Vacuolar H+-ATPase as target to restore cardiac function in the diabetic heart

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Contents

Chapter 1

General introduction

Energy substrate supply and utilization in the healthy adult heart

An optimal contractile function of the heart is required to maintain a sufficient supply of oxygen and nutrients to peripheral tissues [1, 2]. To maintain this contractile function the heart needs to receive a continuous supply of energy, in the form of ATP [3]. Most of this ATP is derived from the mitochondrial oxidation of various energy substrates, in particular (long-chain) fatty acids (FA) and carbohydrates (e.g., glucose and lactate), but also ketone bodies and amino acids (AA) [2] (Figure 1). These latter substrates are commonly referred to as "alternative substrates". It is generally accepted that FA and glucose compete as a major source of acetyl-CoA for entering the TCA cycle and subsequent mitochondrial oxidative energy provision, i.e., ATP [4]. Healthy adult individuals predominantly utilize FA for cardiac energy generation (through β-oxidation, ~60%), while glucose and lactate are responsible for ~25-30% of the total energy production (glycolysis and glucose oxidation) [5, 6]. In addition, the omnivoric character of the heart also implies that the alternative substrates ketone bodies and AA are also being used for cardiac energy provision [2, 4, 7]. In the normal heart, the contribution of these alternative substrates to total ATP production is limited with ketone bodies and AA accounting for 10% and 2% at most of total ATP generated, respectively [4]. However, under specific conditions (e.g., prolonged fasting, exercise), or in disease states (e.g., diabetes mellitus, heart failure) their contribution to myocardial energy provision may become significant [2, 7-9]. According to this premise, it could be anticipated that in situations where the levels of circulating ketone bodies and AA are elevated, such as in diabetes mellitus or heart failure, this increased ketone body and AA oxidation could compete for acetyl-CoA, eventually contributing to an impairment of FA or glucose oxidation [4].

The heart in physiological conditions is flexible to shift between the source of substrates, especially with respect to FA and glucose, but a chronic shift towards a predominant use of FA/ glucose leads to problems. In case of the preferred utilization of FA over glucose, there is a risk that the heart suffers from lipotoxicity, which could elicit major impairments of cardiac functioning such as diabetic cardiomyopathy [2]. An example of such impairments of cardiac functioning is lipid-induced heart failure [e.g., type 2 diabetes (T2D) and diabetic cardiomyopathy] [10].

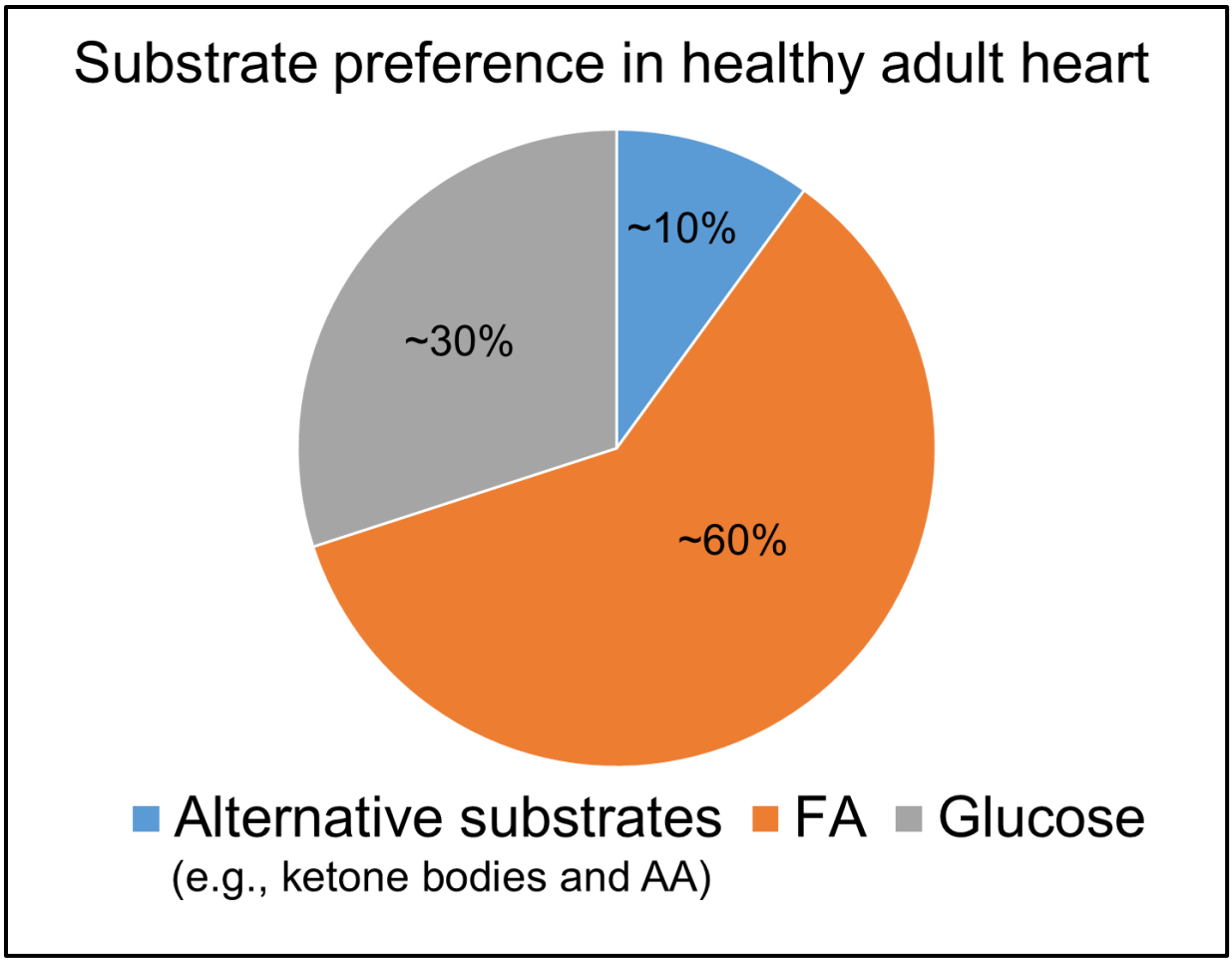

Figure 1: Substrate preference of healthy adult heart.

FA: fatty acids; AA: amino acids.

Type 2 diabetes and diabetic cardiomyopathy

Diabetes is an increasing problem worldwide with 451 million cases in adults between 18 and 99 years in 2017 and an estimated amount of 5 million deaths each year [11, 12] (Figure 2). Part of the diabetes cases are related to type 1 diabetes, where beta cells in the pancreas are not able to produce (sufficient) insulin. However the largest contribution to the diabetes problem arises from T2D [13]. Overconsumption of fatty foods (e.g., saturated fat) and a sedentary life style predispose to a high risk of developing T2D, which is a clinical condition in which insulin resistance in target tissues is a major feature [14].

Notably, one of the major complications in T2D is diabetic cardiomyopathy (e.g., myocardial infarction and heart failure) [15]. In both humans and rodent models, the diabetic cardiomyopathy that develops in the insulin resistant heart is related to intramyocardial accumulation of lipid metabolites (diacylglycerols and/or ceramides) [14, 16]. Indeed, growing

evidence in animal models and humans show that intra-myocardial lipid accumulation and insulin resistance may underlie progressive cardiac contractile dysfunction [16].

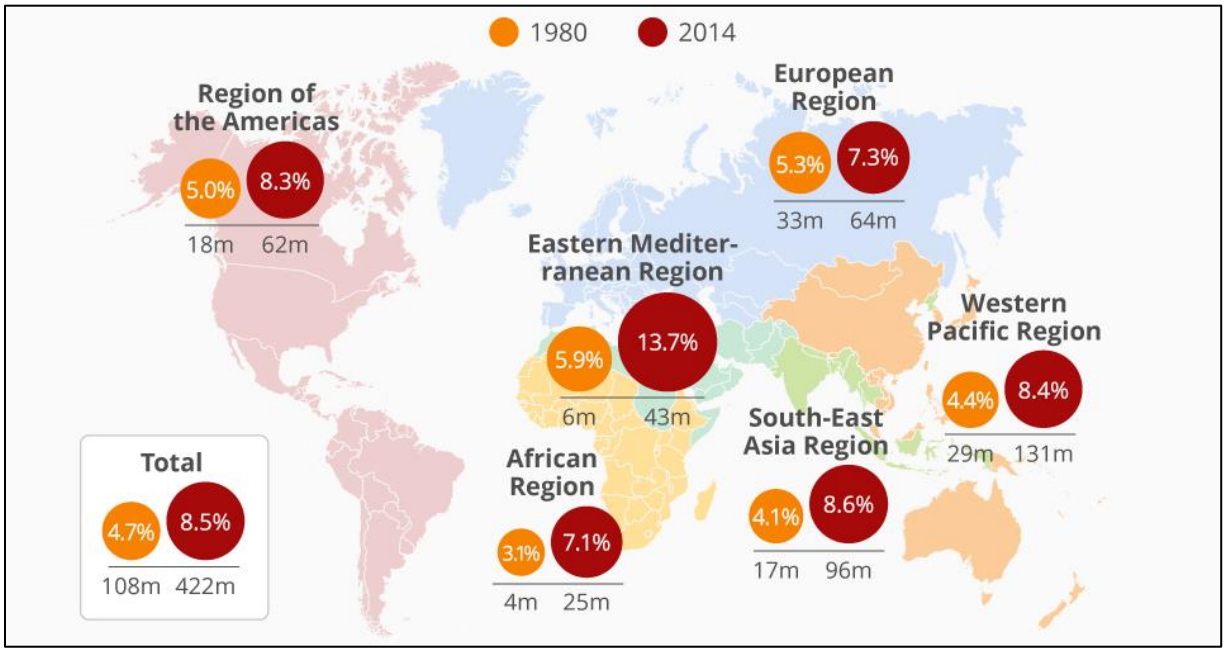

Figure 2: Expanding prevalence of diabetes in the world-wide population.

