行政院國家科學委員會專題研究計畫 期末報告

比較二氫鮫啶類鈣離子通道阻斷劑在脂多醣體/干擾素-γ處理的細胞及內毒素血症小鼠對誘發性一氧化氮合成酶、基質金屬蛋白酵素的表現與 HMGB1 釋放的作用

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計 畫 主 持 人：劉淑芬
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報 告 附 件 ：出席國際會議研究心得報告及發表論文

公 開 資 訊 ：本計畫涉及專利或其他智慧財產權，1 年後可公開查詢

中 華 民 國 102 年 09 月 27 日

中文摘要：目的：發炎是粥狀動脈硬化的重要分子基礎。最近的研究顯示，二氫吡啶類鈣離子通道阻斷劑在血管機能失調實驗模式中具強力抗發炎能力。 本研究的目的是評估新一代二氫吡啶類鈣離子通道阻斷劑 lercanidipine 與 labedipinedilol-A，對於以脂多醣體/干擾素-γ 誘發的血管平滑肌細胞的抗發炎效果及相關作用機轉的探討。
方法和結果：實驗方法包括 MTT、Griess reagent、RT-PCR、ELISA、gelatin zymography、immunocytochemistry 和 Western blotting。實驗結果發現 lercanidipine 與 labedipinedilol-A 可減少脂多醣體/干擾素-γ 刺激血管平滑肌細胞釋放 NO、ROS、TNF-α 和 IL-1β 汹。此外，lercanidipine 與 labedipinedilol-A 減少了脂多醣體/干擾素-γ 誘導的 iNOS 蛋白和 mRNA 表現，並減少 HMGB1 從細胞核易位到細胞質和隨後的胞外釋放量。還有，他們會抑制基質金屬蛋白酶-2/基質金屬蛋白酶-9 的活性，而促進基質金屬蛋白酶-9 的抑制劑-金屬蛋白 組織抑制因子-1 的活性。最後，我們發現 lercanidipine 與 labedipinedilol-A 抑制細胞核因子-κB 核易位和抑制 JNK、P38 MAPK 和 Akt 蛋白質的磷酸化。
結論：lercanidipine 與 labedipinedilol-A 能夠有效抑制 LPS/IFN-γ 誘導的血管平滑肌細胞發炎反應以及抑制 iNOS、COX-2 蛋白表現和 NO、TNF-α 、ROS 的釋放，lercanidipine 與 labedipinedilol-A 也減少了 LPS/IFN-γ 誘導的 MMP-2 和 MMP-9 的酵素活性及蛋白表現量，此作用機轉是經由抑制 LPS/IFN-γ 誘發 MAPKs、Akt、IκBα 的磷酸化進而減少 NF-κB 次單元 p65 的核轉移之訊息傳遞路徑。這些研究結果顯示新一代的二氫吡啶類鈣離子通道阻斷劑 lercanidipine 與 labedipinedilol-A 可經由抗發炎之分子機轉而具有血管保護作用，因此這些成果將提供未來對於發炎性血管疾病如粥狀動脈硬化治療的新方向。

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英文摘要：Objective: Inflammation is an important molecular basis of atherosclerosis. Recent studies have shown that dihydropyridine calcium channel blockers (CCBs) can exert potent anti-inflammatory effects in models of vascular dysfunction. The purpose of the present study was to evaluate anti-inflammatory effects and

mechanisms of lercanidipine and labedipinedilol-A, new generation dihydropyridine CCBs, in rat vascular smooth muscle cells (VSMCs) exposed to lipopolysaccharide (LPS) and interferon-γ (IFN-γ). Methods and results: MTT, Griess reagent, RT-PCR, ELISA, gelatin zymography, immunocytochemistry and Western blotting were employed. We found that lercanidipine and labedipinedilol-A attenuated production of NO, ROS, TNF-α and IL-1β 洶 from LPS/IFN-γ-stimulated VSMCs. In addition, they both diminished the LPS/IFN-γ-induced expression of iNOS protein and mRNA, with attenuation of HMGB1 cytosolic translocation and subsequent extracellular release. Furthermore, they down-regulated MMP-2/MMP-9 activities, while expression of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), an inhibitor of MMP-9, was up-regulated. Finally, we found that lercanidipine and labedipinedilol-A inhibited the nuclear translocation of NF-κB and suppressed the phosphorylation of JNK, p38 MAPK and Akt. Conclusion: Lercanidipine and labedipinedilol-A can exert their anti-inflammatory effects through suppression of NO, ROS, TNF-α and IL-1β 洶 through down-regulation of iNOS, MMP-2/MMP-9, and HMGB1, with inhibition of signaling transduction of MAPKs, Akt/IkB-α and NF-κB pathways. These findings implicate a valuable role of new generation dihydropyridine CCBs lercanidipine and labedipinedilol-A in the treatment of inflammatory vascular diseases.

英文關鍵詞： dihydropyridine calcium channel blockers, vascular smooth muscle cells, lipopolysaccharide/interferon-γ, high mobility group box-1, matrix metalloproteinase, nuclear factor-κB

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結論：lercanidipine 與 labedipinedilol-A 能夠有效抑制 LPS/IFN-γ 誘導的血管平滑肌細胞發炎反應以及抑制 iNOS、COX-2 蛋白表現和 NO、TNF-α、ROS 的釋放，lercanidipine 與 labedipinedilol-A 也減少了 LPS/IFN-γ 誘導的 MMP-2 和 MMP-9 的酵素活性及蛋白表現量，此作用機轉是經由抑制 LPS/IFN-γ 誘發 MAPKs、Akt、IκBα 的磷酸化進而減少 NF-κB 次單元 p65 的核轉移之訊息傳遞路徑。這些研究結果顯示新一代的二氫吡啶類鈣離子通道阻斷劑 lercanidipine 與 labedipinedilol-A 可經由抗發炎之分子機轉而具有血管保護作用，因此這些成果將提供未來對於發炎性血管疾病如粥狀動脈硬化治療的新方向。

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Abstract

Objective: Inflammation is an important molecular basis of atherosclerosis. Recent studies have shown that dihydropyridine calcium channel blockers (CCBs) can exert potent anti-inflammatory effects in models of vascular dysfunction. The purpose of the present study was to evaluate anti-inflammatory effects and mechanisms of lercanidipine and labedipinedilol-A, new generation dihydropyridine CCBs, in rat vascular smooth muscle cells (VSMCs) exposed to lipopolysaccharide (LPS) and interferon-γ (IFN-γ).

Methods and results*:* MTT, Griess reagent, RT-PCR, ELISA, gelatin zymography, immunocytochemistry and Western blotting were employed. We found that lercanidipine and labedipinedilol-A attenuated production of NO, ROS, TNF-α and IL-1β from LPS/IFN-γ-stimulated VSMCs. In addition, they both diminished the LPS/IFN-γ-induced expression of iNOS protein and mRNA, with attenuation of HMGB1 cytosolic translocation and subsequent extracellular release. Furthermore, they down-regulated MMP-2/MMP-9 activities, while expression of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), an inhibitor of MMP-9, was up-regulated. Finally, we found that lercanidipine and labedipinedilol-A inhibited the nuclear translocation of NF-κB and suppressed the phosphorylation of JNK, p38 MAPK and Akt.

Conclusion: Lercanidipine and labedipinedilol-A can exert their anti-inflammatory effects through suppression of NO, ROS, TNF-α and IL-1β through down-regulation of iNOS, MMP-2/MMP-9, and HMGB1, with inhibition of signaling transduction of MAPKs, Akt/IkB-α and NF-κB pathways. These findings implicate a valuable role of new generation dihydropyridine CCBs lercanidipine and labedipinedilol-A in the treatment of inflammatory vascular diseases.

Key words: dihydropyridine calcium channel blockers, vascular smooth muscle cells, lipopolysaccharide/interferon-γ, high mobility group box-1, matrix metalloproteinase, nuclear factor-κB

目　　錄

一、前言 & 研究目的

Inflammation is thought to be one of the major processes that accelerate the progression of atherosclerosis. The inflammatory response involves complicated interactions among immunomodulatory cells, endothelial cells, and vascular smooth muscle cells (VSMCs). In atherosclerosis, the vascular endothelium barrier function become damage and is lost. The underlying vascular smooth muscle is thus exposed and activated by pathogens, resulting in the induction of inducible nitric oxide synthase (iNOS) and other inflammatory genes, leading to vascular collapse [1,2]. Previous studies demonstrated that iNOS expression is increased in VSMCs after exposure to lipopolysaccharide (LPS) or cytokines. Furthermore, the effect of LPS on iNOS expression is strengthened by cytokines [3].

Activation of the transcription factor nuclear factor (NF)-κB is an important step in the development of vascular damage, because it controls the expression of inducible genes, including many inflammatory mediators such as iNOS and cyclooxygenases-2 (COX-2). NF-κB activation is mediated by phosphorylation and subsequent degradation of inhibitory protein kappa B (IκB). NF-κB can then translocate to the nucleus where it facilitates the transcription of many genes, including proinflammatory cytokines, chemokines, and anti-apoptotic factors [4]. Inflammatory mediators stimulate the activation of NF-κB in all vascular cells involved in the development of atherosclerosis, including VSMCs, endothelial cells, and monocyte/macrophages. Therefore, inhibition of NF-κB has potential as a pharmaceutical target for the prevention of vascular events.

Several dihydropyridine calcium channel blockers (CCBs), including amlodipine, benidipine, nicardipine and nifedipine, have been reported to suppress the progression of atherosclerotic lesion formation in atherosclerotic model animals and in hypertension clinical studies [5,6]. CCBs also suppressed neointima thickening in vascular injury model animals [5,7]. Interestingly, most of these studies showed that dihydropyridine CCBs did not affect blood pressure. Based on evidences, in our previous studies, have demonstrated that lercanidipine and labedipinedilol-A, new generation 1,4-dihydropyridine-type CCBs, induce NO production in endothelial cells [8-10] and inhibit VSMCs migration, proliferation and reactive oxygen species generation in *in vitro* studies [11-13]. Wu *et al.* [11] reported that lercanidipine suppressed neointima thicking in the injured carotid artery of Wistar rats. Taken together, these findings support that lercanidipine and labedipinedilol-A may possess anti-inflammatory activities and the therapeutic potential to prevent atherosclerosis. However, whether these new generation dihydropyridine CCBs possess protective effects on LPS/interferon-γ (IFN-γ)-induced vascular inflammation is unknown.

In the present study, we investigated the protective effects of two new generation

dihydropyridine CCBs (lercanidipine and labedipinedilol-A) on rat aortic VSMCs stimulated by LPS/IFN-γ. The anti-inflammatory effects of lercanidipine and labedipinedilol-A were examined and compared with conventional CCB nifedipine, by investigating production of inflammatory mediators (NO, TNF-α, IL-1β and high mobility group box-1 (HMGB1)), protein expression of regulatory enzymes (iNOS, matrix metalloproteinase (MMP)-2 and MMP-9), and activation of transcription factor NF-κB, and related signaling molecules (MAPKs and Akt).

二、研究方法

2.1. Cell culture

VSMCs were isolated from the thoracic aorta of 10-12-week-old male Wistar rats as previously described [12,13]. Culture media were changed every 3 days and passage numbers from 3 to 6 were used for the experiments. To examine the effects of three CCBs, VSMCs were treated with LPS (100 μg/ml) and IFN-γ (100 U/ml) for 24 h in the presence or absence of nifedipine, labedipinedilol-A and lercanidipine (1 and 10 μM).

2.2. Cell viability assay (MTT)

Cell viability was assessed by MTT assay. The assay was performed according to the manufacturer's instructions. The amount of MTT formazan was quantified by determining the absorbance at 540 nm and 630 nm, using an ELISA reader (DYNEX Technologies, Germany).

