Impaired stimulation of glucose transport in cardiac myocytes exposed to Very Low-Density Lipoproteins

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Abstract

We recently observed that free fatty acids impair the stimulation of glucose transport into cardiomyocytes in response to either insulin or metabolic stress. *In vivo*, fatty acids for the myocardium are mostly obtained from triglyceride-rich lipoproteins (chylomicrons and Very Low-Density Lipoproteins). We therefore determined whether exposure of cardiac myocytes to VLDL resulted in impaired basal and stimulated glucose transport. Primary adult rat cardiac myocytes were chronically exposed to VLDL before glucose uptake was measured in response to insulin or metabolic stress, provoked by the mitochondrial ATP synthase inhibitor oligomycin. Exposure of cardiac myocytes to VLDL reduced both insulin- and oligomycin stimulated glucose uptake. The reduction of glucose uptake was associated with a moderately reduced tyrosine phosphorylation of the insulin receptor. No reduction of the phosphorylation of the downstream effectors of insulin signaling Akt and AS160 was however observed. Similarly only a modest reduction of the activating phosphorylation of the AMP-activated kinase (AMPK) was observed in response to oligomycin. Similar to our previous observations with free fatty acids, inhibition of fatty acid oxidation restored oligomycin-stimulated glucose uptake. In conclusions, VLDL-derived fatty acids impair stimulated glucose transport in cardiac myocytes by a mechanism that seems to be mediated by a fatty acid oxidation intermediate. Thus, in the clinical context of the metabolic syndrome high VLDL may contribute to enhancement of ischemic injury by reduction of metabolic stress-stimulated glucose uptake.

Keywords: VLDL; fatty acids; insulin; metabolic stress, glucose transport; cardiac myocytes

Introduction

The metabolic syndrome is a leading cause of mortality and morbidity in the Western hemisphere. The metabolic syndrome increases not only the prevalence but also the severity of myocardial infarction [8]. Stimulation of myocardial glucose transport in response to metabolic stress has been shown to be important for post-ischemic recuperation of function and survival of the myocardium [26, 28]. Thus impairment of the glucose transport response to metabolic stress could underlie the increased susceptibility of the myocardium to ischemia and reperfusion injury. This response shares several mechanisms with the stimulation of glucose transport in response to insulin [21], which is impaired in the metabolic syndrome.

Dyslipidemia is a hallmark of the metabolic syndrome, and includes elevated circulating free fatty acids and very low-density lipoproteins (VLDL) [15]. We have recently shown that chronic exposure of cardiac myocytes to free fatty acids (FFA) resulted in a marked impairment of the stimulation of glucose transport, in response to either insulin or metabolic stress [3]. Notably, the mechanisms appear to be distinct from those by which free fatty acids induce insulin resistance in skeletal muscle myocytes.

VLDL have also been shown to induce insulin-resistance in terms of glucose transport in skeletal muscle cells [24]. Lipoproteins deliver fatty acids to muscle cells, a mechanism that is primordial for energy production by cardiac myocytes [5]; however it could be shown that the lipoprotein receptor interaction, not the provision of fatty acids, mediates insulin resistance in skeletal muscle cells [24]. Therefore, in the present study, we determined whether exposure of cardiac myocytes to VLDL had an impact on basal and stimulated glucose transport.

Materials and Methods

Animals

We obtained male Sprague Dawley rats (100–110 g) from Charles Rivers France (L'Arbresle, France). The ethical committee of the Geneva University School of Medicine and the Geneva State Veterinary Office approved the study protocol, which conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Very low-density lipoproteins

Human very low-density lipoproteins (VLDL) were obtained from the sera of healthy volunteers, by differential centrifugation [14] and suspended in sterile Phosphate Buffer Saline (PBS) containing 10^{-5} M EDTA. VLDL from several donors were pooled, stored at 4°C and used within one week. All donors gave informed consent regarding the utilization of sera, and the Ethical Committee for Human Research of the Geneva University Hospitals approved the protocol.

