國立臺灣大學醫學院藥理學研究所

博士論文

Graduate Institute of Pharmacology

College of Medicine

National Taiwan University

Doctoral Dissertation

AMPK 訊息傳遞路徑在發炎反應中所扮演的角色

Roles of AMP-activated protein kinase signaling axis in inflammation

張美瑩

Mei-Ying Chang

指導教授：林琬琬 博士

Advisor: Wan-Wan Lin, Ph.D.

中華民國 104 年 1 月

Jan, 2015

國立臺灣大學博士學位論文
口試委員會審定書

AMPK 訊息傳遞路徑在發炎反應中所扮演的角色
Roles of AMP-activated protein kinase signaling axis in
inflammation

　　本論文係張美瑩（D94443006）在國立臺灣大學藥理學研究所完成之博士學位論文，於民國 104 年 1 月 16 日承下列考試委員審查通過及口試及格，特此證明

口試委員：

林琬琬 　　　　　　　　（簽名）

（指導教授）
許又豪

蔡　　　

曾賢忠

謝世良

系主任、所長　陳青周　（簽名）
（是否須簽章依各院系所規定）

序與誌謝

　　這些年在實驗室的日子常讓我想起美國作家李察·巴哈筆下的天地一沙鷗強納森，牠有著一般的身體，但是擁有與一般海鷗不同的熱愛飛翔的精神。牠認為飛翔應該不僅僅是往返碼頭、掙一點麵包屑、小魚蝦果腹的手段而已，因此強納森花大量的時間練習高超的飛翔技藝……原來海鷗飛翔那麼艱辛、那麼危險。海上的風既是牠們的助力，卻往往也是牠們最大的阻礙；原來海鷗的飛行並不是完全天生、理所當然；原來海鷗的飛行能力是需要不斷練習的。做實驗也是如此，不斷地嘗試錯誤才會有完美的技術與成果。而近代的女性科學家如巨星般閃耀著前所未有的光芒，這些獨領風騷的成就不知歷經多少次的測試挫敗與灰心……居禮夫人發現鐳；梅特納算出核子分裂的能量；諾耶瑟為愛因斯坦的相對論做出數學公式；柯利揭露了細胞運用食物轉化成能量的過程；喬利爾特－居禮發展出人造放射性；麥克林托克發現跳躍基因；梅爾創造出原子核層模型；李維－蒙塔西妮發現神經生長因子；吳健雄推翻了物理學的宇稱定律；霍德金則首創以分子結構解釋生物結構；愛麗恩以生物工程發展出新的製藥方法；富蘭克林解出 DNA 結構；雅洛創造並推廣內分泌學上廣泛的放射免疫測定技術……等。因此讓我看見以有限生命追尋宇宙永恆真理的偉大與不朽，感動不已。謝謝林琬琬老師教導了我強納森的精神，讓我在研究的路途上越挫越勇，能力日益精進。

　　而當 iPhone 不斷地改良問世的同時，也讓我讚歎著這個並非出身名校卻改變了全世界的賈伯斯……擁有卓爾不凡的能力與熱忱，他教導史丹佛畢業生，只要緊握住自己的心，發揮無限的熱情，措敗只是未來成功的序曲。「Stay Hungry. Stay Foolish. 」求知若渴，虛心若愚。與強納森的精神不謀而合，鼓舞著我們。

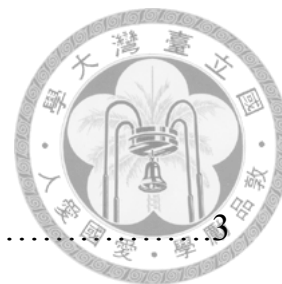　　最後，謝謝謝世良老師、符文美老師、蔡丰喬老師和曾賢忠老師的指導，修正了這本論文的許多小缺點，讓它更完美。也謝謝婷茵學姐與彰勳學長的協助，讓這本論文更增添了不少光彩。

　　當我踏出醫學院大門，回首感謝這棟陪伴我多年、屬於台灣醫學界龍頭的白色巨塔，我期待她再多孕育出一些德才兼備的領袖人物，像顏回那般！

<div align="center">獻給住在我們每個人心中的海鷗強納森</div>

<div align="center">張美瑩　謹誌於</div>

<div align="center">國立台灣大學藥理所</div>

Table of Contents

Abbreviations

|	
ACC	Acetyl-CoA carboxylase
AICAR	5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside
AMPK	AMP-activated protein kinase
COX-2	Cyclooxygenase-2
HUVECs	Human umbilical vein endothelial cells
LKB1	Liver kinase B1
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
Syk	Spleen tyrosine kinase
TAK1	Transforming growth factor beta-activated kinase 1
VSMC	Vascular smooth muscle cells

Abstract

AMP-activated protein kinase (AMPK), a critical signaling molecule for regulating energy homeostasis, might bi-directionally regulate inflammation, and its action mechanism leading to inflammation is not fully understood. We utilized 5-aminoimidazole-4-carboxamide riboside (AICAR) as a pharmacological activator of AMPK to unveil the effects of and signaling cascades mediated by AMPK on cyclooxygenase (COX)-2 gene expression in rat aortic vascular smooth muscle cells (VSMCs), murine macrophage cell line (J774), and human umbilical vein endothelial cells (HUVECs). Biochemical approaches were further conducted to elucidate interactions among signaling molecules. We found that AICAR could induce COX-2 protein expression in the cell types tested. This event was mediated by COX-2 gene transcription, and abrogated by compound C and 5'-iodotubercidin, suggesting the essential role of AMPK in COX-2 induction. Pharmacological and biochemical studies indicated that p38 mitogen activated protein kinase (MAPK) activation is the common downstream signal of AMPK in COX-2 expression in all three cell types. Furthermore, we also found that TAK1 is associated with AMPKα2, and this binding requires an interaction between the kinase domains of both molecules. Notably data of TAK1 phosphorylation indicate that the activating state is enhanced upon AMPK activation *in vivo* and *in vitro*. Our data for the first time prove a pivotal role of TAK1

in the AMPK signaling axis. Such interaction gives AMPK an additional pathway for regulating cellular functions. Via a downstream p38 MAPK signaling cascade, AMPK-dependent TAK1 activation leads to the expression of the inflammatory COX-2 gene in various cell types.
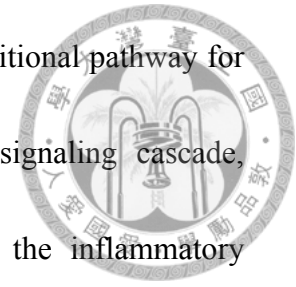
PKC plays a pivotal role in mediating monocyte adhesion; however, the underlying mechanisms of PKC-mediated cell adhesion are still unclear. In this study, we elucidated the signaling network of phorbol ester PMA-stimulated human monocyte adhesion. Our results with pharmacological inhibitors suggested the involvement of AMPK, Syk, Src and ERK in PKC-dependent adhesion of THP-1 monocytes to culture plates. Biochemical analysis further confirmed the ability of PMA to activate these kinases, as well as the involvement of AMPK-Syk-Src signaling in this event. Direct protein interaction between AMPK and Syk, which requires the kinase domain of AMPK and linker region of Syk, was observed following PMA stimulation. Notably, we identified Syk as a novel downstream target of AMPK; AICAR can induce Syk phosphorylation at Ser178 and activation of this kinase. However, activation of AMPK alone, either by stimulation with AICAR or by overexpression, is not sufficient to induce monocyte adhesion. Studies further demonstrated that PKC-mediated ERK signaling independent of AMPK activation is also involved in cell adhesion. Moreover, AMPK, Syk and ERK signaling were also required for PMA

to induce THP-1 cell adhesion to endothelial cells as well as to induce adhesion

response of human primary monocytes. Taken together, we propose a bifurcated

kinase signaling pathway involved in PMA-mediated adhesion of monocytes. PKC

can activate LKB1/AMPK, leading to phosphorylation and activation of Syk, and

subsequent activation of Src and FAK. In addition, PKC-dependent ERK activation

induces a coordinated signal for cytoskeleton rearrangement and cell adhesion. For

the first time we demonstrate Syk as a novel substrate target of AMPK, and shed new

light on the role of AMPK in monocyte adhesion, in addition to its well identified

functions in energy homeostasis.
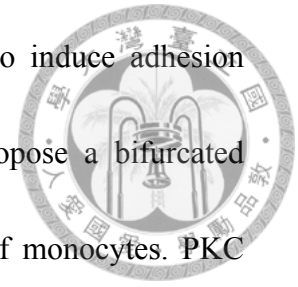
中文摘要

AMPK 是一個調節能量恆定的關鍵訊息分子，它也呈現了雙向調控發炎反應的功能，但它造成發炎的作用機制仍然不是很清楚。我們利用生化的技術將 AICAR 當成是 AMPK 的活化劑來更進一步闡述它在動脈血管平滑肌(VSMCs)、老鼠巨噬細胞(J774)和人類臍帶平滑肌(HUVECs)中分子間的交互作用。我們在幾種細胞測試中發現 AICAR 會誘導 COX-2 的表現。這個作用是藉由 COX-2 基因轉錄而來，而這個現象會被 AMPK 抑制劑:compound C 和 5'-iodotubercidin 所移除。初步推測了 AMPK 在誘導 COX-2 的作用上所扮演的角色。在藥理學上和生化學上的研究證實，在這三種細胞中 COX-2 的產生，p38 MAPK 的活化是位於 AMPK 的下游。進一步地，我們也發現 TAK1 會和 AMPKα2 產生交互作用，而且這二個分子彼此之間是需要各自的 kinase 區域來進行交互作用。更進一步的實驗結果顯示，在體內或體外實驗中，TAK1 的磷酸化現象推測了其活化的階段會加強 AMPK 活化作用。我們的實驗結果首次證實 TAK1 在 AMPK 訊息軸上的重要地位,如此的交互作用賦予了 AMPK 在調節細胞能量的功能之外，又多了另一條路徑。在許多的細胞中，經由一個下游 p38 MAPK 的訊息路徑，再透過 AMPK 使得 TAK1 活化而致使 COX-2 基因的表現。

PKC 在單核球貼附作用扮演了很重要的角色，目前對於 PKC 所傳遞細胞貼附作用的機制仍然不是很清楚。在這個研究中，我們探討了 PMA 所引起人類單核球貼

附作用的訊息網路。我們利用藥理學上的抑制劑，其結果顯示 AMPK、Syk、Src 和 ERK 參與了 PKC 所傳遞的人類單核球貼附到培養盤的過程。生化學上的分析更加確定 PMA 活化了這些激酶的能力以及 AMPK-Syk-Src 這條訊息路徑參與其中。在 PMA 的刺激之下，AMPK 和 Syk 兩個蛋白質直接進行交互作用；AMPK 是需要 kinase 區域，而 Syk 則是需要 linker region 的區域。值得注意的是，我們鑑別出一個 AMPK 新的下游目標，而且 AICAR 可以誘導 Syk 在 Ser 178 的位置磷酸化並使其活化。然而，即使在 AICAR 刺激之下或是藉由蛋白質過量的表現，AMPK 的單獨活化仍不足以使單核球產生貼附的作用。因此，在我們更進一步的研究中顯示：PKC 所傳遞 ERK 這一條路徑(不經由 AMPK 活化的路徑)也參與了細胞的貼附作用。此外，在 PMA 誘導人類單核球和人類初級單核球貼附到內皮細胞是需要 AMPK、Syk、Src 和 ERK 的訊息。總而言之，我們推測有兩條路徑參與 PMA 所傳遞的單核球貼附作用。首先，PKC 可以藉由活化 LKB1/AMPK 而使 Syk 磷酸化和活化，進而使 Src 與 FAK 活化。另外，PKC 所傳遞 ERK 活化的路徑也呈現共同促使細胞骨架重新組合與細胞貼附作用。這是我們第一次證實 Syk 是一個 AMPK 新穎的受質，同時也闡述 AMPK 除了擁有能量恆定的功能外，在單核球貼附作用上也扮演了亮眼的角色。

Chapter 1

Introduction

1-1. Biological function of AMPK in inflammation

AMP-activated protein kinase (AMPK) functions as a metabolic sensor that is activated when cells experience energy-depleting stresses. A cytosolic drop in the ATP:AMP ratio serves as a critical factor in stimulating AMPK activity (Hardie et al., 1998). Physiological and stress conditions known to activate AMPK include exercise, nutritional starvation, heat shock, oxidative stress, and ischemia/hypoxia (Carling, 2004; Steinberg and Kemp, 2009). Through increasing glucose transport, fatty acid oxidation, and glycolysis, AMPK activation inhibits ATP-consuming pathways and stimulates alternative pathways for ATP regeneration (Carling, 2004; Zhang and Zhou, 2009). AMPK recently emerged as an attractive and novel target for treating obesity, type 2 diabetes, and cardiac hypertrophy (Carling, 2004; Steinberg and Kemp, 2009; Zhang and Zhou, 2009).
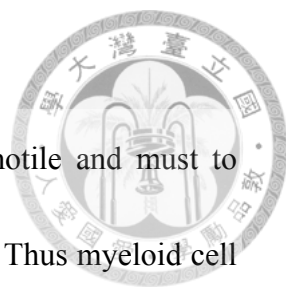
AMPK exists as a heterotrimeric enzyme, consisting of a catalytic subunit (α) and two regulatory subunits (β and γ). AMPK activity is absolutely dependent on the phosphorylation at a major activating site (Thr172) of the α-subunit by the tumor-suppressor kinase, LKB1, and Ca^{2+}/calmodulin-dependent protein kinase kinase (Hardie and Carling, 1997). Studies demonstrated that direct binding of AMP

9

to the γ-subunit of AMPK alters its susceptibility towards phosphorylation by upstream kinases. 5-Aminoimidazole-4-carboxamide riboside (AICAR) is cell membrane-permeable, and after entering cells, it can be converted into 5-aminoimidazole-4-carboxamide ribonucleoside (ZMP) by adenosine kinase. Due to structural similarities, ZMP can mimic AMP in activating AMPK, and thus AICAR is regarded as a pharmacological activator of AMPK (Hardie et al., 1998).
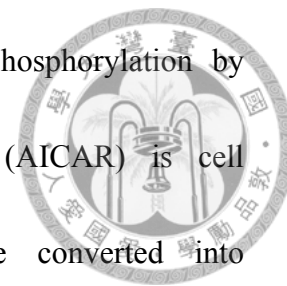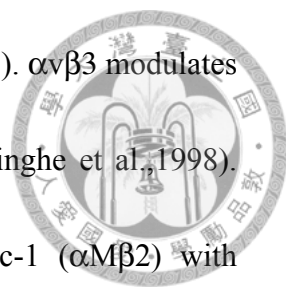
AMPK was also implicated as an anti-inflammatory and anti-cancer target. In endothelial cells, AMPK activity is associated with phosphorylation and activation of endothelial nitric oxide synthase (Zou et al., 2002). Endotoxin lipopolysaccharide-induced expression of proinflammatory molecules and mediators, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, inducible NOS (iNOS), and cyclooxygenase (COX)-2, in primary macrophages, microglia, astrocytes, and mesangial cells were suppressed by AICAR (Giri et al., 2004; Jhun et al., 2004; Kuo et al., 2008; Jeong et al., 2009; Peairs et al., 2009). The constitutive COX-2 expression in colon cancer cells was repressed by AMPK activation (Hwang et al., 2006; Park et al.,2006). Moreover, the benefits of AMPK activation in several inflammatory disease models were also documented (Hallows et al.,2006; Zhao et al., 2008; Myerburg et al., 2009).
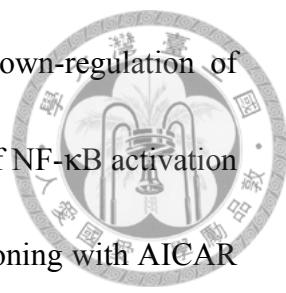
1-2. *AMPK and leukocyte adhesion*

A common aspect of myeloid cells is that they are highly motile and must to traverse multiple tissue barriers in order to perform their functions. Thus myeloid cell adhesion, mediated by specific cell surface molecules, plays a central role in inflammation and a multitude of pathological settings, such as infections, atherosclerosis and arthritis. The recruitment of monocytes from the circulation into the extravascular space involves several steps like rolling, adhesion and transendothelial migration (Paul et al., 2007). To achieve firm leukocyte adhesion, leukocytes collect different inflammatory signals that activate intracellular signaling pathways. Podosomes are ventral adhesion structures prominent in cells of the myeloid lineage. Recently podosomes have gathered attention as important cellular structures that can influence cell adhesion and motility. To date, several signals propagated by adhesive ligands acting on transmembrane receptors and involved in induction of podosome formation and rearrangements include small G proteinsof the Rho family, tyrosine kinases (e.g. Src, Syk, FAK) and cytoskeletal substrates (e.g. paxillin) (Athanassios et al., 2011).

Another key receptor of phagocyte adhesion is integrins whose activation and clustering play a key role to mediate signal transduction pathways essential for leukocyte adhesion. For example, β2 integrins on the leukocyte surface have essential

roles in leukocyte trafficking and function (Tatsuo, 2005). αvβ3 modulates

αLβ2-dependent transendothelial migration of monocyte (Weerasinghe et al.,1998).

Interactions of the monocyte integrins LFA-1 (αLβ2) and Mac-1 (αMβ2) with

ICAM-1, and of VLA-4 (α4β1) with VCAM-1 are crucial steps in forming stable

attachments to endothelial cells. On the other hand, upregulating integrin expression

upon inflammatory environment also accelerates adhesive activity. In this respect,

stimulation with LPS on phagocytes could activate FAK and subsequently integrins,

such as αvβ3, αvβ1 and β2, to generate inside-out signaling for enhanced binding of

its ligands (Mark and Joseph, 1999; Md et al.,2009). In addition, chemokine signals

activate leukocyte integrins and actin remodeling machineries critical for leukocyte

adhesion. In this respect, the chemokine IL-8 promotes leukocyte chemotaxis and has

also been proven to greatly increase the adhesive capacities of monocytes, by

triggering conformational changes in LFA-1 and Mac-1 on rolling monocytes
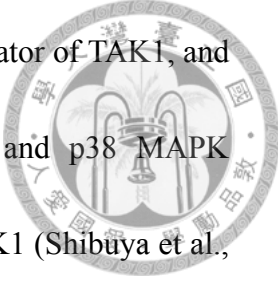
(Janeway et al., 2001).

As mentioned above, even though most AMPK activators exert benefits in

inflammation in terms of their abilities to lower expression of proinflammatory

cytokines and chemokines, the direct role of AMPK in regulating leukocyte adhesion

remains limited. Studies using in vitro model of endothelial-leukocyte interaction

showed that ECs incubated with AICAR inhibited TNF-α-stimulated adhesion of

monocytes. One of the mechanisms of this action is through down-regulation of

expression of endothelial cell adhesion molecules via modulation of NF-κB activation

(Yuan et al., 2011). Another in vivo study indicated that preconditioning with AICAR

is effective to prevent postischemic leukocyte-endothelial cell adhesive interactions

via upregulation eNOS activation in endothelial cells (Gaskin et al., 2007). Recently,

berberine via AMPK signaling was demonstrated to inhibit high glucose-induced

monocyte attachment to endothelial cells (Yiqun et al., 2009). Remarkably all above

studies coincidentally point out that modulation of endothelial cells functions

contribute to the AMPK-mediated inhibition of leukocyte- endothelial cell interaction.

The only one study directly examining action in primary human monocytes revealed

that AMPK mediates rosiglitazone-induced inhibition of monocyte adhesion. Despite

the results on endothelial cells strongly support the anti-adhesive action mediated by

AMPK, the direct action of AMPK in leukocyte adhesive function has not been fully

identified.

1-3.   TAK1

Transforming growth factor-β-activated kinase 1 (TAK1) is a potent activator of

mitogen-activated protein kinases (MAPKs), which include extracellular

signal-regulated kinase (ERK), p38 MAPK, and c-Jun N-terminal kinase (JNK). The

scaffold protein, TAK1-binding protein 1 (TAB1), is a specific activator of TAK1, and

may thus promote MAPK kinase (MKK) autophosphorylation and p38 MAPK

activation via association with the N-terminal kinase domain of TAK1 (Shibuya et al.,

1996). However, a recent study also identified an alternative p38α activation pathway.

