Myonectin Is an Exercise-Induced Myokine That Protects the Heart From Ischemia-Reperfusion Injury

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Rationale: **Physical exercise provides benefits for various organ systems, and some of systemic effects of exercise are mediated through modulation of muscle-derived secreted factors, also known as myokines. Myonectin/C1q (complement component 1q)/TNF (tumor necrosis factor)-related protein 15/erythroferrone is a myokine that is upregulated in skeletal muscle and blood by exercise.**

Objective: **We investigated the role of myonectin in myocardial ischemic injury.**

Methods and Results: **Ischemia-reperfusion in myonectin-knockout mice led to enhancement of myocardial infarct size, cardiac dysfunction, apoptosis, and proinflammatory gene expression compared with wild-type mice. Conversely, transgenic overexpression of myonectin in skeletal muscle reduced myocardial damage after ischemia-reperfusion. Treadmill exercise increased circulating myonectin levels in wild-type mice, and it reduced infarct size after ischemia-reperfusion in wild-type mice, but not in myonectin-knockout mice. Treatment of cultured cardiomyocytes with myonectin protein attenuated hypoxia/reoxygenation-induced apoptosis via S1P (sphingosine-1-phosphate)-dependent activation of cAMP/Akt cascades. Similarly, myonectin suppressed inflammatory response to lipopolysaccharide in cultured macrophages through the S1P/cAMP/Akt-dependent signaling pathway. Moreover, blockade of S1P-dependent pathway reversed myonectin-mediated reduction of myocardial infarct size in mice after ischemia-reperfusion.**

Conclusions: **These data indicate that myonectin functions as an endurance exercise-induced myokine which ameliorates acute myocardial ischemic injury by suppressing apoptosis and inflammation in the heart, suggesting that myonectin mediates some of the beneficial actions of exercise on cardiovascular health. (*Circ Res*. 2018;123:1326-1338. DOI: 10.1161/CIRCRESAHA.118.313777.)**

Key Words: apoptosis ■ exercise ■ inflammation ■ reperfusion injury ■ sphingosine-1-phosphate

Cardiovascular disease, including ischemic heart disease, is the main cause of morbidity and mortality worldwide.[1] Physical exercise benefits a variety of organs, including the cardiovascular system.[2,3] Particularly, endurance exercise training is an effective lifestyle intervention to reduce risk factors for cardiovascular disease.[4] Endurance exercise also improves cardiac function and survival in patients with ischemic heart disease.[5,6] However, the molecular mechanisms of the cardiovascular protective actions of exercise are incompletely understood.

Increasing evidence indicates that skeletal muscle produces a variety of secreted factors, also referred to as myokines, which can directly act on nearby or remote organs.[7,8] Several myokines, including IL (interleukin)-6 and follistatin-like 1, were identified, and some of them are believed to mediate the effects of exercise on systemic metabolic and cardiovascular homeostasis.[7–11] However, nothing is known about the endurance exercise-regulated myokine that affects cardiovascular disorders.

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Myonectin, also known as C1q (complement component 1q)/TNF (tumor necrosis factor)-related protein 15/erythroferrone, is a member of CTRPs (C1q/TNF-related proteins), which are the conserved paralogs of adiponectin containing collagen-like and globular C1q-like domains.[12] Although most of CTRPs are predominantly expressed by adipose tissue, myonectin was originally identified as a myokine that is abundantly expressed in skeletal muscle tissue, in particular, type I muscle fibers.[12,13] Myonectin expression in skeletal muscle and blood is shown to be upregulated by voluntary exercise.[13] It has been reported that

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Novelty and Significance

What Is Known?

- Exercise training has beneficial effects on prevention and treatment of cardiovascular diseases.
- Skeletal muscle can produce various secretory factors, also referred to as myokines, which can affect remote tissues in an endocrine manner.

What New Information Does This Article Contribute?

- Myonectin functions as a myokine which protects the heart from ischemia-reperfusion injury through suppression of cardiomyocyte apoptosis and macrophage inflammatory response.
- Endurance exercise can improve myocardial ischemic damage, at least in part, through upregulation of myonectin.

Physical exercise provides benefits for various organs, including the cardiovascular system, and some of systemic effects of exercise are mediated through modulation of myokine production. To date, nothing is known about the endurance exercise-regulated myokine that can contribute to the development of cardiac diseases. We found that myonectin acts as an endurance exercise-driven myokine which protects the heart from ischemic injury. Mechanistically, myonectin promotes the sphingosine-1-phosphate-dependent activation of cyclic AMP/Akt pathway, thereby contributing to reduction of cardiomyocyte apoptosis and macrophage inflammatory response. Of note, the protective actions of endurance exercise on myocardial ischemic injury are diminished under conditions of myonectin-deficiency. These data indicate that myonectin functions as a cardioprotective myokine, which can partly mediate the cardiovascular benefits of endurance exercise, suggesting that myonectin can represent a novel target molecule for prevention or treatment of cardiac disease.

Nonstandard Abbreviations and Acronyms

AAR	area at risk
Ad-dnAkt	dominant-negative mutant of Akt
CREB	cAMP response element binding protein
GSK-3β	glycogen synthase kinase-3
IA	infarct area
IL	interleukin
LV	left ventricular
MCP-1	monocyte chemoattractant protein 1
S1P	sphingosine-1-phosphate
S1PR1	S1P receptor 1
SphK1	sphingosine kinase-1
TNF	tumor necrosis factor
WT	wild-type

myonectin enhances fatty acid uptake in cultured adipocytes and hepatocytes and suppresses circulating levels of free fatty acids in mice.[14] Furthermore, myonectin has been shown to be an erythroid modulator of iron metabolism and hemoglobin synthesis.[15,16] However, the role of myonectin in cardiovascular disease has not been examined previously. Here, we investigated whether exercise-regulated myokine myonectin modulates acute ischemic injury in the heart.

Methods

The authors declare that all data and methods used in support of the findings presented in this study will be made available from the corresponding author on reasonable request.

An expanded Method section can be found in Online Data Supplement.

Results

Myonectin Protect the Heart From Myocardial Ischemic Injury by Suppressing Apoptosis and Inflammatory Reactions

To investigate the role of myonectin in ischemic heart disease, we analyzed the phenotypic changes in myonectin-knockout mice in a background of C57BL/6. Myonectin expression was undetectable at the transcript and protein levels in soleus in homozygous myonectin-knockout mice (Online Figure IA and IB). Plasma myonectin protein was also undetectable (<0.05 ng/mL) in myonectin-knockout mice by ELISA methods. Myonectin expression was hardly detected in the heart of wild-type (WT) mice and was undetectable in the heart of myonectin-knockout mice by Western blot analysis (Online Figure IB). No significant differences were observed in body weight, the tissue weights of heart, fat and skeletal muscles, and heat weight/body weight between myonectin-knockout and WT mice (Online Table I). In addition, there were no differences in circulating free fatty acids (1.40±0.11 mEq/L in WT mice versus 1.38±0.17 mEq/L in myonectin-knockout mice) and hemoglobin levels (14.2±0.8 g/dL in WT mice versus 14.1±0.5 g/dL in myonectin-knockout mice) between the 2 strains of mice. Thus, myonectin-knockout mice are indistinguishable from WT mice under basal physiological conditions.

Myonectin-knockout and WT mice were subjected to 60 minutes of ischemia and 24 hours of reperfusion. Figure 1A shows representative photographs of heart tissues stained with Evans blue dye to delineate the area at risk (AAR) and 2,3,5-triphenyl tetrazolium chloride to delineate the infarct area (IA). Although the AAR/left ventricular (LV) was the same in myonectin-knockout and WT mice, the IA/AAR and IA/LV ratios in myonectin-knockout mice were increased by 17.1±6.9% and 18.9±4.7%, respectively, compared with those of WT mice (Figure 1A). Circulating level of troponin-I level, an index of myocardial injury, was also significantly higher in myonectin-knockout mice after myocardial ischemia-reperfusion as compared to WT mice (Figure 1B). In contrast, no differences in circulating hemoglobin levels were seen on postoperative day 1 between 2 strains of mice (13.2±0.4 g/dL in WT mice versus 13.4±0.4 g/dL in myonectin-knockout mice).