2.3. Measurement of NO

The accumulation of nitrite, a metabolite of NO as an indirect indicator of NO production, was determined in the cell culture media by Griess reagent (1% sulfanilamide and 0.1% *N*-(1-naphthyl) ethylenediamide in 5% phosphoric acid). The culture supernatants were collected and mixed with an equal volume of Griess reagent and incubated at room temperature for 10 min. The absorbance was measured at 540 nm (OD_{540}).

2.4. Measurement of TNF-α and IL-1β

VSMCs (10^5/ml) were seeded in 24 well plates. The cells were pretreated with three CCBs at the indicated concentrations for 30 min, followed by co-treatment with LPS/IFN-γ for 12 h. Then the cell media were collected, and the concentrations of TNF-α and IL-1β were measured using enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's protocol.

2.5. Gelatin zymography

MMP-2 and MMP-9 activities were detected by gelatin zymography on pre-made 8% polyacrylamide gels containing 0.1% gelatin using 10 μl serum-free media from treated cultures. After electrophoresis, the gel was removed and incubated in 1× Zymogram Renaturing Buffer for 30 min at room temperature with gentle agitation. The gel was equilibrated for 30 min with 1× Zymogram Developing Buffer, and then incubated with fresh 1× Zymogram Developing Buffer overnight. The bands were visualized by staining for 30-60 min with a solution containing 0.1% Coomassie

R-250 in 40% ethanol and 10% acetic acid; followed by destaining for 2 h at room temperature in a solution containing 10% ethanol and 7.5% acetic acid. The images were taken by using UVP Biochemi EC3 imaging system.

2.6. Reverse-transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted with Trizol reagent by a standard protocol (Invitrogen, Carlsbad, Calif). Reverse transcription was performed with 5 μg mRNA and the random primer at 65°C for 5 min, then mixed with Moloney murine leukemia virus reverse transcriptase (RT) to react at 37°C for 1 h to obtain cDNA. Gene amplification was followed with RT-polymerase chain reaction (PCR). Primer sequences were as described: iNOS sense, 5'-CAGGAACCTACCAGCTCACTCT-3'; iNOS antisense, 5'-ACAAGATCAGGAGGGATTTCAA-3'; GAPDH sense, 5'-AACTTTGGCATTGTGGAAGG-3'; and GAPDH antisense, 5'-CCCTGTTGCTGTAGCCGTAT-3'. Reaction cycles for iNOS and GAPDH included 30 cycles of 94°C for 30 s, 61.4°C for 30 s, 72°C for 1 min, and a final incubation at 72°C for 7 min. Polymerase chain reaction products were analyzed on 1.5% agarose gel in the presence of 1 μg/ml of ethidium bromide.

2.7. Preparation of cytosolic and nuclear protein extracts

Separation and preparation of cytoplasmic (CE) and nuclear extracts (NE) were performed using NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. All of the fractionated protein solutions were stored at -80°C until analysis.

2.8. Western blot analysis

Cells were treated with three CCBs at the indicated concentrations for the indicated times and then stimulated with LPS/IFN-γ. The reactions were then terminated by washing with cold PBS for twice. The cells were then harvested and Western analyses were performed as we previously described [13].

2.10. Immunocytochemistry

VSMCs were pretreated with three CCBs (10 μM) for 30 min and then co-treated with LPS/IFN-γ for 2 h or 24 h to detect translocation of NF-κB and HMGB1, respectively. Fixation was performed in 10% formaldehyde for 30 min in 4°C. Preparations were rinsed and incubated with mouse anti-NF-κB and rabbit anti-HMGB1 overnight at 4°C. This was followed by incubation with rhodamine-conjugated secondary antibody and images were collected by confocal laser-scanning microscope (Olympus Fluoview FV1000, Olympus Optical Co, Tokyo,

Japan).

2.11. ROS production

Reactive oxygen species (ROS) generated by VSMCs was assayed using the ROS-sensitive fluorescent dye, 2',7'-dichlorodihydrofluorescin diacetate (DCFH-DA), following a previously described method (13).

2.12. Statistical analyses

Data were expressed as mean ± S.E.M. from at least three independent experiments. Student's t-test was used for determining the significance of differences between two groups, whereas one-way ANOVA was used for multiple comparisons. $P<0.05$ was accepted as the level of statistical significance.

三、結果

3.1. Effects of dihydropyridine CCBs on LPS/IFN-γ-induced production of NO and ROS, and expression of iNOS

As shown in Fig. 1A, treatment of rat VSMCs with LPS/IFN-γ for 24 h increased NO production from 1.08 ± 0.14 μM to 24.26 ± 0.47 μM. Nifedipine, labedipinedilol-A and lercanidipine at the concentration of 10 μM significantly inhibited LPS/IFN-γ-induced NO production in VSMCs by 52.37 ± 6.1%, 79.86 ± 2.3% and 83.13 ± 4.5%, respectively. The potential cytotoxicity of three CCBs was evaluated by the MTT assay after incubating cells for 24 h in the absence or presence of LPS/IFN-γ (Supplemental Fig. 1). We found that cell viabilities were not affected by all these CCBs at the concentrations of 1 μM or 10 μM.

As shown in Fig. 1B, LPS/IFN-γ induced ROS production to 1.9-fold higher than vehicle. In addition, all three CCBs attenuated LPS/IFN-γ-induced ROS production. However, only the ROS reducing effect of nifedipine was PPAR-γ-dependent (Fig. 1B).

We next examined whether the LPS/IFN-γ-induced protein expression and mRNA levels of iNOS were affected by three CCBs. We found that LPS/IFN-γ-induced iNOS protein and mRNA expressions were suppressed by all three CCBs (Fig. 1C). In addition, nifedipine, labedipinedilol-A and lercanidipine at the concentration of 10 μM significantly reduced LPS/IFN-γ-induced iNOS mRNA expression by 61 ± 4.09%, 89 ± 5.93% and 95 ± 6.56%, respectively, indicating that lercanidipine was the most potent inhibitor of NO pathway, followed by labedipinedilol-A and nifedipine.

3.2. Effects of dihydropyridine CCBs on TNF-α and IL-1β

As shown in Table 1, LPS/IFN-γ induced significant production of TNF-α and IL-1β. Nifedipine, labedipinedilol-A or lercanidipine all attenuated LPS/IFN-γ-induced TNF-α production even at the low concentration of 1 μM. The potency of these effects was similar to that on the NO system (lercanidipine > labedipinedilol-A > nifedipine). However, only lercanidipine could suppress LPS/IFN-γ-induced IL-1β production.

3.3. Effects of dihydropyridine CCBs on MMP-1/2/9 and TIMP-1 expressions

MMPs can damage the vascular extracellular matrix and result in a weakening and dilatation of the aortic wall, a hallmark of vascular inflammatory diseases [14,15]. Our results demonstrated that labedipinedilol-A and lercanidipine both inhibited LPS/IFN-γ-induced MMP-2/-9 activity and protein expression, whereas nifedipine only has inhibitory effect on MMP-2 (Fig. 2A and 2B). In addition, we found that all

three CCBs did not affect not MMP-1 expression induced by LPS/IFN-γ (Fig. 2C). We further examined the role of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), a key inhibitor of MMP-9. We found that lecarnidipine and labedipinedilol-A (10 μM) could enhance LPS/IFN-γ-induced TIMP-1 protein expression, while nifedipine could not (Fig. 2C).

3.4. Effects of dihydropyridine CCBs on HMGB1 release and trafficking

Beside its canonical DNA transactions with the nucleus, HMGB1 was recently recognized as an inflammatory mediator of atherosclerosis [16,17]. Given that all three CCBs could inhibit the release of classical cytokines including TNF-α and/or IL-1β, we further investigated whether they could also interferer in the secretion of HMGB1 by LPS/IFN-γ-activated VSMCs. We found that labedipinedilol-A and lercanidipine not only inhibited LPS/IFN-γ-induced HMGB1 cytosolic translocation as shown by Western blotting (Fig. 3A) and immunocytochemistry (Fig. 3B), and but also attenuated extracellular release of HMGB1 (Fig. 3C). However, nifedipine had no such effects.

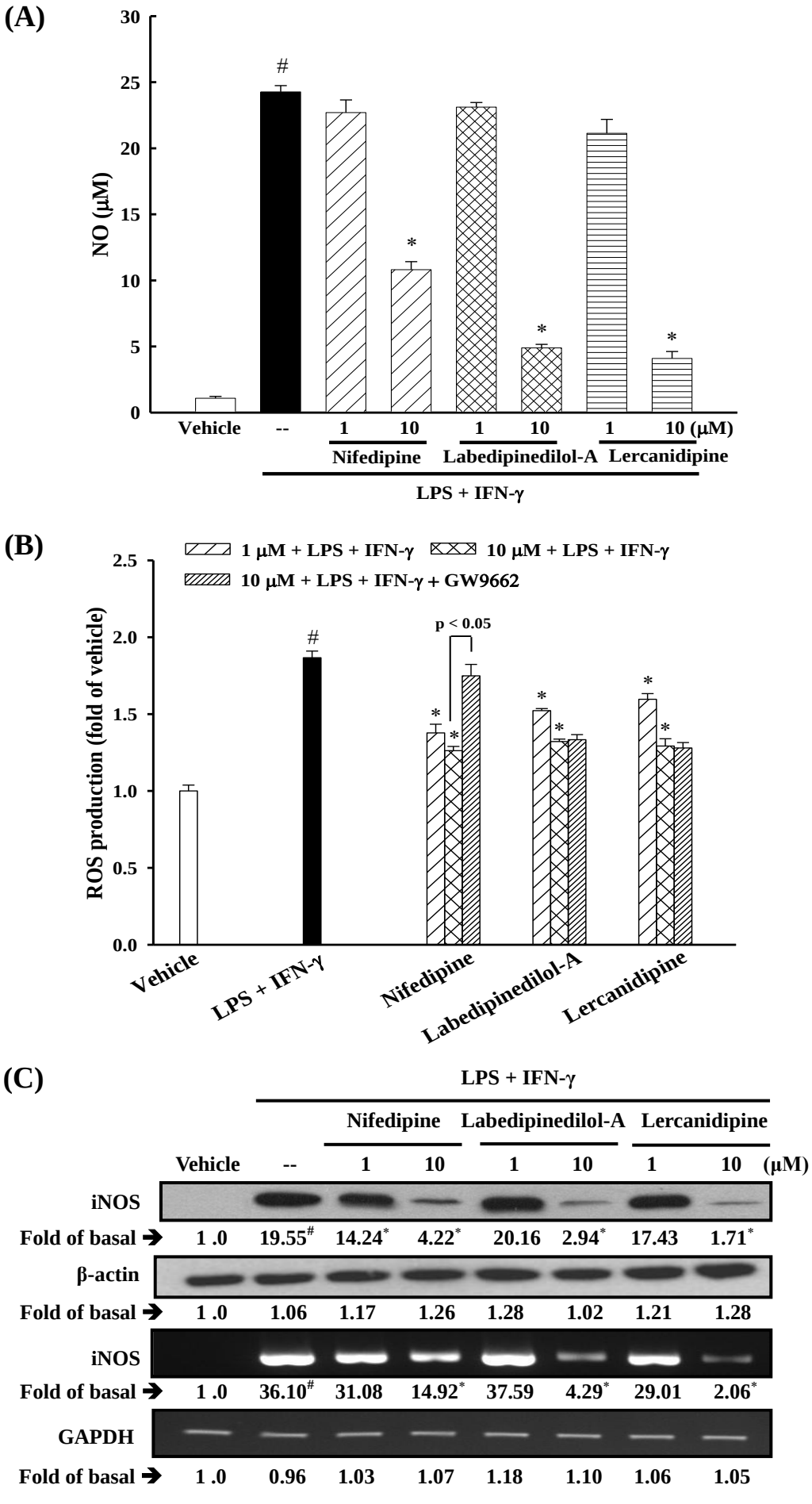
3.5. Effects of dihydropyridine CCBs on the nuclear translocation of NF-κB and phosphorylation of IκB-α

We further investigate the role of the IκB-α/NF-κB pathway in mechanisms for anti-inflammatory effects of dihydropyridine CCBs. As shown in Fig. 4A, all three CCBs (10 μM) attenuated LPS/IFN-γ-induced activation of p65, a subunit of NF-κB. In addition, by immunofluorescence microscopy we demonstrated that they all inhibited LPS/IFN-γ-induced nuclear translocation of p65 (Fig. 4B). Similarly, LPS/IFN-γ induced IκB-α phosphorylation was attenuated by all three CCBs at 10 μM (Fig. 4C).