Adult rat cardiomyocytes culture

Adult rat cardiomyocytes were isolated as previously described [2] by retrograde perfusion of the hearts with collagenase (type II; Worthington) [9, 10]. Cardiomyocytes were separated from non-myocyte cardiac cells by preplating the whole cell suspension for 90 minutes on untreated plastic, to which non-myocyte cells, but not cardiomyocytes, readily adhere. Cardiomyocytes were plated in M199 medium containing 5.5 mM glucose supplemented with 20 mM creatine, 100 μ M cytosine- β -D-arabinofuranoside, 100 nM 9-cis retinoic acid and

20% fetal calf serum (FCS) in dishes previously coated with 0.1% gelatin for 4 h and incubated overnight with complete culture medium. VLDL, PBS and pharmacological agents or their solvents were added to the medium at the time of plating (day 0). The culture medium was renewed every 2-3 days, and subsequent analyses were performed on day 7. At this time point control cardiomyocytes display a well differentiated phenotype with stable insulin responsiveness [25].

Determination of 2-deoxy-D-glucose uptake

Glucose transport was estimated by measuring 2-deoxyglucose (2-DG) uptake, as previously described [2, 20]. Briefly, cardiomyocytes were incubated in M199 containing 10 nM [2,6-³H]-2-DG (ANAWA) (1-2 μ Ci/ml) and 5.5 mM cold glucose at 37°C for 1 h, in the presence or absence of glucose transport agonists. Glucose transport agonists used were insulin (10^{-10} - 10^{-6} M) or oligomycin (10^{-6} M) to induce metabolic stress. Glucose transport in response to hypoxia was also measured in M199, with the 2-DG concentration reduced to 1 nM, to avoid toxic effects of 2-DG over the long incubation periods in hypoxia experiments. Hypoxia was maintained for 6 h in an airtight Billups-Rothenberg chamber flushed with 10% CO₂, 90% N₂; control incubations were conducted for the same time in 5% CO₂ in air at 37°C.

2-DG uptake was stopped by three washes with ice-cold PBS before lysis in 1 ml 0.1 M NaOH. Twenty- μ l aliquots were taken for protein content determination and the remaining NaOH lysate assayed for radioactivity in a TriCARB 1900 TR liquid scintillation analyzer (Packard). Results are expressed as fold stimulation of 2-DG uptake with respect to control unstimulated cardiomyocytes.

Intracellular signaling analysis

After preincubation in serum-free M199 for 30 min, cardiomyocytes were stimulated with glucose transport agonists for 10 min (insulin; 10^{-10} - 10^{-6} M) or 20 min (oligomycin; 10^{-6} M). Stimulations were terminated by three washes in ice-cold PBS before solubilizing cells in 200 μ l lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH7.5), 1 mM EDTA, 0.5% sodium deoxycholate, 1% Igepal CA 630, Halt protease and phosphatase inhibitor Cocktail (Pierce, Thermo Scientific). Proteins (50 μ g) from each sample were separated on SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes. Western Blots were immunoprobed with antibodies for phosphorylated and total signaling intermediates. The following primary antibodies were used: Akt (#9272; 1:1000), phosphoSer⁴⁷³-Akt (#9271; 1:1000), phosphoThr³⁰⁸-Akt (#9275; 1:1000), AMPK α (#2532; 1:1000), phospho Thr¹⁷²-AMPK α (#2531; 1:1000), phospho-(Ser/Thr)-Akt substrate (#9611; 1:1000), phosphoSer²³⁵/Ser²³⁶ S6 ribosomal protein (#4858; 1:1000), S6 ribosomal protein (#2217; 1:1000) and Insulin Receptor β (#3025; 1:1000) from Cell Signaling Technologies, AS160 (#07-741; 1:1000) and phosphotyrosine (#05-777; 1:1000) from Millipore, GLUT1 (#ab652; 1:1000), GLUT4 (#ab654; 1:5000) and GLUT8 (#ab169779; 1: 1000) from Abcam, α -actinin (#A7811; 1:5000) from Sigma-Aldrich. Secondary anti-mouse IgG (#115-035-146; 1:5000) and anti-rabbit IgG (#111-035-003) HRP-conjugated antibodies were from Jackson ImmunoResearch. Densitometric analysis of chemiluminescent signals captured on films was performed using the ImageJ software (National Institutes of Health, <http://rsb.info.nih.gov/ij>). For each separate film, the data were normalized such that the highest intensity, regardless of treatment, was equal to 1.

Analytical methods

Protein concentrations of cell extracts were measured by the bicinchoninic acid assay (MicroBCA Protein Assay Kit, Thermo Scientific Pierce). Free fatty acids concentrations in conditioned culture medium were measured with the Free Fatty Acid Quantitation Kit from Sigma-Aldrich (MAK044) in the colorimetric mode.

