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

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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.

1. Introduction

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 [1]. Physiological and stress conditions known to activate AMPK include exercise, nutritional starvation, heat shock, oxidative stress, and ischemia/hypoxia [2,3]. Through increasing glucose transport, fatty acid oxidation, and glycolysis, AMPK activation inhibits ATP-consuming pathways and stimulates alternative pathways for ATP regeneration [2,4]. AMPK recently emerged as an attractive and novel target for treating obesity, type 2 diabetes, and cardiac hypertrophy [2–4].

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 [5]. Studies demonstrated that direct binding of AMP 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 [1].

AMPK was also implicated as an anti-inflammatory and anti-cancer target. In endothelial cells, AMPK activity is associated with

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phosphorylation and activation of endothelial nitric oxide synthase [6]. 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 [7–11]. The constitutive COX-2 expression in colon cancer cells was repressed by AMPK activation [12,13]. Moreover, the benefits of AMPK activation in several inflammatory disease models were also documented [14–16].

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 [17]. 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 [18]. 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 [19], cardiac fibroblasts [20], skeletal muscle [21], synovial fibroblasts [22,23], osteoblasts [23], and neuroblastoma cells [24]. The downstream events mediated by such a signaling cascade include increased glucose transport [21], fatty acid oxidation [25], Bax translocation to mitochondria [26], IL-6 production [20,22], COX-2 expression [23], inhibition of cancer growth [27], and apoptosis of tumors [28]. In contrast to the positive regulating link as mentioned, a dissociated relationship of both activated kinases during myocardial ischemia [29], skeletal muscle contraction [30], and even a relationship of inhibitory regulation was also demonstrated. For the latter, AMPK was shown to reduce PMA-induced p38 MAPK activation in neutrophils [31].

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. Materials and methods

2.1. Regents

Lipofectamine™ and Plus™ 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). 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).

2.2. 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 [32,33]. 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.

2.3. 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.

2.4. PG measurement

PGI_2 and PGE_2 productions were measured by commercial kits according to the manufacturer's instructions.

2.5. 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).

2.6. Reverse-transcription (RT)-PCR

RT-PCR was conducted as we previously described [34]. 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′).

2.7. 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 [34]. Adenoviruses were propagated in 293F cells, purified by BD-Adeno-X™ Virus Purification kits, and stored at −70 °C. The titration of adenovirus was quantified by Adeno-X™ 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 immunoblotting.

2.8. Statistical evaluation

Values are expressed as the mean ± S.E.M. of at least three experiments. Analysis of variance (ANOVA) was used to assess the statistical significance of the differences, and a p value of <0.05 was considered statistically significant.