3.6. Effects of dihydropyridine CCBs on the activation of PPAR-γ, MAPKs and Akt

Given with our results that ROS-reducing effect of nifedipine is mediated, at least in part, by PPAR-γ (Fig. 1B), we further validated effects on PPAR-γ exerted by dihydropyridine CCBs. As expected, our results demonstrated that only nifedipine could inhibit LPS/IFN-γ-induced down-regulation of PPAR-γ (Fig. 5A).

We finally examined the roles of MAPK and Akt signal transduction in the present study. Our results demonstrated that labedipinedilol-A and lercanidipine could attenuate LPS/IFN-γ-induced phosphorylation of JNK and p38 MAPK, but not ERK 1/2, while nifedipine only inhibited phosphorylation of JNK (Fig. 5B). In addition, we found that all of them reduced the LPS/IFN-γ-elicited Akt phosphorylation.


(A)
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Nifedipine
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LPS + IFN-γ
(B)
1 μM + LPS + IFN-γ
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10 μM + LPS + IFN-γ + GW9662
p < 0.05
ROS production (fold of vehicle)
Vehicle
LPS + IFN-γ
Nifedipine
Labedipinedilol-A
Lercanidipine
(C)
LPS + IFN-γ
Nifedipine
Labedipinedilol-A
Lercanidipine
Vehicle
--
1
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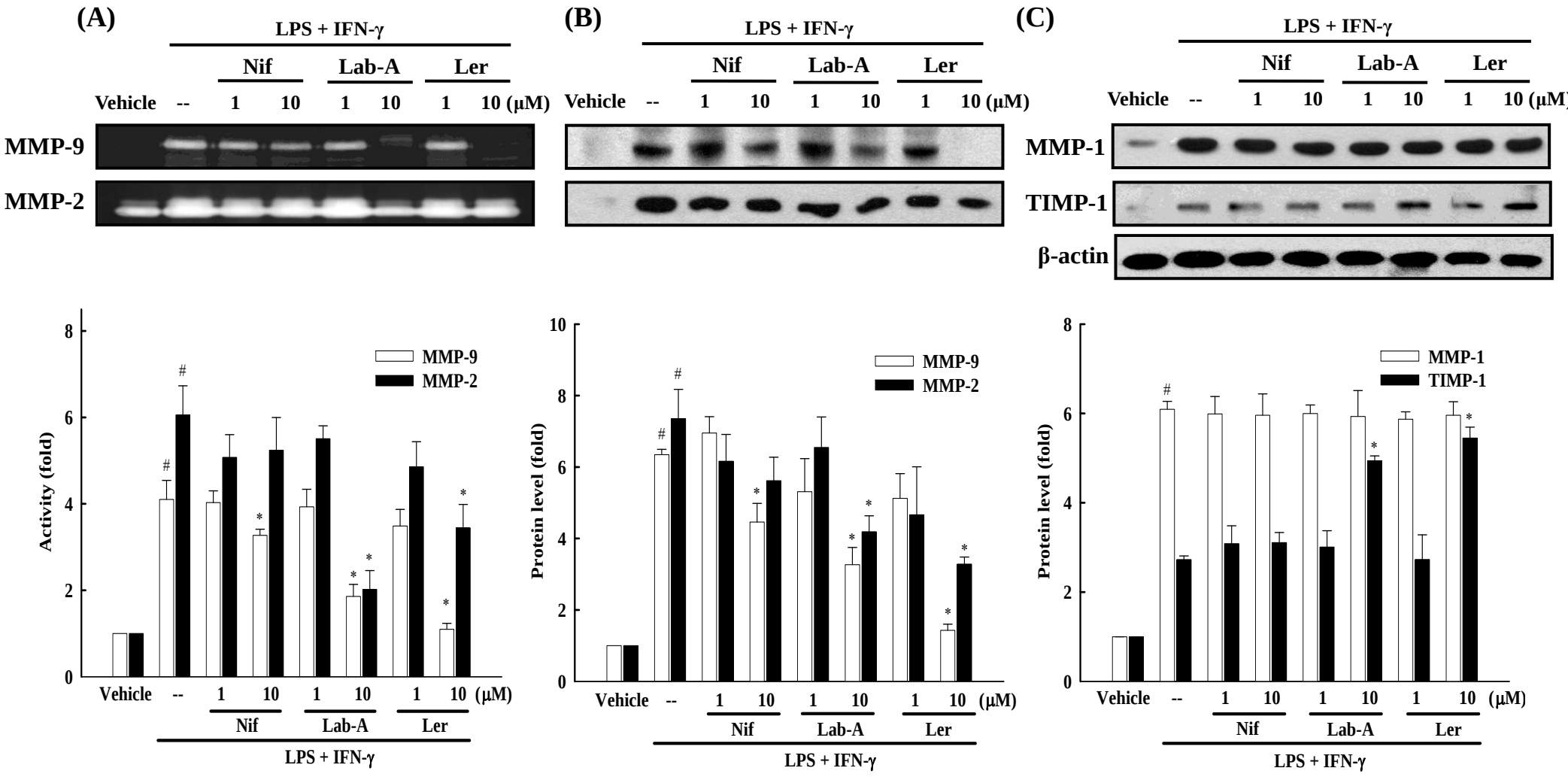
10
(μM)
iNOS
Fold of basal → 1 .0 19.55# 14.24* 4.22* 20.16 2.94* 17.43 1.71*
β-actin
Fold of basal → 1 .0 1.06 1.17 1.26 1.28 1.02 1.21 1.28
iNOS
Fold of basal → 1 .0 36.10# 31.08 14.92* 37.59 4.29* 29.01 2.06*
GAPDH
Fold of basal → 1 .0 0.96 1.03 1.07 1.18 1.10 1.06 1.05

Figure 1


(A)
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TIMP-1
β-actin
Activity (fold)
MMP-9
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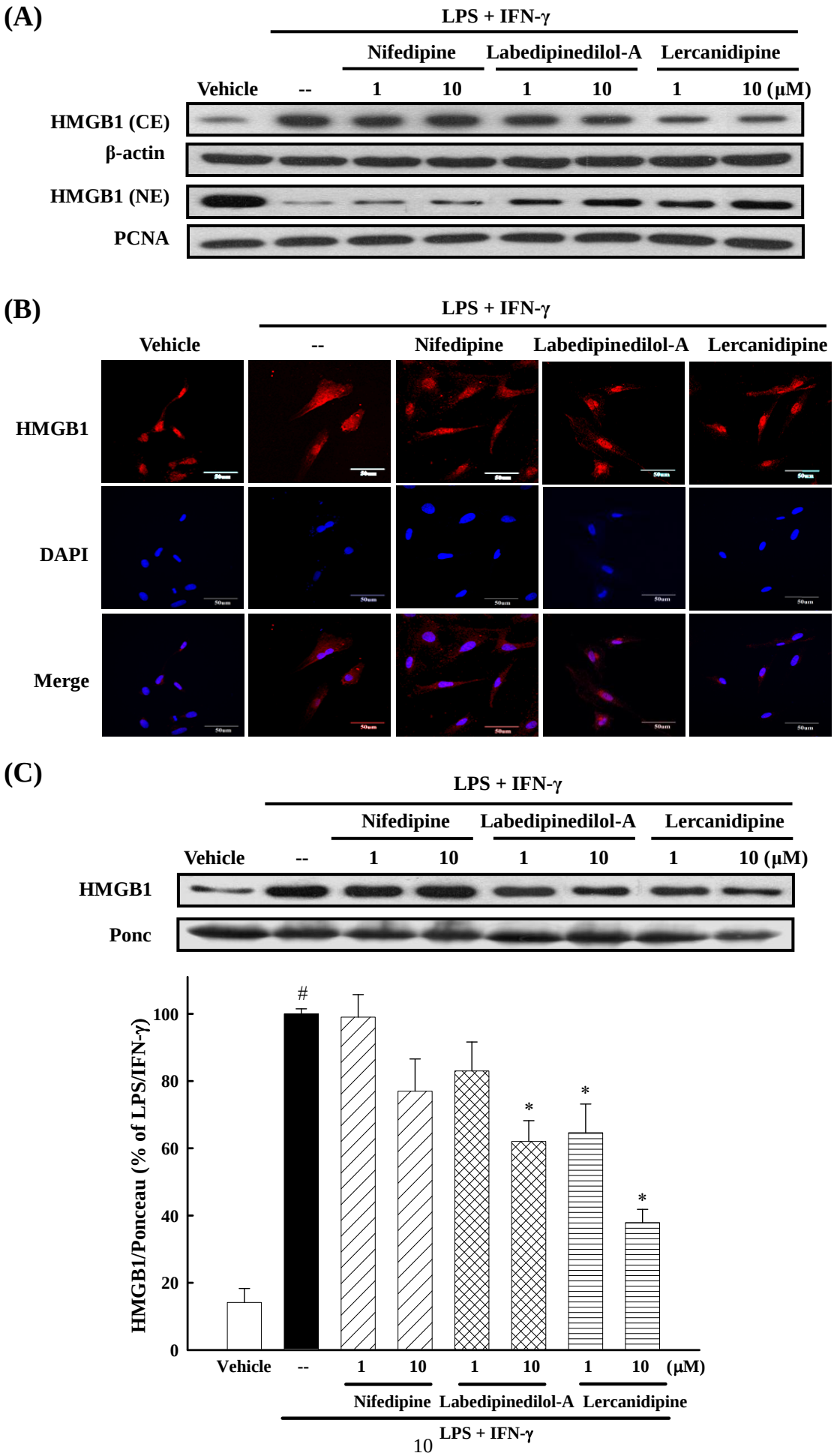
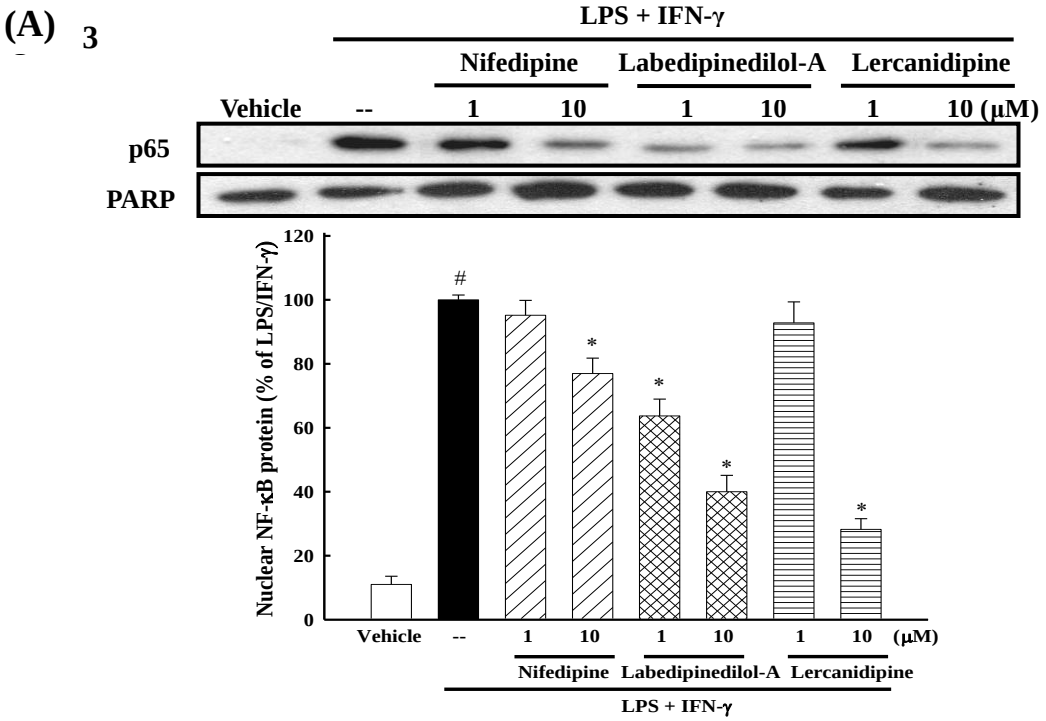
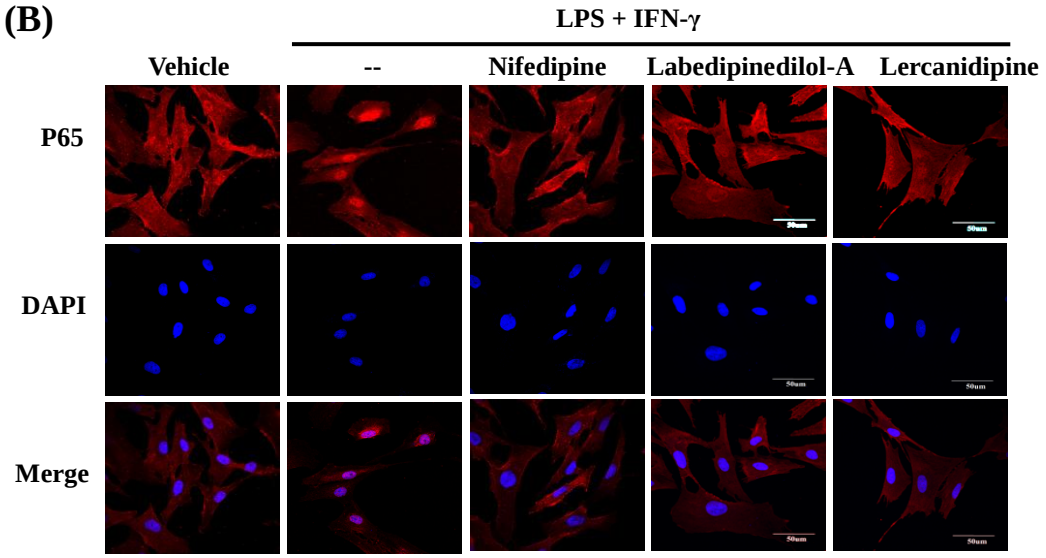
MMP-1
TIMP-1
Vehicle -- 1 10 1 10 1 10 (μM)
Nif
Lab-A
Ler
LPS + IFN-γ

