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의학박사 학위논문

Anti-inflammatory effect of a c-Met inhibitor for the suppression of atherosclerosis via a PPARδ/IL-10 pathway

PPARδ/IL-10 경로를 통한 c-Met 억제제의 항염증 효과가 동맥경화의 억제에 미치는 영향에 관한 연구

2023년 2월

서울대학교 대학원

의학과 외과학전공

박 형 섭

Anti-inflammatory effect of a c-Met inhibitor for the suppression of atherosclerosis via a PPARδ/IL-10 pathway

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이 논문을 의학박사 학위논문으로 제출함
2022년 10월

서울대학교 대학원
의학과 외과학전공
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박형섭의 의학박사 학위논문을 인준함
2023년 1월

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Abstract

Background: Atherosclerosis is a leading cause of cardiovascular disease and the adhesion of inflammatory cells to intimal endothelial cells is a well-known mechanism of atherosclerosis progression. Tyrosine kinases play an important role in inflammation and strategies to control tyrosine kinase activity have been widely used to modulate chronic inflammatory states. In this study we investigated the impact of a c-Met inhibitor capmatinib (CAP) on the suppression of atherosclerotic inflammatory response by investigating CAPs impact on 1) lipopolysaccharide (LPS)-mediated adhesion of human umbilical vein endothelial cells (HUVECs) and THP-1 monocytes in vitro, and 2) progression of atherosclerosis in ApoE knockout mice in vivo.

Methods: HUVECs and THP-1 monocytes were treated with LPS and CAP. Protein expression levels related to endothelial cell adhesion and inflammation were determined using Western blotting. Target protein (PPARδ, IL-10) knockdown was conducted using small interfering (si) RNA transfection. Adhesion between HUVECs and THP-1 cells was assayed using green fluorescent dye. CAPs effect in vivo were tested on ApoE (-/-) mouse fed with western diet. CAP was fed orally for 5 weeks and compared with control, while PPARδ siRNA injection was performed every other day for 5 weeks in another group to verify the PPARδ-related mechanism of CAP.

Results: Through in vitro studies, we found that CAP treatment suppressed cell adhesion between THP-1 monocytes and HUVECs and the expression of adhesive molecules, such as intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin. Moreover, phosphorylation of inflammatory markers, such as NFκB and IκB as well as TNFα and monocyte chemoattractant protein-1 (MCP-1), released from HUVECs and THP-1 monocytes, was decreased by CAP treatment. Treatment with CAP increased PPARδ and IL-10 expression, while siRNA-associated suppression of PPARδ and IL-10 attenuated the effects of CAP on cell adhesion between HUVECs and THP-1 cells and inflammatory responses. Furthermore, PPARδ siRNA suppressed CAP-mediated induction of IL-10 expression. In vivo, CAP abolished the increased expression of ICAM-1, E-selectin and TNFα in ApoE(-/-) atherosclerotic mice, and increased the expression of anti-inflammatory marker IL-10 as well as PPARδ. These effects were reversed in the PPARδ siRNA transfected mouse group.

Conclusion: These findings imply that CAP improves inflamed endothelial-monocyte adhesion in vitro via a PPAR/IL-10-dependent pathway, and this pathway was verified in vivo in an atherosclerotic mouse model. The current study provides insights for a new therapeutic approach in treating atherosclerosis.

Keyword : Capmatinib; Inflammation; PPAR delta; IL-10; c-MET; Atherosclerosis

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Chapter 1. Introduction

Cardiovascular diseases are one of the most common causes of death worldwide and its incidence is continuously increasing. It mainly results from atherosclerosis-mediated complications caused by a chronic inflammatory state, associated with abnormal proliferation of endothelial and vascular smooth muscle cells (VSMC) [1]. Atherosclerosis is characterized by vascular inflammation, endothelial dysfunction, and accumulation of lipid, cholesterol, and calcium in the intima of large- and medium-sized muscular arteries, forming plaques [2]. During the initial stages of atherosclerosis, endothelial dysfunction leads to a decrease in NO-mediated vasodilation, which in turn leads to endothelial activation. This process is followed by an increase in monocyte adhesion and migration, and its interplay with VSMCs leads to plaque formation and rupture [3,4].

Although many current drug therapies related to atherosclerosis mainly target on the reduction of risk factors such as hypertension, diabetes mellitus and dyslipidemia, recent studies have also targeted inflammation as a potential target for suppression of atherosclerosis. Tyrosine kinases are one potential target since drugs targeted in modulating tyrosine kinase activity have been widely used to treat chronic inflammatory states in rheumatologic, cardiovascular and oncologic diseases. Capmatinib (CAP) is a c-Met or hepatocyte growth factor receptor (HGFR) inhibitor, and has

recently received FDA approval for use in treating metastatic non-small cell lung cancer in adults with the MET gene mutation [5]. Although cardiovascular diseases and cancer may seem unrelated at first glance, they share various molecular signaling pathways associated with pathogenic developmental processes, such as genetic alteration, environmental factors, and lifestyle [6]. Notably, cancer development is caused by several molecular mechanisms catalyzed by the same elements for atherosclerosis [7], and a thorough analysis of its molecular mechanisms have clarified significant similarities and have shown evidence of a close relationship [8].

Although CAP is clinically being used as an anticancer drug, it has also been reported in previous studies to attenuate diethylnitrosamine [9] or acetaminophen [10]-induced inflammation in mice through downregulation of serum pro-inflammatory cytokines, such as TNFα and IL-1β. CAP also suppresses inflammation via a PPARδ/p38-dependent pathway, thereby attenuating insulin resistance in skeletal muscle cells [11]. Furthermore, Ahn et al. found that CAP inhibits lipogenesis in 3T3-L1 adipocytes through an AMP-activated protein kinase (AMPK)-dependent mechanism [12]. These studies demonstrate that CAP has anti-inflammatory and anti-metabolic syndrome properties, in addition to its anti-neoplastic properties.

Based on the association between cancer and atherosclerosis and the anti-inflammatory effect of CAP, we performed the current study to demonstrate that CAP attenuates inflammatory responses

in endothelial cells, thereby suppressing its adhesion with monocytes using human umbilical vein endothelial cells (HUVECs) and THP-1 monocytes in vitro. Furthermore, CAP-associated molecular mechanisms are also revealed in vitro and tested in vivo using an ApoE knockout atherosclerotic mouse model.

Chapter 2. Materials and Methods

2.1. Cell culture and treatments

HUVECs (ATCC, Manassas, VA, USA) were grown in M200PRF medium (Invitrogen, Carlsbad, CA, USA) supplemented with a low-serum growth supplement mixture on 0.3 % gelatin-coated culture plates (Invitrogen). THP-1 human monocytes (ATCC, Manassas, VA, USA) were cultivated in RPMI 1640 media containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 g/mL streptomycin (Invitrogen). At 37℃, cells were grown in a humidified environment containing 5% CO2. For all investigations, cells at passages 4–5 were employed. Lipopolysaccharide (LPS) (Sigma, St. Louis, MO, USA) was dissolved in phosphate-buffered saline (PBS; Biosesang, Seoul, Republic of Korea). HUVECs and THP-1 cells were treated with 0–10 nM CAP (Selleckchem, Houston, TX, USA) for 24 h.

2.2. Immunoblotting

Cultured cells were extracted and lysed for 60 min at 4℃ in cell lysis buffer (Intron Biotechnology, Seoul, Republic of Korea). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on equal amounts of proteins (30-40 μg)

using 10% gel. The extracted proteins were separated and put to a nitrocellulose membrane (Amersham Bioscience, Westborough, MA, USA). The transferred membranes were probed with the corresponding primary antibodies, followed by binding with secondary antibodies linked to horseradish peroxidase (Santa Cruz Biotechnology). An enhanced chemiluminescence kit was used to detect immunoreactive signals (Amersham Bioscience).

2.3. Antibodies

The antibodies used were as follows: anti-ICAM-1 (1:1000), anti-VCAM-1 (1:1000), anti-E-selectin (1:1000), anti-phospho NFκB (1:1000), anti-NFκB (1:2500), anti-phospho IκB (1:1500), anti-PPARδ (1:1500), anti-IL-10 (1:1500), and anti-β-actin (1:2000) (Santa Cruz Biotechnology).

2.4. Gene knock-down in cells

The small-interfering RNA (siRNA) oligonucleotides (20 nM) selective for PPARδ (PPARδ siRNA) and IL-10 (IL-10 siRNA) were procured from Santa Cruz Biotechnology. Cultured cells were transfected with PPARδ siRNA or IL-10 siRNA using Lipofectamine 3000 (Invitrogen), according to the manufacturer's directions. In brief, the cells were grown until they were 85% confluent. The cells

were serum-starved for 12 h before being transfected with 20 nM siRNAs. Protein extraction was performed on the transfected cells.