Independent of TAK1 and MKK, TAB1 can directly interact with p38α, leading to its

autophosphorylation (Ge et al., 2002). Recent studies further suggested that AMPK

may participate in the activation of p38 MAPK, while controversial results were

reported. Studies showed that AMPK activation can lead to p38 MAPK activation in

an ischemic heart (Li et al., 2005), cardiac fibroblasts (Du et al., 2005), skeletal

muscle (Xi et al., 2001), synovial fibroblasts (Tang et al., 2007), osteoblasts (Hou et

al., 2008) , and neuroblastoma cells (Jung et al., 2004). The downstream events

mediated by such a signaling cascade include increased glucose transport (Xi et al.,

2001), fatty acid oxidation (Yoon et al.,2006), Bax translocation to mitochondria

(Capano et al.,2006), IL-6 production (Du et al., 2005; Tang et al., 2007), COX-2

expression (Hou et al., 2008), inhibition of cancer growth (Han et al.,2009), and

apoptosis of tumors (Hsu et al., 2010). In contrast to the positive regulating link as

mentioned, a dissociated relationship of both activated kinases during myocardial

ischemia (Jacquet et al.,2007), skeletal muscle contraction (Ho et al., 2007), and even

a relationship of inhibitory regulation were also demonstrated. For the latter, AMPK

was shown to reduce PMA-induced p38 MAPK activation in neutrophils (Alba et al.,2004).

1-4. *Roles of Syk in PKC signaling*

Spleen tyrosine kinase (Syk) is a non-receptor tyrosine kinase, comprising two N-terminal Src homology 2 (SH2) domains, a linker region, and one kinase domain in its C-terminal region (Taniguchi et al., 1991). In last decade, Syk has been widely investigated in association with various immunoreceptors and is demonstrated to play crucial roles in innate and adaptive immunity (Mocsai et al., 2010). In addition, Syk is also involved in the signaling of integrins (such as beta2, beta3 and CD11b) (Abram et al., 2007). Signaling of Syk, typically in coordination with Src kinase, leads to activation of PLCγ and PI3K, which are required for the control of cell adhesion, migration, phagocytosis and aggregation (Berton et al.,2005; Zarbock and Ley, 2011; Kerrigan and Brown, 2011).

Besides the well identified signaling pathway that links Syk indirectly to PKC via PLCγ, which induces phosphoinositide turnover to generate diacylglycerol for PKC activation, direct activation of PKC by Syk was demonstrated. In FcRI-stimulated mast cells, PKCβI and PKCα are activated by Syk-mediated tyrosine phosphorylation

at Tyr662 and Tyr658, respectively (Kawakami et al., 2003). Conversely, some

studies have revealed a pathway where Syk is a downstream signal of PKC.

Incubation of the purified kinase domain of Syk with PKC demonstrates the ability of

PKC isoforms to phosphorylate Syk and enhance its tyrosine kinase activity

(Borowski et al., 1998). Most recently, a study in endothelial cells indicated that

PKCδ-mediated activation of Syk plays an important role in thrombin signaling of

NF-κB activation and intercellular adhesion molecule-1 expression (Bijli et al., 2008).

Thus, there is a complex signaling interplay between PKC and Syk, which is

dependent on cell type and the context of stimulation.
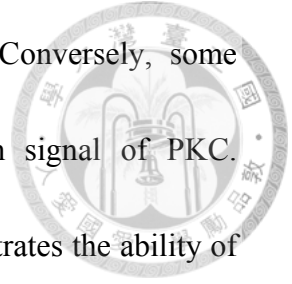
Chapter 2

Specific aims of these studies

2-1. To further explore the actions and molecular mechanisms elicited by AICAR in regulating the inflammatory response, we chose J774 macrophages, vascular smooth muscle cells (VSMCs), and human umbilical vein endothelial cells (HUVECs) as cell models to study AICAR's actions on COX-2 gene expression.

2-2. PKC plays a pivotal role in mediating monocyte adhesion; however, the downstream mechanisms mediating its function are not fully elucidated. Thus, in this study, using phorbol 12-myristate 13-acetate (PMA)-stimulated human monocytic leukemia cell line THP-1 as a model system in most experiments, we investigated the signaling network among PKC, Syk and AMPK, and explored their functional relevance in monocyte adhesion.

Chapter 3

Materials and methods

3-1.   Reagents

Lipofectamine^{TM} and Plus^{TM} reagents were obtained from Invitrogen (Gaithersburg, MD, USA). Antibodies specific for AMPKα, acetyl-CoA carboxylase α (ACCα), TAK1, GFP, and phosphorylated JNK, p38 MAPK, ERK, AMPKα, ACCα, and TAK1 were purchased from Cell Signaling (Beverly, MA, USA). Polyclonal antibodies specific for c-Src (Tyr216), c-Myc, anti-rabbit and anti-mouse antibodies were purchased from Santa Cruz Biotechnology. Antibodies specific for AMPKα, Syk, ERK and phosphorylated AMPKα (Thr172), Syk (Tyr525/526) and ERK1/2 (Thr202/Tyr204) were purchased from Cell Signaling. Antibody against human Src (Tyr419, equivalent to Tyr416 of chicken Src and v-Src) was purchased from Invitrogen. Anti-Flag bead antibody (M2-beads), anti-Flag antibody and antibodies specific for human NLRP3 (clone nalpy3-b) were purchased from Axxora. Anti-ASC and anti-human caspase-1 p10 (A-19) were purchased from Santa Cruz Biotechnology. Anti-processed IL-1β p17 (Asp116) was obtained from Cell Signaling Technology. Other antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Murine IL-1β was purchased from R&D (Minneapolis, MN, USA). U0126, SB203580, AICAR, 5'-iodotubercidin, and compound C were purchased from

Calbiochem (San Diego, CA, USA). SP600125 was purchased from Tocris Cookson (Avon-mouth, UK). Enzyme-linked immunosorbent assay (ELISA) assay kits for prostaglandin (PG)I$_2$ and PGE$_2$ were purchased from Cayman Chemicals (Ann Arbor, MI, USA).
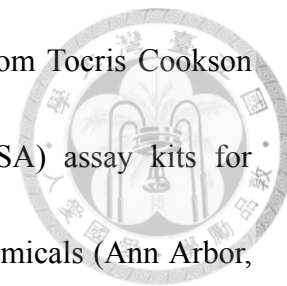
DMEM, RPMI 1640, FBS, penicillin, and streptomycin were obtained from Gibco BRL. The enhanced chemiluminescence detection agents were purchased from Amersham Biosciences. Lipofectamine^{TM} reagents were obtained from Invitrogen. All materials for SDS-PAGE were obtained from Bio-Rad. Ro318220, GF109203X, PMA, Compound C, Syk inhibitors (Syk inhibitor I, Bay 61-3606, Syk inhibitor III), PP2, Go6983, Ro320432, and U0126 were purchased from Calbiochem. LY333531 was purchased from Alexis Biochemicals.

3-2. Cell culture

Primary rat aortic VSMCs, HUVECs, the murine macrophage J774 cell line, human THP-1 monocytes, and 293T cells were isolated and cultured as we previously described (Yang et al., 2005; Wei et al., 2006). Rat aorta, murine bone marrow, and human umbilical cords were prepared according to institute regulations and were approved by the ethics committee of the National Taiwan University College of Medicine. Our experiments conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH

publication no.85-23) as well as the principles outlined in the *Declaration of Helsinki* for use of human tissues.
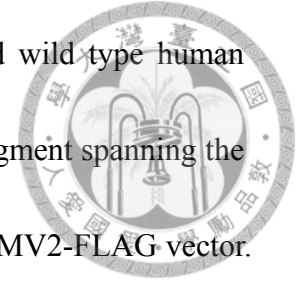
Primary monocytes were prepared from the blood of healthy human donors and cultured in DMEM as described previously (Lee et al., 2008). Experiments of human samples have been approved by Institutional Review Boards (IRB) of National Taiwan University Hospital, Taipei, Taiwan.

3-3. *Plasmids*

The murine COX-2 promoter (-966/+23) was kindly provided by Dr. Byron Wingerd (Michigan State University, East Lansing, MI, USA). All of the Myc-AMPKα2 constructs were gifts from Dr. Kelly A. Wong (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). The regions covering the kinase domain (1~300) and non-kinase domain (301~579) of Flag-tagged mouse TAK1 (1~579) were amplified by a polymerase chain reaction (PCR) and then inserted into the EcoRI/XhoI site of the pcDNA3-Flag vector. GFP-tagged constitutive active Ad-AMPKα2-CA and Ad-DN kinase-dead (K45R) variant of AMPKα2 were gifted by Dr. Benoit Viollet (INSERM, Paris, France), and Dr. Bob Monks (Univ. Pennsylvania, USA), respectively.

Myc-AMPKα2, Myc-AMPKα2 (1-312), Myc-AMPKα2 (1-398), and Myc-AMPKα2 (303-552) were provided from Dr. Kelly A. Wong (Whitehead

20

Institute for Biomedical Research, Cambridge, MA). Flag-tagged wild type human Syk (1-635) was constructed by ligating the PCR-derived DNA fragment spanning the open reading frame region of Syk with the EcoRI/SalI digested pCMV2-FLAG vector. The regions covering 107-635, 260-635 or 1-369 amino acids of hSyk cDNA were amplified by PCR and inserted to EcoRI/SalI digested pCMV2 FLAG to generate the N-SH2-del (dNSH2), SH2-del (dSH2) or kinase domain-del (dKD) of hSyk. The C-SH2-del hSyk (dCSH2) construct was amplified by PCR covering 1-106 and 260-635 amino acids of hSyk. To generate S178A Syk, we used the Quickchange site-directed mutagenesis kit (Stratagene), and serine 178 in the human Syk cloned in the pBlue-script vector was substituted with alanine by changing the codon from ATC to AGC. The mutated primers used were primer 1 (5'-CTC TCG GGA AGA AGC TGA GCA AAT TGT-3') and primer 2 (5'-ACA ATT TGC TCA GCT TCT TCC CGA GAG) for hSyk (S178A) mutation.

3-4. PG measurement

PGI$_2$ and PGE$_2$ productions were measured by commercial kits according to the manufacturer's instructions.

3-5. Immunoblotting and immunoprecipitation

For immunoprecipitation, cells were lysed in 500 μl RIPA lysis buffer and centrifuged at 14,000 rpm and 4 °C for 30 min. The supernatant was pre-cleaned by normal immunoglobulin G (IgG) and 10 μl protein A/G-agarose beads, and the supernatant was incubated with specific antibodies at 4 °C with rocking overnight. Then 10 μl of protein A/G-agarose beads was added and rotated at 4 °C for another 30 min. The immunocomplex was washed three times with cold 150 mM NaCl containing RIPA buffer and twice with 300 mM NaCl containing RIPA buffer. The precipitated complex was subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE).
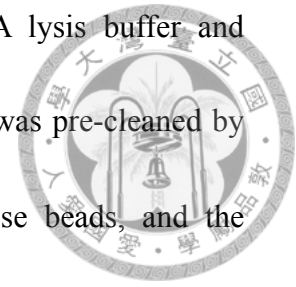
3-6.   *Reverse-transcription (RT)-PCR*

RT-PCR was conducted as we previously described (Chen et al., 2004). Oligonucleotide primers corresponding to COX-2 were used (5'-CAG CAA ATC CTT GCT GTT CC-3' and 5'-TGG GCA AAG AAT GCA AAC ATC-3').

3-7.   *Transient transfection, adenovirus infection and luciferase reporter assay*

We used a reporter assay to assess the promoter activity of the COX-2 gene as we previously described (Chen et al., 2004). Aenoviruses were propagated in 293F cells, purified by BD-Adeno-X^{TM} Virus Purification kits, and stored at -70°C. The

titration of adenovirus was quantified by Adeno-X^{TM} Rapid Titer kit. J774 cells were infected with Ad GFP, Ad AMPKα2-CA or Ad AMPKα2-DN to multiplicity of infection (M.O.I.) at a 20:1 (ifu/ifu) ratio. After 48 h infection, cells were harvested for immunobloting.
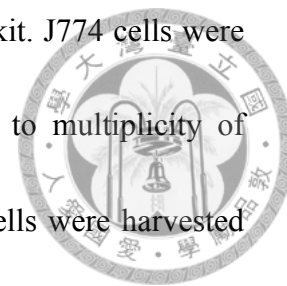
HEK293T cells (5×10^5 cells/well) were transfected using Lipofectamine with 1 μg plasmid for 4 h, and then changed to complete medium. After 24 h transfection, cells were harvested, followed by collection of cell lysates for immunoprecipitation or western blot.

3-8.   *Adhesion Assay*

THP-1 cells (5×10^4 cells) and human primary monocytes (2×10^4 cells) incubated in 96-well plates were pretreated with the indicated kinase inhibitors for 30 min, followed by stimulation with PMA (1-100 nM) for different periods. Nonadherent cells were removed by gentle washing with PBS before fixation with methanol for 10 min, followed by addition of 0.1% (v/v) crystal violet in PBS for 15 min. After washing with distilled water three times, acetic acid was added to a final concentration of 33% (v/v) to solubilize cell lysates, followed by absorbance measurement at 550 nm. In some experiments to determine the adhesive response to HUVEC, THP-1 cells were labeled for 1 h at 37°C with 0.1 μg/ml BCECF-AM,

followed by washing twice with growth medium, and adding to the EC monolayer. The number of adherent cells was determined by measuring the fluorescence intensity.

3-9. Flow Cytometry

THP-1 monocytes were harvested, fixed with 2% paraformaladehyde, stained extracellularly in 1% FBS/PBS with anti-CD11b-FITC, and analyzed by flow cytometry.

3-10. In Vitro Kinase Assay

To determine in vitro kinase activities of Syk, 500 μg of total protein extracts from stimulated cell lysates were pre-cleared for 30 min and then immunoprecipitated with 1 μg anti-Syk antibody overnight, followed by addion of 10 μl protein A/G-agarose beads and rotation at 4°C for another 1 h. The immunocomplexes were washed 3 times with cold lysis buffer (containing 300 mM NaCl) and once with the kinase reaction buffer (20 mM HEPES pH 7.5, 10 mM $MgCl_2$, 5 mM p-nitrophenyl phosphate). The beads were then incubated for 30 min at 30°C in 20 μl kinase reaction buffer supplemented with 10 μCi of γ-^{32}P-ATP. The precipitated complexes were added with 5 μl 5X Laemmli sample buffer and heated at 95°C for 5 min. After

centrifuging the samples, the supernatants were run on 10% SDS-PAGE, followed by

autoradiography.

3-11.   Statistical evaluation

Values were expressed as the mean ± SEM of at least three times of experiments.

ANOVA and Dunnett's tests were used to assess the statistical significance of the

differences, with p values of less than 0.05 being considered statistically significant.

Chapter 4.

Results I.

AICAR induces cyclooxygenase-2 expression through AMP-activated protein kinase-transforming growth factor-activated kinase 1-p38 mitogen activated protein kinase signaling pathway.

第四章：

結果 I.

AICAR 經由 AMPK-TAK1-p38 訊息傳遞路徑來誘導 COX-2 的表現
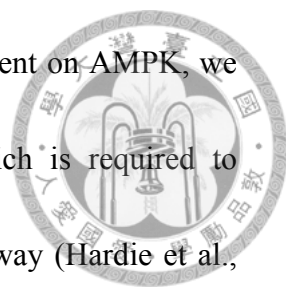
Results I

4-1. AICAR-induced COX-2 gene expression and PGI$_2$ production in VSMCs require AMPK activation

In rat aortic VSMCs after 24 h of treatment, AICAR and IL-1β alone significantly increased PGI$_2$ production (Fig. 1A). The response elicited by 1 mM AICAR exhibited a similar extent of increase as did IL-1β (10 ng/ml). We also found that the co-incubation of AICAR and IL-1β dramatically and synergistically increased PGI$_2$ production. Accordingly the effects of AICAR and IL-1β alone or in combination displayed similar patterns of COX-2 protein expression. Next, we wondered whether COX-2 induction by AICAR resulted from increased gene transcription. Utilizing an RT-PCR analysis, we found that the steady-state level of COX-2 messenger (m)RNA was significantly increased by AICAR (1 mM) after 3 and 6 h of incubation (Fig. 1B). Similar to protein expression, a further increase in mRNA induction was observed upon co-treatment with AICAR and IL-1β for 6 h. Even though AICAR was reported to be cytotoxic to certain cell types (Lopez et al., 2003), measurement of the cell viability by MTT (an index of mitochondrial activity) and crystal violet (an index of protein levels) indicated no cytotoxicity toward rat VSMCs treated with AICAR (3 mM) alone or in combination with IL-1β (10 ng/ml) for 24 h (data not shown).

To determine whether COX-2 induction by AICAR is dependent on AMPK, we utilized 5'-iodotubercidin, an inhibitor of adenosine kinase which is required to convert AICAR into ZMP and activate the AMPK signaling pathway (Hardie et al., 1998). As shown in Fig. 1C, COX-2 protein expression induced by AICAR was inhibited by 5'-iodotubercidin (0.3 or 1 μM); in contrast, IL-1β-induced COX-2 protein expression was not affected. Likewise, compound C (an AMPK inhibitor) pretreatment abolished the COX-2-inducing response of AICAR, but not that of IL-1β. To ensure that AICAR indeed has the ability to phosphorylate and activate AMPK, immunoblotting with a phospho-AMPKα antibody in rat VSMCs treated with AICAR (1 mM) for 15~60 min was performed. Fig. 1D indicates that treatment of AICAR rather than IL-1β led to an obvious increase in AMPKα and ACC phosphorylation.

4-2. *AMPK-dependent induction of COX-2 expression also occurs in macrophages and endothelial cells.*

To further understand if AMPK-dependent COX-2 induction is a cell type-specific phenomenon, we tested murine J774 macrophages and HUVECs. We found that AICAR was able to induce eicosanoid production (PGE_2 in J774 macrophages and PGI_2 in HUVECs) and COX-2 protein expression in both cell types (Fig. 2A, B). Such activation was abrogated when cells were co-treated with
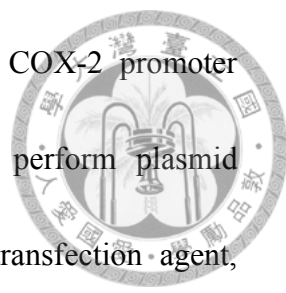
5'-iodotubercidin. Accordingly, AICAR addition increased AMPK phosphorylation in both cell types in a time-dependent manner (Fig. 2C). To confirm the ability of AMPK in COX-2 induction, we further conducted adenovirus-based infection and observed that constitutive active AMPK rather than kinase dead AMPK expression led to an increased COX-2 expression in J774 macrophages (Fig. 2D).
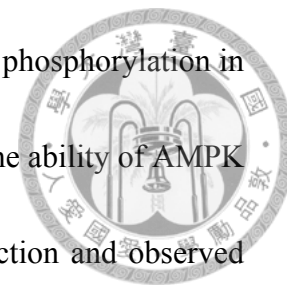
4-3. *Signaling pathways involved in the upregulation of COX-2 by AICAR*

In order to depict MAPK signaling cascades underlying the inductive effects of AICAR on COX-2 protein expression, specific inhibitors of ERK (U0126), p38 MAPK (SB203580), and JNK (SP600125) were examined in VSMCs, J774 macrophages, and HUVECs. We found that in VSMCs, only SB203580 significantly reversed the AICAR-elicited induction of COX-2 protein, suggesting the involvement of p38 MAPK but not ERK or JNK in this event (Fig. 3A). In J774 macrophages, treatment with U0126, SB203580, and SP600125 alone almost completely reduced the effect of AICAR (Fig. 3B). In HUVECs, U0126 had no effect on the AICAR response, while SB203580 and SP600125 inhibited the response of AICAR by 60% and 25%, respectively (Fig. 3C).

To confirm that the effects of MAPK inhibitors on COX-2 protein expression

resulted from gene transcription, we measured their effects on COX-2 promoter activity after AICAR treatment. Since it is quite difficult to perform plasmid transfection in VSMCs even when using the highly efficient transfection agent, LipofectAMINE PLUS, we performed COX-2 promoter experiments in J774 macrophages and HUVECs. In agreement with results of protein changes, we found that all three types of MAPK inhibitors efficiently inhibited AICAR-induced COX-2 promoter activation in J774 macrophages (Fig. 3D, upper panel). In HUVECs, SB203580 and SP600125 exerted effective inhibition, but U0126 did not. Moreover, the inhibitory extent of SB203580 was higher than that of SP600125 (Fig. 3D, lower panel). In both cell types, the increased COX-2 promoter activity caused by AICAR was abolished by the presence of 5'-iodotubercidin.

