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探討抑制人類白血球誘發基質蛋白酵素活化之藥物機轉及其對活體再甦醒性傷害的保護作用之評估(1/2)

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行政院國家科學委員會專題研究計畫期中成果報告

探討抑制人類白血球誘發基質蛋白酵素活化之藥物機轉及其對活體再甦醒性傷害的保護作用之評估(1/2)

The study of inhibitory mechanisms of anti-leukocyte agents on matrix metalloproteinase activation and evaluate the protective effects on resuscitation injury in vivo

計畫編號：NSC 91-2320-B-038-046
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一、中英文摘要

基質金屬蛋白酵素(matrix metalloproteinases, MMPs)能夠催化分解維持組織結構之細胞外基質蛋白(extracellular matrix proteins)，包括基質(ground substances)與結締纖維組織(connective fibers)，因此對於組織之結構重組(remodeling)、修補(repairing)與破壞(destroy)都扮演相當重要之角色。同時MMPs 的含量與活性表現均受到許多方式嚴密地調節控制。根據文獻指出，許多發炎性疾病如類風濕性關節炎的軟骨組織不正常破壞或粥狀動脈血管斑塊組織的剝離均與異常基質崩解作用有關，其主要原因源自單核球或巨噬細胞產生及釋放大量MMPs 所致。

在大規模中藥材萃取物及化學合成等藥物成分篩選實驗下，我們發現其中傳統中藥穿心蓮(*Andrographis paniculata*)所萃取的天然物成分 andrographolide，具有明顯抑制 MMPs 活化之作用。在先導試驗中我們以人類單核球細胞(THP-1 cells)為實驗細胞，分別以不同濃度的 TNF-α或 LPS 處理 24 小時後，以電泳酵素分析法(gelatin zymography)評估可發現到人類單核球細胞能誘發大量 MMPs 的活性，而其中又以 MMP-9 為甚。在電泳酵素分析法下觀察到 andrographolide 有意義地依濃度效應(1-50 μM)抑制 TNF-α或 LPS 誘發人類單核球細胞之 MMP-9 活性。然而在酵素活性分析(MMP-9 activity assay system)下，andrographolide 在濃度 50 μM 時，卻不具有抑制 MMP-9 酵素活性之能力。另外以細胞存活率測定(MTT assay)發現 andrographolide 的抑制作用並非源自細胞之損害。並利用酵素免疫分析測定法(ELISA)，發現 andrographolide 也會抑制 TIMP-1 的含量。

從西方墨點實驗法(Western blot)發現在不同刺激下(如 TNF-α或 LPS)發現 MMP-9 protein 表現量隨 andrographolide 濃度增加而降低，故証實此天然物作用在 MMP-9 蛋白質表現層面。並進一步以 RT-PCR 的實驗分析法加以分析，發現 andrographolide 會抑制 MMP-9 mRNA 的表

現，更深入瞭解細胞轉錄(transcription)之影響程度。同時我們也更進一步探討 andrographolide 在訊息傳遞中作用機轉的方式，從實驗結果得知 andrographolide 會明顯抑制由 TNF-α 刺激所導致 Inhibitor-κB-α (IκB-α) 的降解作用，使 Nuclear factor-κB (NF-κB)無法進入細胞核中與特定 MMP-9 相關的 DNA 序列接合。LPS 則可能並非經 NF-κB 之途徑。在 Mitogen-activated protein kinases (MAPKs) 方面，從目前實驗結果得知 andrographolide 對於 extracellular signal-regulated kinases (ERKs) 並無直接的影響，至於 c-Jun-NH_2-terminal kinase (JNK)雖有初步結果，但仍需進一步探討其影響的程度。

綜合目前實驗的結果發現，天然物 andrographolide 的確具有抑制 MMP-9 表現之活性，而在 TNF-α刺激方面其作用機轉可能主要藉由影響 NF-κB 的訊息傳遞過程。目前本實驗室正進行其它有效成分機轉作用評估，同時也已完成出血性再甦醒性傷害之活體動物模式。第二年將進行相關之活體實驗以瞭解這些成分(如 andrographolide)是否具抗發炎與再甦醒性傷害之療效功能。

關鍵詞：基質金屬蛋白酵素、andrographolide、白血球、nuclear factor kappa-B、再甦醒性傷害

Abstract

Matrix Metalloproteinases (MMPs) could catalyze and degrade extracellar matrix protein (ECM), including ground substances and connecting fibers, which have their function to maintain tissue structure. Thus, it lays certain important roles in tissue structure remodeling repairing and destroys. The levels and activities of MMPs are strictly regulated and controlled in various ways. Many evidence indicated that human monocytes/macrophages synthesize and secrete several MMPs which are structurally related and participate in the degradation of ECM components in either rheumatoid arthritis tissues or atherosclerotic plaques. In general, inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin1β (IL-1β), lipopolysaccharides (LPS), can stimulate inflammatory cells to express MMPs genes and protein, and its activates are also regulated by physically endogenous tissue inhibitor of metalloproteinases (TIMPs), such as TIMP-1 and TIMP-2.

According to previous experiments, we found that andrographolide extracted from Chinese herb *Andrographis paniculata* showed obviously inhibitory effect on MMPs activation. We used human monocyte THP-1 cells in our preliminary experiments and by using different concentration of TNF-α or LPS treatment for 24 hours. We observed that andrographolide concentration-dependently (1-50 μM) inhibit MMP-9 activation induced by TNF-α and LPS significantly in zymography method. In addition, andrographolide did not show inhibitory effect on enzymatic activity of MMP-9 at concentration of 50 μM. Also, we found that the inhibitory effect of andrographolide was not due to impairment of cellular viability by MTT tests. On the other way, andrographolide also inhibited the

TIMP-1 levels by the ELISA analysis.

According to Western blot method, we found that the inhibition on expression of MMP-9 protein is concentration-dependently by andrographolide in various stimulations. This indicated that this natural compound has effect on the protein expression of MMP-9. By using RT-PCR method, we found that andrographolide can inhibit the expression of MMP-9 mRNA, thus has deeper influence on the level of MMP-9 transcription. At the same time, we investigated the mechanism of action of andrographolide in various signaling pathways. We found that it could significantly inhibit the degradation of inhibitor-κB-α (IκB-α) induced by TNF-α, therefore nuclear factor-κB (NF-κB) may not translocate for transcription. However, LPS is possibly not associated with this NF-κB pathway. Furthermore, in mitogen-activate protein kinases (MAPKs) aspect, andrographolide did not show direct influence on phosphorylated activation of extracellular signal-regulated kinases (ERKs), however, it showed slightly affect on c-Jun-NH_2-terminal kinase (JNK) activation. In summary, we found that Chinese herbal compound, andrographolide, with inhibitory effect on MMP-9 expression, and its main mechanism of action might through NF-κB signal pathway on TNF-α stimulation. It will be interesting to investigate its therapeutic profile on inflammatory animal model such as resuscitation injury *in vivo* on the second year.

Keywords: matrix metalloproteinases、andrographolide、leukocyte、nuclear factor kappa-B、 resuscitation injury

二、背景與目的

近年許多文獻指出基質金屬蛋白酵素(matrix metalloproteinses, MMPs)不僅於正常生理具有胚胎發育之再塑作用(embryonic developmental remodeling)，同時在許多病理急慢性發炎疾病如缺血性腦傷害症(focal cerebral ischemia)(Gasche et al., 1999)、急性呼吸窘迫症(acute respiratory distress syndrome) (Martinez-Hernandez and Amenta, 1983)、類風濕性關節炎(Okada et al., 1989)、多發性硬化症(Ozenci et al., 1999)及粥狀動脈硬化症(Galis et al., 1994)，甚至癌症(Cousens et al., 2000)都扮演著重要的角色。特別在胰臟發炎所導致之肺組織衰竭，更可發現白血球釋出的 MMPs 為重要傷害病因。另外在類風濕性關節炎與粥狀動脈硬化症之發炎組織可明顯觀察到單核球或巨噬細胞(monocytes/macrophages)浸潤活化而破壞周邊結締組織(Stetler and Stevenson, 1996)。這種不正常破壞且崩解細胞外基質之作用，主要源自於單核球或巨噬細胞產生及釋放大量基質金屬蛋白酵素(Woessner, 1991)。一般而言，單核球或巨噬細胞並非持續性(constitutive)產生 MMPs，而是當受特定外來發炎介質(如細胞激素)所活化產生。細胞激素(cytokines)如腫瘤壞死因子(tumor necrosis factor, TNF)、淋巴激素(interleukins)與顆粒性白血球巨噬細胞之群落刺激素(GM-colony stimulating factor)均可誘使單核球進入發炎組織且影響單核球產生 MMP 之種類及含量(Jovanovic et al., 2000, Redford et al., 1997)。然而顆粒性白血球在受到發炎介質(如 interleukin-8 ; IL-8 、tumor necrosis

factor；TNF 或 FMLP)刺激下，便立刻釋出儲存之 MMP (Opdenakker et al., 2001)。在活體實驗中，當以 MMP 抑制劑或 TNFα抑制劑處理動物後，可明顯降低肺部微血管滲漏(Keck et al., 2002)或關節組織之黏液水腫、發炎細胞浸潤及黏合分子表現(Shaw et al., 2000)。缺血性腦傷害更可藉 MMP 基因剃除或抑制劑而達到改善腦組織壞死之程度(Asahi et al., 2000)。最近更有文獻報導指出發炎性細胞激素(如 TNF-α及 IL-1β)可刺激人類白血球細胞促進大量 MMP-9 產生與釋放(Saren et al., 1996)。從這些結果更可推斷顆粒性白血球、單核球或巨噬細胞產生的 MMP-9 的確參與於呼吸窘迫症肺部組織或缺血性器官傷害之發炎與破壞反應。

Matrix metalloproteinases簡稱MMPs，為一群結構類似且含鋅(zinc ion)金屬離子之蛋白酵素(Kotra et al., 2001)。因MMPs的催化作用需金屬離子加以活化，並具備催化水解細胞外基質蛋白(extracellular matrix proteins)之能力，且特有基因序列可加以區別相近的MMPs，因而稱之為基質金屬蛋白酵素(Stöcker et al., 1995)。目前為止已有28種不同的MMPs被辨識出來，且持續的在增加(Sternlicht and Werb, 2001)。