Prevalence and number of adults with diabetes in 1980 (orange) and 2014 (red) according to the World Health Organization (WHO). [**Figure 2** taken from McCarthy et al. (2016)] [12].

Role of FA transporter CD36 and glucose transporter GLUT4 in cardiac lipid accumulation and diabetic cardiomyopathy

FA uptake into the heart is regulated by several membrane-associated FA transporters including FA-binding protein at the plasma membrane (FABP-PM), FA transport proteins (FAT) 1-6 , caveolin-1 and FA translocase/cluster of differentiation 36 (FAT/CD36, officially known as scavenger receptor-B2) [17, 18]. From these FA transporters, CD36 is the major contributor to FA uptake in the heart [17]. In line with this, hearts from CD36 knockout mice [19, 20] and cardiomyocytes pre-treated with CD36-blocking antibodies [21] are protected from lipid overload *in vivo* and *in vitro*, respectively, suggesting that CD36 is the key player in regulating FA uptake in lipid-overload situations. Therefore, CD36 is acknowledged as a crucial target for the cardiac lipid metabolism. CD36, a 472-amino acid (88 KDa) membrane protein, found in sarcolemmal and endosomal membranes [18]. Under basal conditions in skeletal

muscle [22] and heart [23], ~50% of CD36 is estimated to be stored in endosomes. Endosomes are acidic organelles, and their luminal acidity is important for CD36 storage [24]. In response to physiological stimuli, such as insulin or contraction, CD36 translocates to sarcolemmal membranes. Specifically, CD36 translocation occurs via vesicle mediated trafficking, which includes vesicle budding from endosomes, translocation along cytoskeletal filaments and vesicle fusion with the sarcolemma [18, 25]. Subsequently, the sarcolemmal localization of CD36 then leads to an increase of FA uptake, e.g., in order to meet the immediate requirement of substrate for muscular energy demand [17].

GLUT4 is responsible for most of stimulus-induced glucose uptake. Similar to CD36, GLUT4 is also mainly located in intracellular stores (endosomes) or the sarcolemma [26]. Moreover, the subcellular GLUT4 localization also partially overlaps with that of CD36 given that the recycling functions of endosomes also contribute to GLUT4 storage [24]. Furthermore, the same physiological stimuli inducing vesicle-mediated CD36 translocation, also have been shown to induce vesicle-mediated GLUT4 translocation, being insulin as well as contraction [18]. When extracellular substrate concentrations are fluctuating within the normal range, CD36 and GLUT4 recycling are similarly regulated. However, in insulin resistance and T2D CD36 permanently relocates to the sarcolemma, while GLUT4 is imprisoned within the endosomes [26]. Recent studies in rats fed a high fat diet and in cardiomyocyte cultures exposed to high palmitate concentrations have indicated that increased CD36 translocation initiates a vicious cycle of increased lipid uptake and lipid accumulation, ultimately culminating into insulin resistance and cardiac dysfunction [27]. These results suggest that lipid-induced CD36 translocation to the sarcolemma is a key early event in diabetic cardiomyopathy. In contrast, in the lipid-overloaded heart, GLUT4 subcellular distribution shows a complete opposite picture, i.e., with decreased sarcolemmal abundance and concomitantly increased presence in intracellular membrane compartments [18, 28]. In this respect, the GLUT4 translocation machinery also undergoes such an alteration, which underlie the intracellular imprisonment of GLUT4 in the lipid-overloaded heart [24]. Yet, based on the time-course of lipid-induced metabolic alterations, the changes in CD36 traffic by far precede the changes in GLUT4 traffic, further supporting CD36 translocation as a key early event in lipid-induced cardiomyopathy. Although this regulatory detailed mechanism has not been settled yet, CD36 is an attractive target to decrease FA uptake in the diabetic heart [29-31]. Similar to previous findings that CD36 ablation in CD36-null mice studies prevents loss of cardiac function in a lipid-induced cardiomyopathy model [19, 20, 32], pharmacological inhibition of CD36 translocation is

expected to be a valuable treatment strategy to counteract impaired lipid accumulation and lipid-induced contractile dysfunction in diabetic cardiomyopathy [33].

V-ATPase regulates CD36-mediated lipid metabolism in diabetic cardiomyopathy

As established earlier, vacuolar H^+-ATPase (v-ATPase) is the intracellular lipid sensor that regulates CD36 trafficking from endosomes to the sarcolemma [27, 33]. In the healthy heart CD36 is mainly stored in endosomal compartments. During lipid oversupply v-ATPase disassembles and becomes dysfunctional, leading to increased CD36 translocation to sarcolemma, as further explained in Figure 3.

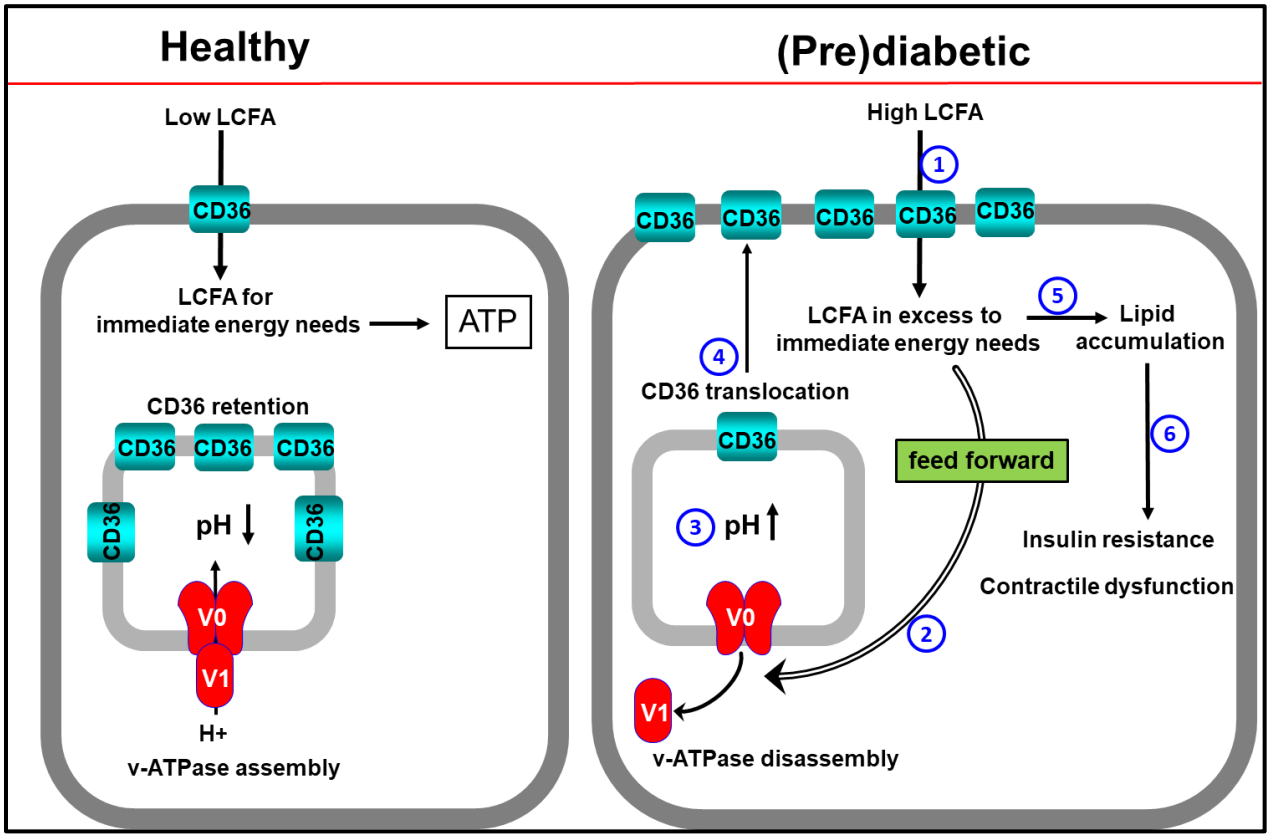

Figure 3. Schematic presentation of FA-induced v-ATPase inhibition.

In the healthy heart, the v-ATPase V_0 sub-complex, which is integral to the endosomal membrane, is assembled with the cytosolic V_1 sub-complex allowing for acidification of the endosomal lumen. In this situation, the FA taken up by the cardiomyocyte is metabolized to meet the immediate energy demand.

In the (pre-)diabetic heart, elevated extracellular FA supply triggers a series of events: (1) Increased CD36-mediated FA uptake results in elevated intramyocellular FA levels. (2) Intracellular FA causes the V_1 and V_0 sub-complexes to dissociate with V_1 being released into the cytoplasm. (3) The disassembly of v-ATPase leads to endosomal alkalinization. (4) Increased endosomal pH triggers the translocation of CD36 vesicles to the sarcolemma. 5) Upon chronic lipid oversupply, where FA uptake surpasses the metabolic needs, 6) further processes are set in motion, specifically the onset of loss of insulin sensitivity and of contractile function. [Figure 3 is adapted from [27]].

Apart from the effect of FA, a possible regulation of v-ATPase by the other cardiac substrates (glucose, AA and ketone bodies) has not yet been clarified in the heart. Evidence from yeast and mammalian kidney cells suggest that glucose starvation contributed to v-ATPase disassembly, whereas high glucose addition may induce v-ATPase assembly [34-37]. Furthermore, v-ATPase was recently identified as a critical component of the amino acid (AA)-sensing machinery that communicates AA availability to mTORC1 [38]. At present, there is no evidence that ketone bodies could have an influence on v-ATPase function. Hence, it would be of high interest to investigate whether the energy substrates glucose, AA, and ketone bodies would have an impact on the functioning of v-ATPase in the heart, either directly or indirectly.

Outline of the thesis

As mentioned above, CD36-mediated lipid accumulation presents a crucial step towards development of diabetic cardiomyopathy, and there is increasing evidence that this maladaptive step is due to the loss of v-ATPase function. Therefore, an important aim of this thesis is to glean strategies to re-assemble v-ATPase in the lipid-overloaded heart.