Figure 2


(A)
LPS + IFN-γ
Nifedipine
Labedipinedilol-A
Lercanidipine
Vehicle
--
1
10
1
10
1
10 (μM)
HMGB1 (CE)
β-actin
HMGB1 (NE)
PCNA
(B)
LPS + IFN-γ
Vehicle
--
Nifedipine
Labedipinedilol-A
Lercanidipine
HMGB1
DAPI
Merge
(C)
LPS + IFN-γ
Nifedipine
Labedipinedilol-A
Lercanidipine
Vehicle
--
1
10
1
10
1
10 (μM)
HMGB1
Ponc
HMGB1/Ponceau (% of LPS/IFN-γ)
0
20
40
60
80
100
#
*
*
*
Vehicle
--
1
10
1
10
1
10
(μM)
Nifedipine
Labedipinedilol-A
Lercanidipine
LPS + IFN-γ


(A)
LPS + IFN-γ
Nifedipine
Labedipinedilol-A
Lercanidipine
Vehicle
--
1
10
1
10
1
10 (μM)
p65
PARP
Nuclear NF-κB protein (% of LPS/IFN-γ)
120
100
80
60
40
20
0
#
*
Vehicle
--
1
10
1
10
1
10
(μM)
Nifedipine
Labedipinedilol-A
Lercanidipine
LPS + IFN-γ


(B)
LPS + IFN-γ
Vehicle
--
Nifedipine
Labedipinedilol-A
Lercanidipine
P65
DAPI
Merge

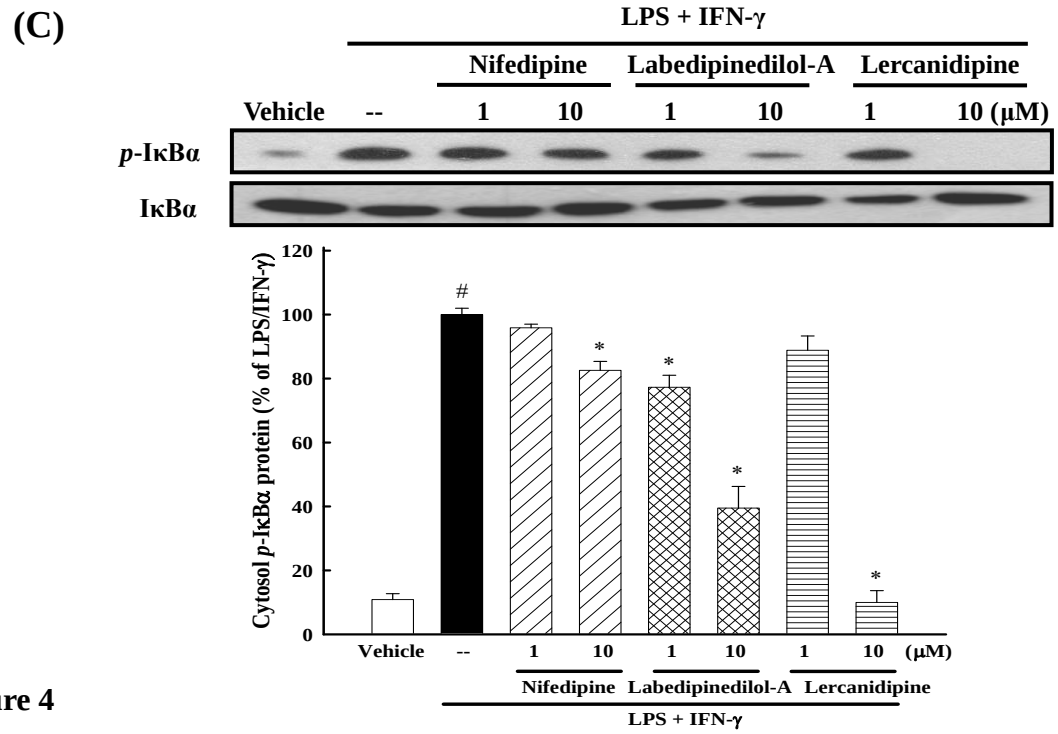
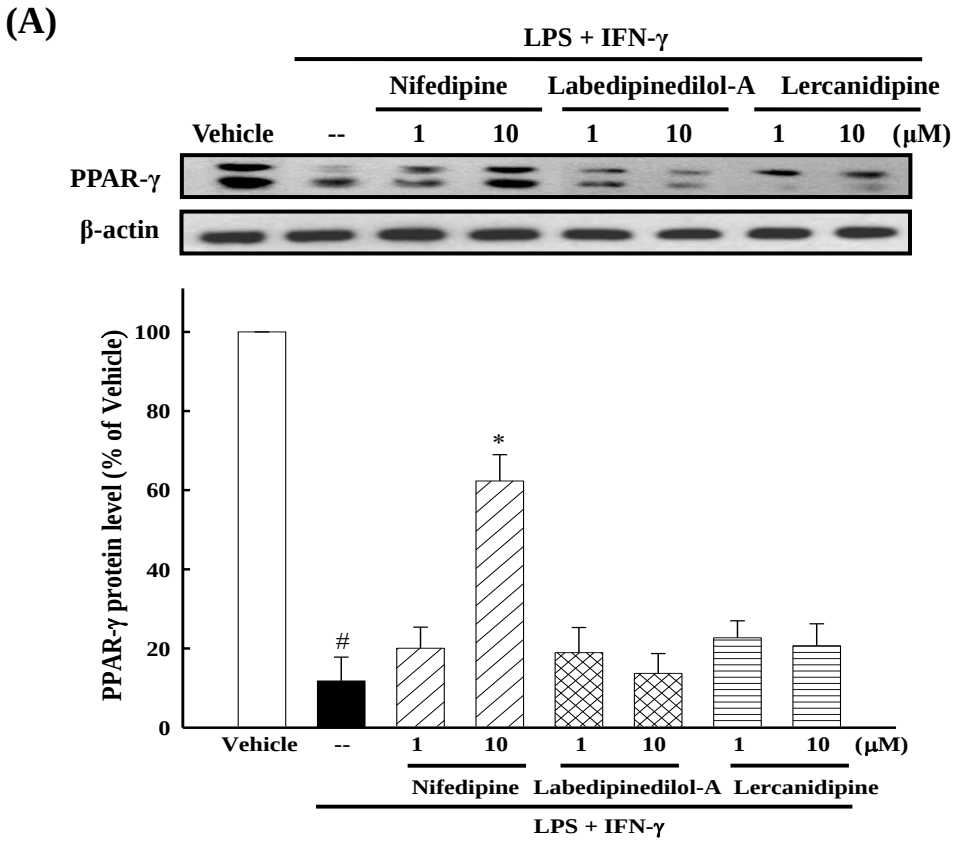

(C)
LPS + IFN-γ
Nifedipine
Labedipinedilol-A
Lercanidipine
Vehicle
--
1
10
1
10
1
10 (μM)
p-IκBα
IκBα
Cytosol p-IκBα protein (% of LPS/IFN-γ)
120
100
80
60
40
20
0
#
*
Vehicle
--
1
10
1
10
1
10
(μM)
Nifedipine
Labedipinedilol-A
Lercanidipine
LPS + IFN-γ

Figure 4


(A)
LPS + IFN-γ
Nifedipine
Labedipinedilol-A
Lercanidipine
Vehicle -- 1 10 1 10 1 10 (μM)
PPAR-γ
β-actin
PPAR-γ protein level (% of Vehicle)
100
80
60
40
20
0
#
*
Vehicle -- 1 10 1 10 1 10 (μM)
Nifedipine Labedipinedilol-A Lercanidipine
LPS + IFN-γ