To test whether myonectin affects cardiac function after ischemia-reperfusion, echocardiographic parameters were assessed in myonectin-knockout and WT mice on postoperative day 7. Myocardial ischemia-reperfusion injury led to increases in LV

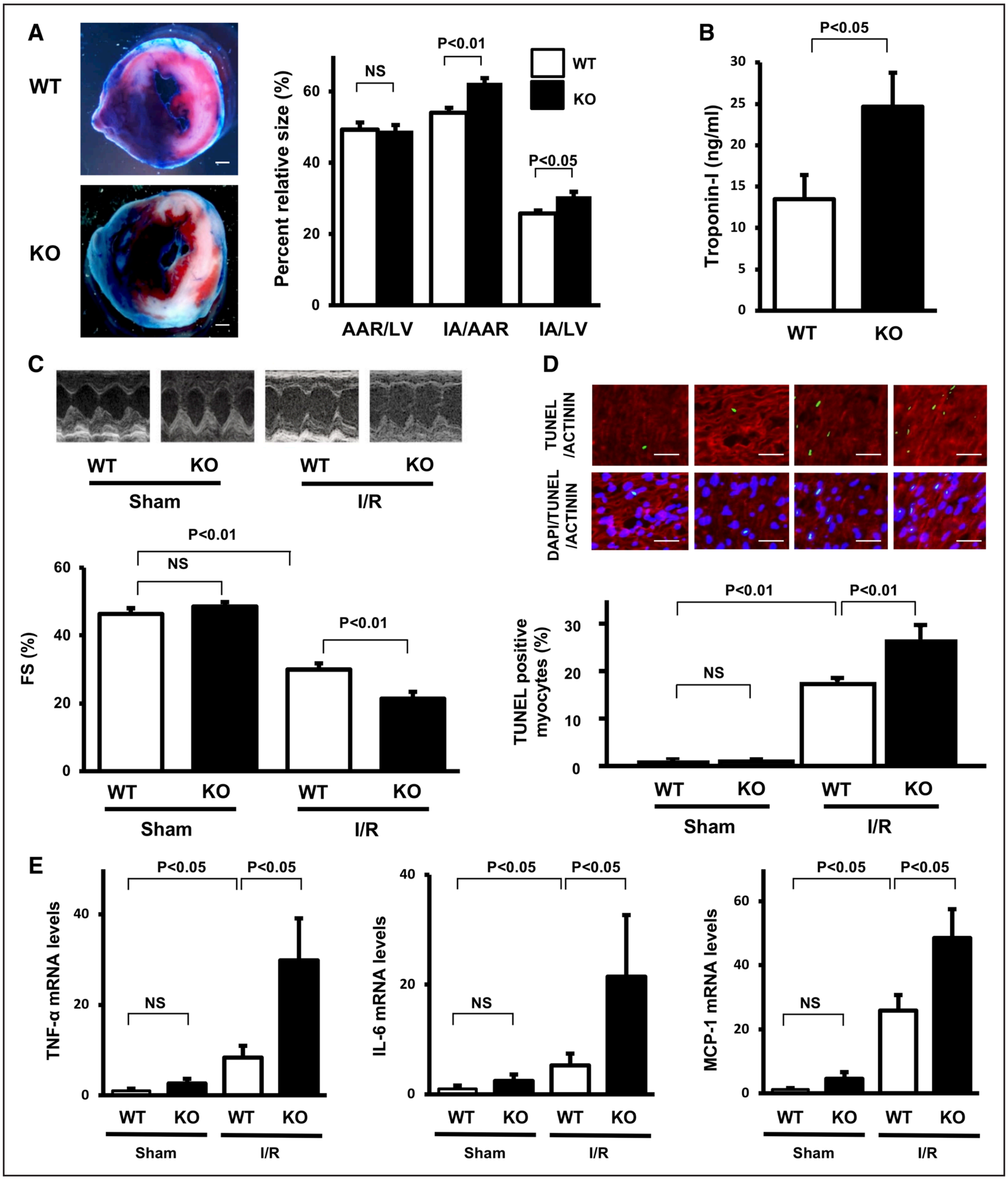

Figure 1. Myonectin-deficiency exacerbates myocardial infarction, apoptosis, and inflammatory response after ischemia-reperfusion (I/R). A, Representative pictures of myocardial tissues from wild-type (WT) and myonectin-knockout (KO) mice at 24 h after I/R (**left**). Blue color represents the nonischemic area. Red indicates the area at risk (AAR) and white indicates the infarct area (IA). Quantification of infarct size in WT (open square, n=6) and myonectin-KO (closed square, n=6) mice (**right**). Left ventricular (LV) area, AAR, and IA were measured. Scale bar=1 mm. **B**, Circulating levels of Troponin-I in myonectin-KO (n=6) and WT mice (n=6) after I/R. **C**, Representative M-mode echocardiograms for myonectin-KO and WT mice at day 7 after sham operation or I/R are shown in **upper**. Quantitative analysis of LV fractional shortening (%FS) in myonectin-KO and WT mice is shown in **lower** (n=8–10 in each group). **D**, Representative photographs of heart sections stained with terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) from WT and myonectin-KO mice at 24 h after sham operation or I/R (**left**). Scale bar=50 μm. Quantitative analysis of apoptotic nuclei in the AAR of myocardium from WT (n=4) and myonectin-KO (n=4) mice after sham operation or I/R (**right**). TUNEL-positive nuclei were counted in several randomly selected fields and expressed as a percentage of the total number of nuclei. **E**, Expression of TNF (tumor necrosis factor)-α, IL (interleukin)-6, and MCP-1 (monocyte chemoattractant protein 1) in myocardium of WT (n=6) and myonectin-KO mice (n=8) at 24 h after sham operation or I/R as determined by real-time polymerase chain reaction methods. DAPI indicates 4',6-diamidino-2-phenylindole; and NS, nonsignificant.

end-diastolic dimension and LV end-systolic dimension and decreases in fractional shortening and ejection fraction in both WT and myonectin-knockout mice, but myonectin-knockout mice showed further increases in LV end-diastolic dimension and LV end-systolic dimension, and further decreases in fractional shortening and ejection fraction as compared to WT mice (Figure 1C;

Online Table II). There were no significant differences in LV end-diastolic dimension, LV end-systolic dimension, fractional shortening, and ejection fraction between myonectin-knockout and WT mice after sham operation.

Enhanced apoptosis is one of the crucial features of the pathological cardiac remodeling in response to ischemia.[17] To investigate the impact of myonectin on myocyte apoptosis in the heart, we stained heart sections with terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL). Figure 1D shows representative fluorescent photographs of TUNEL-positive nuclei in the heart after sham or ischemia-reperfusion surgery. The proportion of TUNEL-positive cells in the AAR of myocardium was significantly higher in myonectin-knockout mice than in WT mice after ischemia-reperfusion injury, whereas little or no TUNEL-positive cells could be detected in the myocardium of WT or myonectin-knockout mice after sham operation (Figure 1D). In addition, myocyte size in the remote zone from infarct hearts was assessed by hematoxylin-eosin staining. No significant differences were observed in cross-sectional areas in the myocardium between WT and myonectin-knockout mice after sham operation or ischemia-reperfusion (Online Figure II).

Because inflammation is considered to cause myocardial injury after ischemia-reperfusion,[18] cardiac levels of proinflammatory mediators including, TNF-α, IL-6, and MCP-1 (monocyte chemoattractant protein 1) were assessed by real-time polymerase chain reaction methods. mRNA levels of TNF-α, IL-6, and MCP-1 in the AAR of myocardium were higher in myonectin-knockout than in WT mice after ischemia-reperfusion, whereas mRNA levels of these proinflammatory genes did not differ between myonectin-knockout and WT mice after sham operation (Figure 1E). Likewise, myonectin-knockout mice showed higher expression levels of the macrophage marker F4/80 in the AAR of myocardium after ischemia-reperfusion by a factor of 2.3±0.4 compared with WT mice (P<0.05). Thus, myonectin may protect the heart from ischemic injury by suppressing apoptosis and inflammatory reactions.

Myonectin Is Involved in the Inhibitory Effect of Endurance Exercise on Infarct Size After Ischemia-Reperfusion

To test the possible involvement of myonectin in the beneficial effect of endurance exercise on ischemic heart, WT and myonectin-knockout mice were run on a treadmill for 60 minutes 5 days a week for 4 weeks, followed by subjection to 60 minutes of myocardial ischemia and 24 hours of reperfusion. Sedentary WT and myonectin-knockout mice were housed in cages without running for the same duration, followed by subjection to myocardial ischemia-reperfusion. Treadmill exercise increased myonectin mRNA levels in soleus muscle of WT mice by a factor of 2.8±0.4 as determined by real-time polymerase chain reaction methods (Online Figure IIIA). Exercise also increased myonectin protein levels in soleus muscle in WT mice by a factor of 2.1±0.2 as determined by Western blot analysis (Online Figure IIIB). Furthermore, exercise increased plasma myonectin levels of WT mice by a factor of 2.5±0.1 (0.87±0.04 µg/mL in sedentary WT mice [n=4)] versus 2.19±0.06 µg/mL in exercised WT mice [n=3]) as assessed by ELISA methods. In contrast, myonectin mRNA expression in the heart in WT mice was markedly lower compared with that in the skeletal muscle, and treadmill exercise did not affect myonectin mRNA expression in the myocardium of WT mice (Online Figure IIIC). Ischemia-reperfusion had no effects on myonectin mRNA expression in myocardium of WT mice (Online Figure IIID). We also isolated cardiac myocytes, fibroblasts, and endothelial cells from sedentary or exercised WT and sedentary myonectin-knockout mice. Treadmill exercise training did not affect mRNA expression of myonectin in cardiac myocytes, fibroblasts, and endothelial cells from WT mice (Online Figure IIIE, IIIF, and IIIG). Moreover, myonectin mRNA expression could not be detected in cardiac myocytes, fibroblasts, and endothelial cells from myonectin-knockout mice (Online Figure IIIE, IIIF, and IIIG). Thus, these data indicate that myonectin is an endurance exercise-induced myokine consistent with a previous report.[13]

Treadmill running exercise significantly reduced the IA/AAR and IA/LV ratios in WT mice by 18.8±2.1% and 14.8±1.7%, respectively without affecting the AAR/LV ratio (Figure 2A). Of importance, treadmill exercise training trended to reduce myocardial infarct size in response to ischemia-reperfusion in myonectin-knockout mice, but this difference was not statistically significant. In addition, treadmill running significantly reduced the frequencies of TUNEL-positive myocytes and mRNA levels of TNF-α and IL-6 in ischemic heart of WT mice (Figure 2B and 2C). In contrast to WT mice, treadmill exercise did not significantly suppress the numbers of TUNEL-positive myocytes and mRNA levels of these proinflammatory genes in the ischemic myocardium of myonectin-knockout mice.

