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行政院國家科學委員會補助專題研究計畫成果報告

探討 Mitogen-Activated Protein Kinase 在氧化低密度脂蛋白及發炎性細胞激素對內皮與巨噬細胞誘發 Matrix metalloproteinase 活化之角色

The studies of the roles of mitogen-activated protein kinase on oxidized LDL- or inflammatory cytokines-induced the activation of matrix metalloproteinase in endothelial cells and macrophages

計畫類別：■個別型計畫　□整合型計畫
計畫編號：NSC 89-2320-B-038-058
執行期間：89 年 8 月 1 日至 90 年 7 月 31 日

計畫主持人：蕭哲志
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執行單位：台北醫學大學醫學系藥理科

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一、中文摘要

基質金屬酵素在許多疾病中扮演相當重要的角色，尤其癌症的轉移與血管斑塊剝離在臨床上更受到重視。本計畫之目的在探討分裂素活化蛋白酵素是否在細胞激素或氧化型人類低密度脂蛋白所誘發細胞基質金屬酵素的表現與活化上所扮演的角色。從實驗結果發現，的確細胞激素（如腫瘤壞死因子）能使單核球細胞引發大量基質金屬酵素的表現與活化，尤其第九型基質金屬酵素的表現特別明顯。我們也發現兩種分裂素活化蛋白酵素抑制劑(PD98059 與 SB203580)二者均能抑制電泳酵素分析之基質金屬酵素的分解作用，且隨濃度增高而抑制性越強。但二者對於第九型基質金屬酵素之酵素活性並不影響。而這些抑制作用也不是源自於細胞傷害。另外，未刺激之人類臍帶靜脈內皮細胞能持續釋放多量的第二型基質金屬酵素，二者抑制劑對此基質金屬酵素之活化並不影響。初步活體大鼠總頸動脈氣球擴張損傷後，血管明顯提高 MMP-9 的生成與釋放。從這些結果可提供有關分裂素活化蛋白酵素在不同血管性細胞基質金屬酵素活化過程時之重要性，以利活體穩定血管(vascular stability)或癌症實驗之參考。

關鍵詞：基質金屬蛋白酵素、分裂素活化蛋白酵素、單核球、內皮細胞

Abstract

Matrix metalloproteinases (MMPs) have the important role in the pathogenesis of atherosclerotic plaque destabilization. The purpose of this study was to determine if mitogen-activated protein kinase might involve in the cytokine- or oxLDL-induced MMPs activation, and thus represents an attractive therapeutic target. Exposure monocyte (THP-1) to TNFα (10 ng/ml) increased MMP-9 protein expression as measured by gelatinolytic activity as determined by zymography. We found that treatment with MAPK inhibitors (PD 98059 or SB 203580) significantly decreased MMP-9 gelatinolytic at a concentration-dependent manner. However, both inhibitors were without any inhibition on MMP-9 activity. These inhibitory actions on gelatinolytic activity by MAPK inhibitors were not mediated by

reduction of cellular viability. Differently, non-stimulated HUVEC could expressed much amount of MMP-2. Such constitutve expression of MMP-2 was no clearly affected by MAPK inhibitors. On the other hand, according to preliminary animal studies, the MMP-9 activity was clearly elevated on the balloon-injuried common carotid artery. These data demonstrate that MAPK play an important role on induction of different MMPs. This newly described action of MAPK inhibition might prove useful to inhibit matrix degradation and to improve vascular stability, or even cancer invasion.

Keywords: matrix metalloproteinase, mitogen-activated protein kinase, monocyte, endothelial cell

