A Novel Cardioprotective Agent in Cardiac Transplantation: Metformin Activation of AMP-Activated Protein Kinase Decreases Acute Ischemia-Reperfusion Injury and Chronic Rejection

Jocelyn T. Chin, Joshua J. Troke, Naoyuki Kimura, Satoshi Itoh, Xi Wang, Owen P. Palmer, Robert C. Robbins, Michael P. Fischbein*

Department of Cardiothoracic Surgery, Stanford University School of Medicine, Stanford, California

The main cause of mortality after the first year from cardiac transplantation is cardiac allograft vasculopathy (CAV†), which leads to chronic rejection of the heart. To improve long-term outcomes in cardiac transplantation, treatments to prevent or diminish CAV are actively being researched. Ischemia-reperfusion (I-R) injury has been shown to be the strongest alloantigen-independent factor in the development of CAV. Here, we investigate the use of metformin in murine cardiac transplantation models as a novel cardioprotective agent to limit acute I-R injury and subsequent chronic rejection. We show that metformin treatment activates AMP-activated kinase (AMPK) in vitro and in vivo. In the acute transplantation model, metformin activation of AMPK resulted in significantly decreased apoptosis in cardiac allografts on postoperative day (POD) 1 and 8. In the chronic transplantation model, metformin pretreatment of allografts led to significantly improved graft function and significantly decreased CAV, as measured on POD 52. Taken together, our results in the acute and chronic rejection studies suggest a potential cardioprotective mechanism for metformin; we demonstrate a correlation between metformin-induced decrease in acute I-R injury and metformin-related decrease in chronic rejection. Thus, one of the ways by which metformin and AMPK activation may protect the transplanted heart from chronic rejection is by decreasing initial I-R injury inherent in donor organ preservation and implantation. Our findings suggest novel therapeutic strategies for minimizing chronic cardiac rejection via the use of metformin- and AMPK-mediated pathways to suppress acute I-R injury.

*To whom all correspondence should be addressed: Michael P. Fischbein, Department of Cardiothoracic Surgery, 300 Pasteur Drive, Falk Cardiovascular Research Building (CVRB), Stanford University, Stanford, CA 94305; Tele: (650) 724-0831; E-mail: mfischbe@stanford.edu.

†Abbreviations: CAV, cardiac allograft vasculopathy; I-R, ischemia-reperfusion; UKPDS, United Kingdom Prospective Diabetes Study; AMPK, AMP-activated protein kinase; AMPKK, AMP-activated protein kinase kinase; LKB1, liver kinase B1; CaMKK, calmodulin-dependent protein kinase kinase; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; PFK2, phosphofructokinase 2; ROS, reactive oxygen species; KD, kinase dead; POD, postoperative day; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; pAMPK, phosphorylated AMPK; SEM, standard error of the mean; SPECT, single proton emission computed tomography; ELISA, enzyme-linked immunosorbent assay; IP, intraperitoneal.

Keywords: Metformin, AMPK, apoptosis, I-R injury, acute rejection, chronic rejection, cardiac transplantation
INTRODUCTION

Almost 6 million people in the United States have heart failure [1], a subset of which does not respond to even maximal medical therapy. The only definitive treatment for these patients with end stage heart failure is cardiac transplantation. The main cause of mortality after the first transplant year is cardiac allograft vasculopathy (CAV), or chronic rejection [1]. In order to improve long-term outcomes in cardiac transplantation, treatments to prevent or diminish CAV are actively being researched. Ischemia-reperfusion (I-R) injury has been shown to be the strongest alloantigen-independent factor in the development of CAV [2,3]. Much attention has been given to metformin and its potential cardioprotective property of decreasing I-R injury. Metformin is one of the most commonly prescribed anti-diabetic drugs for Type 2 diabetes patients. In the United Kingdom Prospective Diabetes Study (UKPDS) of diabetic patients, metformin usage was found to decrease the risk of myocardial infarction by 14 percent and decrease the likelihood of heart failure by 16 percent [4]. These observations were confirmed in isolated rat hearts, where metformin reduced cardiac functional loss and acutely improved cardiac function after ischemia [5]. However, there are currently no defined molecular pathways for metformin’s cardioprotective properties. Recent literature suggests that the therapeutic effects of metformin may be mediated by activation of AMP-activated protein kinase (AMPK) [6], an endogenous signaling enzyme and master regulator of energy homeostasis [7].