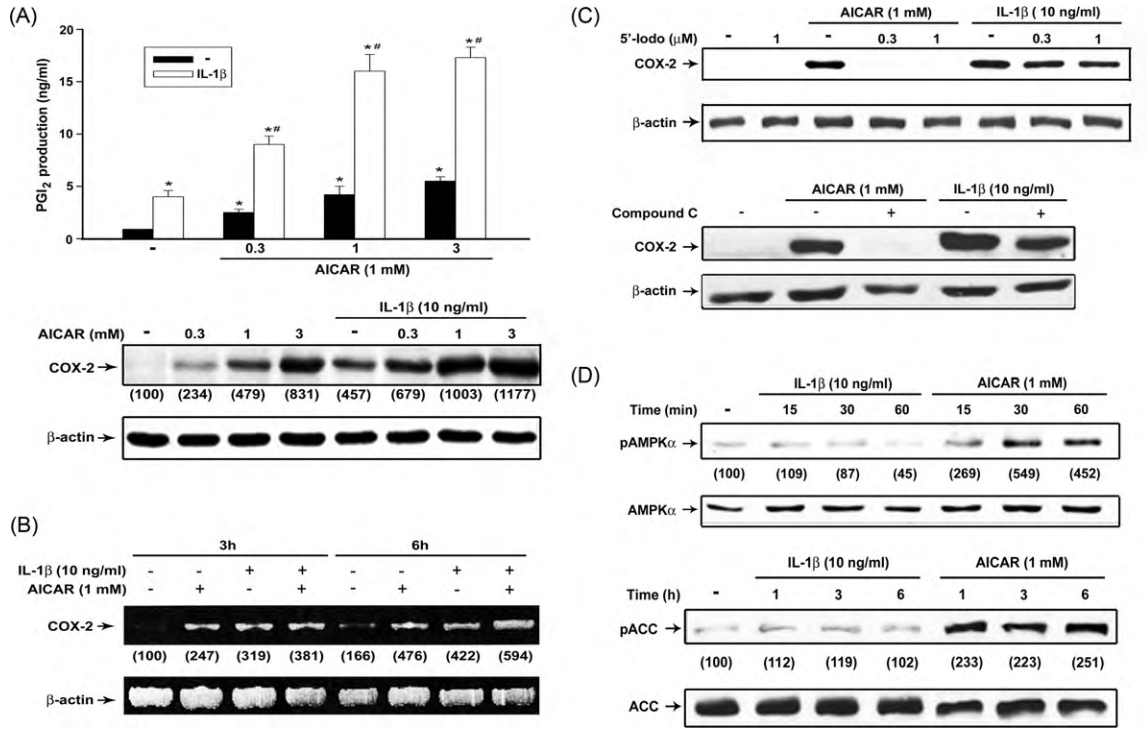

Fig. 1. AICAR induces COX-2 expression in VSMCs. (A) After VSMCs were treated with AICAR (0.3–3 mM) and/or IL-1β (10 ng/ml) for 24 h, culture medium was collected for PGI_2 measurement, and cell lysates were harvested for immunoblotting with a COX-2 antibody. (B) Cells were treated with AICAR (1 mM) 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.

3. Results

3.1. AICAR-induced COX-2 gene expression and PGI_2 production in VSMCs require AMPK activation

Depending on biological setting, controversial effects of AICAR and/or AMPK-dependent signaling on COX-2 expression have been reported [9,10,11–13,23]. To further explore if this outcome possibly relates to the basal or inflammatory state of specific cells tested, we determined the effects of AICAR alone and in combination with proinflammatory cytokine IL-1β on COX-2 expression 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 [35], 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 [1]. 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. Since ACC is a target substrate of AMPK [1,2], its phosphorylation indicates the activation of AMPK by AICAR.

3.2. 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 and 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