To verify the distinctive involvement of MAPKs in AMPK-dependent COX-2 gene expression in various cell types, we determined the effects of AICAR on MAPK activation and the signaling relationship to AMPK. In VSMCs, we found that AICAR obviously increased p38 MAPK phosphorylation, but not ERK or JNK phosphorylation within 1 h (Fig. 4A). In contrast, IL-1β obviously and rapidly induced the phosphorylation of these MAPKs. Furthermore, we found that the time-dependent p38 MAPK activation by AICAR was dramatically reduced by the presence of 5'-iodotubercidin (Fig. 4B). In AICAR-stimulated J774 macrophages, we

found strong increases in p38 MAPK and JNK phosphorylation and a slight increase

in ERK phosphorylation. In the presence of 5'-iodotubercidin, all three MAPK

phosphorylation events were reduced (Fig. 4C, left panel). In HUVECs, AICAR

induced significant p38 MAPK and JNK phosphorylation, but had no effect on ERK

phosphorylation. Again, the activation effects on p38 MPAK and JNK were

diminished by the presence of 5'-iodotubercidin (Fig. 4C, right panel). All these

results suggest that the differential involvement of MAPKs in AICAR-mediated

COX-2 gene expression is associated with cell type-specific regulation of MAPKs by

AMPK.
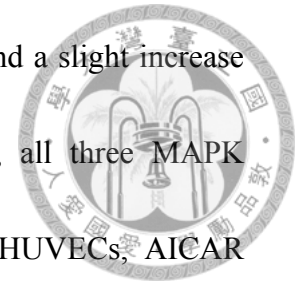
4-4.   *AMPK is associated with TAK1 and its activation*

After finding that p38 MAPK is the common downstream signal of AMPK in

various cell types, we then determined if AMPK can activate TAK1, the upstream

signal kinase of p38 MPAK. In J774 macrophages and HUVECs, we found that TAK1

phosphorylation levels at Thr184 and Thr187 were increased accompanied by the

activation of AMPK following AICAR treatment for 10~60 min (Fig. 5A). Dual

phosphorylation of Thr184/187 residues within the kinase activation loop of TAK1

was reported to be an activation index of TAK1 (Sakurai et al., 2000) . These results

suggest that TAK1 is the downstream mediator linking AMPK to p38 MPAK

activation. Next, in order to verify this suggestion, we determined the in vivo protein

interactions of both kinases. Results of co-immunoprecipitation revealed a binding

interaction between endogenous AMPKα and TAK1 in J774 macrophages, VSMCs,

HUVECs, and THP-1 monocytes (Fig. 5B). Such binding was not affected by

stimulation with AICAR or compound C, suggesting the irrelevance of AMPK

activity in this binding event.

To further analyze the biochemical properties of such protein interactions, we

conducted experiments in 293T cells overexpressing AMPKα and TAK1. When

Flag-tagged TAK1 and Myc-tagged AMPKα2 were expressed in 293T cells, TAK1

was detected to be associated with Myc-AMPKα2 upon immunoprecipitation.

Accordingly, the association between TAK1 and AMPKα2 remained unchanged after

treatment with either AICAR or compound C, regardless of which antibody was used

against TAK1 or AMPKα2 to conduct immunoprecipitation (Fig. 5C). Such AMPK

activity-independent binding was further confirmed by observing the binding of

kinase active and kinase dead AMPK to TAK1 (Fig. 5D). Next, in order to clarify if

activated AMPK leads to the associated TAK1 activation, we determined the TAK1

phosphorylation level in co-immunoprecipitates. As shown in Fig. 5E, upon AMPKα2

co-expression, TAK1 phosphorylation remained negligible (lane 6 vs. lane 2);

however, it markedly increased following stimulation of cells with AICAR (lane 3 vs.

lane 2). This increase in AMPK-co-expressing cells was about 3-fold that observed in cells without AMPK overexpression (lane 7 vs. lane 3). These in vitro results further confirmed the ability of activated AMPK to trigger the associated TAK1 activation.
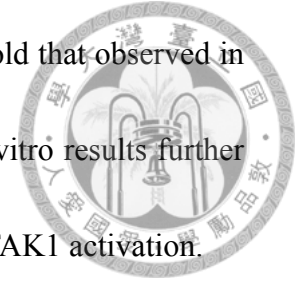
4-5. *Kinase domain of AMPKα2 and TAK1 are necessary for mutual association*

In order to define the domain necessary for the association between AMPKα2 and TAK1, we transfected several Myc-tagged AMPKα2 mutant constructs to confirm their expressions (Fig. 6A). Meanwhile, wild-type (WT) TAK1 was co-expressed with deletion mutants of Myc-AMPKα2 in 293T cells. We found that Myc-AMPKα2 (WT), Myc-AMPKα2 (1~312), and Myc-AMPKα2 (1~398), all containing the kinase domain, could bind to TAK1, while Myc-AMPKα2 (303~552) and Myc-AMPKα2 (386~552) could not (Fig. 6B).

Next, to map the TAK1 interaction region in AMPKα2, 293T cells were transiently transfected with the expression plasmids of Flag-TAK1 (WT), Flag-TAK1 (1~300), or Flag-TAK1 (301~579) with AMPKα2 (Fig. 6C). As a result, interactions of WT TAK1 and Flag-TAK1 (1~300) with AMPKα2 were detected. In contrast, Flag-TAK1 (301~579) could not bind to AMPKα2 (Fig. 6D). These results indicate that the kinase domain of TAK1 is required for the interaction with AMPKα2.

Chapter 5.

Results II

PKC-dependent human monocyte adhesion requires AMPK and Syk activation.

第五章：

結果 II

藉由 PKC 傳導路徑而來的單核球貼附作用需要 AMPK 與 Syk 的活化參與

Results II

5-1. PMA-induced THP-1 monocyte adhesion involves AMPK, Syk and Src

Previous reports have demonstrated that human monocytic THP-1 leukemia cells can be induced to differentiate along the monocytic lineage following exposure to PMA, a potent tumor promoter capable of activating conventional and novel PKC isoforms. PMA treatment resulted in adherence, loss of proliferation, phagocytosis of latex beads, and expression of CD11b and CD14 (Schwende et al., 1996). We found that PMA (100 nM) can enhance THP-1 cell adhesion in time- and concentration-dependent manners (Fig. 7A). To elucidate which PKC isoforms are involved in PMA-induced cell adhesion, selective inhibitors of PKCα (Ro320432), PKCβ (LY333531), or nonselective PKC inhibitors (Ro318220, GF109203X and Go6983) were tested for their abilities to block this effect. PMA-triggered cell adhesion within 4 h was reduced in the presence of all tested inhibitors, indicating that PMA-enhanced cell adhesion is via activation of PKCα and PKCβ (Fig. 7B). Furthermore, results revealed that inhibitors of AMPK (compound C), Syk (Syk inhibitor I) and c-Src (PP2) also exerted a concentration-dependent inhibition of PMA response (Fig. 7C). To rule out the attenuated responses are resulting from the reduced cell viability, the MTT [3-(4,5-cimethylthizazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay, an index of mitochondrial activity, was performed to address this

question. We found that these inhibitors did not affect cell viability after 24 h incubation (data not shown). To understand whether reduced cell adhesion is associated with cell differentiation status, CD11b expression, a marker of macrophages, was examined by flow cytometry. As shown in Fig. 7D, PMA did not alter CD11b expression until 48 h, suggesting that the inhibition of cell adhesion by kinase inhibitors within the 4-h incubation period is not attributed to attenuated differentiation of THP-1 cells into macrophages.

To further identify the downstream signaling elements, we analyzed the phosphorylation status of Syk, Src and AMPK. Phosphorylation of human Syk at Y525/526 (equivalent to mouse Y519/520), Src at Y419 (equivalent to mouse Y418 and chicken Src Y416) and AMPKα at T172 are activation indexes of the respective kinases. Results shown in Fig. 8A revealed that activation of these kinases, as assessed by their active phosphorylation, was rapidly evoked upon PMA (100 nM) treatment. Meanwhile, pan-PKC inhibitors (Ro318220 and GF109203X) abrogated this effect of PMA. These results suggest that AMPK, Syk, and Src are downstream targets of PKC. The ability of PMA to activate AMPK and Syk was further demonstrated by immunofluorescence detection of phosphorylated forms of these proteins (Fig. 8B).

Next we examined whether inhibition of PKC-dependent adhesion by

pharmacological inhibitors is due to off-target effects. Since targeting PKC to the plasma membrane is a prerequisite for activation, we examined the membrane translocation of PKCα and PKCβ after PMA stimulation. As shown in Fig. 8C, neither compound C nor Syk inhibitor I affected the translocation of PKCα and PKCβ, suggesting the specificities of the kinase inhibitors used.

After observing Syk, Src and AMPK phosphorylation and activation are downstream events of PKC, we investigated the order of the signaling cascade. We found that PMA-induced phosphorylation of Src at Tyr419 was dramatically inhibited by PP2, Syk inhibitor I and compound C. Consistent with this finding, results using antibody against Tyr216 phosphorylation on c-Src, which is another index of c-Src activation, also exhibited the same phenomena (Fig. 8D). These data suggest that both AMPK and Syk are upstream signaling molecules mediating Src activation in response to PKC activation.

5-2. *A new cascade of PKC-LKB1-AMPK-Syk-Src signaling in monocyte adhesion*

Continuing to use pharmacological tools to dissect the relationship between AMPK, Syk and Src, we found that PMA-induced AMPK activation was inhibited by compound C, but not by PP2 and Syk inhibitor I. In contrast, PMA-induced Syk

activation was inhibited by Syk inhibitor I and compound C, but not by PP2 (Fig. 9A).

In vitro kinase activity assay confirmed the abilities of Syk inhibitors (Syk inhibitor I,

Bay 61-3606 and Syk inhibitor III) to block PMA-induced Syk autophosphorylation.

Compound C partially decreased this effect, while PP2 failed to affect this event (Fig.

9B). These results suggest that PKC-dependent AMPK activation accounts for Syk,

and subsequently Src, activation.

To verify the finding that AMPK is responsible for Syk activation, we examined

the protein interaction between both molecules. Results indicate that after PMA

stimulation, Syk can associate with AMPK, and this event was reduced by treatment

with compound C, while unaffected by PP2 or Syk inhibitor I (Fig. 9C). These results

indicate the requirement of kinase activity of AMPK for Syk activation.

Given that PKC mediates AMPK activation, we next explored the molecular

mechanism underlying this event. To understand whether AMPK is a direct substrate

of PKC, AMPK was immunoprecipitated, then probed with anti-phosphoserine PKC

substrate Ab. Results revealed that there is no specific signal detected by

anti-phosphoserine PKC substrate in PMA-stimulated AMPK, suggesting that AMPK

is not the substrate for PKC (Fig. 9D, upper panel). Next, we investigated if PKC can

activate upstream activating kinase LKB1. A previous study demonstrated that PKCξ

can regulate AMPK activity indirectly via activation and phosphorylation of LKB1 at

Ser428 (Xie et al., 2008). Data obtained from immunobloting with anti-phosphoserine

PKC substrate Ab indicated that PMA treatment results in phosphorylation of LKB1

in THP-1 monocytes (Fig. 9D, lower panel). Other evidence to support this notion is

the increased LKB1 phosphorylation at Ser428 after PMA stimulation (Fig. 9E).
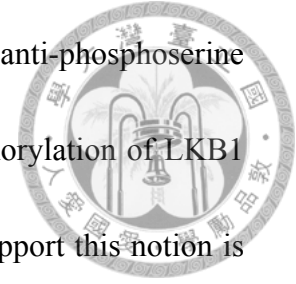
5-3. *Syk is phosphorylated and activated by AMPK*

After observing the association of Syk and AMPK under conditions of PKC

activation, we would like to map their respective interaction domains. To this end, we

made various Flag-tagged DNA constructs of Syk (WT, dNSH2, dCSH2, dSH2, dKD

and SH2 only) (Fig. 10A, upper panel), and verified their expression in HEK293T

cells. Next, we compared the binding of these Syk molecules to AMPKα2 in

HEK293T cells, which do not express endogenous Syk. After immunoprecipitation of

Syk with anti-Flag antibody and detection of AMPKα2 in the immunoprecipitate with

anti-Myc antibody, we found that all deleted Syk proteins except Syk (SH2 only) can

interact with AMPKα2 (Fig. 10A, lower panel). These results suggest that the linker

region of Syk is required for interaction with AMPKα2. Conversely, in order to define

the domain of AMPK necessary for the association with Syk, we tested four

Myc-tagged AMPKα2 constructs (Fig. 10B, upper panel). After immunoprecipitation

of Flag-Syk (WT), we detected the presence of AMPKα2 (1-552), AMPKα2 (1-312),

and AMPKα2 (1-398), but not AMPKα2 (303-552) in the immunoprecipitates (Fig. 10B, lower panel). These results suggest that the kinase domain of AMPK is necessary for the association with Syk.
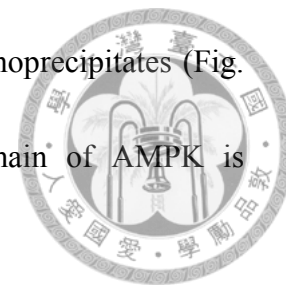
Next, we tested whether AMPK interaction with Syk can induce Syk phosphorylation and activation. After alignment of the consensus phosphorylation motif for AMPK and confirmation using variant synthetic peptides substrates (Weekes et al., 1993; Hurley et al., 2005), we predicted Syk might be phosphorylated by AMPK at Ser178 (Fig. 4C). With the use of site-directed mutagenesis, Ser178 of Syk was mutated into alanine (S178A) to determine its activation status versus wild-type Syk by AMPK. HEK293T cells were transiently transfected with the expression vectors for Flag-Syk (WT) or Flag-Syk (S178A) with AMPKα2, then stimulated with AICAR. Flag-Syk (WT) and Flag-Syk (S178A) immunoprecipitated with Syk antibody were tested for enzyme activity in an *in vitro* autophosphorylation assay. As shown in Fig. 10D, AICAR indeed activated Syk at 15 and 30 min treatment, while Syk mutant (S178A) was not activated under the same conditions. Moreover, ectopic expression of AMPK also caused Syk activation in the absence of AICAR, and this effect was not detected in cells expressing mutant Syk. To confirm this finding, a Syk peptide (CWFHGKISREESEQIV) containing the amino acid sequences of Syk at 167-182 was synthesized as substrate in an *in vitro* kinase assay. We found that

AICAR- and PMA-activated AMPK indeed caused Syk peptide phosphorylation (Fig. 10E). Moreover, expression of Syk (S178A) reduced cell adhesion in response to PMA (Fig. 10F), confirming Syk is a downstream substrate of AMPK.
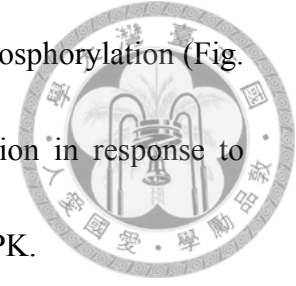
5-4. *AMPK activation itself is not sufficient for monocyte adhesion but increases the adhesive response to PMA*
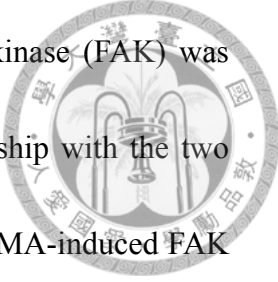
The above findings suggest the crucial role of AMPK in PKC-mediated monocyte adhesion. We explored if AMPK activation itself was sufficient to induce cell adhesion. To this end, we tested the effects of AICAR, an AMPK activator, on monocytes in terms of signaling-associated cell function. As shown in Fig. 11A, AICAR treatment can rapidly induce AMPK, Syk and Src activation, and all these events were abrogated by compound C. Notably, AICAR (0.1 and 0.5 mM) treatment alone did not affect monocyte adhesion, while it did increase cell adhesion elicited by PMA at 10 and 30 nM, but not that at 100 nM (Fig. 11B). The adhesion of total THP-1 cell suspension was fully achieved by PMA (100 nM) stimulation for 4 h, therefore there was not potential for further increase of cell adhesion upon adding AICAR. These results suggest that activation of AMPK by AICAR can enhance the adhesive response. To confirm the results observed with AICAR treatment, we employed a genetic approach. THP-1 cells constitutively expressing active or kinase-dead AMPKα2 exhibited the same extent of cell adhesion in the resting state.

PMA (100 nM)-induced cell adhesion at 1 and 4 h was inhibited by kinase-dead

AMPK. However, cells with active AMPK responded to PMA (100 nM) more rapidly,

and reached the maximal adhesive effect at 1 h (Fig. 11C). These results suggest that

AMPK activity is modulatory but not sufficient for the cell adhesion.
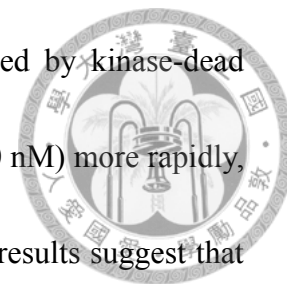
5-5. *PKC-dependent ERK activation contributes to monocyte adhesion*

To determine if other signaling events are required to mediate PKC-dependent

monocyte adhesion, we tested the role of ERK, a major downstream signaling

pathway of PKC, in THP-1 monocyte adhesion. First, we found that U0126 (a specific

inhibitor of MEK/ERK) can inhibit PMA-induced THP-1 monocyte adhesion in a

concentration-dependent manner (Fig. 12A). Despite the ability to block cell adhesion,

U0126 did not affect AMPK, Syk or Src activation induced by PMA (Fig. 12B, upper

panel). Conversely, PMA-induced ERK activation was not altered by the inhibitors of

Syk, Src or AMPK (Fig. 12B, lower panel). Moreover, consistent with our previous

findings in rat vascular smooth muscle cells and HUVEC (Towler and Hardie, 2007),

AICAR itself cannot induce ERK activation (data not shown) or affect PMA-induced

ERK phosphorylation (Fig. 12C). These results suggest a PKC-activated bifurcated

pathway to induce monocyte adhesion through both AMPK-Syk-Src and ERK

signaling cascades. Since protein tyrosine kinase focal adhesion kinase (FAK) was described as an index of cell adhesion, we determined its relationship with the two signaling pathways mediated by PKC as proposed. We found that PMA-induced FAK phosphorylation was attenuated by Syk inhibitor I, PP2 and compound C, but was unaffected by U0126 (Fig. 12D). Thus, we suggest that the FAK-mediated cytoskeletal reorganization for PKC-dependent monocyte adhesion is downstream of AMPK-Syk-Src but not the ERK signaling pathway.

Next, we examined whether the protein kinases mentioned above are also involved in monocyte adhesion to HUVEC, as well as in the case of primary monocytes. We found that SykI, PP2, compound C and U0126 were effective to inhibit the adhesion of THP-1 cells to HUVEC, and Ro318220 can abrogate this response of PMA (Fig. 12E). Similar effects of these kinase inhibitors on PMA-induced adhesion of primary human monocytes to culture plates were observed (Fig. 12F).

Concerned that the adhesion data shown in Fig. 12E was interfered with cytotoxicity of HUVEC, we measured LDH in the culture medium of HUVEC, either in the presence or absence of PMA-stimulated THP-1 for 4 h. As a result, we found no significant difference of LDH level with or without THP-1 adhesion (data not shown). In addition, we also measured TNFα in the culture medium of THP-1 cells to understand if TNFα plays a role for monocyte adhesion to EC. Our data revealed that

after PMA stimulation for 4 h (the time for us to measure cell adhesion), TNFα

concentration (2268 pg/ml, n =3) in the medium was not changed. Moreover, in the

LDH assay we did not observe significant cytotoxicity in HUVEC treated with TNFα

for 4 h (data not shown). These results all together ruled out the intervention of

cellular integrity and autocrine factor in our adhesion data.

