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Original article

Overexpression of AMP-activated protein kinase or protein kinase D prevents lipid-induced insulin resistance in cardiomyocytes

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ABSTRACT

During lipid oversupply, the heart becomes insulin resistant, as exemplified by defective insulin-stimulated glucose uptake, and will develop diastolic dysfunction. In the healthy heart, not only insulin, but also increased contractile activity stimulates glucose uptake. Upon increased contraction both AMP-activated protein kinase (AMPK) and protein kinase D (PKD) are activated, and mediate the stimulation of glucose uptake into cardiomyocytes. Therefore, each of these kinases is a potential therapeutic target in the diabetic heart because they may serve to bypass defective insulin-stimulated glucose uptake. To test the preventive potential of these kinases against loss of insulin-stimulated glucose uptake, AMPK or PKD were adenovirally overexpressed in primary cultures of insulin resistant cardiomyocytes for assaying substrate uptake, insulin responsiveness and lipid accumulation. To induce insulin resistance and lipid loading, rat primary cardiomyocytes were cultured in the presence of high insulin (100 nM; HI) or high palmitate (palmitate/BSA: 3/1; HP). HI and HP each reduced insulin responsiveness, and increased basal palmitate uptake and lipid storage. Overexpression of each of the kinases prevented loss of insulin-stimulated glucose uptake. Overexpression of AMPK also prevented loss of insulin signaling in HI- and HP-cultured cardiomyocytes, but did not prevent lipid accumulation. In contrast, overexpression of PKD prevented lipid accumulation, but not loss of insulin signaling in HI- and HP-cultured cardiomyocytes. In conclusion, AMPK and PKD prevent loss of insulin-stimulated glucose uptake into cardiomyocytes cultured under insulin resistance-inducing conditions through different mechanisms. This article is part of a Special Issue entitled "Focus on Cardiac Metabolism".

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1. Introduction

The insulin resistant heart is characterized by impaired cardiac function and altered substrate preference towards increased long chain fatty acid (LCFA) utilization at the expense of glucose [1]. The

greater influx of LCFA exceeds the mitochondrial β -oxidative capacity [2]. This mismatch between LCFA uptake and oxidation results in the gradual intramyocellular build-up of triacylglycerols (TG) and other LCFA metabolites, such as diacylglycerols (DG) and ceramides. Lipid accumulation leads to decreased insulin signaling, decreased translocation of the major cardiac glucose transporter GLUT4, and, consequently, decreased insulin-stimulated glucose uptake [3]. Over time, both lipid accumulation and insulin resistance contribute to altered energy metabolism and remodeling of the heart, which eventually, in the presence of cardiac stress such as ischemia, may lead to heart failure [4]. Strategies that stimulate glucose uptake, or reduce LCFA uptake, independent of insulin signaling, may serve as strategies to normalize substrate uptake in the insulin resistant heart, and thereby protect against loss of contractile function [5,6].

In the healthy heart, glucose and LCFA uptake are mainly regulated by reversible glucose transporter 4 (GLUT4) and fatty acid transporter CD36

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translocation between intracellular storage compartments (endosomes) and the sarcolemma [1,7]. Insulin, as well as increased contractile activity, independently stimulate reversible GLUT4 and CD36 translocation, and concomitantly enhance glucose uptake and LCFA into cardiomyocytes, respectively [8]. One of the earliest steps in myocellular insulin resistance is permanent relocation of CD36 from endosomes to the sarcolemma [9]. Permanent CD36 relocation causes a chronic increase in myocellular fatty acid uptake and a concomitant increase in cardiac lipid accumulation. This pivotal role of CD36 in cardiac lipid accumulation was confirmed in CD36 knockout mice, which were protected from lipid accumulation during conditions of lipid overload [5,10]. There is evidence for the existence of insulin-responsive CD36 stores within the endosomes, from where CD36 permanently relocates to the cell surface upon the onset of insulin resistance [1]. Consequently, these intracellular stores will be increasingly depleted of CD36, and increasingly unable to deliver CD36 for translocation upon short-term insulin exposure [9]. At early insulin resistance, permanent CD36 relocation is not accompanied by changes in insulin-stimulated GLUT4 translocation [1]. However, during progression of insulin resistance insulin-stimulated GLUT4 translocation will be increasingly impaired, indicating that the shift in CD36 subcellular localization precedes (and might be responsible for) the impairment in GLUT4 translocation. Ultimately in cardiomyocytes with full-blown insulin resistance, CD36 and GLUT4 display a juxtaposed localization with CD36 being at the cell surface, and GLUT4 being intracellular [1].

In view of that insulin signaling is blunted in the insulin resistant heart, recent research has focused on the activation of contraction-induced signaling for the stimulation of glucose uptake [11]. It is well documented that during contraction-induced signaling AMP-activated protein kinase (AMPK) is activated [11]. The activation of AMPK occurs through phosphorylation by upstream kinases, such as LKB1, in combination with binding of AMP to one of the regulatory subunits [11]. In mice overexpressing a dominant-negative mutant of AMPK α , it has been demonstrated that AMPK is necessary for contraction-induced glucose and LCFA uptake [12]. Several AMPK-activating agents, such as the biguanide metformin and resveratrol, currently serve as blood-glucose lowering agents [13]. Although metformin improved glycemic control, it did not improve cardiac function in type 2 diabetes patients [14]. The activation of AMPK by resveratrol was shown to improve health and survival when given to mice on a high-calorie diet [15,16] and to mimic the effects of calorie restriction in obese humans [17].

Recently, we showed that also protein kinase D (PKD) is contraction activated and stimulates glucose uptake in rat cardiomyocytes [18]. The underlying mechanism encompasses contraction-induced reactive oxygen species (ROS) production, binding of PKD to death-activated protein kinase (DAK) followed by the activation of PKD by DAK [19]. The precise interplay between ROS, DAK and PKD1 has not yet been resolved. The activation of PKD becomes apparent by its autophosphorylation at Ser916 [20]. Pharmacological inhibition of PKD activity did not affect AMPK-Thr172-phosphorylation but reduced contraction-induced glucose uptake to basal levels [18]. In addition, it was shown that contraction-activated PKD does not affect LCFA uptake into cardiomyocytes [19]. This makes PKD a new player in the regulation of cardiac metabolism with a different action independent of AMPK.

