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Metformin inhibits inflammatory response via AMPK–PTEN pathway in vascular smooth muscle cells

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ABSTRACT

Atherosclerosis is a chronic inflammation of the coronary arteries. Vascular smooth muscle cells (VSMCs) stimulated by cytokines and chemokines accelerate the inflammatory response and migrate to the injured endothelium during the progression of atherosclerosis.

Activation of AMP activated protein kinase (AMPK), a key sensor maintaining metabolic homeostasis, suppresses the inflammatory response. However, how AMPK regulates the inflammatory response is poorly understood. To identify the mechanism of this response, we focused on phosphatase and tensin homolog (PTEN), which is a negative regulator of inflammation.

We investigated that activation of AMPK-induced PTEN expression and suppression of the inflammatory response through the AMPK–PTEN pathway in VSMCs. We treated with the well-known AMPK activator metformin to induce PTEN expression. PTEN was induced by metformin (2 mM) and inhibited by compound C (10 μM) and AMPK siRNA. Tumor necrosis factor-alpha (TNF-α) was used to induce inflammation. The inflammatory response was confirmed by cyclooxygenase (COX)-2, inducible nitric oxide synthase (iNOS) expression, and activation of nuclear factor (NF)-κB. Metformin suppressed COX-2 and iNOS mRNA and protein expression dose dependently. Treatment with compound C and bpv (pic) in the presence of metformin, iNOS and COX-2 protein expression increased. NF-κB activation decreased in response to metformin and was restored by inhibiting AMPK and PTEN. Inhibiting AMPK and PTEN restored ROS levels stimulated with TNF-α.

Taken together, PTEN could be a possible downstream regulator of AMPK, and the AMPK–PTEN pathway might be important in the regulation of the inflammatory response in VSMCs.

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1. Introduction

Vascular smooth muscle cells (VSMCs) play an important role initiating atherosclerosis. Atherosclerosis includes chronic inflammation of the carotid arteries that occurs by consistent stimulation of proinflammatory molecules such as cytokines, chemokines, and oxidized lipids. During the progression of atherogenesis, VSMCs undergo a modification of phenotype and proliferate and migrate from the media to the intima [1,2].

AMP-activated protein kinase (AMPK) is a key sensor for maintaining energy homeostasis and monitoring cellular energy charge. AMPK activates in cases of metabolic stress or exercise that reduce cellular energy levels, namely increases in the AMP/ATP ratio due to depletion of ATP. Thus, AMPK promotes the ATP-generating pathway and inhibits the ATP-consuming pathway [3]. AMPK is a highly conserved heterotrimeric serine/threonine kinase that consists of a catalytic subunit (α) and two regulatory subunits (β,γ). Phosphorylation of the specific threonine residue (Thr172) on the α subunit is essential for AMPK activity [4].

AMPK has been implicated in the anti-inflammatory effects of various cells. AMPK is crucial for repression of lipopolysaccharide-induced expression of proinflammatory molecules and mediators, such as tumor necrosis factor-alpha (TNF-α, interleukin (IL)-1β, IL-6, inducible nitric oxide synthase (iNOS), and cyclooxygenase (COX)-2 in macrophages, microglia, astrocytes, and mesangial cells [5–9]. AMPK is activated in several inflammatory disease models [10,11].

Phosphatase and tensin homolog (PTEN), a lipid and protein phosphatase, is a tumor suppressor gene that acts as a negative regulator of phosphatidylinositol 3-kinase (PI3K). Phosphatidylinositol 3,4,5 trisphosphate is converted to phosphatidylinositol 4,5 bisphosphate after dephosphorylation by PTEN. PTEN regulates cell proliferation, survival, and growth [12,13]. Reduced PTEN activity promotes a pro-inflammatory response, whereas PTEN overexpression decreases neointimal formation and inflammatory cytokine

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levels [2,14]. Based on these data, AMPK and PTEN show striking similarities in regulating inflammatory responses in addition to modulating reactive oxygen species (ROS) generation and are likely to interact to perform these functions. Indeed, AMPK plays a role in the regulation of antioxidant effects by PTEN in prostate cancer cells [15].