二、緣由與目的

發炎性細胞激素(inflammatory cytokines)或氧化變性之低密度脂蛋白(oxidized low-density lipoprotein)均能刺激發炎性單核球或巨噬相關細胞局部釋放出分解結締組織之 matrix metalloproteinase (MMPs) 酵素，而使得纖維帽(fibrous cap)內纖維結構易於鬆脫(weaken)，呈現不穩定(instability)狀態，進而容易剝離而造成血栓(1, 2)。這些 Matrix metalloproteinses (MMPs)為一群結構類似，催化活性部位含鋅(Zinc ion)金屬離子之蛋白酵素，它們能夠催化分解維持組織結構之細胞外基質蛋白(extracellular matrix proteins, ECM)，如 collagen、elastin、lamin 及 glycoprotein 等。因此對於組織之結構重組(remodeling)、修補 (repairing)與破壞(destroy)都具有相當重要之角色(3)。同時 MMP 的含量與活性表現均受到許多方式嚴密地調節控制(4)。特別在血管斑塊病灶大量釋放與活化 MMPs 遂造成斑塊表面內皮細胞剝蝕脫落(endothelial erosion)而形成血栓(5, 6)。目前已知許多疾病，包括癌細胞轉移(metastasis)、關節炎(arthritis) 等致病機轉均與 MMP 關係密切。

分裂素活化蛋白酵素（mitogen-activated protein kinase, MAPK）屬於 serine-threonine kinase 酵素，此酵素成分為細胞對外界刺激反應作用之重要細胞內訊息介質。目前在文獻上已知哺乳動物細胞中有四種相關 MAPKs 被確認（7, 8）。三種重要的 MAPKs 為：（一） extracellular signal-regulated kinase (ERK1/2)、(二)c-Jun N-terminal kinase (JNK) 與（三）p38 MAPK 等。這些相關酵素可受外來生長因子、自泌素或物理性傷害所活化。尤其 JNK 與 p38MAPK 特別會因熱傷害、紫外線照射與發炎細胞激素所刺激而活化(9)。MAPKs 在細胞訊息傳遞過程中，自身須被磷酸化才具有活化之酵素型態，以催化改變特定轉錄因子的活性，而影響基因的表現(10)。相關文獻也顯示 JNK 的活化的確在氧化性損傷所造成細胞 MMP 之釋放活化占有重要角色(11)。

本計劃之目的在於了解 MAPKs 在發炎性細胞激素或氧化低密度脂蛋白對內皮或單核球（巨噬細胞）產生與活化 MMP 作用機轉之角色，並評估 MAPKs 抑制劑在動物模式粥狀動脈硬化血管剝離預防及治療上(plaque stabilization therapy)之可能性。

三、結果與討論

根據先導實驗結果確立電泳酵素分析法(Zymography)與刺激物（如 TNFα）引發 MMP 之實驗標準條件。由於巨噬細胞源自於單核球細胞，而人類單核球細胞（THP-1）目前廣泛為學界研究 MMP 所應用。實驗中我們發現以 TNFα濃度 10 ng/ml 刺激 24 小時為佳。由電泳酵素分析圖更顯示單核球(THP-1)分解 gelatin 主要以 92 kDa 之 MMP-9 為主，而 72 kDa 之 MMP-2 為次要。

關於 MAPK 抑制劑相關藥物對於細胞 MMP 等之作用，我們先以 PD98059 與 SB203580 等（前者為 MEK 抑制劑，後者為 p38 MAPK 抑制劑）進行實驗。另外以 PMA（positive control）及 Doxycycline 或 Minocycline（negative control）進行相關比較性實驗。電泳酵素分析實驗發現 TNFα 刺激單核球細胞所引起之 gelatin 崩解作用，尤其是 MMP-9 之分解反應，可明顯且有意義被 PD98059 或 SB203580 隨濃度升高所抑制，且完全被

Doxycycline 所抑制。特別是 PD98059 較 SB203580 的抑制程度高，其 IC_{50} 分別為 1.41±0.2 與 5.6±1.3 μM（圖一）。另外，在同樣實驗條件下，PMA 的確可引起大量 MMP-9 崩解反應，尤其活化型(active form)可明顯出現。

另外，根據人類臍帶靜脈內皮細胞（HUVEC）的電泳酵素分析實驗結果知，HUVEC 在未受刺激時，便會釋放大量 72 kDa 之 MMP-2 與少量 92 kDa 之 MMP-9，此結果與其它相關文獻一致。當以 PMA 刺激下，除了 MMP-2 會增加反應與其活化型(active form)出現外，MMP-9 也同時會明顯產生作用，但仍以 MMP-2 為主。PD98059 或 SB203580 二者不影響 MMP-2 之作用。