Chapter 1 provides an overview of current mechanistic insight about the substrate utilization shift in the diabetic heart away from glucose and towards lipids. In particular, it becomes evident that relocation of CD36 to the sarcolemma is dependent on v-ATPase function. This allows us to hypothesize that an intervention capable of increasing v-ATPase function should induce CD36 retention in the endosome, then leading to decreased FA uptake, and restoration of insulin sensitivity and contractile dysfunction in the diabetic heart.

First, given that in yeast and mammalian kidney cells, increased glucose availability is known to increase v-ATPase function, **Chapter 2** describes whether increased glucose availability in cardiomyocytes, which is achieved through (a) high glucose addition or (b) adenoviral overexpression of protein kinase-D1 (a kinase mediating GLUT4 translocation), induces v-ATPase re-assembly. The positive outcomes of these studies provide the first proof-of-principle for testing strategies to counteract lipid-induced insulin resistance and contractile dysfunction via v-ATPase re-assembly.

Next, based on evidence that v-ATPase is required for endosomal localization and activation of the anabolic master-regulator mTORC1 in response to AA, **Chapter 3** reveals the effect of AA on v-ATPase function. We establish a potent AA cocktail (arginine/leucine/lysine combination) to assemble v-ATPase. This AA mixture was added to lipid-overloaded cardiomyocytes, where it successfully re-internalized CD36 to the endosomes, reversed lipid accumulation, and restored insulin-stimulated glucose uptake and contractile function. Therefore, the specific AA mixture, by acting through v-ATPase re-assembly, may offer a simple and effective treatment to protect from lipid-induced cardiomyopathy.

Ketone bodies are rapid fuels of heart and elevated cardiac uptake of ketone bodies might therefore be an adaptive and compensatory response to the impaired cardiac energy metabolism in the diabetic heart [7, 39]. In **Chapter 4** we aim to investigate the effects of ketones on the v-ATPase assembly status. We found, in contrast to glucose and AA (which induce v-ATPase re-assembly), that the ketone 3HB induces v-ATPase disassembly, thereby resulting in loss of endosomal acidification. Subsequently, this 3HB-induced v-ATPase inhibition triggers CD36-mediated FA uptake to induce insulin resistance and contractile dysfunction. By deciphering the mechanism of 3HB-induced insulin resistance via v-ATPase, this study unsheathes the effect of ketone bodies on fatty acid uptake with particular relevance to insulin resistance and the diabetic situation.

Finally, the main findings of this thesis are integrated and further discussed in **Chapter 5**, so that on the basis of these results new insights to the current state of this field are set in a broader perspective.

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Chapter 2

Augmenting vacuolar H^+-ATPase function prevents cardiomyocytes from lipid-overload induced dysfunction

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Running title: Glucose availability induces v-ATPase assembly in cardiomyocytes.

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Abstract

The diabetic heart is characterized by a shift in substrate utilization from glucose to lipids, which may ultimately lead to contractile dysfunction. This substrate shift is facilitated by increased translocation of lipid transporter CD36 (SR-B2) from endosomes to the sarcolemma resulting in increased lipid uptake. We previously showed that endosomal retention of CD36 is dependent on the proper functioning of vacuolar H^+-ATPase (v-ATPase). Excess lipids trigger CD36 translocation through inhibition of v-ATPase function. Conversely, in yeast, glucose availability is known to enhance v-ATPase function, allowing us to hypothesize that glucose availability, via v-ATPase, may internalize CD36 and restore contractile function in lipid-overloaded cardiomyocytes. Increased glucose availability was achieved through (a) high glucose (25 mM) addition to the culture medium or (b) adenoviral overexpression of protein kinase-D1 (a kinase mediating GLUT4 translocation). In HL-1 cardiomyocytes, adult rat and human cardiomyocytes cultured under high-lipid conditions, each treatment stimulated v-ATPase re-assembly, endosomal acidification, endosomal CD36 retention and prevented myocellular lipid accumulation. Additionally, these treatments preserved insulin-stimulated GLUT4 translocation and glucose uptake as well as contractile force. The present findings reveal v-ATPase function as a key regulator of cardiac substrate preference and as a novel treatment approach for the diabetic heart.

Keywords: Vacuolar H+-ATPase; Lipid accumulation; Insulin resistance; Contractile function; Diabetic heart

Abbreviations: aRCMs, adult rat cardiomyocytes; hiPSC-CMs, human induced pluripotent stem cells differentiated into cardiomyocytes; LCFA, long chain fatty acid; v-ATPase, vacuolar H+-ATPase; V_0-a2, a2-subunit of the v-ATPase V_0 super-complex; V_0-d1, d1-subunit of the v-ATPase V_0 super-complex; V_1-B2, B2-subunit of the v-ATPase V_1 super-complex; AdPKD, adenoviral overexpression of protein kinase-D1; AdGFP, adenovirus containing Green Fluorescent Protein; IRAP, insulin-regulated aminopeptidase; IP, immunoprecipitation; CHLQ, chloroquine

Introduction

The diabetic heart is characterized by a shift in substrate utilization from glucose to lipids, resulting in insulin resistance and impaired cardiac contractile function [1, 2]. Notably, this is accompanied by increased abundance of CD36, the predominant cardiac lipid transporter, at the sarcolemma [3]. In the healthy heart, CD36 stimulates long chain fatty acid (LCFA) uptake via reversible translocation from the endosomes to the sarcolemma upon hormonal or mechanical stimuli [1]. Upon long-term overexposure of the heart to lipids, CD36 chronically translocates to the sarcolemma, initiating a vicious cycle of increased lipid uptake and lipid-induced insulin resistance, leading to cardiac dysfunction [4]. Our previous work showed that CD36 was expelled from the endosomes to translocate to the sarcolemma upon treatment of cardiomyocytes with pharmacological agents (e.g., monensin, and bafilomycin-A) causing endosomal alkalinization, indicating that proper functioning of the endosomal proton pump vacuolar H^+-ATPase (v-ATPase) is needed for endosomal CD36 retention [5, 6]. Studies in high-fat diet fed rats demonstrated that increased myocellular CD36 abundance at the sarcolemma of cardiomyocytes, as well as increased LCFA uptake and decreased insulin signaling was due to loss of endosomal acidification, thereby providing a novel link between v-ATPase inhibition and decreased cardiac function in the lipid-overloaded diabetic heart [6]. Taken together, v-ATPase inhibition underlies increased CD36 translocation to the sarcolemma and subsequent development of lipid-induced cardiomyopathy.

As a proton pump that is present in acidic organelles, v-ATPase is responsible for endosomal acidification [7]. v-ATPase is structurally divided into a cytosolic V_1 sub-complex and a transmembrane V_0 sub-complex, encompassing the ATP catalyzing activity and the proton channel, respectively [8]. Studies in yeast and mammalian kidney cells revealed that v-ATPase activity is mainly regulated via assembly and disassembly of the two sub-complexes [9-11]. These assembly/disassembly cycles were found to be regulated by glucose availability, as glucose deprivation caused v-ATPase disassembly, whereas glucose-enriched conditions favored assembly and hence restoration of organellar acidification [9-11]. Upon disassembly of the V_1 and V_0 sub-complexes, such as when cells are subjected to glucose withdrawal, v-ATPase activity declines [12]. Similarly to glucose deprivation, lipid-overload induces v-ATPase disassembly in HL-1 cardiac cells and primary adult rat cardiomyocytes (aRCMs), mechanistically underlying CD36 translocation [6]. Together, these observations suggest that v-ATPase assembly/disassembly cycles are reciprocally regulated by glucose and lipids.

Based on the experiments in yeast where increased glucose availability results in reassembly of v-ATPase [9-11], we hypothesize that reassembly of v-ATPase via forced glucose influx in lipid-overloaded cardiomyocytes will internalize CD36, decrease fatty acid uptake, improve insulin sensitivity, and restore contractile function. To test whether forced glucose uptake can preserve contractile function in lipid-overloaded cardiomyocytes, we applied two conditions: (i) high glucose concentration (25 mM) in the culture media, and (ii) adenoviral overexpression of protein kinase-D1 (PKD1). With respect to condition (i), the increased glucose concentrations will be taken up by GLUT1 and GLUT4, the two most highly expressed glucose transporters in cardiomyocytes, of which GLUT1 mediates basal glucose transport whereas GLUT4 is mainly responsible for insulin or contraction-induced glucose transport [13, 14]. Recent studies showed a direct effect of 25 mM glucose exposure on GLUT1 expression, thus contributing to the increased glucose uptake [15, 16]. Condition (ii) needs further explanation. PKD1, a member of a novel class of Ser/Thr kinases belonging to the PKD family, is specifically involved in regulation of GLUT4 translocation but not CD36 translocation [17]. Accordingly, cardiac-specific overexpression of constitutively active PKD1 in mice resulted in increased GLUT4 translocation and a shift in cardiac substrate preference from fatty acids to glucose [18].

The present data show that applying the two forced glucose uptake conditions to lipid-overloaded cardiomyocytes each resulted in increased v-ATPase assembly, proper endosomal acidification, endosomal CD36 retention, lowered levels of myocellular lipids and normalized contractile function. We further observed partial recovery of insulin-stimulated GLUT4 translocation in the absence of detectable effects on insulin signaling. Accordingly, targeting v-ATPase assembly as a novel strategy to combat lipid-induced cardiomyopathy warrants further investigation.