We hypothesized that AMPK and PTEN serve as key molecules of TNF-α-induced inflammatory signaling and that they control inflammatory responses in VMSCs. To test this, we examined AMPK-induced PTEN expression mediated by metformin. We also determined whether TNF-α-induced inflammatory molecules such as nuclear factor (NF)-κB, iNOS, and COX-2 respond to gain or loss of AMPK and PTEN function.

2. Materials and methods

2.1. Materials

Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were obtained from Hyclone (Logan, UT, USA). The enhanced chemiluminescence (ECL) plus Western blot Detection Reagent was obtained from GE Healthcare (Piscataway, NJ, USA). TNF-α was obtained from R&D Systems (Minneapolis, MN, USA). AMPK, PTEN, AKT siRNAs, and bpv (pic) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-AMPK, anti-phospho-AMPK, anti-ACC, anti-phospho-ACC, anti-PTEN, anti-AKT, anti-phospho AKT, and anti-iκB antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-COX-2 antibody was obtained from Cayman Chemical (Ann Arbor, MI, USA). Anti-iNOS was obtained from BD Biosciences (Franklin Lakes, NJ, USA). Metformin, wortmmanin, and anti-β-actin antibodies were obtained from Sigma–Aldrich (St. Louis, MO, USA). Compound C was obtained from Calbiochem (San Diego, CA, USA). Lipofectamine 2000 and H_2DCFDA were purchased from Invitrogen (Carlsbad, CA, USA). The Dual-Luciferase® Reporter Assay System was obtained from Promega (Madison, WI, USA).

2.2. Cell culture

VSMCs were isolated from thoracic aorta of 11-week-old male Sprague–Dawley rats. The thoracic aorta were removed with connective tissue, cut into 1 mm sections, and maintained in DMEM with 10% FBS and 1% antibiotics (penicillin 10,000 U/ml, amphotericin B 25 μg/ml, and streptomycin 10,000 μg/ml). We used VSMCs from passages 4–8 at 70% confluence in 10 cm dishes, and cell growth was arrested by incubating the cells in serum-free DMEM for 16–24 h prior to use.

2.3. siRNA transfection

Aliquots of 1×10^4 cells were plated on 6-well plates on the day before transfection and grown to about 70% confluence. Then, VSMCs were transfected with AMPK, PTEN and AKT siRNA using Lipofectamine 2000 and incubated in Opti-MEM® I reduced serum medium (Invitrogen) for 4–6 h. The medium was then replaced with DMEM medium containing 10% FBS. Following a 48-h incubation, the protein level was measured using Western blot analysis.

2.4. Western blot analysis

Protein extracts were prepared by lysing cells in RIPA lysis buffer. The protein concentration was quantified with protein assay reagent (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were mixed with 5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer and incubated for 5 min at 100 °C before loading. Total protein samples (40 μg) were subjected to 8% SDS–PAGE for about 1 h 30 min at 100–130 V. The separated proteins were electrophoretically transferred onto a PVDF membrane for 1 h at 100 V. The membranes were blocked with 5% non-fat milk in TBS containing 1% Tween 20 (TBST) for 2 h at room temperature. Then, the membranes were washed three times with TBST for 10 min each. The membranes were incubated with the primary antibodies at a dilution of 1:1000 in PBS containing 2% BSA overnight at 4 °C. After the incubation, the membranes