Myonectin-Transgenic Mice Are Protected From Myocardial Ischemia-Reperfusion Injury

To examine whether increased production of muscle-derived myonectin affects cardiac ischemic injury, we generated transgenic mice expressing the mouse myonectin gene under the control of the MCK (muscle creatine kinase) promoter (myonectin-transgenic mice) in the background of C57BL/6J. Myonectin-transgenic mice showed an increase in the expression of myonectin protein in soleus muscle, but not in heart tissue, compared with littermate control WT mice (Figure 3A). Plasma levels of myonectin were 3.3±0.1-fold higher in myonectin-transgenic mice (3.15±0.10 µg/mL, n=4) than in control WT mice (0.95±0.18 µg/mL, n=4). Under basal conditions, myonectin-transgenic mice are indistinguishable from WT mice as is the case in myonectin-knockout mice (Online Table III). Myonectin-transgenic mice showed marked reductions of IA/AAR and IA/LV ratios by 26.3±5.1% and 28.3±6.0%, respectively, with an accompanying decrease in circulating troponin-I level, as compared to WT mice (Figure 3B and 3C). The proportion of apoptotic myocytes in the ischemic myocardium was significantly lower in myonectin-transgenic mice than in WT mice after ischemia-reperfusion, whereas little or no TUNEL-positive cells could be detected in the heart of WT or myonectin-transgenic mice after sham operation (Figure 3D). Moreover, expression levels of TNF-α and IL-6 in ischemic heart were significantly reduced in myonectin-transgenic compared with WT mice, whereas mRNA levels of these proinflammatory genes in myocardium did not differ between sham-operated myonectin-transgenic and WT mice (Figure 3E). Myocardial interstitial fibrosis was assessed by picro-sirius red staining. Minimal interstitial fibrosis in the

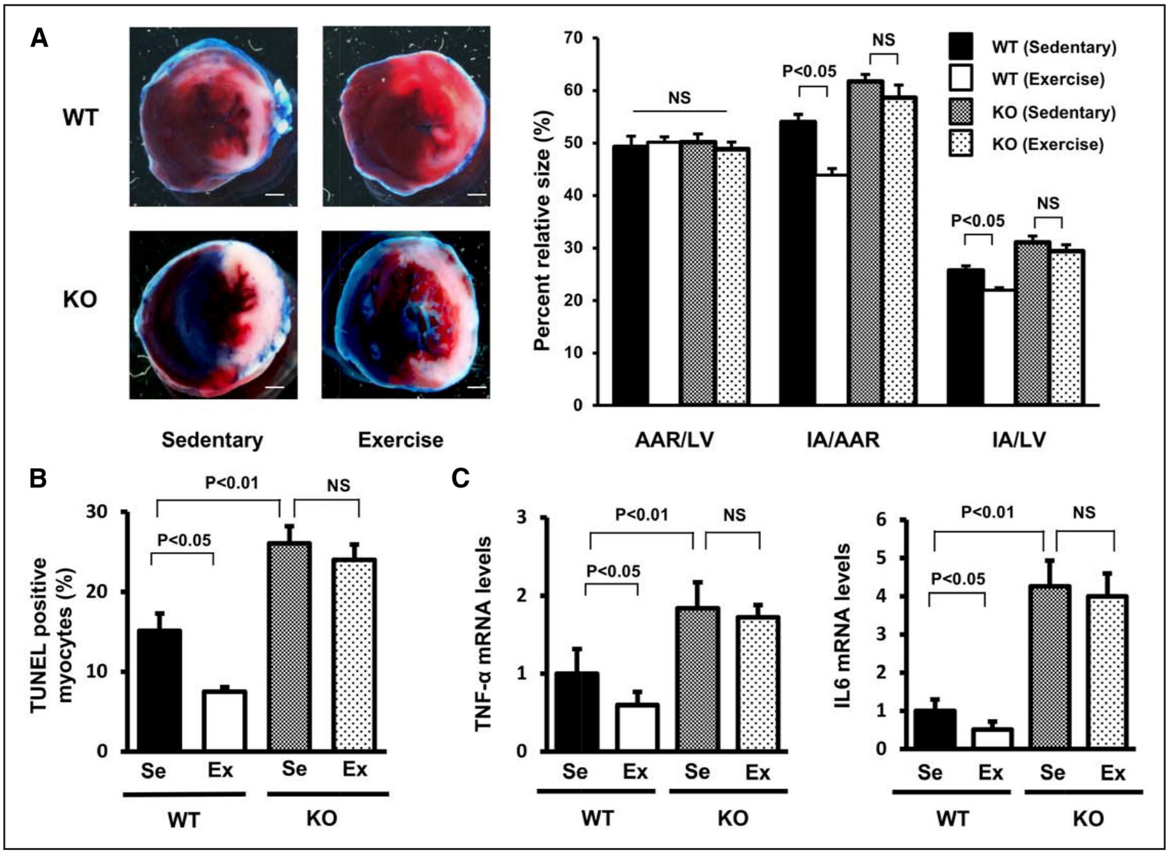

Figure 2. Myonectin is involved in the infarct-sparing effect of endurance exercise. A, Representative photographs of heart tissues from sedentary or exercised wild-type (WT) and myonectin-knockout (KO) mice in response to ischemia-reperfusion (I/R; **left**). Scale bar=1 mm. Quantification of infarct size in sedentary or exercised WT and myonectin-KO mice (sedentary WT mice: n=7, exercised WT mice: n=10, sedentary myonectin-KO mice: n=8, exercised myonectin-KO mice: n=8; **right**). **B**, Quantification of apoptotic nuclei in the area at risk (AAR) in myocardium from sedentary or exercised WT and myonectin-KO mice at 24 h after I/R (sedentary WT mice: n=4, exercised WT mice: n=4, sedentary myonectin-KO mice: n=4, exercised myonectin-KO mice: n=4). Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive nuclei were counted in several randomly selected fields and expressed as a percentage of the total number of nuclei. **C**, Expression of TNF (tumor necrosis factor)-α and IL (interleukin)-6 in myocardium of sedentary or exercised WT and myonectin-KO mice at 24 h after I/R as determined by real-time polymerase chain reaction methods (sedentary WT mice: n=4, exercised WT mice: n=4, sedentary myonectin-KO mice: n=4, exercised myonectin-KO mice: n=4). Results are presented as mean±SE. Ex indicates exercise; IA, infarct area; LV, left ventricular; NS, nonsignificant; and Se, sedentary.

AAR of hearts was detected in WT and myonectin-transgenic mice after ischemia-reperfusion, and no significant differences were observed in fibrotic areas between WT and myonectin-transgenic mice after ischemia-reperfusion or sham operation (Online Figure IV). These data suggest that muscle-derived myonectin may mediate some of beneficial actions of endurance exercise training on ischemic heart.

To test whether systemic administration of myonectin protein could minimize infarct area after myocardial ischemia, we intravenously injected a single dose of recombinant myonectin protein (200 ng/g mouse) or vehicle to WT mice at 5 minutes before the induction of ischemia. Systemic administration of myonectin protein to mice led to a significant reduction in infarct size after ischemia-reperfusion relative to vehicle treatment (Online Figure V).

Myonectin Attenuates Myocyte Apoptosis Through Its Ability to Promote the cAMP-Dependent Activation of Akt

To investigate the effect of myonectin on apoptosis at a cellular level, neonatal rat cardiac myocytes were subjected to H/R (hypoxia/reoxygenation) under conditions of serum deprivation in the presence or absence of recombinant myonectin protein. Treatment with physiological concentrations of myonectin protein dose-dependently suppressed the frequencies of TUNEL-positive cells under conditions of H/R (Figure 4A). We also assessed the phosphorylation of various apoptosis-related molecules in cardiac myocytes following treatment with myonectin. Treatment of cardiac myocytes with myonectin stimulated the phosphorylation of Akt, GSK-3β (glycogen synthase kinase-3), a downstream target of Akt, and CREB (cAMP response element binding protein) with maximal levels occurring at 15 minutes (Figure 4B). In contrast, myonectin did not affect the phosphorylation of ERK (extracellular signal-regulated kinase), p38, and AMPK (AMP-activated protein kinase) in cardiac myocytes.