To understand if integrins might play a role in PMA-induced monocyte adhesion,

we examined the effects of α5β1 inhibitor and αVβ3 blocking antibody (LM609),

which interfere with the interaction through fibronectin and vitronectin respectively,

on PMA-stimulated monocyte adhesion to culture dishes and HUVEC (Yang et al.,

2005; Kurihara et al., 2011). Before this experiment, we first confirmed the effective

concentration (10 mg/ml) of alpha5beta1 inhibitor and LM609 to abrogate

fibronectin- and vitronectininduced cell adhesion (Fig. 12G, left panel). Next, we

observed that both integrin inhibitors can reduce PMA-induced THP-1 adhesion to

culture plate and HUVEC (Fig. 12G, right panel). These results suggest that integrins

are involved in PMA-induced cell adhesion.
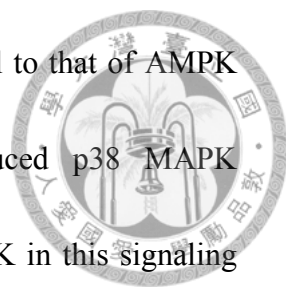
Chapter 6

Discussion

Besides its role in metabolic processes, AMPK was also reported to regulate inflammatory gene transcription. Despite several cellular and animal models having demonstrated that anti-inflammatory effects are mediated by AMPK (Giri et al., 2004; Park et al., 2006; Hallows et al., 2006; Zhao et al., 2008; Jeong et al., 2009), opposite findings of AMPK-dependent inflammatory responses were also documented. Based on controversial findings about AICAR-regulated COX-2 induction, and unclear molecular mechanisms underlying AMPK-dependent p38 MAPK activation, we re-examined this issue in three cell types in this study.
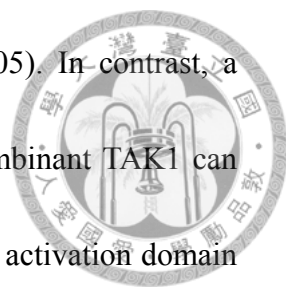
We found that AICAR treatment led to a significant increase in AMPK phosphorylation, which was sufficient to induce COX-2 protein expression in macrophages, VSMCs, and HUVECs. 5'-Iodotubercidin and compound C abolished AICAR-elicited COX-2 promoter activity and protein induction, indicating the requirement of AMPK activity for initiating signaling cascades leading to COX-2 gene expression. Upon exploring the molecular mechanisms underlying COX-2 induction, we found that AICAR exerted a cell type-specific action in inducing ERK, p38 MAPK, and/or JNK activation, and p38 MAPK was the most important and common player for AICAR-induced COX-2 gene upregulation. AICAR rapidly

stimulated p38 MAPK phosphorylation with a time course parallel to that of AMPK

phosphorylation, and 5'-iodotubercidin decreased AICAR-induced p38 MAPK

phosphorylation, suggesting the upstream regulatory role of AMPK in this signaling

pathway. Moreover, our data showed the cell type-specific effects of AMPK on JNK

and ERK. Both kinases were activated by AICAR in J774 macrophages, while only

JNK was activated in HUVECs. In contrast, neither MAPK was activated in VSMCs.

We suggest that different regulatory mechanisms controlled in cell type-specific

manners are involved in the modulation of AMPK-dependent signaling pathways.
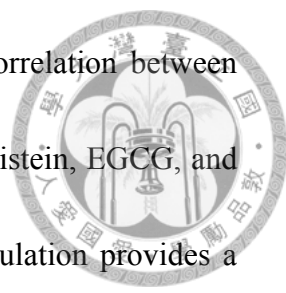
AMPK and p38 MAPK are stress-responsive protein kinases and are simultaneously

involved in ischemia-reperfusion heart injury and glucose utilization (Monick et al.,

2002; Tang et al., 2007) . Both kinases seem to form a reciprocal and complex

functional signaling cascade. The p38 MAPK cascade downstream of AMPK was

implicated in glucose transport (Xi et al., 2001), cytoskeletal rearrangement (Englesbe

et al., 2004) , COX-2 expression (Hou et al., 2008), and IL-6 secretion (Tang et al.,

2007). In contrast, AMPK is likely downstream of p38 MAPK when mediating the

effects of adenosine in glucose utilization in hearts stressed by transient ischemia

(Monick et al., 2002).

Regarding AMPK-dependent p38 MAPK activation, recent evidence showed that

TAB1 is responsible for promoting the recruitment of p38 MAPK to the

TAB1/AMPK-containing macromolecular complex (Li et al., 2005). In contrast, a study claimed an opposite link between AMPK and TAK1. Recombinant TAK1 can phosphorylate AMPKα at Thr-172 in HeLa cells when fused to the activation domain of its binding partner, TAB1 (Miletic et al., 2007) . Another study showed that TAK1 can activate AMPK-dependent cytoprotective autophagy in TRAIL-treated epithelial cells (Park et al.,2007). Both findings support TAK1 being a candidate for an authentic AMPK kinase in mammalian cells. In this study, we did not observe the protein interaction between AMPKα and TAB1 in HUVECs, J774 macrophages, or VSMCs, either in the absence or presence of AICAR stimulation (data not shown). In contrast, we observed for the first time the binding between AMPKα and TAK1 both in vivo and in vitro, and demonstrated AMPK-dependent TAK1 activation. We found that the binding between AMPKα and TAK1 does not rely on AMPK activity, but requires the kinase domains of both protein molecules. These results provide novel insights into the mechanism through which AMPK leads to p38 MAPK activation. Even though our current findings revealed that AMPK can activate TAK1, we still cannot exclude the reverse interaction manner proposed by previous studies by which TAK1 can phosphorylate Thr172 in the activation loop of the AMPK catalytic domain (Miletic et al., 2007).

To date, a few reports suggested that AMPK may be a novel regulatory molecule

in COX-2 expression. In HT-29 colon cancer cells, a negative correlation between AMPK activation and COX-2 expression under treatment with genistein, EGCG, and selenium was reported. Those results imply that COX-2 downregulation provides a mechanism for AMPK to serve as a chemotherapeutic target (Hwang et al., 2006). Our current and previous findings together suggest that AMPK signaling might have a paradoxical intervention in regulating cell functions via COX-2. The suppressive effect of AICAR on COX-2 levels against inflammatory inducers may be involved in its protective action. In contrast, in a basal cellular situation, AMPK-dependent upregulation of COX-2 might exert a homeostatic function through eicosanoid production. In this context, AICAR is possibly involved in controlling blood pressure, platelet aggregation, and anti-adhesion of leukocytes.
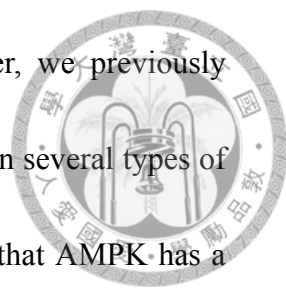
In this study, we demonstrate that PKC, Syk, AMPK, Src and ERK kinases play critical roles in monocyte adhesion, and propose a novel signaling mechanism of this event. Previous studies have implicated PKC and Syk in the adhesive functions of integrins. (Abram and Lowell, 2007; Moreno et al., 2008)   Most findings propose that Syk is the upstream signal to induce PKC activation either indirectly via PLC activation or directly via physical interaction. (Kawakami et al., 2003; Moreno et al., 2008; Mocsai et al., 2010). In contrast, a reverse signaling cascade between PKC and Syk is also demonstrated in endothelial cells where thrombin can induce NF-κB

activation through PKCδ-Syk pathway (Bijli et al., 2008). In this study we unveil a

novel signaling network linking PKC activation to downstream Syk via AMPK.
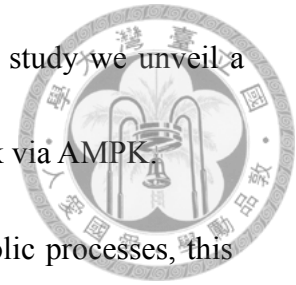
Besides the relatively well identified role of AMPK in metabolic processes, this

energy sensor kinase is also implicated as an anti-inflammatory target. AICAR, the

pharmacological activator of AMPK, has been reported to exert anti-inflammatory

and immunomodulatory effects in various models of inflammation (Yang et al., 2005;

Chang et al., 2010., Kurihara et al., 2011). In endothelial cells, AMPK activity is

associated with phosphorylation and activation of eNOS, resulting in

anti-inflammatory action in the vascular wall (Pula et al., 2005). Endotoxin

lipopolysaccharide-induced expression of proinflammatory molecules and mediators

in primary macrophages, microglia, astrocytes, and mesangial cells are suppressed by

AICAR (Prasad et al.,2006; Mycrburg et al., 2010; Bai et al., 2010). Mechanistic

studies suggest inhibition of NF-κB, c/EBPβ and reactive oxygen species production

contribute to the anti-inflammatory event mediated by AMPK signaling (Zou et al.,

2002; Giri et al., 2004; Jhun et al., 2004; Prsad et al., 2006; Geolotto et al., 2007;

Kuo et al., 2008).

Despite the above reports implicating AMPK as an anti-inflammatory molecule,

contrary findings of AMPK-dependent inflammatory responses are also documented.

In this respect, AICAR and adiponectin can stimulate IL-6 production in fibroblasts
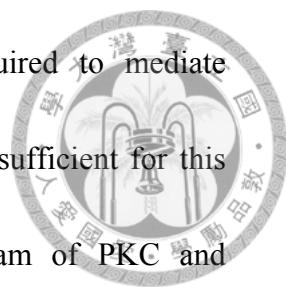
via an AMPK-dependent pathway (Zhao et al., 2008). Moreover, we previously found that AICAR alone can induce cyclooxygenase-2 expression in several types of cells under resting status (Towler et al., 2007). We thus postulate that AMPK has a bi-directional function and might act as an "early warning and protective signal" under sub-pathological stress. Cells under inflammatory stress upon LPS or proinflammatory cytokine stimulation would favor AMPK to play a role in resolving inflammation and tissue protection. In contrast, at a resting basal situation, AMPK-dependent upregulation of cyclooxygenase-2 might exert a homeostatic function through eicosanoid production.

Leukocyte adhesion is the essential step required for circulating monocytes to traverse multiple tissue barriers, and to perform their functions in host defense. Both lower expression of proinflammatory cytokines, chemokines, and adhesion molecules, and upregulation of eNOS activity in endothelial cells might contribute to the AMPK-mediated inhibition of leukocyte-endothelial cell interaction (Prasad et al., 2006; Myerburg et al., 2010; Bai et al., 2010; Kurihara et al.,2011; Bess et al., 2011). Currently only one study has shown the direct role of AMPK in regulating leukocyte adhesion (Chandrasckar et al.,2008). In this study, AMPK was found to mediate rosiglitazone-induced inhibition of fibronectin-mediated adhesion of human

peripheral blood mononuclear cells. The difference between this result and ours might be due to ligand (integrin vs direct PKC activator) and cell type specificity.
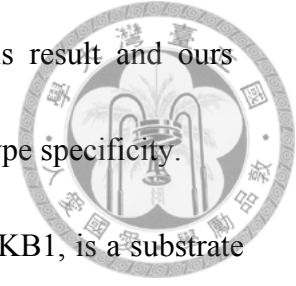
In this study, we proved that the upstream kinase of AMPK, LKB1, is a substrate of PKC in THP-1 monocytes stimulated by PMA. Moreover, we ruled out that AMPK has a direct interaction with PKC. Moreover, we also provide evidence to support that Syk is a novel biological substrate of AMPK, thus extending the functional impact of AMPK in regulation of leukocyte adhesion. First, Syk can directly interact with AMPKα2 via the linker region of Syk and the kinase domain of AMPK. Second, the direct interaction between Syk and AMPKα2 is induced upon AMPK activation. Third, Syk-AMPKα2 interaction leads to AMPK-mediated phosphorylation of Syk at Ser178, which is located at the C terminal region of SH2 domain. Using Syk synthetic peptides as a substrate (CWFHGKISREES̲EQIV), we further confirmed the phosphorylation site by AMPK at the Ser178 residue of Syk. Fourth, phosphorylated Syk in turn exerts its activation and propagates downstream signals to Src and FAK for cell adhesion. The cross-activation of Syk and Src has been demonstrated to exert the cellular functions of immunoreceptors and integrins (Berton et al., 2005). Fifth, using a direct AMPK activator (AICAR) and genetic approach (AMPK-CA and AMPK-DN) we confirmed the role of AMPK in activation of Syk, Src and increase of monocyte adhesion.

It is interesting to note that even though AMPK is required to mediate PKC-dependent monocyte adhesion, AMPK activity alone is not sufficient for this event. In other words, a coordinate signal pathway downstream of PKC and independent of AMPK is required to evoke cell adhesion. In this respect, we identify ERK as the major player in this event. The PKC-mediated AMPK-Syk-Src-FAK pathway proposed above is not affected by ERK inhibitor, while ERK inhibitor can reduce cell adhesion. Moreover, the failure of inhibitors of AMPK, Syk, and Src to affect PMA-induced ERK phosphorylation, and the inability of AICAR to induce ERK activation suggest that a bifurcated signaling pathway is induced and is crucial to PKC-dependent cell adhesion. We propose that to achieve firm leukocyte adhesion, leukocytes need to collect different intracellular signals.

The involvement of ERK in PKC-mediated THP-1 cell adhesion has been documented (Huang et al., 2009). Molecular mechanistic studies provide evidence for the role of ERK signaling in adhesion assembly. In this respect, PMA-induced activated ERKs are localized to focal adhesions, and promote Rho-dependent focal adhesion formation by suppressing p190A RhoGAP (Tang et al., 2007; Ewart et al., 2008). Moreover, some actin-binding proteins as phosphorylation targets of ERKs and contributing to focal adhesion formation have been demonstrated (Besson et al., 2001; Tsai et al., 2010; Pullikuth and Catling, 2010).

In summary, our part I study for the first time prove a pivotal role of TAK1 in the AMPK signaling axis. Such interaction gives AMPK an additional pathway for regulating cellular functions. Via a downstream p38 MAPK signaling cascade, AMPK-dependent TAK1 activation leads to the expression of the inflammatory COX-2 gene in various cell types (Fig. 13). Our part II study suggest that a coordinated signaling between AMPK-Syk-Src-FAK and ERK is involved in PMA-induced monocyte adhesion (Fig. 14). We for the first time unveil a novel signaling between AMPK and Syk, which may shed new light on the roles of AMPK in cell adhesion, in addition to its well identified functions in energy homeostasis, insulin sensitivity and obesity.
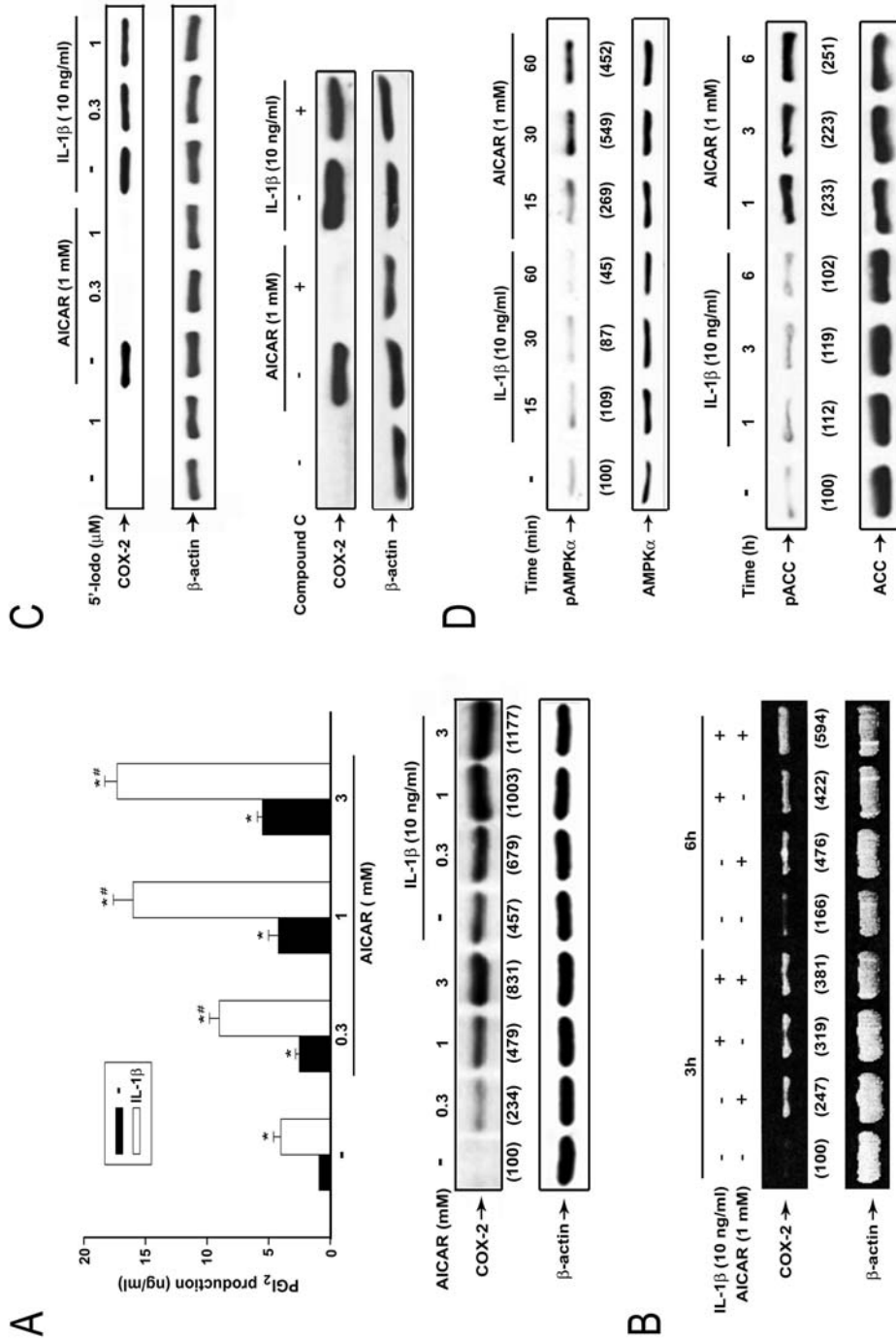
Figure 1

Figure 1. AICAR induces COX-2 expression in VSMCs. (A) After VSMCs were treated with AICAR (0.3~3 mM) and/or IL-1β (10 ng/ml) for 24 h, culture medium was collected for PGI_2 measurement, and cell lysates were harvested for immunoblotting with a COX-2 antibody. **(B)** Cells were treated with AICAR (1 mM) or IL-1β (10 ng/ml) for the indicated time periods, and an RT-PCR analysis of steady-state mRNA levels of COX-2 was carried out. **(C)** Cells were pretreated with 5'-iodotubercidin (0.3, 1 μM) or compound C (30 μM) for 30 min, followed by treatment with AICAR or IL-1β. After 24 h, an immunoblot analysis of COX-2 was carried out. **(D)** Cells were treated with AICAR or IL-1β for the indicated periods, and immunoblotting of AMPKα and ACC was carried out. Numbers in parentheses are percentages of the control level of specific proteins in vehicle-treated cells. The results are representative of three separate experiments. Data in (A) are presented as the mean ± S.E.M. from at least three independent experiments. * $p<0.05$, indicating significant induction of PGI_2. [#] Indicates the synergistic effects of AICAR and IL-1β.