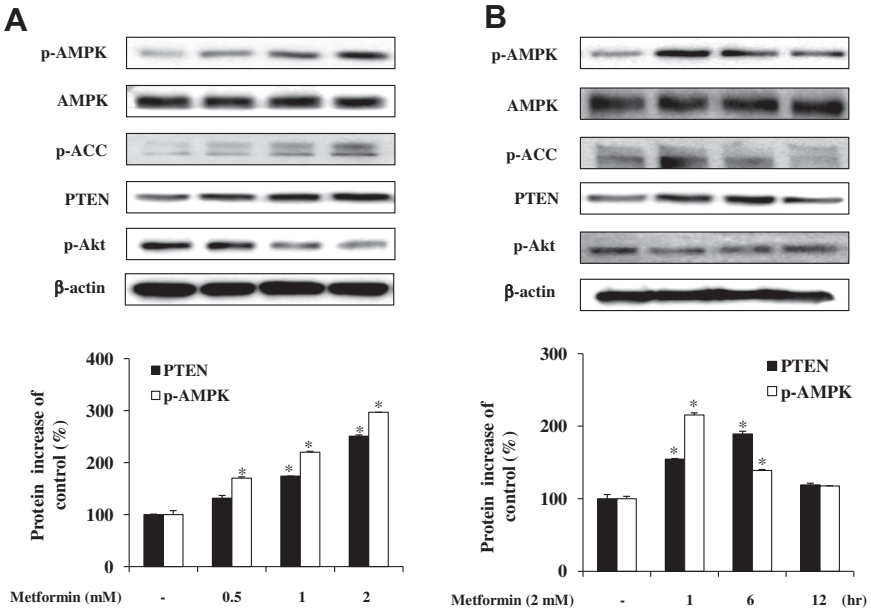

Fig. 1. The effects of metformin on AMP activated protein kinase (AMPK) phosphorylation and phosphatase and tensin homolog (PTEN) expression in vascular smooth muscle cells (VSMCs). AMPK phosphorylation at Thr172 (p-AMPK) and PTEN expression were determined by Western blot and densitometric analysis. (A) VSMCs were incubated with different concentrations of metformin (0.5, 1, and 2 mM) for 1 h. (B) VSMCs were incubated with 2 mM metformin for different time periods (1, 6, and 12 h). Data are represented as the mean ± standard error ($n = 3$) $^{\#}P < 0.05$ compared with control.

were washed three times for 10 min each time and incubated for 1 h at room temperature in TBST containing anti-rabbit IgG (Cell Signaling Technology) or anti-mouse IgG (Santa Cruz Biotechnology). Finally, after three more washes with TBST, the membranes were exposed to ECL or ECL plus Western blot analysis detection reagents.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from VSMCs using a single-step guanidine thiocynate/phenol chloroform extraction procedure with Trizol® reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. RNA was reverse transcribed into single-stranded cDNA by 10 min incubations at 25 °C, 60 min at 37 °C, 60 min at 42 °C, and 5 min at 95 °C in a final volume of 50 μL using TaqMan® Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA). PCR amplification was carried out on cDNA equivalent to 100 ng of starting mRNA using specific oligonucleotide primers for iNOS (sense 5′-ATG GCT TGC CCC TGG AAG TTT CTC-3′, antisense 5′-CCT CTG ATG GTG CCA TCG GGC ATC TG-3′), COX-2 (sense 5′-TTC ACC AGA CAG ATT GCT GGC-3′, antisense 5′-AGT CTG GAG TGG GAG GCA CTT G-3′), and β-actin (sense 5′-TAG GCA GGC CTC TTT TCT CA-3′, antisense 5′-AGA GGG GAC CTG GGT TTA GA-3′). The cDNA was heated for 5 min at 95 °C, then amplified 30 cycles for iNOS (two-temperature PCR at 94 °C for 15 s and 60 °C for 30 s), 28 cycles for COX-2 (94 °C for 1 min and 58 °C for 1 min, 72 °C for 2 min) and 28 cycles for β-actin (94 °C for 45 s, 55 °C for 1 min, and 72 °C for 2 min), followed by a 5 min extension at 72 °C. The predicted sizes of the RT-PCR products for iNOS, COX-2, and β-actin were 830, 530, and 214 bp. The PCR products were separated by electrophoresis on a 1.5% agarose gel containing ethidium bromide. Result was detected with a Fujifilm LAS-3000 Imager (Biocompare, San Francisco, CA, USA).

2.6. Real-time quantitative PCR analysis

The RNA extraction and reverse transcription processes for real-time PCR were conducted in the same way as the RT-PCR procedure. The mixture for quantitative PCR was composed of 1 μL primer, 10.5 μL water, 6.25 μL SYBR mixture and 1 μL cDNA in a final volume of 25 μL. The following primers were used: iNOS (sense 5′-atg gaa cag tat aag gca aac-3′; antisense 5′-gtt tct ggt cga tgt cat gag-3′), COX-2 (sense 5′-AGG AGA GAA AGA AAT GGC TG-3′, antisense 5′- GAG AAC AGA TGG GAT TAC CC-3′), and β-actin was used as in RT-PCR. PCR reactions were carried out at 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and finally at 65 °C for 1 min.

