Int J Physiol Pathophysiol Pharmacol 2009;1:116-126
www.ijppp.org/IJPPP903002

Original Article

A novel AMPK activator from Chinese herb medicine and ischemia phosphorylate the cardiac transcription factor FOXO3

Jingying Wang[1†], Heng Ma[1†], Xiaoyu Zhang[2], Leilei He[1], Jianming Wu[3], Xiaoping Gao[3], Jun Ren[1], Ji Li[1*]

[1]Division of Pharmaceutical Sciences & Center for Cardiovascular Research and Alternative Medicine, University of Wyoming, School of Pharmacy, Laramie, WY 82071; [2]Institute of Physiology, School of Basic Medical Sciences, Lanzhou University, Lanzhou 730000, P.R. China; [3]Department of Research & Development, Kanghong Pharmaceutical Co. Ltd, Chengdu 610000, P.R. China. [†]Equal contribution.

Received March 10, 2009; accepted March 16, 2009; available online March 25, 2009

Abstract: Oleanolic Acid (OA) is a nature product extracted from Chinese Herb Medicine which is traditionally used as treatment of diabetes and ischemic heart diseases. Mounting evidence showed that AMP-activated protein kinase (AMPK) has cardioprotective effect against ischemic injury and the forkhead transcription factor 3 (FOXO3) was recently identified as a downstream target of AMPK. We hypothesize that OA may protect against ischemic dysfunction of cardiomyocytes via activation of AMPK signaling pathway. Male C57BL/6 mice which were subjected to *in vivo* regional cardiac ischemia stimulated AMPK Thr^{172} phosphorylation, as well as phosphorylation of downstream FOXO3 (Ser^{413}) and acetyl CoA carboxylase (ACC). The natural product, OA, significantly stimulated cardiac AMPK activation in cardiomyocyes in time- and dose-dependent manners. The mechanism of AMPK activation by OA may be due to the loss mitochondrial membrane potential ($\Delta\Psi m$) as shown by JC-1 fluorescence assay. Intriguingly, OA as an AMPK activator also triggered FOXO3 (Ser^{413}) phosphorylation in cardiomyocytes. Furthermore, OA treatment can protect cardiomyocytes from contractile dysfunction induced by hypoxia. Taken together, the results indicated that both ischemia and OA stimulated cardiac AMPK phosphorylation, as well downstream FOXO3 phosphorylation. The cardioprotective effect of OA maybe associated with activation of AMPK signaling pathways.

Key words: AMP-activated protein kinase (AMPK), ischemia, Forkhead transcription factor 3 (FOXO3), Oleanolic Acid (OA)

Introduction

Myocardial ischemia is a disorder of cardiac function that occurs when blood supply to myocardium is insufficient. The decrease blood flow may happen with a number of reasons, such as coronary arteriosclerosis, coronary thrombosis (obstruction by a thrombus) and less frequently, the narrowing of arterioles in the heart [1]. Currently, AMP-activated protein kinase (AMPK) pathway was revealed to be one of the signaling pathways that protect against cardiac ischemia [2]. AMPK is a stress sensitive kinase that can be activated by ATP depletion such as hypoxia [3], ischemia [4] and exercise [5]. Activated AMPK can phosphorylate Acetyl-CoA carboxylase (ACC) to inhibit its activity involved in fatty acid synthesis [6]. Other downstream effects of AMPK pathways include glucose uptake [7, 8], glycolysis [9] and fatty acid oxidation [10], which favor the ATP production that supply enough energy for cell living under the stress conditions.

Recent studies have found that FOXO3, one member in the forkhead transcription factors (FOXO) family, is also a direct downstream target of AMPK [11]. Upon phosphorylated by AMPK at Ser^{413}, the transcription activity of FOXO3 is enhanced, which leads to the up-regulation of genes involved in glucose

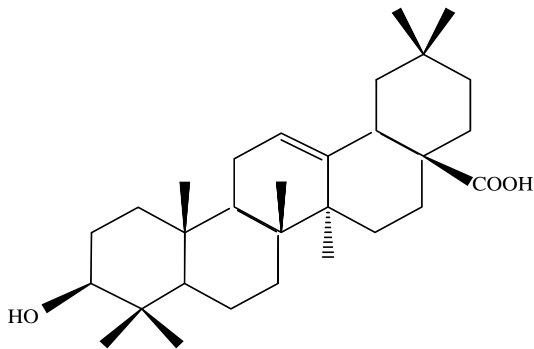
metabolism and stress resistance [11, 12]. Therefore, we are interested in whether the cardiac FOXO3 could be phosphorylated by AMPK during ischemia.

Oleanolic Acid (3b-hydroxy-olea-12-en-28-oic acid, OA) is a natural triterpenoid compound that exists in many herbal medicines [13]. The pharmacological functions of OA include hepatoprotective effects against toxicity [14], anti-tumor initiation and promotion [15] and anti-diabetic disease [16]. Recently, we found that OA played an important role in maintaining energy stores in the heart. Coincidently, it has been suggested that the OA are involved in the regulation of energy homeostasis [16]. However, the molecular mechanisms through which OA exerts its biological effective remain unclear.

The purpose of this study is to investigate whether AMPK directly regulates FOXO3 in the ischemic heart and whether OA can serve as an AMPK activator to protect heart against ischemic injury. The results demonstrated that FOXO3 was phosphorylated with the association of AMPK activation during cardiac ischemia, and OA, the novel AMPK activator could also regulate FOXO3 phosphorylation to exert the cardioprotective effect in hypoxic cardiomyocytes.

Materials and Methods

Structure of Oleanolic acid (OA)



Oleanolic acid (OA)

Animals and cell line

8-12 weeks male C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). All animals were kept in the institutional animal facility at the University of Wyoming with standard laboratory chow diet and tap water. The animal care and treatment was performed in accordance with the guidelines of the University of Wyoming Animal Care and Use Committee. H9c2 cells, a myoblast cell line derived from fetal rat heart, were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY) and 1% penicillin and streptomycin and maintained in 95% air and 5% CO_2 at 37ºC. Cells were passaged regularly and subcultured to 90% confluence before experiments.

