Cyclooxygenase-2 induction by adiponectin is regulated by a sphingosine kinase-1 dependent mechanism in cardiac myocytes

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Abstract

The adipose-derived plasma protein, adiponectin (APN), has various protective effects on cardiovascular diseases. In this study, we show that endogenous APN is required for full cyclooxygenase-2 (COX-2) induction by ischemia-reperfusion injury in the heart in vivo. In rat neonatal cardiac myocytes, APN-induced COX-2 expression was reduced by treatment with a sphingosine kinase-1 (SphK-1) inhibitor or siRNA targeting SphK-1. Treatment with a sphingosine-1-phosphate (S1P) receptor antagonist also diminished COX-2 expression in response to APN stimulation. These findings suggest that APN is a physiological regulator of COX-2 signaling in the heart and that this regulation occurs in part via a SphK-1–S1P receptor dependent mechanism in cardiac myocytes.

Keywords

adiponectin; cyclooxygenase-2; sphingosine kinase-1; cardiac myocytes

1. Introduction

Obesity-related diseases are closely associated with the development of cardiovascular diseases. Adiponectin (APN) is a plasma protein that is primarily produced by adipose tissue [1]. Plasma APN concentrations are decreased in association with obesity, hypertension and ischemic heart diseases. APN displays various protective properties in the context of obesity-related metabolic, inflammatory and cardiovascular diseases [2–6]. We have reported that APN protects the heart from various injuries [5,7] and that it inhibits myocardial ischemia-

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reperfusion injury through both AMP-activated protein kinase (AMPK)- and cyclooxygenase-2 (COX-2)- dependent mechanisms [8].

COX-2, the key enzyme regulating the production of prostaglandins, plays a variety of roles in the cardiovascular system. COX inhibitors are widely used as non-steroidal anti-inflammatory drugs in chronic inflammatory diseases [9]. However, COX-2 selective inhibitors have been associated with an increased risk of cardiovascular events [10]. It has been reported that COX-2 exerts cardioprotective effects via the production of prostaglandin (PG) E_2 synthesis in a model of myocardial ischemia-reperfusion injury [11]. Furthermore, we have recently reported that APN suppresses lipopolysaccharide-induced tumor necrosis factor (TNF)-α production in cardiac cells through COX-2-PGE$_2$-dependent pathways which are independent of AMPK [8]. However, the molecular mechanisms by which APN regulates COX-2 expression in cardiac cells remain unknown.

Sphingosine-1-phosphate (S1P) is generated from sphingosine through phosphorylation by sphingosine kinase (SphK) [12]. S1P has various bioactivities, including the stimulation of angiogenesis and the suppression of apoptosis [13]. It has been reported that S1P increases COX- 2 expression in vascular endothelial cells [14] or smooth muscle cells [15]. These findings led us to speculate that SphK acts as a modulator for APN-COX-2 signaling axis in cardiac myocytes. In this study, we investigated whether APN regulates COX-2 expression through SphK-dependent pathway.

2. Materials and methods

2.1 Mouse model of myocardial ischemia-reperfusion

Wild-type (WT) and APN-deficient (APN-KO) mice in C57/BL6 background were used for this study. Study protocols were approved by the Institutional Animal Care and Use Committee in Boston University. Mice at the ages of 10 to 12 weeks were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally). A left thoracotomy was performed, and the left anterior descending artery (LAD) was visualized under a microscope and ligated with 8-0 silk suture using snare occluder. Mice were subjected to 30 min of LAD ligation, followed by 48 h of reperfusion [8]. Mice were then sacrificed and the hearts were extracted for Western blot analysis.