2.7. Luciferase reporter gene assay

VSMCs were stably transfected with a plasmid containing the luciferase reporter gene linked to NF-κB binding sites (p-NF-κB-luc) to detect activation of NF-κB. The p-NK-κB-luc plasmid was transfected together with the p-RL-Tk-luc plasmid into rat VSMCs

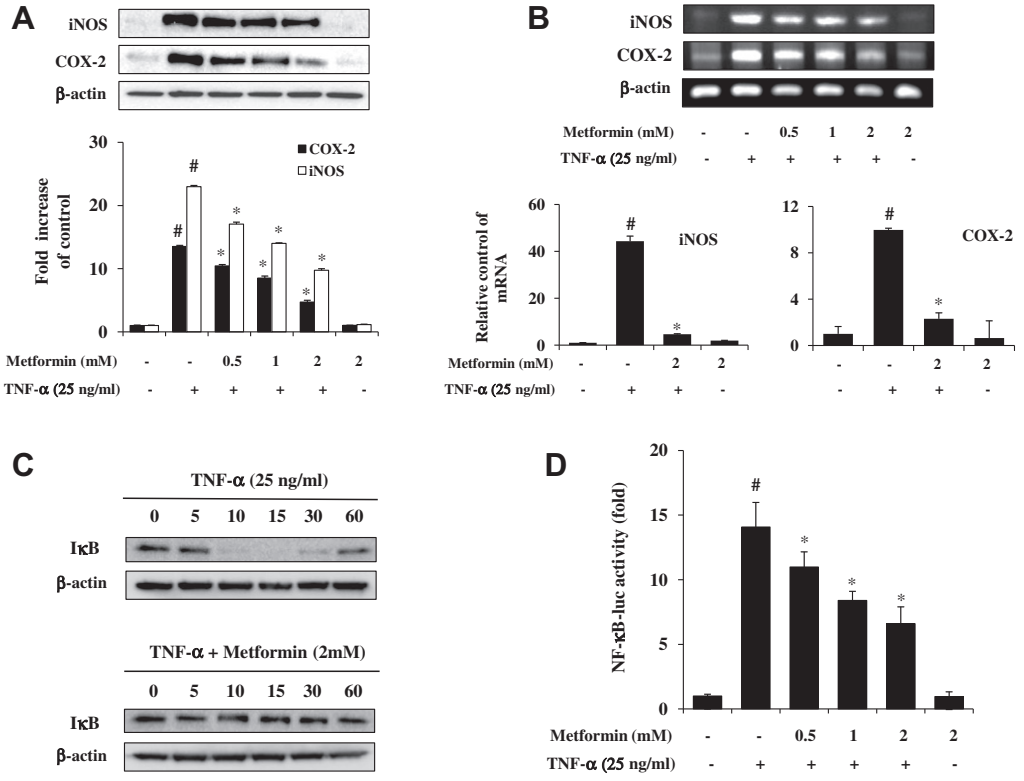

Fig. 2. Inhibitory effects of metformin on tumor necrosis factor (TNF)-α induced inflammatory responses. Vascular smooth muscle cells (VSMCs) were treated with metformin (0.5, 1, and 2 mM) for 1 h in the presence of TNF-α (25 ng/ml). (A and B) Protein and mRNA expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 were determined by Western blot and densitometric analysis, Reverse transcriptase PCR and real-time quantitative PCR analysis. (C) Metformin inhibited I-κB degradation. VSMCs were pre-incubated with metformin for 1 h and stimulated with TNF-α for different time periods. I-κB expression was determined by Western blot analysis. (D) VSMCs were transfected with pNF-κB-luc and the pRL-Tk-luc reporter gene using the DAEA-dextran method. Twenty-four hr after transfection, VSMCs were stimulated with TNF-α and then treated with metformin for 1 h. Data are presented as mean ± standard error ($n = 3$) $^{\#}P < 0.05$ compared with control, $^{*}P < 0.05$ compared with TNF-α.

using the DAEA-dextran method, as described previously [16]. Luciferase activity was measured with the Dual-Luciferase® Reporter Assay System.