In vivo ischemic model

Mice were anesthetized, intubated and ventilated with respirator [17, 18]. The body temperature was maintained at 37 ºC with a heating pad. After left lateral thoracotomy, the left anterior descending (LAD) was occluded for 20 minutes with an 8-0 nylon suture and polyethylene tubing to prevent arterial injury. ECGs confirmed the ischemic hallmark of ST-segment elevation during coronary occlusion (ADInstruments).

Isolation of mouse cardiomyocytes

Cardiomyocytes were enzymatically isolated as described previously [19]. In brief, hearts were removed and perfused with oxygenated (5% CO_2/95% O_2) Krebs-Henseleit bicarbonate (KHB) buffer containing (in mM) 118 NaCl, 4.7 KCl, 1.25 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 10 HEPES and 11.1 glucose. Hearts were then perfused with a Ca^{2+}-free KHB containing Liberase Blendzyme 4 (Hoffmann-La Roche Inc., Indianapolis, IN) for 10 min. After perfusion, left ventricles were removed and minced to disperse cardiomyocytes in Ca^{2+}-free KHB buffer. Extracellular Ca^{2+} was added incrementally back to 1.25 mmol/L. Only rod-shaped myocytes with clear edges were selected for pharmacological test and cell contractility studies.

Assessment of mitochondrial membrane potential (Δψ)

The cardiomyocyte mitochondrial membrane potential was measured by using 5, 5', 6, 6'-Tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazo-locarbocyanine iodide (JC-1, Sigma, St. Louis,

MO). Briefly, JC-1 is a positively charged fluorescent compound which is taken up by mitochondria proportionally to the inner mitochondrial membrane [20]. When a critical concentration is reached, JC-1 monomer forms J-aggregates, altering the fluorescence properties of the compound. Thus, the ratio of red (J-aggregate) green (monomeric JC-1) emission is directly proportional to the mitochondrial membrane potential (Dc). Isolated cardiomyocytes were suspended in HEPES-saline buffer and incubated with 10 mM JC-1 for 10 min at 37 ºC. Fluorescence of each sample was read at excitation wavelength of 490 nm and emission wavelength of 530 nm and 590 nm using a spectrofluorimeter. Results in fluorescence intensity were expressed as 590-to-530-nm emission ratio [21].

Immunoblotting

Immunoblots were performed as previously described [18, 22]. Cell lysate proteins were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. For reprobing, membranes were stripped with 50 mM Tris-HCl, 2% SDS, and 0.1 M β-mercaptoethanol (pH 6.8). Rabbit polyclonal antibodies against phosphor-AMPK, total AMPKα, phosphor-p38, total p38 were purchased from Cell Signaling. Rabbit polyclonal antibody against actin was purchased from Santa Cluz. Rabbit polyclonal antibodies against phosphor-Acetyl-CoA Carboxylase (p-ACC) and total ACC were from Millipore. Mouse polyclonal antibody against phopshor-FOXO3 at Ser^{413} was a kind gift from Dr. Michael E Greenberg at Harvard Medical School [23].

Cardiomyocytes shortening/relengthening measurement

The mechanical properties of ventricular myocytes were assessed using a SoftEdge MyoCam system (IonOptix Corporation,Milton, MA) [24]. In brief, left ventricular myocytes were placed in a chamber mounted on the stage of an inverted microscope (Olympus, IX-70) and incubated at 25°C with a buffer containing (in mM): 131 NaCl, 4 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 Glc, and 10 HEPES, at pH 7.4. The cells were field stimulated with suprathreshold voltage at a frequency of 0.5 Hz (unless otherwise stated), 3 msec duration, using a pair of platinum wires placed on opposite sides of the chamber and connected to an electrical stimulator (FHC Inc, Brunswick, NE, USA). The myocyte being studied was displayed on a computer monitor using an IonOptix MyoCam camera. An IonOptix SoftEdge software was used to capture changes in cell length during shortening and relengthening. Cell shortening and relengthening were assessed using the following indices: peak shortening(PS), the amplitude myocytes shortened on electrical stimulation, indicative of peak ventricular contractility; time-to-PS (TPS), the duration of myocyte shortening, an indicative of systolic duration; time-to-90% relengthening (TR90), the duration to reach 90% relengthening, an indicative of diastolic duration (90% rather 100% relengthening was used to avoid noisy signal at baseline concentration); and maximal velocities of shortening/relengthening, maximal slope (derivative) of shortening and relengthening phases, indicative of maximal velocities of ventricular pressure increase/decrease. In the case of altering stimulus frequency, the steady-state contraction of myocyte was achieved (usually after the first 5–6 beats) before PS amplitude was recorded. For hypoxia studies, cardiomyocytes were exposed to 95% N_2 and 5% CO_2 atmosphere by using an anaerobic chamber.

Statistical Analysis

Data were expressed as means ± SE. Significance was tested by Student's two tail *t* tests with Bonferroni correction or two-way repeated measures ANOVA with post hoc analysis. $p<0.05$ was considered significant.

Results

AMPK phosphorylates Foxo-3 at Ser^{413} in the ischemic heart

We first measured the kinetics of AMPK (Thr^{172}) phosphorylation in mice hearts subjected to *in vivo* regional ischemia. Right after 5 minute LAD ligation, the phosphorylation of AMPK (**Figure 1A**), as well as its downstream target protein Acetyl Co-A carboxylase (ACC) (**Figure 1B**) were immediately increased by 2-fold. Associated with AMPK phosphorylation, the phosphorylation of p38 MAPK was also stimulated by ischemia in time-dependent

