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行政院國家科學委員會專題研究計劃成果報告

全身性發炎反應症候群引起血小板及小神經膠細胞活化的機轉探討：評估高壓氧及抗氧化劑的治療效果

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一、中文摘要

全身性發炎反應症候群 (systemic inflammatory response syndrome, SIRS) 是指宿主遭受到某種病原菌入侵或因機械性、化學性的傷害而引起的初期全身性發炎反應稱之。人類全身性發炎反應可依發生的時間先後順序及病情的嚴重性可約略分為四期：第一期為如前述之SIRS, 接著產生敗血症 (sepsis)，若病情持續惡化可進一步轉變成敗血性休克 (septic shock) 及最後轉變成最具致命性的多重器官衰竭 (multiple organ dysfunction)。有許多因素會引起全身性發炎反應症候群如格蘭氏陰性菌和陽性菌以及一些化學物質如oleic acid等。其中格蘭氏陰性菌及陽性菌是引起SIRS的最主要原因。

至目前為止，一般相信格蘭氏陰性菌 (gram-negative) 及其內毒素 lipopolysaccharide (LPS) 是造成敗血症的主要原因；另外格蘭氏陽性菌 (gram-positive) 所引起的細菌感染亦能引發全身性的細菌感染與敗血性休克。引發敗血症的過程目前被認為是因許多細胞 (如白血球、血小板)被活化產生cytokines (如 IL-1β, PAF, TNFα 等)的結果；在中樞神經系統中，最明顯的反應是小神經膠細胞 (microglia) 的活化；此細胞不論在細胞型態，免疫表型或生理功能上都與單核球/巨噬細胞 (macrophage) 相似。

高壓氧氣治療 (hyperbaric oxygenation) 是指運用大於常壓的氧氣作為臨床治療之用途。高壓氧氣治療目前在臨床上正被廣泛的採用；許多證據顯示高壓氧氣可應用於治療包括減壓症、一氧化碳中毒、傷口癒合、燒燙傷及骨髓炎等臨床疾病。1987年Thom等人初步研究發現，間歇給予高壓氧氣可減低敗血性休克造成的動物死亡率，並認為高壓氧氣應可運用於敗血性休克的治療用途；但是此一臨床運用的系統性研究仍相當缺乏。同時對高壓氧氣應用於敗血性休克治療上的可行性及作用機轉亦不清楚。因此，我們在此計畫中將有系統性地研究高壓氧氣在實驗性敗血性休克老鼠的治療效果及詳細機制。

在過去幾年中，本實驗室對格蘭氏陰性菌內毒素LPS在血小板上的作用機轉已有詳加的研究；另外對格蘭氏陽性菌毒素LTA對血小板的影響，亦有初步的研究結果。因此，在本年度中，我們將把重點放在探討陽性菌毒素LTA抑制血小板凝集作用的分子機轉探討及在活體動物內的作用。

關鍵詞: 全身性發炎反應，LTA,，血小板

Abstract

SIRS (systemic inflammatory response syndrome) was developed to imply a clinical response arising from a non-specific insult and includes two or more defined variables. There is a continuum from the development of SIRS to the onset of sepsis and progression to septic shock and multiple organ dysfunctions. The SIRS caused predominantly by gram-negative and

gram-positive bacteria.

At present, it is widely believed that sepsis is caused predominantly by gram-negative organisms, and endotoxin LPS (lipopolysaccharide), a substance produced by these organisms. However, recent studies show an increasing evidence of gram-positive sources of sepsis. Lipoteichoic acid (LTA), a predominant component associated with the cell wall of gram-positive bacteria, can provoke marked stimulation of sepsis. Sepsis is believed to result from a complex mechanism involving activation of a number of cells, most notably leukocytes, platelets and mcroglia. Microglia are like macrophages, and reside in the CNS.