Statistics

Data are presented as mean $\pm$ SD, with the number of separate experiments indicated at the foot of each column. A few experiments were not replicated, with the results shown as triplicate determinations within a single experiment. Data were compared by two-way ANOVA (Prism 5, GraphPad Software) followed by Bonferroni's post hoc test. Dose-response curves for insulin were fitted with sigmoid curves by the least squares method. Differences were considered significant when $p < 0.05$.

Results

Impaired stimulation of glucose uptake in cardiac myocytes exposed to VLDL

We first measured glucose uptake in cardiac myocytes exposed to VLDL at a concentration of 80 μ g proteins/mL of culture medium. This concentration is at the higher end of the normal VLDL concentration range in healthy humans and is about half the concentration in normal rats [16]. Glucose transport agonists were insulin, at 10^{-8} and 10^{-6} M, oligomycin at 10^{-6} M (Figure 1a) and 6 hours of hypoxia (Figure 1b). Exposure to VLDL reduced basal and stimulated glucose uptake in response to all three stimuli. To further ascertain that the reduction of insulin-stimulated glucose transport was a reduction in insulin responsiveness, not sensitiveness, we constructed a dose-response curve for insulin (Figure 1c). The results showed no increase in the EC_{50} for insulin in cardiac myocytes exposed to VLDL as compared to control cardiac myocytes (C: $1.2 \cdot 10^{-10}$ M; 95 % confidence interval $2.8 \cdot 10^{-11}$ to $5 \cdot 10^{-10}$ M; VLDL: $6.7 \cdot 10^{-11}$ M; 95 % confidence interval $1.5 \cdot 10^{-12}$ to $3 \cdot 10^{-9}$ M)

Because of the endothelial barrier the cardiac myocytes *in vivo* are certainly never directly exposed to such high concentrations of VLDL. Therefore we assessed whether lower concentrations of VLDL would have similar effects on glucose transport. Indeed concentrations of VLDL as low as 5 μ g proteins/mL significantly impaired stimulation of glucose transport in response to either insulin (Figure 1d) or oligomycin (Figure 1e). Further experiments were performed with VLDL concentrations around 40 μ g/mL.

Several isoforms of glucose transporters are expressed in the myocardium; GLUT4 is the predominant isoform, with GLUT1 and GLUT8 also being significantly expressed [1]. Western Blot experiments showed that neither GLUT1 nor GLUT4 expression was affected by incubation with VLDL; however there was a significant reduction of GLUT8 expression by 40% in cardiac myocytes exposed to VLDL (Figure 1f).

Insulin and metabolic stress signaling in cardiac myocytes exposed to VLDL

To try to explain the reduction of stimulated glucose transport in cardiac myocytes exposed to VLDL, we investigated several steps of the classical signaling pathways leading to the stimulation of glucose transport (Figure 2) [21]. As expected insulin induced tyrosine phosphorylation of its receptor's β subunit and phosphorylation of Akt on residues Ser473 and Thr308. Oligomycin induced activating phosphorylation of AMPK α on Thr172, and both agonists induced phosphorylation of the Rab GTPase-activating protein AS160 on Ser and Thr residues (Figure 2). Remarkably, there were only minor reductions in the phosphorylation of these

key signaling intermediates. Only the reduction of IR β tyrosine phosphorylation in VLDL-exposed cardiac myocytes reached significance by ANOVA analysis. In addition we probed the phosphorylation of the S6 ribosomal protein on Ser 235 and 236, as a read-out for mTORC1 complex activity. The basal level of phosphorylation was high, as expected in cardiac myocytes cultured in a serum-rich medium. Phosphorylation of Ser235/236 was slightly but significantly increased by insulin, but was not significantly altered in VLDL-exposed cardiac myocytes.