Results

Forced Glucose Uptake Re-assembles v-ATPase in Lipid-overloaded Cardiomyocytes

Our earlier studies showed that lipid-overexposure (or high-palmitate treatment) inhibits v-ATPase function via dissociation of the soluble V_1 sub-complex from the endosomal membrane-bound V_0 sub-complex, thereby providing a mechanistic explanation for lipid-induced endosomal alkalinization [6]. Using a subcellular fractionation method, we confirmed

in aRCMs **(Figure 1A)** and HL-1 cells **(Figure S1A)** that in all the conditions the V_0-a2 subunit, as part of the membrane-bound V_0 sub-complex, was localized at the membrane, whereas upon lipid-overexposure the V_1-B2 subunit, as indicator of the soluble V_1 sub-complex, was shifted from the endosomal membrane to the cytoplasmic fraction. Upon high glucose treatment, V_1-B2 was redistributed back to the membrane fraction **(aRCMs: Figure 1A; HL-1 cells: Figure S1A)**. In a complementary approach, we studied the influence of forced glucose influx on v-ATPase V_0/V_1 assembly by applying glucose addition followed by IP of v-ATPase sub-complexes. Using antibodies against the d1 subunit of the V_0 super-complex (V_0-d1) or V_1-B2 as part of the soluble V_1 super-complex of v-ATPase **(Figure 1B-C)**, we verified that lipid-overexposure conditions lead to a lower degree of co-IP with the other subunits as compared to control condition, which indicated increased V_0/V_1 disassembly upon lipid overload. Conversely, the degree of co-immunoprecipitation with the other subunits (e.g., V_0-d1 or V_1-B2) increased upon-treatment of lipid-overloaded cells with high glucose, indicating that forced glucose influx reassembled V_0/V_1 status in lipid-overloaded cardiomyocytes **(Figure 1B-C)**. Together, these findings show that increased glucose influx promotes re-assembly of v-ATPase in lipid-overloaded cardiomyocytes.

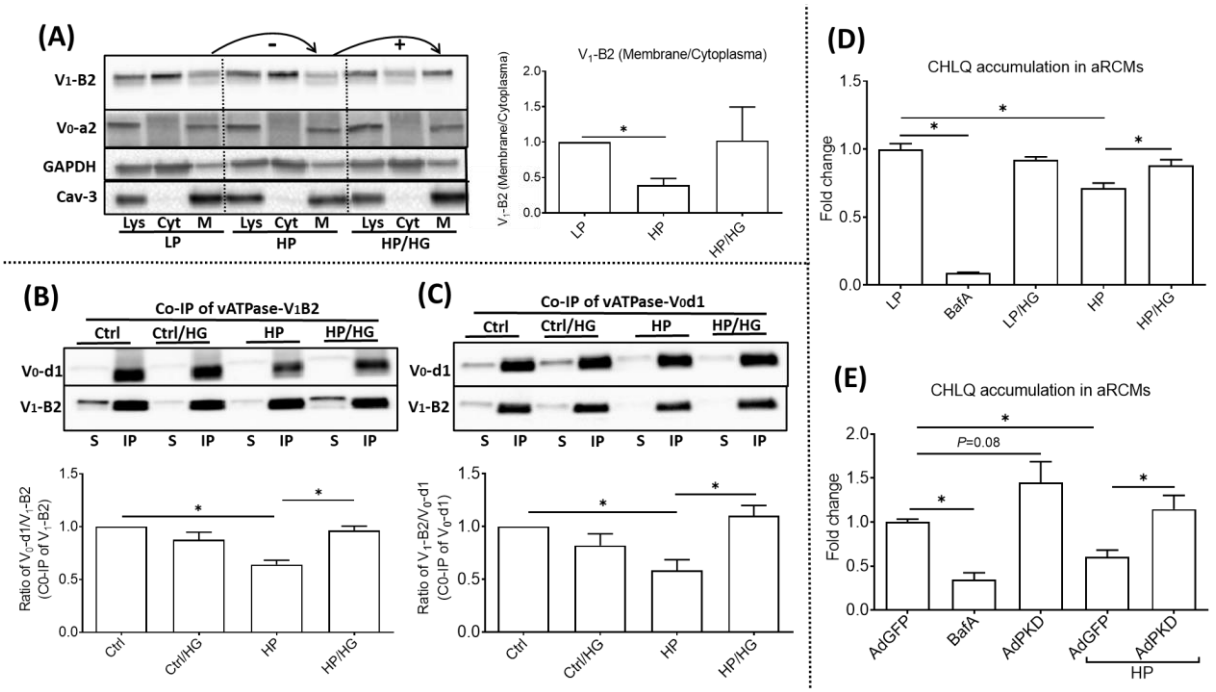

Figure 1. Status and activity of v-ATPase in lipid-overexposed cardiomyocytes.

(A) Fractionation in adult rat cardiomyocytes (aRCMs): aRCMs were incubated for 24h with either low palmitate (LP, palmitate/BSA ratio 0.3:1), high palmitate (HP, palmitate/BSA ratio 3:1), or HP with the addition of 25mM glucose (HP/HG). Contents of v-ATPase subunit a2 (V_0-a2) and subunit B2 (V_1-B2) were assessed by Western blotting in total cell lysates (Lys), in the cytoplasmic fraction (Cyt) and in the membrane fraction (M). GAPDH and Caveolin-3 were detected as loading control for V_1-B2 and V_0-a2, respectively. Representative blots and quantifications of three independent experiments are shown.

(B-C) Immunoprecipitation (IP) of v-ATPase subunit d1 (V_0-d1) or subunit B2 from HL-1 cells after incubation for 24 h with either basal (Ctrl) medium, Ctrl medium supplemented with 25mM glucose (Ctrl/HG), HP medium containing 500µM palmitate and 100 nM insulin, or HP medium supplemented with 25mM glucose (HP/ HG). IP samples were immunoblotted with antibodies against v-ATPase subunits V_0-d1 and V_1-B2 (n=4).

(D-E) Chloroquine (CHLQ) accumulation in lipid-overexposed aRCMs: **(D)** aRCMs were incubated for 24h with LP medium (LP), LP supplemented with 100nM Bafilomycin-A (BafA), LP/HG, HP, and HP/HG; **(E)** aRCMs were incubated for 48h with either LP medium with the addition of 120µl AdGFP (AdGFP), AdGFP supplemented with 100nM Bafilomycin-A (BafA), LP medium with the addition of 120µl AdPKD (AdPKD), HP with the addition of 120µl AdGFP (AdGFP/HP), or HP with the addition of 120µl AdPKD (AdPKD/HP). After the culturing of all conditions above, cells were sub for [^{3}H] CQ accumulation assay last 20 min. Values are displayed as mean ± SEM (n=4). $^*p<0.05$ were considered statistically significant.

Reassembly of V_0/V_1 Restores Endosomal Acidity in Lipid-overloaded Cardiomyocytes

To further investigate whether forced glucose influx (via high glucose or AdPKD overexpression) can restore proper endosomal acidification, we measured [^{3}H]CHLQ accumulation as an indicator of v-ATPase activity. Pharmacological inhibition of v-ATPase by bafilomycin-A (BafA) caused >80% decrease of v-ATPase function in both aRCMs **(Figure 1D-E)** and HL-1 cells **(Figure S1B-C)**, consistent with our previous findings [6], while v-ATPase function was also reduced by >30% in lipid (high-palmitate)-overloaded aRCMs **(Figure 1D-E)** and >50% in lipid-overloaded HL-1 cells **(Figure S1B-C)**. When high-palmitate exposure was combined with high glucose treatment or AdPKD treatment, v-ATPase function was not significantly decreased compared to the "LP' or "AdGFP' condition.

Increased Endosomal Acidification Induces Endosomal CD36 Retention and Decreases Lipid Accumulation in Lipid-overloaded Cardiomyocytes

Our earlier results showed that v-ATPase inhibition leads to increased CD36 translocation from endosomes to the sarcolemma [6]. Here, we further assessed whether the effects of forced myocardial glucose influx on v-ATPase function could be further extended to the regulation of CD36 translocation. HP treatment of HL-1 cells lead to a ~2-fold upregulation in basal cell surface CD36 content, indicative of CD36 translocation **(Figure 2A)**. A short-term stimulation by insulin in this condition of lipid overload had no additive effect on cell surface CD36 content indicating insulin resistance **(Figure 2A and Figure S2)**. Treatment of palmitate-overexposed HL-1 cells with high glucose prevented the lipid-overload induced CD36 translocation to the sarcolemma and restored insulin-induced CD36 translocation in this condition **(Figure 2A and Figure S2)**.

Furthermore, we tested the effect of increased glucose influx on cellular lipid accumulation (e.g., triacylglycerol content). As expected, myocellular triacylglycerol content was increased (i.e., HL-1 cells; by 1.8-fold; aRCMs: by 3.2-fold) in lipid-overloaded cardiomyocytes **(Figure 2B-C)**. High glucose treatment of these cells significantly prevented this increase. Collectively, these findings indicate that the beneficial effects of forced glucose uptake on v-ATPase function in lipid-overloaded cells extend to the endosomal retention of CD36, and to a decrease in triacylglycerol accumulation.