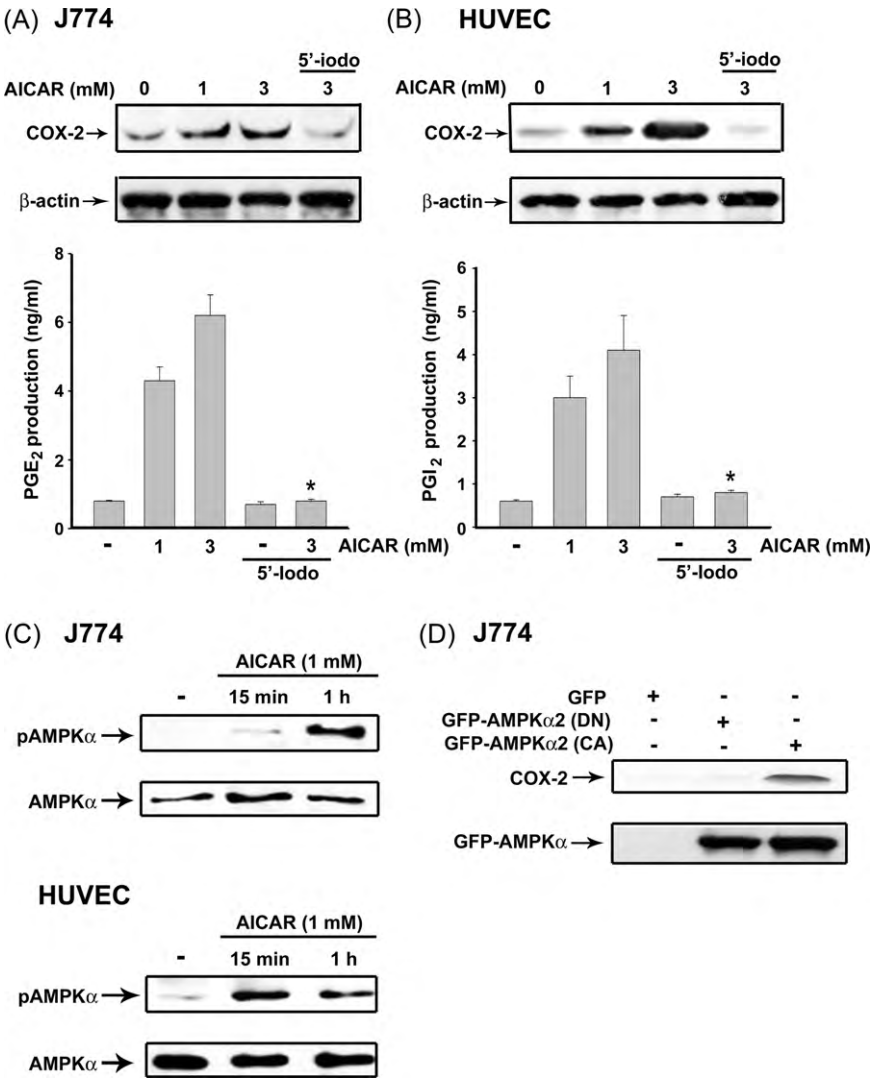

Fig. 2. AICAR induces COX-2 expression in J774 and HUVECs through the AMPK pathway. J774 macrophages (A) and HUVECs (B) were pretreated with 5′-iodotubercidin (1 μM) for 30 min, followed by treatment with AICAR (1 or 3 mM). After 24 h, culture media were used for PGE_2 or PGI_2 measurements, and cell 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.

AMPK expression led to an increased COX-2 expression in J774 macrophages (Fig. 2D).