Role of free fatty acids and of fatty acid oxidation in the effects of VLDL

VLDL are important purveyors of free fatty acids to the myocardium, in the form of triglycerides, to be locally hydrolyzed to FFA by the heart-specific lipoprotein lipase (LPL) [6, 23]. We previously demonstrated that exposure to FFA reduced insulin- and oligomycin-stimulated glucose uptake in cardiac myocytes [3]. To evaluate the importance of FFA release from VLDL in the effects of VLDL, we inhibited LPL activity with the pharmacological agents tetrahydrolipstatin (THL) [23] or GSK 264220A (GSK) [11]. VLDL released FFA in the culture medium only in the presence of cardiac myocytes, to reach a concentration of FFA in the medium of approximately $109 \pm 2 \mu\text{M}$ (mean $\pm$ SEM; $n = 8$) after two days of incubation. Coincubation of cardiac myocytes with VLDL and 10^{-5} M THL reduced the release of FFA to reach a concentration of $48 \pm 2 \mu\text{M}$. By comparison the concentration of FFA in normal medium containing 20% FCS was found to be $40 \mu\text{M}$ and the concentration used in our previous study to inhibit glucose transport [3] was $400 \mu\text{M}$.

Inhibition of LPL with THL increased both basal and stimulated glucose transport in cardiac myocytes exposed or not to VLDL, to levels even higher than in control cardiac myocytes (Figure 3a). This effect was associated with a marked increase in Thr¹⁷² phosphorylation of AMPK α , indicating AMPK activation (Figure 3b).

Inhibition of LPL with $10 \mu\text{M}$ GSK was not as efficient as with THL, reducing FFA concentration in the medium from 128 ± 10 to $106 \pm 9 \mu\text{M}$, but did not increase AMPK α phosphorylation (Figure 3d). Glucose uptake was marginally increased in cardiac myocytes incubated with VLDL and GSK as compared to VLDL alone, and remained lower than in control cardiac myocytes (Figure 3e), possibly owing to incomplete LPL inhibition. Similar to THL, GSK by itself had a positive effect on glucose transport, though not as large as that of THL and not statistically significant.

In our previous study we observed that oligomycin-stimulated glucose uptake could be restored in cardiac myocytes exposed to FFA if fatty acid oxidation was either inhibited or stimulated [3]. We repeated these experiments in cardiac myocytes exposed to VLDL. Indeed, we found that blockade of fatty acid oxidation with etomoxir improved oligomycin-stimulated glucose uptake, but failed to ameliorate insulin-stimulated glucose uptake (Figure 3e). We had previously observed that etomoxir by itself had no effect on insulin-stimulated glucose uptake and a slightly inhibitory effect on oligomycin-stimulated glucose uptake [3]. In contrast to the previous study, stimulation of fatty acid oxidation by the PPAR δ -agonist GW0742 failed to restore oligomycin-stimulated glucose uptake in cardiac myocytes exposed to VLDL (Figure 3f). Again we had previously shown that GW0742 alone had no effect on oligomycin-stimulated glucose uptake, and GW0742 action was demonstrated by stimulation of fatty acid oxidation [3]. Finally we could also replicate an as yet unexplained observation made in FFA-exposed cardiac myocytes [3] and show that pharmacological inhibition of PPAR γ activity with GW9662 improved VLDL-reduced oligomycin-stimulated glucose uptake (Figure 3g). A separate

set of experiments demonstrated that GW9662 by itself had no impact on stimulated glucose transport (data not shown).

Role of cholesterol in the effects of VLDL

VLDL deliver not only triglycerides to the myocardium, but also cholesteryl esters [23]. Because we were unable to manipulate the cholesterol content of VLDL without disrupting VLDL structure and stability, we directly manipulated the cardiomyocytes cholesterol with empty or cholesterol-loaded methyl- β -cyclodextrin [29]. Cholesterol depletion with unloaded methyl- β -cyclodextrin had no effect on basal or insulin-stimulated glucose transport, but reduced oligomycin-stimulated glucose transport (Figure 3h). On the other hand, augmentation of the cholesterol content restored oligomycin-stimulated glucose transport, but was also without any effect on basal or insulin-stimulated glucose transport.