MMPs的主要結構骨架至少可分為三部分，此基本構造以matrilysin (MMP-7)為例(Gaire et al., 1994)，包含第一段為signal sequence、第二段為含游離的thiol (SH) group之propeptide，及一段含鋅離子之催化區，而綜觀MMPs序列中最長且複雜者為92 kDa gelatinase B (MMP-9)，其結構除了有 matrilysin 的構造外，還具備 collagen-binding fibronectin type II inserts、hinge region 及 hemopexin/vitronectin domain。至於MMPs具有的其它特徵包括 furin-susceptible site、transmembrane domain、cytoplasmic tail、glycophosphatidyl inositol-anchoring domain、cysteine/proline及interleukin-1 receptor等。一般而言，不同類型之細胞有其特殊分泌之MMPs，如分離出的人類臍帶靜脈內皮細胞，主要以持續性 (constitutive) 分泌 MMP-2 為主 (De Lorenzo et al., 2000)。而活化的人類單核球或巨噬細胞，其分泌之主要形式為誘發性(inducible) MMP-9為主，而藉此破壞基底膜(basement membrane)並進行移走溢出(extravasation) (Lepidi et al., 2001)。根據人類染色體基因圖譜得知，不同MMPs之基因序列位於不同之染色體上，如MMP-2位於第16號染色體，而MMP-9則在第20號染色體，均藉由特定轉錄因子活化啟動promoter序列調控其基因的表現，而產生大量酵素原(zymogens or proenzymes)。多數生成的MMPs為不具活性的酵素原，當受外來或內生性作用以催化氮端含游離的thiol (SH) group之propeptide斷裂反應後，便能提升MMPs的催化活性(Harper et al., 1971)。

MMPs 最初被定義為一群酵素可以被金屬螯合劑與內生性抑制劑所抑制，且其未活化型可受有機汞所活化，而其所能催化水解之細胞外基質蛋白至少一種以上，例如 collagen、elastin 及 laminin 等，所以對於組織之結構重組(remodeling)、修補(repairing)與破壞(destroy)上扮演著相當重要的角色(Woessner et al., 1991)。此外，MMPs 可藉分解細胞表面蛋白分子(如 TNF-α) 及其它 pericellular non-matrix proteins，進而調控細胞生理活性(Sternlicht et al., 2000)。因此，MMPs 修改許多作用性或結構性蛋白，以影響生理及病理過程，

包括 embryonic development、tissue morphogenesis、wound repair、inflammatory diseases 及 cancer (Nelson et al., 2000)。另一方面，根據臨床文獻指出在失血性休克（hemorrhagic shock）與再甦醒（resuscitation）處理後，可引起廣泛發炎反應與介質的產生，造成組織損傷，嚴重更導致多重器官衰竭（multiple organ failure）而造成死亡(Regel et al., 1996)。在失血缺氧隨後再甦醒處理所導致的氧化迫傷與白血球活化是引發全身性發炎反應（systemic inflammatory response）的重要原因。缺血性腦傷害更可藉 MMP 基因剃除或抑制劑而達到改善腦組織壞死之程度(Asahi et al., 2000)。最近更有文獻報導指出在活體實驗中，當以 MMP 抑制劑處理動物後，可明顯降低多重器官衰竭所造成的肺部微血管滲漏傷害(Keck et al., 2002)。

Andrographolide 由中藥穿心蓮(*Andrographis paniculata*)的葉子中所萃取出來(Lu et al., 1981)，為一種具雙環二十碳的酮類(bicyclic diterpenoid lactone)化合物(Figure 1)。穿心蓮在一般民間中醫用於治療感冒、細菌感染或發炎疾病(如關節炎等)，用途非常廣泛。自從 andrographolide 被萃取出來之後，陸續有學者針對此藥物進行抗發炎性研究，也發現的確可影響 iNOS 的表現(Chiou et al., 1998)，進而引起我們對 andrographolide 抗發炎作用中有關 MMPs 方面的興趣，並加以探究其可能作用機轉與治療性。

實驗目的

在各種發炎疾病狀態下，MMPs 於疾病過程中扮演著一個重要調節及作用的角色。如前所述，細胞激素與細菌性內毒素會刺激 MMPs 的產生而造成分解結締組織，此種情況會造成各種不同之病理傷害。在本研究中，以人類單核球細胞(THP-1)為實驗細胞，藉以瞭解 andrographolide 與其它有效成分對 TNF-α及 LPS 所誘發 MMP-9 protein 的活性及表現的影響性，並探討其中所牽涉的藥理作用機轉。更進一步我們將了解 andrographolide 等成分在細胞訊息傳遞中的NF-κB 或 MAPK 等機制之影響程度。並藉以評估 andrographolide 等成分在再甦醒性傷害、粥狀動脈硬化血管剝離或關節炎的治療可能性。

三、實驗結果

1. 探討 Andrographolide 對人類單核球細胞(THP-1 cells)以 TNF-α或 LPS 誘發產生 MMP-9 酵素活性之作用

我們首先設立電泳酵素分析法(gelatin zymography)與刺激物引發 MMP-9 之實驗標準條件。由於巨噬細胞源自於單核球，而人類單核球細胞(THP-1)為目前研究 MMPs 所廣泛應用，因此，我們分別投與不同濃度之發炎性細胞激素 TNF-α或細菌性內毒素 LPS 處理 THP-1 細胞 24 小時後，利用電泳酵素分析法可發現單核球細胞能誘發大量 MMP-9 的活性，而非 MMP-2 活性。特別是在 TNF-α濃度為 10 ng/ml 或 LPS 濃度為 10 ng/ml 時，得到的效果最明顯。此外，在 TNF-α或 LPS 作用下，細胞濃度以 1 $\times 10^6$ cell/ml 所得效果最適切。依上述適當實驗條件下，由電泳酵素分析法可得知 andrographolide 隨濃度上升(1 μM、5 μM、10 μM、20 μM 及 50 μM)而抑制 TNF-α或 LPS 所誘發 MMP-9 的活性。而 andrographolide 對 TNF-α刺激作用的抑制百分率(In %)分別為 18.8 ± 9.8 % (5 μM)、60.8 ± 6.2 % (10 μM)、76.2 ± 8.0 % (20 μM)

與 116.4 ± 11.2 % (50 μM)，其抑制 50 %反應之濃度(IC_{50})為 9.66 ± 2.24 μM (n = 4～5，Figure 2)。另外 andrographolide 對 LPS 刺激作用的抑制百分率(In %)分別為 23.0 ± 3.6 % (1 μM)、62.0 ± 4.2 % (5 μM)、89.3 ± 9.9 % (10 μM)與 116.8 ± 12.5 % (20 μM)，其抑制 50 %反應之濃度(IC_{50})為 2.24 ± 0.36 μM (n = 3～4，Figure 3)。此外我們以已知 MMP-9 抑制劑 Doxycycline (50 μM, negative control)及 MMP-9 促進劑 PMA (10 ng/ml, positive control)分別進行此實驗，可發現 TNF-α或 LPS 刺激 THP-1 細胞所引起之 gelatin 崩解作用，特別是 MMP-9 之分解反應，可明顯且有意義的被 Doxycycline 所抑制。在同樣實驗條件下，PMA 的確能引發大量 MMP-9 的釋放(data not show)。

2. 探討 Andrographolide 對 THP-1 cells 的細胞毒性

為了證明 andrographolide 抑制 MMP-9 的活性及表現，並非由此成分所引發的細胞毒性，而導致 MMP-9 的含量減少。因此先將 THP-1 細胞(1 $\times 10^6$ cells/ml)種於 24 well 的培養盤，以 andrographolide (5 μM、10 μM、20 μM 及 50 μM)處理細胞至 22 小時，再加入 MTT 試劑處理兩小時，利用存活細胞的粒線體內酵素將 MTT 試劑還原成 formazan 紫色結晶，再用 DMSO 將結晶溶解後測 550 nm 的吸光值，作為存活細胞的含量。由實驗結果發現 andrographolide 濃度在 5 μM (1.22 ± 0.03)、10 μM (1.35 ± 0.02)及 20 μM (1.28 ± 0.05)對細胞存活率沒有影響(resting：1.26 ± 0.04)，而濃度達到 50 μM (0.94 ± 0.09)時，雖會降低細胞存活率，但仍僅影響約 15 %的存化程度(n = 3～4, Figure 4)。

3. 探討 Andrographolide 對 MMP-9 酵素本身活性之影響

首先將 THP-1 細胞(1 $\times 10^6$ cells/ml)種於 10 cm dish 的培養盤，以 10 ng/ml 之 PMA 處理細胞至 24 小時，吸至離心管中，離心後，利用酵素活性套組分析(MMP-9 activity assay kit system)，我們以濃度 50 μM 的 andrographolide 處理時發現，andrographolide 並不會直接影響 MMP-9 本身的酵素活性(resting：5.9 ±0.47 equivalent MMP-9 ng/ml、50 μM：5.2 ± 0.61 equivalent MMP-9 ng/ml, n = 2～3)，由此了解 andrographolide 的作用並不藉抑制 MMP-9 本身的酵素活性而達成，而可能是抑制 MMP-9 蛋白質的表現，深入細胞轉錄之影響作用，或影響訊息傳遞如 Nuclear factor-κB (NF-κB)、Mitogen-activated protein kinases (MAPKs)等的活化，或藉由其它訊息傳遞方式以影響 MMP-9 之產生與活化。

4. 探討 Andrographolide 對 THP-1 cells 以 TNF-α或 LPS 誘發釋放 MMP-9 蛋白表現之作用

Figure 2、Figure 3 已證實 TNF-α及 LPS 的確能誘導 MMP-9 的活性增加，而 andrographolide 則可抑制此作用，並由 Figure 4 證實 andrographolide 對 MMP-9 活性抑制，並非由此成分所引發的細胞毒性，而導致 MMP-9 的含量減少。為了瞭解 andrographolide 抑制 MMP-9 的產生是否藉由減少蛋白的表現，因此我們使用西方點

墨法(Western blot)分析 andrographolide 對 MMP-9 蛋白表現的作用。由 Figure 5 及 Figure 6 得知，THP-1 細胞於 resting 培養24小時後，取細胞萃取物(lysate)進行實驗後，發現幾乎偵測不到 92 kDa 的 MMP-9 蛋白(lane 1)。然而以 TNF-α 刺激 24小時後，細胞萃取物有大量 MMP-9 被誘導產生(Figure 10, lane 2)。當以不同濃度 5 μM (lane 3)、10 μM (lane 4)、20 μM (lane 5)與 50 μM (lane 6)之 andrographolide 處理後，可發現 TNF-α 引發 MMP-9 的蛋白表現隨 andrographolide 的濃度增加而減少。接著以 LPS 刺激 THP-1 細胞 24小時後，細胞萃取物有大量 MMP-9 被誘導產生(Figure 6, lane 2)。當以不同濃度 1 μM (lane 3)、5 μM (lane 4)、10 μM (lane 5)與 20 μM (lane 6)之 andrographolide 處理後，可發現 LPS 引發 MMP-9 的蛋白表現亦隨 andrographolide 的濃度增加而減少。