2.5. Enzyme linked immunosorbent assay (ELISA)

The culture supernatant of cells was stored at -70℃ for further ELISA. The TNFα and MCP-1 as well as IL-10 levels in the cell culture media were quantified using ELISA kits (R&D Systems, Minneapolis, MN, USA) for each protein following the manufacturer's instructions.

2.6. Cell adhesion assay between HUVECs and THP-1 monocytes

LPS (200 ng/mL) and CAP (0-5 nM) were given to HUVECs for 24 h. Then, the treated HUVECs were co-cultured with THP-1 monocytes that had been tagged with the green fluorescent dye by incubating with 10 μg/mL 2,7-bis (2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethylester (Invitrogen) for 30 min at 37℃. THP-1 cells were washed twice with PBS after co-culture with HUVECs for 30 min. Adhered THP-1 monocytes (green dots) were calculated as follows: adhered THP-1 cells (%): [(number of THP-1 cells co-cultured with HUVECs) - (number of washed out THP-1 cells)] / (number of THP-1 cells co-cultured with

HUVECs) × 100. Number of THP-1 cells co-cultured with HUVECs was 1×10^4.

2.7. In vivo atherosclerotic mouse model

Eight-week-old ApoE knockout mice (C57BL/6) were fed with western diet (WD) for 5 weeks to create an in vivo model of atherosclerosis. To verify the effect of CAP, CAP (1mg/kg) was fed orally during the 5 weeks (CAP group), while in another group, injections of PPARδ siRNA (20nM) were performed every other day in the tail vein for suppression of PPARδ expression (PPARδ siRNA group). Based on these treatment protocols, the mice were divided into 5 groups: 1) ApoE (-/-), 2) ApoE (-/-) + WD, 3) ApoE (-/-) + WD + CAP, 4) ApoE (-/-) + WD + CAP + PPARδ siRNA, and 5) control (normal B6 mouse). After 5 weeks, blood was drawn for total cholesterol analysis and the mice were sacrificed. The aorta was harvested, and histologic analysis as well as tissue expression of endothelial and inflammatory markers was performed using protocols previously mentioned.

2.8. Statistical analyses

The data is provided as a set of relative values. Analyses were conducted using one-way ANOVA, which was followed by post hoc analysis (Tukey test). All statistical analyses were conducted using

GraphPad Prism version 7 for Windows (La Jolla, CA, USA). Results are presented as the fold of the highest values (means ± SEM) or the absolute values. At least three replicates of each experiment were performed.

Chapter 3. Results

3.1. CAP inhibits the adhesion between monocytes and endothelial cells and the expression of adhesive molecules in endothelial cells

First, we performed a cell viability assay in HUVECs and THP-1 cells to determine the treatment concentration of CAP. Cell viabilities were not affected by 0-5 nM CAP. However, a significant decrease in viable cells was noticed at 10 nM CAP in both cell types (Fig. 1A). Adhesion and migration of monocytes are known to be enhanced at the beginning of atherosclerotic plaque formation [13]. Thus, we used cell culture to investigate the impact of CAP on the adhesion between endothelial cells and monocytes. We confirmed that LPS upregulated the adhesion of THP-1 monocytes to HUVECs. However, CAP treatment reversed dose-dependently this change (Fig. 1B). In HUVECs, CAP treatment dose-dependently inhibited LPS-induced expressions of adhesive molecules, such as ICAM-1, VCAM-1, and E-selectin (Fig. 1C).

3.2. CAP suppresses inflammatory responses in LPS-treated endothelial cells and monocytes

Inflammation is a crucial factor in the progression of atherosclerosis [14]. Treatment with CAP attenuated LPS-induced inflammatory markers, such as phosphorylated NFκB and IκB expression in HUVECs and THP-1 cells (Fig. 2A). Furthermore, LPS mediated the release of pro-inflammatory cytokines, such as TNFα and MCP-1, to culture media of HUVECs and THP-1 monocytes, and its effects were again attenuated by administration of CAP (Fig. 2B).

3.3. PPARδ is involved in CAP's impact on atherosclerotic response

PPARδ exerts suppressive effects on atherogenic inflammation [15]. We found that treatment with CAP enhanced the expression of PPARδ in HUVECs and THP-1 monocytes (Fig. 3A). siRNA-associated suppression of PPARδ abolished the effects of CAP on LPS-mediated attachment of THP-1 monocytes on HUVECs and adhesion molecule expression in HUVECs (Fig. 3B and C). Moreover, the inhibitory effects of CAP on LPS-induced NFκB and IκB phosphorylation were reversed by PPARδ siRNA (Fig. 3D). Additionally, PPARδ siRNA abolished the impact of CAP on the release of TNFα and MCP-1 to culture media of LPS-treated HUVECs and THP-1 monocytes (Fig. 3E).

3.4. PPARδ/IL-10 signaling participates in the effects of CAP on monocytes and endothelial cells

IL-10 is a cytokine with anti-inflammatory properties, which appears to play a preventive role in the development of atherosclerosis [16]. Treatment with CAP increased IL-10 expression in HUVECs and THP-1 cells as well as the release of IL-10 (Fig. 4A). IL-10 siRNA reversed CAP-mediated suppression of adhesion of THP-1 monocytes to HUVECs (Fig. 4B and C) and inflammatory responses (Fig. 4D and E) in these two cell types. In addition, siRNA for IL-10 did not affect PPARδ expression, whereas PPARδ siRNA suppressed CAP-induced IL-10 expression in HUVECs (Fig. 4F), demonstrating that PPARδ exerts its effect on Il-10 and not vice versa.

3.5. CAP suppresses inflammatory response through PPARδ/IL-10 signaling in an ApoE knockout atherosclerotic mouse model

Our in vitro study results demonstrate that CAP increases expression of PPARδ, which in turn increases expression of anti-inflammatory IL-10, thus suppressing inflammation as represented by decreased interaction of HUVECs and THP-1 monocytes after LPS-stimulation (Fig. 5). To demonstrate this mechanism of action

in vivo, CAP was administered to ApoE knockout mice fed with western diet to induce an atherosclerotic response. The total cholesterol levels analyzed from blood samples obtained at 5 weeks revealed that there was a significant increase in cholesterol levels in the ApoE(−/−)+ WD group compared to control, which represents the successful formation of atherosclerosis (Fig 6A). The expression of adhesion molecules ICAM−1 and E−selection, and inflammatory marker TNFα was significantly increased in the aorta of ApoE(−/−) fed with western diet [ApoE(−/−)+ WD group], but was significantly reduced after administration of CAP (CAP group). However, this effect of CAP was abolished in mice administered with PPARδ siRNA (PPARδ siRNA group) (Fig 6B−C). On the other hand, the expression of anti−inflammatory cytokine IL−10 was low in the ApoE(−/−)+ WD group, but was significantly increased in the CAP group and was again significantly decreased in the PPARδ siRNA group (Fig. 6D). Additionally, PPARδ levels were also increased in the CAP group, while it was totally decreased in the PPARδ siRNA group, showing that PPARδ was effectively suppressed in aortic tissue by injection of siRNA (Fig 6E). In terms of histologic analysis, although there was no definite formation of atherosclerotic plaques on H&E stain, there was a tendency for formation of foam cells in the aortic walls of ApoE(−/−)+ WD group and PPARδ siRNA group mice, which was not so evident in the CAP group (Fig 6F). Based on these in vivo results, it can be inferred that CAP exerts its anti−inflammatory effect on atherosclerosis through a PPARδ/IL−10 pathway.

Chapter 4. Discussion

Atherosclerosis is the leading cause of vascular death around the world, and it is a severe health problem with poor prognosis and large socioeconomic burden [17]. Inflammation is a major contributor to the development of atherosclerosis by generating arterial plaques [18]. Macrophage infiltration (primarily derived from circulating monocytes) leads to atherosclerotic plaque destabilization, causing upregulation of pro-inflammatory cytokines and lytic proteins, thereby rupturing the plaque's fibrous cap [19]. Therefore, proper inflammation management could be a therapeutic strategy for treating atherosclerosis and its complications. In the presence of LPS, we discovered that CAP treatment reduced THP-1 monocyte adherence to HUVECs and inhibited the expression of adhesion molecules. Treatment of HUVECs and THP-1 cells with CAP attenuated LPS-induced inflammatory responses, such as NFκB and IκB phosphorylation, as well as TNFα and MCP-1 secretion. These results may reveal that CAP attenuates the inflammatory response and adhesion between endothelial cells and monocytes, a typical pathological phenomenon associated with atherosclerosis, showing the potential of CAP as a therapeutic agent for atherosclerosis.