2.8. Measurement of intracellular reactive oxygen species (ROS)

TNF-α-induced ROS generation was measured using 2′-7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA). After the indicated time periods, the medium was changed with phenol red-free DMEM medium, and ROS generation was detected using live imaged laser scanning and fluorescence-activated cell sorting (FACS) analysis. For the FACS analysis, 10 μM of H_2DCF-DA was added for 30 min in a dark incubator. The cells were washed twice and suspended in 500 μL PBS. DCF fluorescence was detected using a FACSDiva version 6.1.1 flow cytometer. For live imaged laser scanning, 10 μM H_2DCF-DA was added immediately prior to exposure to the laser. ROS generation was detected from the oxidation of DCF fluorescence using a fluorescein isothiocyanate (FITC) filter set.

2.9. Statistical analysis

All data are presented as mean ± standard error. Differences between data sets were assessed by one-way analysis of variance or Student's *t*-test as appropriate. A $P < 0.05$ was considered statistically significant.

3. Results

3.1. Metformin induces AMPK phosphorylation and PTEN expression in VSMCs

Metformin was used to determine whether activating AMPK induces PTEN expression. VSMCs were exposed to various concentrations of metformin (0.5, 1, and 2 mM) for 1 h. Protein level of phospho-AMPK increased following metformin treatment and induced PTEN expression dose dependently. Metformin also elevated the protein levels of phospho-ACC but decreased phospho-Akt, a major downstream regulator of AMPK and PTEN (Fig. 1A). AMPK was fully phosphorylated at 1 h but PTEN expression began at 1 h but was at its maximum at 6 h (Fig. 1B), indicating that PTEN expression was affected by AMPK activation at 1 h. TNF-α did not change AMPK phosphorylation or PTEN expression levels (data not shown).

3.2. Metformin attenuates the TNF-α induced inflammatory response

Many studies have reported that metformin has anti-inflammatory effects. Thus, we examined the effects of metformin on the inflammatory response. We confirmed the inflammatory response using NF-κB activation and iNOS and COX-2 expression levels as mediators of inflammation. VSMCs were stimulated with TNF-α (25 ng/ml) for 4 h and then treated with various concentrations