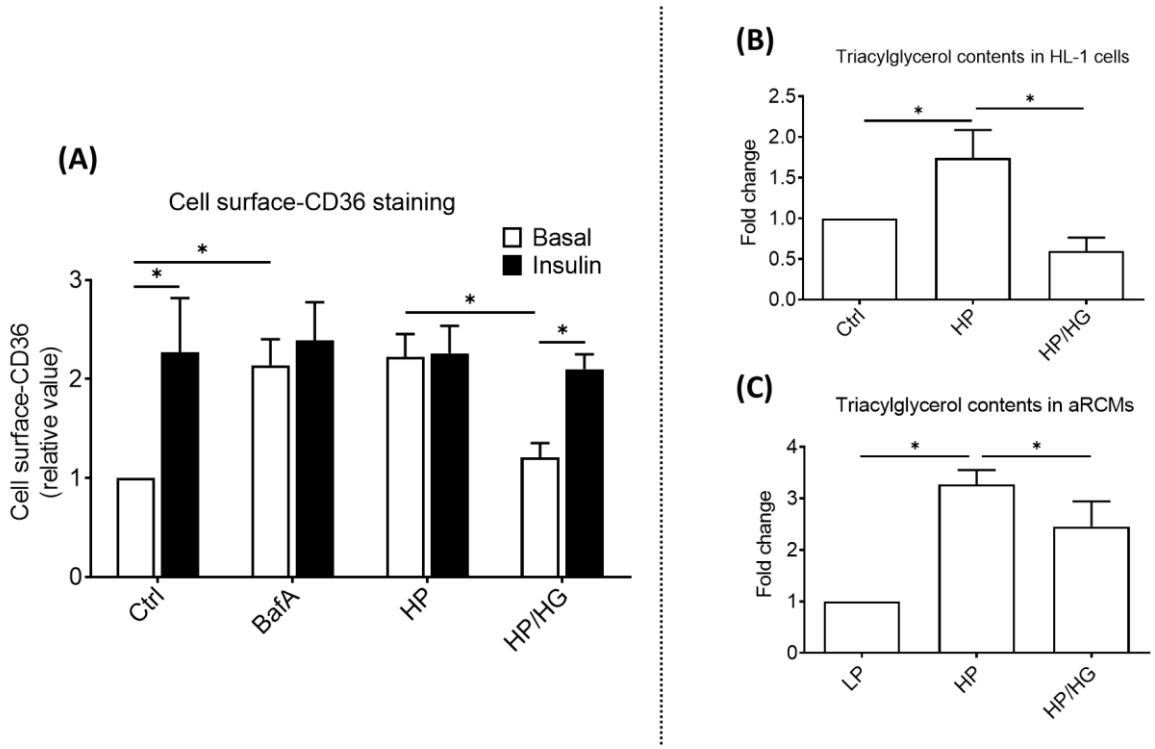

Figure 2. Cell surface-CD36 staining and triacylglycerol contents in lipid-overexposed cardiomyocytes.

(A) Cell surface CD36 staining of HL-1 cells: Prior to CD36 cell surface staining, HL-1 cells were treated for 24h either with control (Ctrl) medium, Ctrl medium containing 100nM Bafilomycin-A (BafA), high palmitate medium containing 500µM palmitate and 100nM insulin (HP), or HP medium with 25mM glucose (HP/HG). Subsequently, cells were stimulated either without or with 200 nM insulin for 30 min and immunochemically stained for cell surface CD36 content (n=3).

(B-C) Triacylglycerol contents in lipid-overexposed cardiomyocytes: **(B)** HL-1 cells were incubated for 24h with either Ctrl medium, HP medium, or HP/HG (n=5); **(C)** aRCMs were incubated for 24h with either LP medium, HP medium, or HP/ HG (n=7). Values are displayed as mean ± SEM. $^{*}p<0.05$ were considered statistically significant.

Reassembly of v-ATPase Does Not Promote Insulin Sensitivity in Lipid-overloaded Cardiomyocytes

For evaluation of insulin signaling, phosphorylation levels of Akt (pAkt Ser473) and of ribosomal protein S6 (pS6 Ser235/236), were assessed. In agreement with previous observations [6], lipid-overload in aRCMs and HL-1 cells induced a loss of insulin-stimulated phosphorylation of Akt and S6. However, both glucose uptake-enforcing treatments **(aRCMs: Figure 3A-F; HL-1 cells: Figure S3A-F)** did not restore this lipid-induced loss of insulin-stimulated Akt phosphorylation and S6 phosphorylation.

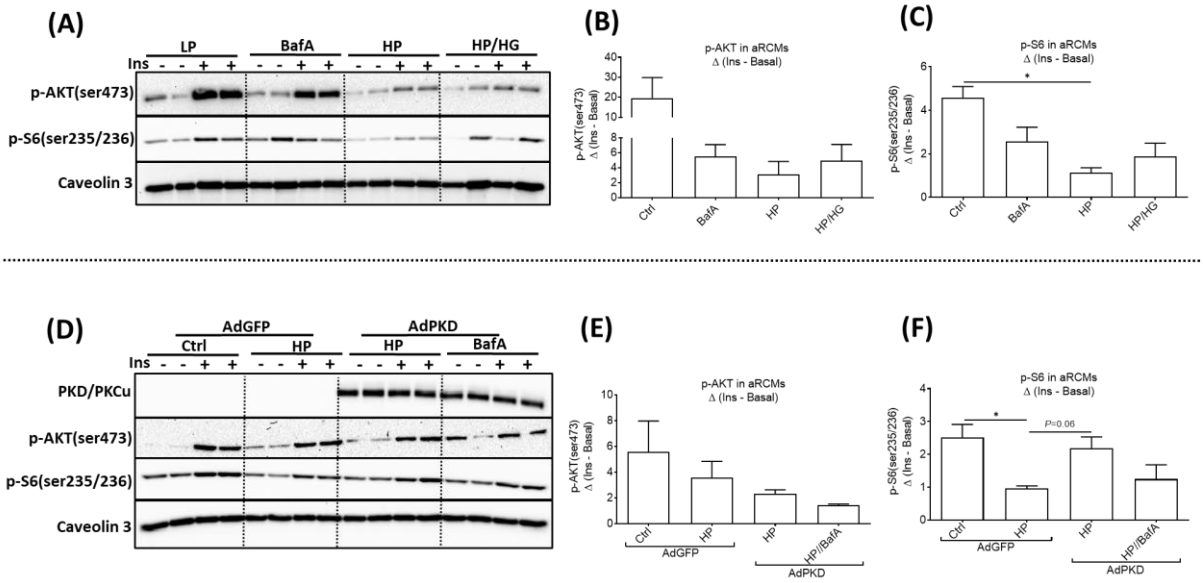

Figure 3. Insulin sensitivity in lipid overloaded aRCMs.

(A-C) aRCMs were incubated for 24h with either low palmitate (LP), LP medium containing 100nM Bafilomycin-A (BafA), high palmitate (HP), or HP with addition of 25mM glucose (HP/HG) for 24h. Subsequently, cells were stimulated either with or without 100 nM insulin for 30 min and harvested for western blotting analysis. **(A)** Representative blots. **(B-C)** Quantification of the level of pAKT and pS6 (n=3).

(D-F) aRCMs were incubated for 24h in LP medium with either the addition of 120µl AdGFP (AdGFP), HP with the addition of 120µl AdGFP (AdGFP/HP), LP with the addition of 120µl AdPKD (AdPKD), HP with the addition of 120µl AdPKD (AdPKD/HP), or AdPKD/HP medium containing 100nM Bafilomycin-A (BafA) (AdPKD/HP+BafA). Subsequently, cells were stimulated either with or without 100 nM insulin for 30 min and harvested for western blotting analysis. **(D)** Representative blots. **(E-F)** Quantification of the level of pAKT and pS6 (n=3). Caveolin 3 was detected as loading control. Values are displayed as mean ± SEM. $^{*}p<0.05$ were considered statistically significant.

Reassembly of v-ATPase Improves Insulin Stimulated-GLUT4 Translocation and Glucose Uptake in Lipid-overloaded Cardiomyocytes

A surface biotinylation assay was used to investigate whether insulin-stimulated GLUT4 translocation would be improved in lipid-overloaded cardiomyocytes upon treatment with either high glucose exposure or AdPKD overexpression. Translocation of GLUT4 (reflected by IRAP) was largely decreased upon HP treatment and almost entirely re-installed in lipid-overloaded aRCMs under glucose addition **(Figure 4A-B, and Figure S4A)** and AdPKD overexpression **(Figure 4C-D and Figure S4B)**.

A radiolabeled glucose analog ([^{3}H]deoxyglucose) was used to investigate whether both treatments could preserve insulin-stimulated glucose uptake in lipid-overloaded cardiomyocytes. PKD overexpression caused an increase in glucose uptake (1.8 fold in aRCMs and 1.4-fold in HL-1 cells), which is due to increased insulin-independent GLUT4 translocation [18]. As expected, insulin-stimulated glucose uptake was almost entirely abolished in high palmitate-treated cells **(aRCMs: Figure 4E-F and Figure S4C-D; HL-1 cells: Figure S5A-D).** In both cell models, this loss of insulin-stimulated glucose uptake was partly corrected by each of the treatments **(aRCMs: Figure 4E-F and Figure S4C-D; HL-1 cells: Figure S5A-D).** Taken together, at the level of insulin-stimulated GLUT4 translocation as well as at the

level of insulin-stimulated glucose uptake, both treatments proved to be at least partly effective in restoring the relatively large insulin effect of the low-palmitate condition.

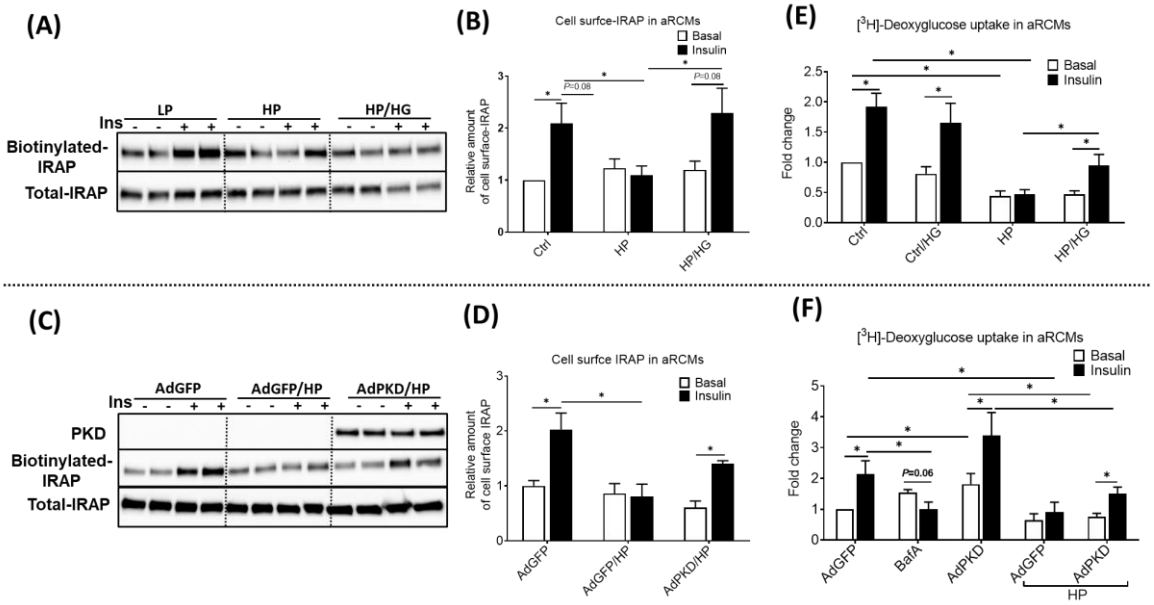

Figure 4. Insulin-stimulated GLUT4 translocation and glucose uptake in lipid-overexposed aRCMs.

