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

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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

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

COX-2, the key enzyme regulating the production of prostaglandins (PGs), 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 PG E2 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-PGE2-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 (SIP) is generated from sphingosine through phosphorylation by sphingosine kinase (SphK) [12]. SIP has various bioactivities, including the stimulation of angiogenesis and the suppression of apoptosis [13]. It has been reported that SIP 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.
inhibitor (SKI) (Cayman, MI, USA), 0.01, 0.1 and 1.0 μM VPC23019
(Avanti Polar Lipids, AL, USA), an antagonist of SIP receptors 1 and
3, or vehicle was performed for 1 h 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 re-
ported [6]. Briefly, the cardiac myocytes were transfected for 24 h with
100 nM of each siRNA by using RNAiMAX reagent (Invitrogen, CA,
USA) in OptiMEM (Invitrogen) 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 h later after siRNA transfection.

2.4. Measurements of the levels of mRNA expression
Total RNA was extracted from cardiac myocytes with RNAeasy mi-
cro kit (QIAGEN, CA, USA). cDNA synthesis from total RNA was
performed using a cDNA synthesis kit (QuantiTect Reverse Transcrip-
tion Kit, QIAGEN) according to the manufacturer’s instructions.
Transcript expression levels of SphK-1 and GAPDH were quantified by
Cycler QO Real-Time PCR Detection Systems (BIORAD, CA,
USA) using SYBR Green Master Mix (Applied Biosystems, CA,
USA). Transcript levels of SphK-1 were adjusted relative to the expres-
sion of GAPDH. The sequence of PCR primer is as follows; 5’-
GCAAGGCTCTGAAGCTCTTT-3’ for SphK-1 and 5’-TCAAGAAAGGTTGGGAGAGAGCAG3’
and 5’-AGTGAGAGATGGGAA TCTT-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 re-
solved on SDS–PAGE (BIORAD), followed by electrophoretic trans-
fer PVDF membranes (Hybond C228, USA) in OptiMEM (Invitrogen) according to the manufacturer’s
instructions, and then membranes were exposed to COX-2 antibody (Cayman) and
then treated with APN (30 μg/ml) or vehicle for 18 h. Cell extracts were
then treated with APN (30 μg/ml) or vehicle for 18 h. Cell extracts were
analyzed in Western blotting. Values are expressed relative to the
vehicle-treated cultures and expressed as means ± S.E.M.

2.6. Statistical analysis
Data are presented as means ± S.E.M. Statistical analysis was per-
formed by a 2-tailed Student’s t-test or ANOVA analysis. A value of
P < 0.05 were 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 attenu-
ated 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 hu-
man APN. APN significantly increased COX-2 expression in
neonatal rat cardiac myocytes [8] (Fig. 1B). To test whether

![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 the sham-operated WT mice. Values are expressed as means ± S.E.M.* 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 h prior to APN treatment. Cells were then treated with APN (30 μg/ml) or vehicle for 18 h. Cell extracts were analyzed in Western blotting. Values are expressed relative to the vehicle-treated cultures and expressed as means ± S.E.M. ** P < 0.01, n = 8 in each group.](image-url)

APN augments COX-2 expression through a SphK-1-depen-
dent 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 be-
tween the presence or absence of SKI under basal conditions,
APN-induced COX-2 upregulation was diminished by 38% by
preincubation with SKI (Fig. 1B). To corroborate these findings,
knockdown experiments were performed using siRNA against
SphK-1. SphK-1 mRNA levels were reduced by 68% by treat-
ment 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 SIP
receptor antagonist
SIP acts as a ligand for SIP receptors, a family of specific-G-
protein-coupled receptors [18], and SIP receptors are ex-
pressed by cardiomyocytes [19]. Indeed, SIP upregulated
COX-2 expression in cardiac myocytes, and SIP-induced
COX-2 expression was reduced in a dose-dependent manner
by the addition of VPC23019, which antagonizes SIP receptors
1 and 3 (Fig. 3A). Thus, we assessed whether APN-induced
COX-2 upregulation is mediated by SIP receptors in cardiac
myocytes using VPC23019. Pretreatment with 0.1 and
1.0 μM, but not 0.01 μM, 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
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 APN 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 SIP receptor antagonist also repressed COX-2 upregulation by APN. These data suggest that APN increases COX-2 expression through a SphK-1-SIP receptor pathway. SIP is a sphingolipid metabolite that regulates diverse biological processes in a variety of cell types [20]. Whereas SIP 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 APN has anti-inflammatory and anti-atherogenic actions [2,6].

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