Figure 2

A
J774

AICAR (mM) 0 1 3 | 5'-iodo 3

COX-2 →

β-actin →

PGE$_2$ production (ng/ml)

AICAR (mM): − 1 3 | − 3
5'-Iodo

*

B
HUVEC

AICAR (mM) 0 1 3 | 5'-iodo 3

COX-2 →

β-actin →

PGI$_2$ production (ng/ml)

AICAR (mM): − 1 3 | − 3
5'-Iodo

*

C
J774

AICAR (1 mM)
−   15 min   1 h

pAMPKα →

AMPKα →

HUVEC

AICAR (1 mM)
−   15 min   1 h

pAMPKα →

AMPKα →

D
J774

GFP   +   −   −
GFP-AMPKα2 (DN)   −   +   −
GFP-AMPKα2 (CA)   −   −   +

COX-2 →

GFP-AMPKα →
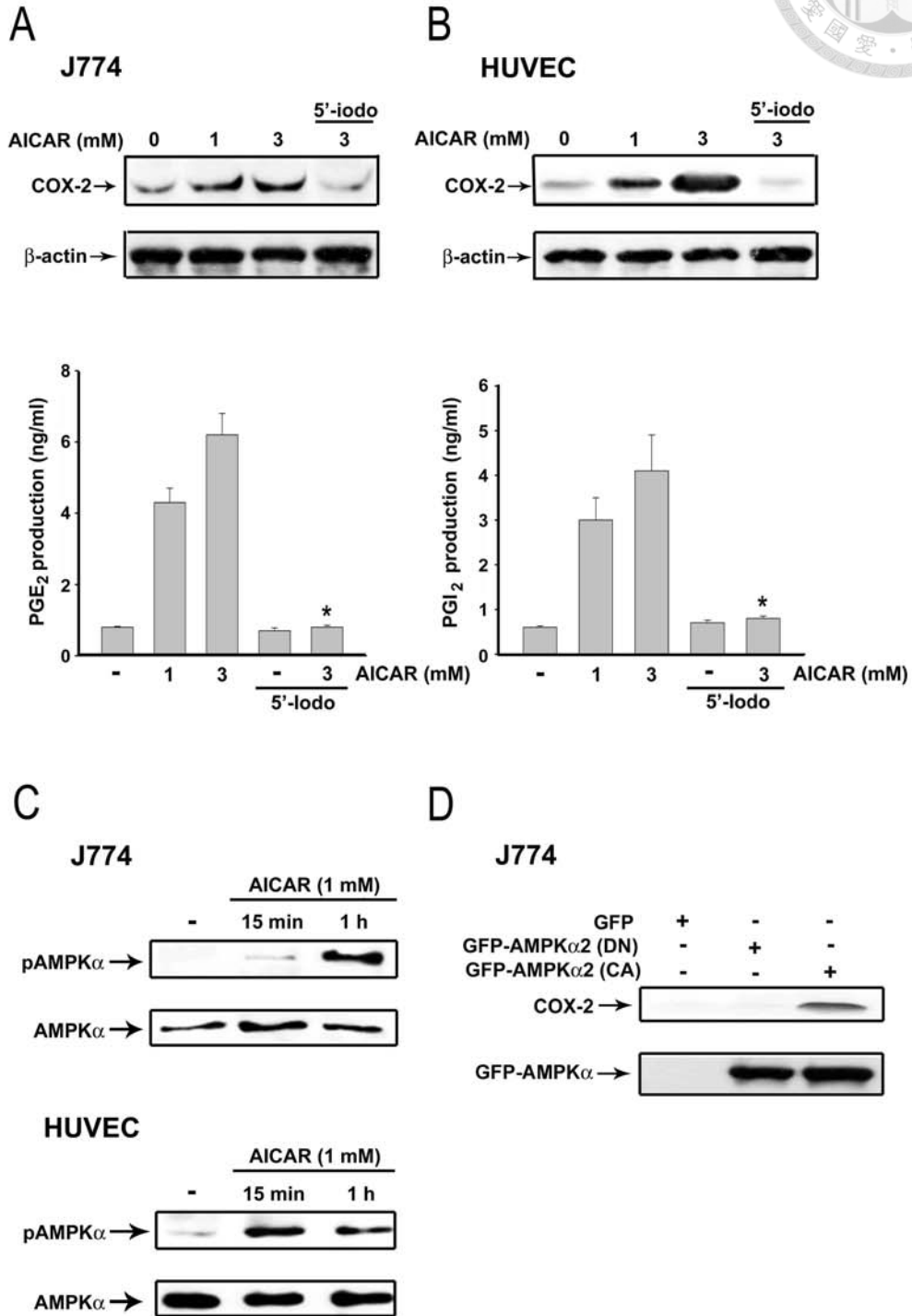
Figure 2. AICAR induces COX-2 expression in J774 and HUVECs through the AMPK pathway. J774 macrophages **(A)** and HUVECs **(B)** were pretreated with 5'-iodotubercidin (1 µM) for 30 min, followed by treatment with AICAR (1 or 3 mM). After 24 h, culture media were used for PGE_2 or PGI_2 measurements, and cell lysates were subjected to an immunoblot analysis of COX-2. Data of prostaglandin production are presented as the mean ± S.E.M. from at least three independent experiments. * $p<0.05$, indicating significant inhibition of the prostaglandin response by 5'-iodotubercidin. **(C)** In some experiments, cells were treated with AICAR (1 mM) for 15 min or 1 h, and cell lysates were prepared to conduct immunoblotting of AMPKα. **(D)** J774 cells were infected with adenovirus expressing constitutive active or kinase dead AMPK. After 48 infection, COX-2 expression was determined. The results are representative of three separate experiments.

Figure 3

A VSMC

B J774

C HUVEC

D J774
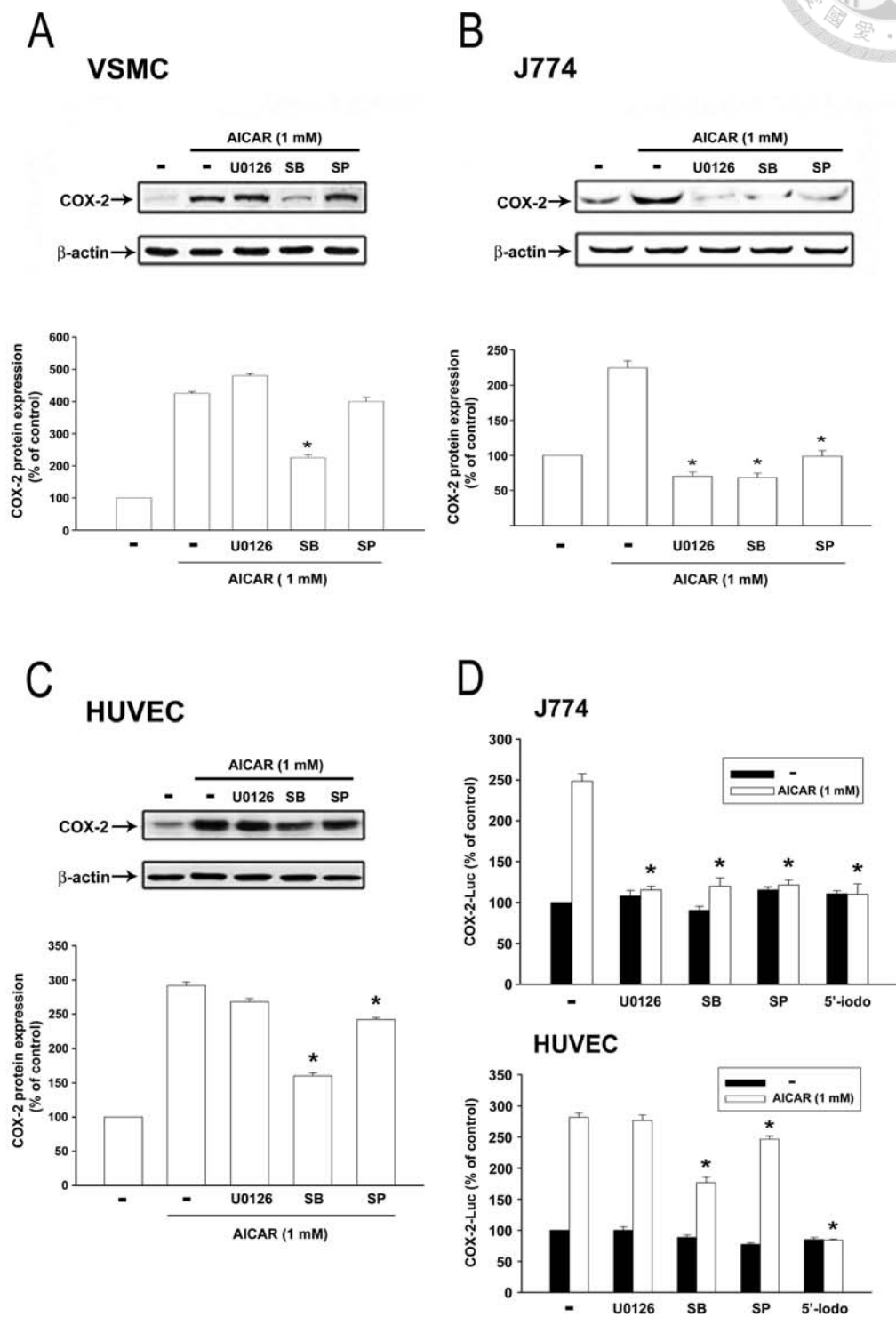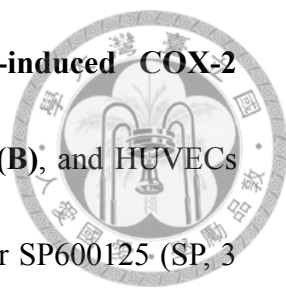
Figure 3. Differential involvement of MAPKs in AICAR-induced COX-2 expression is cell type specific. VSMCs **(A)**, J774 macrophages **(B)**, and HUVECs **(C)** were pretreated with U0126 (1 μM), SB203580 (SB, 3 μM), or SP600125 (SP, 3 μM) for 30 min, followed by treatment with AICAR (1 mM) for 24 h. The protein level of COX-2 was determined by immunoblotting. Traces shown are representative of three separate experiments, and the mean ± S.E.M. from three independent experiments is shown in the vertical bar chart. **(D)** J774 macrophages and HUVECs were transfected with the COX-2 promoter, followed by treatment with specific inhibitors of MAPKs or 5'-iodotubercidin at concentrations described above for 30 min prior to the addition of AICAR (1 mM). After 24 h of incubation, reporter activity was measured, and results are expressed as a percentage of the control group without inhibitor pretreatment. * $p<0.05$, indicating significant inhibition of AICAR's effects by the inhibitors.

Figure 4

A

		IL-1β (10 ng/ml)			AICAR (1 mM)		
Time (min)	-	15	30	60	15	30	60
pERK	(100)	(171)	(255)	(230)	(87)	(77)	(92)
ERK							
pp38	(100)	(124)	(270)	(150)	(161)	(178)	(245)
p38							
pJNK	(100)	(249)	(208)	(110)	(107)	(130)	(120)
JNK							

B

			AICAR (1 mM)					
			30 min		60 min		120 min	
5'-Iodo (1 μM)	-	+	-	+	-	+	-	+
pp38	(100)	(115)	(332)	(172)	(387)	(175)	(311)	(200)
p38								

C

5'-Iodo	-	-	-	+	-	-	-	+
AICAR (1 mM)	-	+	+	+	-	+	+	+
Time (min)	0	30	60	60	0	30	60	60
pERK	(100)	(198)	(171)	(101)	(100)	(98)	(102)	(103)
ERK								
pp38	(100)	(562)	(316)	(118)	(100)	(280)	(153)	(115)
p38								
pJNK	(100)	(504)	(405)	(132)	(100)	(245)	(475)	(265)
JNK								

J774   **HUVEC**

Figure 4. AMPK mediates different MAPK activations in VSMCs, J774 macrophages, and HUVECs. (A) VSMCs were treated with AICAR or IL-1β for the indicated time periods. After stimulation, cell lysates were subjected to an immunoblot analysis of ERK, p38, and JNK. **(B)** VSMCs were pretreated with 5'-iodotubercidin for 30 min followed by AICAR (1 mM) for different periods and then harvested. Total cell lysates were prepared for immunoblotting with an antibody specific for p38. **(C)** A similar approach as described for VSMCs was conducted in J774 macrophages (left panel) and HUVECs (right panel). Numbers in parentheses are percentages of the control level of a specific protein in vehicle-treated cells. The results are representative of three separate experiments.
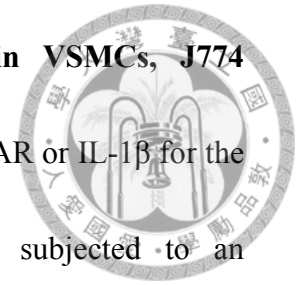
Figure 5
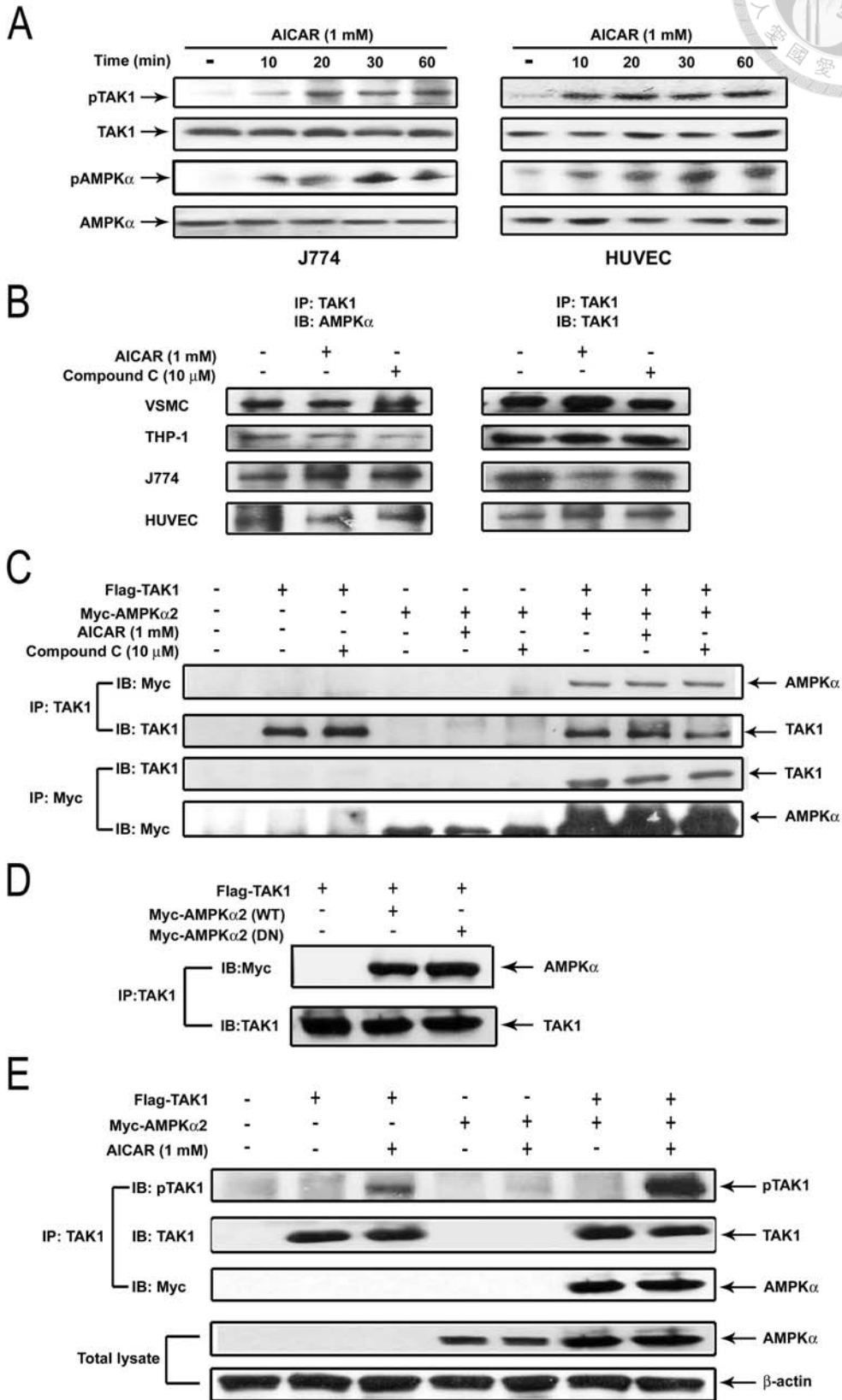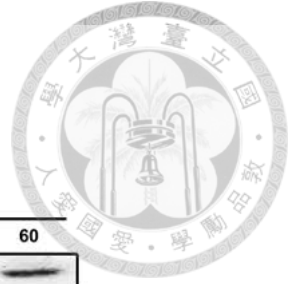
Figure 5. AMPK is associated with TAK1 and induces TAK1 activation. (A) J774

macrophages and HUVECs were treated with AICAR for different time intervals, and

protein levels of phosphorylated TAK1 and AMPKα were determined. **(B)** Different

cell types were treated with AICAR or compound C for 30 min. Supernatants of cell

lysates were immunoprecipitated with a TAK1 antibody, and immunocomplexes were

subjected to immunoblotting with anti-AMPK (left panel) or anti-TAK1 (right panel)

antibody. **(C)** Expression plasmids for Myc-AMPKα2 and Flag-TAK1 were

co-transfected into 293T cells. Cells were then stimulated with AICAR, compound C,

or vehicle for 30 min. Aliquots of lysates were immunoprecipitated with an

anti-TAK1 antibody, and the immunoprecipitates were immunoblotted with an

anti-AMPKα or anti-TAK1 antibody (upper panel). In some experiments,

immunoprecipitation with an anti-Myc antibody followed by immunoblotting with an

anti-AMPKα or anti-TAK1 antibody was also performed (lower panel). **(D)** 293T

cells were co-transfected with Flag-TAK1 and Myc-AMPKα2 of wild type (WT) or

dominant negative (DN) form. Immunoprecipitation was performed with anti-TAK1

antibody followed by immunobloting with either anti-Myc antibody or anti-TAK1

antibody. **(E)** Similar experiments were performed as described in (C), and TAK1

phosphorylation was determined with an anti-p-TAK antibody in immunoprecipitates

(upper panel). Aliquots of the lysates were also immunoblotted with an anti-AMPK or
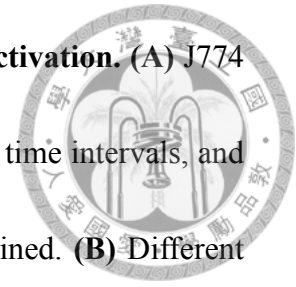
anti-β-actin antibody (lower panel). IP, immunoprecipitation; IB, immunoblot. Data

shown are representative of three independent experiments.
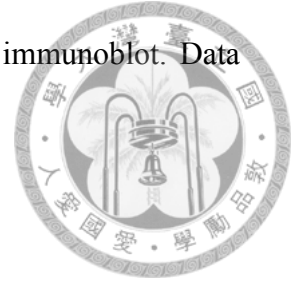
Figure 6
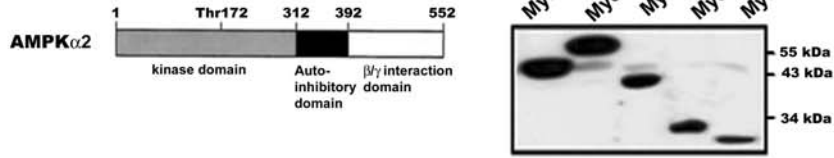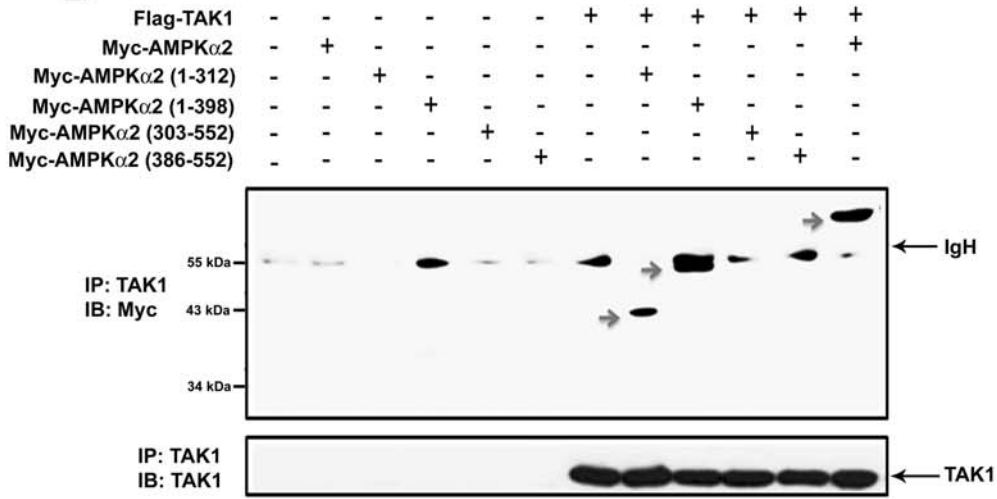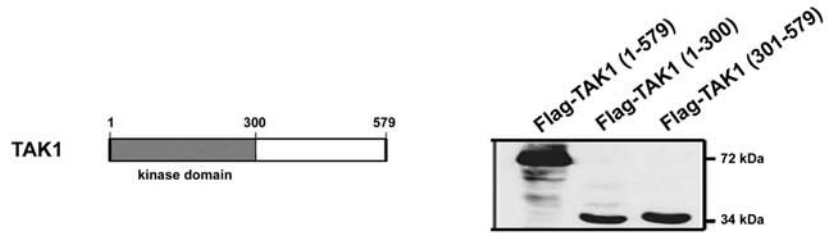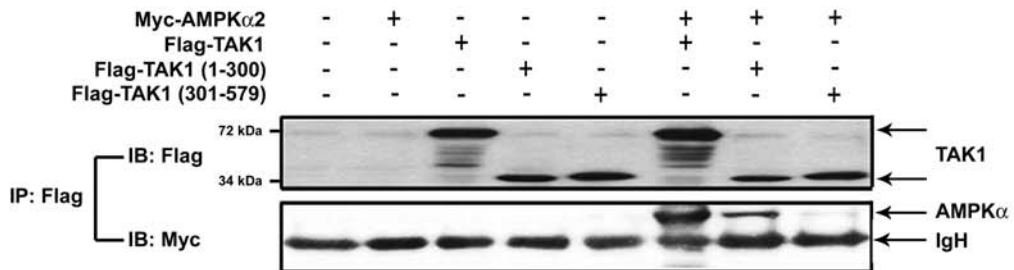
Figure 6. Interactive binding regions of AMPKα2 and TAK1. (A) Different