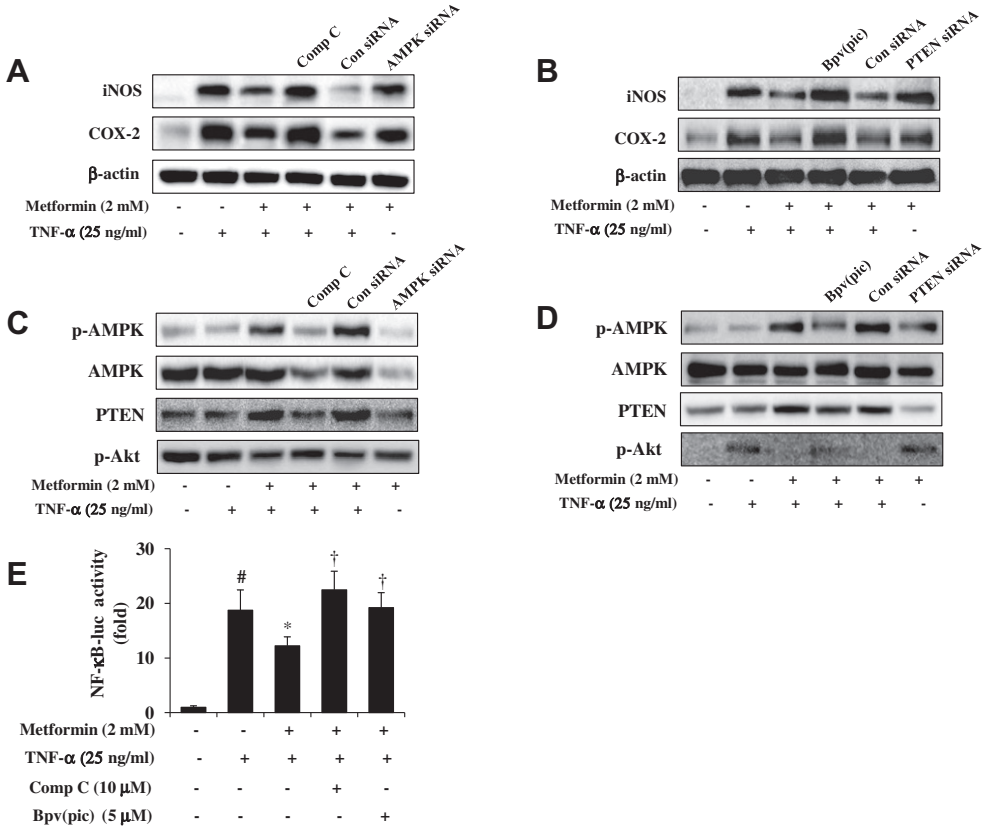

Fig. 3. Inhibiting AMP activated protein kinase (AMPK) and phosphatase and tensin homolog (PTEN) restored the tumor necrosis factor (TNF)-α-induced inflammatory response. AMPK and PTEN were inhibited by compound C, bpv (pic), and siRNA. Vascular smooth muscle cells (VSMCs) were stimulated with TNF-α for 4 h and then compound C (10 μM) was added 1 h earlier than metformin. bpv (pic) (5 μM) was added together with metformin for 1 h. AMPK and PTEN siRNAs were transfected 3 days before treatment with TNF-α and metformin. (A and B) Inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 expression as well as (C and D) AMPK phosphorylation and PTEN expression were determined by Western blot analysis. (E) VSMCs were transfected with pNF-κB-luc and the pRL-Tk-luc reporter gene using the DAEA-dextran method. Twenty-four hours after transfection, VSMCs were stimulated with TNF-α and bpv (pic) for 1 h. Compound C was added 1 h earlier. Data are presented as mean ± standard error ($n = 3$) $^{\#}P < 0.001$ compared with control, $^{*}P < 0.05$ compared with TNF-α and $^{\dagger}P < 0.05$ compared with TNF-α + metformin.

of metformin (0.5, 1, and 2 mM). As expected, metformin attenuated TNF-α-induced iNOS and COX-2 protein expression (Fig. 2A). We also detected iNOS and COX-2 mRNA expression using RT-PCR and real-time quantitative PCR analysis, and mRNA level decreased following metformin treatment (Fig. 2B). Furthermore, metformin blocked TNF-α-induced I-κB degradation and NF-κB luciferase reporter gene activity (Fig. 2C and D). These results indicate that metformin derived AMPK activation and PTEN expression regulated the inflammatory response.

3.3. Inhibition of AMPK and PTEN restores the TNF-α-induced inflammatory response

To investigate whether the inflammatory response is regulated by a AMPK and PTEN dependant pathway, we first examined how inhibiting AMPK and PTEN affected iNOS and COX2 expression. VSMCs were prepared on 6-well plates and transfected with siRNA to genetically inhibit AMPK and PTEN. At 48 h after transfection, VSMCs were stimulated with TNF-α for 4 h and then treated with compound C (a specific AMPK inhibitor) and bpv (pic) (a PTEN inhibitor). Compound C and AMPK siRNA recovered iNOS and COX-2 protein expression reduced by metformin. Inhibiting PTEN restored iNOS and COX-2 protein expression (Fig. 3A and B). Next, we examined the effect of inhibiting AMPK and PTEN on NF-κB luciferase reporter gene activity. The decrease in NF-κB activity caused by metformin was regained by inhibiting AMPK and PTEN (Fig. 3E). Thus, AMPK and PTEN participated in regulation of the inflammatory response. We next investigated the regulatory mechanism of the inflammatory response between AMPK and PTEN. Interestingly, inhibiting AMPK decreased PTEN expression and inhibiting PTEN also decreased AMPK activation (Fig. 3C and D). Based on this result, AMPK and PTEN clearly interacted, but we did not identify the mechanism in the present study.