Discussion

The simplest explanation for the inhibition of VLDL on glucose transport in cardiac myocytes is that these effects are due to free fatty acids obtained through LPL-mediated lipolysis of VLDL triglycerides. This interpretation is supported by the observation that pharmaceutical inhibition of LPL improves VLDL-impaired glucose transport. The concentrations of FFA measured in the VLDL-conditioned medium were much lower than the concentrations of exogenous FFA in the culture medium used in previous experiments, but the actual concentrations in the vicinity of the cardiac myocytes could actually be higher, and the FFA locally released by LPL could be immediately taken up by the cardiac myocytes. This interpretation is supported by the observations that maneuvers that were shown to restore FFA-impaired glucose transport [3] have similar impact on VLDL-impaired glucose transport; these include fatty acid oxidation blockade, PPAR δ activation or PPAR γ antagonism. Unfortunately this interpretation could not be supported by the most straightforward experiment, i.e. lipoprotein lipase inhibition. Indeed, pharmaceutical inhibition of LPL with either THL or GSK 264220A resulted in stimulation glucose transport activity in control cardiomyocytes as well as in cardiomyocytes exposed to VLDL, thus making interpretation of these results difficult. Remarkably, the stimulation of glucose transport was more pronounced with THL than GSK, as was LPL inhibition. In this respect it is relevant to note that cardiac-specific knock-out of LPL increases basal and insulin-stimulated myocardial glucose transport *in vivo* [4]. In our *in vitro* experiments stimulation of glucose transport occurred with LPL inhibitors even in the absence of LPL substrates, suggesting that LPL may interact with the regulation of glucose transport independently of its effects on fatty acid metabolism.

There are also minor discrepancies suggesting a more complex scenario. First, the impacts of FFA and VLDL on cell signaling are dissimilar: FFA mostly impact on Akt phosphorylation on residues Thr³⁰⁸ and Ser⁴⁷³, whereas there exists only a trend in that direction with VLDL. In both cases it should be noted that the apparent reduction in canonical insulin and AMPK cell signaling is disproportionally modest as compared to the impairment of glucose transport. Second, treatments with either the PPAR δ agonist GW0742 or the PPAR γ inhibitor GW9662 both fully restored oligomycin-stimulated glucose uptake in FFA-exposed cardiac myocytes, but were only partially effective on VLDL-exposed cardiac myocytes, or even not effective at all in the case of GW0742.

Two characteristics of the lipid makeup of VLDL could account for these minor discrepancies. First, the fatty acid composition of triglycerides within VLDL is complex, presumably much more so than the simple 1:1 mix

of palmitate and oleate used in our previous FFA exposure experiments. Therefore, other FFA could have slightly different effects on glucose transport and cell signaling. Second, in addition to fatty acids, VLDL deliver cholesterol to the myocardium, by a transfer mechanism independent of lipoprotein lipase activity [23]. Indeed, the cholesterol content of the plasma membrane of adipocytes has been shown to impact directly on GLUT4 translocation, independently of insulin signaling [17]. As we were unable to manipulate the cholesterol content of VLDL without disrupting VLDL structure and stability, we could not address the question directly. Through an indirect approach of altering the cardiomyocytes cholesterol content with empty or cholesterol-loaded methyl- β -cyclodextrin, we concluded that the provision of cholesterol to cardiomyocytes from VLDL was unlikely to significantly affect glucose transport.

In cardiomyocytes, stimulated glucose transport is mostly mediated by the GLUT4 isoform of facilitative glucose transporters, less so for basal glucose transport, mediated in part by GLUT1. Indeed we observed by specific inhibition of GLUT4 with indinavir [22] that at least 80% of the stimulated and about 40% of the basal glucose transport is GLUT4-mediated in our model (unpublished observations). As total expression of neither GLUT1 nor GLUT4 was reduced in cardiomyocytes exposed to VLDL, inhibition of glucose transport in cardiomyocytes exposed to VLDL is most likely caused by impaired translocation of GLUT4 than by impaired expression of glucose transporters. However, GLUT4 density at the cell surface could not be evaluated in this model. In contrast GLUT8 expression was significantly reduced in cardiomyocytes exposed to VLDL. The function and contribution of GLUT8 to glucose transport in the myocardium remain obscure [27]. This isoform is only found in intracellular compartments, not at the cell surface [13], and therefore should not contribute significantly to transarcolemmal glucose transport. It was initially hypothesized that, similar to GLUT4, GLUT8 could translocate to the cell surface a response to a specific stimulus. Although insulin-stimulated translocation of endogenous GLUT8 to the plasma membrane of blastocysts has been described [7], this finding could not be reproduced in other cell types. Notably, mice deficient in GLUT8 display a very mild phenotype with normal glucose homeostasis [19].