Because both AMPK and PKD mediate contraction-stimulated glucose uptake, both kinases are potential targets to preserve glucose uptake in the insulin-resistant heart. How, and if, AMPK and PKD can restore cardiac lipid loading and insulin resistance, is not known yet. Based on earlier findings described above, we propose that AMPK and PKD differently prevent development of insulin resistance and lipotoxicity. Therefore, cardiomyocytes were cultured under lipid overloading and insulin resistance-inducing conditions [21], and then subjected to adenoviral overexpression of AMPK or PKD.

2. Materials and methods

2.1. Materials

Palmitic acid and [3 H]deoxyglucose were obtained from GE Healthcare (Little Chalfont, UK). Laminin and insulin were purchased from Sigma (Saint Louis, USA). Bovine serum albumin (BSA) (fraction V), dependent on the application, was derived from MP Biomedicals (Irvine, USA) (for cell isolation and incubation purposes), or from Sigma (other purposes). Collagenase type II was from Worthington (Freehold, USA).

2.2. Experimental animals

Male Lewis rats, 200–250 g, were purchased from Charles River laboratories and used for culturing isolated cardiomyocytes. All animal procedures were approved by the Experimental Animal Committee of Maastricht University, NL.

2.3. Cardiomyocyte isolation

Rat cardiomyocytes were isolated using a Langendorff perfusion system, as previously described [22] with the only difference being the sterile conditions that were taken into account for rat cardiomyocyte isolations.

2.4. Adenovirus amplification

Control adenovirus containing enhanced green fluorescent protein (GFP) (control), a recombinant adenovirus encoding a truncated form of AMPK α 2 (AMPK α 2 1–312) and GFP (AdAMPK) [23,24], and a recombinant adenovirus encoding full-length wild-type mouse PKD1 and EGFP (AdPKD) [25] were used. Adenoviruses were amplified in HEK293 cells and purified over CsCl₂ gradients as previously described by Luo et al. [26]. Optimal multiplicity of infection (MOI) was determined

Fig. 1. Western blot images of cultured primary rat cardiomyocytes transfected with adenovirus containing GFP (control), constitutively active (CA) AMP-activated protein kinase (AMPK) and protein kinase D (PKD). Cardiomyocytes were cultured for 2 days in control/low palmitate medium, and subsequently used for measurement of (A) total AMPK total PKD, (B) AMPK-Thr172 phosphorylation, PKD-Ser916 phosphorylation, (C) acyl-CoA carboxylase (ACC)-Ser79 phosphorylation, and troponinI (TnI)-Ser22/23 phosphorylation. Representative blots are shown. Phosphorylated endogenous and CA-AMPK were detected 62 at 35 kDa, respectively.

Fig. 2. Effect of overexpression of adenoviral (Ad) constitutively active AMP-activated protein kinase (AMPK) or protein kinase D (PKD) on metabolic parameters in cultured primary rat cardiomyocytes. Cardiomyocytes were cultured in control/low palmitate medium (LP) in the absence (Basal) or presence of adenovirus (Ad) containing constitutively active AMP-activated protein kinase (AMPK) or protein kinase D (PKD). Upon 2 days of culturing, cells were used for the measurement of uptake of (A) [3 H]deoxyglucose and (B) [14 C] palmitate, as well as the measurement of (C) triacylglycerol stores via HPTLC, (D) protein presence of CD36 and GLUT4, (E) Akt-Ser473 phosphorylation and (F) GSK3 β -Ser9 phosphorylation via Western blotting. For the experiments displayed in panels A, B, E and F, the cells underwent a 30 min recovery period after the culturing, and subsequent short-term (15 min) insulin (100 nM) stimulation. Basal (open bars) and insulin-stimulated (striped bars). Statistical analysis was done by one-way ANOVA with Bonferroni post-tests and Student's *t*-test, * Insulin effect $p < 0.05$, ^ adenovirus effect $p < 0.05$. $n = 6-12$.

by fluorescence microscopy and Western blotting for downstream targets of both AMPK and PKD (Supplementary Figs. 1–2). MOI 40 was chosen for AdAMPK and MOI 10 was chosen for AdPKD.

2.5. Culturing of rat cardiomyocytes

After isolation of cardiomyocytes, 200,000 cells were seeded per well in laminin coated 6-well plates and after 90 min adhesion medium (control medium without Ca^{2+}) was replaced with control medium (20 μM palmitate, palmitate:BSA 0.3:1), high insulin medium (100 nM) or high palmitate medium (200 μM , palmitate:BSA 3:1). Cells were cultured according to the method described by Volz et al. [27] in modified serum-free medium M199 containing carnitine, creatine and taurine [27]. Culturing adult cardiac myocytes does not affect their insulin responsiveness [28]. During the experiment the cells were maintained in an incubator (37 °C, 5% CO_2) and were used for experiments 48 h after seeding and transduction. Primary rat cardiomyocytes were transduced with control, AdAMPK, or AdPKD 90 min after seeding the cardiomyocytes. Cardiomyocytes were cultured for 48 h because of optimal combination of MOI and transduction time (Supplementary Figs. 1–2).

2.6. Measurement of substrate uptake

Uptake of [$1\text{-}^{14}\text{C}$]palmitate (in complex with BSA) and [3H] deoxyglucose into cardiac myocytes was measured as previously described for cardiomyocytes in culture [29]. In short, after 2 days of culturing, cardiomyocytes were washed and were allowed to recover for 30 min from short-term insulin effects. Namely, 30 min of insulin withdrawal appear sufficient to re-establish low glucose and LCFA uptake rates into cardiomyocytes and other insulin-sensitive cells upon prior short-term insulin addition (see [21] and [30]). Hence, 30 min withdrawal of insulin-containing culture media is sufficient to truly assess “basal” conditions. Afterwards, one portion of the cells was exposed to insulin (100 nM) for 15 min to be able to compare basal uptake with insulin-stimulated uptake. Then, radioactively-labeled glucose and palmitate were added for 10 min, cells were washed, lysed and radioactivity was measured with a β -counter (Fig. 3A for a graphical description of this method).

2.7. Triacylglycerol stores

Intramyocellular lipids were determined after 2 days of culturing in lysed cells as described previously [31]. Triacylglycerol bands were detected with a Molecular Imager (ChemiDoc XRS, BioRad) and analyzed with Quantity One® (BioRad).