3.4. Metformin diminishes intracellular ROS through AMPK and PTEN

Because ROS generation is closely correlated with TNF-α-induced NF-κB activation [17], we verified the effect of metformin on TNF-α-induced ROS generation. VSMCs were transfected with AMPK and PTEN siRNA 48 h before being stimulated with TNF-α. Metformin, compound C, and bpv (pic) were added for different time periods, and ROS were detected using flow cytometry and a live imaging laser scanning system. ROS level increased significantly after stimulation with TNF-α and metformin. Inhibiting AMPK and PTEN restored ROS levels as much as being stimulated with TNF-α (Fig. 4A and B). These finding indicate that AMPK and PTEN regulated the inflammatory response as well as ROS production in VSMCs.

4. Discussion

The aim of the present study was to determine whether AMPK or PTEN regulate the TNF-α-induced inflammatory response in VSMCs. Atherosclerosis is an important cardiovascular disease worldwide and results from inflammation occurring in carotid arteries, due to various pathologies such as obesity and senescence [1]. Abnormal proliferation of VSMCs is also suggested as a potential cause of atherosclerotic lesions [18]. VSMCs migrate from the intima to the media due to exposure to inflammatory mediators such as TNF-α, IL-1β, free fatty acids, and endotoxins, which strongly influences the production of atherosclerotic plaque [19].