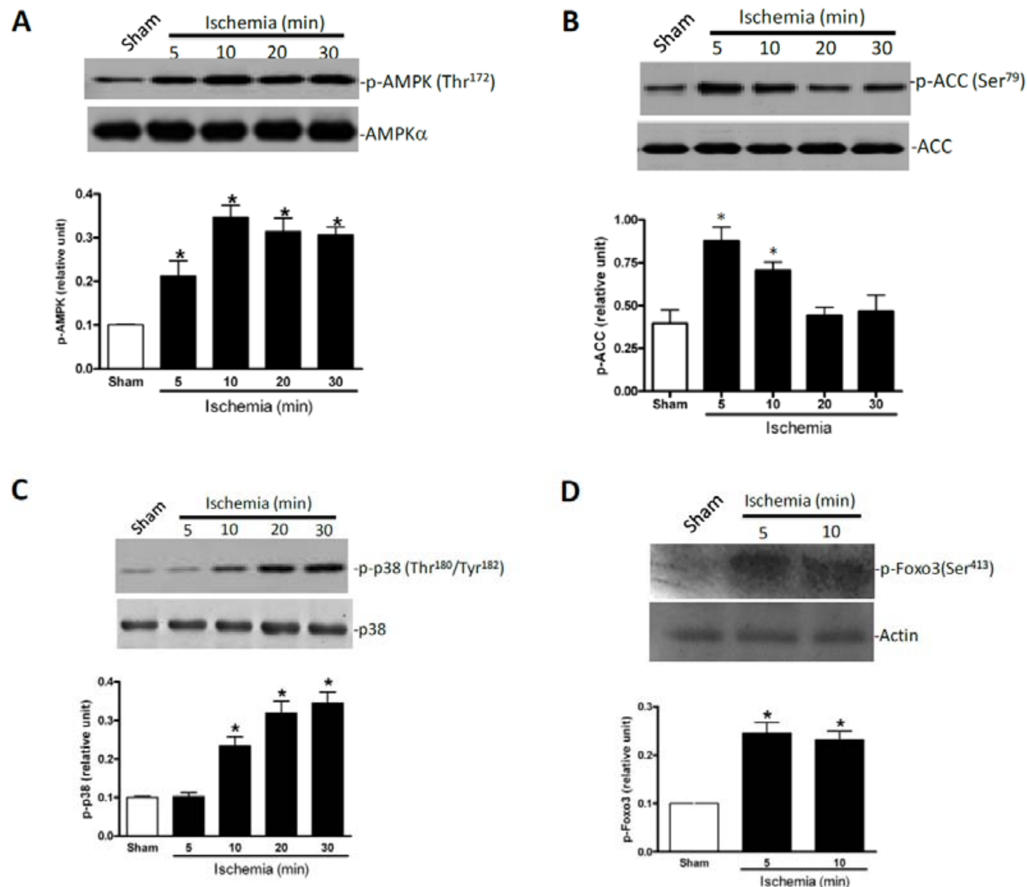


Figure 1. The cardiac AMP-activated protein kinase (AMPK) signaling pathway was activated during in vivo regional ischemia. Representative immunoblots of ischemic heart homogenate phosphorylated AMPK at Thr172 (p-AMPK) and total AMPK α subunit (AMPKα). Phosphorylated AMPK was quantified relative to total AMPKα. In vivo regional ischemia stimulated phosphorylation of (A) AMPK (Thr172), (B) ACC (Ser79), (C) p38 MAPK (Thr180/Tyr182) and (D) FOXO3 (Ser413) in a time-dependent manner. Values are means ± SE (n=3). *p<0.05 vs. sham.

manner (**Figure 1C**) [17]. Intriguingly, the FOXO3 phosphorylation (Ser^{413}) was significantly increased during ischemia in an initial parallel with AMPK phosphorylation (**Figure 1D**). The results indicate that ischemia triggered AMPK activation can stimulate FOXO3 phosphorylation at Ser^{413} in heart.

OA stimulates AMPK signaling pathway in H9c2 cells

Oleanolic Acid (OA) is a nature product extracted from Chinese herb medicine which is traditionally used as a treatment of type 2 diabetes [16]. Our previous study found that OA could protect against ischemia injury in rat heart by attenuating ST segment elevation (Wu et al, unpublished data). Recently, more evidence showed that AMP-activated protein kinase (AMPK) has cardioprotection against ischemic injury [2] and the forkhead transcription factor 3 (FOXO3) was identified as a downstream target of AMPK [11]. To define the mechanism involved in the cardioprotective effect of OA, the H9c2 myoblast cells were treated with different OA dosage for 20 min. The results demonstrated that when OA concentration reached 100 μM, the phosphorylation of AMPK and p38 MAPK was activated (**Figure 2A and 2B**). Accordingly, we also examined the FOXO3 (Ser^{413}) phosphorylation level in the cells treated with high dose of OA, and as shown in **Figure 2C**, the phosphorylation of FOXO3 (Ser^{413}) is initiated. Taken together, our data suggests that natural product OA can activate AMPK