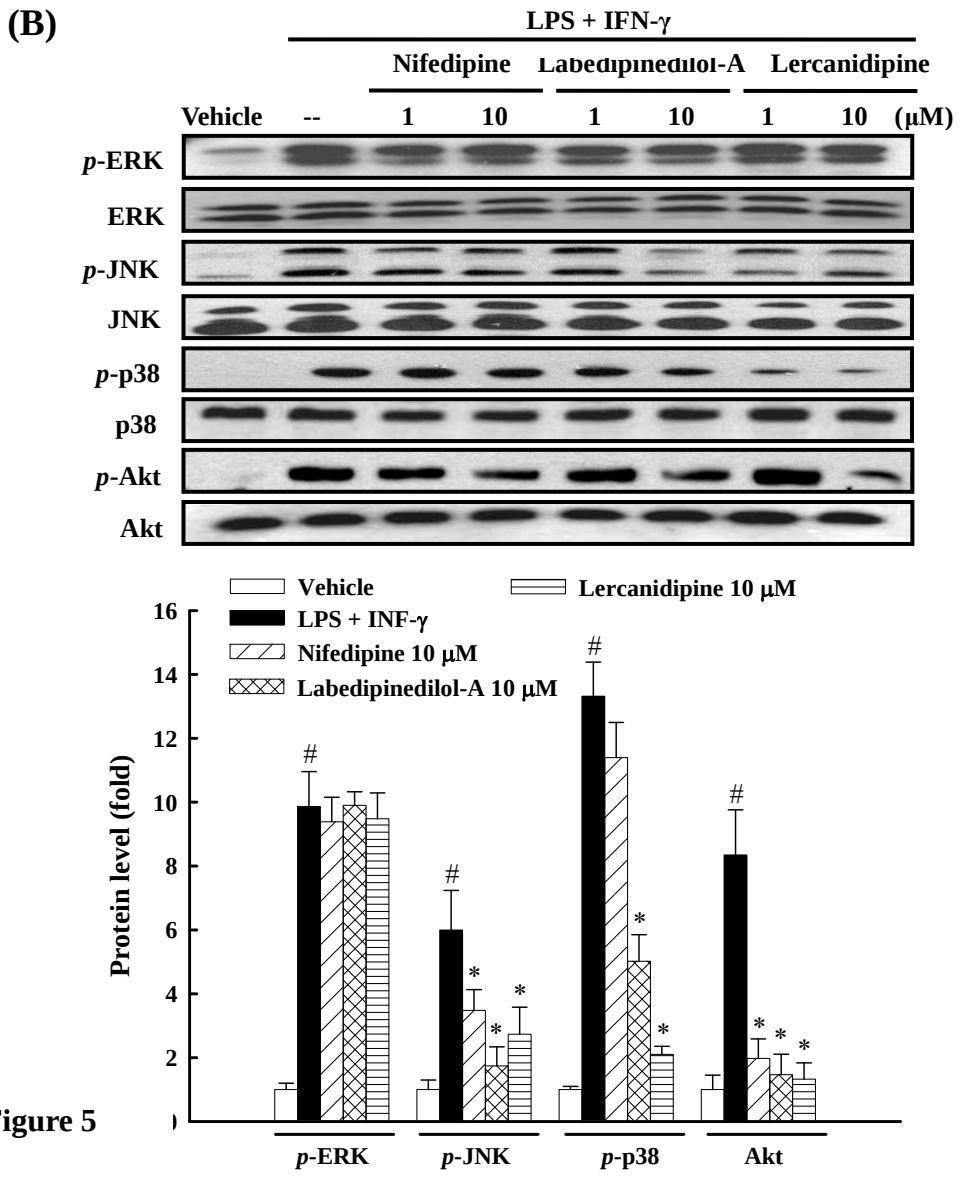

(B)
LPS + IFN-γ
Nifedipine Labedipinedilol-A Lercanidipine
Vehicle -- 1 10 1 10 1 10 (μM)
p-ERK
ERK
p-JNK
JNK
p-p38
p38
p-Akt
Akt
Vehicle
LPS + INF-γ
Nifedipine 10 μM
Labedipinedilol-A 10 μM
Lercanidipine 10 μM
Protein level (fold)
16
14
12
10
8
6
4
2
0
#
*
p-ERK
p-JNK
p-p38
Akt

Figure 5

Legends for figures

Fig. 1. Effects of nifedipine, labedipinedilol-A and lercanidipine on NO, ROS, and iNOS expression. VSMCs were pretreated with three calcium channel blockers for 30 min and then co-stimulated with LPS/IFN-γ for 24 h. (A) All three CCBs attenuated LPS/IFN-γ-induced NO production. (B) The LPS/IFN-γ-induced ROS production was inhibited by all three CCBs, but only the effect of nifedipine was reversed by PPAR-γ inhibitor, GW-9662. (C) All three CCBs down-regulated LPS/IFN-γ-induced iNOS protein expression. In addition, nifedipine, labedipinedilol-A and lercanidipine at the concentration of 10 μM reduced LPS/IFN-γ-induced iNOS mRNA expression by 61 ± 4.09%, 89 ± 5.93% and 95 ± 6.56%, respectively, indicating that lercanidipine as the most potent inhibitor of NO pathway. Each value represents the mean ± S.E.M. of three independent experiments, with triplicate determinations in each experiment. $^{\#}$ $P < 0.05$ compared with the vehicle; * $P < 0.05$ compared with LPS + IFN-γ alone.

Fig. 2. Effects of nifedipine, labedipinedilol-A and lercanidipine on MMP-2, MMP-9, MMP-1 and TIMP-1. (A and B) Labedipinedilol-A and lercanidipine both inhibited LPS/IFN-γ-induced MMP-2/-9 activity (A) and protein expression (B), whereas nifedipine only has inhibitory effect on MMP-2. (C) All three CCBs did not affect MMP-1 expression induced by LPS/IFN-γ. However, lecarnidipine and labedipinedilol-A (10 μM) but not nifedipine could enhance LPS/IFN-γ-induced TIMP-1 protein expression. Each value represents the mean ± S.E.M. of three independent experiments, with triplicate determinations in each experiment. $^{\#}$ $P < 0.01$ compared with the vehicle; * $P < 0.05$ compared with LPS + IFN-γ alone.

Fig. 3. Effects of nifedipine, labedipinedilol-A and lercanidipine on nuclear translocation of HMGB1. (A) Cells were subjected to nuclear (NE)/cytosol extracts (CE) and immunoblotted against HMGB1 antibody. Western blotting shows that labedipinedilol-A and lercanidipine but not nifiedipine could inhibit LPS/IFN-γ-induced HMGB1 cytosolic translocation as shown by decreased cytosolic fraction and increased nuclear fraction. (B) Consistent results were validated by confocal microscope showing that labedipinedilol-A and lercanidipine could result in nuclear translocation of HMGB1. Scale bar: 50μM. (C) Western blotting shows that labedipinedilol-A and lercanidipine also attenuated extracellular release of HMGB1 induced by LPS/IFN-γ. Each value represents the mean ± S.E.M. of three independent experiments, with triplicate determinations in each experiment. $^{\#}$ $P < 0.01$ compared with the vehicle; * $P < 0.05$ compared with LPS + IFN-γ alone.

Fig. 4. Effects of nifedipine, labedipinedilol-A and lercanidipine on NF-κB

translocation and phosphorylation of IκB-α. (A) The nuclear fractions were used to analyze the content of NF-κB. Western blotting shows that all three CCBs attenuated LPS/IFN-γ-induced NF-κB activation at 10 μM. (B) Confocal microscopy demonstrates that they all inhibited LPS/IFN-γ-induced nuclear translocation of p65, a subunit of NF-κB, as shown by the location of anti-p65 stain within the nucleus stained with DAPI. Scale bar: 50μM. (C) The cytosolic fractions were used to analyze the content of IκB-α and phosphorylated IκB-α. Western blotting shows that LPS/IFN-γ induced IκB-α phosphorylation was attenuated by all three CCBs at 10 μM. Each value represents the mean ± S.E.M. of three independent experiments, with triplicate determinations in each experiment. $^{\#}$ $P < 0.01$ compared with the vehicle ; * $P < 0.05$ compared with LPS + IFN-γ alone.

Fig. 5. Effects of nifedipine, labedipinedilol-A and lercanidipine on activations of. PPAR-γ, MAPKs and Akt. (A) Western blotting shows that only nifedipine could inhibit LPS/IFN-γ-induced down-regulation of PPAR-γ. (B) Labedipinedilol-A and lercanidipine both attenuated LPS/IFN-γ-induced phosphorylation of JNK and p38 MAPK, but not ERK 1/2. Nifedipine only inhibited phosphorylation of JNK. However, they all reduced LPS/IFN-γ-elicited Akt phosphorylation. The total MAPK levels were used as internal controls. Similar results are obtained from three independent experiments. $^{\#}$ $P < 0.01$ compared with the vehicle ; * $P < 0.05$ compared with LPS + IFN-γ alone.

四、討論

An expanding body of literature has demonstrated that dihydropyridine CCBs have the pleiotropic ability to protect against endothelial injury, inactivate macrophages and improve VSMCs dysfunction to prevent the progression of atherosclerosis [5], making dihydropyridine CCBs unique and intriguing targets of investigations in cardiovascular pharmacology.

Common molecular signatures of inflammatory responses include production of early and late proinflammatory cytokines such as TNF-α, IL-1β, IL-6 and HMGB1. HMGB1 is a nuclear protein secreted from activated monocytes/macrophage and epithelial cells after proinflammatory stimuli such as LPS and TNF-α [18,19]. In atherosclerotic lesions, HMGB1 protein is expressed in endothelial cells, VSMCs and macrophages and may play a crucial role in the progression of atherosclerosis [16,17]. HMGB1 interacts with specific receptors including RAGE (receptor for advanced glycation end products), toll-like receptor (TLR)-2, and TLR-4. The binding of HMGB1 to its receptors results in the activation of several kinases such as ERK 1/2, JNK and p38 MAPK, which ultimately leads to the activation of NF-κB-dependent genes [20]. The present study indicates that lercanidipine possesses anti-inflammatory properties by inhibiting the production of NO, TNF-α and IL-1β. Moreover, our data also suggest that lercanidipine can act as an anti-inflammatory agent through the inhibition of HMGB1 translocation and subsequent secretion by VSMCs. The effects of nifedipine and labedipinedilol-A were similar, but differential effects were observed in HMGB1 translocation. The exact mechanism by which lercanidipine and labedipinedilol-A inhibited the HMGB1 translocation and release is not clear. Nevertheless, previous studies have shown that the inhibitory effects of lercanidipine and labedipinedilol-A on HMGB1 release may be mediated through different mechanisms, e.g. inhibition of TNF-α and IL-1β or iNOS/NO [21,22].

NO is an important regulator of vascular function. In addition, MMPs also play important role in vascular remodeling and subsequent pathological events in the progression of coronary arterial diseases and atherosclerosis [14]. Increased expression/activity of MMPs, especially MMP-2, plays a role in the vascular alterations induced by hypertension, and increased oxidative stress is a major factor activating MMPs. MMP-9 is also important for the development of arterial lesions by promoting VSMC migration and proliferation [15]. Therefore, the inhibition of MMP-2 and MMP-9 appear to be the appropriate target for the development of anti-atherogenic agents. Our results demonstrated that the activities and expressions of both MMP-2 and MMP-9 were inhibited by lercanidipine and labedipinedilol-A, whereas nifedipine only decreased MMP-9 activity and protein expression. An important extracellular mechanism for the control of MMP activity is via the binding

to TIMP and forming a 1:1 enzyme-inhibitor complex. Our results that TIMP-1, as the major inhibitor for MMP-9, was enhanced by lercanidipine and labedipinedilol-A, provides potential mechanisms for the decreased MMP-9 activity and synergistic effect on MMP-9 inhibition. Furthermore, the over-expression of TIMP-1 has been shown to reduce the neointimal formation in human saphenous veins and to prevent atherosclerotic lesions in apolipoprotein E-deficient mice [23,24]. Therefore, our results implicate a novel mechanism for the anti-atherosclerotic effect of dihydropyridine CCBs by increasing the expression of TIMP-1.