To test whether Akt participates in antiapoptotic effects of myonectin, cardiac myocytes were transduced with adenoviral vectors expressing HA (human influenza hemagglutinin)-tagged dominant-negative mutant of Akt (Ad-dnAkt) or control β-galactosidase. Transduction with Ad-dnAkt suppressed myonectin-induced phosphorylation of GSK-3β in cardiac myocytes (Figure 4C). Ad-dnAkt also reversed the inhibitory effects of myonectin on myocyte apoptosis under conditions

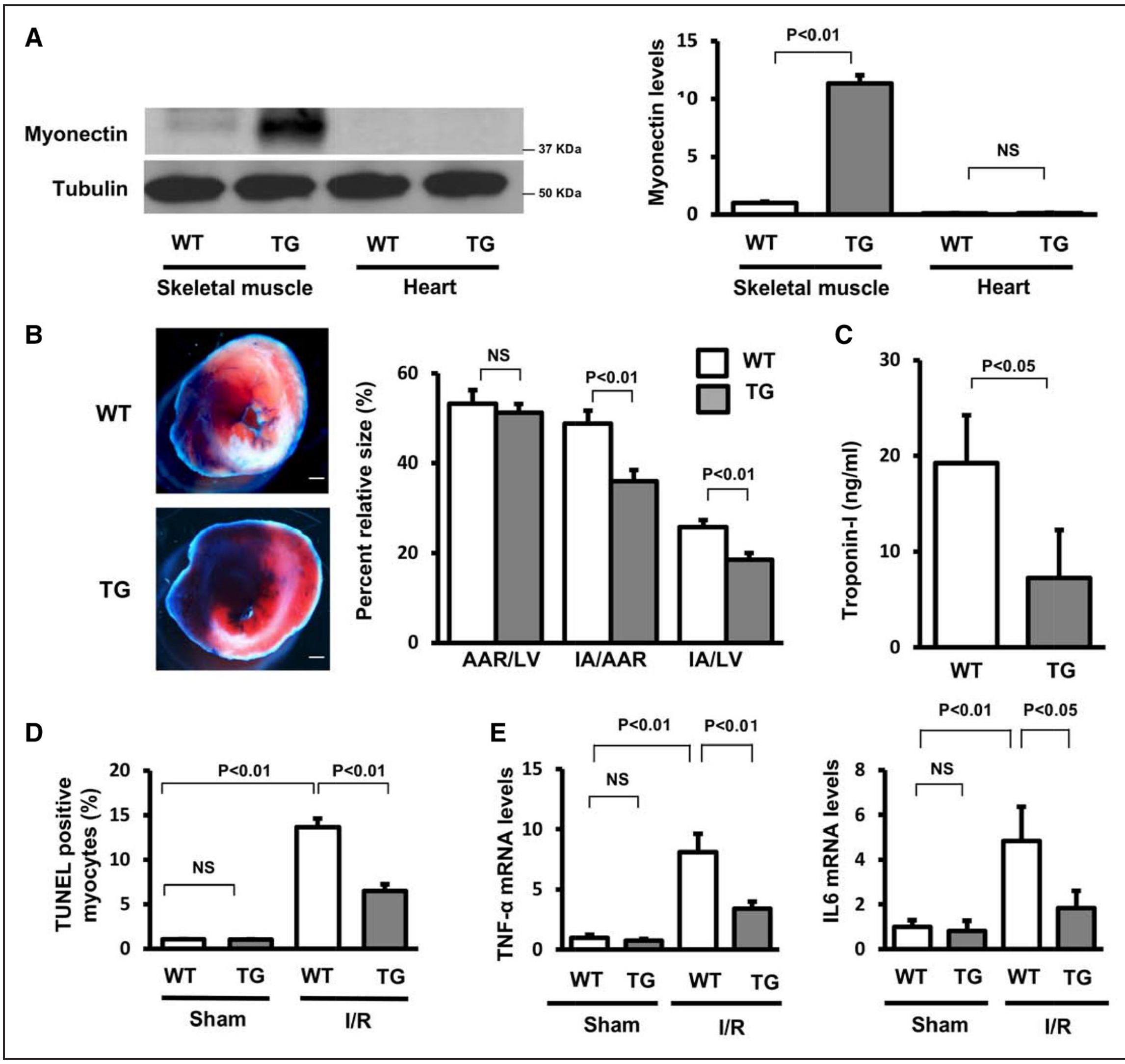

Figure 3. Myonectin-transgenic (TG) mice show reduced cardiac injury after ischemia-reperfusion (I/R). A, Myonectin protein expression in skeletal muscle (soleus) and heart tissue of wild-type (WT) and myonectin-TG mice. **B**, Representative pictures of myocardial tissues from WT and myonectin-TG mice at 24 h after I/R (**left**). Scale bar=1 mm. Quantification of infarct size in WT and myonectin-TG mice (n=8; **right**). **C**, Circulating levels of Troponin-I in WT and myonectin-TG after I/R (n=6). **D**, Quantitative analysis of apoptotic nuclei in the area at risk (AAR) of myocardium from WT (n=4) and myonectin-TG (n=4) mice hearts after sham operation or I/R. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive nuclei were counted in several randomly selected fields and expressed as a percentage of the total number of nuclei. **E**, Expression of TNF (tumor necrosis factor)-α and IL (interleukin)-6 in myocardium of WT (n=4) and myonectin-TG mice (n=4) at 24 h after sham operation or I/R as determined by real-time polymerase chain reaction methods. Results are presented as mean±SE. IA indicates infarct area; LV, left ventricular; and NS, nonsignificant.

of H/R (Figure 4D). These findings indicate that Akt activation is required for antiapoptotic actions of myonectin in cultured cardiac myocytes. To test whether cAMP signaling is involved in the effect of myonectin on apoptosis, cardiac myocytes were pretreated with the adenylate cyclase inhibitor SQ22536 or vehicle followed by stimulation with myonectin protein or vehicle. Pretreatment with SQ22536 diminished myonectin-stimulated phosphorylation of Akt and CREB in cardiac myocytes (Figure 4E). Pretreatment with SQ22536 also reversed the inhibitory effects of myonectin on H/R-induced apoptosis (Figure 4F). Therefore, myonectin can attenuate myocyte apoptosis, at least in part, through its ability to promote the cAMP-dependent activation of Akt.

Autophagy was also evaluated by measuring the LC3II (microtubule-associated protein light chain 3)/LC3I ratio in cardiac myocytes in vitro and in heart tissue in vivo as quantified by Western blot analysis. Cardiac myocytes were deprived of serum under conditions of normoxia or H/R in the presence or absence of myonectin protein. Treatment with myonectin did not affect the ratio of LC3II/LC3I in cardiac myocytes under normoxic conditions. H/R increased the LC3II/LC3I ratio in cardiac myocytes, and treatment with myonectin suppressed the LC3II/LC3I ratio in cardiac myocytes under conditions of H/R (Online Figure VIA). We also assessed the ratio of LC3II/LC3I in the hearts from WT and myonectin-knockout mice. Ischemia-reperfusion increased the LC3II/LC3I ratio in the hearts in both WT and myonectin-knockout mice, but the myonectin-knockout mice showed a further increase in myocardial LC3II/LC3I ratio after ischemia-reperfusion as compared to WT mice (Online Figure VIB). No significant differences were observed in LC3II/LC3I ratio in the hearts between WT and myonectin-knockout