deletion mutant constructs of AMPKα were transfected into 293T cells. Immunoblots

of input lysates with an anti-Myc antibody for the Myc-tagged AMPKα2

(Myc-AMPKα2, Myc-AMPKα2 (1~312), Myc-AMPKα2 (1~398), Myc-AMPKα

(303~552), and Myc-AMPKα2 (386~552) are shown. **(B)** Flag-TAK1 was expressed

with or without Myc-AMPKα2 mutant constructs in 293T cells. Immunoprecipitation

with an anti-TAK1 antibody was done, and the product was subjected to an

immunoblot analysis with an anti-Myc or anti-TAK1 antibody. **(C)** Input lysates were

determined by Western blots with an anti-Flag antibody for the Flag-tagged TAK1

full-length and mutant constructs. **(D)** Myc-AMPKα2 was expressed with deletion

mutants of Flag-TAK1 in 293T cells. Immunoprecipitation with an anti-Flag antibody

and then an immunoblot analysis with an anti-Myc or anti-TAK1 antibody were

conducted. IgH, heavy chain of immunoglobulin. Data shown are representative of

two independent experiments.
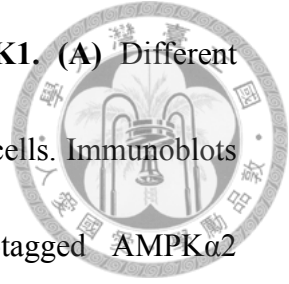
Figure 7
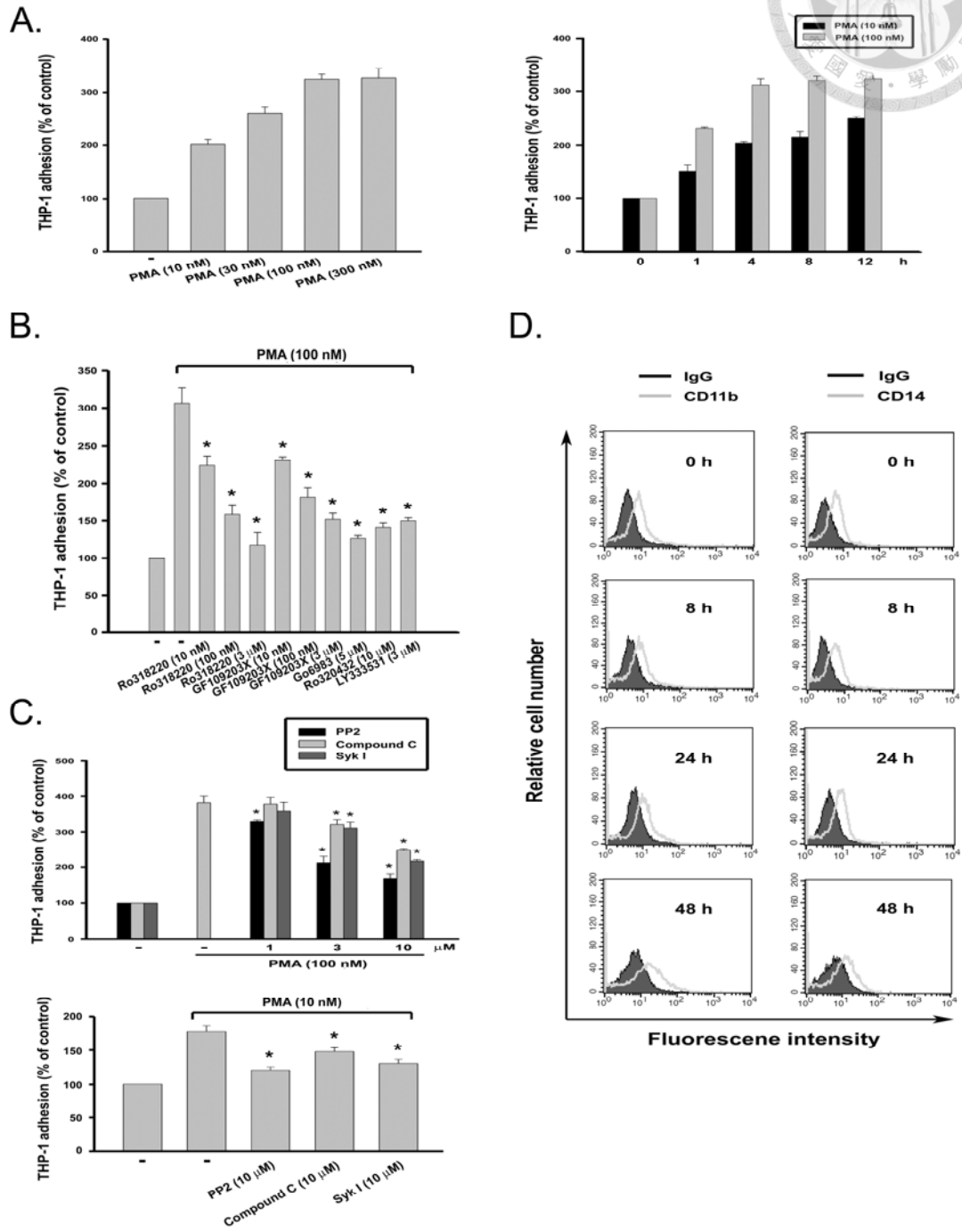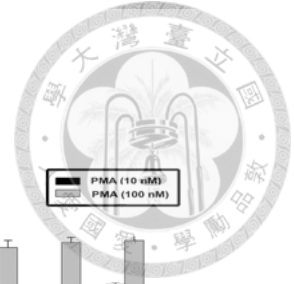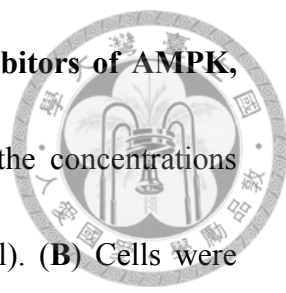
Figure 7. PMA-induced monocyte adhesion is blocked by inhibitors of AMPK, Syk and c-Src. (**A**) THP-1 cells were incubated with PMA at the concentrations indicated for 4 h (left panel) or for different periods (right panel). (**B**) Cells were treated with Ro318220, GF109203X, Go6983, Ro320432, or LY333531 (each at 3 μM) for 30 min prior to the addition of PMA and then incubated for another 4 h. (**C**) All inhibitors at the concentrations indicated were used to pretreat cells 30 min before PMA incubation for 4 h. Cell adherence was determined by crystal violet staining and quantified by measuring the absorbance at 550 nm. *p<0.05 as compared to the PMA response with vehicle treatment. (**D**) After PMA treatment, THP-1 cells were stained with FITC-conjugated CD11b antibody for flow cytometry. The cell population with CD11b expression was measured.

Figure 8
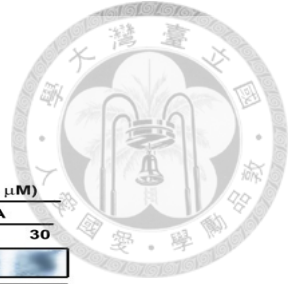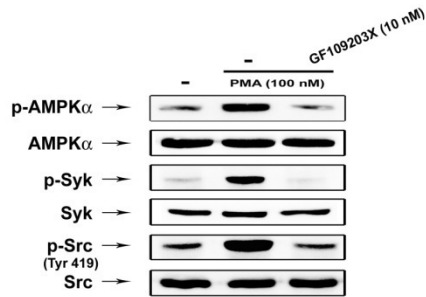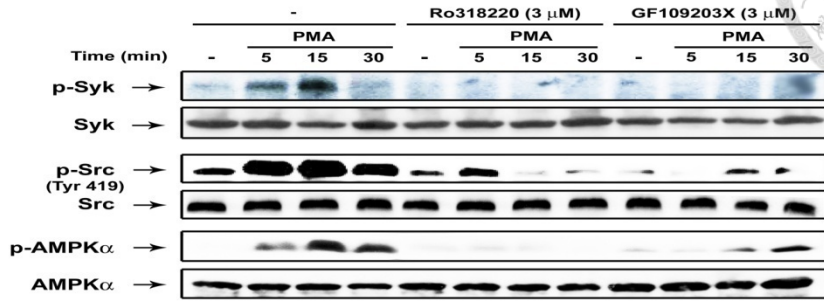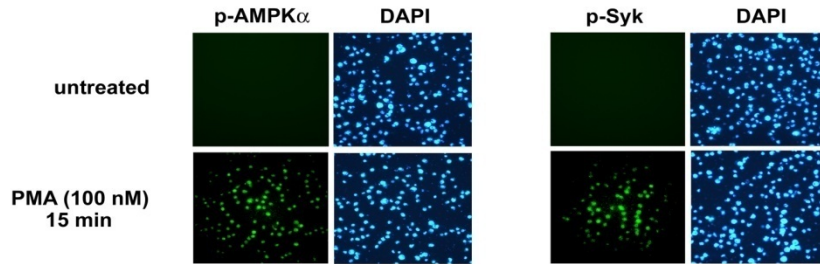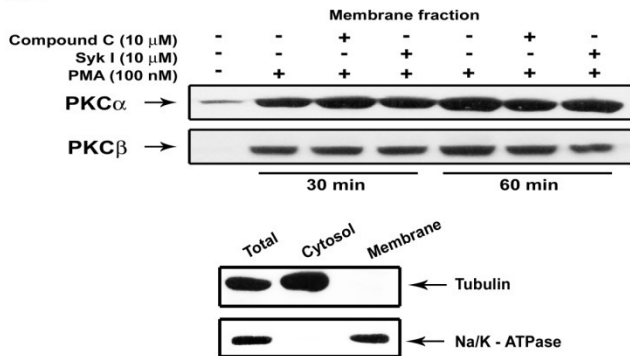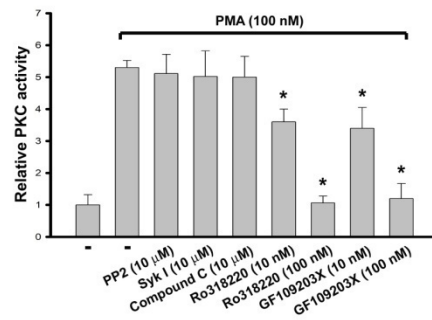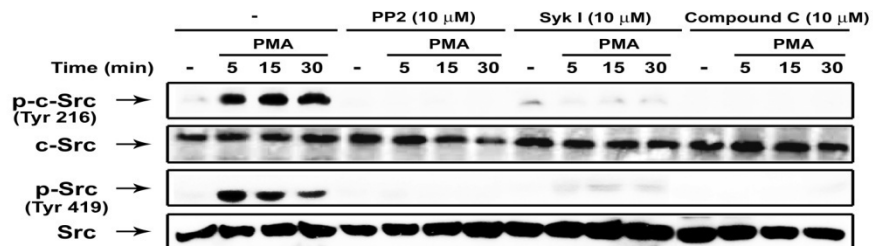
Figure 8. PKC-dependent AMPK and Syk activation mediate Src activation. (A) THP-1 cells were incubated with PMA (100 nM) and the indicated PKC inhibitors for various periods, followed by immunobloting. (**B**) THP-1 cells were treated with PMA for 15 min; phosphorylation of AMPK and Syk were analyzed by immunofluorescence staining. DAPI staining was used to determine nuclear localization. (**C**) After drug treatment as indicated, membrane fractions were harvested for immunoblotting of PKCα and PKCβ. (**D**) THP-1 cells were pretreated with inhibitors for 30 min, followed by treatment with PMA (100 nM) for the indicated time intervals, and then immunoblotting of protein phosphorylation on Src (Tyr 419) and c-Src (Tyr 216).
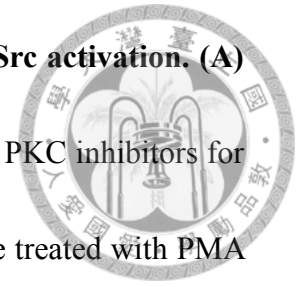
Figure 9
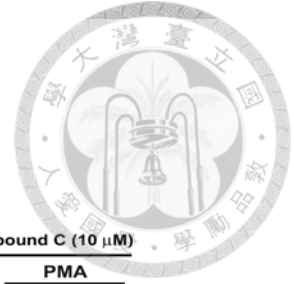
A.
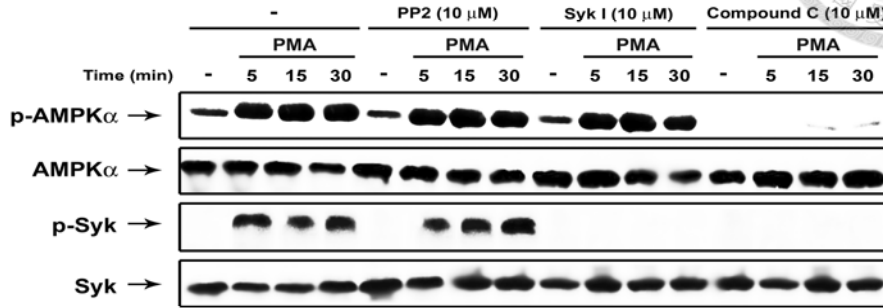
B.
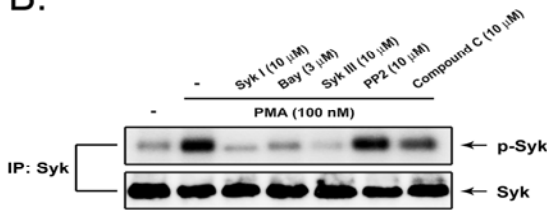
C.
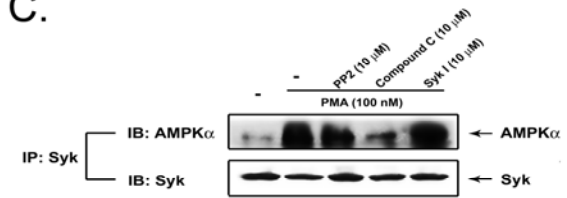
D.
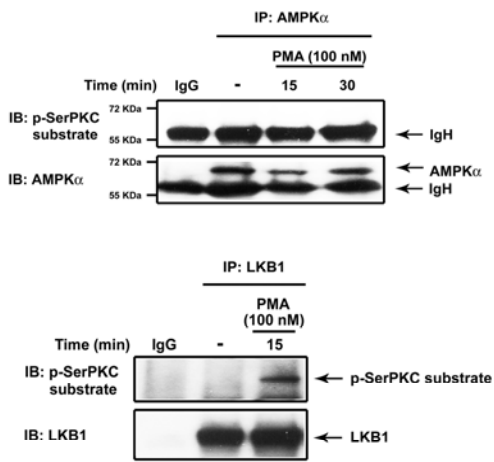
E.
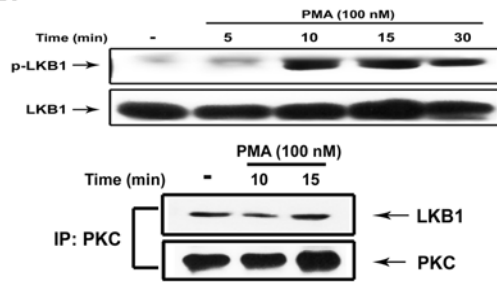
F.
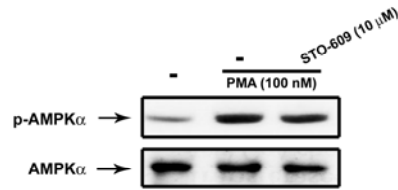
Figure 9. PKC induces the LKB1-AMPK-Syk signal pathway. (**A**) THP-1 cells were treated with inhibitors for 30 min, followed by PMA (100 nM) for the indicated time periods. Immunoblotting using antibodies against AMPK and Syk were conducted. (**B-C**) After pretreatment with inhibitor for 30 min and PMA treatment for 10 min, cell lysates were immunoprecipitated with Syk antibody. Syk enzymatic activity was determined by autophosphorylation (**B**) and AMPK association with Syk was determined by immunoblotting (**C**). (**D**) After PMA treatment, immunoprecipitation with control IgG, anti-AMPKα (upper panel) or anti-LKB1 (lower panel) antibody was subjected to immunoblot analysis with anti-phospho-serine PKC substrate antibody. (**E**) Immunoblotting with antibodies against phospho-LKB1 and total LKB1 was conducted.

Figure 10

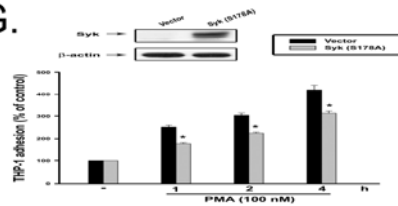**Figure 10. Binding domains between Syk and AMPK and AMPK-mediated Syk phosphorylation.** (**A**) HEK293T were transfected with Myc-AMPKα2 and various deletion constructs of Flag-Syk. Cell lysates were immunoprecipitated with anti-Flag antibody and then subjected to immunoblot analysis with anti-Myc or anti-Flag antibody. (**B**) Plasmids of Flag-Syk and myc-AMPKα2 with various deletions were transfected into HEK293T cells. Immunoprecipitates with anti-Flag antibody were subjected to immunoblot analysis with anti-Myc and -Syk antibodies. (**C**) Alignment of the consensus recognition motifs for AMPK and sequences around sites to be phosphorylated by AMPK on physiological substrates. The serine residues phosphorylated were indicated by the arrow. In the consensus recognition motif, Φ refers to a hydrophobic residue and β to a basic residue. (**D**) Myc-AMPKα2 was expressed with Flag-Syk (WT) or Flag-Syk (S178A) construct in HEK293T. Afterwards, cells were treated with AICAR for 15 or 30 min. Immunoprecipitate with Syk antibody was then subjected to *in vitro* kinase assay. (**E**) THP-1 cells were treated as indicated, and immunocomplexes with AMPK antibody were evaluated in the kinase assay using synthetic Syk peptide containing S178 as a substrate. (**F**) THP-1 cells were transfected with vector or Syk (S178A) plasmids for 48 h, followed by PMA treatment and adhesion assay. *p<0.05 as compared to the control PMA response.
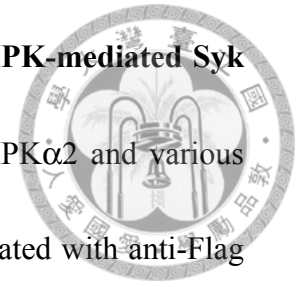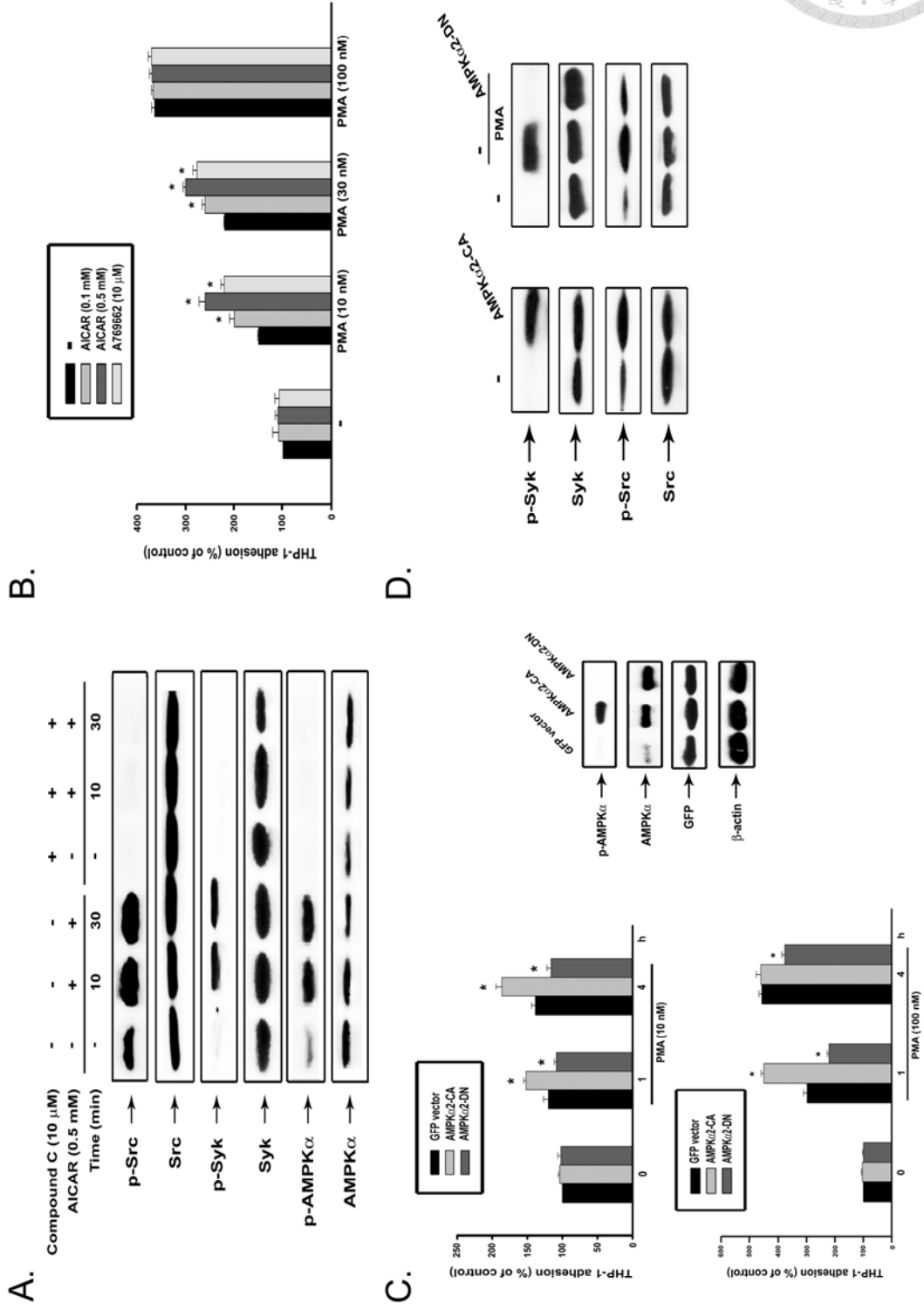
Figure 11

Figure 11. AICAR induces Syk and c-Src activation, and enhances cell adhesion upon PMA stimulation. (A) After treatment with agents as indicated, cell lysates were subjected to immunoblotting. **(B)** THP-1 cells were co-incubated with AICAR and/or PMA for 4 h, followed by cell adhesion assay. **(C)** THP-1 cells were infected with adenovirus expressing constitutively active or kinase dead AMPK. After 48 h infection, THP-1 cells were harvested and tested in the adhesion assay. The expression and activity of AMPK were determined by immunobloting. *$p<0.05$ as compared to the PMA response without AICAR or AMPK adenovirus infection.