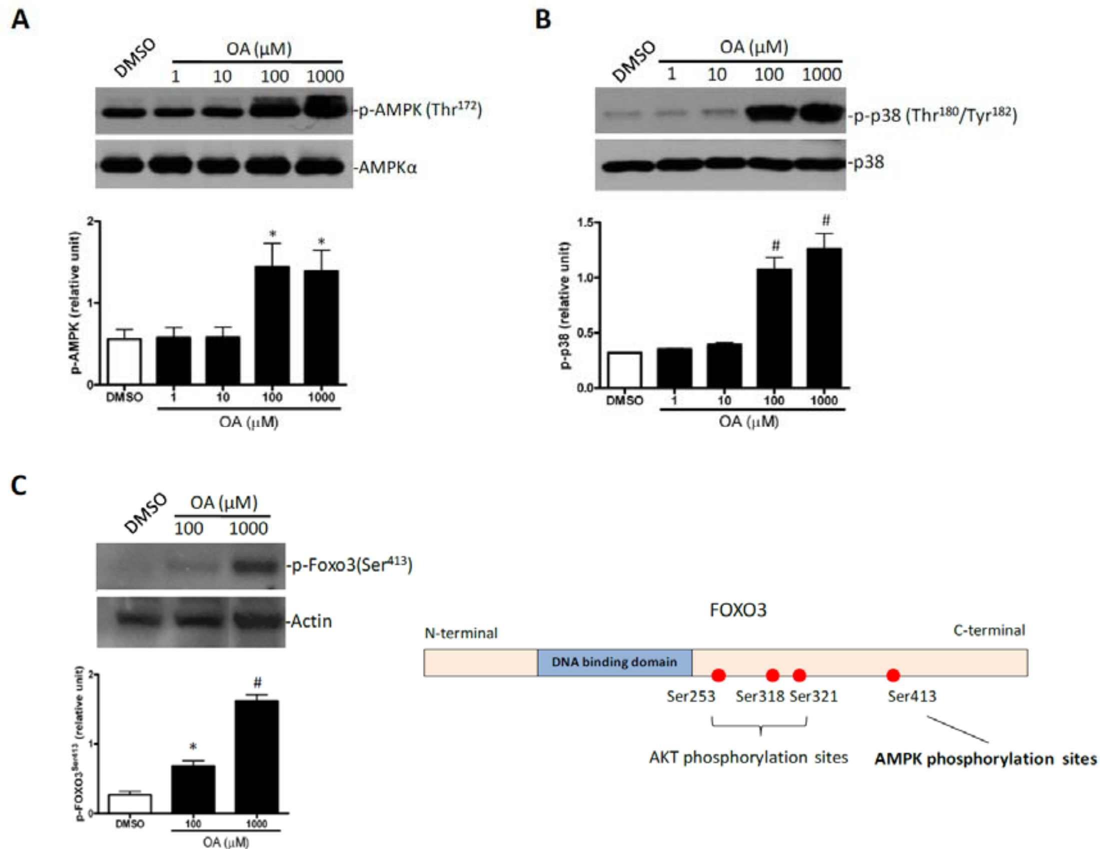


Figure 2. OA stimulated AMPK signaling pathway in H9c2 cells. (A) Phosphorylation of AMPK at Thr172 (p-AMPK) and (B) p38 at Thr180 and Tyr182 (p-p38) in H9c2 cells treated with different dosage of OA. Phosphorylated AMPK or p38 MAPK was quantified relative to total AMPKα or p38 MAPK, respectively. (C) Phosphorylation of FOXO3 at Ser413 in OA treated H9c2 cells. FOXO3 phosphorylation was quantified relative to the amount of loading control actin. Values are means ± SE (n=3). #p<0.01 vs. control (DMSO), *p<0.05 vs. control (DMSO), respectively.

signal pathway and stimulates the phosphorylation of FOXO3 (Ser^{413}) in H9c2 myoblast cells.

OA stimulates cardiac AMPK activation in cardiomyocytes

To determine whether OA can stimulate AMPK activation in heart, we isolated cardiomyocytes from mouse heart and treated with different OA dosage for different time periods. The results showed that OA acutely and robustly stimulated cardiac AMPK phosphorylation once the concentration reached 10 μM (**Figure 3A**). Moreover, OA stimulates cardiac AMPK activation in a time-dependent manner as well (**Figure 3B**). Following 5 min incubation with OA, AMPK phosphorylation was initiated and it reached to the highest point when incubated with OA for 20 min (**Figure 3B**). In parallel with AMPK activation, the phosphorylation of ACC and p38 MAPK were also induced by OA treatment (**Figure 3C and 3D**). Taken together, OA triggers cardiac AMPK activation in both dose and time-dependent manner and markedly increase ACC and p38 MAPK phosphorylation as well.

OA can reduce the mitochondrial membrane potential of cardiomyocytes

Konrad and colleagues have reported that Troglitazone, the anti-diabetic drug, enhances glucose uptake by acutely activating AMPK signaling pathway through decreasing mitochondrial membrane potential (Δψ) [21].