(A-D) Insulin-stimulated GLUT4 translocation in lipid-overexposed aRCMs: **(A)** Representative blotting and **(B)** quantification of Insulin-regulated aminopeptidase (IRAP, which reflects GLUT4 trafficking) from biotin-labeled (A upper panel) and total lysate fractions (A lower panel) of aRCMs, which are incubated for 24 h with low palmitate (LP), high palmitate (HP), and HP+25mM glucose (HP/HG), followed by 30 min of (-/+) insulin (100 nM) incubation prior to biotin labeling and lysis (n=4). **(C)** Representative blotting and **(D)** quantification of protein kinase-D1 (PKD) (A upper panel) and IRAP from biotin-labeled (A middle panel) and total lysate fractions (A lower panel) of aRCMs, which are incubated for 48 h with LP containing 120µl Adenovirus Green Fluorescent Protein (AdGFP), HP containing 120µl AdGFP (AdGFP/HP), and HP containing 120ul Adenovirus PKD (AdPKD/HP), followed by 30 min of (-/+) insulin (100 nM) incubation prior to biotin labeling and lysis (n=4). Quantification of the amount of cell surface IRAP using BioRad Quantity One software.

(E-F) Insulin-stimulated glucose uptake in lipid-overexposed aRCMs: **(E)** aRCMs were incubated for 24h with either LP, LP/HG, HP, or HP/HG, followed by 30 min of (-/+) insulin (100 nM) incubation prior to [^{3}H] deoxyglucose labeling (n=6); **(F)** aRCMs were incubated for 48h with AdGFP, AdGFP/HP, AdPKD, AdPKD/HP, or AdPKD/HP medium containing 100nM Bafilomycin-A (AdPKD/HP+BafA), followed by 30 min of (-/+) insulin (100 nM) incubation prior to [^{3}H] deoxyglucose labeling (n=4). Values are displayed as mean ± SEM. $^*p<0.05$ were considered statistically significant.

Reassembly of v-ATPase Restores Contractile Function in Lipid-overloaded Cardiomyocytes

Given our previous finding that high lipid exposure causes contractile dysfunction, we next tested if high glucose exposure could restore such loss. Indeed, as seen previously [6], lipid induced-v-ATPase inhibition significantly decreased sarcomere shortening **(Figure 5A)**, but it had no significant influence on contraction acceleration time (e.g., time to peak) and the duration of relaxation [e.g., decay time to 50% percent (RT50), and decay time to 90% percent (RT90)] in lipid-overloaded aRCMs **(Figure 5B-D).** Notably, treatment of these lipid-overloaded aRCMs with high glucose could restore a decrease of sarcomere shortening (**Figure 5A**), also without changes in both the contraction acceleration time (e.g., time to peak) (**Figure 5B**) and the duration of relaxation (e.g., RT50 and RT90) (**Figure 5C-D)**. Collectively, treatment of these lipid-overloaded aRCMs with high glucose could restore their contractile function.

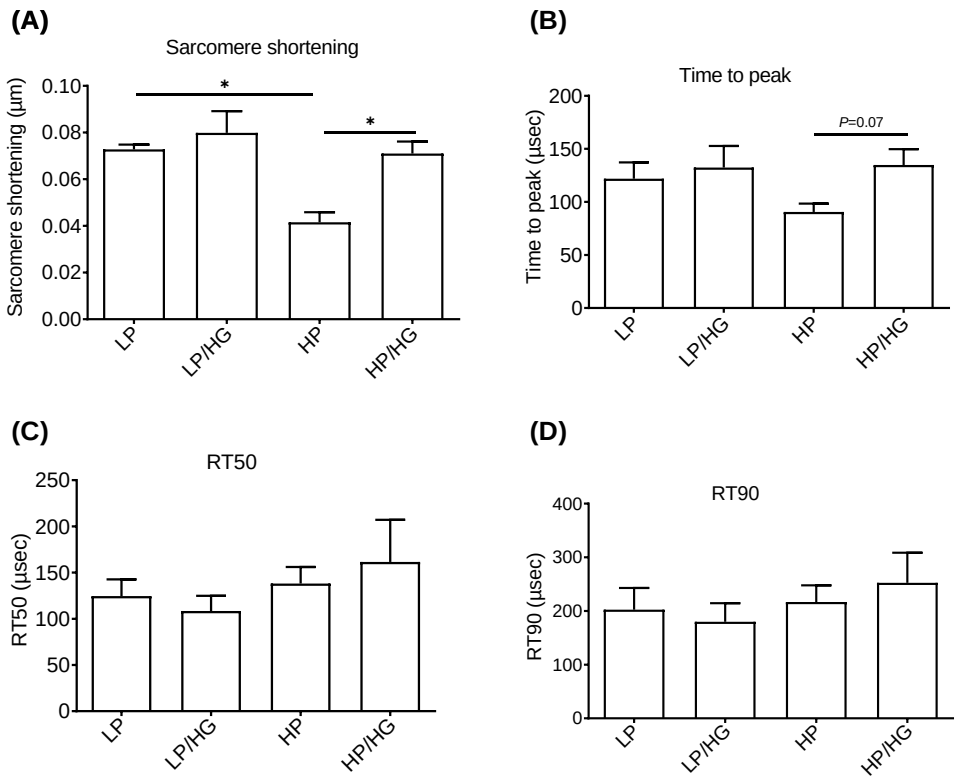

Figure 5. Contractile function in lipid overloaded aRCMs.

(A-D) aRCMs were incubated for 24 h in either low palmitate medium (LP), LP medium with the addition of 25mM glucose (LP/HG), high palmitate medium (HP), or HP with the addition of 25mM glucose (HP/HG). Parameters of contraction amplitude and kinetics were determined upon 1 Hz electrostimulation: **(A)** sarcomere shortening; **(B)** time to peak; **(C)** decay time to 50% percent (RT50); **(D)** decay time to 90% percent (RT90). Values are displayed as mean ± SEM (n=3; imaging of 10 cells/condition). $^{*}p<0.05$ were considered statistically significant.

Restoration of v-ATPase Function Re-balances Energy Substrates in Human iPSC-Cardiomyocytes

To further confirm whether the rebalancing of cellular energy substrate metabolism via restoration of v-ATPase function also occurs in human iPSC-cardiomyocytes, hiPSC-CMs were subsequently used for this study. These hiPSCs were first characterized for their pluripotency prior to cardiomyocyte differentiation and as shown in **Figure S6A-B**, the pluripotent markers (e.g., Nanog and Oct4) were highly expressed in hiPSCs. Additionally, the karyotyping results of hiPSCs from the Department of Clinical Genetics, Maastricht UMC+, also demonstrated that there were no chromosomal abnormalities observed in these cells **(Figure S7)**.

Similar to that observed in rodent cardiomyocytes **(aRCMs: Figure 4E-F and Figure S4C-D; HL-1 cells: Figure S5)**, hiPSC-CMs developed the key features of insulin resistance upon high-palmitate culturing: loss of insulin-stimulated LCFA and glucose uptake and increased basal LCFA uptake **(Figure 6B-C and Figure S6C-D)**. Moreover, hiPSCs-CM cultured with high-palmitate display loss of v-ATPase function **(Figure 6A)**. When high-palmitate exposure was combined with high glucose treatment, v-ATPase function, insulin-stimulated LCFA- and glucose uptake were restored **(Figure 6A-C)**. Most importantly, these findings strongly support the observations seen in rodent cardiomyocytes and provide substantial evidence that the mechanism of increased glucose availability-driven v-ATPase assembly to re-balance substrate uptake in lipid-overloaded cardiomyocytes is conserved between species.

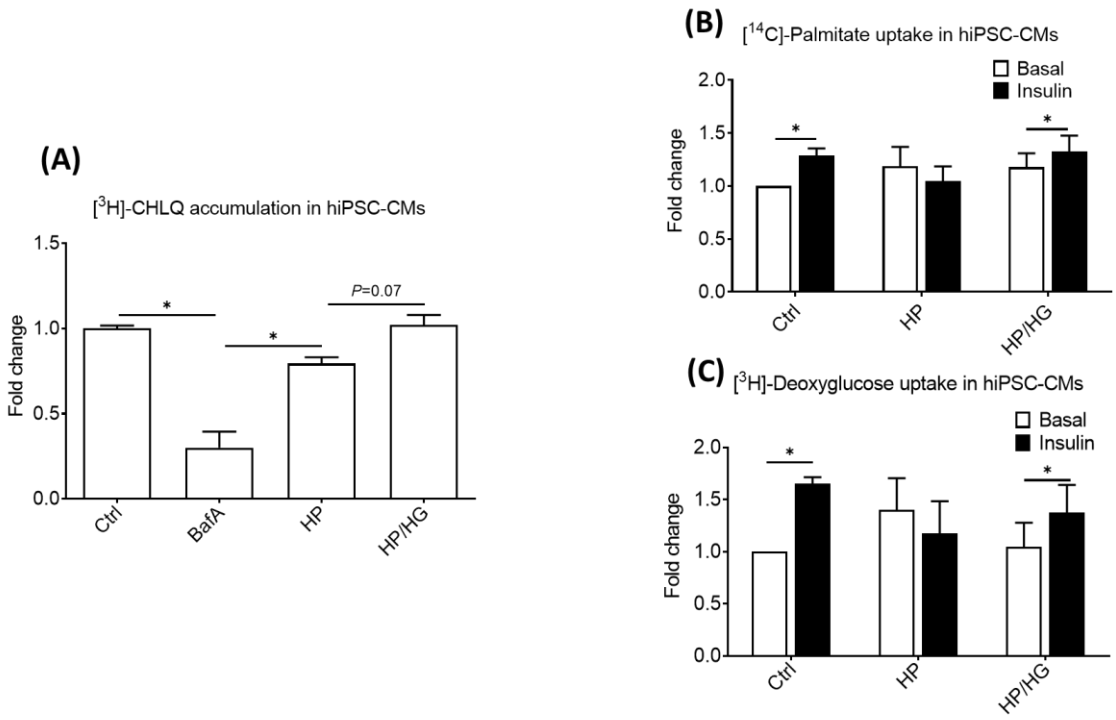

Figure 6. Chloroquine (CHLQ) accumulation, glucose- and fatty acid uptake in lipid-overexposed human iPSC-derived cardiomyocytes (hiPSC-CMs).