2.8. Protein detection

Rat cardiomyocytes were assayed after 2 days of culturing and a recovery of 30 min from acute insulin effects. Afterwards, cells were exposed to insulin (100 nM) for 15 min to be able to compare basal-phosphorylation to insulin-stimulated phosphorylation. Cells were lysed in a sample buffer and used for protein detection by SDS-polyacrylamide gel electrophoresis, followed by Western blotting, as previously described [32]. Primary antibodies against phospho-Ser473-Akt, Akt, phospho-Ser9-GSK3 β , GSK3 β , phospho-Thr172-AMPK, AMPK, phospho-Ser916-PKD/PKC- μ , PKD/PKC- μ , and phospho-Ser22/23-TnI from Cell Signaling (Danvers, MA), CD36 from GenTex Inc. (San Antonio, TX, USA), GLUT4 from Abcam (Cambridge, USA), phospho-Ser79-ACC from Upstate and caveolin3 from BD Transduction Laboratories. Western blot images were analyzed with a Molecular Imager (ChemiDoc XRS, BioRad) and quantified with Quantity One® (BioRad).

2.9. Statistics

All data are presented as means $\pm$ S.E.M. Statistical difference between groups was evaluated by one-way ANOVA, 2-way ANOVA or Student's *t*-test by using GraphPad Prism. *P* values equal to or less than 0.05 were considered significant.

Fig. 3. Effects of AdAMPK or AdPKD treatment on substrate uptake and lipid content in cardiomyocytes cultured under insulin resistance-inducing conditions. Cardiomyocytes were cultured in control/low palmitate medium (LP), or in media containing high insulin (HI) or high palmitate (HP) concentrations in the absence (Basal) or presence of adenovirus (Ad) containing constitutively active AMP-activated protein kinase (AMPK) or protein kinase D (PKD). Upon 2 days of culturing, cells were allowed to recover for 30 min prior to short-term (15 min) insulin (100 nM) addition and subsequent measurement of uptake of (A) [3H]deoxyglucose and (B) [^{14}C] palmitate. (C) Alternatively, upon 2 days of culturing, cardiomyocytes were lysed, and triacylglycerol stores were measured by HPTLC. Statistical analysis was done by 2-way ANOVA with Bonferroni post-tests, $p < 0.05$, * versus LP in the same group. Statistical analysis was done by one-way ANOVA with Bonferroni post-tests and Student's *t*-test, * Insulin effect $p < 0.05$, ^ adenovirus effect $p < 0.05$, # medium effect $p < 0.05$, $n = 8\text{--}12$.

3. Results

3.1. Effect of AMPK or PKD overexpression on substrate uptake, insulin responsiveness and lipid storage in control cells

Cardiomyocytes were cultured for 48 h because of optimal combination of multiplicity of infection (MOI) and transfection time (Supplementary Figs. 1–2). Overexpression of the truncated constitutively active form of AMPK α 2 did not affect endogenous total AMPK levels (Figs. 1A–B) or phosphorylation of the downstream substrate of PKD, troponin I (TnI), but potentially increased phosphorylation of its main subcellular target acetyl CoA carboxylase (ACC) (Fig. 1C). Overexpression of full length PKD (Figs. 1A–B) potentially increased TnI phosphorylation, but did not affect downstream signaling of AMPK, i.e., phosphorylation of ACC (Fig. 1C). This indicates that both kinases were successfully overexpressed in our cardiomyocyte cultures, and furthermore, confirms earlier findings that PKD and AMPK function in separate pathways [18,19].

Cardiomyocytes cultured for 48 h in control medium, and transduced with control adenovirus, displayed insulin-stimulated glucose (1.8-fold) and palmitate uptake (1.5-fold) (Figs. 2A–B), in agreement with our previous short-term substrate uptake studies with suspensions of freshly isolated cardiomyocytes [32]. These results indicate that insulin responsiveness was not lost due to culturing or viral transduction.

Overexpression of either AMPK or PKD increased basal glucose uptake (Fig. 2A) (2.3-fold and 3.3-fold, respectively). The insulin-stimulated component of glucose uptake was retained upon overexpression of either kinase (Fig. 2A). AdAMPK, but not AdPKD, significantly increased basal palmitate uptake into cardiomyocytes, in agreement with earlier findings that AMPK is dedicated to both LCFA and glucose uptake, but that PKD is selective for glucose uptake [12,19] (Fig. 2B). However, insulin-stimulated palmitate uptake did not reach significance in AdAMPK and AdPKD-treated cells. This likely is an experimental limitation of palmitate uptake measurements in cultures of cardiomyocytes; in long-term cultures palmitate uptake rates are lower in comparison to freshly isolated cardiomyocyte suspensions, so that it is difficult to establish significance of a moderate effect on top of another moderate effect. Furthermore, AdAMPK or AdPKD did not significantly alter intramyocardial TG content in basally cultured cardiomyocytes (Fig. 2C), or alter expression of the substrate transporters GLUT4 and CD36 (Fig. 2D).

To examine insulin responsiveness at the level of the insulin-signaling cascade we measured insulin-stimulated Akt-phosphorylation and insulin-stimulated GSK3 β -phosphorylation (Figs. 2E–F). Transducing cardiomyocytes with AdAMPK significantly increased basal and insulin-stimulated Akt-phosphorylation (3.9 and 3.0-folds, respectively), as well as GSK3 β -phosphorylation (2.5 and 1.9-folds, respectively). In contrast, AdPKD was without an effect (Figs. 2E–F). These changes in insulin-stimulated Akt phosphorylation could not be ascribed to alterations in protein expression of the loading control, total Akt and GSK3 β (Supplementary Figs. 3–4).

Altogether these results show that AdAMPK and AdPKD indeed both enhance glucose uptake, while the cardiomyocytes remain insulin responsive. In addition, AdAMPK, but not AdPKD, enhances LCFA uptake, Akt phosphorylation and GSK3 β -phosphorylation.

3.2. Establishment of insulin resistant cardiomyocytes: effects of high insulin and high palmitate on substrate uptake, insulin responsiveness and lipid storage

For induction of myocellular insulin resistance, cardiomyocytes were exposed to high insulin (HI) or high palmitate (HP)-containing media. HI or HP-medium-cultured cardiomyocytes showed no change in basal glucose uptake (Fig. 3A). However, they did show an increase in basal palmitate uptake (amounting to 1.5-fold and 1.8-fold, respectively; Fig. 3B). In agreement with previous works [33,34] HI-medium entirely blocked insulin-stimulated glucose uptake. Insulin-stimulated glucose uptake was also abrogated in cardiomyocytes cultured in

HP-medium (Fig. 3A). In addition, insulin-stimulated palmitate uptake was lost in HI- and HP-medium-cultured cardiomyocytes (Fig. 3B). Both HI- and HP-media increased TG stores 1.9-fold and 2.9-fold, respectively (Fig. 3C).