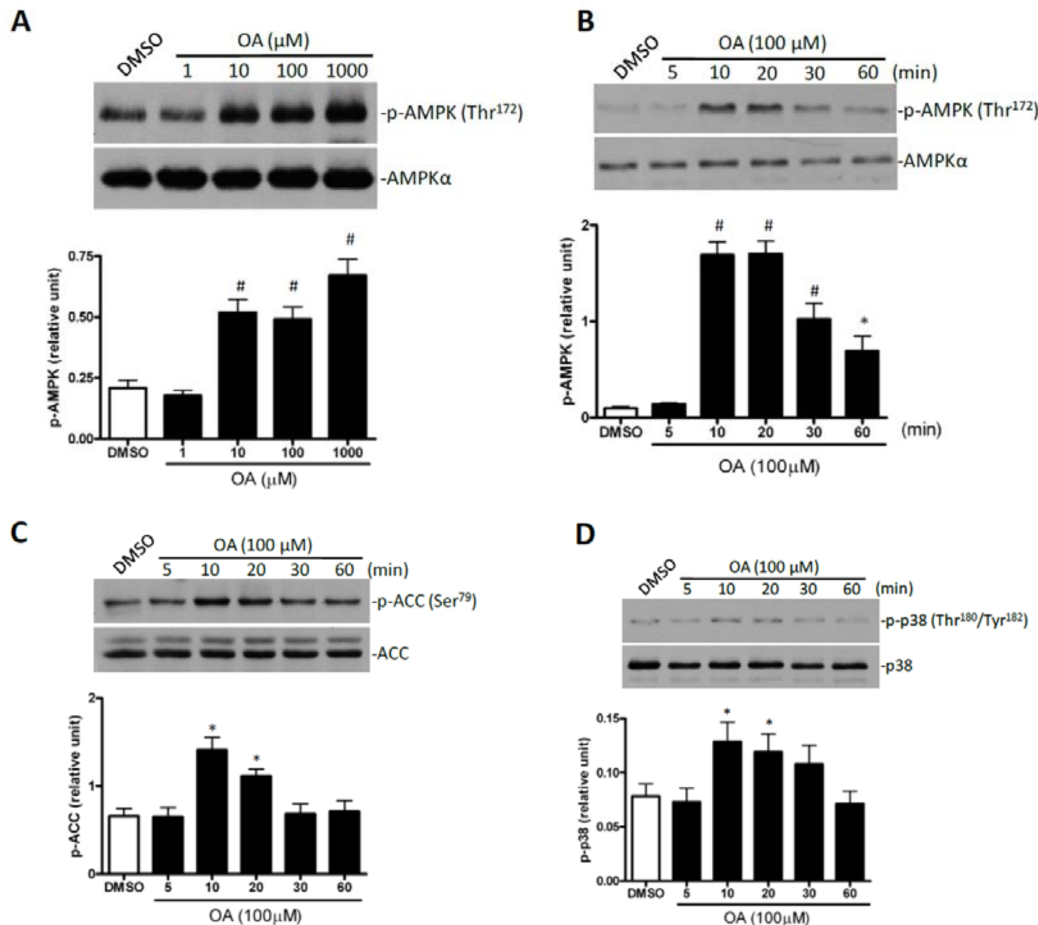


Figure 3. OA activated AMPK signaling pathway in isolated mouse cardiomyocytes. OA stimulated AMPK phosphorylation in (A) dose-dependent and (B) time-dependent manner. Phosphorylation of (C) ACC (Ser79) and (D) p38 MAPK (Thr180/Tyr182) were activated by OA in time dependent manner. Values are means ± SE (n=3). #p<0.01 vs. control (DMSO); *p<0.05 vs. control (DMSO).

To figure out the mechanism of OA stimulating AMPK activation, we used JC-1 assay to measure mitochondrial membrane potential (Δψ) in cardiomyocytes. JC-1 is a fluorescent dye to indicate loss of mitochondrial membrane potential (Δψ) [21]. It can exist as monomer in the cytoplasm showing green and meanwhile, accumulate as aggregates in mitochondria showing red. When mitochondrial membrane potential (Δψ) collapses, JC-1 cannot accumulate in mitochondria, which leads to the decrease of red fluorescence but an increase of green fluorescence. Presented as the ratio of red/green fluorescence, the result illustrated that OA treatment significantly reduced the mitochondrial membrane potential (ΔΨm) of isolated adult mouse cardiomyocytes in a dose-dependent manner, with a 2.8-fold decrease of JC-1 590/530 nm ratio in 1 mM OA treatment group compared to 0.1 mM OA treatment group (**Figure 4**). These results indicate that the activation of AMPK signaling by OA was via reducing mitochondrial membrane potential in cardiomyocytes.

OA ameliorates cardiomyocyte contractile dysfunction induced by hypoxia

To determine whether the activation of AMPK signaling as well as FOXO3 phosphorylation stimulated by OA contributes to the resistance to ischemic injury, we investigated the contractility of cardiomyocyte when exposed to hypoxia atmosphere. The mechanical properties were obtained under extracellular

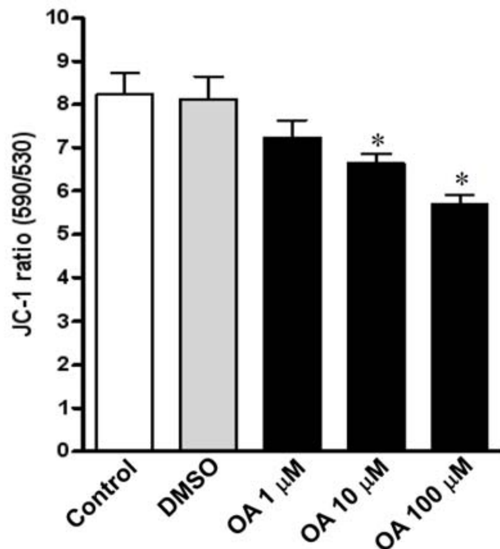


Figure 4. OA reduced mitochondrial membrane potential (Δψ) in cardiomyocytes. Mitochondrial membrane potential (Δψ) was measured by JC-1 fluorescence assay. The result was presented as the ratio of red/green fluorescence measured at 590nm and 530 nm respectively. Values are means ± SE (n=3). *$p<0.05$ vs. control (DMSO).

Ca^{2+} of 1.0 mM and a stimulus frequency of 0.5 Hz [19]. As shown in Figure 5, OA treatment did not affect cardiomyocyte contractile function under the normal condition. However, during hypoxic exposure, the cardiomyocytes displayed sever impaired peak shortening and reduced maximal velocity of shortening/ relengthening (+/-dL/dt) compared to the cells with OA (100μM) treatment (Figure 5). Moreover, hypoxia caused the prolonged time-to-PS (TPS) and time-to-90% relengthening (TR_{90}) of cardiomyocytes, however, OA (100 μM) markedly inhibited the hypoxia-induced cardiac dysfunction. Taken together, the results suggest that OA, by initiating AMPK signaling and FOXO3 phosphorylation, is benefit to the cardiac protection against the cardiomyocyte dysfunction induced by hypoxia.

Discussion

In this study, we illustrated that cardiac FOXO3 would be phosphorylated by AMPK during ischemia, which could expend the downstream effects of AMPK, such as mediating gene expression in response to ischemic stress. Moreover, we found a natural compound, Oleanolic Acid, could stimulate AMPK signaling pathway in cardiomyocytes by decreasing mitochondrial membrane potential. The activation of AMPK by OA exerts the cardioprotective effects against cell injury and dysfunction induced by hypoxia.

FOXO3 is one of the forkhead transcription factors that mediate the expression of genes involved in the regulation of glucose metabolism, stress resistance, cell proliferation and apoptosis [11, 12, 25]. FOXO3 was previously identified to be regulated by many protein kinases including AKT and JNK [26]. As targeted by AKT, FOXO3 is translocated from nucleus to cytoplasm, which results in the inhibition of FOXO3 transcriptional activity [26]. Different from AKT phosphorylation site, AMPK can recognize and phosphorylate FOXO3 at six novel sites, and among those six sites, Ser^{413} is the residue that may be most efficiently phosphorylated by AMPK [11]. Treated with AMPK pharmacological stimuli 2DG, the phosphorylation of endogenous FOXO3 at Ser^{413} is increased in cells [11]. However, whether the activation of AMPK under the physiological stress such as ischemia can stimulate FOXO3 phosphorylation is not well understood. In our study, we found that during in vivo regional ischemia, the phosphorylation of FOXO3 at Ser^{413} is increased in heart along with AMPK activation. This suggests that ischemia activated AMPK can also phosphorylate FOXO3 (Ser^{413}) to enhance its transcriptional activity. The intensified FOXO3 activity will lead to the up-regulation of genes involved in the regulation of glucose metabolism and oxidative stress [11], which will further mediate cell survival under ischemic stress.