以間接酵素方法，測定 MMP-9 之酵素活性。根據初步實驗成果顯示以 PMA 刺激單核球細胞所獲得的培養液(conditioned medium)含高量 MMP-9 酵素活性。PD98059 或 SB203580 二者在 10 μM 濃度下均不影響 MMP-9 之酵素活性（圖二）。為確定測試成分抑制電泳酵素分析之效果非源自於細胞傷害，所以也利用 MTT 方法進行細胞存活實驗。從結果顯示不論是單核球或內皮細胞以測試成分高濃度 10 μM 處理時，並不具有細胞致毒作用（圖三、四）。

另外，在初步活體動物實驗發現，大鼠以氣球擴張術損傷血管三週後，受損的總頸動脈組織均質液其 MMP-9 酵素活性較正常血管均質液有義意增高約五倍以上（圖五）。

我們目前實驗正進行以 PD98059 或 SB203580 對於 oxidized cholesterol 或 oxidized LDL 影響單核球或內皮細胞釋放 MMPs 的程度。同時針對 MAPK 機轉之探討也進行蛋白質磷酸化的 Western blotting 分析。初步至少由 gelatin 分解與 MMP-9 酵素活性之結果，我們推測 PD98059 與 SB203580 可能直接影響 MMP-9 生成。當然下一步有關 TIMP 之定量也將進行。另外我們也正嘗試其它 cytokines 以了解對 monocyte 或 HUVEC 所誘發 MMP 作用。為了解粥狀動脈硬化症斑塊形成中 MAPK 在 MMP 生成之角色，目前我們先以大鼠總頸動脈氣球擴張術損傷血管，發現這種 neointima 生成具明顯 MMP-9 的生成與釋放。未來將以此實驗方式進行高血脂倉鼠的氣球擴張術，以符合粥狀動脈硬化症的動物模式。

四、計畫成果自評

此研究計劃為本人第二次取得之國科會的研究計劃，而相關之研究設備較有改善。但仍須藉用其它單位的儀器進行實驗時較為不便。兩次計劃綜合所購置之螢光倒立顯微鏡，確實改善了實驗的品質與進度。本實驗室保持了兩種細胞的培養，其中包括人類初級細胞與細胞株（內皮細胞與單核球細胞）。在本次計劃所發現 PD98059 與 SB203580 對不同細胞釋放 MMP 的影響性，說明 MAPKs 的確在 cytokine 刺激下參與重要訊息傳遞的角色。同時也設立了 MMP 之酵素分析法與 RT-PCR，由於後者為初步階段，故未呈現結果。關於粥狀動脈硬化症的動物模式的設立，本實驗室嘗試先以設立大鼠總頸動脈氣球擴張術，再應用於高血脂倉鼠，以符合粥狀動脈硬化症的病理要求。目前本實驗室已完成設立大鼠總頸動脈氣球擴張術。未來將嘗試以水溶性 MAPK 抑制劑，以探討倉鼠活體內粥狀動脈斑塊之剝離穩定性。