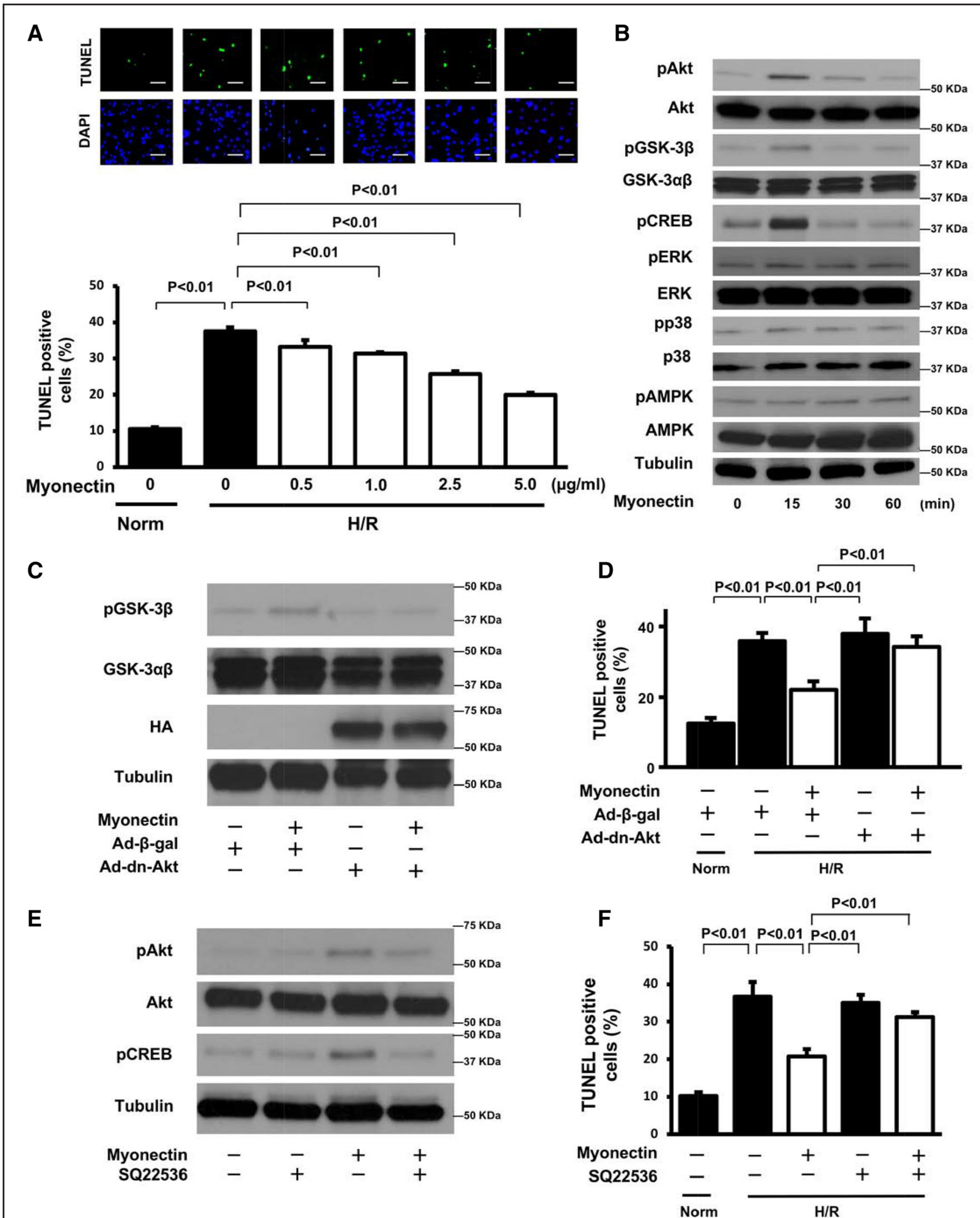

Figure 4. Myonectin attenuates cardiomyocyte apoptosis. A, Representative photomicrographs of terminal deoxynucleotidyl transferase-mediated dUTP nick-end end labeling (TUNEL)-positive cardiac myocytes. Rat neonatal cardiac myocytes were treated with myonectin (0.5, 1.0, 2.5, or 5.0 μg/mL) or vehicle in serum-free media under 36 h of normoxic conditions or 12 h of hypoxic conditions followed by 24 h of reoxygenation. Apoptotic nuclei were identified by TUNEL staining (green), and total nuclei were identified by DAPI (4',6-diamidino-2-phenylindole) counterstaining (blue). Scale bar=25 μm. Quantitative analysis of TUNEL-positive cells under normoxia or hypoxia-reoxygenation (H/R) conditions. **B**, Myonectin-stimulated phosphorylation signaling in cardiac myocytes. Changes in the phosphorylation levels of Akt (pAkt), GSK-3β (glycogen synthase kinase-3; pGSK-3β), CREB (cAMP response element binding protein; pCREB), ERK (extracellular signal-regulated kinase; pERK), p38 (pp38), and AMPK (AMP-activated protein kinase; pAMPK) after myonectin treatment (5 μg/mL) were determined by Western blot analysis. Representative blots are shown from 3 independent experiments. **C** and **D**, Effect of Akt inactivation on myonectin-stimulated pGSK-3β (**C**) and survival (**D**) of cardiac myocytes. Cardiac myocytes were transduced with adenoviral vectors expressing HA (human influenza hemagglutinin)-tagged dominant-negative mutant of Akt (Ad-dnAkt) or control β-galactosidase (Ad-β-gal) at an MOI (multiplicity of infection) of 25 for 24 h. Cardiac myocytes were treated with myonectin (5 μg/mL) or vehicle for 15 min, and pGSK-3β was analyzed by Western blotting. Representative blots are shown from 4 independent experiments (**C**). After transduction with adenoviral vectors, cardiac myocytes were cultured in the presence of myonectin (5.0 μg/mL) or vehicle under conditions of H/R, and TUNEL-positive nuclei were quantified (n=4; **D**). **E**, Effect of SQ22536 on myonectin-stimulated pAkt and CREB. Cardiac myocytes were pretreated with SQ22536 (10 μM) or vehicle for 60 min and stimulated with myonectin (5.0 μg/mL) or vehicle for 60 min. Representative blots are shown from 4 independent experiments. **F**, Effect of SQ22536 on myonectin-mediated inhibition of myocyte apoptosis. After pretreatment with SQ22536 (10 μM) or vehicle for 60 min, cardiac myocytes were cultured in the presence or absence of myonectin (5.0 μg/mL) under conditions of H/R (n=4).

mice after sham operation. These results suggest that myonectin can regulate cardiac autophagy activation under conditions of hypoxia or ischemia.

cAMP-Dependent Activation of Akt is Essential for the Anti-Inflammatory Actions of Myonectin in Macrophages

Because macrophage is one of the most important cells that produce proinflammatory cytokines during myocardial ischemia-reperfusion,[19] we examined the effects of myonectin on inflammatory responses of macrophages. RAW264.7 macrophages and murine peritoneal macrophages were pretreated with myonectin protein or vehicle followed by stimulation with lipopolysaccharide. Myonectin treatment dose-dependently reduced lipopolysaccharide-stimulated mRNA expression of TNF-α, IL-6, and MCP-1 in RAW264.7 macrophages (Figure 5A). Likewise, treatment with myonectin dose-dependently attenuated lipopolysaccharide-stimulated expression of TNF-α, IL-6, and MCP-1 in cultured peritoneal macrophages (Online Figure VII).

The transcription factor, NF-κB (nuclear factor-κB), is a key mediator of inflammatory response in various cells, including macrophages.[20] Thus, we investigated the effect of myonectin on NF-κB phosphorylation in response to lipopolysaccharide. Stimulation of RAW264.7 macrophages with lipopolysaccharide resulted in induction of NF-κB phosphorylation, which was suppressed by myonectin pretreatment (Figure 5B).

Myonectin stimulated the phosphorylation of Akt and CREB in macrophages with maximal levels occurring at 60 minutes (Figure 5C). To test the contribution of Akt to the anti-inflammatory actions of myonectin, RAW264.7 macrophages were transduced with Ad-dnAkt or adenoviral vector expressing β-galactosidase. Ad-dnAkt suppressed myonectin-induced phosphorylation of GSK-3β in macrophages (Figure 5D). Transduction with Ad-dnAkt reversed the inhibitory actions of myonectin on lipopolysaccharide-induced TNF-α expression in macrophages (Figure 5E). Treatment of RAW264.7 macrophages with myonectin also increased the intracellular cAMP levels (Figure 5F). Pretreatment with SQ22536 significantly reversed myonectin-mediated suppression of lipopolysaccharide-induced expression of TNF-α in cultured macrophages (Figure 5G). Pretreatment with SQ22536 also diminished myonectin-stimulated phosphorylation of Akt and CREB in macrophages (Figure 5H). These data suggest that cAMP-dependent activation of Akt is essential for the anti-inflammatory actions of myonectin in macrophages.

Ischemia-reperfusion increased the phosphorylation levels of Akt and CREB in sedentary WT hearts, and these inductions were further enhanced in the myocardium of exercised WT mice (Online Figure VIIIA). In contrast, the phosphorylation levels of Akt and CREB were similar in myocardium between sedentary and exercised WT mice after sham operation. Furthermore, the phosphorylation levels of Akt and CREB were significantly lower in myonectin-knockout hearts after ischemia-reperfusion compared with WT hearts, whereas the phosphorylation levels of Akt and CREB in myocardium did not differ between WT and myonectin-knockout mice after sham operation (Online Figure VIIIB). Thus, myonectin can act as a positive regulator of cAMP-dependent Akt activation in ischemic heart.

Sphingosine-1-Phosphate Is Involved in the Beneficial Actions of Myonectin on Macrophage Inflammation, Myocyte Apoptosis, and Ischemic Heart

Because S1P (sphingosine-1-phosphate) plays important roles in modulating inflammation and apoptosis through its association with S1P receptors,[21,22] we assessed the possible contribution of S1P in anti-inflammatory actions of myonectin. Treatment of cultured macrophages with myonectin increased S1P levels in media (Figure 6A). Pretreatment with VPC23019, an antagonist of S1PR1 (S1P receptor 1) and S1PR3, blocked myonectin-mediated suppression of lipopolysaccharide-induced TNF-α expression in macrophages (Figure 6B). Pretreatment of cultured macrophages with VPC23019 abolished the stimulatory effects of myonectin on cAMP accumulation (Figure 6C). Moreover, pretreatment with VPC23019 attenuated myonectin-induced phosphorylation of Akt and CREB in macrophages (Figure 6D). Consistent with these observations, S1P stimulated the phosphorylation of Akt and CREB in cultured macrophages (Online Figure IXA). Pretreatment with SQ22536 or VPC23019 reversed the suppressive effects of S1P on lipopolysaccharide-induced TNF-α expression in macrophages (Online Figure IXB). S1P is synthesized from sphingosine by SphK1 (sphingosine kinase-1).[21,23] To test the participation of SphK1 in the anti-inflammatory actions of myonectin in vitro, macrophages were transfected with siRNA (small interfering RNA) against SphK1 or unrelated control siRNA. Transfection of macrophages with siRNA targeting Sphk1 resulted in 75.5±3.9 % reduction of Sphk1 mRNA expression compared with control siRNA (Online Figure X). Knockdown of Sphk1 reversed myonectin-mediated suppression of lipopolysaccharide-induced TNF-α expression in macrophages (Figure 6E). These data indicate that myonectin-stimulated secretion of S1P contributes to activation of cAMP/Akt signaling pathway through S1P receptors, thereby resulting in resolution of macrophage inflammatory response.

Furthermore, treatment of cardiac myocytes with myonectin significantly increased S1P levels (Figure 7A). Treatment with VPC23019 reversed the inhibitory effects of myonectin on H/R-induced cardiomyocyte apoptosis (Figure 7B). Treatment of cardiac myocytes with myonectin increased the intracellular cAMP levels, and pretreatment with VPC23019 abolished the stimulatory effects of myonectin on cAMP accumulation in cardiac myocytes (Figure 7C). Moreover, pretreatment with VPC23019 diminished myonectin-stimulated phosphorylation of Akt and CREB in cardiac myocytes (Figure 7D). Knockdown experiments were also performed with siRNAs targeting S1PR1 or S1PR3 because S1PR1 and S1PR3 are highly expressed in cardiac myocytes. After transfection of cardiac myocytes with siRNAs against S1PR1 or S1PR3, S1PR1 mRNA was decreased by 78.1±2.0 %, and S1PR3 mRNA was decreased by 78.9±0.8 % (Online Figure XB and XC). The myonectin-induced phosphorylation levels of Akt and CREB in cardiac myocytes were reduced by knockdown of S1PR3 but not by ablation of S1PR1 (Figure 7E). Furthermore, knockdown experiments were performed with siRNA against SphK1. Treatment of cardiomyocytes with siRNA targeting Sphk1 resulted in 72.8±1.5 % reduction of Sphk1 mRNA expression compared with control siRNA (Online Figure XD).