5. 探討 Andrographolide 對 THP-1 cells 以 TNF-α或 LPS 誘發釋放 TIMP-1 蛋白表現之作用

許多發炎性細胞激素 TNF-α或細菌性內毒素 LPS 均會影響 MMP-9 之活化，同時 MMPs 也會受生理性之組織抑制劑如 TIMP-1 及 TIMP-2 所調節(Denhardt et al., 1993)。其中 TIMP-1 在人類單核球細胞中主要與 MMP-9 活性調節有所關聯(Leber et al., 1998)。因此我們便將實驗所取得之上清液(supernetant)來進行 TIMP-1 蛋白表現的實驗。我們以酵素免疫分析測定法(ELISA)來觀察，發現 THP-1 細胞在未經刺激時，便有些許的 TIMP-1 蛋白的表現(Figure 7, lane 1：218.2 ± 10.1 ng/10^6 cells, n = 5；Figure 8, lane 1：280.4 ± 11.5 ng/10^6 cells, n = 5)，經 TNF-α刺激後(Figure 7)，則可明顯見到 TIMP-1 蛋白的表現增加 270.2 ± 10.5 ng/10^6 cells (lane 2, n = 5)。若經不同濃度 10 μM (lane 3)、20 μM (lane 4)與 50 μM (lane 5)之 andrographolide 處理後，則以 TNF-α 刺激後的 TIMP-1 蛋白表現，隨 andrographolide 的濃度增加而減少 200.8 ± 27.0 ng/10^6 cells (lane 3, n = 4)、187.3 ± 13.9 ng/10^6 cells (lane 4, n = 6)、134.3 ± 10.7 ng/10^6 cells (lane 5, n = 4)。經 LPS 刺激後(Figure 8)，則亦可明顯見到 TIMP-1 蛋白的表現 323.2 ± 9.4 ng/10^6 cells (lane 2, n = 5)。若經不同濃度5 μM (lane 3)、10 μM (lane 4)與 20 μM (lane 5)之 andrographolide 處理後，則以 LPS 刺激後的 TIMP-1 蛋白表現，亦隨 andrographolide 的濃度增加而減少 261.0 ± 15.1 ng/10^6 cells (lane 3, n = 3)、250.0 ± 21.7 ng/10^6 cells (lane 4, n = 4)、217.3 ± 5.2 ng/10^6 cells (lane 5, n = 3)。

6. 探討 Andrographolide 對由 TNF-α誘導 THP-1 cells 引發 MMP-9 mRNA 之影響

萃取細胞內全部 RNA，利用反轉錄酵素(reverse transcriptase)將 RNA 轉換成 cDNA，於核酸循環加熱器進行聚合酵素鏈鎖反應(簡稱 PCR)。利用 MMP-9 和 GAPDH 的專一性引子(primer)放大 MMP-9 及 GAPDH 的 cDNA 產物。以 1 %洋菜膠(agarose gel)進行電泳分析。實驗結果顯示(Figure 9)，以 TNF-α (10 ng/ml)處理 6 小時後，MMP-9 mRNA 明顯地被誘發出來(lane 2)，而 andrographolide 在 20 μM 時，即有意義的減少 MMP-9 mRNA 的表現，減少

約 90 % (lane 3)。至於 housekeeping gene，GAPDH，則作為 internal control，藥物對其無明顯影響。

7. 探討 Andrographolide 對 THP-1 cells 以 TNF-α或 LPS 誘導引發 IκB-α降解

依據研究指出，TNF-α 作用於發炎細胞大都經由 NF-κB/IκB 或 mitogen-activated protein kinases (MAPKs)等路徑，進而造成轉錄因子(transcription factors)的活化。其相關轉錄因子主要以 NF-κB 與 AP-1 為主。而 LPS 刺激細胞後，釋放出部分的內生性調節因子，主要為細胞激素，如 Interleukin-1 (IL-1)、tumor necrosis factor-α (TNF-α)等，間接造成轉錄因子的活化。首先，我們利用 Western blot 來了解是否 andrographolide 作用在 IκB 的磷酸化之分解過程，而影響 NF-κB 之活化。我們首先以 TNF-α刺激觀察不同時間點 resting 5 分鐘(lane 1)、resting 15 分鐘(lane 2)、5 分鐘(lane 3)、15 分鐘(lane 4)與 30 分鐘(lane 5)之 IκB-α 蛋白表現(Figure 10)。實驗發現，TNF-α於 15 分鐘時，IκB-α有最明顯減少，隨後又逐漸回到基準值(basal level)，接著再以 LPS 刺激，觀察不同時間點 resting 5 分鐘(lane 1)、resting 15 分鐘(lane 2)、5 分鐘(lane 3)、15 分鐘(lane 4)與 30 分鐘(lane 5)之 IκB-α 蛋白表現(Figure 11)。實驗發現，IκB-α並無明顯改變。我們根據前述結果，將細胞以 TNF-α 刺激 15 分鐘進行實驗(Figure 12)，發現以不同濃度 10 μM (lane 3)、20 μM (lane 4)與 50 μM (lane 5)之 andrographolide 處理後，IκB-α 可回到原正常表現量。因此我們推論 andrographolide 可能經由抑制 IκB-α protein 的降解來減少 NF-κB 移至核內與其特定的 DNA 序列接合，而達到抑制 TNF-α 刺激所造成 MMP-9 的表現。另外，抑制 LPS 刺激所造成 MMP-9 的表現之原因，則需在進一步加以探討。

8. 探討 Andrographolide 對 THP-1 cells 以 TNF-α或 LPS 誘發釋放 ERK、JNK 等 MAPKs 活化的作用

MAPKs 在細胞訊息傳遞過程中，須自身被磷酸化才具活化之酵素型態，進而轉移(translocation)進入細胞核，且活化轉錄因子(AP-1)以進行基因表現(Roger et al., 1994)。其中的 ERK 及 JNK 易受發炎性細胞激素(cytokines)而活化，所以我們觀察 andrographolide 在它們的抑制機轉上所扮演的角色。我們利用 Western blot 來了解是否 andrographolide 抑制 ERK 及 JNK 的磷酸化之過程，而影響活化轉錄因子(AP-1)以進行基因表現。我們首先以 TNF-α刺激觀察不同時間點 resting 5 分鐘(lane 1)、resting 15 分鐘(lane 2)、5 分鐘(lane 3)、15 分鐘(lane 4)與 30 分鐘(lane 5)之 ERK 蛋白表現(Figure 13)。實驗發現，TNF-α刺激於 15 分鐘時，ERK 有最明顯增加，隨後又逐漸回到基準值(basal level)。我們根據前述結果，將細胞以 TNF-α 刺激 15 分鐘進行實驗(Figure 14)，發現以不同濃度 10 μM (lane 3)、20 μM (lane 4)與 50 μM (lane 5)之 andrographolide 處理後，ERK 的表現量無明顯直接的影響，至於 JNK 則有某種程度上的影響(data not show)。接著再以 LPS 刺激15 分鐘進行實驗(Figure 15)，發現以不同濃度5 μM (lane 3)、10 μM (lane 4)與 20 μM (lane 5)之 andrographolide 處理後，ERK 的表現量亦無明顯直接的影響。因此我們推

論 andrographolide 可能經由抑制部分 MAPKs 的磷酸化，來減少活化轉錄因子(AP-1)與其特定的DNA序列接合以進行基因表現，而達到抑制 TNF-α 或 LPS 刺激所造成 MMP-9 的表現，則需在進一步加以探討。

四、討論

單核球是白血球中最大的一種，約佔白血球總量的 2-10 %。由骨髓至組織的發生過程中，自骨髓中的多元性幹細胞(pluripotent stem cells)先分化成 monoblast 後繼續成長為前單核球(promonocyte)，最後形成單核球(monocyte)而釋放至血液循環中。當進入網狀內皮組織或周邊組織時，遂分化成特定巨噬細胞(specific macrophage)。許多文獻指出，在粥狀動脈硬化(atherosclerosis)及風濕性關節炎(rheumatoid arthritis)等發炎反應時，單核球會經內皮細胞移動進入血管下組織(vascular subendothelium) (Aikawa et al., 1998；Zhang et al., 1999；Close, 2001)。此單核球移動作用，除需藉由強效的化學趨向物質(chemoattractant)來進行，如 monocyte chemotactic protein-1 (MCP-1)外，同時亦伴隨著釋放基質金屬蛋白酵素(matrix metalloproteinases, MMPs)以局部或廣泛性破壞細胞外基質(extracellular matrix) (Watanabe et al., 1993；Adams and Shaw, 1994)。在本實驗中所使用的人類單核球細胞株(human acute monocytic leukemia cell line；THP-1 cells)，是由白血病患者所取出之單核球加以培養所得。

近年來許多文獻報導指出類風濕性關節炎或血管斑塊不正常破壞且崩解基質之作用，主要是因單核球或巨噬細胞受特定外來發炎介質(如細胞激素，TNF-α或 IL-1)或外來病理因子(如 LPS)活化，而產生及釋放大量催化性基質金屬蛋白酵素(MMPs) (Heller et al., 1994；Cannon et al., 1990；Remick et al., 1990)。此外，由關節炎病人之滑液檢體，更可發現大量 MMP-9 mRNA 的存在(Cawston et al., 1998)。許多證據顯示發炎性細胞激素(如 TNF-α 或 IL-1β)或細菌性內毒素(如 LPS)，均可刺激人類單核球細胞促進大量 MMP-9 產生及釋放，但同時亦會合成及釋放生理性組織抑制劑(如 TIMP-1 及 TIMP-2)以適度調節酵素活性(Shapiro et al., 1992；Denhardt et al., 1993)。在人類單核球細胞中，MMP-9 的活性調節主要與 TIMP-1 有所關連(Leber et al., 1998)，其碳端基團(C-terminal domain)會與 proMMP-9 形成一複合物(complex)。

自從 andrographolide 由中藥穿心蓮(*Andrographis paniculata*)的葉子中所萃取出來之後，陸續有學者針對此藥物進行抗發炎性研究，也發現的確可影響 iNOS 的表現(Chiou et al., 1998)，進而引起我們對 andrographolide 抗發炎中有關 MMPs 方面的興趣，並加以探究其機轉。因此我們想進一步瞭解 andrographolide 能否抑制 MMP-9 的產生。本實驗以不同濃度 andrographolide (1、5、10、20 及 50 μM)來觀察 THP-1 細胞對由 TNF-α或 LPS 刺激而產生 MMP-9 活性的影響程度。經 Zymography 實驗結果證實 andrographolide 呈現有意義之抑制效果，且其 IC_{50} 值分別為 9.66 ± 2.24 μM 及 2.24 ± 0.36 μM。另外，為了證明 andrographolide 抑制 MMP-9 的活性及表現，並非經細胞毒性而導致之結果，我們由 MTT 實驗獲知即使濃度達到