Hyperbaric oxygenation (HBO) involves the use of oxygen under pressure greater than that found on earth's surface at sea level. HBO has been applied as an adjunct treatment for a variety of clinical problems such as decompression sickness, carbon monoxide poisons, burn injury and wound healing. On the other hand, Thom (1987) proposed that HBO might be beneficial in septicemia by a study showing that intermittent HBO reduced the mortality in experimental polymicrobial sepsis. Unfortunately, there is lacking of systematic studies that are designed to evaluate the therapeutic values of HBO in septic shock. However, the role of HBO plays in the treatment of septic shock is not clear and warrants further investigations.

In the past few years, we have studied the inhibitory mechanisms of LPS in agonist-induced platelet aggregation. Furthermore, we also accomplished the preliminary studies of the influence of LTA on platelets. Therefore, this project will further explore the detailed mechanisms of LTA in platelet aggregation in vitro and in vivo experiments.

Key words: SIRS, LTA, platelet

二、緣由與目的

全身性發炎反應症候群 (systemic inflammatory response syndrome, SIRS)是指宿主遭受到某種病原菌入侵或因機械性、化學性的傷害而引起的初期全身性發炎反應稱之(Davies and Hagen, 1997)。人類全身性發炎反應可依發生的時間先後順序及病情的嚴重性可約略分為四期：第一期為如前述之SIRS, 接著產生敗血症(sepsis), 若病情持續惡化可進一步轉變成敗血性休克(septic shock)及最後轉變成最具致命性的多重器官衰竭 (multiple organ dysfunction)。有許多因素會引起全身性發炎反應症候群如胰臟炎(pancreatitis), 燒傷(burn)及組織創傷(trauma), 格蘭氏陰性菌和陽性菌以及一些化學物質如oleic acid等(Yasuhara and Muto, 1998)。其中以格蘭氏陰性菌及陽性菌所造成的敗血症在SIRS中所佔的比例最大，也是引起SIRS的最主要原因。細菌感染(特別是陰性菌)常引起敗血性休克(septic shock)；而臨床上的病徵常因感染的時期不同和發病過程不同而有不同的臨床病徵表現(Bone, 1991)。較常見且具高致命性的反應如引起低血壓；因微血管通透增加而引起的滲漏(microvascular leaky)，心肌失去功能 (myocardial dysfunction)，瀰漫性血管內凝血(DIC, disseminated intravascular coagulation)等；最後，常導致許多體內器官的衰竭如腎皮質壞死 (renal cortical necrosis) 而死亡 (Parrilo et al., 1990)。雖然引起敗血性休克的病理因素非常複雜，但目前已知寄主的發炎反應乃是造成休克及多重器官衰竭的主要原因 (Raij et al., 1977; Dunn, 1991)。細菌體特別是格蘭氏陰性菌 (gram-negative) 其細胞壁外層之成份LPS (lipopolysaccharide) 為其主要的毒性來源 (Dunn, 1991),會刺激寄主而引起許多發炎媒介物的產生；包括各種不同的cytokines (如腫瘤壞死因子, TNFα), prostaglandins, platelet activating factor (PAF)及 kinins等等 (Dunn, 1991)。當這些發炎物質累積到足夠量時，便會釋放進入全身循環中而影響到如心臟、血管等主要器官 (Gaynor et

al., 1970)。至於LPS是透過何種次級媒介物(secondary mediator) 而造成體內如此複雜的病理現象，則目前尚未完全清楚。若能進一步確認這些媒介物的角色及其引發的可能作用機轉，則將有助於瞭解敗血症之種種不同的病理作用。

雖然幾年對於格蘭氏陰性菌所引起的敗血性休克已獲得廣泛的研究與證實，然而對於格蘭氏陽性菌所引起的敗血性休克則已開始被大家所重視。這幾年研究發現由格蘭氏陽性菌所引起的細菌感染亦能引發全身性細菌感染與敗血性休克(Bone, 1994)。研究陽性菌最困難的一點是在不同的菌種間其細胞壁的成份各有不同。其中LTA為陽性菌的細胞壁中最重要的成份之一；另外，LTA在細菌生長時亦擔任重要角色。最近的研究顯示LTA在臨床實驗上可對動物及人體造成發熱、白血球減少(leukocytopenia)、低血壓(hypotension)、體內器官受損、血小板減少症(thrombocytopenia), 瀰漫性血管內凝血 (disseminated intravascular coagulation, DIC)等相關敗血性休克的病理現象 (Bone, 1994)。