As shown in Figure 1, AMPK is a heterotrimeric molecule that sensitively detects changes in the AMP/ATP ratio. Upon binding two molecules of AMP, AMPK is activated through phosphorylation by several upstream kinases (AMPKK), including LKB1 (liver kinase B1) and CaMKK (calmodulin dependent protein kinase kinase) [8]. AMPK is activated by stressors that increase the AMP/ATP ratio, such as glucose deprivation, ischemia and hypoxia, and exercise and skeletal muscle contraction. Once activated, AMPK has a diverse array of effects, including, but not limited to, inhibition of biosynthesis (hepatic fat synthesis, muscle glycogen synthesis, and protein synthesis), cell cycle arrest in G1 through activation of p53-p21 system, and upregulation of catabolism (skeletal muscle glucose uptake, glycolysis in cardiomyocytes, and fatty acid oxidation) [8].

AMPK activation by metformin has been observed in hepatocytes, skeletal muscle, and cardiomyocytes [9]. In LKB1 knockout mice, metformin is not as efficacious in lowering blood glucose [10], suggesting that AMPK activation is necessary for metformin’s therapeutic actions. In cultured bovine aortic endothelial cells, metformin dose-dependently activates AMPK by increasing the phosphorylated, active form, and increasing the association of AMPK with LKB1, its upstream kinase [11]. Therefore, metformin is likely to increase AMPK activation by facilitating the phosphorylation process. The interplay of metformin and AMPK yields cardioprotective results. In cultured canine cardiomyocytes, metformin prevents cell death through AMPK activation. Similarly, in dog models of heart failure, metformin promotes phosphorylation of AMPK, decreases apoptosis, and generally improves function of the failing hearts [12]. The cardioprotective benefits of metformin are mimicked with addition of AMPK agonist AICAR [6] and abrogated in AMPK knockout mice. Consequently, in the context of heart disease, metformin’s cardioprotective benefits may be mediated by AMPK.

There has been extensive research elucidating AMPK’s role in cardioprotection from I-R injury. During periods of hypoxia, AMPK directly phosphorylates cardiac phosphofructokinase 2 (PFK2) and increases uptake of glucose through Glut4 translocation, stimulating glycolysis in the cardiomyocytes [10]. Besides serving as a substrate for glycolysis, the increased glucose flux also can stimulate the pentose phosphate pathway to generate NADPH and reduced glutathione, which neutralize the reactive oxygen species (ROS) generated in I-
R injury [13]. Indeed, in transgenic mice with a kinase dead (KD) AMPK, the heart functions normally before ischemia, but during ischemia, \textit{in vitro} KD hearts cannot uptake glucose and perform glycolysis. Furthermore, the KD hearts have increased apoptosis and necrosis compared to control hearts [14]. In another transgenic mouse study, isolated hearts from transgenic mice with dominant negative \( \alpha \) subunits of AMPK have impaired left ventricular recovery of systolic function after I-R injury [15]. Therefore, AMPK protects hearts from I-R injury by sustaining energy supply during ischemic stress and reducing the likelihood of cardiomyocyte cell death.

The cardioprotective effects of metformin and AMPK may also be applicable in cardiac transplantation and chronic organ rejection. AICAR (an AMPK agonist), applied before and during cardiac transplantation, resulted in decreased patient mortality and myocardial injury, as well as improved clinical outcomes [9]. We hypothesize that metformin protects the allograft by activating AMPK. AMPK can prevent cardiomyocyte death from I-R injury by sustaining energy supply during ischemic stress, thereby reducing apoptosis in allografts. By decreasing initial I-R injury, metformin and AMPK activation may then lead to decreased CAV and improved chronic rejection outcomes. Metformin is a novel agent to reduce chronic rejection due to CAV by limiting I-R related cell death. Metformin preconditioning and control of acute cardiomyocyte cell death in allografts has the potential to delay development of chronic cardiac rejection, by means other than immune suppression.