Because NF-κB is a critical transcription factor in transactivating inflammatory genes, including iNOS and MMP-9 [25,26], it appears to be a promising molecular target in treating inflammatory vascular diseases. Another key element to the regulation of cytokine production is the MAPKs, which are strictly related with the activation of NF-κB [27]. Accordingly, inhibitors of p38 MAPK and NF-κB block the production of IL-1, TNF-α and HMGB1 at the transcriptional and translational levels, being effective in the treatment of chronic inflammatory diseases [28]. Additionally, it has been reported that IκB kinase can be a substrate of Akt and thus activation of Akt may stimulate NF-κB activity [29]. Our data demonstrated that labedipinedilol-A and lercanidipine attenuated LPS/IFN-γ-activated JNK, p38 MAPK and Akt phosphorylation, whereas nifedipine inhibited the activation of JNK and Akt pathway. In addition, they all inhibited LPS/IFN-γ-induced activation of the downstream IκB-α/NF-κB pathway. These data strengthen the notion that anti-inflammatory effects of dihydropyridine CCBs are potentially mediated through the MAPKs/Akt and IκB-α/NF-κB signaling pathways.

Oxidative stress and the production of ROS have been implicated in the pathogenesis of atherosclerosis. Our results showed that nifedipine, labedipinedilol-A and lercanidipine significantly inhibited the ROS production, which is similar with other CCBs such as azelnidipine, felodipine, benidipine, and manidipine [5]. We have recently reported that the anti-oxidative effects of labedipinedilol-A may be mediated through down-regulation of the NAD(P)H oxidase subunits (Nox1 and Rac1) [13]. On the other hand, lercanidipine suppressed the proliferation of VSMCs via inhibiting ROS production [11]. In the present study we further elucidated that in LPS/IFN-γ-treated VSMCs, nifedipine could up-regulate PPAR-γ and its ROS-reducing effect is PPAR-γ dependent. This finding is in line with a recent study showing that nifedipine can reduce oxidative stress through PPAR-γ activation in macrophages and suppress atherogenesis in apoE$^{-/-}$ mice [31].

Differential effects were detected among nifedipine, lecarnidipine and labedipinedilol-A in the present study but the mechanisms is not completely understood. We found that the anti-inflammatory effects of labedipinedilol-A and

lecarnidipine were similar, but lecarnidipine seems to possess more potent inhibitory effects against the production of pro-inflammatory mediators including NO, ROS, iNOS, TNF-α, IL-1β and HMGB1. These effects are potentially mediated through attenuation of the MMP pathway, MAPKs/Akt transduction and NF-κB signaling. Intriguingly, nifedipine has no effect on IL-1β production, MMP-2, TIMP-1 expression and HMGB1 translocation, but may convey anti-oxidative ability through up-regulation of PPAR-γ. On the basis of the present results, we cannot draw any definite conclusion on the mechanisms underlying differential effects of these three CCBs. However, we speculate that the lipophilicity may play a key role in explaining the differences. Comparisons of anti-inflammatory effects between the dihydropyridines and other L-type CCBs have not been well addressed. Diltiazem has been shown to exert anti-atherosclerotic effects mediated through the inhibition of dendritic cell-endothelial cell interaction by inhibiting NF-κB activity and IL-12 expression [32]. Verapamil has been reported to reduce lipopolysaccharide-induced PYK2 phosphorylation, nuclear translocation of AP-1 and TNF-α production in macrophage [33]. Our results suggest that in VSMCs dihydropyridines CCBs have anti-inflammatory effects though a wide array of pathways and whether these effects can be extrapolated into inflammatory cells may warrant further investigations.

In conclusion, our findings provide new insights of anti-inflammatory effects of new generation dihydropyridine CCBs and suggest their potential roles in the treatment of vascular inflammatory diseases such as atherosclerosis. Further studies are warranted to validate their *in vivo* and clinical effects.

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Lercanidipine and labedipinedilol-A attenuate lipopolysaccharide/interferon-γ-induced inflammation in rat vascular smooth muscle cells through inhibition of HMGB1 release and MMP-2, 9 activities

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ABSTRACT

Objective: Inflammation is an important molecular basis of atherosclerosis. Recent studies have shown that dihydropyridine calcium channel blockers (CCBs) can exert potent anti-inflammatory effects in models of vascular dysfunction. The purpose of the present study was to evaluate anti-inflammatory effects and mechanisms of lercanidipine and labedipinedilol-A, new generation dihydropyridine CCBs, in rat vascular smooth muscle cells (VSMCs) exposed to lipopolysaccharide (LPS) and interferon-γ (IFN-γ).

Methods and results: MTT, Griess reagent, RT-PCR, ELISA, gelatin zymography, immunocytochemistry and Western blotting were employed. We found that lercanidipine and labedipinedilol-A attenuated production of NO, ROS and TNF-α from LPS/IFN-γ-stimulated VSMCs. In addition, they both diminished the LPS/IFN-γ-induced expression of iNOS protein and mRNA, with attenuation of HMGB1 cytosolic translocation and subsequent extracellular release. Furthermore, they down-regulated MMP-2/MMP-9 activities, whereas expression of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), an inhibitor of MMP-9, was up-regulated. Finally, we found that lercanidipine and labedipinedilol-A inhibited the nuclear translocation of NF-κB and suppressed the phosphorylation of JNK, p38 MAPK and Akt.

Conclusion: Lercanidipine and labedipinedilol-A can exert their anti-inflammatory effects through suppression of NO, ROS and TNF-α through down-regulation of iNOS, MMP-2/MMP-9, and HMGB1, with inhibition of signaling transduction of MAPKs, Akt/IkB-α and NF-κB pathways. These findings implicate a valuable role of new generation dihydropyridine CCBs lercanidipine and labedipinedilol-A for the treatment of inflammatory vascular diseases.

1. Introduction

Inflammation is thought to be one of the major processes that accelerate the progression of atherosclerosis. The inflammatory response involves complicated interactions among immunomodulatory cells, endothelial cells, and vascular smooth muscle cells (VSMCs). Endothelial damage is one of the hallmark features of atherosclerosis. The underlying vascular smooth muscle is thus exposed and activated by pathogens, resulting in the induction of inducible nitric oxide synthase (iNOS) and other inflammatory genes, with subsequent vascular remodeling and obliteration [1,2]. Previous studies demonstrated that iNOS expression is increased in VSMCs after exposure to lipopolysaccharide (LPS) or cytokines. Furthermore, the effect of LPS on iNOS expression is strengthened by cytokines [3].

Activation of the transcription factor nuclear factor (NF)-κB is an important step in the development of vascular damage, because it controls the expression of inducible genes, including many inflammatory mediators such as iNOS and cyclooxygenases-2 (COX-2). NF-κB can be then translocated to the nucleus where it facilitates the transcription of many genes, including proinflammatory cytokines, chemokines, and anti-apoptotic factors [4]. Inflammatory mediators stimulate the activation of NF-κB in all

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appears to be the appropriate target for the development of anti-atherogenic agents. Our results demonstrated that the activities and expressions of both MMP-2 and MMP-9 were inhibited by lercanidipine and labedipinedilol-A, whereas nifedipine only decreased MMP-9 activity and protein expression. An important extracellular mechanism to control the activity of MMP is to bind TIMP and form a 1:1 enzyme–inhibitor complex. Thus, the over-expression of TIMP-1 has been shown to reduce the neointimal formation in human saphenous veins and to prevent atherosclerotic lesions in apolipoprotein E-deficient mice [23,24]. Our results showed that TIMP-1, the major inhibitor for MMP-9, was enhanced by lercanidipine and labedipinedilol-A. Therefore, these data implicate a novel mechanism for the anti-atherosclerotic effect of dihydropyridine CCBs by increasing the expression of TIMP-1.

NF-κB is a critical transcription factor in transactivating inflammatory genes, which include iNOS and MMP-9 [25,26]. It then appears to be a promising molecular target for the treatment of inflammatory vascular diseases. Being strictly related with the activation of NF-κB, MAPK is another key element to regulate the production of cytokine [27]. Accordingly, inhibitors of p38 MAPK and NF-κB block the production of IL-1, TNF-α and HMGB1 at the transcriptional and translational levels, indicating their effectiveness to treat chronic inflammatory diseases [28]. Beside, activation of Akt may stimulate NF-κB activity since IκB kinase is a substrate of Akt [29]. Our data demonstrated that labedipinedilol-A and lercanidipine attenuated LPS/IFN-γ-activated JNK, p38 MAPK and Akt phosphorylation, whereas nifedipine inhibited the activation of JNK and Akt pathway. In addition, they all inhibited LPS/IFN-γ-induced activation of the downstream IκB-α/NF-κB pathway. These data strengthen the notion that anti-inflammatory effects of dihydropyridine CCBs are potentially mediated through the MAPKs/Akt and IκB-α/NF-κB signaling pathways.

Oxidative stress and the production of ROS have been implicated in the pathogenesis of atherosclerosis. Our results showed that nifedipine, labedipinedilol-A and lercanidipine significantly inhibited the ROS production, indicating a similarity of lercanidipine and labedipinedilol-A to other dihydropyridine CCBs such as azelnidipine, felodipine, benidipine, and manidipine [5]. We have recently reported that the anti-oxidative effects of labedipinedilol-A may be mediated through down-regulation of the NAD(P)H oxidase subunits (Nox1 and Rac1) [13]. On the other hand, lercanidipine suppressed the proliferation of VSMCs via inhibiting ROS production [11]. In the present study we further elucidated that in LPS/IFN-γ-treated VSMCs, nifedipine could up-regulate PPAR-γ and its ROS-reducing effect is PPAR-γ dependent. This finding is in line with a recent study showing that nifedipine reduces oxidative stress through PPAR-γ activation in macrophages and suppresses atherogenesis in apoE$^{-/-}$ mice [30].

Differential effects were detected among nifedipine, lercanidipine and labedipinedilol-A in the present study. We found that the anti-inflammatory effects of labedipinedilol-A and lercanidipine were similar, but lercanidipine seems to possess more potent inhibitory effects against the production of pro-inflammatory mediators including NO, ROS, iNOS, TNF-α, IL-1β and HMGB1. These effects are potentially mediated through attenuation of the MMP pathway, MAPKs/Akt transduction and NF-κB signaling. Intriguingly, nifedipine has no effect on IL-1β production, MMP-2, TIMP-1 expression and HMGB1 translocation, but may convey anti-oxidative ability through up-regulation of PPAR-γ. On the basis of the present results, we cannot draw any definite conclusion on the mechanisms underlying differential effects of these three CCBs. However, we speculate that the lipophilicity may play a key role in explaining the differences.

Comparisons of anti-inflammatory effects between the dihydropyridines and other L-type CCBs have not been well addressed. Diltiazem has been shown to exert anti-atherosclerotic effects mediated through the inhibition of dendritic cell–endothelial cell interaction by inhibiting NF-κB activity and IL-12 expression [31]. Verapamil has been reported to reduce LPS-induced PYK2 phosphorylation, nuclear translocation of AP-1 and TNF-α production in macrophage [32]. Our results suggest that in VSMCs dihydropyridines CCBs demonstrate anti-inflammatory effects though a wide array of pathways. However, whether these effects can be extrapolated into inflammatory cells may warrant further investigations.

In conclusion, our findings provide new insights of anti-inflammatory effects of new generation dihydropyridine CCBs and suggest their potential roles in the treatment of vascular inflammatory diseases such as atherosclerosis. Further studies are warranted to validate their *in vivo* and clinical effects.

Acknowledgments

This work was supported in part by grants from the National Science Council (NSC 99-2320-B-037-015-MY3 and NSC 101-2320-B-041-003) and Chia-Nan University of Pharmacy and Science (CN 10029), Taiwan. We would also like to thank Li-Ying Chen and Yi-Chun Hsieh for their excellent technical support.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2012.12.005.