50 μM 時，細胞存活率也不會有明顯的減少，亦即本實驗使用藥物濃度範圍內對細胞不具毒性。

由 Western blot 實驗得知，andrographolide 可明顯抑制由 TNF-α 或 LPS 所誘發釋出的 MMP-9，而其細胞內之蛋白質也明顯降低。由 ELISA 實驗得知，雖然 andrographolide 也會影響釋出細胞外的 TIMP-1，但此結果不影響 andrographolide 對於抑制 MMP-9 蛋白表現之作用。綜合上述，我們推論 andrographolide 可能抑制之機轉點在 MMP 蛋白質轉譯(translation)之上游(upstream)。在真核生物中，細胞基因的調節相當複雜，需要許多蛋白酵素及因子參與此過程，才可表現出最終的功能性蛋白(functional protein)。根據人類染色體基因圖譜得知，MMP-9 之基因序列主要位於第 20 號染色體之 20q11.2 至 q13.1 上。一般而言，基因訊息經特定調控轉錄(transcription)解碼形成 mRNA，mRNA 再經轉譯(translation)以製造特定蛋白。經 RT-PCR 實驗過程中，我們發現 andrographolide 的確能降低由 TNF-α 刺激所誘發的 MMP-9 的 mRNA 之產生。因此進一步再推論其中 MMP-9 基因的轉錄上游過程受到相關之抑制作用。

根據過去研究，TNF-α作用在發炎細胞，主要藉由細胞膜表面之特殊接受體 TNFR1 (p55)或 TNFR2 (p75)，再經由不同之細胞內訊息傳遞，如 NF-κB/IκB 或 mitogen-activated protein kinases (MAPKs)等路徑，進而造成轉錄因子(transcription factor)的活化，而表現出許多作用性產物。轉錄因子的活化為基因轉錄作用之主要關鍵點(Nathan and Xie, 1994)，其相關轉錄因子主要以 NF-κB 與 AP-1 為主。很多研究也證實 LPS 除了活化 NF-κB 也會增加 AP-1 這個轉錄因子的活性(Galdiero M et al., 2002)。這兩者為調節 MMP 及 TIMP genes 表現的重要轉錄因子(Fisher et al., 1996；Logan et al., 1996)。所以我們假設 Andrographolide 可能透過 NF-κB/Rel system 與 MAPKs/AP-1 路徑影響 MMP-9 蛋白的表現。因此我們想加以探討 andrographolide 是否經由抑制 IκB-α的降解或降低 MAPKs 活化表現而降低 MMP-9 蛋白的表現與產生。

NF-κB 首次被發現於成熟的 B lymphocytes 細胞核內，它與 Kappa light chain enhancer region 上的特定序列 5'-GGGACTTTCC-3' 結合 (Sen and Baltimore, 1986)。然而在陸續的研究指出，幾乎所有細胞皆存有 NF-κB 這個轉錄因子。當細胞受外界刺激(如 TNF-α、LPS、H_2O_2 等)，NF-κB/Rel system 便會快速參與誘導型的基因表現，因此大部分被 NF-κB/Rel system 所活化的基因都會調控免疫及發炎的過程。根據文獻指出，NF-κB 主要受 IκB-α (IκB 包含 IκB-α、IκB-β、IκB-γ 及 IκB-δ) 調控 (Thanos and Maniatis, 1995)。雖然促使 NF-κB 活化的機轉不盡然十分瞭解，但一般相信將 IκB-α在 Ser-32 或 Ser-36 磷酸化，可導致 IκB-α降解，此為促使 NF-κB 活化的重要過程(Brown et al., 1995；Emery et al., 2001)。由 Western blot 分析結果顯示，TNF-α作用 15 分鐘之後可引發 THP-1 細胞內 IκB-α的降解，且在處理三十分鐘後，IκB-α又會漸漸回復到基準值。而 LPS 作用後，在三十分鐘內並無意義上的影響，因為 LPS 作用較 TNF-α慢，

未來將探討一小時與兩小時之時間點，其與 NF-κB 之相關性則需再進一步加以探討。當 THP-1 細胞以 andrographolide 投與後，發現 andrographolide 可明顯抑制由 TNF-α刺激所導致的 IκB-α降解。因此，我們推測 andrographolide 主要可能經由抑制 IκB-α的降解，而減少由 TNF-α所引發的游離態 NF-κB 進入細胞核與相關之 DNA 序列接合(如 MMP-9 之 promoter)，以減少 MMP-9 的基因表現。唯 andrographolide 是否作用於 IκB-α上游之酵素 IKK-β kinase 或影響 NF-κB 之轉位作用(translocation)，其真正機轉仍須進一步探討。

另外，我們也探討 andrographolide 是否也會經由影響 MAPKs 活化表現以影響 AP-1 之 c-fos 或 c-jun 的磷酸化，而減少 MMP-9 蛋白的產生。Mitogen-activated protein kinase (MAPK)屬於 serine-threonine kinase，可經由細胞表面接受生物訊號，如細胞激素(cytokine)、細菌性內毒素(endotoxin)或生長因子(growth factor)。近來主要被研究之 MAPKs 為下列三種，包含 extracellular signal-regulated kinase (ERK 1/2)、c-Jun N-terminal kinase (JNK)與 p38 MAPK 等。這些相關酵素可受外來生長因子與物理性傷害等刺激因素而導致活化。較特別的是其中 JNK 及 p38 MAPK 易受如紫外線照射、熱傷害(heat shock)、高張環境(hyperosmolarity)與發炎性細胞激素(cytokines)等而活化(Kyriakis et al., 1996)。MAPKs 在訊息傳遞過程中，必須由上游之不同 MAP kinase kinase (MEK)分別將其磷酸化，轉為活化態之蛋白酵素，才可進一步調控轉錄因子進行基因表現(Roger et al., 1994)。此外，依據最近研究報告指出活性氧屬(reactive oxygen species, ROS)，如 superoxide 為細胞引發 MMPs 產生及活化之重要因素。因 ROS 可能直接參與 MMPs 產生的訊息傳遞，也可能間接影響傳遞中 JNK、NF-κB 等活性(Rajagopalan et al., 1996)。由 Western blot 分析結果顯示，TNF-α作用 15 分鐘之後會導致 THP-1 細胞內 ERK 1/2 的增加，且在處理 30 分鐘後，ERK 1/2 又會漸漸回復到基準值。當 THP-1 細胞以 andrographolide 投與後，發現 andrographolide 對於由 TNF-α刺激所誘發的 ERK 1/2 增加並無明顯的抑制作用，而 LPS 作用後亦無意義上的影響。因此，我們推測 andrographolide 可能經由抑制 MAPK 其它相關的路徑，而減少由 TNF-α或 LPS 所引發 MMP-9 蛋白的表現，其與 JNK、p38 MAPK 之相關性則需再進一步加以探討。

本論文主要證實了 andrographolide 可抑制人類單核球細胞受 TNF-α或 LPS 所誘發的 MMP-9 表現。根據 andrographolide 抑制 TNF-α刺激 THP-1 細胞誘發 MMP-9 酵素活性、protein 表現含量及 mRNA 表現等的證據顯示，MMP-9 基因的轉錄可被 andrographolide 抑制。藉由 Western blot 實驗分析 IκB-α降解程度，以及 ERK 1/2 和 JNK 磷酸化含量變化情形。可知 andrographolide 能透過抑制 IκB-α的降解，使 MMP-9 基因表現減少，但 ERK 1/2 磷酸化含量變化並不明顯。而 LPS 刺激 MMP-9 基因表現可能尚有其它路徑，例如藉由 early growth response gene product-1 (Egr-1)等(Mausumee Guha et al., 2001)，仍須進一步研究探討。

五、結論

綜合以上結果，研究證實

andrographolide 的確可以抑制由腫瘤壞死因子(TNF-α)或脂多醣體(LPS)誘發人類單核球細胞(THP-1 cells)所釋放的 MMP-9 蛋白質表現，且此抑制效果並非來自於細胞毒性。我們亦證實 andrographolide 對 MMP-9 抑制細胞外基質降解的能力，與內生性組織抑制劑(如 TIMP-1)的作用無關。另外，我們亦證實 andrographolide 抑制 TNF-α誘發 THP-1 細胞所釋放的 MMP-9 可能經由壓制 NF-κB 活化步驟來達成，而不與細胞訊息相關的 ERK 路徑有關(Figure 16)。未來我們將進一步探討 MAPKs 其它及相關訊息路徑。另外，本實驗室也已完成出血性再甦醒性傷害之活體動物模式(Figure 17)。藉此初步結果在第二年將更進一步進行 andrographolide 等成分之活體實驗，以評估作為發炎疾病 (如再甦醒性傷害) 的可能預防或治療性藥物。

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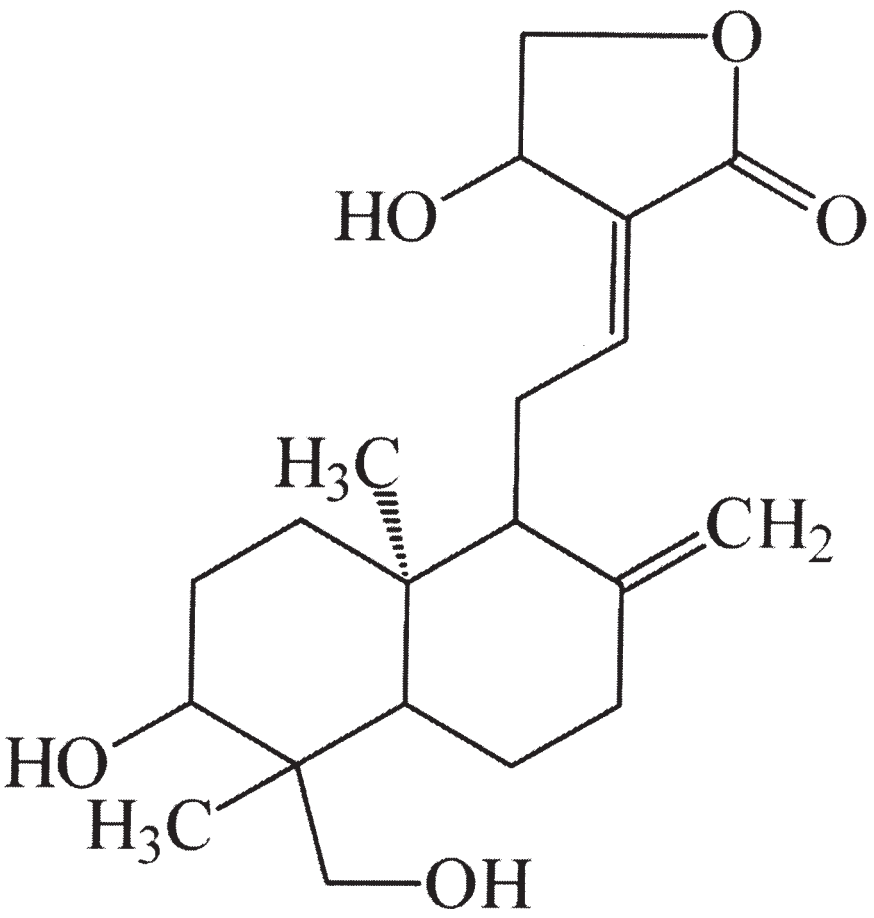