**MATERIALS AND METHODS**

**Acute Transplantation Study**

All animal protocols were approved by the Administrative Panel on Laboratory Animal Care at Stanford University and followed the NIH and USDA Guidelines for the Care and Use of Animals in Research. Our Stanford protocol number is 21536. FVB/NJ donor hearts were heterotopically transplanted into C57BL/6J mice (MHC I and II mismatch). There were three treatment conditions: metformin (Bristol-Myers Squibb, New York City, NY), AICAR/AMPK agonist (Sigma-Aldrich, St. Louis, MO), or PBS control/vehicle (Sigma-Aldrich, St. Louis, MO). FVB/NJ donor hearts were first preconditioned with their respective treatment solution (metformin, AICAR, or PBS control). Treatment included: 1) an intraperitoneal (IP) injection in the donor animal 1 hour prior to surgery; 2) vena cava injection 2 minutes before donor organ procurement; and 3) immersion of donor organ in treatment solution during the cold-ischemia time (approximately 30 minutes). Following the heterotopic heart transplant, recipient mice were given IP injection of treatment solution every other day. Donor hearts were harvested on postoperative day (POD) 1 and 8 (\( n = 4 \) for each time point and treatment condition). Protein extract was collected through cell lysis, centrifugation, and protease inhibitor treatment. Experimental method is summarized in Figure 2.
Chronic Transplantation Study

To assess CAV development, we performed murine heterotopic heart transplantsations using a MHC Class II mismatch model. B6.C-H2-Ab1bm12/KhEg-Mc1r+/-J donor hearts were heterotopically transplanted into littermate control C57BL/6J mice. Graft viability and beat score was assessed by direct abdominal palpation daily. Allografts were harvested on POD 52, with n = 6 for each group. Morphometric analysis was performed using Image J software (NIH) to assess luminal narrowing (intimal proliferation). Percent luminal narrowing = (area bounded by internal elastic lamina — area bounded by lumen)/area bounded by internal elastic lamina * 100 percent.

SDS-PAGE and Western Blot Analysis

Protein concentrations from graft protein extracts were determined through the bicinchoninic acid assay (ThermoScientific Pierce, Waltham, MA) according to manufacturer protocol. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 10 µg of protein from each graft per well. Western blot was performed with the following primary antibodies: rabbit α vinculin (Sigma-Aldrich, St. Louis, MO), rabbit α AMPK, rabbit α phospho-AMPK, mouse α caspase-9, mouse α caspase-8, and rabbit α Bax (Cell Signaling Technologies, Beverly, MA). Quantification by densitometry was performed using Image J.

Other Protein Assays

To quantify cell death, histone-associated DNA levels in the protein extracts were measured using the cell death detection ELISA (Roche, Indianapolis, IN) using 10 µg of protein in duplicates, according to manufacturer protocol. To assay caspase-3 activity in protein extracts, the caspase-3 fluorometric assay (R&D Systems, Minneapolis, MN) was performed using 100 µg of protein in duplicates. The reporter molecule 7-amino-4-trifluoromethyl coumarin is cleaved by active caspase-3, resulting in a fluorochrome that emits fluorescence at 505 nm when excited at 400 nm.

In Vitro Studies

A well-developed cell culture system was used for studying I-R injury. Immortalized cardiomyocytes (HL-1 cells) were pre-treated with combinations of metformin, AICAR, Compound C (AMPK antagonist), and PBS vehicle and then exposed to hypoxic conditions with 1 percent O₂, 5 percent CO₂, and 94 percent N₂ for 45 minutes. Cell lysates were captured at 24 hours, and protein extract was collected through cell lysis, centrifugation, and protease inhibitor treatment.

Statistical Analysis

Statistical tests of differences between the treatment groups were performed with
two tailed, unpaired Student’s T Test without the assumption of equal variances, with p < 0.05 considered significant. All data were presented as mean ± standard error (SEM).
fects of metformin on AMPK activation in the context of heart transplantations. Western blot analysis illustrated that metformin and AICAR treatment of the allograft increased active phosphorylated AMPK levels (Figure 3b), while total AMPK levels did not change with treatment. This result indicates that metformin activates AMPK signaling \textit{in vivo}, just as it does \textit{in vitro}.

**Activation of AMPK by Metformin or AICAR Modulates Apoptosis in Cardiac Grafts at POD 8**

We next explored the specific pathways by which metformin and AMPK may modulate acute rejection. The two pathways studied were intrinsic apoptosis (through mitochondrial release of cytochrome C) and extrinsic apoptosis (through Fas death ligand). Each pathway was studied using a representative protein marker(s) (Figure 3c).

To analyze the intrinsic apoptosis pathways in the donor heart, we performed Western blots for Bax and cleaved caspase-9. Bax plays a role in the formation of mitochondrial ion channels and subsequent cytochrome C release. Thus, Bax is pro-apoptotic, promoting the intrinsic apoptosis pathway. Both metformin- and AICAR-treated grafts exhibited decreased Bax compared to vehicle-treated grafts (Figure 3d), implying decreased activation of the intrinsic apoptosis pathway.