2.2 Cell culture and treatment

Primary neonatal rat ventricular cardiac myocytes were isolated and cultured as previously described [16]. In brief, cardiac myocytes were treated with serum-free Dulbecco's Modified Eagle medium (DMEM) for 24 hours prior to each experiment, and then treated with or without human recombinant APN (30 µg/ml) for 18 hours or sphingosine-1-phosphate (1.0µM) for 6 hours (BIOMOL International, L.P., PA, USA). Recombinant human APN from baculovirus-insect cell expression system was obtained from Nosan Corp. [6]. Preincubation with 10µM sphingosine kinase inhibitor (SKI) (Cayman, MI, USA), 0.01, 0.1 and 1.0µM VPC23019 (Avanti Polar Lipids, AL, USA), an antagonist of S1P receptors 1 and 3, or vehicle was performed for 1 hour prior to APN treatment.

2.3 RNA interference experiments

The siRNA directed to SphK-1 and non-targeting siRNA control were purchased from Dharmacon (siGENOME SMART Pool and ON-TARGETplus siCONTROL Non-Targeting siRNA Pool, CO, USA), and transfection with this agent was performed as previously reported [6]. Briefly, the cardiac myocytes were transfected for 24 hours with 100 nM of each siRNA by using RNAiMAX reagent (Invitrogen, CA, USA) in OptiMEM (Invitrogen, CA, USA) according to the manufacturer's instructions, and then medium was changed to fresh serum-

free DMEM. Experiments with cardiac myocytes transfected with siRNA were performed 48 hours later after siRNA transfection.

2.4 Measurements of the levels of mRNA expression

Total RNA was extracted from cardiac myocytes with RNeasy micro kit (QIAGEN, CA, USA). cDNA synthesis from total RNA was performed using a cDNA synthesis kit (QuantiTect Reverse Transcription Kit, QIAGEN, CA, USA) according to the manufacturer's instructions. Transcript expression levels of *SphK-1* and *GAPDH* were quantified by iCycler iQ Real-Time PCR Detection Systems (BIO-RAD, CA, USA) using SYBR Green Master Mix (Applied Biosystems, CA, USA). Transcript levels of *SphK-1* were adjusted relative to the expression of *GAPDH*. The sequence of PCR primer are as follows; 5'-GCAAGGCTCTGAAGCTCTTT-3' and 5'-CCCAGTACCCAGTTCTTCT-3' for SphK-1 and 5'-TCAAGAAGGTGGTGAAGCAG- 3' and 5'-AGGTGGAAGAATGGGAGTTG -3' for GAPDH.

2.5 Western blotting

Protein was extracted from cardiac myocytes with cell lysis buffer (Cell Signaling, MA, USA). The method of protein extraction from mice hearts was previously described in detail [17]. Whole cell lysate were resolved on SDS polyacrylamide gels (BIO-RAD, CA, USA), followed by electrophoretic transfer PVDF membranes (Hybond™-ECL, Amersham Biosciences, NJ, USA). Immunoreactive bands were visualized using ECL or ECL-PLUS chemiluminescence reagent (Amersham Biosciences, NJ, USA). The membranes were exposed to COX-2 antibody (Cayman, MI, USA) and α-tubulin antibody (Calbiochem, CA, USA), and then exposed to anti-rabbit or anti-mouse secondary antibodies conjugated with horseradish peroxidase, respectively. The band density was quantified by using Image J 1.38X (http://rsb.info.nih.gov/ij/). All of them were normalized to α-tubulin.

2.6 Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was performed by a 2-tailed Student's t test or ANOVA analysis. A value of $P < 0.05$ was accepted as statistically significant.

3. Results

3.1 APN signaling is required for full COX-2 induction following acute ischemic injury in the heart

WT and APN-KO mice underwent myocardial ischemia-reperfusion injury or sham surgery, and COX-2 protein levels in the heart were assessed. Little or no expression of COX-2 protein could be detected by Western blotting in hearts of WT and APN-KO mice that had undergone sham surgery. Ischemia-reperfusion markedly increased the expression of COX-2 in WT mice hearts, but this upregulation was attenuated in the APN-KO hearts (Fig. 1A).