With respect to insulin signaling, insulin-stimulated Akt and GSK3 β phosphorylations were completely lost in HI-medium-cultured cardiomyocytes, and markedly reduced (by 42% and 31%, respectively; $p < 0.05$) in HP-medium-cultured cardiomyocytes (Figs. 4A–B).

Taken together, both HI- and HP-media induce lipid accumulation and insulin resistance in cardiomyocytes. A key underlying mechanism is the permanent relocation of the fatty acid transporter CD36 from intracellular stores to the sarcolemma [21], which causes chronically elevated basal LCFA uptake. The surplus of incoming LCFA will enhance their deposition into TGs and other LCFA metabolites leading to inhibition of insulin signaling downstream of the insulin-receptor substrate and, consequently, to inhibition of insulin-induced glucose and LCFA uptake, according to the lipid-induced insulin resistance theory of Shulman [35]. Hence, HI and HP-medium-cultured cardiomyocytes prove to be models for lipid overloaded insulin resistant cardiomyocytes.

3.3. Effect of AMPK overexpression on substrate uptake, lipid storage and insulin responsiveness

Overexpression of AMPK enhanced basal glucose uptake not only in cardiomyocytes in control medium but also in cardiomyocytes cultured in HI or HP-medium. Additionally, AdAMPK prevented the loss in insulin-induced glucose uptake into cardiomyocytes in both culturing conditions (Fig. 3A). However, AdAMPK did not decrease the elevated basal palmitate uptake (Fig. 3B) or TG accumulation (Fig. 3C) in cells cultured in either experimental medium, nor was it able to retain the insulin-stimulated component of palmitate uptake (Fig. 3B).

Similarly to insulin-stimulated glucose uptake, culturing cardiomyocytes with AdAMPK prevented inhibition of insulin-stimulated Akt and GSK3 β phosphorylations in both experimental media (Figs. 4A–B). Altogether, we show that AdAMPK treatment prevents the loss of insulin-mediated Akt signaling and of glucose uptake in cardiomyocytes cultured under insulin resistance-inducing conditions, but it does not prevent lipid accumulation.

3.4. Effect of PKD overexpression on substrate uptake, lipid storage and insulin responsiveness

The effects of AdPKD treatment on glucose uptake into HI/HP-media-cultured cardiomyocytes were similar to those of AdAMPK; increased basal glucose uptake and prevention of loss of insulin-stimulated glucose uptake, but no lowering of basal LCFA uptake or retention of insulin-stimulated palmitate uptake (Figs. 3A–B). Despite the inability of AdPKD to decrease basal palmitate uptake, AdPKD markedly reduced the storage of TG in cardiomyocytes cultured in both insulin resistance-inducing media (Fig. 3C).

With respect to insulin signaling, AdPKD incompletely preserved insulin-stimulated phosphorylation of Akt and GSK3 β under insulin resistance-inducing conditions, in contrast to full preservation of insulin-stimulated glucose uptake. Specifically, in HP-medium-cultured cardiomyocytes, insulin-stimulated Akt-phosphorylation was retained upon AdPKD treatment (Fig. 4A). However, AdPKD treatment did not preserve insulin-stimulated Akt-phosphorylation in HI-medium-cultured cardiomyocytes, nor did AdPKD preserve insulin-stimulated GSK3 β -phosphorylation under both insulin resistance-inducing culturing conditions (Figs. 4A–B). These latter findings are in line with the lack of an effect of AdPKD on basal and insulin-stimulated Akt-phosphorylation and GSK3 β -phosphorylation in insulin-sensitive cardiomyocytes (Figs. 2E–F). A possible explanation for the observation that a significant increase in Akt phosphorylation upon insulin-stimulation of HP medium-cultured AdPKD-treated cardiomyocytes is

Fig. 4. Effects of AdAMPK or AdPKD treatment on insulin signaling in cardiomyocytes cultured under insulin resistance-inducing conditions. Cardiomyocytes were cultured in control/low palmitate medium (LP), or in media containing high insulin (HI) or high palmitate (HP) concentrations in the absence (Basal) or presence of adenovirus (Ad) containing constitutively active AMP-activated protein kinase (AMPK) or protein kinase D (PKD). Upon 2 days of culturing, cells were allowed to recover for 30 min prior to short-term (15 min) insulin (100 nM) addition. Immediately thereafter, cells were lysed in a sample buffer and used for Western detection of (A) Ser473-Akt phosphorylation and (B) Ser9-GSK3 β phosphorylation. Statistical analysis was done by one-way ANOVA with Bonferroni post-tests and Student's *t*-test, * Insulin effect $p < 0.05$, ^ adenovirus effect $p < 0.05$, $n = 6-10$.

paralleled by a non-significantly increased GSK3- β phosphorylation probably may lie in the non-linearity of signaling responses. Taken together, overexpression of PKD prevents the loss of insulin-stimulated glucose uptake and it prevents myocellular lipid loading.

4. Discussion

AMPK and PKD are two contraction-activated kinases. Both kinases are also essential for the contraction-stimulation of glucose uptake. Especially this last property makes both kinases attractive targets to improve glucose uptake dynamics in the lipid overloaded heart on a path to develop insulin resistance.

In this study, we have used primary cultures of cardiomyocytes. Although cardiomyocytes in culture display lower basal metabolic rates than the intact working heart model, metabolic responses to hormonal and pharmacological stimuli are preserved [32,36]. In a recent study [21], we have established that these cells will go through the same sequence of events upon a lipid oversupply to develop insulin resistance as compared to the heart in vivo upon feeding rodents a high fat diet (although on a shorter time span, i.e., ≤ 48 h versus

≥ 4 weeks): permanent CD36 relocation to the sarcolemma; chronically increased LCFA uptake, accumulation of LCFA metabolites, impairment of insulin signaling and of insulin-stimulated glucose uptake, and finally contractile dysfunction [6,9]. We have also shown that in skeletal muscle of obese individuals the increase in sarcolemmal CD36 abundance is causal to the development of insulin resistance [37]. Assuming that CD36 relocation will similarly occur in the heart of obese individuals, the used model of cultured cardiomyocytes might have clinical relevance for studies on the mechanism of lipid-induced insulin resistance.