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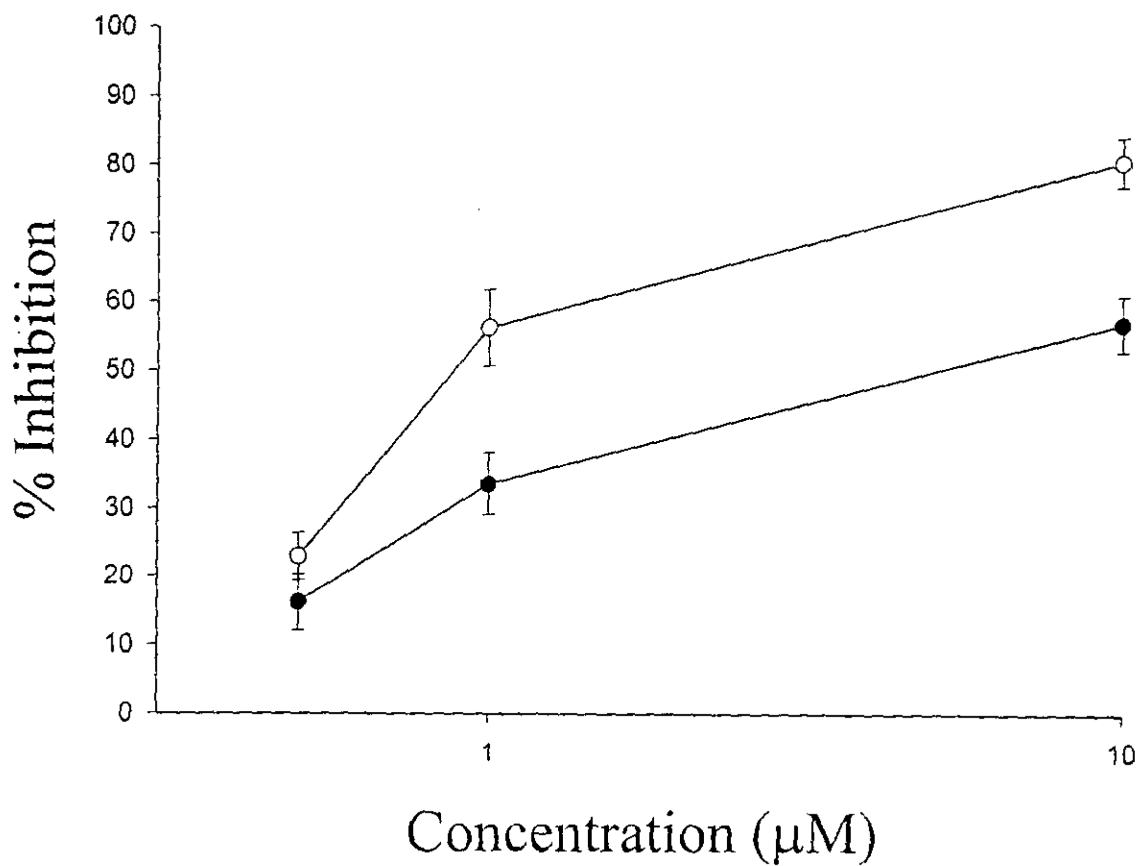

Figure 1. Effects of MAPK inhibitors on TNFα (10 ng/ml)-induced MMP-9 secretion by monocytes (THP-1 cells). THP-1 cells (2×10^6/ml) were incubated with MAPK inhibitors (PD98059, open circle or SB203580, filled circle) for 30 min, then stimulated with TNFα for 24 hours. The cell supernatants were then removed, added to the SDS sample buffer and incubated for 10 min at room temperature, then analyzed by zymography on 10 % acrylamide-0.1 % gelatin gels. Densitometric analysis of gelatinolytic activity (MMP-9) were done. Percent inhibition are expressed as mean ± S.E.M. (n = 3).