3.2 APN-induced COX-2 upregulation is reduced by SphK-1 inhibition

To elucidate the mechanism of COX-2 regulation by APN, cultured cardiac myocytes were treated with recombinant human APN. APN significantly increased COX-2 expression in neonatal rat cardiac myocytes [8] (Fig. 1B). To test whether APN augments COX-2 expression through a SphK-1-dependent pathway, cardiac myocytes were pretreated with a SphK-1 inhibitor (SKI) followed by incubation with APN or vehicle. While no differences in COX-2 expression were observed between the presence or absence of SKI under basal conditions, APN-induced COX-2 upregulation was diminished by 38% by preincubation with SKI (Fig. 2). To corroborate these findings, knockdown experiments were performed using siRNA against SphK-1. SphK-1 mRNA levels were reduced by 68% by treatment with siRNA for

SphK-1 compared to control RNA (Fig. 2A). Knockdown of SphK-1 suppressed APN-stimulated COX-2 expression by 43% compared to control RNA (Fig. 2B).

3.3 APN-induced COX-2 expression is diminished by S1P receptor antagonist

S1P acts as a ligand for S1P receptors, a family of specific-G-protein-coupled receptors [18], and S1P receptors are expressed by cardiomyocytes [19]. Indeed, S1P upregulated COX-2 expression in cardiac myocytes, and S1P-induced COX-2 expression was reduced in a dose-dependent manner by the addition of VPC23019, which antagonizes S1P receptor 1 and 3 (Fig. 3A). Thus, we assessed whether APN-induced COX-2 upregulation is mediated by S1P receptors in cardiac myocytes using VPC23019. Pretreatment with 0.1 and 1.0µM, but not 0.01µM, VPC23019 diminished APN-induced COX-2 expression (Fig. 3B).

4. Discussion

The present study demonstrates the existence of an APN – COX-2 regulatory axis in vivo. Previously we reported that the cardioprotective actions of APN overexpression could be partially blocked by a COX-2 inhibitor [8]. To extend these observations, we tested whether adiponectin-deficiency would reduce the induction of cardiac COX-2 by ischemia-reperfusion injury in vivo. APN-KO hearts showed reduced COX-2 induction by ischemia-reperfusion compared to WT hearts, indicating APN is necessary for full COX-2 expression in the heart. These findings are of importance because they demonstrate that endogenous adiponectin participates in the myocardial COX-2 response to injury.

This study also elucidates aspects of the mechanism by which APN regulates COX-2 in neonatal rat cardiac myocytes. APN-induced COX-2 expression was considerably suppressed by incubation with SKI or siRNA of SphK-1. A S1P receptor antagonist also repressed COX-2 upregulation by APN. These data suggest that APN increases COX-2 expression through a SphK-1-S1P receptor pathway. S1P is a sphingolipid metabolite that regulates diverse biological processes in a variety of cell types [20]. Whereas S1P levels are controlled by activities of SphK-1 and SphK-2, SphK-1 is the dominant isoform in heart tissue [21]. Deficiency of SphK-1 has been shown to exaggerate cardiac cell apoptosis in oxidative stress [22] or hypoxia and glucose deprivation [23].

In contrast to these protective actions of SphK-1, Kase and colleagues have recently reported that globular APN promotes an inflammatory state in endothelial cells through SphK-1-dependent NF-κB activation [24]. In contrast, others have reported that APN suppresses TNF-α induced NF-κB activation and subsequent endothelial adhesion molecules in endothelial cells [25], and it is well established that adiponectin has anti-inflammatory and anti-atherogenic actions [2,6].