In order to force cultured cardiomyocytes to develop lipid-induced insulin resistance, we have exposed these cells to two different culturing conditions (HI or HP), similarly as recently reported [21]. This results in the inhibition of insulin signaling and of insulin-induced glucose uptake. In order to test the cardioprotective actions of AMPK and PKD under both in vitro conditions of lipid-induced insulin resistance, we adenovirally overexpressed each of the kinases in cardiomyocytes simultaneously with the start of the exposure of these cells to HI/HP media. In these experiments we showed that overexpression of each of the kinases prevents the loss of insulin-induced glucose uptake. However, the underlying mechanism used

by these kinases to preserve insulin-induced glucose uptake appeared to be different.

4.1. How does AMPK overexpression preserve insulin-stimulated glucose uptake in cardiomyocytes cultured under insulin resistance-inducing conditions?

Overexpression of the catalytic subunit of AMPK α 2 in basally cultured cells elicited increases in basal glucose and palmitate uptake as well as in insulin signaling, without alterations in insulin-responsiveness of the uptake of both substrates or in lipid accumulation. None of the alterations in the substrate uptake could be ascribed to changes in protein presence of substrate transporters, indicating that AdAMPK likely exerts these effects through translocation of these transporters. Hence, AdAMPK will induce both GLUT4 and CD36 translocation.

Earlier, we established that in AMPK α 2-knockout cardiomyocytes, contraction-induced glucose and LCFA uptake were abolished [12,19], thereby indicating an essential role of this kinase in both contraction-induced uptake processes. Consequently, AMPK overexpression, mimicking contraction-induced AMPK activation, indeed stimulates uptake of both substrates. Another previous observation of us included that contraction-activated substrate uptake is additive to, and therefore independent from, insulin-stimulated substrate uptake [38]. This previous finding is in line with the presently observed lack of an effect of AdAMPK on insulin-stimulated substrate uptake. However, AdAMPK further improves insulin signaling. Similar AMPK-mediated effects on insulin signaling have been observed previously by using AMPK activators like metformin, phenformin or oligomycin [33,34]. More specifically, it was shown in 3T3 adipocytes that the AMPK activator AICAR directly induces Akt-phosphorylation via a PI3K dependent pathway and, that AICAR enhances insulin-mediated Akt-phosphorylation [39]. Moreover, in a leukemia cell line it was shown that AICAR treatment leads to Akt phosphorylation via direct AMPK down regulation of mTOR and activation of IRS-1 signaling [40]. However, some other studies have shown the opposite effect, an inhibition of Akt-phosphorylation upon AMPK activity [41,42]. The reason underlying this discrepancy is unclear.

Combined, the apparent absence of an effect of AdAMPK on insulin-stimulated glucose uptake in the presence of enhanced AMPK-mediated insulin signaling in basally cultured cardiomyocytes can be explained by the notion that insulin stimulation of Akt in the absence of AdAMPK is already maximally effective in recruiting the entire intracellularly stored insulin-responsive GLUT4 and CD36 pools to the sarcolemma. This is in agreement with the hypothesis that in normo-insulin-sensitive cells the insulin signaling pathway is not the rate-limiting step in insulin-stimulated glucose uptake [43]. Hence, further stimulation of insulin signaling will have no extra effect on the stimulation of glucose and LCFA uptake. The AdAMPK-mediated increase in LCFA uptake without an increase in lipid deposition indicates that all the extra incoming LCFA are efficiently directed towards β -oxidation, in agreement with our previous observations [38].

Under insulin resistance-inducing conditions, likely the most important AdAMPK action is the prevention of the loss in insulin-induced glucose uptake into cardiomyocytes. Furthermore, AdAMPK enhanced basal glucose uptake, just like in basally cultured cells. However, AdAMPK did not alter the elevated basal LCFA uptake in cells cultured in either experimental medium, nor was it able to retain the insulin-stimulated component of LCFA uptake.

The AdAMPK-mediated retention of insulin-stimulated glucose uptake in cardiomyocytes under insulin resistance inducing conditions is accompanied by the retention of insulin signaling but not by the normalization of low myocellular TG levels. Hence, DG and ceramide levels, in equilibrium with the TG, would also not be decreased.

Consequently, the blockade of insulin signaling at the levels of IRS1 (through DG-induced PKC-mediated Ser-phosphorylation) and Akt (direct inhibition by ceramide) remains. However, AdAMPK, just as the above mentioned insulin-sensitizing pharmacological AMPK activators, will likely disrupt the negative feedback loop in insulin signaling by the inhibition of IRS-1-Ser-phosphorylation via the mTOR/p70S6K axis [43]. Apparently this insulin-sensitizing action of AMPK effectively counteracts the negative effects of lipids on insulin signaling.

The lack of an effect of AdAMPK on the elevated basal LCFA uptake rates in cardiomyocytes under insulin resistance-inducing conditions, is in line with, and likely the cause of the lack of an effect of AdAMPK on myocellular lipid accumulation. The explanation of a lack of an effect of AdAMPK in cardiomyocytes under insulin resistance-inducing conditions, in contrast to stimulatory action in basally cultured cells could include that basal uptake into insulin resistant cardiomyocytes is already maximally elevated.

It is not known yet why AdAMPK prevents the HP/HI-induced loss of insulin-stimulated glucose uptake but not that of the insulin-stimulated LCFA uptake. However, it is known that in normal insulin-sensitive cardiomyocytes different subcellular components (e.g., soluble NSF attachment protein receptor (SNARE) proteins, and cytoskeletal actin, see [29,44]) are involved in insulin-stimulated glucose versus LCFA uptake. For example, a recent study showed that berberine, an AMPK activator, was also able to enhance insulin-stimulated GLUT4 translocation and glucose uptake in insulin resistant cells by the improvement of insulin-induced actin reorganization [45]. However, actin reorganization is not involved in insulin-induced CD36 translocation and LCFA uptake [44]. These studies suggest that various subcellular trafficking components are involved in the regulation of cardiomyocyte metabolism and are able to discriminate between glucose and fatty acid uptake. Then, only those components specifically involved in insulin-stimulated glucose uptake would be a target of AMPK in insulin resistant cells.

Altogether, we conclude that AdAMPK treatment prevents the loss of insulin-mediated signaling and glucose uptake in cardiomyocytes cultured under insulin resistance-inducing conditions, but does not prevent lipid accumulation.

4.2. How does PKD overexpression preserve insulin-stimulated glucose uptake in cardiomyocytes cultured under insulin resistance-inducing conditions?