PPARδ, a ligand-activated transcription factor, is a member of the nuclear hormone receptor superfamily [20]. It is essential for the control of cellular energy metabolism and inflammation [21]. In this

context, Zingarelli et al. have found that PPARδ activation by a specific PPARδ ligand attenuates inflammation via suppression of NFκB-mediated pathway in sepsis models [22]. Telmisartan inhibits the pro-inflammatory effects of homocysteine on HUVECs via the PPARδ-dependent pathway [23]. Lee et al. have demonstrated that kynurenic acid improves inflammatory responses in HUVECs through PPARδ/heme oxygenase-1 (HO-1) signaling [24]. Therefore, these studies propose PPARδ as a therapeutic target for inflammatory metabolic diseases, including atherosclerosis [15]. In our study, we found that CAP treatment increased PPARδ expression in HUVECs and THP-1 monocytes, and suppression of PPARδ expression by siRNA abolished the effects of CAP on LPS-mediated cell adhesion between HUVECs and THP-1 monocytes and inflammatory responses. These results show that PPARδ contributes to the suppressive effects of CAP on LPS-induced monocyte-endothelial adhesion and inflammation in HUVECs and THP-1 monocytes.

IL-10, an anti-inflammatory cytokine, blocks the synthesis of pro-inflammatory cytokines in inflamed monocytes [25]. Several studies have shown that IL-10 has an anti-atherogenic property. For instance, recombinant IL-10 inhibits monocyte adherence to endothelial cells via suppression of cell adhesion molecules [26,27]. Recombinant IL-10 administration decreased stent-implantation-mediated intimal hyperplasia in hypercholesterolemic animal models [28]. Conversely, IL-10-deficient mice demonstrated the development of severe atherosclerosis evidenced by increased T-

cell infiltration and reduction of collagen content in atherosclerotic lesions [16]. The current study found that treating HUVECs and THP-1 monocytes with CAP increased cellular IL-10 expression and IL-10 release to culture media. Similar to PPARδ, IL-10 siRNA abolished the effects of CAP on HUVECs and THP-1 cells in the presence of LPS. Furthermore, siRNA for PPARδ supressed CAP-induced IL-10 expression, whereas IL-10 siRNA did not influence the induction of PPARδ expression by CAP. These results indicate that the PPARδ-regulated IL-10 axis has an impact on the effects of CAP on monocyte-endothelial adhesion and inflammation.

Based on our in vitro data, we inferred that the anti-inflammatory effect of CAP works through a PPARδ/IL-10 pathway (graphically represented in figure 5). This anti-inflammatory effect on atherosclerosis was checked in vivo using an ApoE (-/-) atherosclerotic mouse model, and analysis of aortic tissue showed similar results to that of our in vitro data. The increase in ICAM-1, E-selectin and TNFα in ApoE(-/-) atherosclerotic mice was suppressed in mice fed with CAP, while this decrease from CAP administration was abolished in mice injected with PPARδ siRNA, showing that CAP exerts its effect through PPARδ. Additionally, IL-10 expression was elevated only after CAP administration, and again abolished after PPARδ siRNA injection, demonstrating that PPARδ exerts its effect through IL-10. Our histological analysis of the aortic tissue failed to show formation of atherosclerotic plaques, probably due to the short experimental period of 5 weeks since other studies have reported successful plaque formation after 16

weeks [29,30]. However, there was a tendency for formation of foam cells (lipid-laden macrophages) in the atherosclerotic mice group, which is known as an early finding of atherosclerosis. Such findings were not so evident in the CAP group, but was visible in the PPARδ siRNA injection group, demonstrating a potential effect of CAP on atherosclerosis suppression in vivo. It should be noted that biological responses tend to occur earlier than histologic changes, which may be the reason why at 5 weeks we found significant changes in the expression of molecular markers but histologic changes were not so evident. Therefore, the short period of experimentation was a limitation of our study, and we suggest that histologic changes would be more likely to appear with longer study periods.

Chapter 5. Conclusion

In the current study, we have demonstrated several novel findings: 1) CAP treatment suppressed the adhesion between HUVECs and THP-1 monocytes and expression of adhesion molecules in HUVECs; 2) Treatment with CAP suppressed inflammatory responses in HUVECs and THP-1 monocytes in the presence of LPS; 3) Treatment of CAP dose-dependently increased PPARδ and IL-10 expression in both cell types; 4) siRNA for PPARδ or IL-10 abolished the effects of CAP on the adhesion and inflammatory responses between HUVECs and THP-1 monocytes under LPS conditions; 5) The anti-inflammatory effect of CAP through a PPARδ/IL-10 pathway was verified in vivo in an atherosclerotic mouse model.

Based on these results, we have demonstrated that the c-Met inhibitor capmatinib exerts an anti-inflammatory effect through a PPARδ/IL-10 signaling pathway, which in turn attenuates atherosclerotic response in vitro (HUVECs) and in vivo (ApoE knockout mice). Considering the role of inflammation in the progression of atherosclerosis, modulation of inflammation using tyrosine kinase inhibitors may be an effective strategy in the treatment of atherosclerosis, capmatinib being a potential drug of choice.

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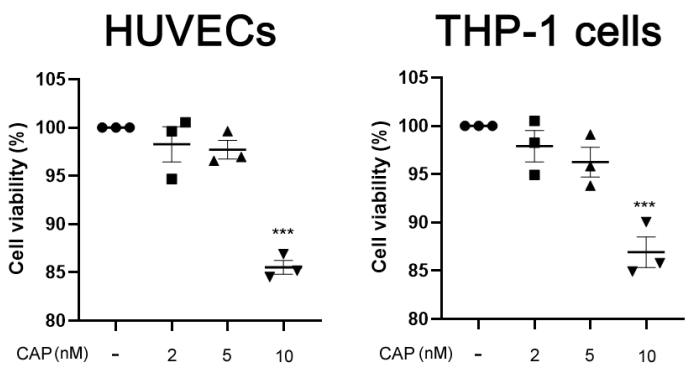
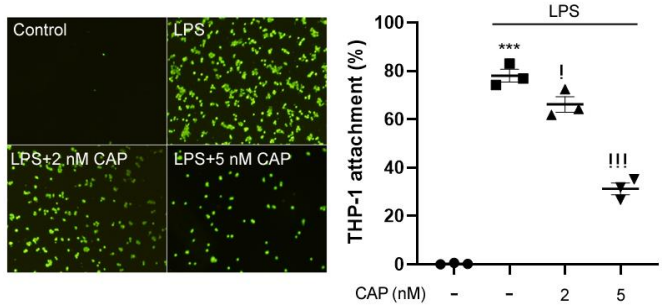
Figures

Fig. 1. CAP reduces monocyte adhesion on endothelial cells as well as adhesion molecule expression in endothelial cells. (A) MTT assay in HUVECs and THP-1 cells treated with 0-10 nM CAP for 24 h. (B) THP-1 cell adhesion assay and (C) Western blotting of ICAM-1, VCAM-1, and E-selectin in HUVECs treated with LPS (200 ng/mL) and 0-5 nM CAP for 24 h. ***P<0.001 when compared to control. !!!P<0.001, !!P<0.01, and !P<0.05 when compared to LPS.

(A)



(B)


Control
LPS
LPS+2 nM CAP
LPS+5 nM CAP
LPS
THP-1 attachment (%)
100
80
60
40
20
0
***
!
!!!
CAP (nM) - - 2 5

(C)