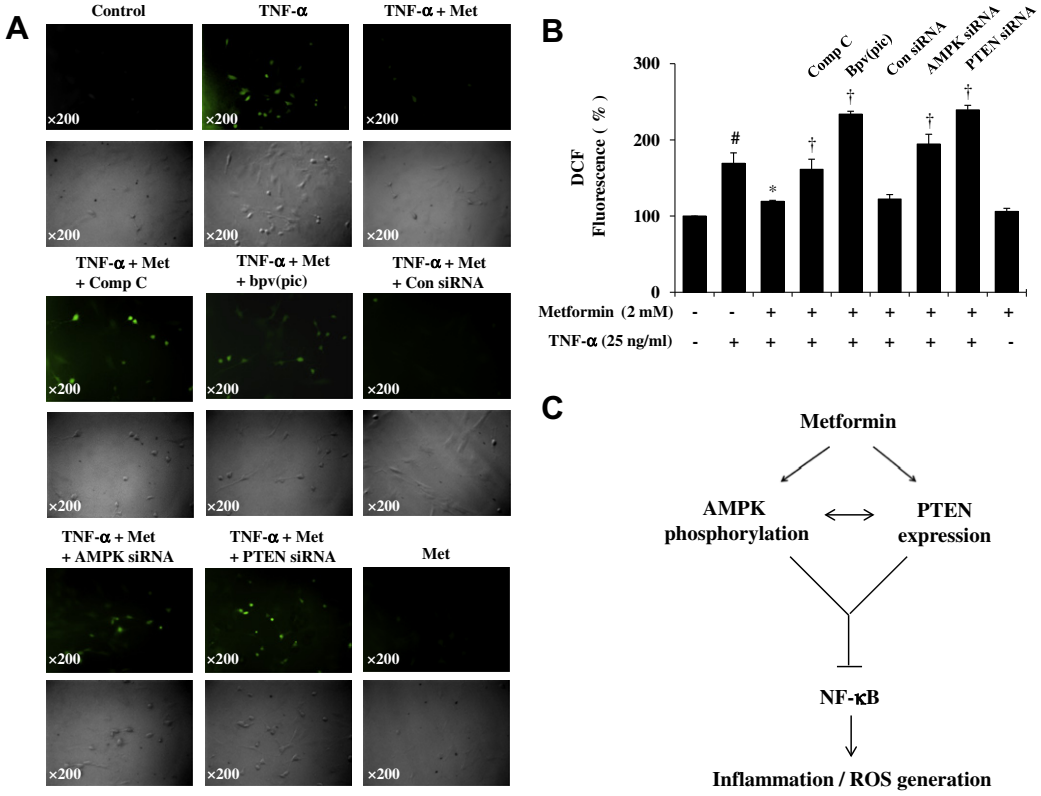

Fig. 4. The effects of AMP activated protein kinase (AMPK) and phosphatase and tensin homolog (PTEN) on intracellular reactive oxygen species (ROS) generation. Vascular smooth muscle cells (VSMCs) were stimulated with tumor necrosis factor (TNF)-α for 4 h and then treated with compound C for 2 h. Bpv (pic) and metformin were added for 1 h. AMPK, PTEN, and control siRNA were transfected 3 days before the treatments with TNF-α and metformin. Intracellular ROS were determined by (A) live imaged laser scanning and (B) flow cytometric analyses. (C) AMPK-PTEN signaling model induced by metformin. Data are presented as mean ± standard error ($n = 3$) $^{\#}P < 0.001$ compared with control, $^{*}P < 0.001$ compared with TNF-α and $^{\dagger}P < 0.005$ compared with TNF-α + metformin.

Previous studies have documented that inflammatory cytokines affect VSMC proliferation [20].

AMPK monitors metabolic homeostasis and modulates inflammatory responses in macrophages, microglia, astrocytes, and mesangial cells [5–9]. AMPK inhibits expression of adhesion molecules via negative regulation of MAPK, AKT, and the NF-κB signaling pathways in VSMCs, and α1-AMPK antagonizes free fatty acid-induced inflammation in macrophages [21]. Many studies support the finding that AMPK has anti-inflammatory effects. However, the particular signaling cascade has not been identified.

PTEN, a tumor suppressor gene, antagonizes PI3 kinase during conversion of PIP3 to PIP2 by dephosphorylating the 3-phosphate of PIP3. PTEN affects cell survival, growth, and proliferation by overexpressing AKT and mTOR [22]. PTEN also attenuates formation of atherosclerotic lesions in high fat-fed rabbits and upregulates the NF-κB-dependent cytokine family in PTEN-depleted SMCs [2,23]. Furthermore, PTEN is a possible downstream regulator of AMPK and has antioxidant effects in prostate cancer cells [15]. However, metformin regulates insulin signaling through AMPK mediated PTEN downregulation in preadipocyte 3T3-L1. According to this article, metformin activates AMPK phosphorylation but decreases PTEN expression [24].

Our data demonstrate that AMPK activation and PTEN expression elicited by metformin may obstruct the inflammatory response. Metformin showed anti-inflammatory effects by sustaining NF-κB activity through the PI3K and AKT pathway [25]. Thus, to confirm that AMPK and PTEN acted dependently, we determined that iNOS and COX-2 expression as well as NF-κB activity were recovered by inhibiting AMPK and PTEN expression using compound C, bpv (pic), and AMPK and PTEN siRNAs. Based on these data, we suggest that AMPK and PTEN are directly involved in regulating the inflammatory response. Moreover repressing AMPK downregulated PTEN expression. We expected that PTEN could be a potential downstream regulator of AMPK and investigated AMPK activity after inhibiting PTEN expression. Intriguingly, inhibiting PTEN also reduced AMPK activation and ACC activation, one of the major downstream regulators of AMPK. These results indicate that AMPK and PTEN interacted to regulate inflammation though PTEN and AMPK.

Inflammatory mediators are implicated in the production of ROS. Oxidative stress affects injured vessels, which develops into inflammation. In contrast, inflammation can increase ROS on atherosclerotic lesions. This vicious cycle leads not only to cardiovascular disease but also myocardial infarction, stroke, and heart failure. Thus, reducing oxidative stress is important to control inflammation [26]. Studies suggest that ROS increase by cytokines on atherosclerotic plaques [27]. Reducing the generation of ROS is connected with the AMPK and PTEN signaling pathways [15,28,29]. In the present study, TNF-α-induced ROS production decreased following AMPK and PTEN treatment and ROS generation was restored when AMPK and PTEN were inhibited. These findings suggest that AMPK and PTEN are crucial negative regulators of inflammation. Furthermore, they are expected to play a significant role in the treatment of inflammation-related cardiovascular diseases such as atherosclerosis.

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