Figure 1. Chemical structures of andrographolide

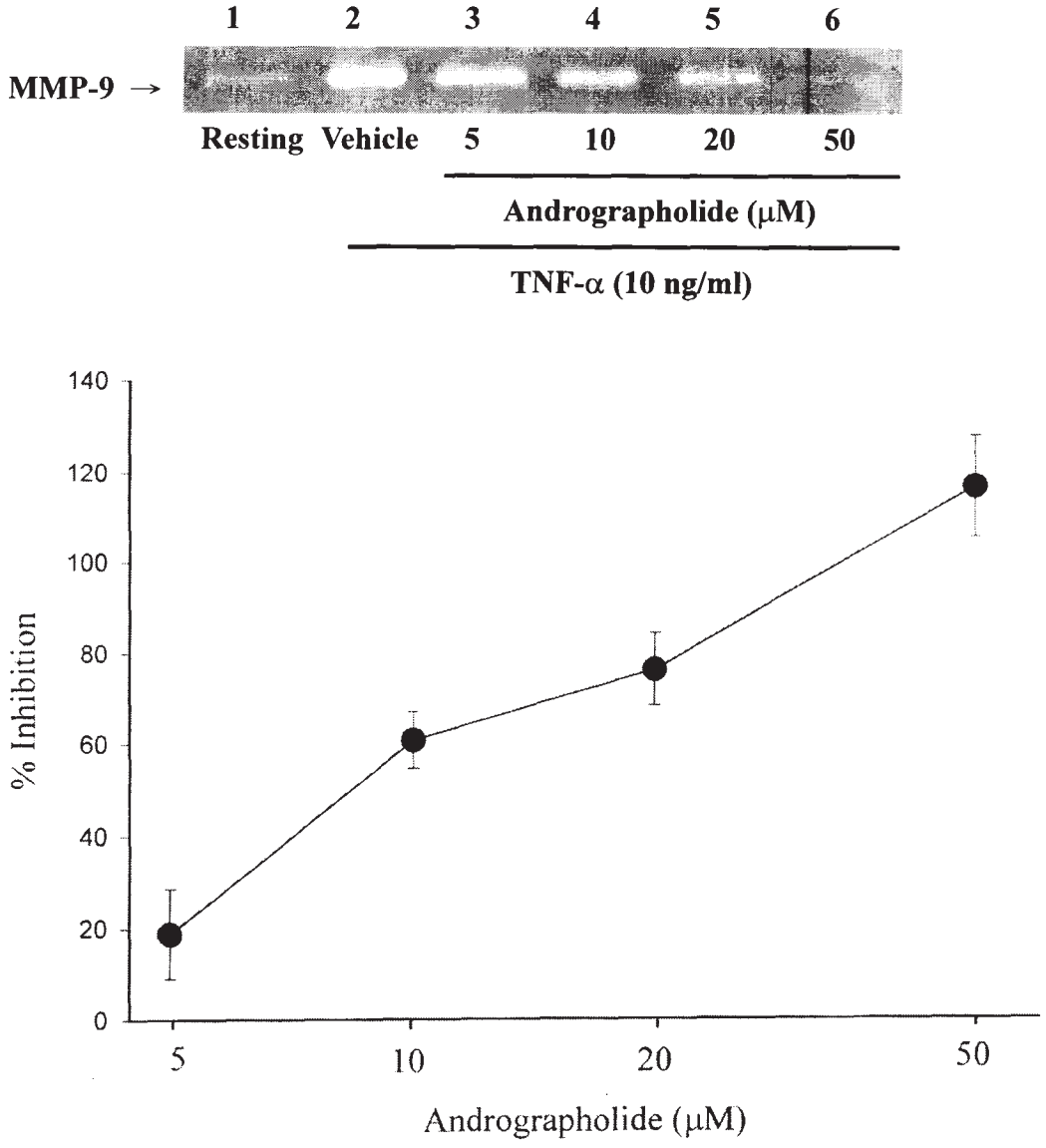

Figure 2. Effect of andrographolide on TNF-α-induced enzymatic activity of matrix metalloproteinase-9 (MMP-9) in THP-1 cells. THP-1 cells (1×10^6 cell/ml) were dispensed on 24-well plates till 70-80% confluent condition and treated with TNF-α (10 ng/ml) for 24 hrs as indicated. Cells were treated with the indicated concentrations of andrographolide (lane 3, 5 μM; lane 4, 10 μM; lane 5, 20 μM; lane 6, 50 μM) or vehicle (lane 2) for 15 minutes before treatment with TNF-α. Cell-free supernatants were then assayed for MMP-9 activity by gelatin zymography, as detailed in "Methods" (lane 1, control). Percent inhibition is presented as mean ± S.E.M. of four to five independent experiments.

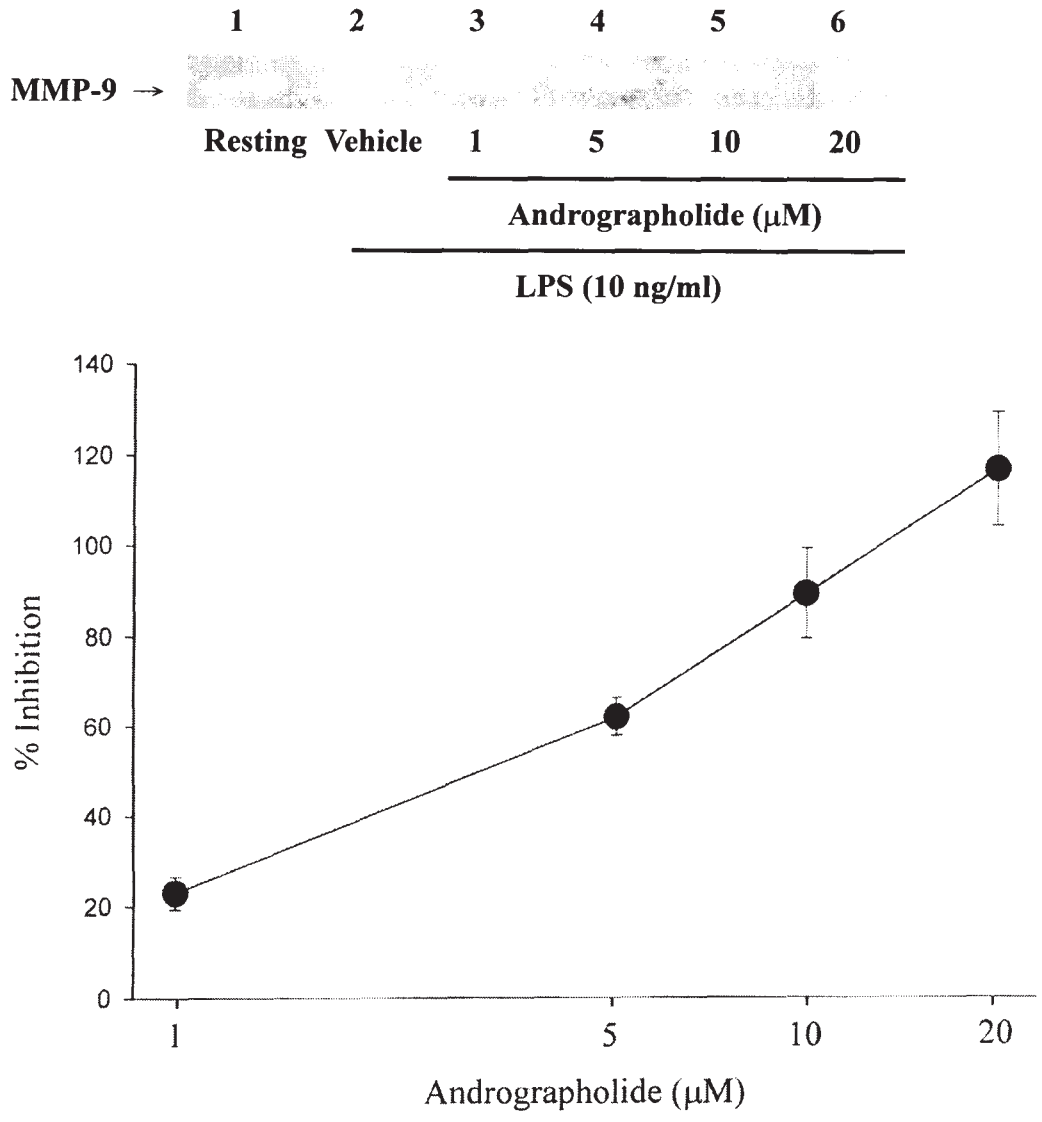

Figure 3. Effect of andrographolide on LPS-induced enzymatic activity of matrix metalloproteinase-9 (MMP-9) in THP-1 cells. THP-1 cells (1×10^6 cell/ml) were dispensed on 24-well plates till 70-80% confluent condition and treated with LPS (10 ng/ml) for 24 hrs as indicated. Cells were treated with the indicated concentrations of andrographolide (lane 3, 1 μM; lane 4, 5 μM; lane 5, 10 μM; lane 6, 20 μM) or vehicle (lane 2) for 15 minutes before treatment with LPS. Cell-free supernatants were then assayed for MMP-9 activity by gelatin zymography, as detailed in "Methods" (lane 1, control). Percent inhibition is presented as mean ± S.E.M. of three to four independent experiments.

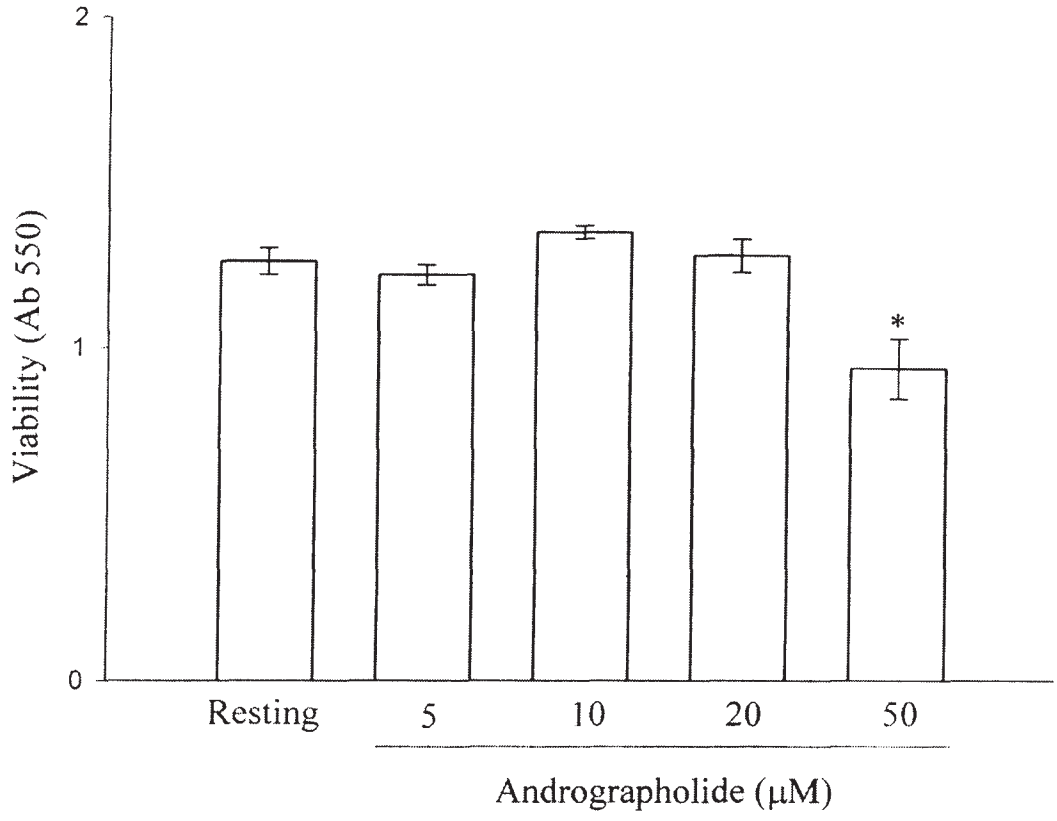

Figure 4. Cytotoxicity of andrographolide on THP-1 cells. THP-1 cells were treated with different concentration of andrographolide (5, 10, 20, 50 μM) and incubated for 24 hrs. Cell viability was measured by a colorimetric assay at 550 nm based on the ability of mitochondria to reduce the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) in viable cells. Percentage of viability is presented as mean ± S.E.M. of three to four independent experiments. ∗: $P < 0.05$ as compared with the resting.