(A) CHLQ accumulation in hiPSC-CMs cultured for 20 h in either control medium (Ctrl), Ctrl with addition of 100 nM Bafilomycin-A (BafA), high palmitate medium (HP), or HP with the addition of 25mM glucose (HP/HG). **(B-C)** Palmitate- and glucose uptake in in hiPSC-CMs treated without/with 100 nM insulin for 30 min, determined by means of [^{14}C]palmitate and [^{3}H]deoxyglucose uptake, respectively. Values are displayed as mean ± SEM (n=3). $^{*}p<0.05$ were considered statistically significant.

Discussion

In this study, we investigated whether forced glucose uptake would improve insulin resistance and contractile dysfunction in lipid-overloaded cardiomyocytes and whether this is accompanied by re-assembly of v-ATPase. The following main observations were made **(Figure 7)**: high glucose addition and AdPKD overexpression stimulated assembly of v-ATPase, endosomal acidification, endosomal CD36 retention, and insulin-stimulated glucose uptake, inhibited lipid accumulation, and promoted contractile force in lipid-overloaded cardiomyocytes, but did not restore insulin signaling.

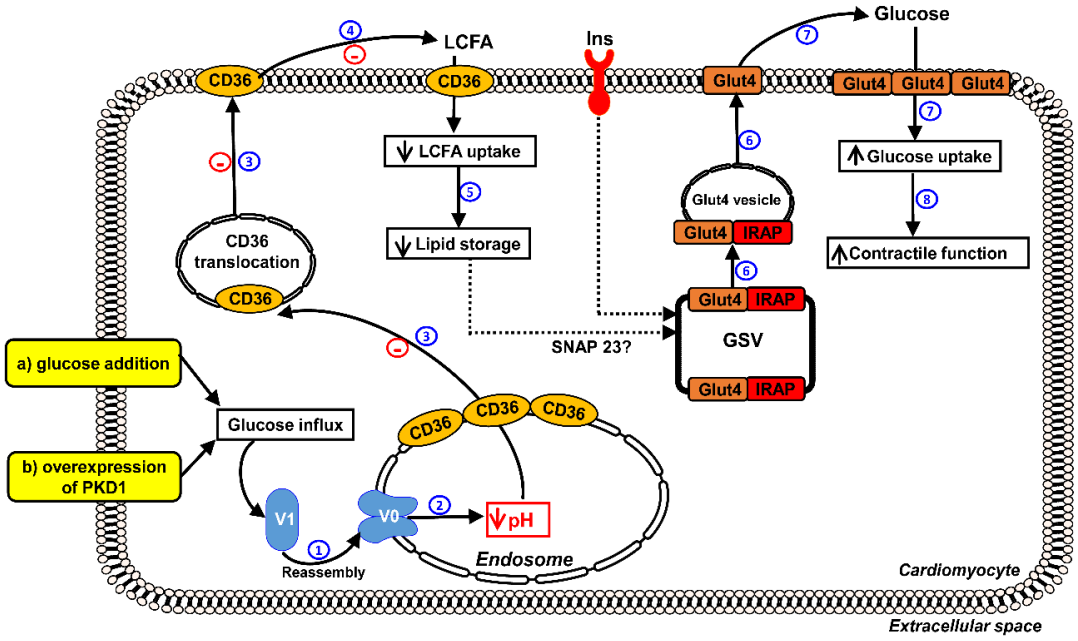

Figure 7. Schematic hypothesis of reassembly of v-ATPase as a target to restore contractile function in lipid-overloaded cardiomyocytes.

When LCFA supply is high, CD36 translocation from the endosome to the sarcolemma is stimulated. Furthermore, the v-ATPase V_0 sub-complex, which is integral to the endosomal membrane, is disassembled from the cytosolic V_1 sub-complex contributing to endosomal alkalinization. In this situation, the supply of LCFA will be in excess to the immediate energy demand, resulting in insulin resistance and impaired cardiac contractile function. Therefore, proper endosomal acidification mediated by v-ATPase is essential for CD36 retention.

Elevated glucose influx triggers a series of following events: **(1)** both treatments (e.g., glucose addition and overexpression of PKD1) promote the V_1 and V_0 sub-complexes to reassemble, V_1 is therefore integrated to the membrane. **(2)** Reassembly of v-ATPase leads to endosomal acidification. **(3)** Endosomal acidification triggers CD36 retention to the endosome as well as Glut4 translocation to the sarcolemma. **(4-5)** The inhibition of CD36 translocation decreases LCFA uptake, thereby inhibiting lipid accumulation. **(6-7)** Decreased lipid droplet numbers leads to redistribution of SNAP23 to the GLUT4 storage vesicle (GSV) to become available for insulin-stimulated GLUT4 translocation and insulin-stimulated glucose uptake, therefore shifting energy substrate preference from LCFA to glucose. **(8)** Rebalancing of energy substrate utilization restores contractile function in lipid-overloaded cardiomyocytes.

As established earlier, lipid overload in cardiomyocytes leads to disassembly of the V_0/V_1 holo-complex [6]. The consequent inhibition of v-ATPase function causes CD36 translocation

to the sarcolemma, leading to the well-described shift of substrate uptake from glucose to lipids, as seen in the diabetic heart [1, 6]. Given that v-ATPase responds to the availability of lipids by dissociation of the super-complexes thereby decreasing its pumping function, v-ATPase could be seen as a lipid sensor. Vice versa, it has been shown in yeast that glucose can increase v-ATPase function by restoring the assembly of its super-complexes [8, 9]. Here, we set out to investigate the effect of forced glucose uptake in lipid-overloaded mammalian cardiomyocytes, which is a setting where glucose and lipids would compete for v-ATPase assembly and activity. Indeed, the present study shows that lipid-induced dissociation of v-ATPase was partially prevented by forced glucose uptake. Consistent with this, forced glucose uptake also prevented the loss of v-ATPase activity, decreased CD36 translocation and cellular lipid accumulation, and reinstalled insulin-stimulated glucose uptake as well as contractile function. Reassembly of v-ATPase is therefore deemed as a suitable target to counteract lipid accumulation in the diabetic heart.

High glucose treatment by itself, especially in a diabetic setting, would be a truly counter-intuitive strategy to combat lipid-induced insulin resistance. Therefore, we would like to stress that glucose addition was chosen to provide proof-of-principle for testing strategies to counteract lipid-induced contractile dysfunction via v-ATPase re-assembly. The other strategy to enforce glucose uptake, i.e., PKD1 overexpression, would be more favorable in the diabetic setting because it stimulates glucose entry into cardiomyocytes via induction of contraction-responsive GLUT4 not CD36 translocation, which would even contribute to lowering the circulating glucose levels [18]. This favorable anti-diabetic action would be even greater when PKD1 would induce GLUT4 translocation also in skeletal muscle, but the latter is currently undefined. Hence, as a future step in therapeutic strategies against lipid-induced insulin resistance, specific PKD1 activators could be employed to coerce myocytes to specifically take up glucose for subsequent v-ATPase reassembly. A number of compounds, such as α-adrenergic agonists, are known to induce PKD1 activation [19], but also to induce a variety of other signaling pathways with rather undesirable side actions such as cardiac hypertrophy. Although specific PKD1 activators have not been identified to date, it's development would hold promise for future therapy in diabetic heart.

Surprisingly, we did not observe positive effects of forced glucose uptake on insulin-stimulated Akt phosphorylation. This raises the question on how glucose uptake-enforcing treatments can restore insulin-stimulated glucose uptake in the lipid-overloaded heart in the absence of restoration of insulin signaling. A possible explanation includes that lipids

negatively impact on insulin-stimulated glucose uptake in the heart independently of impairment of insulin-stimulated Akt activation. In this respect, an alternative mechanism of lipid-induced impairment of insulin-stimulated glucose uptake involves the competition between GLUT4 vesicles and lipid droplets for the same SNARE proteins. Recently, it has been revealed that lipid droplets use SNARE proteins for dynamic fission/fusion processes [20]. One of the SNARE proteins shared by GLUT4 vesicles and lipid droplets is SNAP23 [20]. Using this information, the following scenario could take place when the lipid-overloaded cells are forced to take up glucose. First, v-ATPase would re-assemble leading to endosomal re-acidification, CD36 endocytosis, and decreased myocellular lipid uptake. The consequential decline in lipid droplets would then make SNAP23 re-available for insulin-stimulated GLUT4 translocation without the need for stimulation through Akt signaling.

Yet there would still be one unresolved issue with this SNARE competition-driven scenario in lipid-overloaded cells: there is still a ~50% decrease in insulin-stimulated Akt phosphorylation, which is upstream of insulin-stimulated GLUT4 translocation (via phosphorylation and inhibition of AS160), and as mentioned, not restored upon PKD1 overexpression. This issue can be circumvented when it would be assumed that the partial impairment of Akt activation would not be rate limiting for insulin-stimulated GLUT4 translocation, as has been suggested by us earlier [4].