A limitation of this study model is the direct contact of VLDL with cardiac myocytes. *In vivo* the endothelial barrier would largely, but not entirely limit such contact. Nevertheless we believe our data are relevant for the following reasons: 1. The inhibitory effects of VLDL on glucose transport appear already with very low concentrations of the lipoproteins (Figure 1D); 2. Extravasation of VLDL is not unheard of, in the context of inflammation which prevails in the metabolic syndrome [12] and 3. The expression of the VLDL receptor in cardiac myocytes [18], not only endothelial cells, suggests direct interactions *in vivo*, if not with native VLDL, at least with triglyceride-rich lipoproteins remnants [6].

In conclusion, it appears that the exposure of cardiac myocytes to VLDL replicates several aspects of the negative effects of FFA on basal and stimulated glucose transport. Provision of FFA from VLDL through LPL-mediated lipolysis presumably explains most of these findings; yet other effects of VLDL unrelated to FFA delivery cannot be excluded.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figures legends

Fig. 1 Impaired stimulation of glucose uptake in cardiac myocytes exposed to VLDL

Cardiac myocytes were exposed for 7 days to VLDL or PBS and glucose uptake measured in unstimulated myocytes or in response to insulin, oligomycin or hypoxia. a: effects of 80 $\mu\text{g/mL}$ VLDL on basal and insulin- or oligomycin-stimulated glucose uptake. b: effects of 80 $\mu\text{g/mL}$ VLDL on glucose uptake in response to hypoxia. c: dose-response curves for insulin in cardiomyocytes exposed to 40 $\mu\text{g/mL}$ VLDL. d-e: Dose-response for the effects of VLDL on basal and insulin- or oligomycin-stimulated glucose uptake. f: GLUT1, GLUT4 and GLUT8 expression by Western Blot; representative Western Blots are shown on the right, α -actinin is shown as a loading control. For all panels: *: significant ($p < 0.05$) effect of insulin or oligomycin; #: significant effect of VLDL. Experiments depicted in panels c, d and e were not replicated and are shown as triplicate determinations within a single experiment.

Fig. 2 Insulin and metabolic stress signaling in cardiac myocytes exposed to VLDL

Cardiac myocytes were exposed for 7 days to VLDL or PBS, and thereafter stimulated for 10 min with 10^{-8} – 10^{-6} M insulin or for 20 min with 10^{-6} M oligomycin. Phosphorylation of key intermediate proteins in insulin and AMPK signal transduction was determined by Western Blot and quantified. For each phosphoprotein indicated on top of panels, density of the phosphorylated protein band was normalized to that of the total protein band. For all panels: *: significant ($p < 0.05$) effect of insulin or oligomycin; #: significant effect of VLDL. Significance over bracket symbols (pY IR β) indicate that the group is different as a whole by ANOVA, but that the Bonferroni's post hoc test found no significant difference in individual comparisons. The right panel shows a representative Western Blot for each protein.

Fig. 3 Role of free fatty acids, and of fatty acid oxidation in the effects of VLDL

Cardiac myocytes in primary culture were exposed for 7 days to VLDL (40 $\mu\text{g/mL}$) or PBS in the presence or absence of the lipoprotein lipase inhibitors tetrahydrolipstatin (THL, 10^{-5} M, panels a and b) or GSK 264220A (GSK, 10^{-3} M, panel c and d), the fatty acid oxidation inhibitor etomoxir (10^{-5} M, panel e), the PPAR δ agonist GW0742 (10^{-6} M; panel f) or the PPAR γ inhibitor GW9662 (10^{-6} M; panel g). Thereafter glucose uptake was measured in unstimulated cardiac myocytes or in response to insulin (10^{-6} M; grey bars) or oligomycin (10^{-6} M; black bars). Panels b and c: state of phosphorylation of AMPK α in cardiac myocytes exposed for 7 days to VLDL or PBS in the presence or absence of THL or GSK; representative Western Blots are shown on the right. h: Cardiac myocytes in primary culture were exposed for 7 days to methyl- β -cyclodextrin (M β CD, 0.5 mM) or cholesterol-loaded methyl- β -cyclodextrin (M β CD /cholesterol). For all panels: *: significant ($p < 0.05$) effect of insulin or oligomycin; #: significant effect of VLDL, §: significant effect of compound added to VLDL. Significances over bracket symbols (panels a and c) indicate that the group is different as a whole by ANOVA, but that the Bonferroni's post hoc test found no significant difference in individual comparisons.

Figure 1

Figure 2

Figure 3