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附件五

國科會補助專題研究計畫出席國際學術會議心得報告

日期：102 年 09 月 27 日

計畫編號	NSC 101－2320－ B－041－003		
計畫名稱	比較二氫吡類鈣離子通道阻斷劑在脂多醣體/干擾素-γ 處理的細胞及內毒素血症小鼠對誘發性一氧化氮合成酶、基質金屬蛋白酵素的表現與 HMGB1 釋放的作用		
出國人員姓名	劉淑芬	服務機構及職稱	嘉南藥理科技大學藥學系副教授
會議時間	2013 年 07 月 21 日至 2013 年 07 月 26 日	會議地點	伯明罕 英國
會議名稱	(中文) 第 37 屆國際生理科學聯合會大會 (英文) 37th International Union of Physiological Sciences		
發表題目	(中文) Lercanidipine 與 labedipinedilol-A 透過抑制脂多醣體/干擾素-γ 誘發的血管平滑肌細胞之 HMGB1 釋放和 MMP-2, 9 的活性而具有抗發炎作用 (英文) Lercanidipine and labedipinedilol-A play anti-inflammatory role through inhibition of lipopolysaccharide/interferon-γ-induced HMGB1 release and MMP-2, 9 activities in vascular smooth muscle cells		

一、參加會議經過

2013 年 7 月 21 日至 26 日，第 37 屆國際生理科學聯合會大會（37th IUPS Congress）在英國伯明罕舉行，共有來自世界的 2000 多名各國的專家學者出席，在會場也見到多位台灣學者參加本次國際會議，並與世界各國學者相互交流認識。由於本人首次以脂多醣體/干擾素-γ 刺激血管平滑肌細胞進行抗發炎的新藥開發相關研究，因此報名

與會學習這個領域的新知。本校一同前往的有施美份教授，搭乘國泰航空公司班機從高雄小港機場出發飛往香港再轉機到英國倫敦希斯洛機場。

7 月 21 日下午 15:00 開始辦理報到，下午 17:30 有 1 場 Opening lecture- Physiology moves back onto centre stage: a new synthesis with evolutionary biology，下午 19：00 舉行開幕隨後於晚上 20：00 舉辦歡迎晚宴與會學者歡聚一堂。主辦單位除了早、午場的學術報告外，另安排了壁報展示，提供與會學者進行壁報討論，提供餐點讓學者在輕鬆愉悅的心情下進行學術交流。本次大會安排了 4 天 Poster Session 的時段，由於本人此次的研究是以脂多醣體/干擾素-γ 刺激血管平滑肌細胞誘發發炎反應進行抗發炎的藥物篩選，因為被歸類於 Vascular Biology。在這 6 天我和許多國際學者相互討論交流研究心得，感到獲益匪淺。

二、與會心得

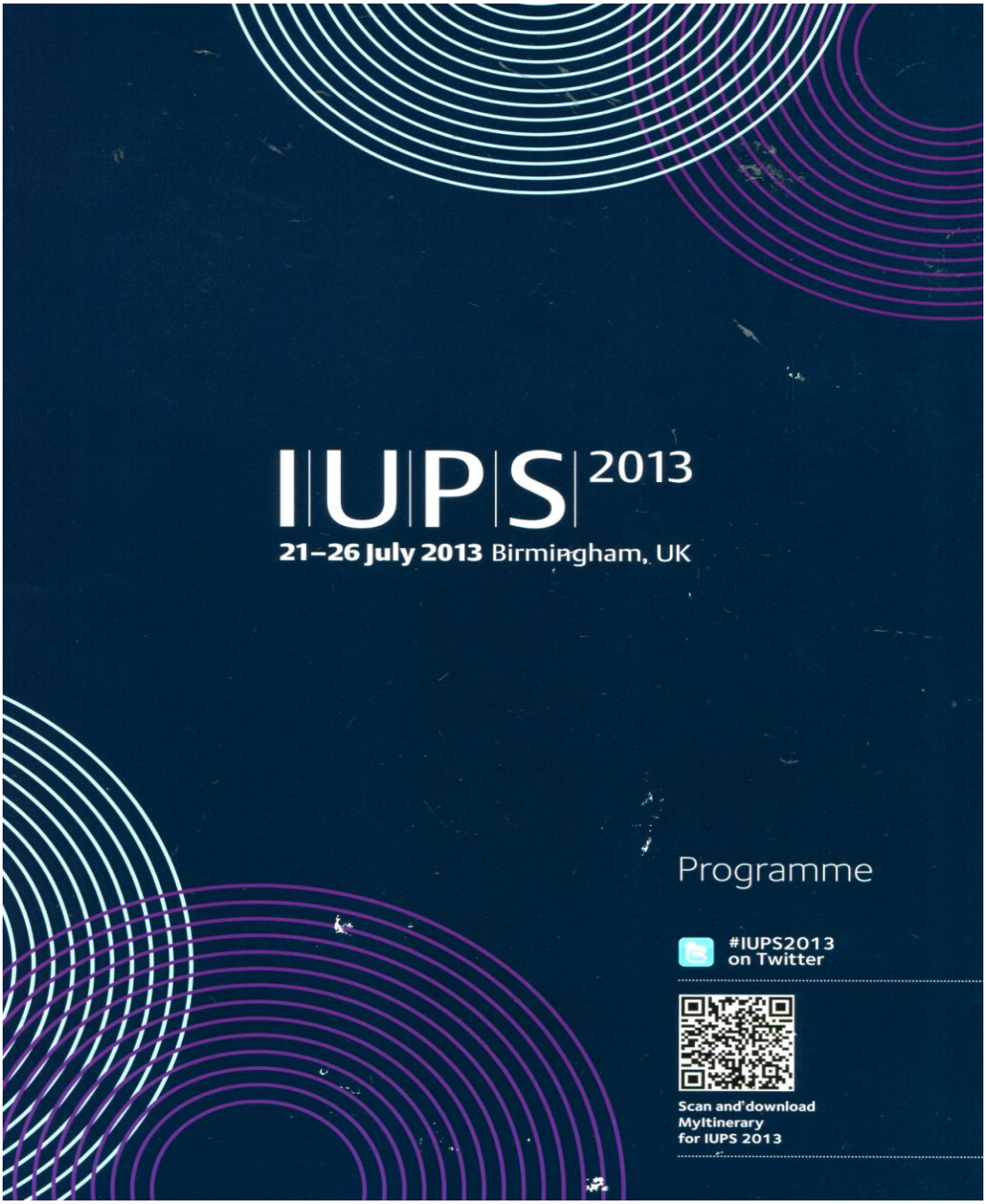
本次大會中有 2 場 Special Lectures，主題分別為 ”Recent advances in Renin-Angiotensin System and implications in health and disease” 與 ” TRPV1 channels: from molecules to physiology”最令人印象深刻。其中，在 Recent advances in Renin-Angiotensin System and implications in health and disease 的報告中指出，腎素–血管收縮素系統（RAS）是人體調節血壓和血容量的重要系統，但在高血壓、粥狀動脈硬化和心臟衰竭等多種心血管疾病時 RAS 過度激活，RAS 中的血管收縮素轉換酶（ACE）將血管收縮素 I 轉化為血管收縮素 II，後者是誘發和加重粥狀動脈硬化的重要因子。血管收縮素轉換酶 2（ACE2）是近年來發現的與 ACE 結構相似的一種金屬蛋白酶，可將血管收縮素 II 分解為血管收縮素 1-7，後者可拮抗血管收縮素 II，但是否具有抗粥狀動脈硬化的作用尚不明了。於是利用攜帶小鼠 ACE2 基因的腺病毒載體，應用於粥狀動脈硬化成熟斑塊兔模型的治療。結果顯示，ACE2 過表達可顯著減少斑塊內巨噬細胞和脂質含量，增加斑塊內平滑肌細胞和膠原纖維的含量，首次證實，ACE2 過表達可穩定粥狀動脈硬化斑塊。

另外，Dr. Tamara Rosenbaum 在 7 月 25 日早上的 keynotes 發表關於 TRPV1 channels: from molecules to physiology 的研究，TRPV1 全名是 transient receptor potential vanilloid type 1，屬於 transient receptor potential (TRP) nonspecific cation channel family (短暫受體電位非特異性陽離子通道)。它們分布在各式各樣組織的感覺神經纖維末梢上，例如眼睛，皮膚，血管，關節腔，肌肉與韌帶，及呼吸道、泌尿道、消化道等等。這些感覺細胞的本體則位於脊髓後根神經節或顱神經節中，統稱為 polymodal nociceptor。TRP 是一種多因受體。它接收許多性質迥異的、非特異性的激活因素以打開陽離子通道。這些激活因素目前已知包括有溫度(>43℃)，物理/機械性的傷害(mechanical)，酸/氫離子(hydrogen ion)，多種自然界物質如辣椒、mustard、大蒜 (allicin)，以及許多的具有刺激性的化學物質如丙烯醛等。這些刺激或大或小，形成分散但可重複累積的陽離子流。最終可以誘發該神經細胞向 efferent 端釋放出 Substance P，Neurokinin，與 CGRP 等神經胜肽，在組織間(結膜、肺泡、腸道)造成 neurogenic inflammation，並同時在 afferent 端則釋出 Glutamate 及 CGRP，向中樞傳遞痛覺。偏頭痛研究的動物模型中，常用辣椒的主要刺激性成分辣椒素 (capsaicin 注射來取代電擊之類的破壞性組織傷害。Capsaicin 便是透過與 TRPV1 結合造成我們熟知的疼痛與灼熱的感覺。有趣的是，在活化 TRPV1 後，會先出現灼熱痛覺與短暫痛覺敏感，接下來就跟著一段痛覺減低期，然後痛覺再慢慢回復。而在重複刺激 TRPV1 之後，抑制痛的效果會相應的延長，形成了長期的止痛的效

果。這時神經細胞內 SP 與 CGRP 的基因表現降低，神經末梢上 TRPV1 受體的表現也產生變動，進一步調節了神經細胞的疼痛感受力與傳達力。而除了 Capsaicin 與酸 (氫離子)等因子的直接作用之外，TRPV1 的表現也可以被 prostaglandins 與 bradykinin 等發炎介質調控。

在會場也見到許多位台灣學者參加本次國際會議，並且與歐美學者相互交流認識。

三、發表論文全文或摘要



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論文摘要

Proof

CONTROL ID: 1646692
CURRENT THEME OR SYMPOSIUM: Vascular & Smooth Muscle Physiology
Topic GC: Vascular Biology
PRESENTATION TYPE: General Communication
TITLE: Lercanidipine and labedipinedilol-A play anti-inflammatory role through inhibition of lipopolysaccharide/interferon-γ-induced HMGB1 release and MMP-2, 9 activities in vascular smooth muscle cells

AUTHORS (FIRST NAME, LAST NAME): Shu-Fen Liou[1], Mei-Fen Shih[1], Chiu-Lan Chen[1]
INSTITUTIONS (ALL): 1. Department of Pharmacy, Chia-Nan University of Pharmacy and Science, Tainan, Taiwan, Tainan, Taiwan.

