 12) : Sensitization to OVA + airway challenge with PBS
Group 3 (N = 16): Sensitized with OVA + airway challenge with OVA
Group 4 (N = 12): Sensitizatiion with OVA + pretreated with aerosolized 5% DPCPX
        (A1 receptor antagonist) + airway challenge with OVA
Group 5 (N = 12): Sensitization with OVA + pretreated with aerosolized 3% KF17837
        (A2a-selective antagonist) + airway challenge with OVA
Group 6 (N = 12) : Sensitization with OVA + pretreated with aerosolized 3% DPSPx
        (nonselective A2 antagonist) + airway challenge with OVA

Noninvasive determination of airway responsiveness to the allergen:
Airway responsiveness was assessed using a whole-body plethysmograph (Buxco). From the box pressure signals, the enhanced pause (Penh) can be calculated (Hamelmann.1997). Animals were placed in the plethysmograph, baseline values were recorded, and then mice are exposed to nebulized PBS for 3 minutes. Animals were provoked with 5% OVA for 20 minutes, and airway responsiveness is monitored for the next 10 consecutive hours.

BAL and tissue procurement after EPR and LPR:
Using high-dose pentobarbital anesthesia, 6 mice in groups 1-3 were sacrificed after EPR to 5% OVA (10 min after the first peaking of Penh within 5-30 min). The other 5 mice were sacrificed after LPR (1 hour after the second peaking of Penh at approximately 6-7 hours after OVA). Tracheostomy was performed immediately and lung lavage (PBS 1 mL, 37°C) will be collected through a tracheal tube.

Determination of adenosine level in the BAL during EPR and LPR:
Adenosine was measured in 50 µl of the fluid using a Waters high-performance liquid chromatography (HPLC) system with a Waters ultraviolet light detector set at a wavelength of 254 nm.

A2b receptor mRNA and protein analysis: The relative amount of A2b receptor was determined using the Perkin-Elmer ABI 7700 real-time sequence detector according

to the instructor manual. The Cot values of these genes were subtracted by the Cot values of the housekeeping gene (GADPH) for semi-quantification of their expressions. <u>Western blotting</u>: Nitrocellulose membranes were probed with A2b receptor rabbit anti-mouse polyclonal antibody (All from alpha Diagnostics, USA). Membranes will then be washed. PBS supplemented with 0.1% Tween 20 and incubated with peroxidase-conjugated anti-rabbit or anti-mouse IgG (Amersham) for 1 h at 37^0C. Peroxidase activity will be developed using the enhanced chemiluminescence technique (ECL, Amersham) Radiograms bands will be scanned with a scanning densitometer.

<u>Statistical analysis:</u>

ANOVA was used to determine the levels of difference between all groups. Comparisons for all pairs were performed by Tukey-Kramer HSD test. Statistical significance was set at $P < 0.05$. Values for all measurements are expressed as the mean $\pm$ SEM.

四、結果

Firstly, we have successfully created an animal model of asthma in balb/c mice, which was characterized by AHR to both methacholine and AMP and significant eosinophilia after OVA challenge in the BAL fluid and bronchial tissues. Moreover, In our initial experiments, the LPR was not obvious in 50% of mice if the concentration of OVA used in the allergen challenge was 1%. We therefore increased the concentration of OVA to 5% and both EPR and LPR could be observed in more than 90% of sensitized mice. The levels of adenosine in the BAL fluid during EPR and LPR

Secondly, we found that inhalation of OVA caused a significant increase in the concentration of adenosine in BAL fluid in sensitized mice as compared to control groups (Gr 1 and Gr 2). This increase was slightly attenuated by pretreatment of inhaled DPCPX (A1 receptor antagonist) (Gr 4) and DPSPX (nonselective A2 antagonist) (Gr 6).

We also found that the OVA-induced EPR could be attenuated by pretreatment with aerosolized DPCPX (A1 receptor antagonist) and DPSPX (nonselective A2 antagonist), but not by pre-inhalation of KF17837 (A2a-selective antagonist) in sensitized mice. However, none of the three agents could affect the OVA-induced LPR in sensitized mice. In summary, these data suggest that the A1 and A2b adenosine receptor may be involved, at least partially, in the mechanism of allergen-induced bronchoconstriction and AHR to adenosine in sensitized mice. The A3 receptor specific agonist failed to inhibit OVA-induced EPR and LPR.

The real-time PCR showed that, in comparison to baseline expression levels, the A2b receptor mRNA did not increase during EPR but increased during LPR (3.2±0.6 folds). The protein levels correlated with the results of mRNA changes. The densitometry showed that A2b receptor protein increased 2.1± 0.7 folds during the LPR. *In situ* hibridization was not successful due to experimental problems. We could not detect the presence of A2a and A3 receptor mRNA and protein expression in the murine lung tissues.

五、討論

Our data suggest that adenosine may participate in the early and late phases of

antigen-induced bronchoconstriction. These results were compatible with a recent paper showing that adenosine potentiates the airway narrowing induced by OVA challenge and that this effect may develop through facilitation of the release of bronchoconstrictor mediators.

Treatment with antisense oligodeoxynucleotide targeting the adenosine A_1 receptor desensitized the allergic rabbits to subsequent challenge with either adenosine or allergen (Nyce, 1997). However, the evidence presented so far does not preclude the contribution of more than one adenosine receptor in asthma, or the possibility that nonadenosinergic mechanisms contribute to the antiasthmatic effects of enprofylline and other methylxanthines. According to the above results, it might be reasonable to conclude that adenosine A1 and A2a receptor antagonists might not be effective anti-inflammatory drugs in this animal model of asthma since they could not inhibit the LPR. On the other hand, expression of adenosine A3 receptor has been documented in the eosinophils, which are important in the pathogenesis of LPR of asthmatic responses. Both *in vitro* and *in vivo* studies have established that activation of A_3 receptors results in mast-cell degranulation and/or enhancement of mast degranulation in response to allergen in a variety of rodent species. However, A_3-receptor protein was not found in association with human lung mast cells [18], thus questioning its relevance in the mechanism of adenosine-induced bronchoconstriction in humans. To our knowledge, the effects of selective A3R antagonists on asthmatic responses have not been clinically evaluated. However, our data suggest that A3R antagonist may not be very effective using this model of murine model of asthma.

五、參考文獻 (References)

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