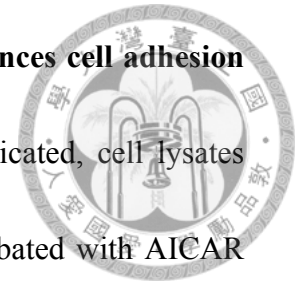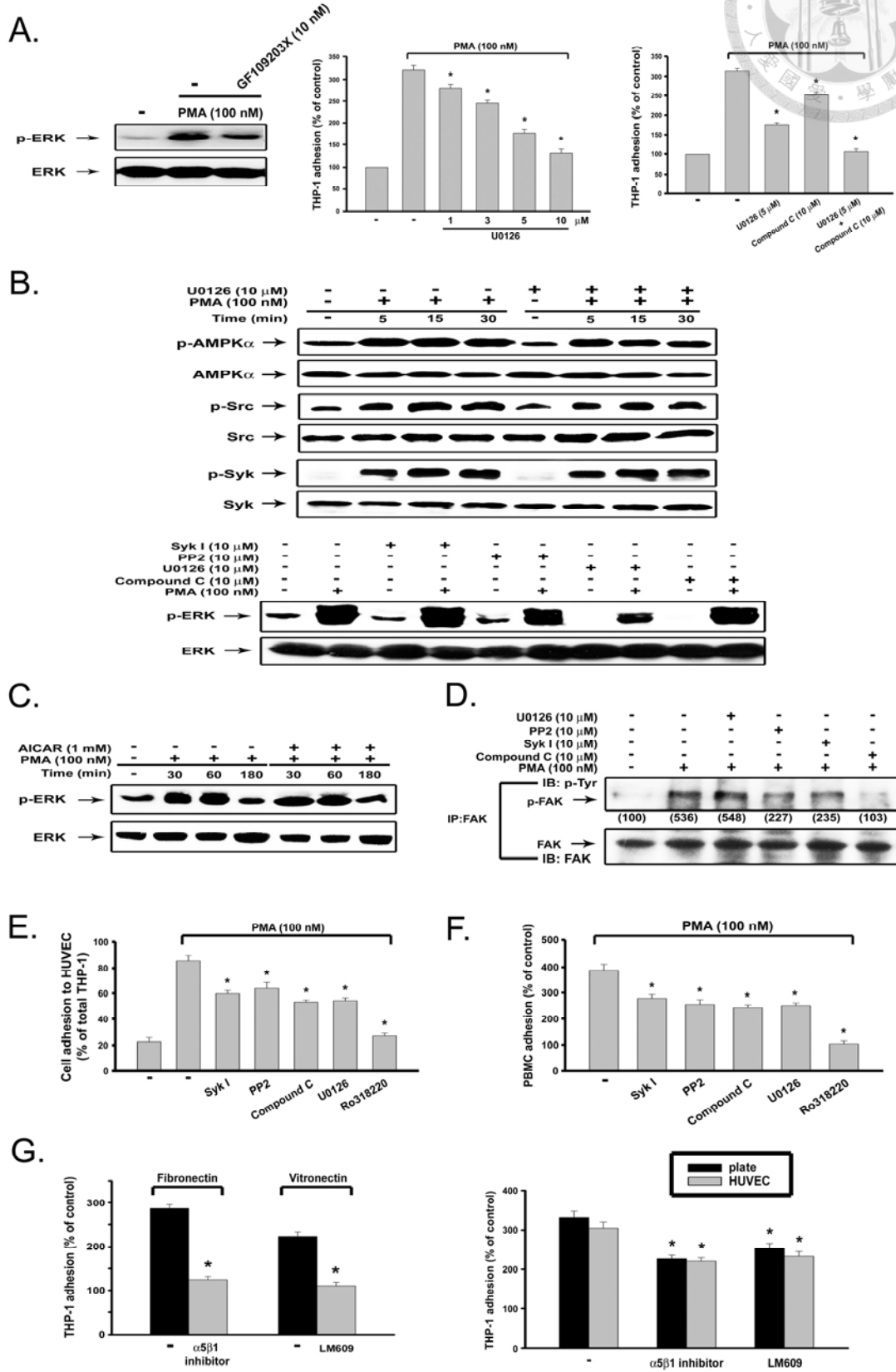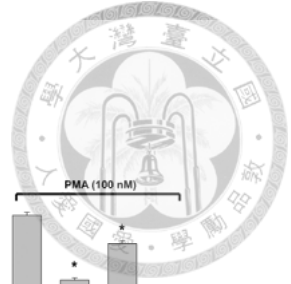# Figure 12

Figure 12. ERK activity independent of AMPK-Syk-Src signaling participates in PKC-mediated monocyte adhesion. (**A**) After pretreatment with U0126 for 30 min, THP-1 cells were stimulated with PMA for 4 h, and cell adhesion was determined. (**B-D**) THP-1 cells were treated with PMA in the presence of vehicle or pharmacological agents for 10 min (lower panel of **B**, **D**) or indicated time periods (upper panel of **B**, **C**). Immunobloting with specific antibodies was conducted. FAK activation was determined by immunoprecipitation with FAK-specific antibody and immunoblotting with phospho-tyrosine antibody (**D**). (**E**) THP-1 cells pre-labeled with BCECF were treated with SykI, PP2, compound C, U0126 (each at 10 μM), or Ro318220 (3 μM) for 30 min prior to the addition of PMA (100 nM). After 4 h incubation, cell adhesion was determined. (**F**) Human primary monocytes were similarly treated with inhibitors and PMA, and cell adhesion was determined. (**G**) PMA-induced cell adhesion to matrix-coated culture plates, in the absence or presence of α5β1 inhibitor and αVβ3 blocking antibody (LM609), was determined (left panel). In some experiments, after pretreatment with integrin inhibitors for 30 min, THP-1 cells were stimulated with PMA (100 nM) for 4 h, and cell adhesion to either the culture plate or to HUVEC was determined (right panel).

Figure 13
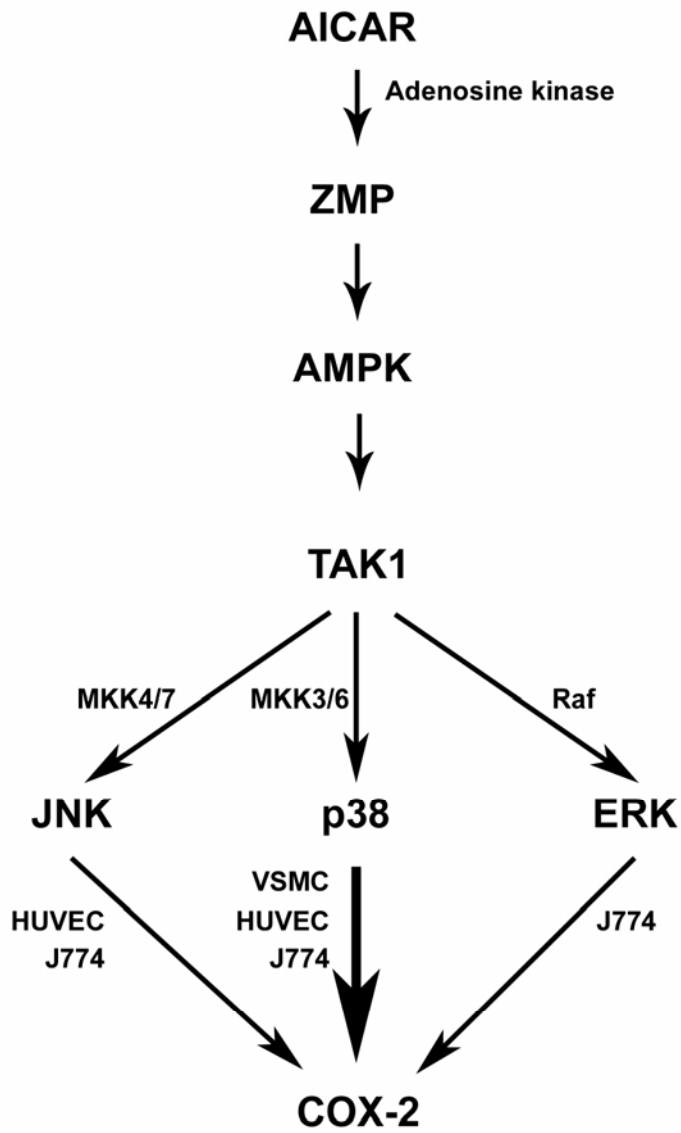
Figure 13. Conclusions

Our present study proposes a novel mechanism for AICAR of upregulating COX-2 gene expression. Via direct interaction with TAK1 and induction of TAK1-dependent p38 MAPK signaling cascade, AMPK activation leads to the expression of the inflammatory COX-2 gene in various cell types. These findings may shed new light on the roles of AMPK in inflammatory responses. Also, the present information suggests that AMPK may play a new important role in inflammatory diseases, in addition to its therapeutic potential to treat metabolic diseases, including diabetes, obesity, and insulin resistance.

Figure 14

PMA

↓

PKC

↓

LKB1

↓

AMPK

↓

Syk

↓

Src

↓

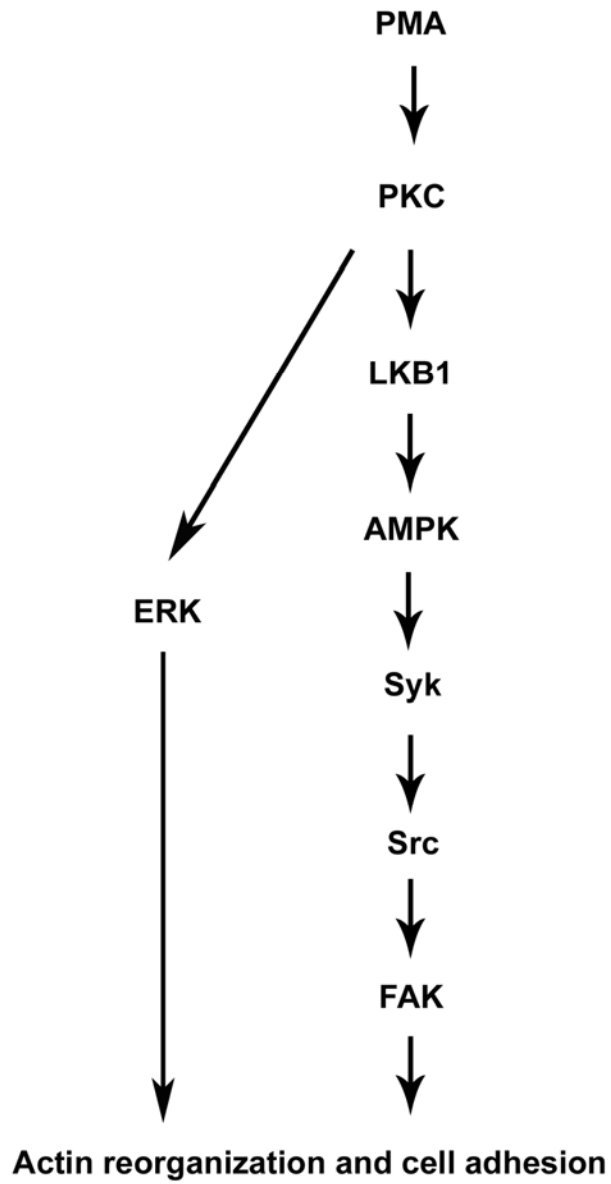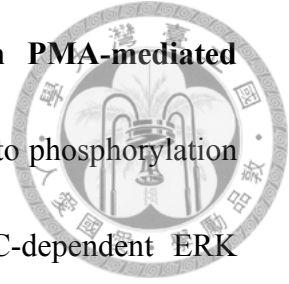FAK

ERK

Actin reorganization and cell adhesion

Figure 14. A bifurcated signaling pathway is involved in PMA-mediated adhesion of monocytes. PKC can activate LKB1/AMPK, leading to phosphorylation and activation of Syk, then Src and FAK subsequently. PKC-dependent ERK activation induces a coordinated signal for cytoskeleton rearrangement and cell adhesion.

Chapter 8
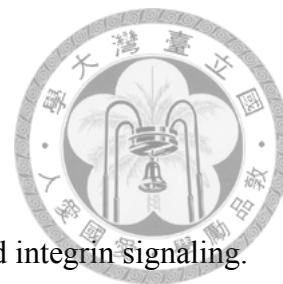
References

Abram CL, Lowell CA (2007) Convergence of immunoreceptor and integrin signaling.

Immunol Rev 218: 29-44.

Alba G, El Bekay R, Alvarez-Maqueda M, Chacon P, Vega A, Monteseirin J, Santa

Maria C, Pintado E, Bedoya FJ, Bartrons R, Sobrino F. (2004). Stimulators of

AMP-activated protein kinase inhibit the respiratory burst in human neutrophils.

FEBS Lett 573:219-25.

Athanassios Dovas, Dianne Cox (2011). Signaling networks regulating leukocyte

podosome dynamics and function. Cell Signal. 23(8): 1225–1234.

Bai A, Ma AG, Yong M, Weiss CR, Ma Y, et al. (2010). AMPK agonist downregulates

innate and adaptive immune responses in TNBS-induced murine acute and

relapsing colitis. Biochem Pharmacol 80: 1708-1717.

Berton G, Mocsai A, Lowell CA (2005). Src and Syk kinases: key regulators of

phagocytic cell activation. Trends Immunol 26: 208-214.

Bess E, Fisslthaler B, Fromel T, Fleming I (2011). Nitric oxide-induced activation of

the AMP-activated protein kinase alpha2 subunit attenuates IkappaB kinase

activity and inflammatory responses in endothelial cells. PLoS One 6: e20848.

Besson A, Davy A, Robbins SM, Yong VW (2001). Differential activation of ERKs to

focal adhesions by PKC epsilon is required for PMA-induced adhesion and migration of human glioma cells. Oncogene 20: 7398-7407.
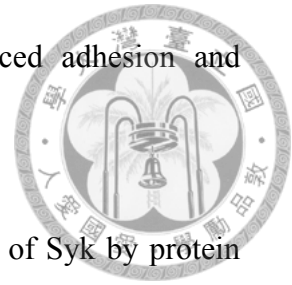
Bijli KM, Fazal F, Minhajuddin M, Rahman A (2008). Activation of Syk by protein kinase C-delta regulates thrombin-induced intercellular adhesion molecule-1 expression in endothelial cells via tyrosine phosphorylation of RelA/p65. J Biol Chem 283: 14674-14684.

Borowski P, Heiland M, Kornetzky L, Medem S, Laufs R (1998). Purification of catalytic domain of rat spleen p72syk kinase and its phosphorylation and activation by protein kinase C. Biochem J 331 (Pt 2): 649-657.

Bryant C, Fitzgerald KA. (2009). Molecular mechanisms involved in inflammasome activation. Trends Cell Biol. 19:455-464.

Capano M, Crompton M. (2006). Bax translocates to mitochondria of heart cells during simulated ischaemia: involvement of AMP-activated and p38 mitogen-activated protein kinases. Biochem J 395:57-64.

Carling D. (2004). The AMP-activated protein kinase cascade--a unifying system for energy control. Trends Biochem Sci 29:18-24.

Ceolotto G, Gallo A, Papparella I, Franco L, Murphy E, et al. (2007). Rosiglitazone reduces glucose-induced oxidative stress mediated by NAD(P)H oxidase via AMPK-dependent mechanism. Arterioscler Thromb Vasc Biol 27: 2627-2633.

Chandrasekar B, Boylston WH, Venkatachalam K, Webster NJ, Prabhu SD, et al. (2008). Adiponectin blocks interleukin-18-mediated endothelial cell death via APPL1-dependent AMP-activated protein kinase (AMPK) activation and IKK/NF-kappaB/PTEN suppression. J Biol Chem 283: 24889-24898.
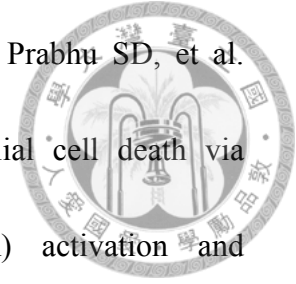
Chang MY, Ho FM, Wang JS, Kang HC, Chang Y, et al. (2010). AICAR induces cyclooxygenase-2 expression through AMP-activated protein kinase-transforming growth factor-beta-activated kinase 1-p38 mitogen-activated protein kinase signaling pathway. Biochem Pharmacol 80: 1210-1220.

Chen JC, Huang KC, Wingerd B, Wu WT, Lin WW. (2004). HMG-CoA reductase inhibitors induce COX-2 gene expression in murine macrophages: role of MAPK cascades and promoter elements for CREB and C/EBPbeta. Exp Cell Res 301:305-19.

Dheepika Weerasinghe, Kevin P. McHugh, Frederick P. Ross, Eric J. Brown, Roland H. Gisler, and Beat A. Imhof. (1998). A role for the αvβ3 integrin in the transmigration of monocytes. J Cell Biol. 27;142(2):595-607.

Du JH, Xu N, Song Y, Xu M, Lu ZZ, Han C, Zhang YY. (2005). AICAR stimulates IL-6 production via p38 MAPK in cardiac fibroblasts in adult mice: a possible role for AMPK. Biochem Biophys Res Commun 337:1139-44.

Englesbe MJ, Deou J, Bourns BD, Clowes AW, Daum G. (2004). Interleukin-1beta

inhibits PDGF-BB-induced migration by cooperating with PDGF-BB to induce cyclooxygenase-2 expression in baboon aortic smooth muscle cells. J Vasc Surg. 39:1091-6.

Ewart MA, Kohlhaas CF, Salt IP (2008). Inhibition of tumor necrosis factor alpha-stimulated monocyte adhesion to human aortic endothelial cells by AMP-activated protein kinase. Arterioscler Thromb Vasc Biol 28: 2255-2257.

Fisslthaler B, Fleming I (2009). Activation and signaling by the AMP-activated protein kinase in endothelial cells. Circ Res 105: 114-127.

Gaskin FS, Kamada K, Yusof M, Korthuis RJ. (2007). 5'-AMP-activated protein kinase activation prevents postischemic leukocyte-endothelial cell adhesive interactions. Am J Physiol Heart Circ Physiol. 292(1):H326-32.