在陽性菌感染引起敗血症的過程中，其中一項明顯的變化為造成血小板減少症 (thrombocytopenia)；其所造成的原因是否與陰性菌感染是一樣的，目前不得而知。雖然血小板的生合成可能造成一部份的原因；但最重要的原因可能是增加血小板在體內的消耗有關。LTA對血小板的作用於1977年便有學者指出：Streptococcus的LTA在in vitro的實驗中會抑制血小板的凝集反應(Beachey et al., 1977)；在1990年亦有學者發現Staphylococcus之LTA會影響fibrin與platelet之間的附著(Chugh et al., 1990)；至於LTA對於血小板的詳細作用機轉至今尚未有完整的報告；因此本計劃擬對LTA抑制血小板凝集反應作進一步研究。

三、結果與討論

由本研究結果顯示，LTA (0.5-1.0 μg/ml) 會以dose-dependent的方式明顯的抑制由各種活化劑(如collagen, thrombin等)所引起的血小板凝集反應(Fig. 1)；另外，LTA抑制血小板凝集反應也呈現time-dependent的方式，隨著incubation的時間增加 (10-70 min)其抑制效果也跟著增加，在60 min呈最大的抑制反應 (Fig. 2)。在進一步探討其抑制血小板凝集反應的作用機轉分析，我們發現LTA會明顯的促進血小板cyclic AMP的形成 (Table 1)；在未活化的血小板細胞內cyclic AMP的含量非常低 (29.4± 1.5 pmol/10^9 platelets),在投與PGE_1 (10 μM) (一種adenylate cyclase的活化劑)後，會明顯的促進cyclic AMP的形成 (120.8± 18.8 pmol/10^9 platelets)；在投與LTA (0.5和1.0 μg/ml)至血小板懸浮液後，LTA會明顯的促進cyclic AMP的形成，特別是在1.0 μg/ml 的劑量下作用更明顯(68.7 ± 4.4 pmol/10^9 platelets) (Table 1)。另一方面，LTA對血小板cyclic GMP的影響方面，研究發現 LTA不會影響血小板細胞內cyclic GMP的形成 (Table 1)。由此顯示LTA抑制血小板凝集反應可能與cyclic GMP無關。

Cyclic AMP的增加可抑制許多血小板活化的訊息傳遞路徑，如抑制 $[Ca^{+2}]i$ mobilization及protein kinase C的活化；protein kinase C的活化在血小板凝集過程中扮演一非常重要的訊息傳遞角色；如protein kinase C可促進血小板細胞內47-kDa蛋白質磷酸化，進一步強化血小板凝集反應。本實驗利用Bio-imaging analyzer system (FAL 2000, Fuji, Japan)進一步探討LTA對PKC引起 47-kDa蛋白質磷酸化的影響；由結果顯示LTA在0.5和1.0 μg/ml的劑量下會明顯的抑制PDBμ (30 nM),一種protein kinase C的活化劑，所引起的47-kDa蛋白質的磷酸化反應，其抑制程度分別為14 % 和30 % 左右 (Table 2)。另外，LTA (0.5 和 1.0 μg/ml) 亦會明顯抑制PDBμ (30 nM)所引起的血小板凝集反應，其抑制程度分別為25 % 和35 % (Fig. 3)。