3.3. 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

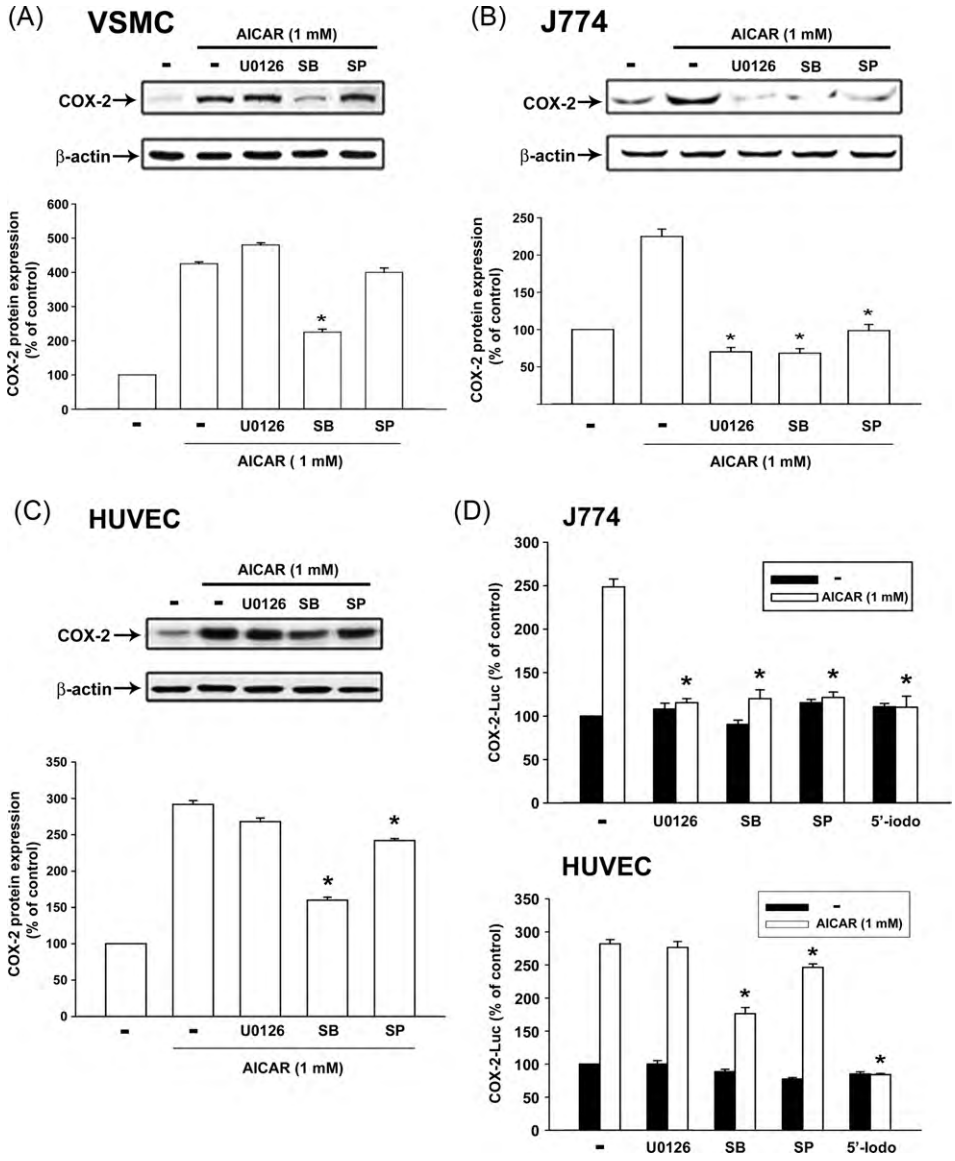

Fig. 3. Differential involvement of MAPKs in AICAR-induced COX-2 expression is cell type-specific. VSMCs (A), J774 macrophages (B), and HUVECs (C) were pretreated with U0126 (1 μM), SB203580 (SB, 3 μM), or SP600125 (SP, 3 μM) for 30 min, followed by treatment with AICAR (1 mM) for 24 h. The protein level 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.

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.

3.4. AMPK is associated with TAK1 for 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 [36]. These results suggest that TAK1 is the downstream molecule linking AMPK to p38 MAPK 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 significantly 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