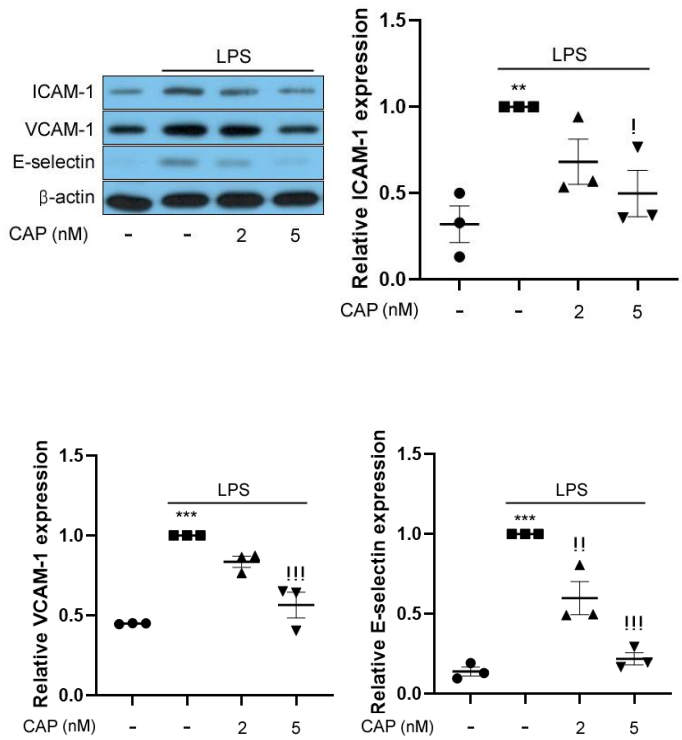
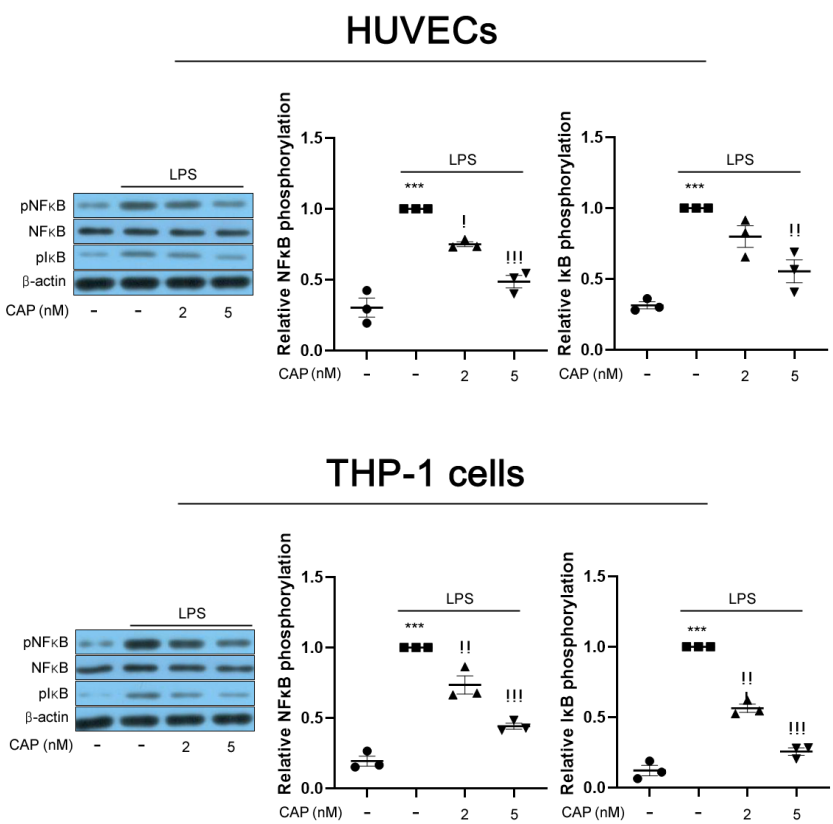

LPS
ICAM-1
VCAM-1
E-selectin
β-actin
CAP (nM) - - 2 5
Relative ICAM-1 expression
1.5
1.0
0.5
0.0
**
!
Relative VCAM-1 expression
***
!!!
Relative E-selectin expression
!!

Fig. 2. CAP suppresses inflammatory responses in LPS-treated HUVECs and THP-1 monocytes. (A) Western blot analysis of phosphorylated NFκB and IκB in HUVECs and THP-1 cells treated with LPS (200 ng/mL) and 0-5 nM CAP for 24 h. (B) ELISA of TNF and MCP-1 in culture media of HUVECs and THP-1 cells treated with LPS (200 ng/mL) and 0-5 nM CAP for 24 h. ***P<0.001 when compared to control. !!!P<0.001, !!P<0.01, and !P<0.05 when compared to LPS.

(A)



(B)

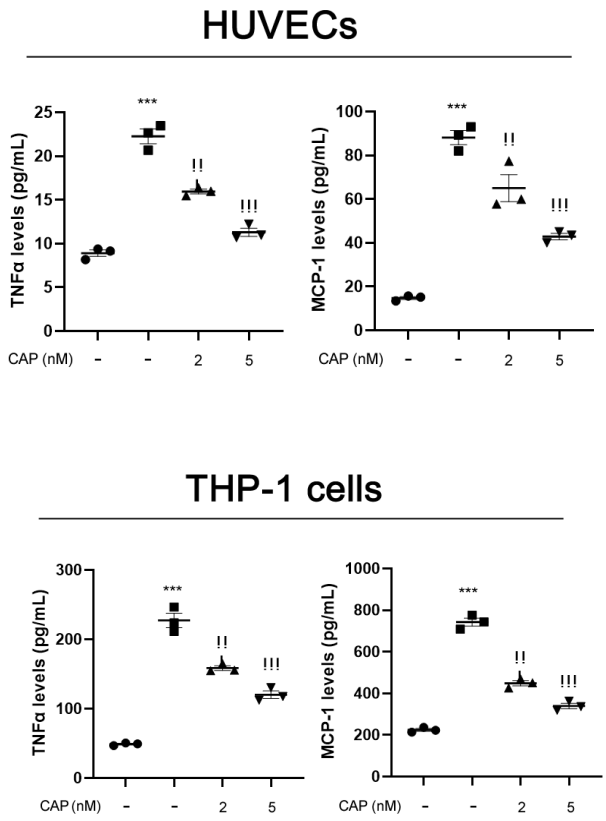
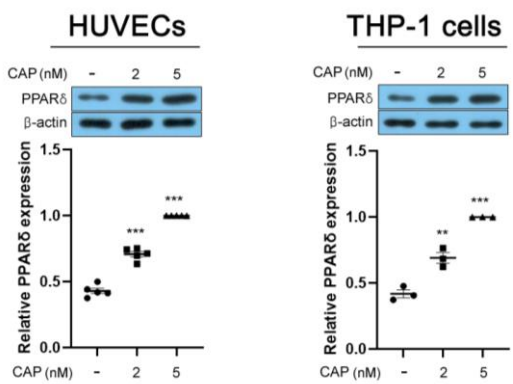
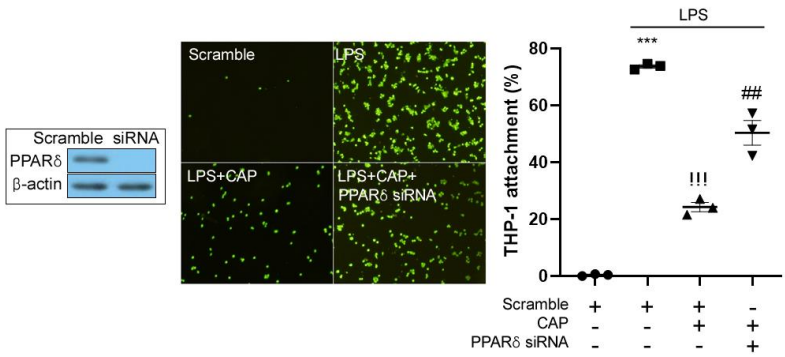

HUVECs
TNFα levels (pg/mL)
MCP-1 levels (pg/mL)
CAP (nM)
- - 2 5
***
!!
!!!
THP-1 cells
TNFα levels (pg/mL)
MCP-1 levels (pg/mL)
CAP (nM)
- - 2 5

Fig. 3. CAP attenuates the adhesion between HUVECs and THP-1 monocytes as well as inflammation through PPARδ-dependent signaling. (A) Western blot analysis of PPARδ in HUVECs and THP-1 cells treated with 0-5 nM CAP for 24 h. (B) THP-1 cell adhesion assay and (C) Western blotting of ICAM-1, VCAM-1, and E-selectin in scrambled or PPARδ siRNA (20 nM)-transfected HUVECs treated with LPS (200 ng/mL) and CAP (5 nM) for 24 h. (D) Western blot analysis of phosphorylated NFκB and IκB in scrambled or PPARδ siRNA (20 nM)-transfected HUVECs and THP-1 cells treated with LPS (200 ng/mL) and CAP (5 nM) for 24 h. (E) ELISA of TNF and MCP-1 in culture media of scrambled or PPARδ siRNA (20 nM)-transfected HUVECs and THP-1 cells treated with LPS (200 ng/mL) and CAP (5 nM) for 24 h. ***P<0.001 and **P<0.01 when compared to control. !!!P<0.001, !!P<0.01, and !P<0.05 when compared to LPS. ###P<0.001, ##P<0.01, and #P<0.05 when compared to LPS plus CAP.

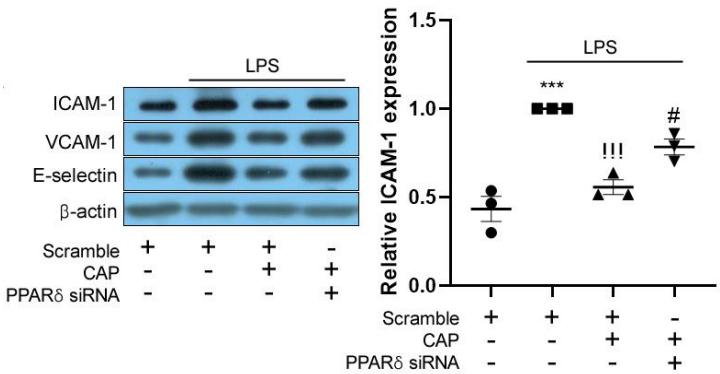
(A)