In basally cultured cells, AdPKD enhanced basal glucose uptake without altering insulin-stimulated glucose uptake, LCFA uptake, myocellular TG content and insulin signaling. This selective effect of AdPKD on the stimulation of glucose uptake (and not on the LCFA uptake) confirms our earlier findings in PKD knockout mice in which contraction-induced glucose uptake was abolished but contraction-induced LCFA uptake was retained [19]. AdPKD did not alter GLUT4 expression, indicating that the increase in basal glucose uptake is due to GLUT4 translocation, in agreement with our earlier observations [12,19]. Hence, unlike AMPK, PKD is selectively involved in the regulation of glucose uptake. Furthermore, PKD is known to be activated by contraction [18,19], and not by insulin, and is therefore not involved in the regulation of insulin signaling.

Under both insulin resistance-inducing conditions, AdPKD treatment, similarly to AdAMPK treatment, prevented the loss in insulin-stimulated glucose uptake. Also the other effects of AdPKD on substrate uptake under insulin resistance-inducing conditions were similar to those of AdAMPK: increased basal glucose uptake and no reduction of elevated basal LCFA uptake or retention of insulin-stimulated LCFA uptake. This same action pattern of AdPKD and AdAMPK in cardiomyocytes cultured under insulin resistance-inducing conditions is surprising given the operability of each of these kinases in different signaling pathways.

In contrast to AdAMPK, AdPKD treatment did not completely prevent loss of insulin signaling in cardiomyocytes under insulin resistance-inducing conditions, but lowered the elevated lipid levels to normal proportions. Hence, the preventive action of AdPKD on insulin-stimulated glucose uptake could not be explained by preservation of insulin signaling. This raises two questions: can reduction of lipid storage by itself preserve insulin-stimulated glucose uptake despite impairment of insulin signaling? Second, how can AdPKD treatment reduce lipid stores given its disability to reduce enhanced basal LCFA uptake?

To start out with the latter question, the ability of AdPKD to reduce lipid stores in insulin resistant cardiomyocytes is in contrast to the lack of a reduction in basal LCFA uptake by AdPKD treatment and to the proposed non-involvement of PKD in the regulation of LCFA uptake [19]. In addition, recent studies with cardiospecific PKD knock-out mice showed that PKD is not involved in LCFA oxidation [19]. Since the role of PKD in cellular metabolism is a relatively novel research subject, downstream PKD targets have been incompletely charted. Perhaps AdPKD would increase TG hydrolysis by increasing the expression of TG hydrolases, such as ATGL or HSL, and thereby reduce lipid deposition. Despite that there is no evidence for this yet, this explanation gains in likelihood by the recent observation that PKD is a key regulator of the dynamics of lipoprotein lipase [46], since this shows that PKD controls enzymes involved in cardiomyocyte lipid handling.

With respect to the first question, excess lipids are known to interfere with insulin signaling [34], and consequently, reduction of lipids should deblock insulin signaling. How then can AdPKD-mediated reduction of myocellular lipids in the absence of preservation of insulin signaling account for preservation of insulin-induced glucose uptake in cardiomyocytes under insulin resistance-inducing conditions? Part of an explanation could include SNARE proteins involved in insulin-stimulated GLUT4 translocation downstream of insulin signaling [47]. Some of these SNARE proteins, e.g., SNAP23, also regulate the dynamics of lipid droplets [48]. It is proposed that lipid droplets compete with the insulin-stimulated GLUT4 translocation process for SNAP23, and excess myocellular lipids would shift SNAP23 away from insulin-stimulated GLUT4 translocation [48]. Then, reduction in lipid storage would make SNAP23 available to re-participate in insulin-stimulated GLUT4 translocation.

Altogether, we conclude that in cardiomyocytes cultured under insulin resistance-inducing conditions, AdPKD treatment prevents lipid accumulation and insulin-induced glucose uptake, but does not prevent loss of insulin-mediated Akt/GSK3 β signaling.

4.3. Conclusion

In the present study, we explored the potential of AMPK or PKD overexpression to prevent loss of insulin-stimulated glucose uptake in cardiomyocytes incubated under insulin resistance-inducing conditions. The results can be summarized as follows; lipid loading and insulin resistance were induced by exposure to HI or HP medium. AdAMPK as well as AdPKD treatment prevented loss of insulin-stimulated glucose uptake in cardiomyocytes cultured in both culturing conditions, but they exerted this beneficial effect through different mechanisms. AdAMPK, but not AdPKD, prevents the loss in insulin-mediated Akt signaling. Conversely, AdPKD, but not AdAMPK prevents lipid accumulation.

Recently we established that culturing cardiomyocytes in either of both HI and HP media results in severe impairment of contractile function [21]. However, we do not know yet whether AdAMPK and/or AdPKD treatment would prevent the loss of contractility during culturing under both insulin resistance-inducing conditions. In that same recent study [21], we also found a close correlation of changes in lipid accumulation with changes in sarcomere shortening: in the tested conditions, a maladaptive increase in lipid content accompanied

a decrease in sarcomere shortening, and a normalization of lipids accompanied a normalization of contractility. With respect to insulin signaling, a decrease in this parameter correlated with a decrease in contractile function. However, it appeared to be possible to preserve contractile function while insulin signaling remained impaired [21]. From this, we may predict that AdPKD treatment, selectively preserving low myocellular lipid levels, would also preserve sarcomere shortening. However, in the case of AdAMPK treatment, selectively preserving insulin signaling, we are less certain as to whether contractile function is preserved. The close correlation between myocellular lipids and cardiomyocyte contractility could be a direct effect of lipids on the mechanics of the contraction, whereby large lipid droplets in between the contractile fibers would spatially inhibit the contractility.

It is important to stress that all the effects of AdAMPK or AdPKD on glucose uptake, insulin responsiveness and lipid content in cardiomyocytes were observed in both culturing conditions, thereby suggesting that the beneficial effects of AdAMPK and AdPKD on cardiomyocyte substrate uptake are not model-related but may be of general applicability in the insulin resistant setting. Future experiments should show if their combined action would be even more beneficial for the preservation of cardiomyocyte substrate uptake under insulin resistance-inducing conditions. In addition, it would be interesting to measure cardiomyocyte contractility after overexpression of AMPK or PKD since culturing these cells in HI- or HP-medium, and also increased glucose utilization, is known to decrease their contractile capacity [21].

Finally, it was revealed that not only AMPK, but also PKD, is a suitable target for the prevention of maladaptive changes in cardiac substrate uptake leading to cardiomyopathy and loss of function. Next to the known AMPK activators (metformin, AICAR, resveratrol), perhaps also PKD activators would be potential insulin resistance therapeutics. Several hormones and pharmacological agents, such as endothelin and phenylephrine have been shown to activate PKD via α -adrenergic signaling in a G-protein coupled-receptor manner [49]. Hence, all these agents have a large spectrum of actions, in which PKD only forms a small component. Therefore, it would be of interest to search for pharmacological agents that would more specifically activate PKD.