ABSTRACT BODY: Inflammation is an important molecular basis of atherosclerosis. Several reports have revealed that dihydropyridine calcium channel blockers (CCBs) exert anti-inflammatory effects on various vascular cells. The purpose of the present study was to evaluate anti-inflammatory effects and mechanisms of lercanidipine and labedipinedilol-A, new generation dihydropyridine CCBs, in rat vascular smooth muscle cells (VSMCs) exposed to lipopolysaccharide (LPS) and interferon-γ (IFN-γ). MTT, Griess reagent, RT-PCR, ELISA, gelatin zymography, immunocytochemistry and Western blotting were employed. We found that lercanidipine and labedipinedilol-A attenuated production of NO, ROS and TNF-α from LPS/IFN-γ-stimulated VSMCs. In addition, they both diminished the LPS/IFN-γ-induced expression of iNOS protein and mRNA, with attenuation of HMGB1 cytosolic translocation and subsequent extracellular release. Furthermore, they down-regulated MMP-2/MMP-9 activities, whereas expression of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), an inhibitor of MMP-9, was up-regulated. Finally, we found that lercanidipine and labedipinedilol-A inhibited the nuclear translocation of NF-κB and suppressed the phosphorylation of JNK, p38 MAPK and Akt. Lercanidipine and labedipinedilol-A can exert their anti-inflammatory effects through suppression of NO, ROS and TNF-α through down-regulation of iNOS, MMP-2/MMP-9, and HMGB1, with inhibition of signaling transduction of MAPKs, Akt/IkB-α and NF-κB pathways. These findings implicate a valuable role of new generation dihydropyridine CCBs lercanidipine and labedipinedilol-A for the treatment of inflammatory vascular diseases.

Hall 3, ICC *Denotes presenting author

TUESDAY

ime-dependent structural and unctional changes in the spinal ord injured rat bladder PCB394 VS
sannah Gray*, Niamh McKerr, Barbara las, Celia Cruz, Francisco Cruz, Gordon cMurray, Karen McCloskey

vivo irradiation negatively pacts neurogenic and agonist-oked contractions in the mouse adder PCB395 VS
nagh McDonnell*, Karl Butterworth, vin Prise, Karen McCloskey

nvastatin inhibits human ometrial contractility by echanisms independent of olesterol depletion and HMG-A reductase inhibition PCB396 VS
cent Pang*

pid dilatation induced by ease of muscle compression PCB397 VS
co Turturici*, Silvestro Roatta

role of TRPM8 channels in mal rat bladder function PCB398 VS
hew King*, Orla Murray, Susannah Louise Johnston, Karen McKloskey, Johnson

cts of chronic administration irgin coconut oil on the liovascular profile in middle d male rats PCB399 VS
ya Thongsepee*, Chaiweewan kul

itory mechanism of *T*-type channel inhibitor, mibefradil oltage-dependent K⁺ nels in coronary arterial oth muscle cells PCB400 VS
un Na*, Da Hye Hong, Won Sun Park

Expression of smooth muscle-specific ion channels in TGF-β1-induced differentiation of human adipose-derived mesenchymal stem cells PCB401 VS
Youn Kyoung Son*, Da Hye Hong, Won Sun Park

The effect of PI3 kinase inhibitor LY294002 on voltage-dependent K⁺ channels in rabbit coronary arterial smooth muscle cells PCB402 VS
Da Hye Hong*, Sung Hun Na, Youn Kyoung Son, Won Sun Park

Decreased expression of ATP-sensitive K⁺ channel in aortic smooth muscle during isoproterenol-induced left ventricular hypertrophy PCB403 VS
Youn Kyoung Son*, Da Hye Hong, Won Sun Park

Physiological dynamics in the reversal of the Fåhraeus effect in the venous microcirculation of the human finger pulp PCB404 VS
William Murphy, Susan Browne*, Ricardo Segurado, Brigid Gallagher, Ciaran Murphy, Emma Tong

Lercanidipine and labedipinedilol-A play anti-inflammatory role through inhibition of lipopolysaccharide/interferon-γ-induced HMGB1 release and MMP-2, 9 activities in vascular smooth muscle cells PCB405 VS
Shu-Fen Liou*, Mei-Fen Shih, Chiu-Lan Chen

Effect of azelastine on cardiac action potential duration and hERG K⁺ channel current PCB406 VS
Mi-Hyeong Park*, Hyun Jo Su

Differential effects of environmental toxicants, PCB 126 and PCB 77, on cardiac electrophysiology PCB407 VS
Mi-Hyeong Park*, Su-Hyun Jo

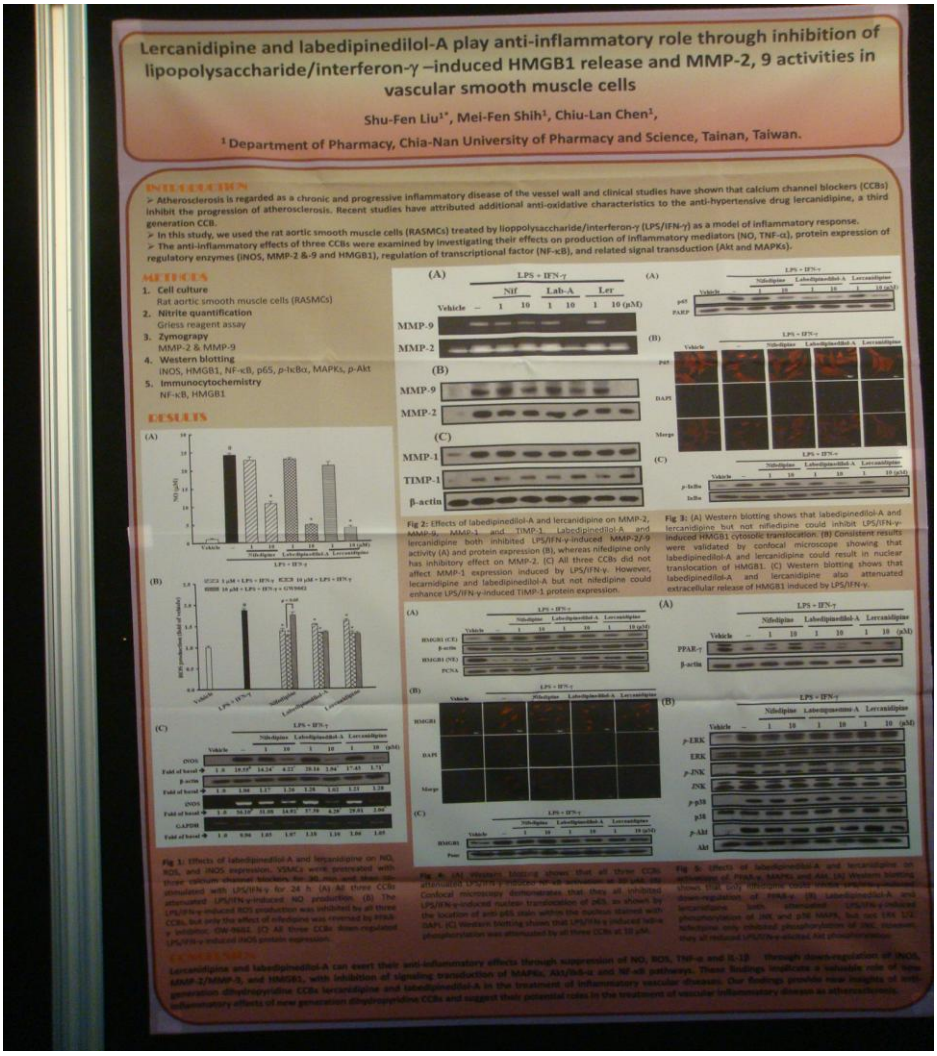
Novel approach to inhibition of neointimal hyperplasia in arteriovenous fistulae PCB408 VS
Richard Corbett*, Nicolò Demicheli, Lorenza Grechy, Francesco Iori, Jeremy Crane, Neill Duncan, Peter Vincent, Colin Caro

Perivascular adipose tissue inhibits thoracic aorta endothelial function through Cav-1-dependent inhibition of nitric oxide production PCB409 VS
Shiu-Jen Chen*, Hui-Hsin Lee, Cheng-Ming Tsao, Chin-Chen Wu

PKG signalling mediates perivascular adipose tissue function of up- and downstream of secreted adipokines via regulation of the expression and signalling of adiponectin PCB410 VS
Sarah Withers*, Laura Simpson, Matthias Werner, Anthony Heagerty

Local cleavage of endothelial cell junctional adhesion molecule (JAM)-C by neutrophil elastase governs development of systemic inflammation PCB411 VS
Bartomeu Colom*, Jennifer Bodkin, Mathieu Voisin, David Leinster, Michel Aurrand-Lions, Triantafyllos Chavakis, Beat Imhof, Sussan Nourshargh

Impact of gap junction-mediated information transfer on vessel tone and structure of microvascular networks PCB412 VS
Bianca Nitzsche, Bettina Reglin, Timothy Secomb, Axel Radlach Pries, Michael Hoepfner, Martin Maibier*



四、建議

參加這次的學會時與一些其他國家的相關研究領域學者進行討論，趕到獲益匪淺，一些改進的建議對自己的研究有所幫助，很感謝國科會經費補助，希望將來能有機會能再參與類似的國際會議，也希望國科會能繼續補助研究學著參與國際會議。世界各國最近幾年生物醫學發展隨著經濟提升，發展非常快速，不僅歐美領導，日本、韓國都有令人羨慕之成果。隨著經濟發展，鄰近的中國、越南、馬來西亞、泰國學術研究水準大幅提高，足以作為我們強力競爭對手。

五、攜回資料名稱及內容

IUPS 2013 Programme
IUPS 2013 Abstract Book

六、其他

國科會補助計畫衍生研發成果推廣資料表

日期:2013/09/27

國科會補助計畫	計畫名稱: 比較二氫　啶類鈣離子通道阻斷劑在脂多醣體/干擾素-γ處理的細胞及內毒素血症小鼠對誘發性一氧化氮合成酶、基質金屬蛋白酵素的表現與HMGB1釋放的作用
	計畫主持人: 劉淑芬
	計畫編號: 101-2320-B-041-003-　　學門領域: 藥學

無研發成果推廣資料

101 年度專題研究計畫研究成果彙整表

計畫主持人：劉淑芬	計畫編號：101-2320-B-041-003-
計畫名稱：比較二氫鮫啶類鈣離子通道阻斷劑在脂多醣體/干擾素-γ處理的細胞及內毒素血症小鼠對誘發性一氧化氮合成酵、基質金屬蛋白酵素的表現與 HMGB1 釋放的作用	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	1	1	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	1	1	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	1	1	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	無

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：■已發表 □未發表之文稿 □撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：(以 100 字為限)

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3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）(以 500 字為限)

在本計劃我們發現 lercanidipine 與 labedipinedilol-A 能夠有效抑制 LPS/IFN-γ 誘導的血管平滑肌細胞發炎反應以及抑制 iNOS、COX-2 蛋白表現和 NO、TNF-α 、ROS 的釋放，lercanidipine 與 labedipinedilol-A 也減少了 LPS/IFN-γ 誘導的 MMP-2 和 MMP-9 的酵素活性及蛋白表現量，此作用機轉是經由抑制 LPS/IFN-γ 誘發 MAPKs、Akt、IκBα 的磷酸化進而減少 NF-κB 次單元 p65 的核轉移之訊息傳遞路徑。配合先前的離體細胞實驗成果顯示，lercanidipine 與 labedipinedilol-A 可誘導血管內皮細胞 NO 生成和抑制血管平滑肌細胞的遷移、增生和活性氧屬的產生。另外，在活體動物實驗中也證實了 lercanidipine 在頸動脈受損的 Wistar 大鼠可抑制血管內膜增厚。綜合離體細胞與活體動物的實驗成果，證實 lercanidipine 與 labedipinedilol-A 具有增進血管功能與抑制發炎反應之重要角色，補充 lercanidipine 與 labedipinedilol-A 可經由抗發炎之分子機轉而具有血管保護作用，因此這些成果將提供未來對於發炎性血管疾病如粥狀動脈硬化之治療與研究提供新的方向。