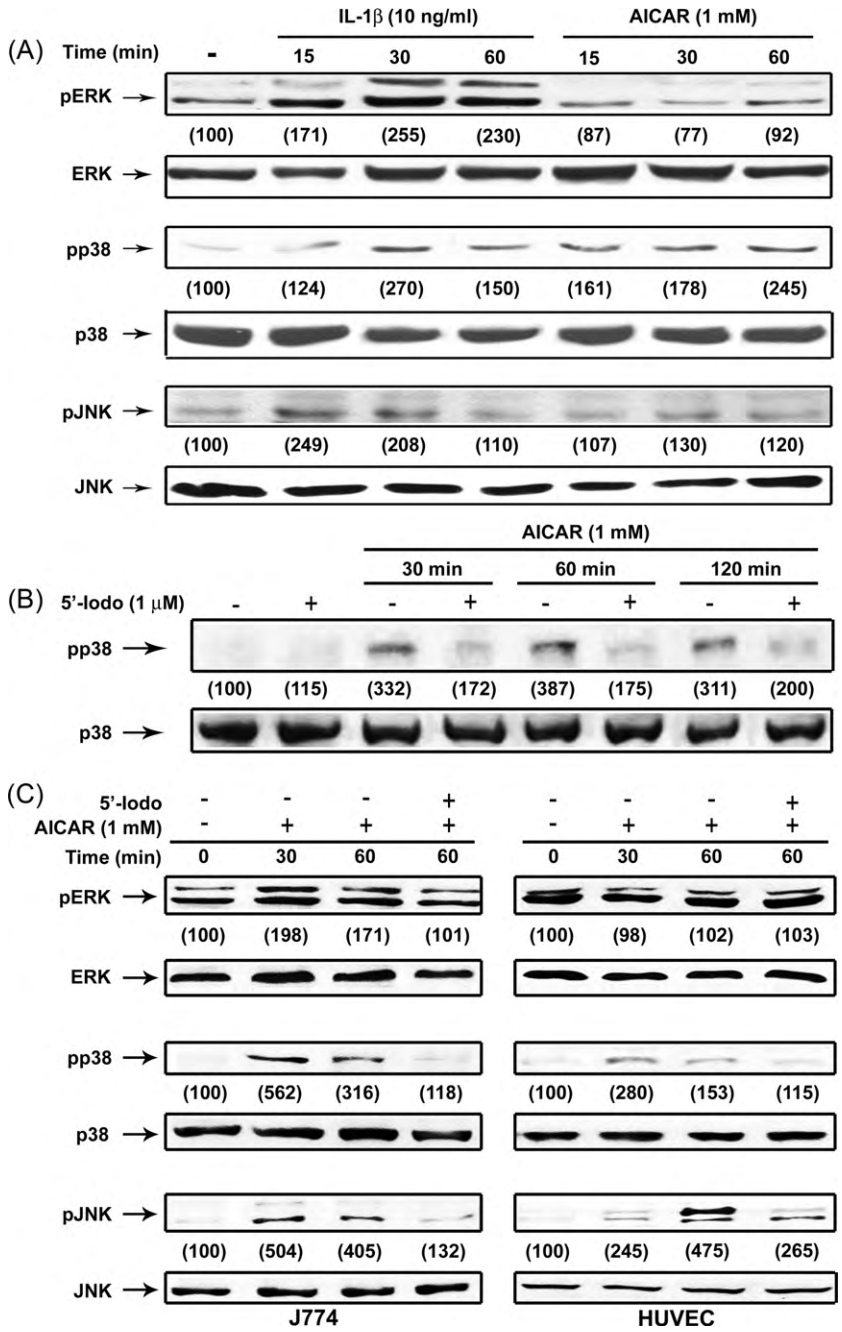

Fig. 4. AMPK mediates different MAPK activations in VSMCs, J774 macrophages, and HUVECs. (A) VSMCs were treated with AICAR or IL-1β for the indicated time periods. After stimulation, cell lysates were subjected to an immunoblot analysis of ERK, p38, and JNK. (B) VSMCs were pretreated with 5′-iodotubercidin 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.

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.