Furthermore, we also indentified a novel AMPK activator, Oleanolic Acid (OA). OA is a natural product that is exacted from Chinese herbal medicine which was traditionally used for hepatoprotection and anti-tumor [27]. It has also been illustrated that OA exert strong anti-HIV effect [28]. Recently, OA was reported to regulate blood glucose level in streptozotocin-induced type 1 diabetic model [16], which indicated that it might play a role in modulating energy homeostasis. Moreover, we found that OA exhibits cardioprotection through reducing ST segment elevation during cardiac ischemia induced by acute Isoproterenol treatment. Therefore, we

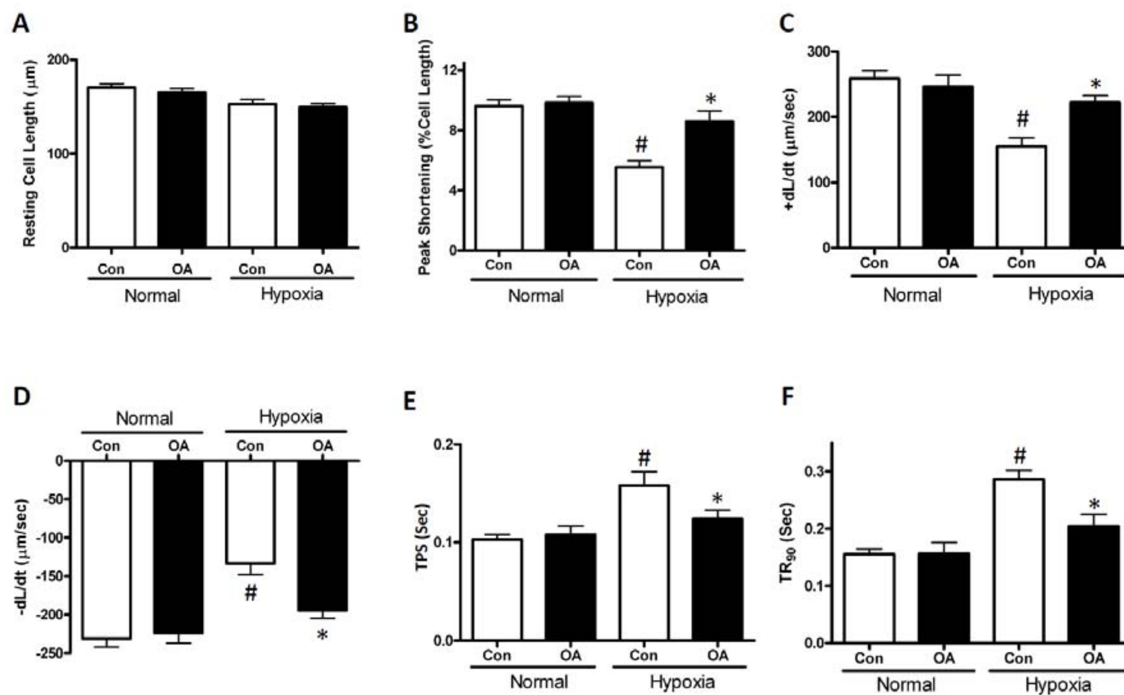


Figure 5. Contractile properties of cardiomyocytes from control and OA treatment after exposed to hypoxia. (A) Resting cell length; (B) Peak shortening (PS, normalized to cell length); (C) Maximal velocity of shortening (+dL/dt) and (D) relengthening (-dL/dt); (E) Time-to-PS (TPS); (F) Time-to-90% relengthening (TR90). Values are means±SE, n = 50-60 cells per group, #p<0.01 vs. Con (normal), *p<0.05 vs. Con (hypoxia), respectively.

suspected whether OA could stimulate AMPK activation, a very important kinase that mediates glucose metabolism to protect against cardiac ischemia injury [4]. Our data suggests that the acute OA (100 μM) treatment can trigger AMPK phosphorylation in both H9c2 cells and adult mouse cardiomyocytes, and the activation of AMPK signaling was further confirmed via the phosphorylation of ACC, one of the AMPK direct downstream targets. Initiation of AMPK signaling pathway by OA would lead to several downstream effects such as mediating glucose transporter, GLUT4, translocation onto the cell membrane to enhance glucose uptake [18], which might be a potential explanation for the role OA played in modulating energy homeostasis.

In addition to ACC phosphorylation, the AMPK activation by OA can stimulate FOXO3 phosphorylation at Ser^{413} as well. As discussed above, phosphorylation of the AMPK targeted site will lead to a gain of function in FOXO3, which will promote the expression of those genes participating in energy metabolism to help cell survive in stress conditions such as ischemia. Moreover, we also observed p38 MAPK phosphorylation associated with AMPK activation in both ischemic heart and OA treated cardiomyocytes. Our previous work has demonstrated that AMPK activation during ischemia can foster p38 MAPK phosphorylation by increasing its recruitment to TAB1, the scaffold protein involved in p38 MAPK activation [17]. The p38 MAPK pathway was also suggested to contribute to AMPK activation of glucose transport [17]. However, the phosphorylation of AMPK was rapidly decreased once reperfusion occured (data not shown), while p38 phosphorylation would still remain at a high level [29]. So we did not exclude the possibility that p38 MAPK can be activated by OA via other signaling pathways besides in AMPK dependent manner. Therefore, to investigate the mechanism of p38 MAPK activation in OA treated cells, further studies with the use of AMPK inhibitor, compound C, need to be performed to confirm

whether it is via the AMPK signaling or other upstream stimuli.