Correlating with the Bax results, Western blot showed that metformin- and AICAR-treated hearts had lower levels of cleaved caspase-9 than vehicle-treated hearts (Figure 3e). To rule out contributions from extrinsic apoptosis, we performed a Western blot for cleaved caspase-8 but did not observe any trends or patterns. In particular, the levels of cleaved caspase-8 did not vary between groups (data not shown). Taken together, the results imply that metformin and AICAR treatments down-regulate intrinsic apoptosis in allografts.

To quantify the downstream effects of inhibiting intrinsic apoptosis, caspase-3 activity was measured in allografts using a fluorometric activity assay. Caspase-3 is a downstream executor protease for apoptotic pathways, so increased caspase-3 activity should signify more robust apoptosis. The results of the caspase-3 activity assay demonstrated that metformin and AICAR significantly decrease caspase-3 activity, as compared to the vehicle, by $73.41 \pm 18.86$ percent ($p = 0.03$) and $45.64 \pm 20.17$ percent ($p = 0.05$), respectively (Figure 3f). Thus metformin and AICAR decrease biomarkers of intrinsic apoptosis and caspase-3 activity.

**Metformin Treatment Decreases Apoptosis Acutely After Transplantation**

Having shown that metformin and AICAR similarly decrease intrinsic apoptosis in cardiac allografts, we next investigated the cardioprotective benefit of metformin on an acute rejection time course. To directly assess acute cell death in grafted hearts, we performed a cytoplasmic histone-associated DNA fragment cell death detection ELISA (Roche). The colorimetric signal detected in this ELISA corresponds to histone-associated DNA fragments in the sample, which are indicative of many different types of cell death. On POD 1 of the time course, metformin-treated grafts showed a significant reduction ($68.00 \pm 17.18$ percent) in cell death compared to the control ($p = 0.007$). On POD 8, metformin-treated grafts showed a $63.80 \pm 35.76$ percent reduction compared to the control ($p = 0.07$) (Figure 4a). Therefore, 1 day and 8 days after transplantation, metformin treatment exerted a protective effect on the allograft by decreasing the amount of DNA fragmentation, a sign of cell death.

However, the ELISA may not have been able to differentiate between apoptosis and necrosis, so we also performed a caspase-3 fluorescent activity assay. On both POD 1 and POD 8, metformin-treated grafts exhibited significantly decreased caspase-3 activity compared to the vehicle-treated grafts (POD 1: $44.83 \pm 7.57$ percent, $p = 0.001$; POD 8: $73.41 \pm 18.86$ percent, $p = 0.03$) (Figure 4b). Therefore, acutely after transplantation on POD 1 and 8, metformin treatment confers a cardioprotective effect on the graft by significantly decreasing apoptosis.
Metformin Treatment Decreases CAV Chronically After Transplantation

After establishing metformin’s cardio-protective mechanism in the acute rejection time course, we used a chronic rejection model to study the long-term effects of metformin treatment of the allograft. In this model, animals treated with metformin had a significant increase in their cardiac graft beating score (Figures 5a, b) and a significant decrease in CAV development (Figure 5c), as measured by luminal narrowing ($46.77 \pm 3.63$ percent vs. $67.95 \pm 4.42$ percent compared to vehicle, respectively, $p < 0.001$). The long-term effect of metformin pretreatment of a cardiac allograft is improved beating function and decreased CAV and chronic rejection.

**DISCUSSION**

Here, we show that metformin results in AMPK activation *in vitro*. Although cardiomyocyte cell culture is not an *in vitro* model of transplantation, we use it to prove, in a defined system, that metformin leads to increased pAMPK levels. Additionally, we show that the effects of metformin on pAMPK can be decreased by Compound C (an ATP-competitive inhibitor) and that metformin’s effects are mimicked by AICAR (an AMPK activator). In cardiac allografts, metformin and AICAR also behave similarly, leading to increased pAMPK but not changing total AMPK levels. Thus, metformin seems to increase AMPK signaling by phosphorylation and activation of AMPK, and not by increasing total AMPK levels.

We also show that metformin’s cardio-protection is mimicked by AMPK activation through AICAR. In cardiac allografts at POD 8, both metformin and AICAR treatment resulted in decreased cleaved, active caspase-9 and pro-apoptotic Bax but did not affect caspase-8 levels. We conclude that the decrease in cell death from metformin and AMPK activation results from a change in intrinsic, rather than extrinsic, apoptosis (or immune-mediated rejection).