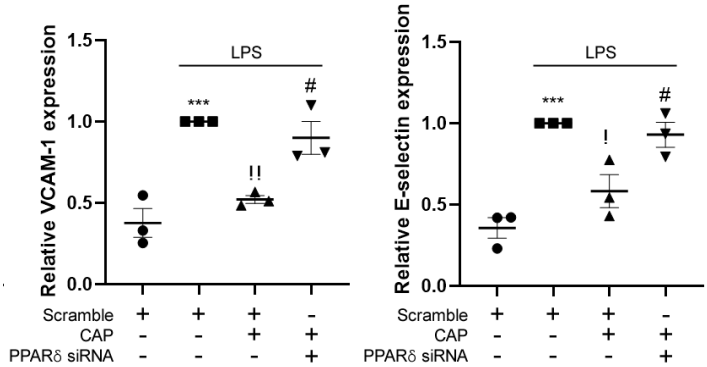


(B)

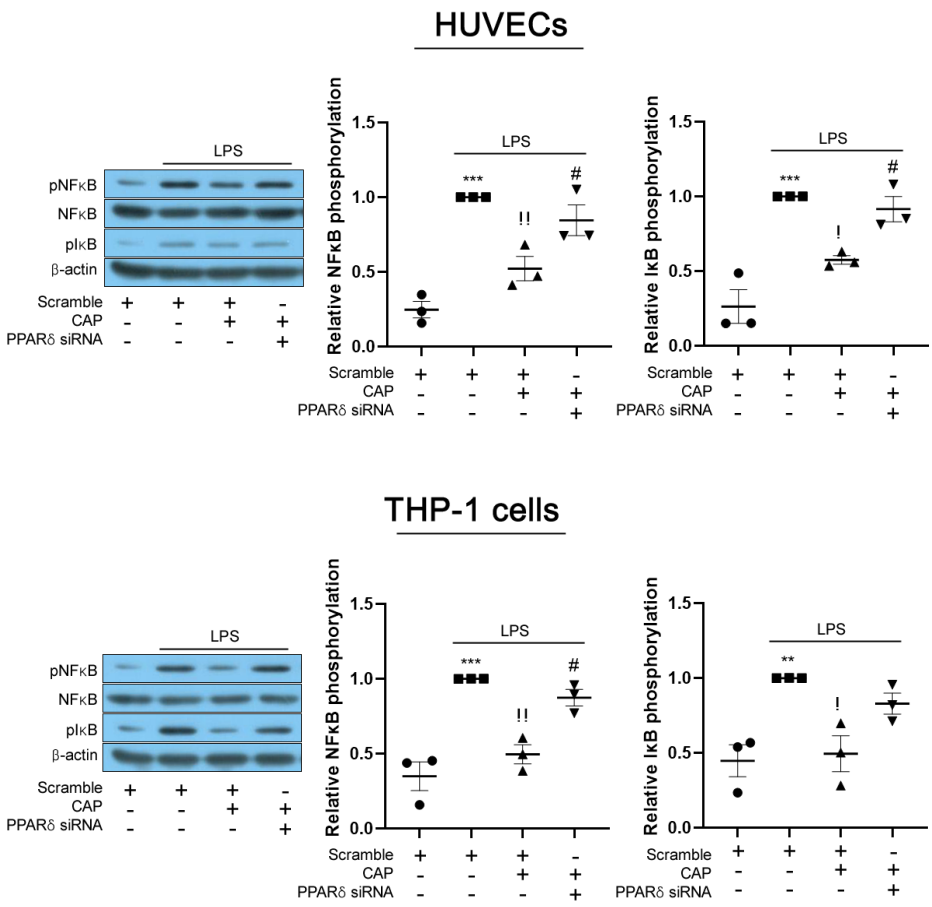

Scramble siRNA
PPARδ
β-actin
Scramble
LPS
LPS+CAP
LPS+CAP+
PPARδ siRNA
LPS
THP-1 attachment (%)
***
!!!
##
Scramble
CAP
PPARδ siRNA

(C)


LPS
ICAM-1
VCAM-1
E-selectin
β-actin
Scramble
CAP
PPARδ siRNA
Relative ICAM-1 expression
***
!!!
#


Relative VCAM-1 expression
***
!!
#
Relative E-selectin expression
***
!
#
LPS
Scramble
CAP
PPARδ siRNA

(D)


HUVECs
LPS
pNFκB
NFκB
pIκB
β-actin
Scramble + + + -
CAP - - + +
PPARδ siRNA - - - +
Relative NFκB phosphorylation
Relative IκB phosphorylation
***
!!
!
#
THP-1 cells
**

(E)

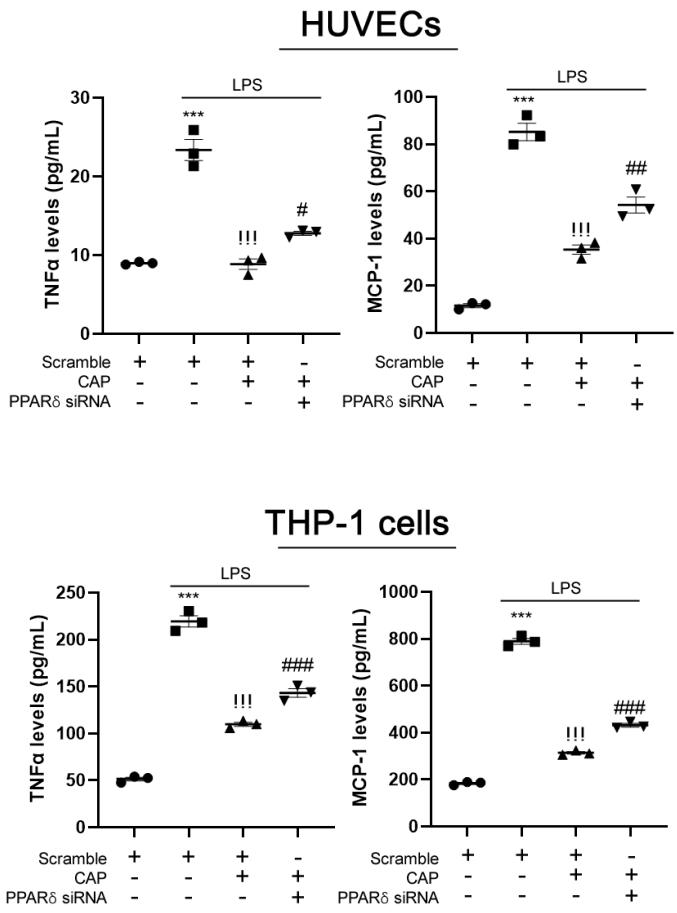
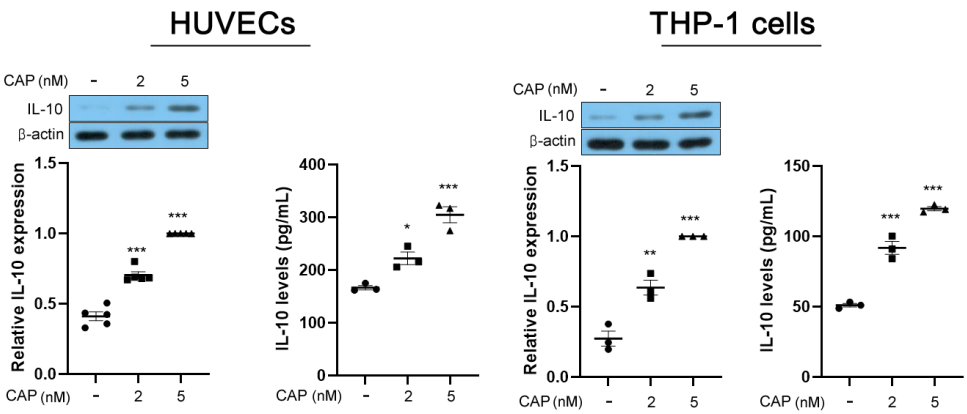
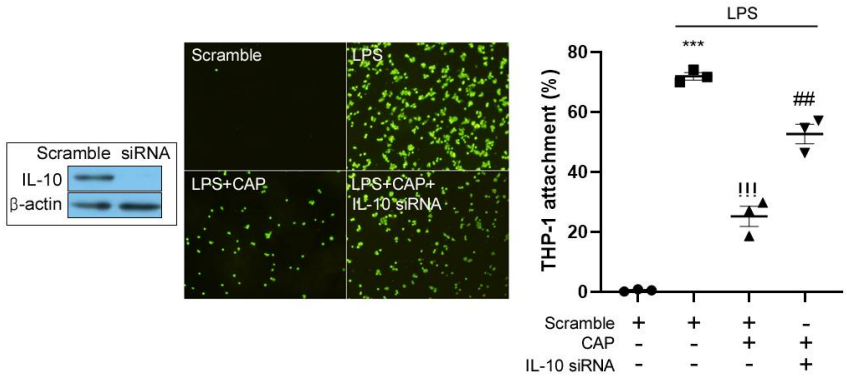

HUVECs
LPS
TNFα levels (pg/mL)
MCP-1 levels (pg/mL)
***
!!!
#
##
Scramble
CAP
PPARδ siRNA
THP-1 cells
###