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Disclosures

None.

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References

- [1] Glatz JFC, Luiken JJFP, Bonen A. Membrane fatty acid transporters as regulators of lipid metabolism: implications for metabolic disease. *Physiol Rev* 2010;90:367–417.
- [2] Bonen A, Holloway GP, Tandon NN, Han XX, McFarlan J, Glatz JF, et al. Cardiac and skeletal muscle fatty acid transport and transporters and triacylglycerol and fatty acid oxidation in lean and Zucker diabetic fatty rats. *Am J Physiol Regul Integr Comp Physiol* 2009;297:R1202–12.
- [3] Borradaile NM, Schaffer JE. Lipotoxicity in the heart. *Curr Hypertens Rep* 2005;7:412–7.

- [4] Taegtmeyer H, McNulty P, Young ME. Adaptation and maladaptation of the heart in diabetes: part I: general concepts. *Circulation* 2002;105:1727–33.
- [5] Steinbusch LK, Luiken JJ, Vlasblom R, Chabowski A, Hoebers NT, Coumans WA, et al. Absence of fatty acid transporter CD36 protects against Western-type diet-related cardiac dysfunction following pressure overload in mice. *Am J Physiol Endocrinol Metab* 2011;301:E618–27.
- [6] Glatz JF, Bonen A, Ouwens DM, Luiken JJ. Regulation of sarcolemmal transport of substrates in the healthy and diseased heart. *Cardiovasc Drugs Ther* 2006;20:471–6.
- [7] Zorzano A, Sevilla L, Camps M, Becker C, Meyer J, Kammermeier H, et al. Regulation of glucose transport, and glucose transporters expression and trafficking in the heart: studies in cardiac myocytes. *Am J Cardiol* 1997;80:65A–76A.
- [8] Lopaschuk GD, Ussher JR, Folmes CD, Jaswal JS, Stanley WC. Myocardial fatty acid metabolism in health and disease. *Physiol Rev* 2010;90:207–58.
- [9] Coort SL, Hasselbaink DM, Koonen DP, Willems J, Coumans WA, Chabowski A, et al. Enhanced sarcolemmal FAT/CD36 content and triacylglycerol storage in cardiac myocytes from obese Zucker rats. *Diabetes* 2004;53:1655–63.
- [10] Yang J, Sambandam N, Han X, Gross RW, Courtois M, Kovacs A, et al. CD36 deficiency rescues lipotoxic cardiomyopathy. *Circ Res* 2007;100:1208–17.
- [11] Steinberg GR, Kemp BE. AMPK in health and disease. *Physiol Rev* 2009;89:1025–78.
- [12] Habets DD, Coumans WA, El Hasnaoui M, Zarrinpashneh E, Bertrand L, Viollet B, et al. Crucial role for LKB1 to AMPK α 2 axis in the regulation of CD36-mediated long-chain fatty acid uptake into cardiomyocytes. *Biochim Biophys Acta* 2009;1791:212–9.
- [13] Hawley SA, Ross FA, Chevzoff C, Green KA, Evans A, Fogarty S, et al. Use of cells expressing gamma subunit variants to identify diverse mechanisms of AMPK activation. *Cell Metab* 2010;11:554–65.
- [14] Jonker JT, Lamb HJ, van der Meer RW, Rijzewijk LJ, Menting LJ, Diamant M, et al. Pioglitazone compared with metformin increases pericardial fat volume in patients with type 2 diabetes mellitus. *J Clin Endocrinol Metab* 2009;95:456–60.
- [15] Baur JA, Pearson KJ, Price NL, Jamieson HA, Lerin C, Kalra A, et al. Resveratrol improves health and survival of mice on a high-calorie diet. *Nature* 2006;444:337–42.
- [16] Um JH, Park SJ, Kang H, Yang S, Foretz M, McBurney MW, et al. AMP-activated protein kinase-deficient mice are resistant to the metabolic effects of resveratrol. *Diabetes* 2010;59:554–63.
- [17] Timmers S, Konings E, Bilet L, Houtkooper RH, van de Weijer T, Goossens GH, et al. Calorie restriction-like effects of 30 days of resveratrol supplementation on energy metabolism and metabolic profile in obese humans. *Cell Metab* 2011;14:612–22.
- [18] Luiken JJ, Vertommen D, Coort SL, Habets DD, El Hasnaoui M, Pelsers MM, et al. Identification of protein kinase D as a novel contraction-activated kinase linked to GLUT4-mediated glucose uptake, independent of AMPK. *Cell Signal* 2008;20:543–56.
- [19] Dirckx E, Schwenk RW, Coumans WA, Hoebers N, Angin Y, Viollet B, et al. Protein kinase-D1 is essential for contraction-induced glucose uptake, but is not involved in fatty acid uptake into cardiomyocytes. *J Biol Chem* 2011;287:5871–81.
- [20] Ryckx A, De Kimpe L, Mikhalep S, Vantus T, Seufferlein T, Vandenheede JR, et al. Protein kinase D: a family affair. *FEBS Lett* 2003;546:81–6.
- [21] Angin Y, Steinbusch LK, Simons PJ, Greulich S, Hoebers NT, Douma K, et al. CD36 inhibition prevents lipid accumulation and contractile dysfunction in rat cardiomyocytes. *Biochem J* 2012;448:43–53.
- [22] Luiken JJ, van Nieuwenhoven FA, America G, van der Vusse GJ, Glatz JF. Uptake and metabolism of palmitate by isolated cardiac myocytes from adult rats: involvement of sarcolemmal proteins. *J Lipid Res* 1997;38:745–58.
- [23] Foretz M, Ancellin N, Andreelli F, Saintillan Y, Grondin P, Kahn A, et al. Short-term overexpression of a constitutively active form of AMP-activated protein kinase in the liver leads to mild hypoglycemia and fatty liver. *Diabetes* 2005;54:1331–9.
- [24] Crute BE, Seefeld K, Gamble J, Kemp BE, Witters LA. Functional domains of the α 1 catalytic subunit of the AMP-activated protein kinase. *J Biol Chem* 1998;273:35347–54.
- [25] Cuello F, Bardswell SC, Haworth RS, Yin X, Lutz S, Wieland T, et al. Protein kinase D selectively targets cardiac troponin I and regulates myofilament Ca^{2+} sensitivity in ventricular myocytes. *Circ Res* 2007;100:864–73.