Gross levels of Bax, as detected by Western blot, may not be the most accurate method for estimating intrinsic apoptosis because Bax can exist in three different forms: free in the cytosol, as a homodimer in the outer mitochondrial membrane, and as a heterodimer with Bcl-2 in the outer mitochondrial membrane. Under normal conditions, Bax is mainly found in the cytosol. However, under apoptotic stimulation, Bax undergoes conformational change and is in-
serted into the outer mitochondrial membrane, where it forms a pro-apoptotic homodimer that induces pore formation. However, if Bax forms a heterodimer with Bcl-2, Bax is incapacitated and cannot increase mitochondrial membrane permeability. Therefore, we also used a caspase-3 activity assay to quantify the apoptotic activity in the graft lysate. Both metformin and AICAR treatment led to significantly reduced caspase-3 activity, and thus significantly reduced apoptosis, as compared to the vehicle. However, we cannot rule out the possibility that metformin may protect the heart in ways beyond AMPK pathway modulation. Because metformin has been shown to activate AMPK and treatment with the AMPK agonist AICAR results in a reduction in apoptosis similar to that with metformin treatment, it follows that AMPK activation may play a role in metformin’s cardioprotective effects.

The cardioprotective effect of metformin is not likely immune-mediated, since caspase-8 levels and extrinsic apoptosis are unchanged. Rather, the cardioprotective benefit of metformin likely stems from protection of the graft from initial I-R injury during the transplantation procedure. However, there have been reports implicating metformin in the inhibition of proinflammatory responses, such as nuclear factor-κB, in vascular wall cells [16]. Hence, an alternative mode of metformin’s cardioprotection may be through suppression of inflammatory immune responses. To rule out the possibility that metformin works via an allostrogen-dependent route, future experiments should also assess grafts for inflammatory cytokines by ELISA and inflammatory infiltrates by flow cytometry and histology.

Figure 5. Chronic transplantation study: Improvement in graft function and decrease in CAV from metformin treatment.

a) Time course of cardiac graft beating score up to POD 51 for control and metformin-treated grafts. b) Average of cardiac graft beating scores for POD 10, POD 30, and POD 52. Error bars are SEM. n=6 for each group. *p<0.05. c) Percent luminal narrowing for control and metformin-treated grafts on POD 52. Error bars are SEM. n=6 for each group. *p<0.05.
From the acute transplantation time course, we conclude that both POD 1 and POD 8 metformin-treated grafts resulted in significantly decreased caspase-3 activity compared to the control. Additionally, the level of apoptosis in the graft was not different between POD 1 and POD 8, despite the extra injections of treatment for the grafts harvested on POD 8. It is unclear whether the extra treatments are necessary for metformin to have therapeutic effect. Based on our hypothesis that metformin protects the heart not by suppressing immune-mediated rejection but by decreasing I-R injury, we would expect that only the first metformin treatment at the time of surgery is necessary to exert a cardioprotective benefit. Therefore, in our chronic transplantation study, we only treated the graft during the operation and did not treat the recipient animal with postoperative metformin. Even after 52 days, the benefit of metformin was still appreciable, as suggested by decreased CAV and increased beat scores. This supports our hypothesis that metformin protects grafts from initial I-R injury from the transplantation procedure. Notably, the improvement in beat scores and CAV were achieved with only one dose of metformin and no immunosuppressive regimens.

In this study, we correlate a decrease in initial I-R injury and apoptosis with decreased CAV and increased chronic graft function, as a result of metformin treatment. However, we do not show direct causation since we use different models for the chronic and acute studies. In future experiments, we plan to measure in vivo apoptosis through annexin V-SPECT at acute time points and follow recipient animals to a chronic time point to assess CAV.

Another possible area of future study is the effect of metformin and AMPK on ROS-induced necrosis. Indeed, apoptosis and necrosis can both be stimulated by the inflammatory cytokine tumor necrosis factor-α, which is upregulated in I-R injury. ROS-induced necrosis is another way by which I-R injury contributes to development of CAV. AMPK has been shown to decrease the amount of ROS generated during periods of hypoxic stress, so it is possible that metformin also protects the heart from chronic rejection and CAV by downregulating ROS-induced necrosis. Future studies should examine whether metformin diminishes ROS-induced necrosis, a mechanism through which metformin may decrease CAV. Our results allow for this possibility since we show in the acute time course that metformin treatment decreases histone-associated DNA fragments, which may indicate both apoptosis and necrosis.