3.5. Kinase domains 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).

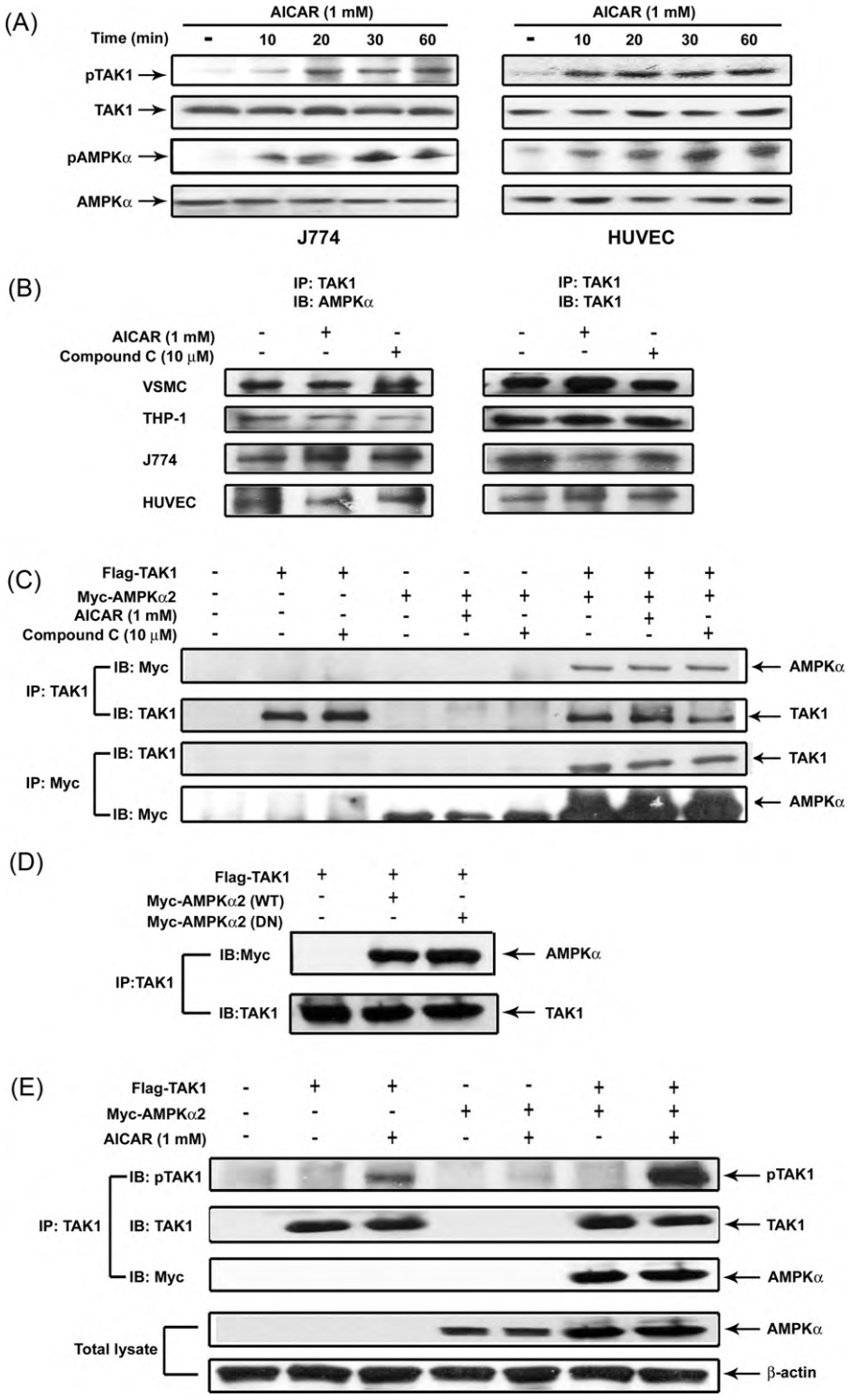

Fig. 5. AMPK is associated with TAK1 and induces TAK1 activation. (A) J774 macrophages and HUVECs were treated with AICAR for different time intervals, and protein levels of phosphorylated TAK1 and AMPKα were determined. (B) Different cell types were treated with AICAR or compound C for 30 min. Supernatants 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 immunoblotting 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.