由此證實LTA的確可抑制由protein kinase C所引起的血小板活化反應。格蘭氏陰性菌內毒素LPS亦可抑制血小板凝集反應，由我們之前的研究顯示此抑制作用可能跟活化nitric oxide/cyclic GMP的路徑有關 (Sheu et al., 1999)。因此，我們亦想進一步證實LTA是否亦會活化nitric oxide的形成；由結果顯示LTA即使在1.0 μg/ml的劑量下，亦不會造成血小板NO的活化 (Table 3)。因此雖然LTA和LPS都會抑制血小板凝集反應，但此兩毒素的作用機轉所有不同，LPS會經由增加 NO/cyclic GMP的路徑來抑制血小板凝集作用；而LTA則可能經由增加cyclic AMP來抑制血小板凝集反應。

四、計畫成果自評

本研究計劃目前已完成當初申請計劃時的內容；且本結果我們亦已發表在國際著名學術期刊。因此，我們算是達到當初所設定的目標。

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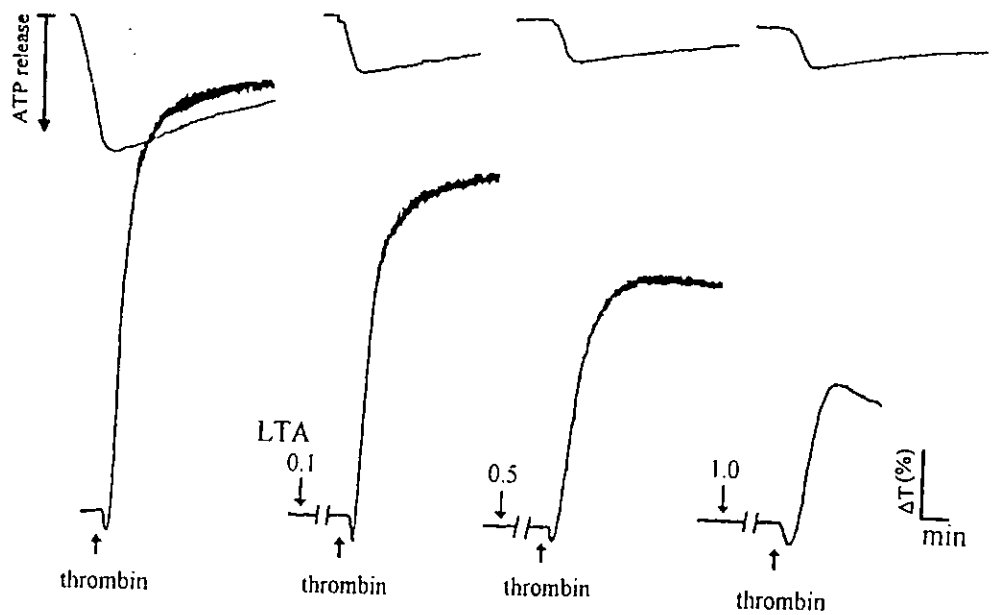

Fig. 1. Tracing curves of LTA on thrombin (0.05 U/mL)-induced aggregation in washed human platelet suspensions. Platelets were preincubated with LTA (0.1–1.0 μg/mL) and stirred for 10 min, then thrombin was added to trigger aggregation (upward tracings) and ATP release (downward tracings). A luciferin–luciferase mixture (20 μL) was added 1 min before the thrombin to measure the ATP release reaction. The curves are representative examples of four similar experiments.