The loss of mitochondrial membrane potential ($\Delta\psi$) was identified in the cardiomyocytes treated with OA, which is in consistent with the findings of ATA, the oleanolic acid derivative. ATA was reported to reduce $\Delta\psi$ in HeLa cells [30]. Here our results showed $\Delta\psi_m$ was gradually decreased as OA concentration increased, which might be the reason for AMPK activation, because mitochondrial membrane depolarization will result in the depletion of cellular ATP. With the rise of AMP/ATP ratio, AMP will bind to the γ subunit of AMPK to cause the conformational change, and as a consequence, the phosphorylation of AMPK at Thr^{172} on the α subunit will be potentiated [31]. In spite of this, the principle of mitochondrial membrane depolarization caused by OA is still uncovered. It has been revealed that FOXO3, upon activated by AMPK, can up-regulate the expression of the uncoupling protein, *Ucp2* [11]. However, this could not be the major issue for the acute loss of mitochondrial membrane potential, thus the reason might lie in the inhibition of electron transfer chain (ETC) on the mitochondrial inner membrane. Still, further work need to be carried out to confirm this. On the other hand, the loss of mitochondrial membrane potential might be considered to be relevant to the events of pro-apoptosis such as the opening of mitochondrial transition pore and release of cytochrome C. However, in this study, the collapse mitochondrial membrane potential induced by OA was acute (within an hour), and the concentration were inside the therapeutic scale, thus serious cell injury and apoptosis were unlikely to occur at this situation. Therefore, the rapid loss of mitochondrial membrane potential is an indicator to reveal the change of intracellular energy status induced by OA, which might be the mechanism of AMPK activation by OA.

Some recent studies have illustrated that OA protects against cardiac ischemia injury by reducing the lactate dehydrogenase (LDH) release and by improving mitochondrial antioxidant mechanism [32, 33]. Our results also revealed that OA could ameliorate hypoxia-induced cardiomyocyte dysfunction, such as depressed peak shortening, reduced maximal velocity of shortening/relengthening, and prolonged duration of contraction in response to hypoxia treatment. The improvement of single cardiomyocyte contractile properties might contribute to the recovery of heart function during post ischemic period. These cardioprotective effects of OA against ischemia injury might partially attribute to the activation of AMPK signaling pathways in cardiomyocytes.

In conclusion, the results indicate that, ischemic activation of AMPK phosphorylates cardiac FOXO3 (Ser^{413}) and the novel AMPK activator, OA, stimulates AMPK downstream targets phosphorylation, i.e. acetyl-CoA carboxylase (ACC), p38 MAPK and FOXO3 (Ser^{413}) as well. The potential mechanism of cardiac AMPK signaling pathway activated by OA might through the reduction of mitochondrial membrane potential ($\Delta\Psi m$) of cardiomyocytes. The activation of AMPK signaling may contribute to the beneficial role of OA in protection against cardiac dysfunction induced by hypoxia.

Acknowledgments

This work was supported by grants from a pilot grant of NIH, University of Wyoming Northern Rockies Regional IDeA Networks for Biomedical Research Excellence (INBRE), 5P20RR016474, R03AG028163, American Heart Association (AHA) National SDG0835168N, and American Federation for Aging Research (AFAR) 08007.

Please address correspondence to: Ji Li, PhD, Division of Pharmaceutical Sciences, University of Wyoming, School of Pharmacy, Laramie, WY 82071, USA, Tel: 307-766-6172, Fax: 307-766-2953, E-mail: jli6@uwyo.edu

References

[1] Ferdinandy P, Schulz R and Baxter GF. Interaction of cardiovascular risk factors with myocardial ischemia/reperfusion injury, preconditioning, and postconditioning. Pharmacol Rev 2007; 59: 418-458.

[2] Young LH, Li J, Baron SJ and Russell RR. AMP-activated protein kinase: a key stress signaling pathway in the heart. Trends Cardiovasc Med 2005; 15: 110-118.

[3] Zou MH, Hou XY, Shi CM, Kirkpatick S, Liu F, Goldman MH and Cohen RA. Activation of 5'-AMP-activated kinase is mediated through c-Src and phosphoinositide 3-kinase activity during hypoxia-reoxygenation of bovine aortic endothelial cells. Role of peroxynitrite. J Biol Chem 2003; 278: 34003-34010.