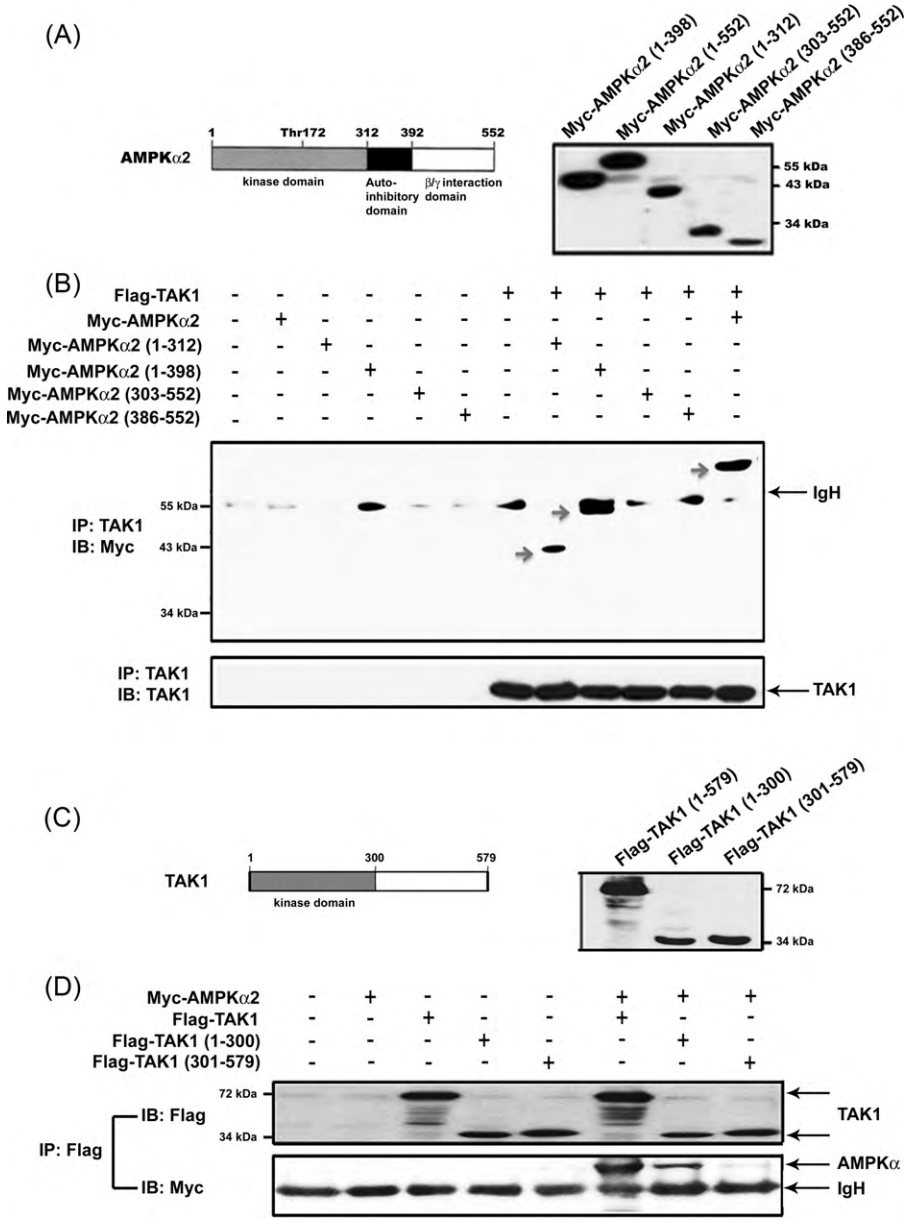

Fig. 6. Interactive binding regions of AMPKα2 and TAK1. (A) Different deletion mutant constructs of AMPKα were transfected into 293T cells. Immunoblots of input lysates with an anti-Myc antibody for the Myc-tagged AMPKα2 Myc-AMPKα2, Myc-AMPKα2 (1–312), Myc-AMPKα2 (1–398), Myc-AMPKα (303–552), and Myc-AMPKα2 (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.

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.

4. 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 [7,10,13–15], opposite findings of AMPK-dependent inflammatory responses were also documented [20,22,23]. Based on controversial findings of AICAR-regulated COX-2 induction [9–13,23], 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 controlling COX-2 gene expression in a cell type-specific manner are involved in the modulation of AMPK-dependent signaling pathways. Regarding to the dependency of each MAPK on COX-2 gene transcription, it was also demonstrated previously to be cell type-specific. In this context, p38 was demonstrated to play a central role in inflammatory gene regulation, including COX-2 in the three cell types tested in this study [34,37–40]. On the other hands, roles of JNK and ERK in COX-2 gene transcription are more dependent on cell types [34,39,40].

AMPK and p38 MAPK are stress-responsive protein kinases and are simultaneously involved in ischemia-reperfusion heart injury and glucose utilization [21,41]. Both kinases seem to form a reciprocal and complex functional signaling cascade. The p38 MAPK cascade downstream of AMPK was implicated in glucose transport [21], cytoskeletal rearrangement [42], COX-2 expression [23], and IL-6 secretion [22]. In contrast, AMPK is likely downstream of p38 MAPK when mediating the effects of adenosine in glucose utilization in hearts stressed by transient ischemia [41].