Fig. 4. PPARδ/IL-10 pathway contributes to the suppressive effects of CAP on atherosclerotic responses. (A) Western blot analysis of IL-10 in HUVECs and THP-1 cells treated with 0-5 nM CAP for 24 h. (B) THP-1 cell adhesion assay and (C) Western blotting of ICAM-1, VCAM-1, and E-selectin in scrambled or IL-10 siRNA (20 nM)-transfected HUVECs treated with LPS (200 ng/mL) and CAP (5 nM) for 24 h. (D) Western blot analysis of phosphorylated NFκB and IκB in scrambled or IL-10 siRNA (20 nM)-transfected HUVECs and THP-1 cells treated with LPS (200 ng/mL) and CAP (5 nM) for 24 h. (E) ELISA of TNFα and MCP-1 in culture media of scrambled or IL-10 siRNA (20 nM)-transfected HUVECs and THP-1 cells treated with LPS (200 ng/mL) and CAP (5 nM) for 24 h. (F) Western blotting of IL-10 in scrambled or PPARδ siRNA (20 nM)-transfected HUVECs and THP-1 cells treated with CAP (5 nM) for 24 h, and western blotting of PPARδ in scrambled or IL-10 siRNA (20 nM)-transfected HUVECs and THP-1 cells treated with CAP (5 nM) for 24 h. *P<0.05 and **P<0.01 when compared to control. !!!P<0.001, !!P<0.01, and !P<0.05 when compared to LPS or CAP. ###P<0.001, ##P<0.01, and #P<0.05 when compared to LPS plus CAP.

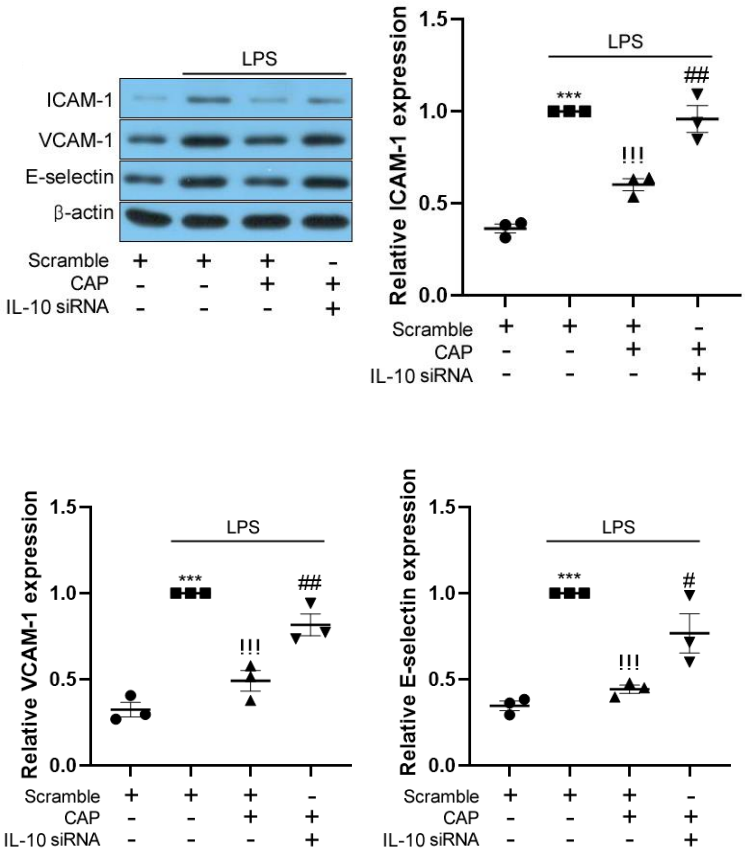
(A)


HUVECs
THP-1 cells
CAP (nM) - 2 5
IL-10
β-actin
Relative IL-10 expression
IL-10 levels (pg/mL)
CAP (nM) - 2 5

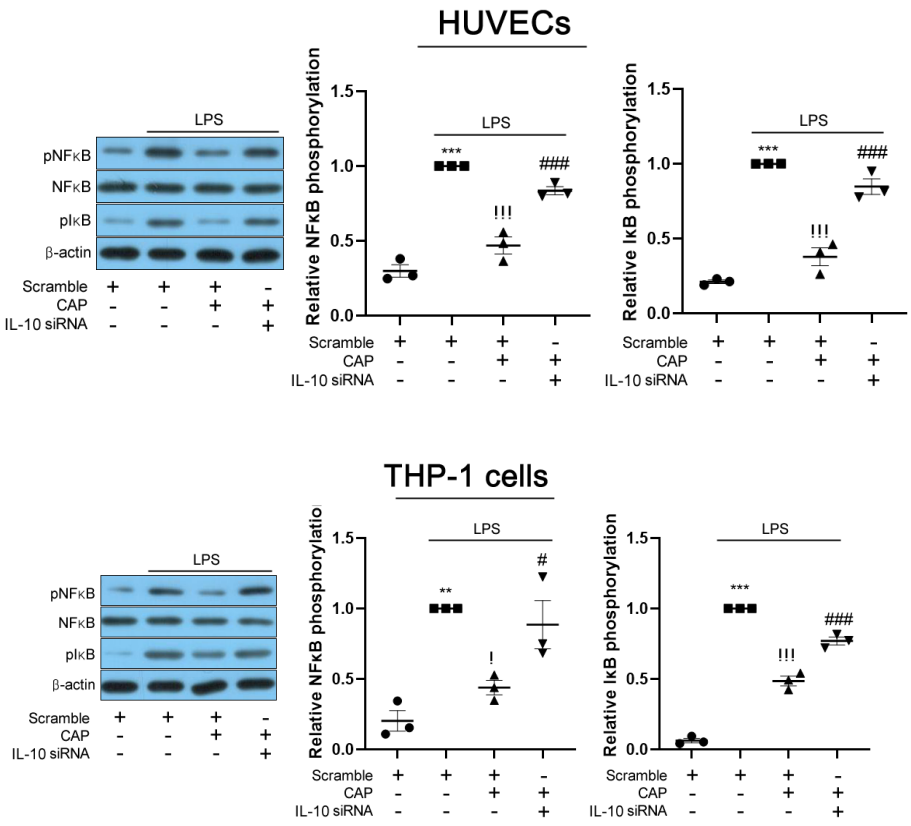
(B)


Scramble siRNA
IL-10
β-actin
Scramble
LPS
LPS+CAP
LPS+CAP+
IL-10 siRNA
LPS
THP-1 attachment (%)
Scramble + + + -
CAP - - + +
IL-10 siRNA - - - +

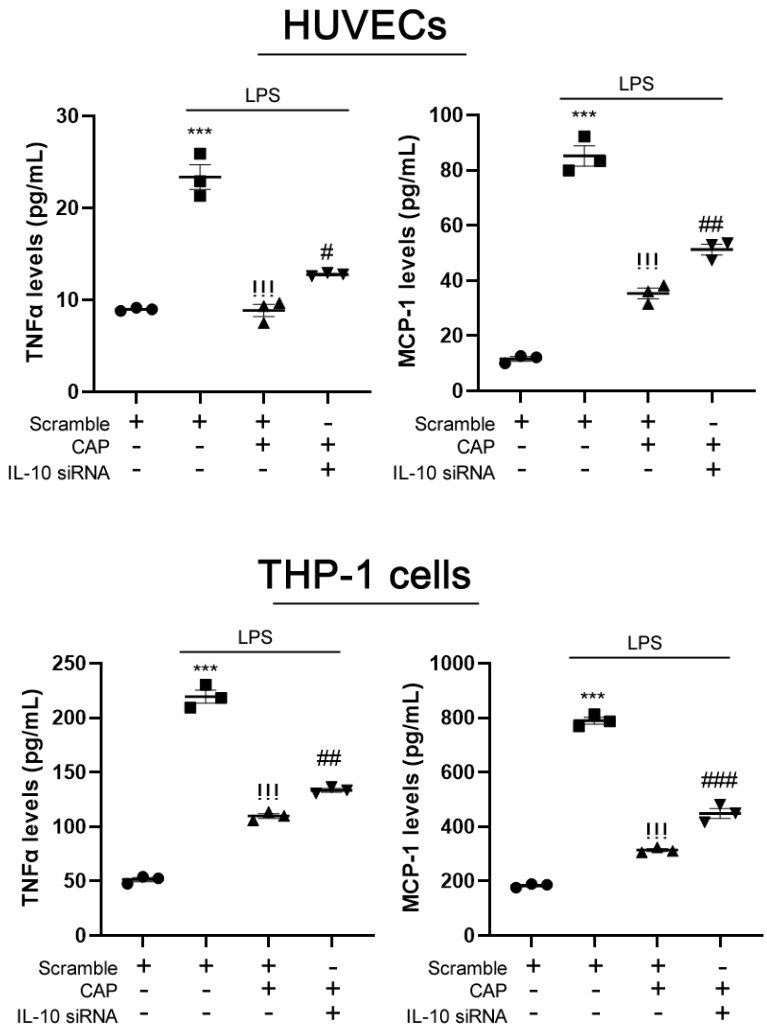
(C)