Ge B, Gram H, Di Padova F, Huang B, New L, Ulevitch RJ, Luo Y, Han J. (2002). MAPKK-independent activation of p38alpha mediated by TAB1-dependent autophosphorylation of p38alpha. Science 295:1291-4.

Giri S, Nath N, Smith B, Viollet B, Singh AK, Singh I. (2004). 5-aminoimidazole -4-carboxamide-1-beta-4-ribofuranoside inhibits proinflammatory response in glial cells: a possible role of AMP-activated protein kinase. J Neurosci 24:479-87.

Hallows KR, Fitch AC, Richardson CA, Reynolds PR, Clancy JP, Dagher PC, Witters LA, Kolls JK, Pilewski JM. (2006). Up-regulation of AMP-activated kinase by

dysfunctional cystic fibrosis transmembrane conductance regulator in cystic fibrosis airway epithelial cells mitigates excessive inflammation. J Biol Chem 281:4231-41.
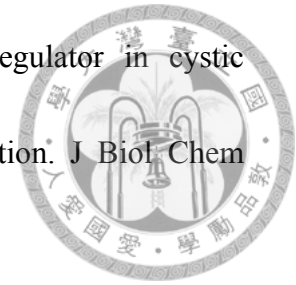
Han JH, Ahn YH, Choi KY, Hong SH. (2009). Involvement of AMP-activated protein kinase and p38 mitogen-activated protein kinase in 8-Cl-cAMP-induced growth inhibition. J Cell Physiol 218:104-12.

Hardie DG, Carling D, Carlson M. (1998). The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? Annu Rev Biochem 67:821-55.

Hardie DG, Carling D. (1997). The AMP-activated protein kinase--fuel gauge of the mammalian cell? Eur J Biochem 246:259-73.

Herrero-Martin G, Hoyer-Hansen M, Garcia-Garcia C, Fumarola C, Farkas T, Lopez-Rivas A, Jaattela M. (2009). TAK1 activates AMPK-dependent cytoprotective autophagy in TRAIL-treated epithelial cells. EMBO J 28:677-85.

Ho RC, Fujii N, Witters LA, Hirshman MF, Goodyear LJ. (2007). Dissociation of AMP-activated protein kinase and p38 mitogen-activated protein kinase signaling in skeletal muscle. Biochem Biophys Res Commun 362:354-9.

Hou CH, Tan TW, Tang CH. (2008). AMP-activated protein kinase is involved in COX-2 expression in response to ultrasound in cultured osteoblasts. Cell Signal 20:978-88.
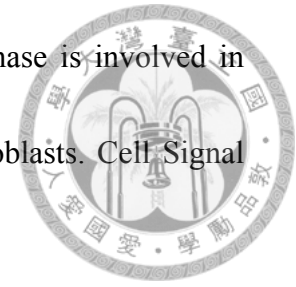
Hsu YC, Meng X, Ou L, Ip MM. (2010). Activation of the AMP-activated protein kinase-p38 MAP kinase pathway mediates apoptosis induced by conjugated linoleic acid in p53-mutant mouse mammary tumor cells. Cell Signal 22:590-9.

Hurley RL, Anderson KA, Franzone JM, Kemp BE, Means AR, et al. (2005). The Ca2+/calmodulin-dependent protein kinase kinases are AMP-activated protein kinase kinases. J Biol Chem 280: 29060–29066.

Hwang JT, Kim YM, Surh YJ, Baik HW, Lee SK, Ha J, Park OJ. (2006). Selenium regulates cyclooxygenase-2 and extracellular signal-regulated kinase signaling pathways by activating AMP-activated protein kinase in colon cancer cells. Cancer Res 66:10057-63.

Hai CM, Gu Z (2006). Caldesmon phosphorylation in actin cytoskeletal remodeling. Eur J Cell Biol 85: 305-309.

Hardie DG (2004). The AMP-activated protein kinase pathway--new players upstream and downstream. J Cell Sci 117: 5479-5487.

Huang NL, Chiang SH, Hsueh CH, Liang YJ, Chen YJ, et al. (2009). Metformin inhibits TNF-alpha-induced IkappaB kinase phosphorylation, IkappaB-alpha

degradation and IL-6 production in endothelial cells through PI3K-dependent AMPK phosphorylation. Int J Cardiol 134: 169-175.
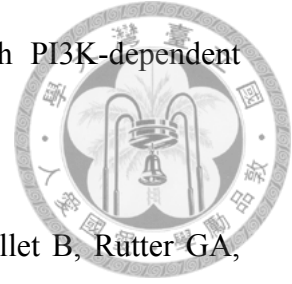
Jacquet S, Zarrinpashneh E, Chavey A, Ginion A, Leclerc I, Viollet B, Rutter GA, Bertrand L, Marber MS. (2007). The relationship between p38 mitogen-activated protein kinase and AMP-activated protein kinase during myocardial ischemia. Cardiovasc Res 76:465-72.

Janeway, C. A., Jr, P. Travers, M. Walport, M. Shlomchik. (2001). Innate Immunity. Immunobiology: The Immune System in Health & Disease 5th ed. Garland Publishing, New York.

Jaswal JS, Gandhi M, Finegan BA, Dyck JR, Clanachan AS. (2007). p38 mitogen-activated protein kinase mediates adenosine-induced alterations in myocardial glucose utilization via 5'-AMP-activated protein kinase. Am J Physiol Heart Circ Physiol 292:H1978-85.

Jeong HW, Hsu KC, Lee JW, Ham M, Huh JY, Shin HJ, Kim WS, Kim JB. (2009). Berberine suppresses proinflammatory responses through AMPK activation in macrophages. Am J Physiol Endocrinol Metab 296:E955-64.

Jhun BS, Jin Q, Oh YT, Kim SS, Kong Y, Cho YH, Ha J, Baik HH, Kang I. (2004). 5-Aminoimidazole-4-carboxamide riboside suppresses lipopolysaccharide -induced TNF-alpha production through inhibition of phosphatidylinositol

3-kinase/Akt activation in RAW 264.7 murine macrophages. Biochem Biophys Res Commun318:372-80.
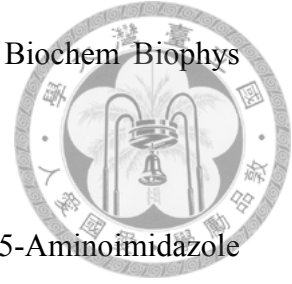
Jung JE, Lee J, Ha J, Kim SS, Cho YH, Baik HH, Kang I. (2004). 5-Aminoimidazole -4-carboxamide-ribonucleoside enhances oxidative stress-induced apoptosis through activation of nuclear factor-kappaB in mouse Neuro 2a neuroblastoma cells. Neurosci Lett 354:197-200.

Jhun BS, Jin Q, Oh YT, Kim SS, Kong Y, et al. (2004) 5-Aminoimidazole -4-carboxamide riboside suppresses lipopolysaccharide-induced TNF-alpha production through inhibition of phosphatidylinositol 3-kinase/Akt activation in RAW 264.7 murine macrophages. Biochem Biophys Res Commun 318: 372-380.

Kawakami Y, Kitaura J, Yao L, McHenry RW, Newton AC, et al. (2003). A Ras activation pathway dependent on Syk phosphorylation of protein kinase C. Proc Natl Acad Sci U S A 100: 9470-9475.

Kelley LC, Hayes KE, Ammer AG, Martin KH, Weed SA (2010). Cortactin phosphorylated by ERK1/2 localizes to sites of dynamic actin regulation and is required for carcinoma lamellipodia persistence. PLoS One 5: e13847.

Kerrigan AM, Brown GD (2011). Syk-coupled C-type lectins in immunity. Trends Immunol 32: 151-156.

Kuo CL, Ho FM, Chang MY, Prakash E, Lin WW (2008). Inhibition of

lipopolysaccharide-induced inducible nitric oxide synthase and cyclooxygenase-2 gene expression by 5-aminoimidazole-4-carboxamide riboside is independent of AMP-activated protein kinase. J Cell Biochem 103: 931-940.
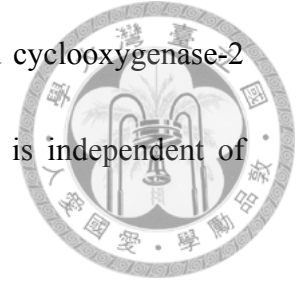
Kurihara Y, Nakahara T, Furue M (2011). alphaVbeta3-integrin expression through ERK activation mediates cell attachment and is necessary for production of tumor necrosis factor alpha in monocytic THP-1 cells stimulated by phorbol myristate acetate. Cell Immunol. 270: 25-31.

Lee YM, Lee JO, Jung JH, Kim JH, Park SH, Park JM, Kim EK, Suh PG, Kim HS. (2008). Retinoic acid leads to cytoskeletal rearrangement through AMPK-Rac1 and stimulates glucose uptake through AMPK-p38 MAPK in skeletal muscle cells. J Biol Chem 283:33969-74.

Li J, Miller EJ, Ninomiya-Tsuji J, Russell RR, Young LH. (2005). AMP-activated protein kinase activates p38 mitogen-activated protein kinase by increasing recruitment of p38 MAPK to TAB1 in the ischemic heart. Circ Res 97:872-9.

Lopez JM, Santidrian AF, Campas C, Gil J. (2003). 5-Aminoimidazole -4-carboxamide riboside induces apoptosis in Jurkat cells, but the AMP-activated protein kinase is not involved. Biochem J 370:1027-32.

Mark A. Williams and Joseph S. Solomkin. (1999). Integrin-mediated signaling in human neutrophil functioning. J. Leukoc. Biol. 65: 725–736.

Md. Monowar Aziz, Shunji Ishihara, Yoshiyuki Mishima, Naoki Oshima, Ichiro Moriyama, Takafumi Yuki, Yasunori Kadowaki, Mohammad Azharul Karim Rumi, Yuji Amano and Yoshikazu Kinoshita. (2009). MFG-E8 attenuates intestinal inflammation in murine experimental colitis by modulating osteopontin-dependent $\alpha_v \beta_3$ integrin signaling. J. Immuno, 182: 7222–7232.

Mocsai A, Ruland J, Tybulewicz VL (2010). The SYK tyrosine kinase: a crucial player in diverse biological functions. Nat Rev Immunol 10: 387-402.

Murdoch C, Tazzyman S, Webster S, Lewis CE (2007). Expression of Tie-2 by human monocytes and their responses to angiopoietin-2. J Immunol 178: 7405-7411.

Myerburg MM, King JD, Jr., Oyster NM, Fitch AC, Magill A, et al. (2010). AMPK agonists ameliorate sodium and fluid transport and inflammation in cystic fibrosis airway epithelial cells. Am J Respir Cell Mol Biol 42: 676-684.

Miletic AV, Graham DB, Montgrain V, Fujikawa K, Kloeppel T, Brim K, Weaver B, Schreiber R, Xavier R, Swat W. (2007). Vav proteins control MyD88-dependent oxidative burst. Blood. 109:3360-8.

Mitchell JA, Warner TD. (2006). COX isoforms in the cardiovascular system: understanding the activities of non-steroidal anti-inflammatory drugs. Nat Rev Drug Discov. Jan;5(1):75-86.

Momcilovic M, Hong SP, Carlson M. (2006). Mammalian TAK1 activates Snf1 protein kinase in yeast and phosphorylates AMP-activated protein kinase in vitro. J Biol Chem 281:25336-43.

Monick MM, Robeff PK, Butler NS, Flaherty DM, Carter AB, Peterson MW, Hunninghake GW. (2002). Phosphatidylinositol 3-kinase activity negatively regulates stability of cyclooxygenase 2 mRNA. J Biol Chem. 277:32992-3000.

Myerburg MM, King Jr JD, Oyster NM, Fitch AC, Magill A, Baty CJ, Watkins SC, Kolls JK, Pilewski JM, Hallows KR. (2010). AMPK agonists ameliorate sodium and fluid transport and inflammation in CF airway epithelial cells. Am J Respir Cell Mol Biol. Am J Respir Cell Mol Biol. 42(6):676-84.

Paul F. Bradfield, Christoph Scheiermann, Sussan Nourshargh, Christiane Ody, Francis W. Luscinskas, G. Ed Rainger, Gerard B. Nash, Marijana Miljkovic-Licina, Michel Aurrand-Lions, and Beat A. Imhof (2007). JAM-C regulates unidirectional monocyte transendothelial migration in inflammation. Blood 110:7 2545–2555.

Park IJ, Hwang JT, Kim YM, Ha J, Park OJ. (2006). Differential modulation of AMPK signaling pathways by low or high levels of exogenous reactive oxygen species in colon cancer cells. Ann N Y Acad Sci 1091:102-9.

Park YS, Kim J, Misonou Y, Takamiya R, Takahashi M, Freeman MR, Taniguchi N. (2007). Acrolein induces cyclooxygenase-2 and prostaglandin production in

human umbilical vein endothelial cells: roles of p38 MAP kinase. Arterioscler Thromb Vasc Biol. 27:1319-25.

Peairs A, Radjavi A, Davis S, Li L, Ahmed A, Giri S, Reilly CM. (2009). Activation of AMPK inhibits inflammation in MRL/lpr mouse mesangial cells. Clin Exp Immunol 156:542-51.

Pilon G, Dallaire P, Marette A. (2004). Inhibition of inducible nitric-oxide synthase by activators of AMP-activated protein kinase: A new mechanism of action of insulin-sensitizing drugs. J Biol Chem 279:20767-74.

Prasad R, Giri S, Nath N, Singh I, Singh AK (2006). 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside attenuates experimental autoimmune encephalomyelitis via modulation of endothelial-monocyte interaction. J Neurosci Res 84: 614-625.

Pula G, Crosby D, Baker J, Poole AW (2005). Functional interaction of protein kinase Calpha with the tyrosine kinases Syk and Src in human platelets. J Biol Chem 280: 7194-7205.

Pullikuth AK, Catling AD (2010). Extracellular signal-regulated kinase promotes Rho-dependent focal adhesion formation by suppressing p190A RhoGAP. Mol Cell Biol 30: 3233-3248.

Sakurai H, Miyoshi H, Mizukami J, Sugita T. (2000). Phosphorylation-dependent activation of TAK1 mitogen-activated protein kinase kinase kinase by TAB1. FEBS Lett 474:141-5.

Salminen A, Hyttinen JM, Kaarniranta K (2011). AMP-activated protein kinase inhibits NF-κB signaling and inflammation: impact on healthspan and lifespan. J. Mol. Med. 89:667-76.

Schwende H, Fitzke E, Ambs P, Dieter P (1996). Differences in the state of differentiation of THP-1 cells induced by phorbol ester and 1,25-dihydroxyvitamin D3. J Leukoc Biol 59: 555-561.

Shibuya H, Yamaguchi K, Shirakabe K, Tonegawa A, Gotoh Y, Ueno N, Irie K, Nishida E, Matsumoto K. (1996). TAB1: an activator of the TAK1 MAPKKK in TGF-beta signal transduction. Science 272:1179-82.

Steinberg GR, Kemp BE (2009). AMPK in Health and Disease. Physiol Rev 89: 1025-1078.

Tang CH, Chiu YC, Tan TW, Yang RS, Fu WM (2007). Adiponectin enhances IL-6 production in human synovial fibroblast via an AdipoR1 receptor, AMPK, p38, and NF-kappa B pathway. J Immunol 179: 5483-5492.

Taniguchi T, Kobayashi T, Kondo J, Takahashi K, Nakamura H, et al. (1991). Molecular cloning of a porcine gene syk that encodes a 72-kDa protein-tyrosine kinase showing high susceptibility to proteolysis. J Biol Chem 266: 15790-15796.

Tatsuo Kinashi (2005). Intracellular signalling controlling integrin activation in lymphocytes. *Nature Rev Immuno.* **5:** 546-559.

Towler MC, Hardie DG (2007) AMP-activated protein kinase in metabolic control and insulin signaling. Circ Res 100: 328-341.

Tsai JS, Chen CY, Chen YL, Chuang LM (2010). Rosiglitazone inhibits monocyte/macrophage adhesion through de novo adiponectin production in human monocytes. J Cell Biochem 110: 1410-1419.

Wang YH, Yan ZQ, Shen BR, Zhang L, Zhang P, et al. (2009). Vascular smooth muscle cells promote endothelial cell adhesion via microtubule dynamics and activation of paxillin and the extracellular signal-regulated kinase (ERK) pathway in a co-culture system. Eur J Cell Biol 88: 701-709.

Weekes J, Ball KL, Caudwell FB, Hardie DG (1993). Specificity determinants for the AMP-activated protein kinase and its plant homologue analysed using synthetic peptides. FEBS Lett 334: 335-339.

Wei CY, Chou YH, Ho FM, Hsieh SL, Lin WW. (2006). Signaling pathways of LIGHT induced macrophage migration and vascular smooth muscle cell proliferation. J Cell Physiol 209:735-43.

Xie Z, Dong Y, Scholz R, Neumann D, Zou MH (2008) Phosphorylation of LKB1 at serine 428 by protein kinase C-zeta is required for metformin-enhanced activation

of the AMP-activated protein kinase in endothelial cells. Circulation 117: 952-962.

Xi X, Han J, Zhang JZ. (2001). Stimulation of glucose transport by AMP-activated protein kinase via activation of p38 mitogen-activated protein kinase. J Biol Chem 276:41029-34.

Yang CR, Hsieh SL, Ho FM, Lin WW. (2005). Decoy receptor 3 increases monocyte adhesion to endothelial cells via NF-kappa B-dependent up-regulation of intercellular adhesion molecule-1, VCAM-1, and IL-8 expression. J Immunol 174:1647-56.

Yiqun Wang, Yu Huang, Karen S.L. Lam, Yiming Li, Wing Tak Wong, Hongying Ye,Chi-Wai Lau, Paul M. Vanhoutte, and Aimin Xu. (2009). Berberine prevents hyperglycemia-induced endothelial injury and enhances vasodilatation via adenosine monophosphate-activated protein kinase and endothelial nitric oxide synthase Cardio Res 82, 484–492.

Yoon MJ, Lee GY, Chung JJ, Ahn YH, Hong SH, Kim JB. (2006). Adiponectin increases fatty acid oxidation in skeletal muscle cells by sequential activation of AMP-activated protein kinase, p38 mitogen-activated protein kinase, and peroxisome proliferator-activated receptor alpha. Diabetes 55:2562-70.

Zarbock A, Ley K (2011). Protein tyrosine kinases in neutrophil activation and recruitment. Arch Biochem Biophys 510: 112-119.
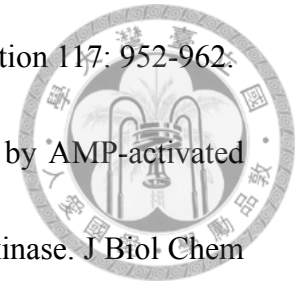
Zhang BB, Zhou G, Li C. (2009). AMPK: an emerging drug target for diabetes and the metabolic syndrome. Cell Metab 9:407-16.
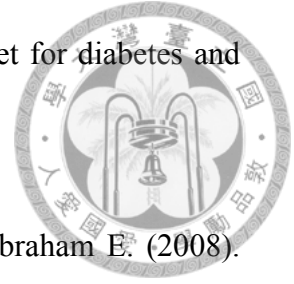
Zhao X, Zmijewski JW, Lorne E, Liu G, Park YJ, Tsuruta Y, Abraham E. (2008). Activation of AMPK attenuates neutrophil proinflammatory activity and decreases the severity of acute lung injury. Am J Physiol Lung Cell Mol Physiol 295:L497-504.

Zou MH, Hou XY, Shi CM, Nagata D, Walsh K, Cohen RA. (2002). Modulation by peroxynitrite of Akt- and AMP-activated kinase-dependent Ser1179 phosphorylation of endothelial nitric oxide synthase. J Biol Chem 277:32552-7.

Chapter 9

Publication
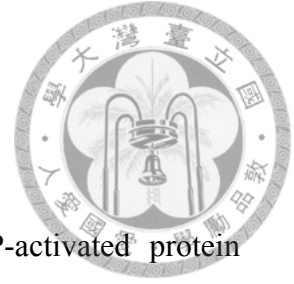
1. AICAR induces cyclooxygenase-2 expression through AMP-activated protein kinase-transforming growth factor-activated kinase 1-p38 mitogen activated protein kinase signaling pathway. *Biochem Pharm* **80** (2010) 1210-1220.

2. PKC-dependent human monocyte adhesion requires AMPK and Syk activation. *PLoS one* **7** (2012) e40999