[4] Russell RR, 3rd, Li J, Coven DL, Pypaert M,

Zechner C, Palmeri M, Giordano FJ, Mu J, Birnbaum MJ and Young LH. AMP-activated protein kinase mediates ischemic glucose uptake and prevents postischemic cardiac dysfunction, apoptosis, and injury. J Clin Invest 2004; 114: 495-503.
[5] Coven DL, Hu X, Cong L, Bergeron R, Shulman GI, Hardie DG and Young LH. Physiological role of AMP-activated protein kinase in the heart: graded activation during exercise. Am J Physiol Endocrinol Metab 2003; 285: E629-636.
[6] Kudo N, Gillespie JG, Kung L, Witters LA, Schulz R, Clanachan AS and Lopaschuk GD. Characterization of 5'AMP-activated protein kinase activity in the heart and its role in inhibiting acetyl-CoA carboxylase during reperfusion following ischemia. Biochim Biophys Acta 1996; 1301: 67-75.
[7] Li J, Hu X, Selvakumar P, Russell RR, 3rd, Cushman SW, Holman GD and Young LH. Role of the nitric oxide pathway in AMPK-mediated glucose uptake and GLUT4 translocation in heart muscle. Am J Physiol Endocrinol Metab 2004; 287: E834-841.
[8] Russell RR, 3rd, Bergeron R, Shulman GI and Young LH. Translocation of myocardial GLUT-4 and increased glucose uptake through activation of AMPK by AICAR. Am J Physiol 1999; 277: H643-649.
[9] Jorgensen SB, Nielsen JN, Birk JB, Olsen GS, Viollet B, Andreelli F, Schjerling P, Vaulont S, Hardie DG, Hansen BF, Richter EA and Wojtaszewski JF. The alpha2-5'AMP-activated protein kinase is a site 2 glycogen synthase kinase in skeletal muscle and is responsive to glucose loading. Diabetes 2004; 53: 3074-3081.
[10] Hardie DG and Carling D. The AMP-activated protein kinase--fuel gauge of the mammalian cell? Eur J Biochem 1997; 246: 259-273.
[11] Greer EL, Oskoui PR, Banko MR, Maniar JM, Gygi MP, Gygi SP and Brunet A. The energy sensor AMP-activated protein kinase directly regulates the mammalian FOXO3 transcription factor. J Biol Chem 2007; 282: 30107-30119.
[12] Greer EL, Dowlatshahi D, Banko MR, Villen J, Hoang K, Blanchard D, Gygi SP and Brunet A. An AMPK-FOXO pathway mediates longevity induced by a novel method of dietary restriction in C. elegans. Curr Biol 2007; 17: 1646-1656.
[13] Price KR, Johnson IT and Fenwick GR. The chemistry and biological significance of saponins in foods and feedingstuffs. Crit Rev Food Sci Nutr 1987; 26: 27-135.
[14] Kim KA, Lee JS, Park HJ, Kim JW, Kim CJ, Shim IS, Kim NJ, Han SM and Lim S. Inhibition of cytochrome P450 activities by oleanolic acid and ursolic acid in human liver microsomes. Life Sci 2004; 74: 2769-2779.
[15] Niikawa M, Hayashi H, Sato T, Nagase H and Kito H. Isolation of substances from glossy privet (Ligustrum lucidum Ait.) inhibiting the mutagenicity of benzo[a]pyrene in bacteria. Mutat Res 1993; 319: 1-9.
[16] Gao D, Li Q, Li Y, Liu Z, Liu Z, Fan Y, Han Z, Li J and Li K. Antidiabetic potential of oleanolic acid from Ligustrum lucidum Ait. Can J Physiol Pharmacol 2007; 85: 1076-1083.
[17] Li J, Miller EJ, Ninomiya-Tsuji J, Russell RR, 3rd and Young LH. AMP-activated protein kinase activates p38 mitogen-activated protein kinase by increasing recruitment of p38 MAPK to TAB1 in the ischemic heart. Circ Res 2005; 97: 872-879.
[18] Miller EJ, Li J, Leng L, McDonald C, Atsumi T, Bucala R and Young LH. Macrophage migration inhibitory factor stimulates AMP-activated protein kinase in the ischaemic heart. Nature 2008; 451: 578-582.
[19] Dong F, Zhang X, Yang X, Esberg LB, Yang H, Zhang Z, Culver B and Ren J. Impaired cardiac contractile function in ventricular myocytes from leptin-deficient ob/ob obese mice. J Endocrinol 2006; 188: 25-36.
[20] Smiley ST, Reers M, Mottola-Hartshorn C, Lin M, Chen A, Smith TW, Steele GD, Jr. and Chen LB. Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation JC-1. Proc Natl Acad Sci U S A 1991; 88: 3671-3675.
[21] Konrad D, Rudich A, Bilan PJ, Patel N, Richardson C, Witters LA and Klip A. Troglitazone causes acute mitochondrial membrane depolarisation and an AMPK-mediated increase in glucose phosphorylation in muscle cells. Diabetologia 2005; 48: 954-966.
[22] Zhao P, Xiao X, Kim AS, Leite MF, Xu J, Zhu X, Ren J and Li J. c-Jun Inhibits Thapsigargin-Induced ER Stress Through Up-Regulation of DSCR1/Adapt78. Exp Biol Med (Maywood) 2008; 233: 1289-1300.
[23] Brunet A, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y, Tran H, Ross SE, Mostoslavsky R, Cohen HY, Hu LS, Cheng HL, Jedrychowski MP, Gygi SP, Sinclair DA, Alt FW and Greenberg ME. Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science 2004; 303: 2011-2015.
[24] Li Q, Ceylan-Isik AF, Li J and Ren J. Deficiency of insulin-like growth factor 1 reduces sensitivity to aging-associated cardiomyocyte dysfunction. Rejuvenation Res 2008; 11: 725-733.
[25] Mammucari C, Schiaffino S and Sandri M. Downstream of Akt: FoxO3 and mTOR in the regulation of autophagy in skeletal muscle. Autophagy 2008; 4: 524-526.
[26] Kharas MG, Deane JA, Wong S, O'Bosky KR, Rosenberg N, Witte ON and Fruman DA. Phosphoinositide 3-kinase signaling is essential for ABL oncogene-mediated transformation of B-lineage cells. Blood 2004; 103: 4268-4275.
[27] Liu J. Pharmacology of oleanolic acid and

ursolic acid. J Ethnopharmacol 1995; 49: 57-68.
[28] Kashiwada Y, Wang HK, Nagao T, Kitanaka S, Yasuda I, Fujioka T, Yamagishi T, Cosentino LM, Kozuka M, Okabe H, Ikeshiro Y, Hu CQ, Yeh E and Lee KH. Anti-AIDS agents. 30. Anti-HIV activity of oleanolic acid, pomolic acid, and structurally related triterpenoids. J Nat Prod 1998; 61: 1090-1095.
[29] Talmor D, Applebaum A, Rudich A, Shapira Y and Tirosh A. Activation of mitogen-activated protein kinases in human heart during cardiopulmonary bypass. Circ Res 2000; 86: 1004-1007.
[30] Sun HX, Zheng QF and Tu J. Induction of apoptosis in HeLa cells by 3beta-hydroxy-12-oleanen-27-oic acid from the rhizomes of Astilbe chinensis. Bioorg Med Chem 2006; 14: 1189-1198.
[31] Baron SJ, Li J, Russell RR, 3rd, Neumann D, Miller EJ, Tuerk R, Wallimann T, Hurley RL, Witters LA and Young LH. Dual mechanisms regulating AMPK kinase action in the ischemic heart. Circ Res 2005; 96: 337-345.
[32] Du Y and Ko KM. Oleanolic acid protects against myocardial ischemia-reperfusion injury by enhancing mitochondrial antioxidant mechanism mediated by glutathione and alpha-tocopherol in rats. Planta Med 2006; 72: 222-227.
[33] Senthil S, Sridevi M and Pugalendi KV. Cardioprotective effect of oleanolic acid on isoproterenol-induced myocardial ischemia in rats. Toxicol Pathol 2007; 35: 418-423.