LPS
ICAM-1
VCAM-1
E-selectin
β-actin
Scramble + + + -
CAP - - + +
IL-10 siRNA - - - +
Relative ICAM-1 expression
1.5
1.0
0.5
0.0
***
!!!
##
Relative VCAM-1 expression
***
!!!
##
Relative E-selectin expression
***
!!!
#

(D)


HUVECs
LPS
pNFκB
NFκB
pIκB
β-actin
Scramble + + + -
CAP - - + +
IL-10 siRNA - - - +
Relative NFκB phosphorylation
Relative IκB phosphorylation
1.5
1.0
0.5
0.0
***
!!!
###
THP-1 cells
**
!
#

(E)


HUVECs
LPS
TNFα levels (pg/mL)
MCP-1 levels (pg/mL)
Scramble
CAP
IL-10 siRNA
THP-1 cells

(F)

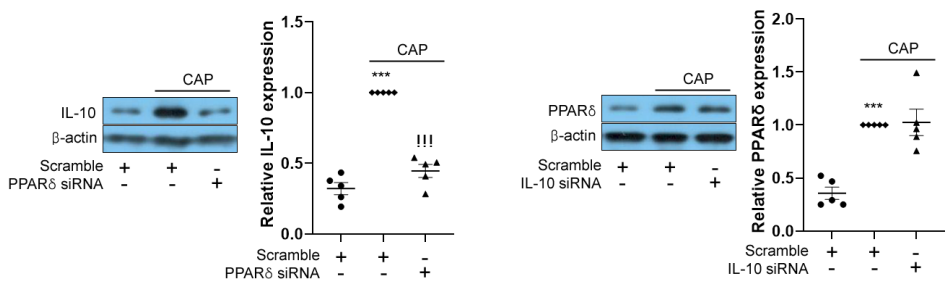

CAP
IL-10
β-actin
Scramble
PPARδ siRNA
Relative IL-10 expression
PPARδ
IL-10 siRNA
Relative PPARδ expression

Fig 5. Schematic diagram of the effects of CAP on the adhesion between endothelial cells and monocytes, and its related pathways.

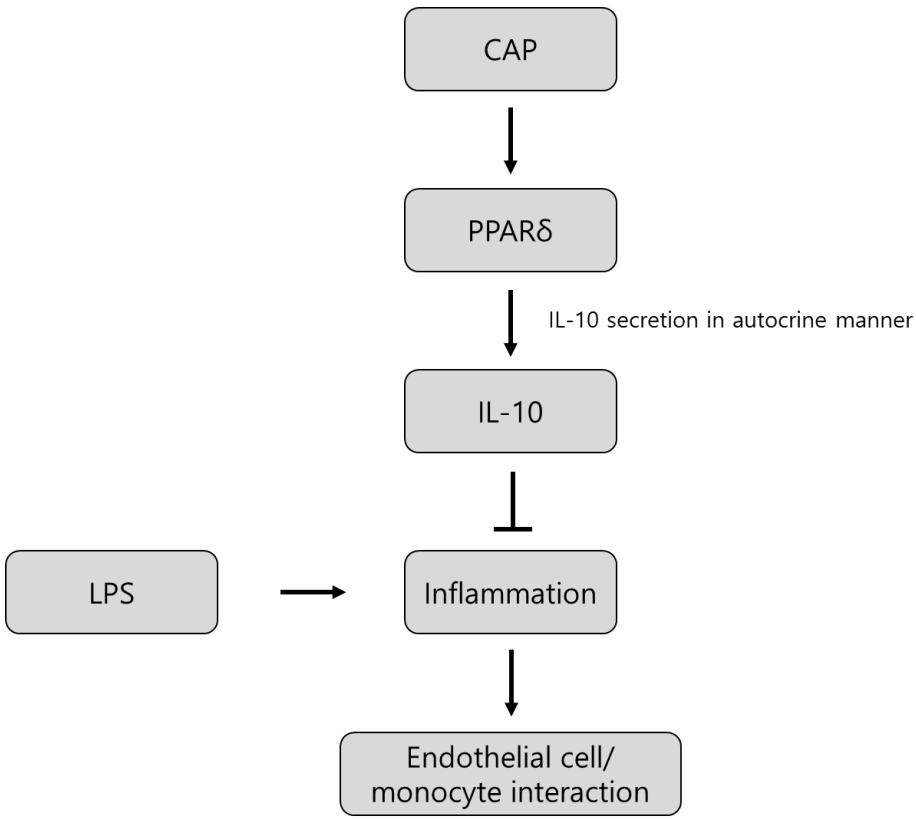
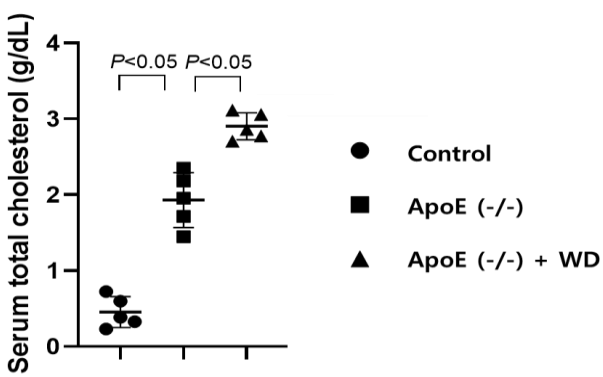
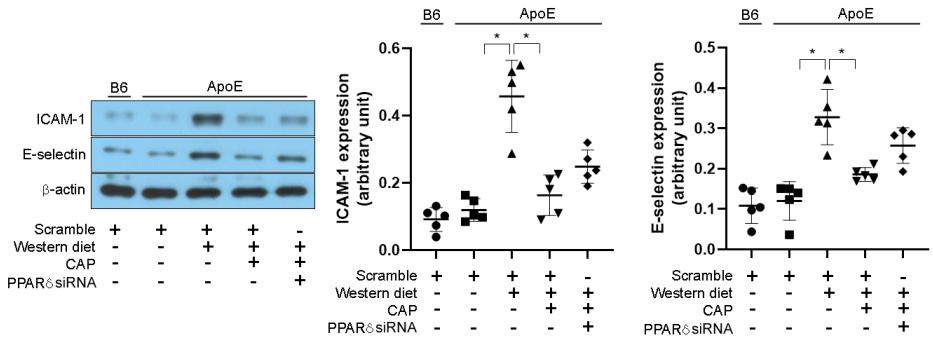


Fig. 6. Suppressive effects of CAP on ApoE (−/−) atherosclerotic mice through PPARδ/IL−10 pathway. (A) Total blood cholesterol levels (g/dL) from the ApoE (−/−) mice fed with western diet compared to controls at 5 weeks. (B) Western blot analysis of ICAM−1 and E−selectin from aorta of scrambled or PPARδ siRNA (20nM)−transfected ApoE (−/−) mice with or without western diet and CAP (5nM). ELISA of (C) TNF and (D) IL−10 from aorta of scrambled or PPARδ siRNA (20nM)−transfected ApoE (−/−) mice with or without western diet and CAP (5nM). (E) Western blot analysis of PPARδ from aorta of scrambled or PPARδ siRNA (20nM)−transfected ApoE (−/−) mice with or without western diet and CAP (5nM). (F) Representative H&E stains of aorta from control, ApoE (−/−) mouse without western diet, ApoE (−/−) with western diet, ApoE (−/−) with western diet and CAP, and ApoE (−/−) with western diet and CAP transfected with PPARδ siRNA(20nM). Black arrows demonstrate formation of foam cells (100x magnification). *P<0.05.

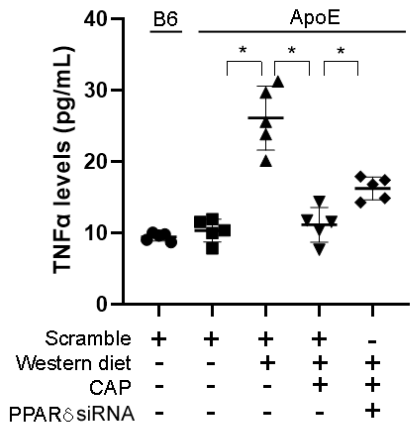
(A)



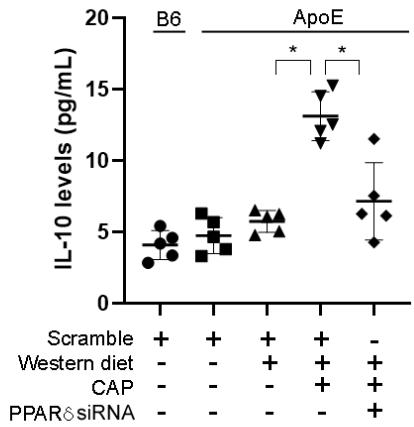
(B)