Taken together, the results of this study represent a novel approach to improving chronic outcomes in cardiac transplantation without immunosuppression. We demonstrate that metformin activates AMPK, which then prevents acute cell death in cardiac allografts by primarily suppressing intrinsic apoptosis due to I-R injury incurred from the transplantation procedure. The pathway modulated by AMPK is likely intrinsic apoptosis, rather than extrinsic apoptosis, since the cardioprotective mechanism of metformin and AMPK is maintenance of the energy supply during ischemic stress. Metformin’s suppression of I-R injury in the acute rejection model correlates with a metformin-associated decrease in CAV in the chronic rejection model. These results suggest that metformin’s long-term graft cardioprotection may stem from decreasing initial I-R injury, and not from immunosuppression. Meanwhile, AMPK, the key regulatory protein under investigation, may be involved in many diseases, such as obesity, cancer, diabetes, and heart disease. Thus, new insights regarding the relationship between AMPK, metformin, and intrinsic apoptosis will not only enhance the long-term efficacy and safety of heart transplants, but will also contribute to progress in many medical research areas.

REFERENCES

1. Hunt SA, Abraham WT, Chin MH, et al. ACC/AHA 2005 Guideline Update for the Diagnosis and Management of Chronic Heart Failure in the Adult. Circulation. 2005;112:154.

2. Murata S, Miniati DN, Kown MH, et al. Superoxide Dismutase Mimetic M40401 Reduces Ischemia-Reperfusion Injury and Graft Coronary Artery Disease in Rodent Cardiac Allografts. Transplantation. 2004;78(8):1166-71.
3. Tanaka M, Mokhtari G, Terry R, et al. Prolonged Cold Ischemia in Rat Cardiac Allografts Promotes Ischemia-Reperfusion Injury and the Development of Graft Coronary Artery Disease in a Linear Fashion. J Heart Lung Transplant. 2005;24:1906-14.
4. Kirpichinkov D, McFarlane S, Sowers J. Metformin: An Update. Ann Intern Med. 2002;137:25-33.
5. Legtenberg R, Houston R, Oeseburg B, et al. Metformin Improves Cardiac Functional Recovery After Ischemia in Rats. Horm Metab Res. 2002;34:182-5.
6. Davis B, Xie Z, Viollet B, et al. Activation of the AMP-Activated Kinase by Antidiabetes Drug Metformin Stimulates Nitric Oxide Synthesis In Vivo by Promoting the Association of Heat Shock Protein 90 and Endothelial Nitric Oxide Synthase. Diabetes. 2006;55:496-505.
7. Calvert J, Gundewar S, Jha S, et al. Acute Metformin Therapy Confers Cardioprotection Against Myocardial Infarction Via AMPK–eNOS–Mediated Signaling. Diabetes. 2008;57:696-705.
8. Hardie D, Hawley S, Scott J. Acute AMP-activated protein kinase – development of the energy sensor concept. J Physiol. 2006;574:7-15.
9. Kwong A, Howie J, Petrie J, et al. AMP-activated protein kinase pathway: a potential therapeutic target in cardiometabolic disease. Clin Sci. 2009;116:607-20.
10. Towler M, Hardie D. AMP-Activated Protein Kinase in Metabolic Control and Insulin Signaling. Circ Res. 2007;100:328-41.
11. Zou M, Kirkpatrick S, Davis B, et al. Activation of the AMP-activated Protein Kinase by the Anti-diabetic Drug Metformin in Vivo. J Biol Chem. 2004;279:43940-51.
12. Sasaki H, Asanuma H, Fujita M, et al. Metformin Prevents Progression of Heart Failure in Dogs: Role of AMP-Activated Protein Kinase. Circulation. 2009;119:2568-77.
13. Viollet B, Athea Y, Mounier R, et al. AMPK: Lessons from transgenic and knockout animals. Front Biosci. 2009;14:19-44.
14. Russell R, Li J, Coven D, et al. AMP-activated protein kinase mediates ischemic glucose uptake and prevents posts ischemic cardiac dysfunction, apoptosis, and injury. J Clin Invest. 2004;114:495-503.
15. Xing Y, Musi N, Fujii N, et al. Glucose Metabolism and Energy Homeostasis in Mouse Hearts Overexpressing Dominant Negative α2 Subunit of AMP-activated Protein Kinase. J Biol Chem. 2003;278:28372-7.
16. Isoda K, Young JL, Zirlik A, et al. Metformin Inhibits Proinflammatory Responses and Nuclear Factor-κB in Human Vascular Wall Cells. Atheroscler Thromb Vasc Biol. 2006;26:611-7.