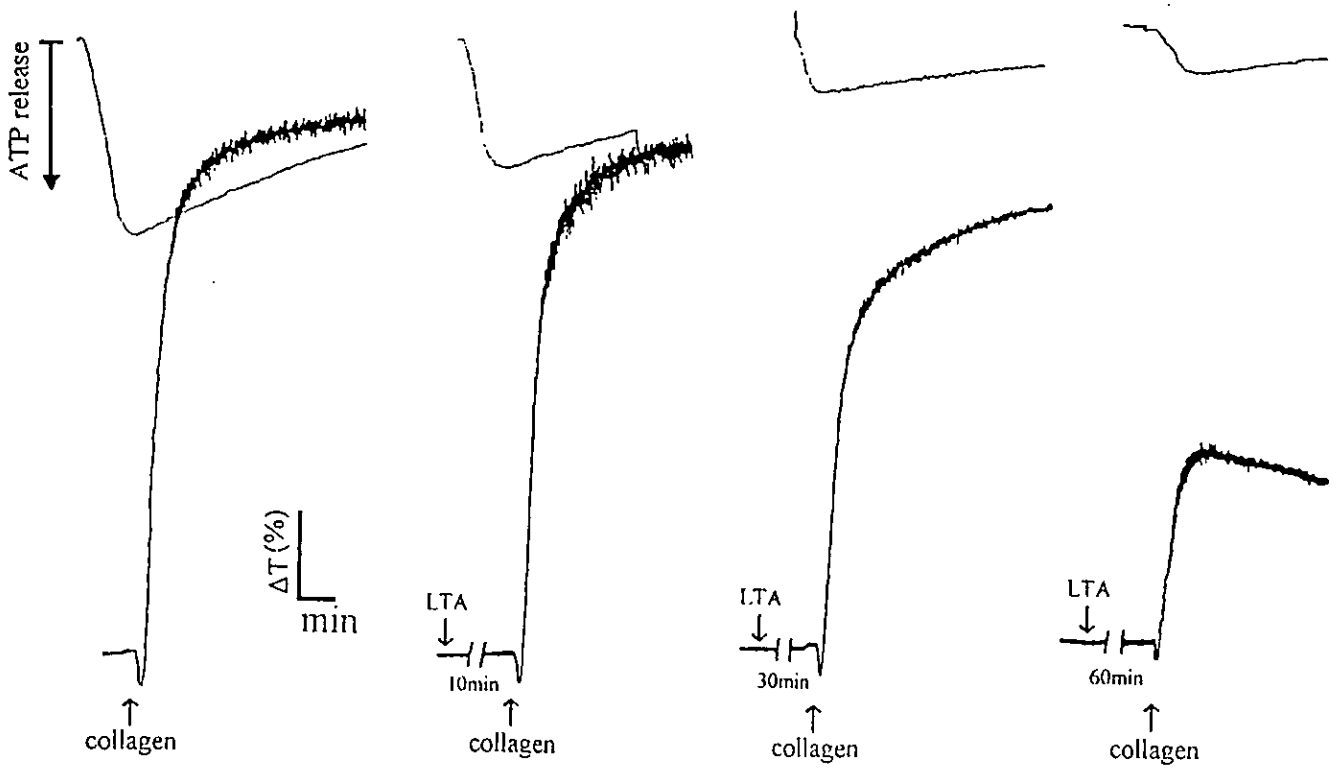

Fig. 2. Time–inhibition curves of LTA on collagen-induced platelet aggregation in human platelet suspensions. Platelets were preincubated with LTA (0.5 μg/mL) and incubated for 10–60 min at 37°C, respectively. Collagen (1.0 μg/mL) was then added to trigger aggregation. The curves are representative examples of four similar experiments.