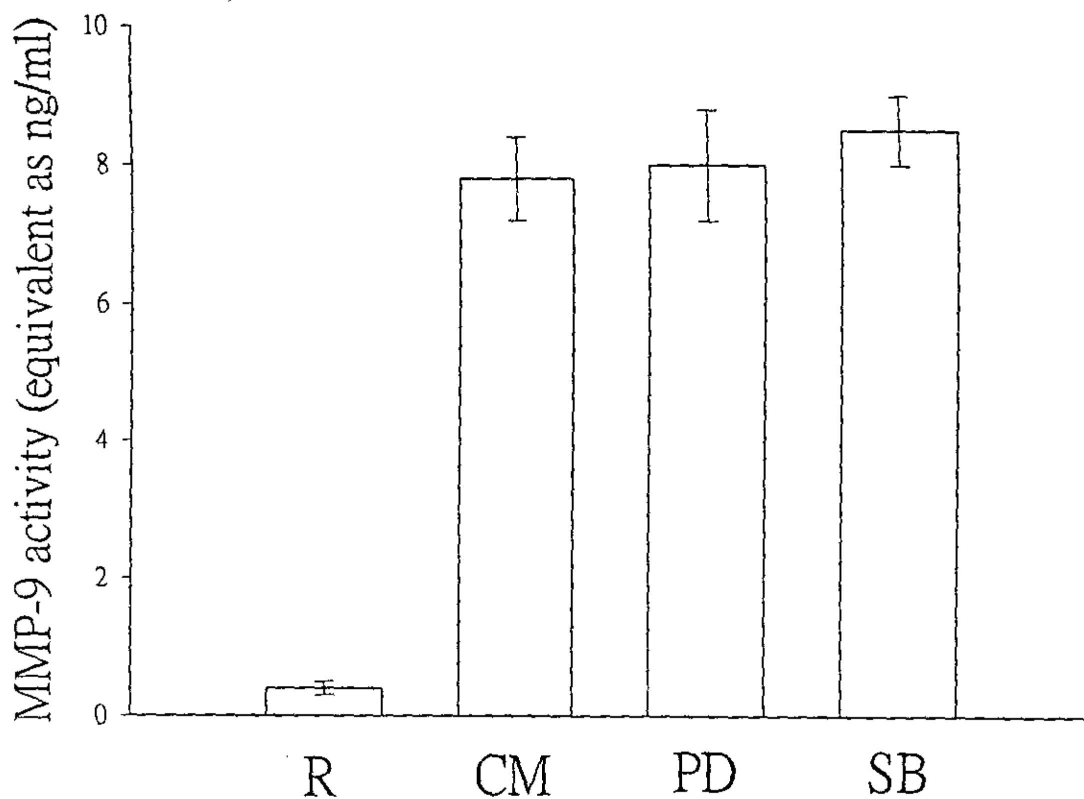

Figure 2. Effects of MAPK inhibitors on PMA (10 ng/ml)-induced MMP-9 activity of THP-1 cells. PMA-stimulated cell conditioned medium were treated with MAPK inhibitors (PD98059 or SB203580), then detect the enzymatic activity (MMP-9). Culture medium were collected at 24 hours for measurement of MMP-9 activity expression by ELISA reader. Untreated THP-1 cells (R). Before activity test, conditioned medium were treated with DMSO (CM), PD98059 (PD, 10 μM) or SB203580 (SB, 10 μM), respectively. Data are expressed as mean ± S.E.M. (equivalent as ng/ml) (n = 3).