B6
ApoE
ICAM-1
E-selectin
β-actin
Scramble + + + + -
Western diet - - + + +
CAP - - - + +
PPARδsiRNA - - - - +
ICAM-1 expression (arbitrary unit)
E-selectin expression (arbitrary unit)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
*

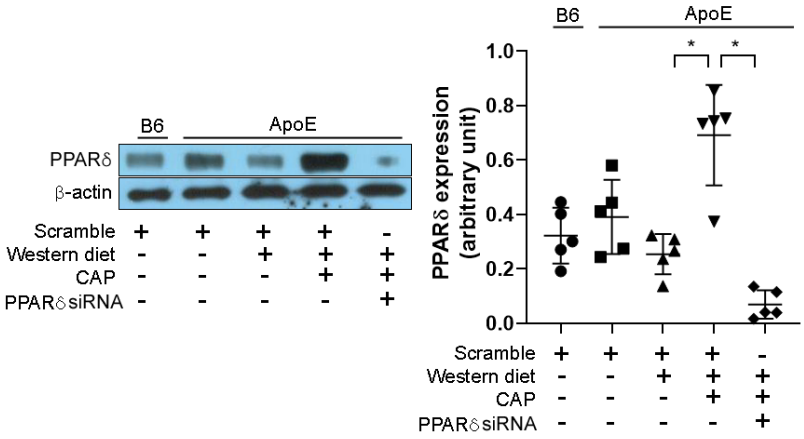
(C)


B6
ApoE
TNFα levels (pg/mL)
0
10
20
30
40
*
Scramble + + + + -
Western diet - - + + +
CAP - - - + +
PPARδsiRNA - - - - +

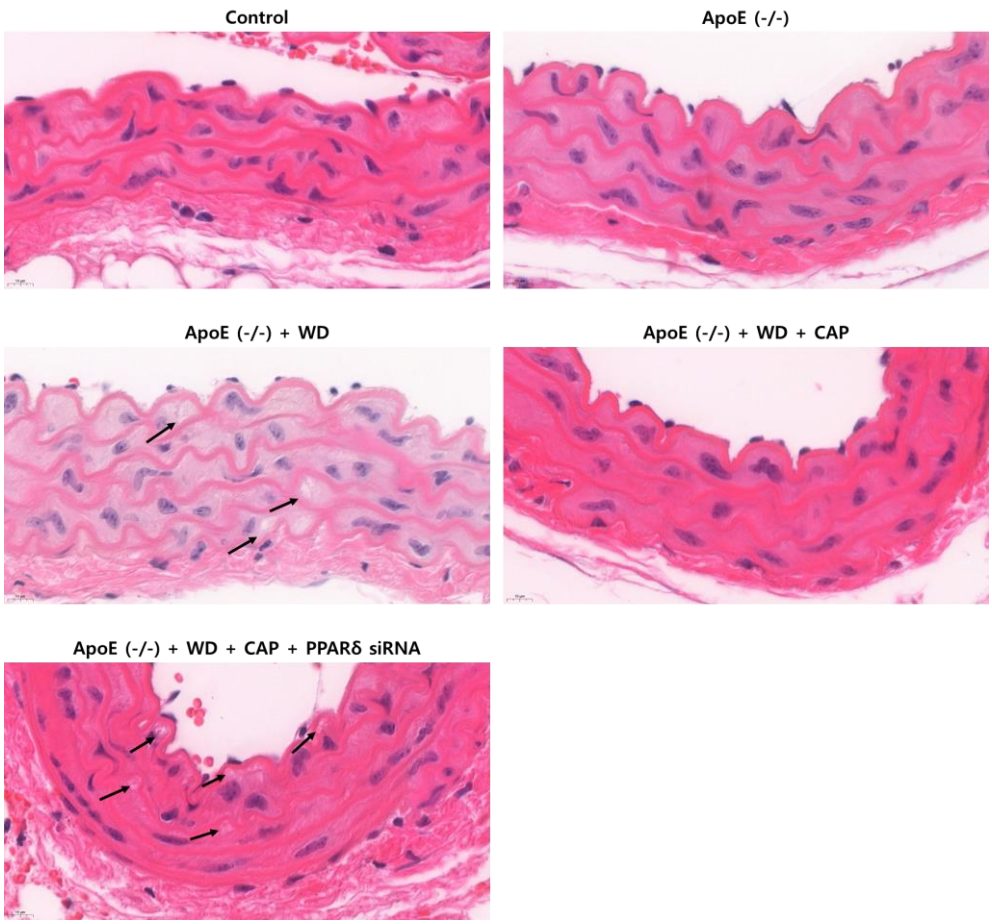
(D)


B6
ApoE
IL-10 levels (pg/mL)
0
5
10
15
20
*
Scramble + + + + -
Western diet - - + + +
CAP - - - + +
PPARδsiRNA - - - - +

(E)


B6
ApoE
PPARδ
β-actin
Scramble + + + + -
Western diet - - + + +
CAP - - - + +
PPARδ siRNA - - - - +
B6
ApoE
PPARδ expression (arbitrary unit)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Scramble + + + + -
Western diet - - + + +
CAP - - - + +
PPARδ siRNA - - - - +

(F)


Control
ApoE (-/-)
ApoE (-/-) + WD
ApoE (-/-) + WD + CAP
ApoE (-/-) + WD + CAP + PPARδ siRNA

국문초록

PPARδ/IL-10 경로를 통한 c-Met 억제제의 항염증 효과가 동맥경화의 억제에 미치는 영향에 관한 연구

서울대학교 대학원
의학과 외과학전공
박 형 섭

배경: 동맥경화는 심혈관질환의 주요 원인으로 알려져 있으며, 염증세포의 혈관 내피세포 안착은 동맥경화의 진행에 있어 매우 중요한 기전이다. Tyrosine kinase는 염증 반응에 중요한 역할을 하는 것으로 알려져 있으며, 이에 tyrosine kinase을 조절하는 전략을 통해 다양한 만성 염증성 질환을 치료하고 있다. 본 연구에서는 c-Met inhibitor인 capmatinib (CAP)의 동맥경화성 염증반응 억제 효과를 확인하기 위하여 1) LPS에 의해 유발되는 인간 탯줄정맥 내피세포 (HUVEC)와 THP-1 단핵구의 부착에 대한 억제 효과를 확인하고, 2) 동맥경화 모델인 ApoE knockout mouse에서 동맥경화의 억제 효과를 확인하고자 한다.

방법: HUVEC과 THP-1 단핵구를 배양하여 LPS처리 후 CAP를 투여하였다. 이에 따른 단백질 구현은 Western blot을 통해 확인하였으며, 목표 단백질의 녹다운 (knockdown)을 위해 짧은 간섭 RNA (siRNA) 형질주입(transfection) 기법을 사용하였다. HUVEC과 THP-1 세포간 부착 여부는 초록 형광 염색을 이용하여 확인하였다. CAP의 효과를 in

vivo에서 확인하기 위해 ApoE knockout mouse에 western diet을 5주간 복용시키고, 1개 군에서는 CAP를 경구로 복용시켰으며, 다른 1개 군에서는 CAP를 경구로 복용시킴과 동시에 PPARδ siRNA를 2일에 한번 꼬리정맥을 통해 주입하였다.

결과: In vitro에서는 CAP에 의해 HUVEC과 THP-1 세포의 부착이 억제되었으며, 이 과정에서 세포부착분자인 ICAM-1, VCAM-1 및 E-selectin의 감소가 확인되었다. 또한 CAP에 의해 NFκB, IκB, TNFα 및 MCP-1과 같은 염증 관련 인자들의 억제 효과를 HUVEC과 THP-1 세포에서 확인하였다. 또한 CAP에 의해 PPARα 및 IL-10 표현의 증가를 확인하였으며, si-RNA에 의해 PPARα 및 IL-10을 억제시킨 경우 CAP의 효과 (HUVEC 및 THP-1 세포 부착 및 염증 반응 억제)가 소실하였다. 아울러 CAP로 유도된 IL-10 표현이 PPARα siRNA에 의해 감소하는 현상을 확인하였다. In vivo에서는 ApoE (-/-) 동맥경화 쥐에서 증가되어 있던 ICAM-1, E-selectin 및 TNFα가 CAP 투여로 감소 효과를 확인하였으며, 반대로 항염증반응 표지자인 IL-10은 증가 소견을 보였다. 이런 효과는 PPARδ siRNA를 주입한 쥐에서는 소실되는 효과를 확인하였다.

결론: 위 결과에서 CAP는 염증으로 유발된 혈관내피세포와 단핵구의 부착을 억제시키는 효과가 있음을 확인하였으며, 이 과정이 PPARα/IL-10 경로가 관여함을 확인하였다. 본 연구를 통해 c-Met inhibitor의 동맥경화 억제 효과와 작용기전을 규명하였으며, 추후 동맥경화의 유효한 치료 전략으로 활용 가능할 것으로 기대된다.

주요어: Capmatinib; 염증; PPAR delta; IL-10; c-MET; 동맥경화
학번: 2017-31009