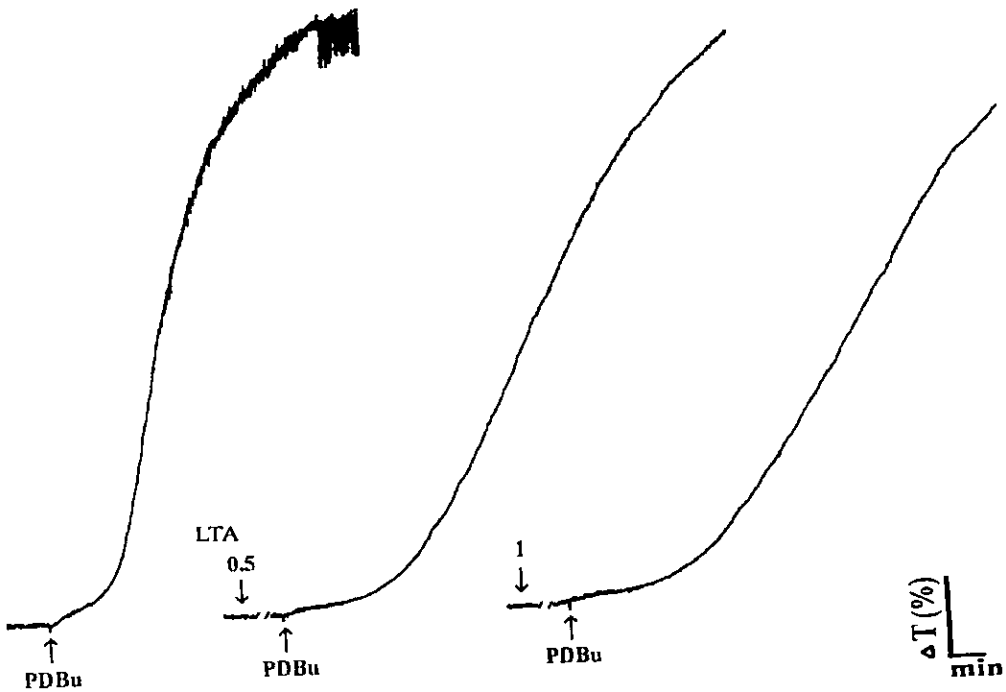

Fig. 3 Effect of LTA on PDBu-induced platelet aggregation in human platelets. Platelets were preincubated with LTA (0.5 and 1.0 μg/ml) for 10 min, then PDBu (0.03 μM) was added to trigger platelet aggregation. The curves are representative examples of four similar experiments.

Table 1. Effect of LTA, prostaglandin E_1, and nitroglycerin on cyclic AMP and cyclic GMP formation in washed human platelets.

	Dose (μg/ml)	Cyclic AMP (pmol/10^9 platelets)	Cyclic GMP (pmol/10^9 platelets)
Resting		29.4 ± 1.5	8.6 ± 0.5
Prostaglandin E_1	10 μM	120.8 ± 18.8*	———
Nitroglycerin	10 μM	———	16.1 ± 0.8*
LTA	0.5 μg/ml	35.0 ± 2.2	8.3 ± 0.2
	1.0 μg/ml	68.7 ± 4.4*	7.6 ± 1.0

Platelet suspensions were preincubated with LTA (0.5 and 1 μg/ml) for 10 min at 37℃. Addition of prostaglandin E_1 and nitroglycerin in platelet suspensions serves as a positive control. Data are presented as means ± S.E.M. (n=4). * $P <$ 0.001 as compared with the resting groups.

Table 2. Effect of LTA on PDBu-induced 47-kDa protein phosphorylation.

	Dose	FSL/mm^2	Inhibition (%)
Resting	——	129.2 ± 5.7	____
PDBu	0.03 (μM)	461.4 ±10.4*	____
+ LTA	0.5 (μg/ml)	396.0 ± 4.9$^\#$	~ 14 %
	1.0	318.7 ± 13.0$^\#$	~ 30 %

[^{32}P]-labeled platelets were preincubated with LTA (0.5 and 1.0 μg/ml) for 10 min 37℃, then PDBu was added to trigger 47-kDa protein phosphorylation. Data are presented as means ± S.E.M. (n=4). * $P < 0.001$ as compared with the resting group. $^\#$ $P < 0.001$ as compared with the PDBu group. FSL, photostimulated luminescence.

Table 3. Effect of LTA and collagen on nitrate formation in washed human platelets.

	Dose (μg/ml)	Nitrate (μM)
Resting	———	4.7 ± 0.8
Collagen	5	12.1 ± 0.4*
LTA	0.5	4.3 ± 0.3
	1	4.9 ± 0.5

Washed human platelets were preincubated with various concentrations of LTA (0.5 and 1 μg/ml) for 10 min at 37℃. Addition of collagen (5 μg/ml) in platelet suspensions serves as a positive control. Data are presented as means ±S.E.M. (n=4). * $P < 0.001$ as compared with the resting groups.