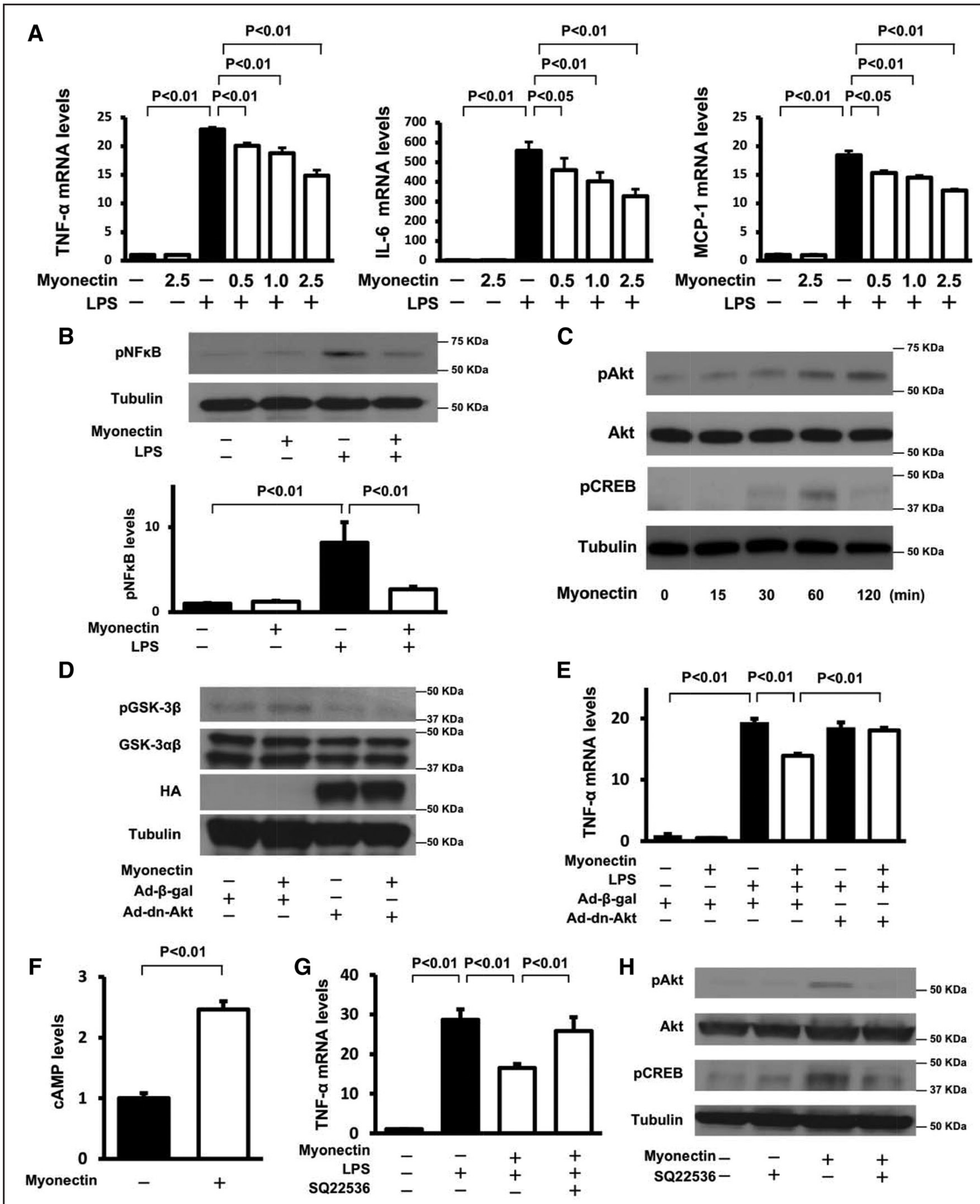

Figure 5. Myonectin attenuates macrophage inflammatory response. A, Effect of myonectin on lipopolysaccharide (LPS)-induced expression of TNF (tumor necrosis factor)-α, IL (interleukin)-6, and MCP-1 (monocyte chemoattractant protein 1) in RAW264.7 macrophages. Cells were pretreated with myonectin (0.5, 1.0, or 2.5 μg/mL) or vehicle for 60 min and stimulated with LPS (100 ng/mL) or vehicle for 6 h. mRNA expression of TNF-α, IL-6, and MCP-1 was measured by real-time polymerase chain reaction methods (n=6). **B**, Effect of myonectin on NF-kB (nuclear factor kB) phosphorylation (pNF-κB) in response to LPS in RAW264.7 macrophages. Cells were pretreated with myonectin (5.0 μg/mL) or vehicle for 60 min followed by stimulation with LPS (100 ng/mL) or vehicle for 30 min (n=4). pNF-κB was determined by Western blot analysis. Immunoblots were normalized to α-tubulin signal. **C**, Time-dependent changes in the phosphorylation of Akt (pAkt) and CREB (cAMP response element binding protein; pCREB) in RAW264.7 macrophages following stimulation with myonectin as determined by Western blot analysis. Representative blots are shown from 4 independent experiments. **D** and **E**, Effect of Akt inactivation on GSK-3β (glycogen synthase kinase-3) phosphorylation (pGSK-3β; **D**) and LPS-induced expression of TNF-α (**E**) after treatment with myonectin. RAW264.7 macrophages were transduced with adenoviral vectors expressing HA (human influenza hemagglutinin)-tagged dominant-negative mutant of Akt (Ad-dnAkt) or control β-galactosidase (Ad-β-gal) at an MOI (multiplicity of infection) of 25 for 24 h. Macrophages were treated with myonectin (5.0 μg/mL) or vehicle for 60 min, and pGSK-3β was analyzed by Western blotting. Representative blots are shown from 3 independent experiments (**D**). After transduction with adenoviral vectors, RAW264.7 macrophages were treated with myonectin (5.0 μg/mL) or vehicle, followed by stimulation with LPS or vehicle for 6 h (n=4; **E**). **F**, Effect of myonectin on the intracellular cAMP levels in media of in RAW264.7 macrophages. RAW264.7 were treated with myonectin (5.0 μg/mL) or vehicle for 60 min (n=4). **G**, Effect of SQ22536 on myonectin-mediated suppression of LPS-induced TNF-α expression in RAW264.7 macrophages. After pretreatment with SQ22536 (10 μM) or vehicle, cells were treated with myonectin (5.0 μg/mL) or vehicle, followed by stimulation with LPS or vehicle for 6 h (n=6). **H**, Effect of SQ22536 on myonectin-stimulated pAkt and pCREB in RAW264.7 macrophages. RAW264.7 macrophages were pretreated with SQ22536 (10 μM) or vehicle for 60 min and stimulated with myonectin (5.0 μg/mL) or vehicle for 60 min. Representative blots are shown from 4 independent experiments. Results are shown as the mean±SE.