S1P functions both as an intracellular second messenger and an extracellular ligand of a family of five S1P receptors [26]. S1P receptor 1, 2 and 3 are expressed in adult mammalian cardiomyocytes [27,28]. It has been reported that VPC23019, a S1P receptor 1 and 3 antagonist, reduces the pro-survival effects of SphK-1 in cardiac cells [23]. It has also been shown that S1P exerts protective effects on cardiomyocytes survival during hypoxia through phosphatidylinositol 3-kinase-Akt signaling via S1P receptor 1 [23,28]. In this study we showed that VPC23019 inhibited both S1P-induced and APN-induced COX-2 upregulation in cardiac myocytes. Thus, APN may stimulate S1P receptors via S1P production, in an autocrine or paracrine manner, resulting in stimulation of COX-2 expression.

In summary, it is shown that APN-induced COX-2 expression in the heart is partially dependent on SphK-1. Because COX-2 is cardioprotective, the ability of APN to stimulate SphK-1-

COX-2 signaling in cardiac myocytes may contribute to its ability to protect the heart from injury.

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Abbreviations

APN, Adiponectin; SKI, sphingosine kinase inhibitor; APN-KO, APN-deficient; WT, wild-type; SphK-1, sphingosine kinase-1; S1P, sphingosine-1-phosphate; COX-2, cyclooxygenase-2; AMPK, AMP-activated protein kinase; TNF-α, tumor necrosis factor-α; PG, prostaglandin.
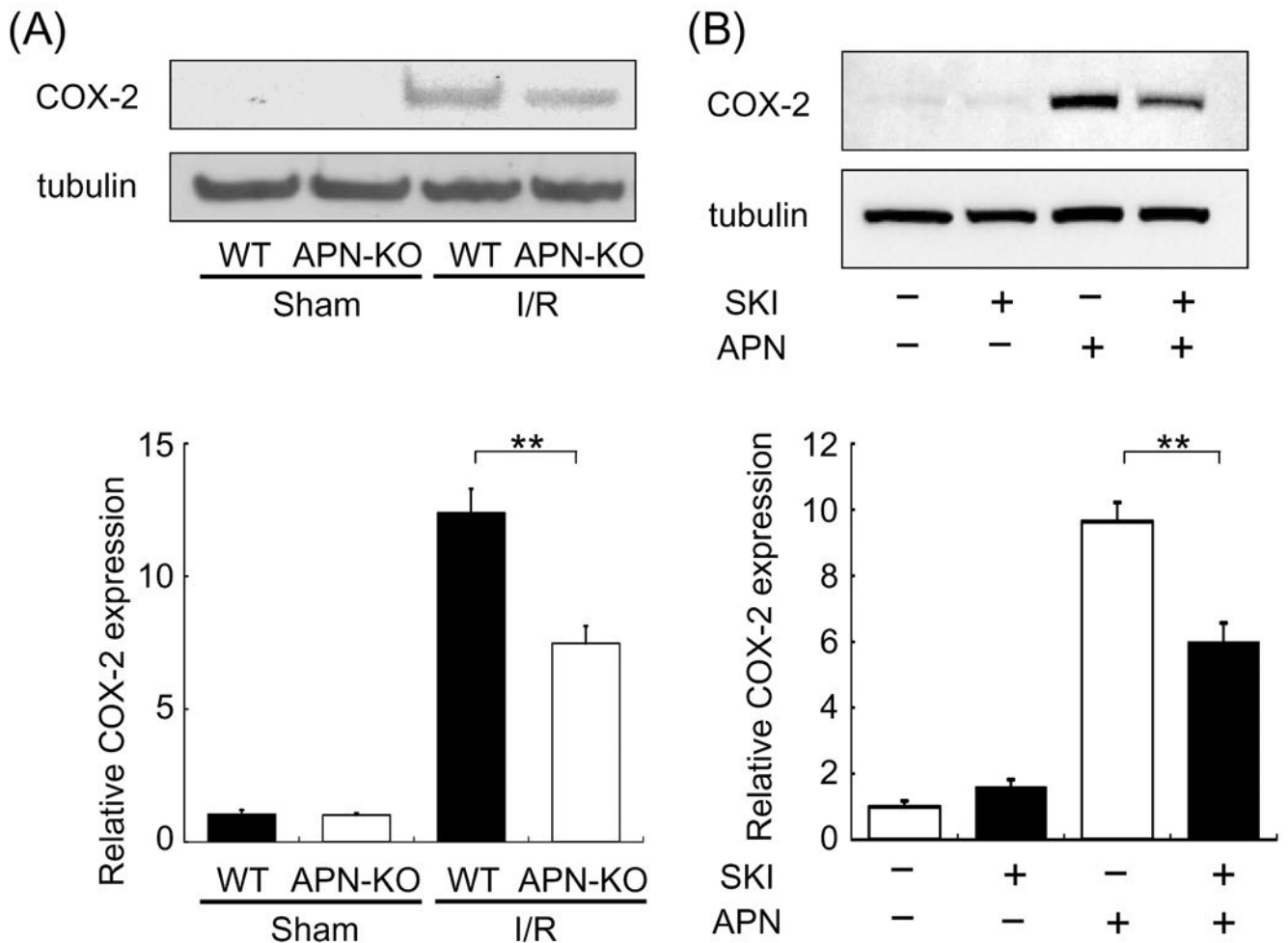
Fig. 1.
APN regulation of COX-2 in the heart and cultured cardiac myocytes. (A) APN is required for full induction of COX-2 by ischemia-reperfusion in heart. The expression of COX-2 protein in heart tissues from WT and APN-KO mice at 48 h after sham operation or ischemia-reperfusion was analyzed by Western blotting. The expression levels of COX-2 were quantified and expressed relative to sham-operated WT mice. Values are expressed as means ± SEM. **P<0.01, *n*=4 in each group. (B) SphK-1 inhibition reduces COX-2 induction by APN in cardiac myocytes. Cardiac myocytes were pretreated with 10 µM SKI for 1 hour prior to APN treatment. Cells were then treated with APN (30 µg/ml) or vehicle for 18 hours. Cell extracts were analyzed in Western blotting. Values are expressed relative to the vehicle-treated cultures and expressed as means ± SEM.. *P<0.01, *n*=8 in each group.