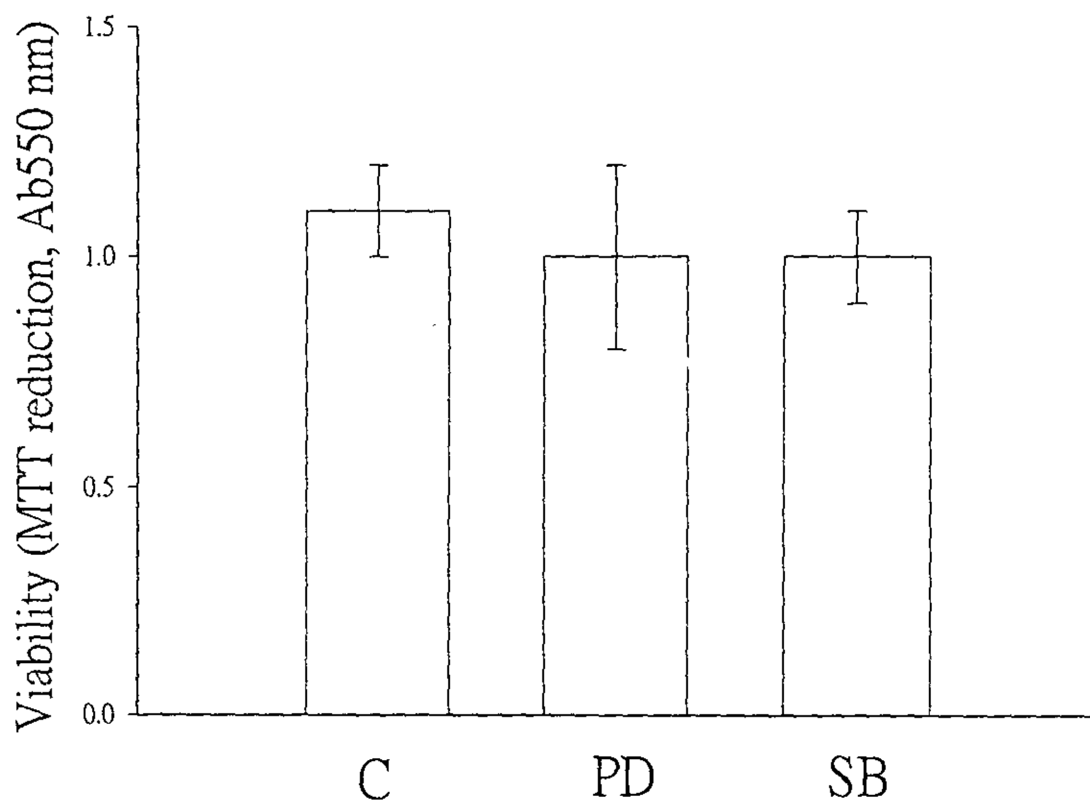

Figure 3. Effects of MAPK inhibitors on THP-1 viability. THP-1 were 80-90 % confluent and treated with MAPK inhibitors (PD98059, PD or SB203580, SB) for 22 hours, then changed medium to MMT (final concentration, 0.5 mg/ml) medium. After 2 hours, cells were washed and dissolved with DMSO. Using the spectrophotometric method by ELISA reader to analyze the absorption at 550 nm. Absorption values were expressed as mean ± S.E.M. (n = 3).