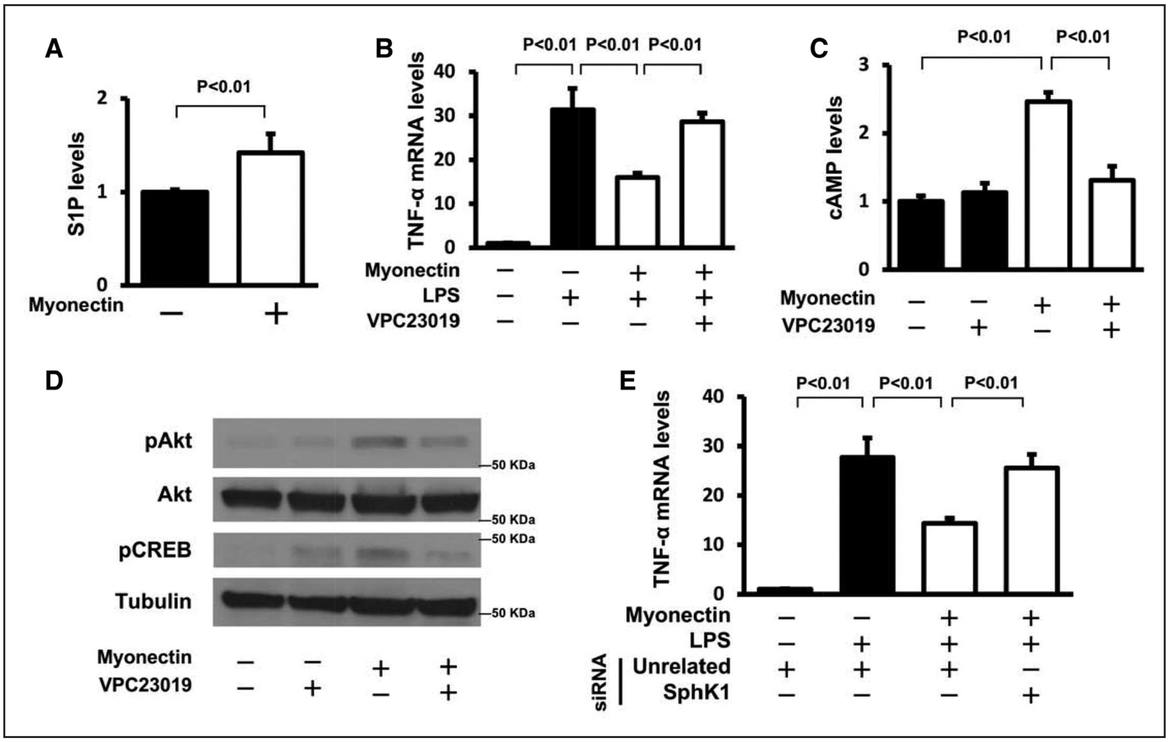

Figure 6. S1P (sphingosine-1-phosphate) is involved in the anti-inflammatory actions of myonectin in macrophages. A, Effect of myonectin on S1P levels in media of RAW264.7 macrophages. RAW264.7 macrophages were treated with myonectin (5.0 μg/mL) or vehicle for 60 min. S1P levels in media of RAW264.7 macrophages were measured by LC-MS/MS (liquid chromatography/mass spectrometry; n=6). **B**, Effect of VPC23019 on myonectin-mediated inhibition of TNF (tumor necrosis factor)-α expression in macrophages. After pretreatment with VPC23019 (1 μM) or vehicle, cells were treated with myonectin (5.0 μg/mL) or vehicle, followed by stimulation with lipopolysaccharide (LPS) or vehicle for 6 h (n=6). **C** and **D**. Effect of VPC23019 on myonectin-induced accumulation of cAMP (**C**) and myonectin-stimulated phosphorylation of Akt (pAkt) and CREB (cAMP response element binding protein, pCREB; **D**) in RAW264.7 macrophages. RAW264.7 macrophages were pretreated with VPC23019 (1 μM) or vehicle for 60 min and stimulated with myonectin (5.0 μg/mL) or vehicle for 60 min. Representative blots are shown from 4 independent experiments. **E**, Effect of knockdown of SphK1 (sphingosine kinase-1) on myonectin-mediated inhibition of TNF-α expression in RAW264.7 macrophages. After transfection with siRNA (small interfering RNA) targeting SphK1 or unrelated siRNA, cells were treated with myonectin (5.0 μg/mL) or vehicle, followed by stimulation with LPS or vehicle for 6 h (n=6). Results are presented as mean±SE.

Ablation of Sphk1 blocked the inhibitory effects of myonectin on H/R-induced cardiomyocyte apoptosis (Figure 7F). Therefore, it is likely that myonectin can attenuate myocyte apoptosis through its ability to promote cAMP/Akt signaling pathway, at least in part, through the S1PR3.

We also assessed cardiac levels of S1P and cAMP in sedentary or exercised WT and myonectin-knockout after ischemia-reperfusion injury. Treadmill exercise significantly increased the levels of S1P and cAMP in ischemic heart of WT mice. In contrast to WT mice, treadmill exercise had no effects on S1P and cAMP levels in ischemic heart of myonectin-knockout mice (Online Figure XIA and B). Thus, these data suggest that myonectin may mediate some of stimulatory effects of endurance exercise on S1P and cAMP production in ischemic heart.

Finally, we examined whether the S1P-dependent pathway is involved in myonectin-mediated cardioprotection in vivo. We intraperitoneally injected VPC23019 or vehicle into WT and myonectin-transgenic mice before induction of myocardial ischemia. Administration of VPC23019 reduced phosphorylation levels of Akt and CREB in both WT and myonectin-transgenic mice (Figure 8A). Furthermore, administration of VPC23019 to myonectin-transgenic mice significantly reversed the reduction of myocardial infarct size in response to ischemia-reperfusion (Figure 8B). In contrast, VPC23019 trended to increase myocardial infarct size in WT mice following ischemia-reperfusion, but this difference was not statistically significant. These in vivo data suggest that S1P-dependent pathway is involved in myonectin-induced reduction of myocardial ischemic injury. Taken together, myonectin functions as an exercise-induced myokine that prevents acute ischemic injury in the heart by its ability to reduce cardiomyocyte apoptosis and macrophage inflammation through the S1P/cAMP/Akt-dependent mechanisms (Figure 8C).

Discussion

The present study provides the first evidence that myonectin functions as an endurance exercise-driven myokine which provides cardioprotection. We found that circulating and muscle myonectin levels are increased by endurance treadmill exercise, which is effective in reducing acute myocardial ischemic injury in WT mice. Myonectin-deficiency led to exacerbation of myocardial infarction, cardiac dysfunction, apoptosis, and inflammation after ischemia-reperfusion, whereas overexpression of myonectin in skeletal muscle resulted in a marked improvement of cardiac ischemic injury. Of note, the effects of treadmill exercise on improving cardiac damage were diminished under conditions of myonectin-deficiency. Thus, it is conceivable that endurance exercise confers resistance to myocardial ischemic damage, at least in part, through upregulation of muscle-derived myonectin.

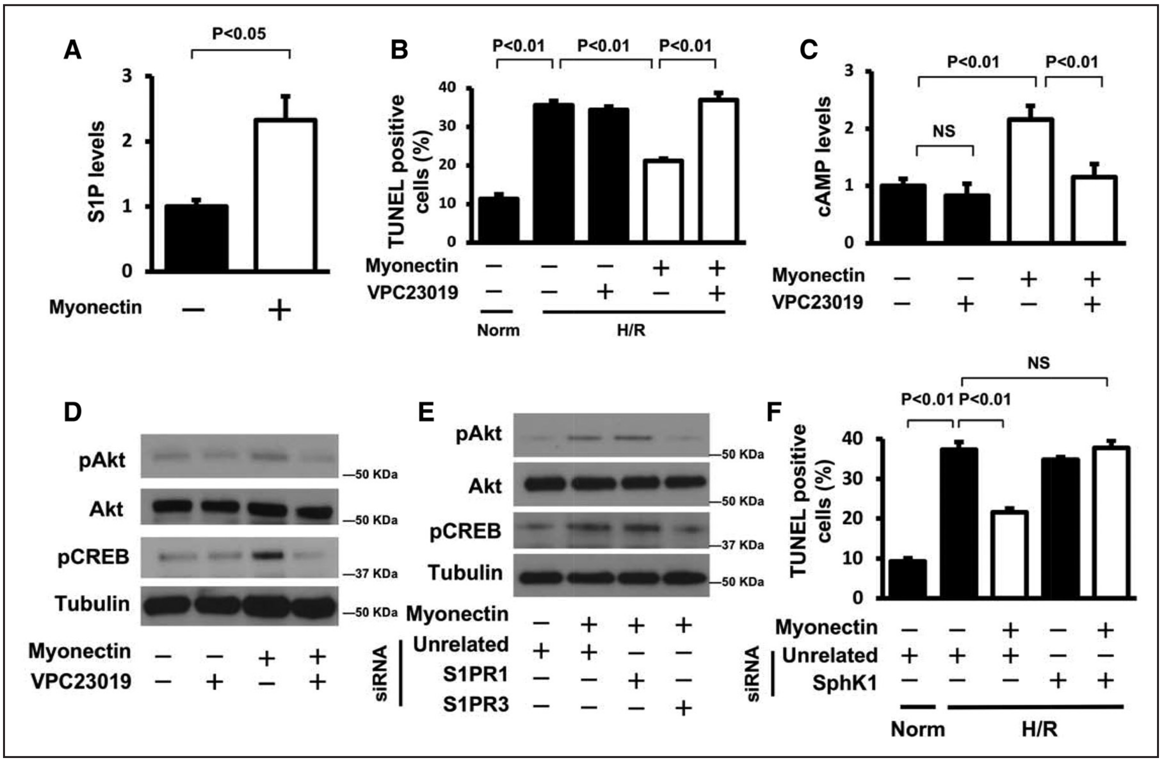

Figure 7. S1P (sphingosine-1-phosphate) is involved in the antiapoptotic actions of myonectin in cardiac myocytes. **A**, Effect of myonectin on S1P levels in media of cardiac myocytes. Cardiac myocytes were treated without myonectin (5.0 µg/mL) or vehicle for 15 min. S1P levels in media of cardiac myocytes were measured by LC-MS/MS (liquid chromatography/mass spectrometry; n=4). **B**, Effect of VPC23019 on myonectin-induced inhibition of myocyte apoptosis. After pretreatment with VPC23019 (1 µM) or vehicle for 60 min, cardiac myocytes were cultured in the presence or absence of myonectin (5.0 µg/mL) under conditions of hypoxia/reoxygenation (H/R; n=4). **C** and **D**, Effect of VPC23019 on myonectin-induced accumulation of cAMP (**C**) and myonectin-stimulated phosphorylation of Akt (pAkt) and CREB (cAMP response element binding protein, pCREB; **D**) in cardiac myocytes. Cardiac myocytes were pretreated with VPC23019 (1 µM) or vehicle for 60 min and stimulated with myonectin (5.0 µg/mL) or vehicle for 60 min. Representative blots are shown from 4 independent experiments. **E**, Effect of knockdown of S1PR1 (S1P receptor 1) or S1PR3 on myonectin-stimulated pAkt and pCREB in cardiac myocytes. After transfection with siRNA (small interfering RNA) targeting S1PR1 or S1PR3, or unrelated siRNA, cardiac myocytes were treated with myonectin (5.0 µg/mL) or vehicle for 60 min. Representative blots are shown from 4 independent experiments. **F**, Effect of knockdown of SphK1 (sphingosine kinase-1) on myonectin-induced inhibition of myocyte apoptosis. After transfection with siRNA targeting SphK1 or unrelated siRNA, cardiac myocytes were cultured in the presence or absence of myonectin (5.0 µg/mL) under conditions of H/R (n=4). Results are presented as mean±SE. NS indicates nonsignificant; and TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