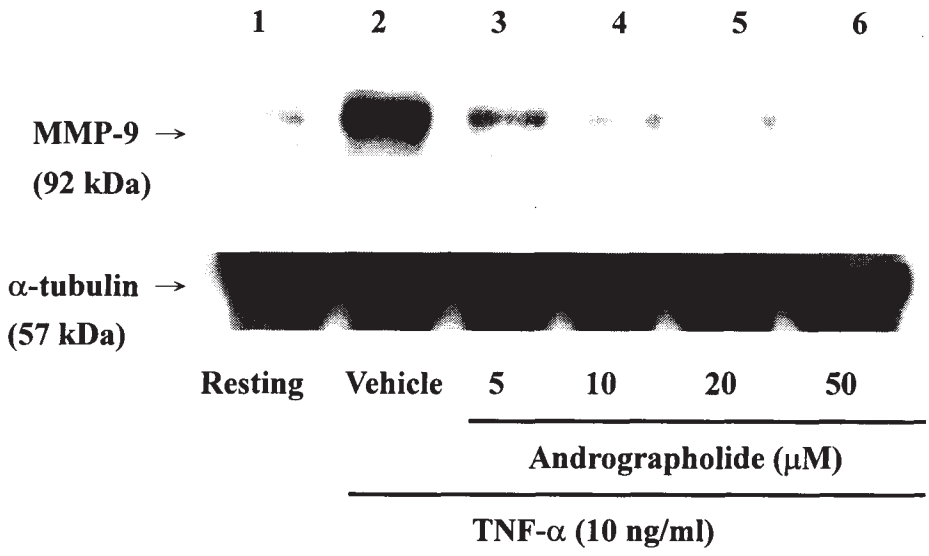

Figure 5. Effect of andrographolide on TNF-α-induced production of matrix metalloproteinase-9 (MMP-9) from conditioned medium of THP-1 cells. THP-1 cells (1×10^6 cell/ml) were dispensed on 24-well plates till 70-80 % confluent condition and treated with andrographolide (lane 3, 5 μM; lane 4, 10 μM; lane 5, 20 μM; lane 6, 50 μM) or vehicle (lane 2) for 15 min before treatment with TNF-α (10 ng/ml) for 24 hrs. Then supernatants were obtained and analysed for MMP-9 protein expression by Western blot (lane 1, control). The data are representative example of three experiments.

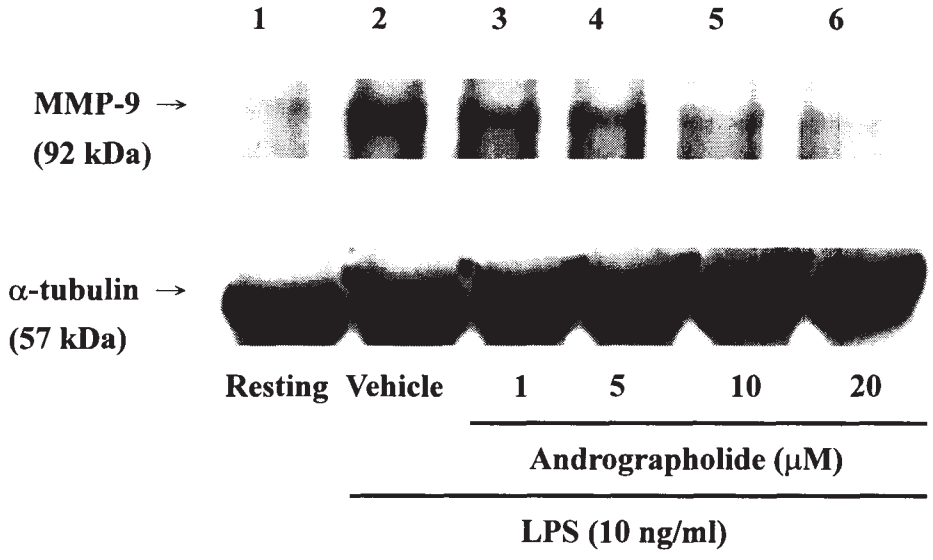

Figure 6. Effect of andrographolide on LPS-induced production of matrix metalloproteinase-9 (MMP-9) from conditioned medium of THP-1 cells. THP-1 cells (1×10^6 cell/ml) were dispensed on 24-well plates till 70-80 % confluent condition and treated with andrographolide (lane 3, 1 μM; lane 4, 5 μM; lane 5, 10 μM; lane 6, 20 μM) or vehicle (lane 2) for 15 min before treatment with LPS (10 ng/ml) for 24 hrs. Then supernatants were obtained and analysed for MMP-9 protein expression by Western blot (lane 1, control). The data are representative example of three experiments.

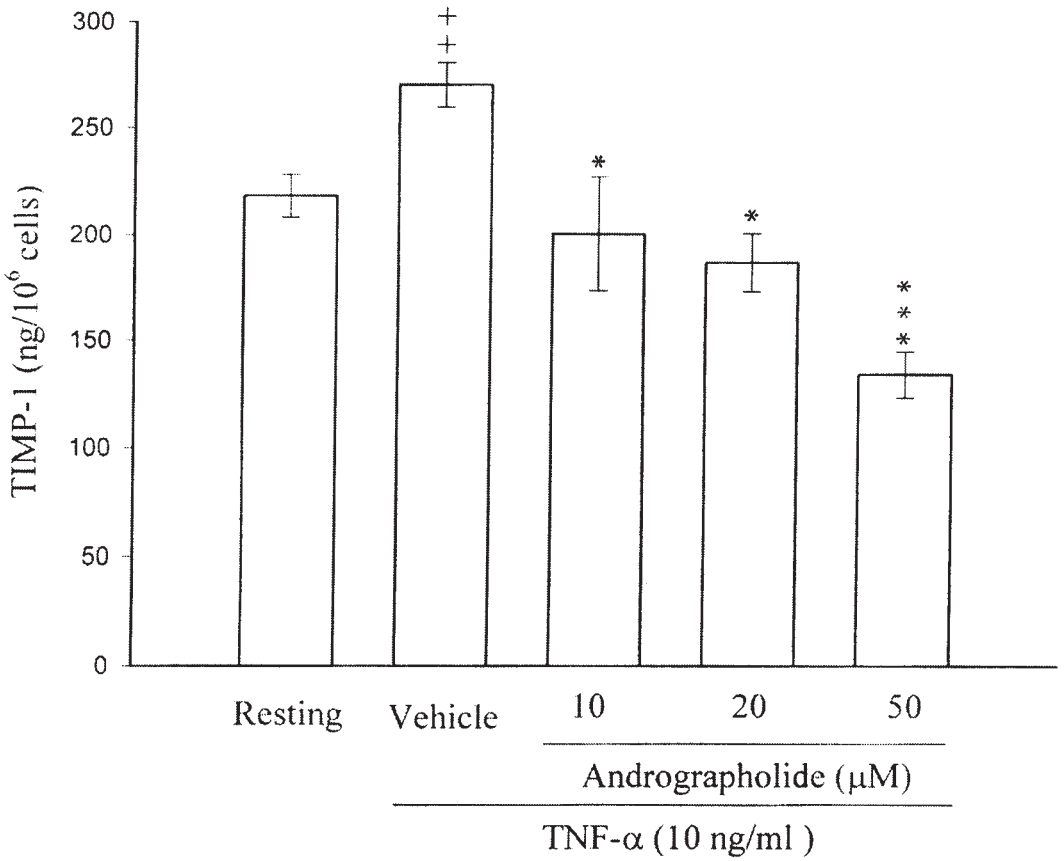

Figure 7. Effect of andrographolide on TNF-α-induced production of tissue inhibitor of metalloproteinase-1 (TIMP-1) from conditioned medium of THP-1 cells. THP-1 cells (1×10^6 cell/ml) were dispensed on 24-well plates till 70-80 % confluent condition and treated with different concentration of andrographolide (10, 20, 50 μM) for 15 min before treatment with TNF-α (10 ng/ml) for 24 hrs. Then supernatants were obtained and analysed for TIMP-1 protein expression by ELISA. Percentage of viability is presented as mean ± S.E.M. of four to five independent experiments. $++$: $P < 0.01$ as compared with the resting; $*$: $P < 0.05$, $***$: $P < 0.001$ as compared with the control.