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 [19]. 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 [43]. Another study showed that TAK1 can activate AMPK-dependent cytoprotective autophagy in TRAIL-treated epithelial cells [44]. 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 [43].

To date, a few reports suggest that AMPK may be a novel regulatory molecule in COX-2 expression. In mouse mesangial [11] and HT-29 colon cancer cells [12,13], AICAR and some AMPK activators (e.g. genistein, EGCG, and selenium) were shown to induce COX-2 downregulation via an AMPK-dependent mechanism. Mechanistic studies suggest inhibition of NF-κB and c/EBPβ contribute to the anti-inflammatory event mediated by AMPK signaling [7,9]. In addition, AMPK-mediated iNOS inhibition primarily resulting from the post-transcriptional regulation was also proposed [45]. Even though AMPK-dependent inhibition of COX-2 or other inflammatory gene expression has been proposed [10–13], AICAR might exert COX-2 regulation via an AMPK-independent mechanism [9]. In our previous study we demonstrated the ability of AICAR to directly interrupt DNA binding of several transcriptional factors upon LPS stimulation. Therefore, current and previous findings together suggest that AMPK signaling might have a paradoxical intervention in regulating cell functions via COX-2. We speculate inflammatory status, for example upon LPS or proinflammatory cytokine stimulation and under high activity of specific signal cascades for inducing inflammatory response, would favor AMPK to play a role in solving inflammation and tissue protection. In contrast, in a basal cellular situation, AMPK-dependent upregulation of COX-2 might exert a homeostatic function through eicosanoid production. As we know, appropriate basal levels of various eicosanoids are required to maintain normal physiological function in many aspects, including in controlling blood pressure, platelet aggregation, and anti-adhesion of leukocytes [46]. Therefore the context-dependent roles of AMPK further imply the crucial and multiple signaling mechanisms played by this protein kinase.

5. Conclusions

Our present study proposes a novel mechanism for AICAR in upregulating COX-2 gene expression, and a summary scheme in view of signal cascade from AMPK to COX-2 induction was shown in Fig. 7. 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

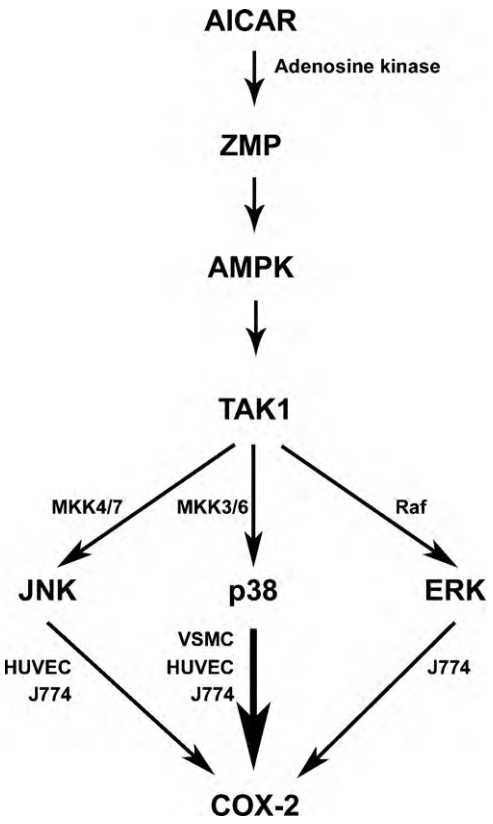

Fig. 7. A scheme for AMPK-dependent COX-2 induction. The AMPK activator AICAR induces COX-2 induction primarily via a common signal cascade of AMPK-TAK1-p38 MAPK in various cell types.

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.

Conflict of interest

The authors declare that they do not have any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning this submitted work that could inappropriately influence, or be perceived to influence, our work.

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