The ability of myonectin to protect the myocardium from ischemic injury is likely because of its ability to stimulate Akt-dependent signaling within heart. Akt is shown to protect against myocardial ischemic injury.[24] It has also been shown that Akt reduces apoptosis of cardiac myocytes and inflammatory responses of macrophages.[24,25] Our data demonstrated that myonectin promoted Akt activation in cultured myocytes and macrophages, as well as in ischemic hearts of mice. Inhibition of Akt activity also blocked the suppressive effects of myonectin on macrophage inflammation and cardiomyocyte apoptosis. Thus, myonectin may suppress myocyte apoptosis and macrophage inflammatory response, at least in part, via activation of Akt, thereby leading to reduction of cardiac injury. Consistent with these findings, myonectin has been shown to promote Akt activation in cultured hepatocytes and in mouse liver, leading to suppression of autophagy.[14] Taken together, these observations indicate that the myonectin-Akt regulatory axis may play a crucial role in various disease processes.

S1P has been shown to exert antiapoptotic and anti-inflammatory actions in various type of cells and play an important role in preventing myocardial ischemic injury.[26,27] Our data showed that myonectin increased S1P levels in media of cultured macrophages and cardiomyocytes. Blockade of S1P signaling reversed the inhibitory effects of myonectin on myocyte apoptosis and macrophage inflammatory response. The current study also demonstrated that inhibition of cAMP-dependent signaling blocked myonectin-mediated activation of Akt, as well as myonectin-induced reduction of myocyte apoptosis and macrophage inflammation. Moreover, inhibition of S1P pathway diminished the stimulatory effects of myonectin on cAMP-dependent activation of Akt. Similarly, the addition of exogenous S1P attenuated macrophage inflammatory response via activation of cAMP and Akt signaling. Thus, it is conceivable that myonectin stimulates the secretion of S1P from macrophages and cardiac myocytes in an autocrine or paracrine manner, and promotes intracellular cAMP-dependent Akt signaling cascade, thereby, leading to resolution of inflammation and apoptosis. Our data also indicated that myonectin positively regulated the phosphorylation of CREB and Akt in ischemic heart of mice. Of note, the myocardial infarct-sparing effect of myonectin in vivo was diminished by inhibition of S1P pathway. These data suggest that the ability of myonectin to prevent acute cardiac injury is likely to be mediated, at least in part, through its ability to stimulate the S1P-cAMP-Akt signaling pathway within the heart.

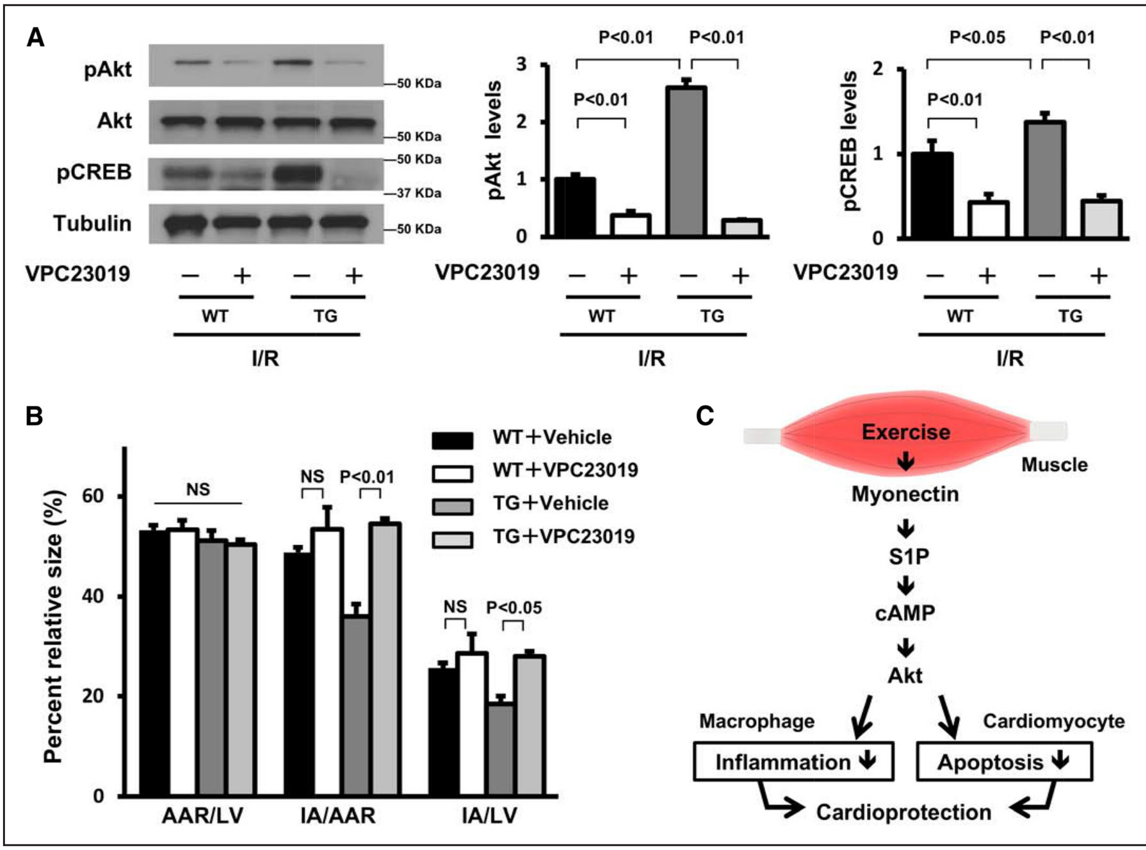

Figure 8. Role of S1P (sphingosine-1-phosphate) signaling in myonectin-mediated cardioprotection in vivo. Wild-type (WT) and myonectin-transgenic (TG) mice were intraperitoneally treated with VPC23019 (1.0 µg/g mouse) or vehicle, followed by subjection to ischemia-reperfusion (I/R). **A**, Phosphorylation of Akt (pAkt) and CREB (cAMP response element binding protein; pCREB) in ischemic heart. Representative blots are shown from 4 independent experiments. Relative phosphorylation levels of Akt and CREB were quantified using the computerized digital image analysis system. Immunoblots were normalized to α-tubulin signal. **B**, Quantitative analysis of infarct size in WT and myonectin-TG mice treated with VPC23019 (1.0 µg/g mouse) or vehicle after I/R. The infarct area (IA), area at risk (AAR), and left ventricular (LV) areas were measured (n=4). **C**, Proposed scheme for the mechanism by which myonectin protects the heart from ischemic injury. Myonectin functions as an exercise-induced myokine that prevents acute ischemic injury in the heart by its abilities to reduce cardiomyocyte apoptosis and macrophage inflammation through the S1P/cAMP/Akt-dependent mechanisms. Results are presented as mean±SE. NS indicates nonsignificant.

Exercise therapy, such as endurance and resistance training, is believed to improve quality of life, cardiovascular and metabolic function, and prognosis in patients with various cardiovascular diseases, including ischemic heart disease.[2,4–6,28] Several myokines, including IL-6 and IL-15, are reported to act as contraction-inducible factors and modulate metabolic function.[7,11] Skeletal muscle hypertrophy, that is related to resistance training, leads to increased production of follistatin-like 1, which improves endothelial cell function, vascular injury, and myocardial ischemic damage.[29–31] Compared with these myokines, our data suggest that myonectin is a unique endurance exercise-induced myokine which exerts salutary actions on ischemic injury in the heart. Thus, it is possible that the approaches aimed at increasing circulating myonectin levels can mimic the cardioprotective effects of exercise.

Myonectin is a member of adiponectin paralogs CTRPs. Adiponectin is an adipose-specific secretory protein which can protect against myocardial ischemic injury.[32] It has been shown that adiponectin can directly affect intracellular signaling pathways in ischemic heart in an endocrine manner, at least in part, through its ability to attenuate myocyte apoptosis by binding to its receptor T-cadherin.[33] Our data suggest that, like adiponectin, myonectin can modulate the anti-inflammatory and antiapoptotic signaling cascades in the myocardium in an endocrine fashion. However, nothing is known about the binding protein or receptor of myonectin. Thus, future studies will be required to clarify the myonectin receptor-mediated intracellular signaling pathways in cardiovascular tissues.

In conclusion, our data showed that myonectin reduces cardiomyocyte apoptosis and macrophage inflammatory response through the S1P-dependent activation of cAMP/Akt pathway, thereby contributing to improvement of myocardial ischemia-reperfusion injury. Thus, it is likely that myonectin-S1P regulatory axis acts as a novel link between skeletal muscle and heart and plays a crucial role in preventing cardiac ischemic injury. Our observations also indicate that myonectin serves as a cardioprotective myokine, which can partly mediate the cardiovascular benefits of endurance exercise, suggesting that myonectin can represent a novel target molecule for prevention or treatment of cardiac diseases that are improved by physical exercise.

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Disclosures

None.

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