(A)

(B)
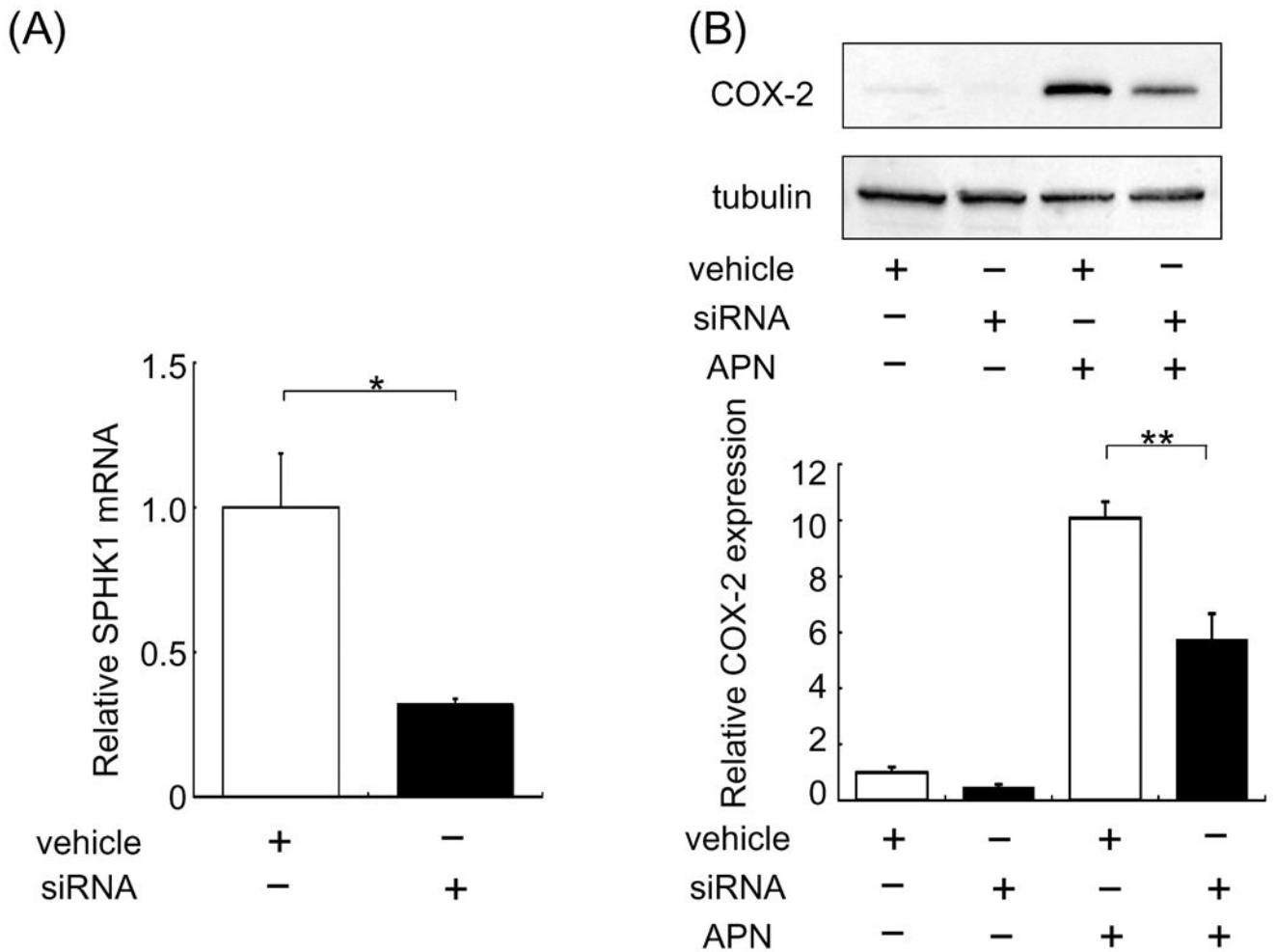
Fig. 2.
Knockdown of SphK-1 reduces COX-2 induction by APN in cardiac myocytes.
Cardiomyocytes were transfected with 100nM siRNA of SphK-1. (A) SphK-1 mRNA levels
were reduced following treatment with siRNA directed toward SphK-1. (B) Treatment with
siRNA against SphK-1 diminished APN-induced COX-2 upregulation on cardiac myocytes.
Forty-eight hours after siRNA transfection, cardiac cells were treated with APN (30 µg/ml) or
vehicle for 18 hours. Values are expressed relative to vehicle-treated cultures and reported as
means ± SEM. *$P < 0.05$, **$P < 0.01$, (A) $n=4$ in each group, (B) $n=6$ in each group.
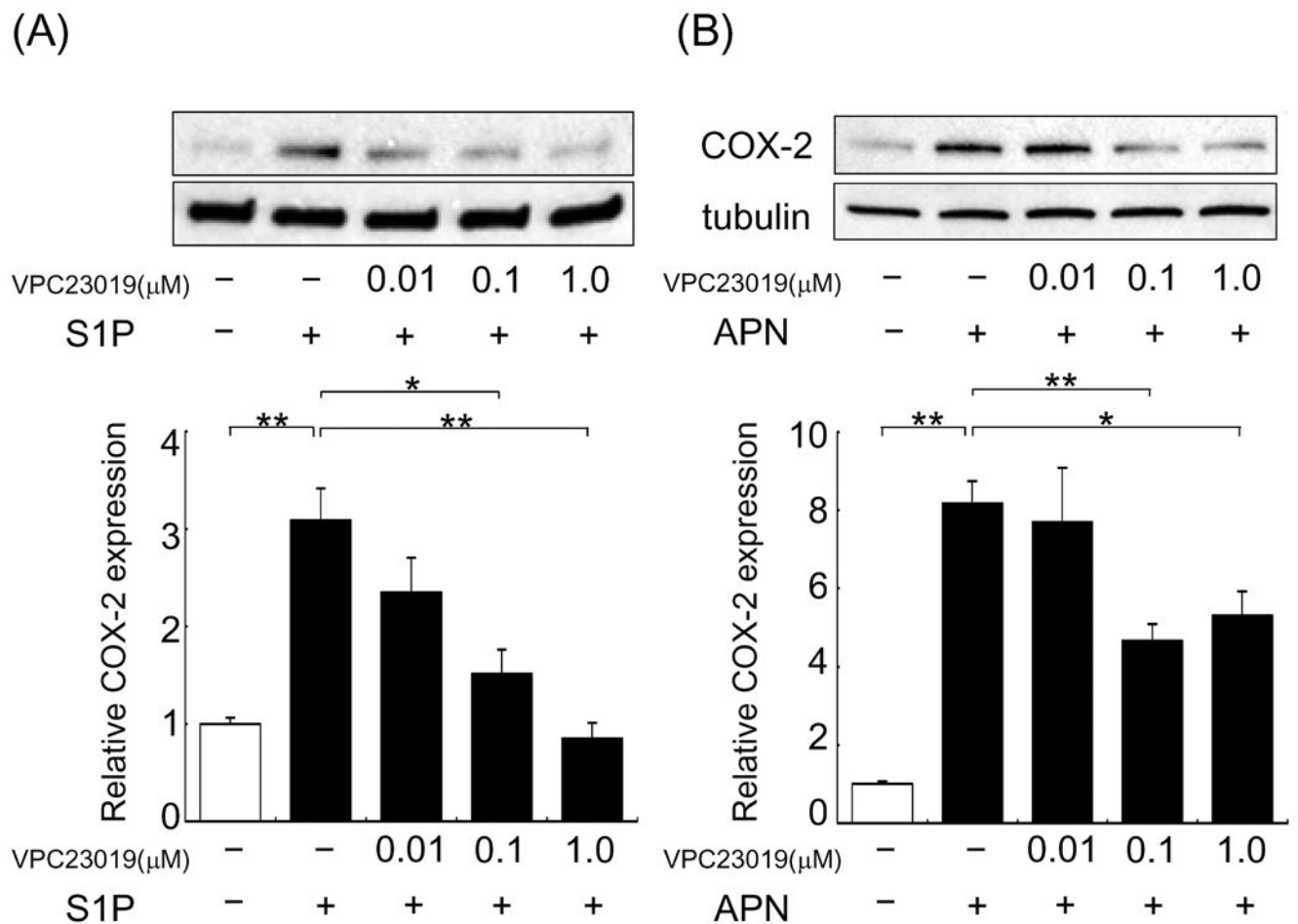
Fig. 3.
S1P receptor inhibition reduces COX-2 upregulation by APN or S1P in cardiac myocytes. (A) S1P receptor-inhibition reduces COX-2 upregulation by S1P. Cardiac myocytes were pretreated with VPC23019 or vehicle for 1 hour prior to incubation with S1P or vehicle for 6 hours. Values are expressed relative to vehicle-treated cultures and reported as means ± SEM. *P<0.05, **P<0.01 (A) n=3 in each group. (B) S1P receptor inhibition reduces COX-2 upregulation by APN in cardiac myocytes. Cardiac myocytes were pretreated with 0.01, 0.1 and 1.0μM VPC23019 or vehicle for 1 hour prior to incubation with APN (30 μg/ml) or vehicle for 18 hours. Values are expressed relative to vehicle-treated cultures and reported as means ± SEM. *P<0.05, **P<0.01, n= 3–8 in each group.