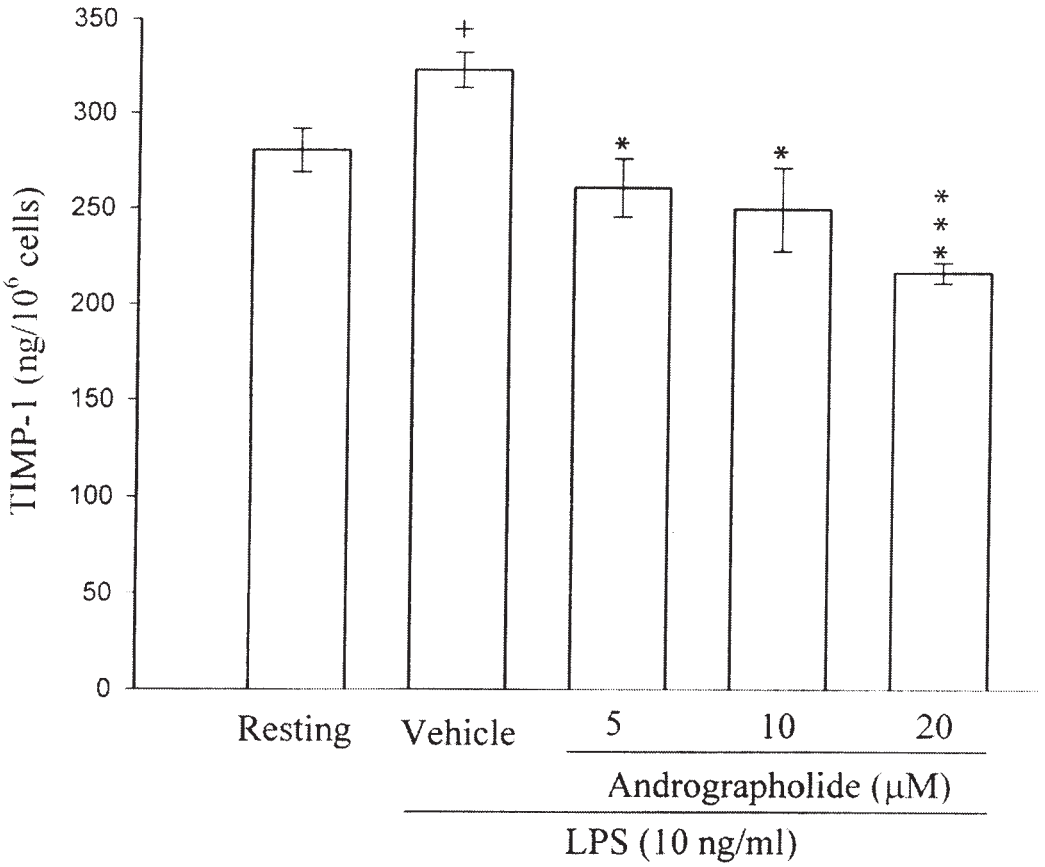

Figure 8. Effect of andrographolide on LPS-induced production of tissue inhibitor of metalloproteinase-1 (TIMP-1) from conditioned medium of THP-1 cells. THP-1 cells (1×10^6 cell/ml) were dispensed on 24-well plates till 70-80 % confluent condition and treated with different concentration of andrographolide (5, 10, 20 μM) for 15 min before treatment with LPS (10 ng/ml) for 24 hrs. Then supernatants were obtained and analysed for TIMP-1 protein expression by ELISA. Percentage of viability is presented as mean ± S.E.M. of three to five independent experiments. +: $P < 0.05$ as compared with the resting; ∗: $P < 0.05$, ∗∗∗: $P < 0.001$ as compared with the control.

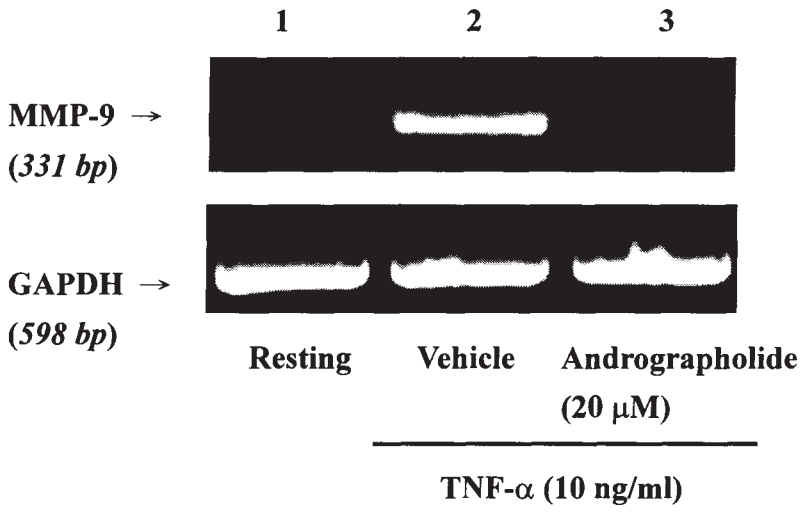

Figure 9. RT-PCR analysis demonstrating the effect of andrographolide on TNF-α-induced MMP-9 mRNA expression in THP-1 cells. Cells were treated with andrographolide (lane 3, 20 μM) or vehicle (lane 2) for 15 min before treatment with TNF-α (10 ng/ml) for 6 hrs. Following by extraction of total RNA and analysis of mRNA levels of MMP-9 and GAPDH. RT-PCR technique was performed as described in "Methods" (lane 1, control). GAPDH levels normalized the amount of cDNA temple used in each PCR reaction. The data are representative example of three experiments.

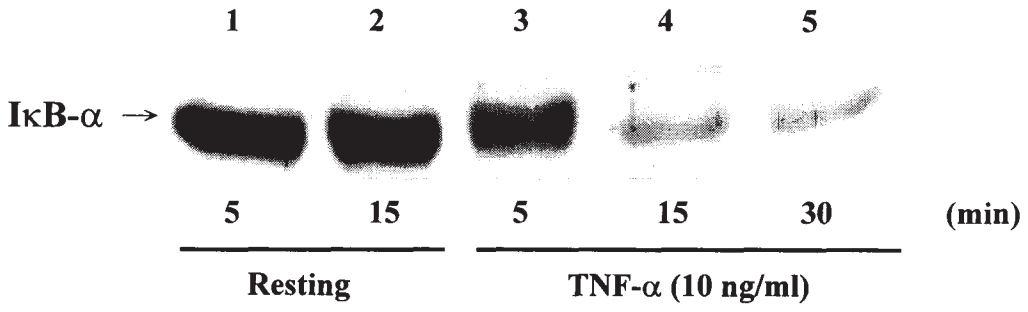

Figure 10. Western blot analysis demonstrating the time course on degradation of immunoreactive IκB-α in THP-1 cells (1×10^6 cell/ml). THP-1 cells were dispensed on 6-well plate till 70-80 % confluent condition and treated with TNF-α (lane 3, 5 min; lane 4, 15 min; lane 5, 30 min) or vehicle (lane 1, control 5 min; lane 2, control 15 min) as indicated. The data are representative example of three experiments.

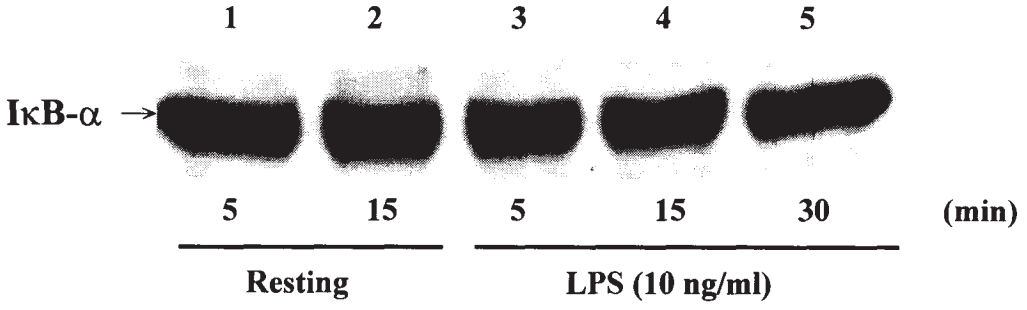

Figure 11. Western blot analysis demonstrating the time course on degradation of immunoreactive IκB-α in THP-1 cells (1×10^6 cell/ml). THP-1 cells were dispensed on 6-well plate till 70-80 % confluent condition and treated with LPS (lane 3, 5 min; lane 4, 15 min; lane 5, 30 min) or vehicle (lane 1, control 5 min; lane 2, control 15 min) as indicated. The data are representative example of three experiments.

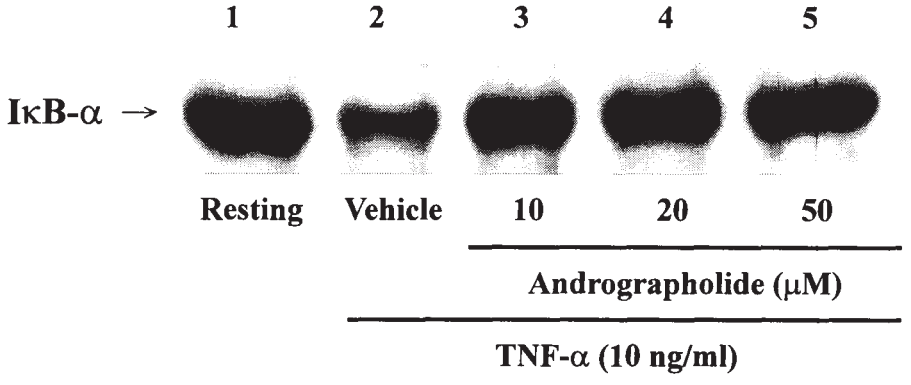

Figure 12. Effect of andrographolide on degradation of immunoreactive IκB-α in THP-1 cells. THP-1 cells (1×10^6 cell/ml) were dispensed on 6-well plate till 70-80 % confluent condition and treated with TNF-α (10 ng/ml) for 15 min as indicated. Cells were treated with andrographolide (lane 3, 10 μM; lane 4, 20 μM; lane 5, 50 μM) or vehicle (lane 2) for 15 min before treatment with TNF-α. Then cells were obtained and analysed for IκB-α protein expression by Western blot (lane 1, control). The data are representative example of three experiments.

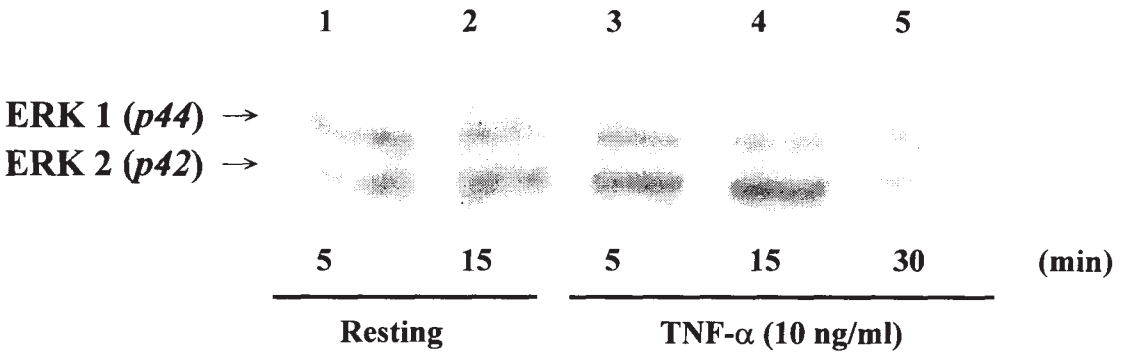

Figure 13. Western blot analysis demonstrating the time course on degradation of immunoreactive ERK 1/2 in THP-1 cells (1×10^6 cell/ml). THP-1 cells were dispensed on 6-well plate till 70-80 % confluent condition and treated with TNF-α (lane 3, 5 min; lane 4, 15 min; lane 5, 30 min) or vehicle (lane 1, control 5 min; lane 2, control 15 min) as indicated. The data are representative example of three experiments.