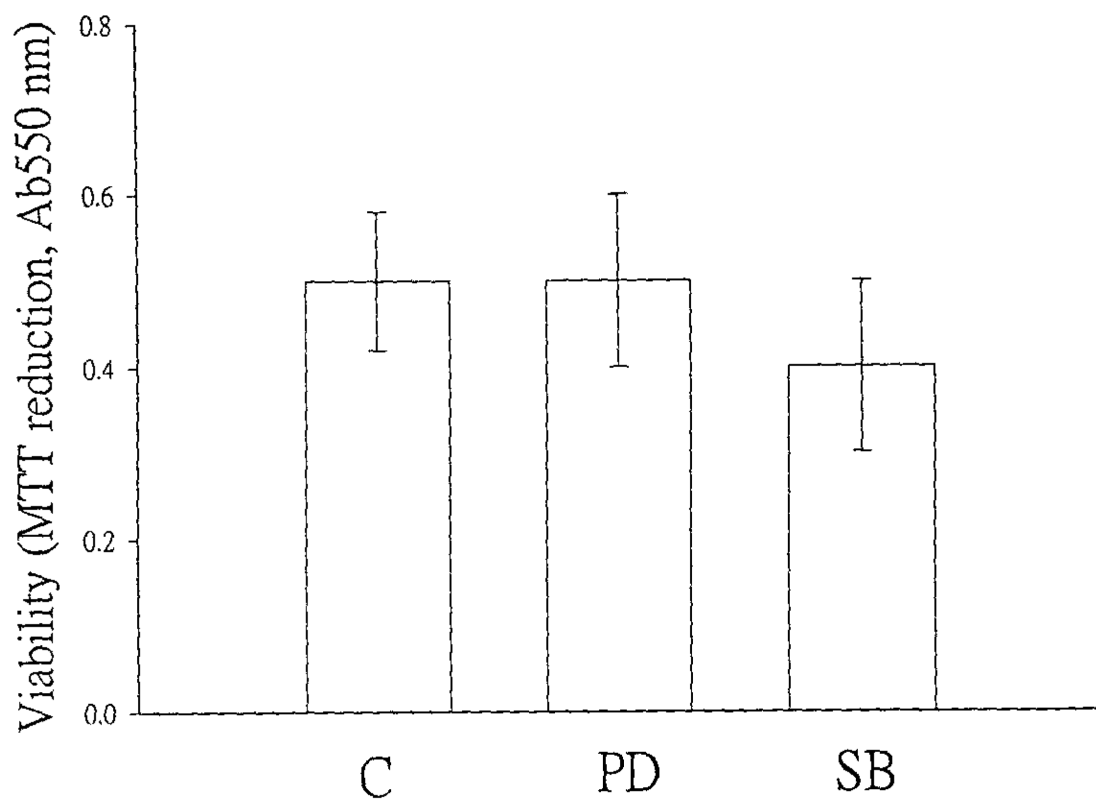

Figure 4. Effects of MAPK inhibitors on HUVEC viability. HUVECs were 80-90 % confluent and treated with MAPK inhibitors (PD98059, PD or SB203580, SB) for 22 hours, then changed medium to MMT (final concentration, 0.5 mg/ml) medium. After 2 hours, cells were washed and dissolved with DMSO. Using the spectrophotometric method by ELISA reader to analyze the absorption at 550 nm. Absorption values were expressed as mean ± S.E.M. (n = 3).

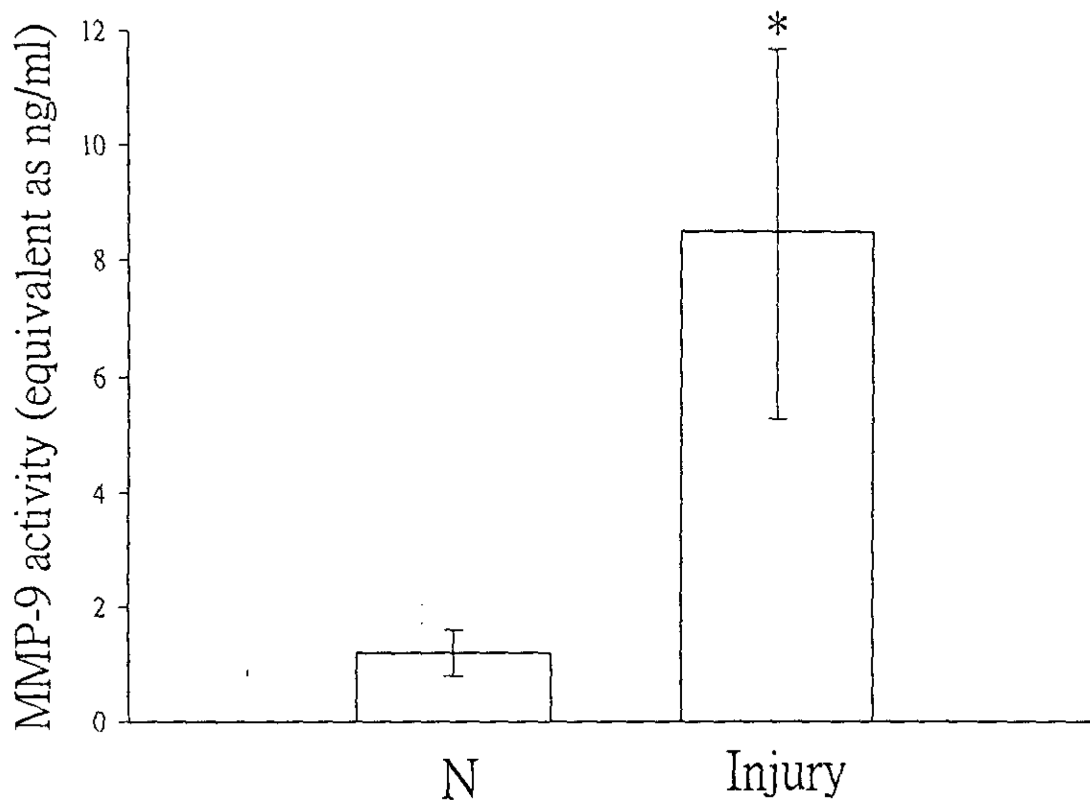

Figure 5. Effects of vascular balloon-injury on tissue MMP-9 activity . One side of commen carotid artery of Wistar rat were damaged by 2F Fogarty embolectomy catheter, then 3 weeks latter, detect the enzymatic activity (MMP-9) of vascular homogenates (Injury). The measurement of MMP-9 activity was by ELISA reader. Untreated normal artery was also analyzed (R). Data are expressed as mean ± S.E.M. (equivalent as ng/ml) (n = 3-4). $*P < 0.05$ when compared with the normal condition.