- [26] Luo J, Deng ZL, Luo X, Tang N, Song WX, Chen J, et al. A protocol for rapid generation of recombinant adenoviruses using the AdEasy system. *Nat Protoc* 2007;2:1236–47.
- [27] Volz A, Piper HM, Siegmund B, Schwartz P. Longevity of adult ventricular rat heart muscle cells in serum-free primary culture. *J Mol Cell Cardiol* 1991;23:161–73.
- [28] Eckel J, van Echten G, Reinauer H. Adult cardiac myocytes in primary culture: cell characteristics and insulin-receptor interaction. *Am J Physiol* 1985;249:H212–21.
- [29] Schwenk RW, Dirckx E, Coumans WA, Bonen A, Klip A, Glatz JF, et al. Requirement for distinct vesicle-associated membrane proteins in insulin- and AMP-activated protein kinase (AMPK)-induced translocation of GLUT4 and CD36 in cultured cardiomyocytes. *Diabetologia* 2010;53:2209–19.
- [30] van Oort MM, van Doorn JM, Bonen A, Glatz JF, van der Horst DJ, Rodenburg KW, et al. Insulin-induced translocation of CD36 to the plasma membrane is reversible and shows similarity to that of GLUT4. *Biochim Biophys Acta* 2008;1781:61–71.
- [31] Alkhateeb H, Chabowski A, Glatz JF, Luiken JJ, Bonen A. Two phases of palmitate-induced insulin resistance in skeletal muscle: impaired GLUT4 translocation is followed by a reduced GLUT4 intrinsic activity. *Am J Physiol Endocrinol Metab* 2007;293:E783–93.
- [32] Luiken JJ, Koonen DP, Willems J, Zorzano A, Becker C, Fischer Y, et al. Insulin stimulates long-chain fatty acid utilization by rat cardiac myocytes through cellular redistribution of FAT/CD36. *Diabetes* 2002;51:3113–9.
- [33] Bertrand L, Ginion A, Beauloye C, Hebert AD, Guigas B, Hue L, et al. AMPK activation restores the stimulation of glucose uptake in an in vitro model of insulin-resistant cardiomyocytes via the activation of protein kinase B. *Am J Physiol Heart Circ Physiol* 2006;291:H239–50.
- [34] Brindley DN, Kok BP, Kienesberger PC, Lehner R, Dyck JR. Shedding light on the enigma of myocardial lipotoxicity: the involvement of known and putative regulators of fatty acid storage and mobilization. *Am J Physiol Endocrinol Metab* 2010;298:E897–908.
- [35] Shulman GI. Cellular mechanisms of insulin resistance. *J Clin Invest* 2000;106:171–6.
- [36] Fischer Y, Rose H, Kammermeier H. Highly insulin-responsive isolated rat heart muscle cells yielded by a modified isolation method. *Life Sci* 1991;49:1679–88.
- [37] Bonen A, Parolin ML, Steinberg GR, Calles-Escandon J, Tandon NN, Glatz JF, et al. Triacylglycerol accumulation in human obesity and type 2 diabetes is associated with increased rates of skeletal muscle fatty acid transport and increased sarcolemmal FAT/CD36. *FASEB J* 2004;18:1144–6.
- [38] Luiken JJ, Coort SL, Willems J, Coumans WA, Bonen A, van der Vusse GJ, et al. Contraction-induced fatty acid translocase/CD36 translocation in rat cardiac myocytes is mediated through AMP-activated protein kinase signaling. *Diabetes* 2003;52:1627–34.
- [39] Tao R, Gong J, Luo X, Zang M, Guo W, Wen R, et al. AMPK exerts dual regulatory effects on the PI3K pathway. *J Mol Signal* 2010;5:1.
- [40] Leclerc GM, Leclerc GJ, Fu G, Barredo JC. AMPK-induced activation of Akt by AICAR is mediated by IGF-1R dependent and independent mechanisms in acute lymphoblastic leukemia. *J Mol Signal* 2010;5:15.
- [41] Tzatsos A, Tschlis PN. Energy depletion inhibits phosphatidylinositol 3-kinase/Akt signaling and induces apoptosis via AMP-activated protein kinase-dependent phosphorylation of IRS-1 at Ser-794. *J Biol Chem* 2007;282:18069–82.
- [42] Ning J, Clemmons DR. AMP-activated protein kinase inhibits IGF-I signaling and protein synthesis in vascular smooth muscle cells via stimulation of insulin receptor substrate 1 S794 and tuberous sclerosis 2 S1345 phosphorylation. *Mol Endocrinol* 2010;24:1218–29.
- [43] Liu LZ, Cheung SC, Lan LL, Ho SK, Xu HX, Chan JC, et al. Berberine modulates insulin signaling transduction in insulin-resistant cells. *Mol Cell Endocrinol* 2010;317:148–53.
- [44] Ginion A, Auquier J, Benton CR, Mouton C, Vanoverschelde JL, Hue L, et al. Inhibition of the mTOR/p70S6K pathway is not involved in the insulin-sensitizing effect of AMPK on cardiac glucose uptake. *Am J Physiol Heart Circ Physiol* 2011;301:H469–77.
- [45] Steinbusch LK, Wijnen W, Schwenk RW, Coumans WA, Hoebers NT, Ouwens DM, et al. Differential regulation of cardiac glucose and fatty acid uptake by endosomal pH and actin filaments. *Am J Physiol Cell Physiol* 2010;298:C1549–59.
- [46] Kim MS, Wang F, Puthanveetil P, Kewalramani G, Hosseini-Behesti E, Ng N, et al. Protein kinase D is a key regulator of cardiomyocyte lipoprotein lipase secretion after diabetes. *Circ Res* 2008;103:252–60.
- [47] St-Denis JF, Cabaniols JP, Cushman SW, Roche PA. SNAP-23 participates in SNARE complex assembly in rat adipose cells. *Biochem J* 1999;338(Pt 3):709–15.
- [48] Boström P, Andersson L, Rutberg M, Perman J, Lidberg U, Johansson BR, et al. SNARE proteins mediate fusion between cytosolic lipid droplets and are implicated in insulin sensitivity. *Nat Cell Biol* 2007;9:1286–93.
- [49] Bossuyt J, Chang CW, Helmstadter K, Kunkel MT, Newton AC, Campbell KS, et al. Spatiotemporally distinct protein kinase D activation in adult cardiomyocytes in response to phenylephrine and endothelin. *J Biol Chem* 2011;286:33390–400.