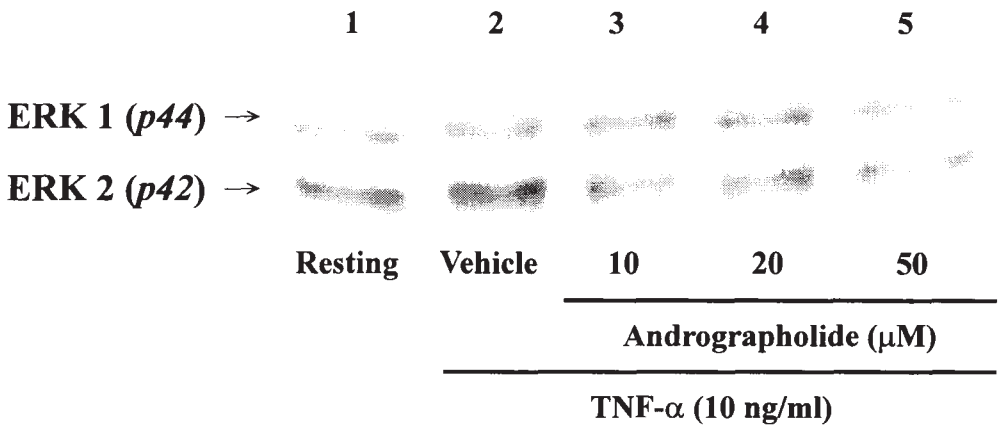

Figure 14. Effect of andrographolide on degradation of immunoreactive ERK 1/2 in THP-1 cells. THP-1 cells (1×10^6 cell/ml) were dispensed on 6-well plate till 70-80 % confluent condition and treated with TNF-α (10 ng/ml) for 15 min as indicated. Cells were treated with andrographolide (lane 3, 10 μM; lane 4, 20 μM; lane 5, 50 μM) or vehicle (lane 2) for 15 min before treatment with TNF-α. Then cells were obtained and analysed for ERK 1/2 protein expression by Western blot (lane 1, control). The data are representative example of three experiments.

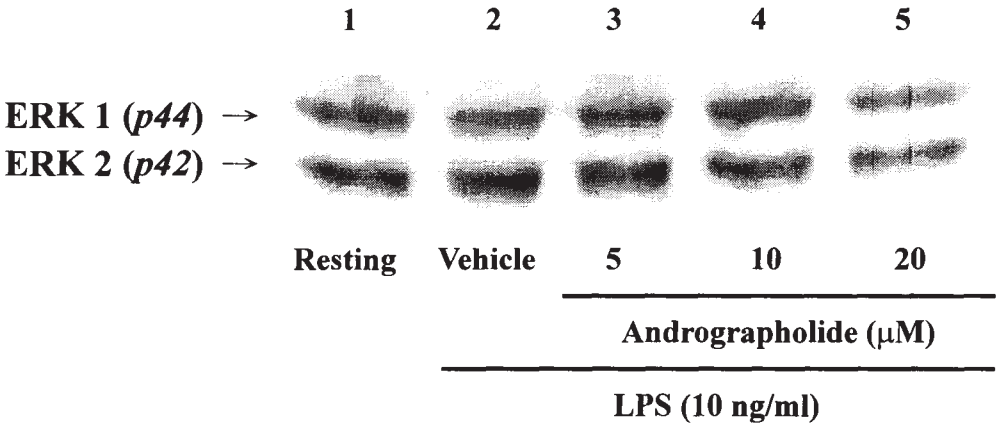

Figure 15. Effect of andrographolide on degradation of immunoreactive ERK 1/2 in THP-1 cells. THP-1 cells (1×10^6 cell/ml) were dispensed on 6-well plate till 70-80 % confluent condition and treated with LPS (10 ng/ml) for 15 min as indicated. Cells were treated with andrographolide (lane 3, 5 μM; lane 4, 10 μM; lane 5, 20 μM) or vehicle (lane 2) for 15 min before treatment with LPS. Then cells were obtained and analysed for ERK 1/2 protein expression by Western blot (lane 1, control). The data are representative example of three experiments.

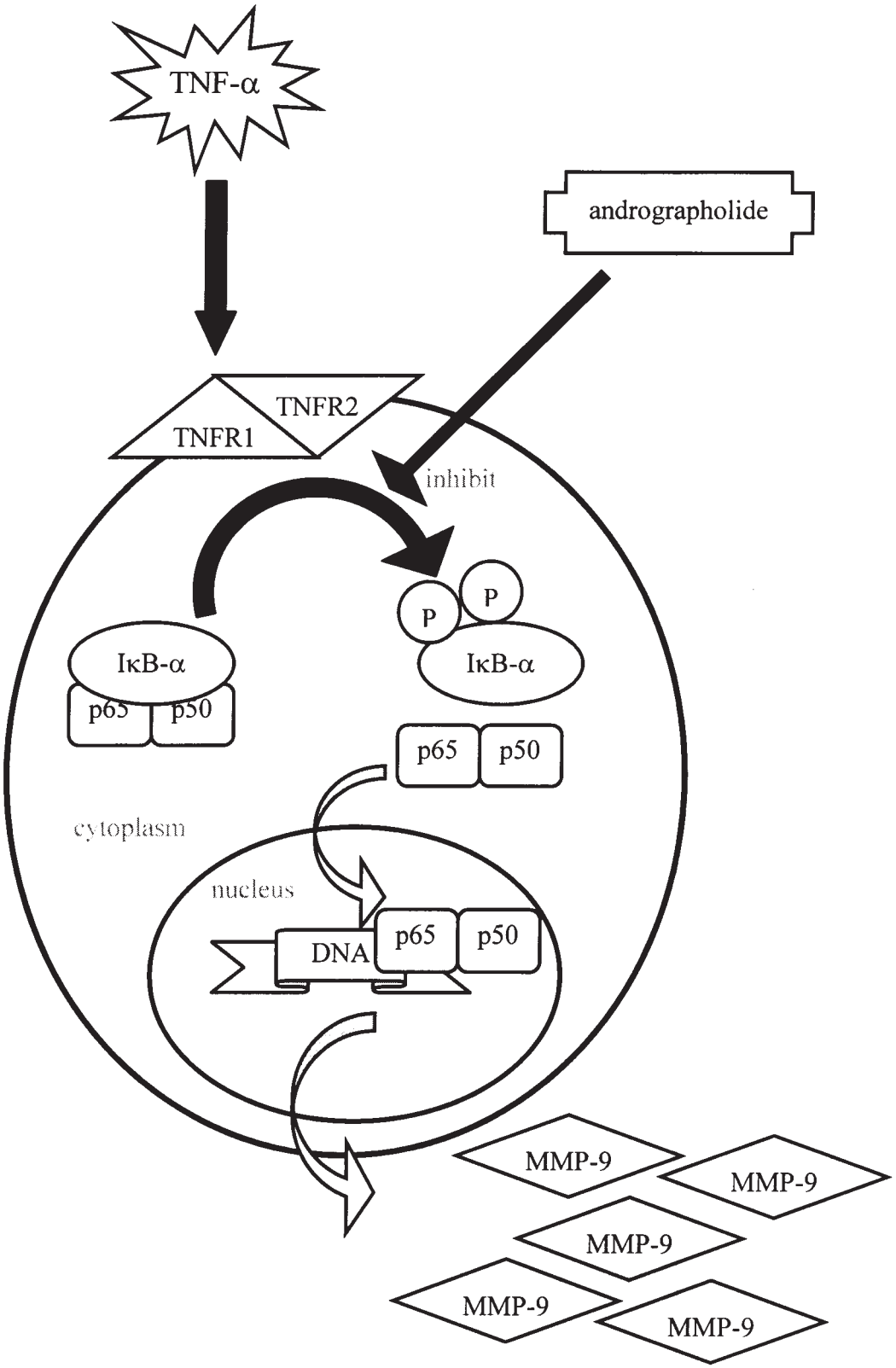

Figure 16. We found that Chinese herbal compound, andrographolide, with inhibitory effect on MMP-9 expression, and its main mechanism of action might through NF-κB signal pathway on TNF-α stimulation.

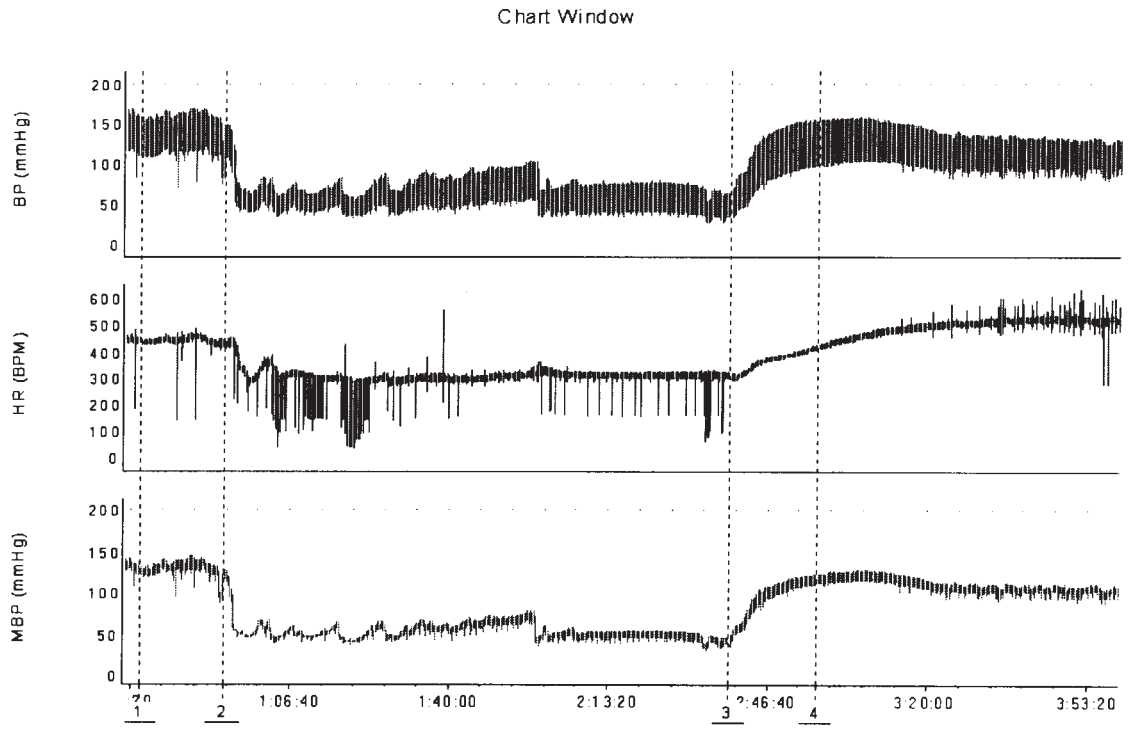

Figure 17. Hemorrhagic shock protocol. Wistar rats were hemorrhaged to a mean arterial pressure (MAP) of 40-50 mmHg and were given saline after 60 min of shock for 1 to 4 hours. The recordings were typical assays of four independent experiuments.