Materials and Methods

Antibodies

Primary antibodies used in Western blotting analysis were rabbit anti- PKD/PKC-μ, rabbit anti- phospho-Ser473-AKT (pAKT), rabbit anti- phospho-Ser235/236-S6 (pS6), rabbit anti- IRAP, and rabbit anti-GAPDH (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-ATP6V_0A2 (V_0-a2), rabbit anti-ATP6V_0D1 (V_0-d1), rabbit anti-ATP6V_1B2 (V_1-B2) (Abcam, Cambridge, UK), mouse anti-CD36 (MO25) (a generous gift from Dr. N. Tandon), mouse anti- caveolin-3 (BD Transduction Laboratories, Lexington, KY, USA). Primary antibodies were detected by either anti-rabbit secondary antibody for PKD/PKC-μ, pAKT, pS6, IRAP and GAPDH (Cell Signaling Technology) or anti-rabbit secondary antibody for V_0-a2, V_0-d1, and V_1-B2 (Dako Corp., Carpinteria, CA, USA), or anti-mouse secondary antibody for caveolin-3 (BD Transduction Laboratories Dako Corp., Carpinteria, CA, USA).

Isolation and Culturing of Primary Rat Cardiomyocytes

Male Lewis rats, 250-340 grams, were purchased from Charles River laboratories, and were maintained at the Experimental Animal Facility of Maastricht University. Animals were housed in a controlled environment (21-22°C) on a 12:12h light dark cycle (light from 0700 to 1900h) and had free-access to food and tap water. All experiments were performed according to Dutch regulations and approved by the Maastricht University Committee for Animal Welfare.

Adult rat cardiomyocytes (aRCMs) were isolated by using a Langendorff perfusion system, as previously described [21]. Briefly, after isolation of cardiomyocytes, cells were seeded on laminin pre-coated plates. After a 2h adhesion period the medium was replaced by either low palmitate medium (LP, 20μM palmitate, palmitate/BSA ratios of 0.3:1), LP with the addition of 25mM glucose (LP/HG), high palmitate medium (HP, 200μM palmitate, palmitate/BSA ratios of 3:1), or HP with the addition of 25mM glucose (HP/HG) for 24h. Cells were cultured as previously described [6].

Culturing of HL-1 Cardiomyocytes

HL-1 cells were kindly provided by Dr. W. Claycomb (Louisiana State University, New Orleans, LA, USA) and cultured as previously described [6]. Briefly, HL-1 cells were either treated with control (Ctrl) medium, Ctrl medium containing 100nM Bafilomycin-A (BafA), HP medium containing 500μM palmitate and 100nM insulin (HP), or HP medium with 25mM glucose addition (HP/ HG) for 24 h.

Human Induced Pluripotent Stem Cell (hiPSC) Maintenance and Differentiation into Cardiomyocytes (hiPSC-CMs)

Skin fibroblasts from healthy adult male individuals were collected and hiPSCs were generated by episomal reprogramming at the Stem Cell Technology Centre, Radboudumc (Nijmegen, Netherlands). The cells were maintained in Essential 8 medium (Thermofisher Scientific, Massachusetts, USA) under feeder-free conditions. Prior to cardiomyocyte differentiation, the cells were passaged with 0.5mM of EDTA solution (Promega, Wisconsin, USA), counted with a cell-counter and seeded in Essential 8 medium containing 10μM ROCK inhibitor (Stem Cell Technologies, Vancouver, Canada) on Matrigel (Corning Inc., New York, USA)-coated 24-well and 12-well plates. This was denoted as day 4 and the medium was changed daily until the cells reached 80–90% confluency. At day 1 of differentiation, Essential 8 medium was removed and replaced with Cardiomyocyte Medium A (Thermofisher Scientific, Massachusetts, USA). The cells were incubated for 2 days prior to a media change to Cardiomyocyte Medium B (Thermofisher Scientific, Massachusetts, USA). At day 5, the media

was then replaced with Cardiomyocyte Maintenance Media (Thermofisher Scientific, Massachusetts, USA) for 2 days and replaced every 2 days until day 14. To purify the cardiomyocyte population, metabolic selection was performed using RPMI 1640 without glucose (Thermofisher Scientific, Massachusetts, USA) containing 4 mM sodium lactate (Sigma Aldrich, Missouri, USA) for 5 days and thereafter the cells were further maintained in Cardiomyocyte Maintenance Media for an additional 5 days. Chloroquine accumulation, fatty acid and glucose uptake assays were subsequently performed on the metabolically-selected hiPSC-CMs.

Adenovirus Amplification

Adenovirus Containing Green Fluorescent Protein (AdGFP, as control) and a recombinant adenovirus encoding full-length wild-type mouse PKD1 (AdPKD) were kindly provided by Dr. L. Bertrand's lab (Pole de Recherche Cardiovasculaire, Institut de Recherche Expérimentale et Clinique, UCLouvain, Brussels, Belgium). Briefly, adenoviruses were amplified in HEK-293 cells and then purified over CsCl2 gradients as previously described [22]. Optimal multiplicity of infection (MOI) was determined by fluorescence microscopy, and MOI 10 was therefore chosen for AdPKD [22].

For the transfection in aRCMs, cells were transfected with either AdGFP or AdPKD for 48h after a 2h adhesion period. Additionally, for the transfection in HL-1 cells, they were also transfected with AdGFP or AdPKD. After 32 h, the medium was changed into FCS- and norepinephrine-free medium and cells were kept overnight. Subsequently, cells were used for measurements of insulin signaling, substrate uptake, or cellular chloroquine accumulation.

Measurement of v-ATPase Disassembly/Assembly

Two methods were applied to measure disassembly, namely, immunoprecipitation (IP) (i) and subcellular fractionation (ii).

(i) IP: the method of IP was conducted as previously described [6]. v-ATPase d1 (V_0-d1, an indicator of cytosolic V_1 sub-complex) and v-ATPase B2 (V_1-B2, an indicator of the membrane-bound V_0 sub-complex) proteins were detected by Western blotting.

(ii) Subcellular fractionation: the method of subcellular fractionation was also conducted as previously described [6]. For subcellular fractionation, V_0-a2, V_0-d1, and V_1-B2, GAPDH, and caveolin-3 proteins were detected by Western blotting.

Measurement of Cellular Chloroquine (CHLQ) Accumulation As Readout of v-ATPase Function.

CHLQ accumulation in HL-1 cells, aRCMs, and hiPSC-CMs was measured as previously described [5, 6].

Determination of Content of CD36 at Cell Surface

Colorimetric detection of sarcolemmal CD36 was carried out using a HRP-linked secondary antibody, as previously described [23].

Quantification of Triacylglycerol Contents

Quantification of triacylglycerol in both HL-1 cells and aRCMs was performed using a Triglyceride Assay Kit (ab65336, Abcam, San Francisco, CA, USA) following the manufacturer's instructions.

Determination of Insulin Sensitivity

After culturing, aRCMs and HL-1 cells were exposed to insulin (aRCMs, 100nm; HL-1 cells, 200 nM) for 30 min to be able to compare basal-phosphorylation to insulin-stimulated phosphorylation. Afterwards, these cells were lysed in sample buffer and used for protein detection by SDS-polyacrylamide gel electrophoresis, followed by Western blotting. PKD/PKC-μ, phospho-Ser473-Akt (pAKT), phospho-Ser235/236-S6 (pS6), and caveolin-3 proteins were detected by Western blotting.

Surface Biotinylation Assay

Surface-protein biotinylation was measured as previously described [24] with the modifications described below. After culturing with high palmitate medium or the transfection with AdPKD, aRCMs were incubated for 30min with (or without) 100nm insulin. Subsequently the cells were biotinylated with the cell-impermeable reagent sulfo-NHS-LC-biotin (0.5 mg/ml dissolved in M199 medium, Thermo Fisher Scientific, Fremont, CA, USA) for 5 min at 37°C. The rest steps of surface-protein biotinylation assay were carried out as previously described [24]. Insulin-regulated aminopeptidase protein (IRAP, which reflects GLUT4 trafficking) was detected by Western blotting.

Measurement of Substrate Uptake

[^{3}H]deoxyglucose uptake into suspensions of cardiomyocytes was measured as previously described [25]. Uptake of substrate into HL-1 cells and into cultured aRCMs and hiPSC-CMs, all cell models seeded on pre-coated glass slides, was measured as previously described [26].

Measurement of Cardiomyocytic Contraction Dynamics

Contractile properties of aRCMs were assessed at 1 Hz field stimulation using a video-based cell geometry system to measure sarcomere dynamics (IonOptix, Milton, MA, USA). From the digitized recordings acquired with IonWizard acquisition software, the following parameters were calculated: sarcomere shortening, time to peak, and decay time. This was conducted as previously described [4].

Statistical Analysis

All data are presented as means ± SEM. Statistical analysis was performed by using a two-sided Student's t-test with GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA). *P*-values of less than 0.05 are considered statistically significant.

Conclusions

In summary, the overall findings provide solid evidence that v-ATPase is both a lipid sensor and a glucose sensor in the heart, making v-ATPase a key regulator of cardiac substrate preference. The present findings as observed in rodents also extend to the human setting, given that the enforcement of glucose uptake also preserves v-ATPase activity and insulin-stimulated glucose uptake treatment in hiPSC-CMs upon over-exposure of these cells to lipids. Hence, the regulation of v-ATPase assembly/disassembly may offer a suitable target to combat cardiac dysfunction elicited by lipotoxic conditions.

Author's Contributions: S.W., Y.L. and J.L. analyzed data, S.W., L.W., J.L. and Y.L performed the experiments, J.L., D.M., M.N. and J.G., designed the experiments, S.W., J.L., D.M., M.N., J.G. and J.G., wrote the manuscript, L.W ., A.S. and G.A., provided technical assistance for the study. All authors discussed the data and commented on the manuscript before submission.

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Conflicts of Interest: The authors declare no conflict of interest.

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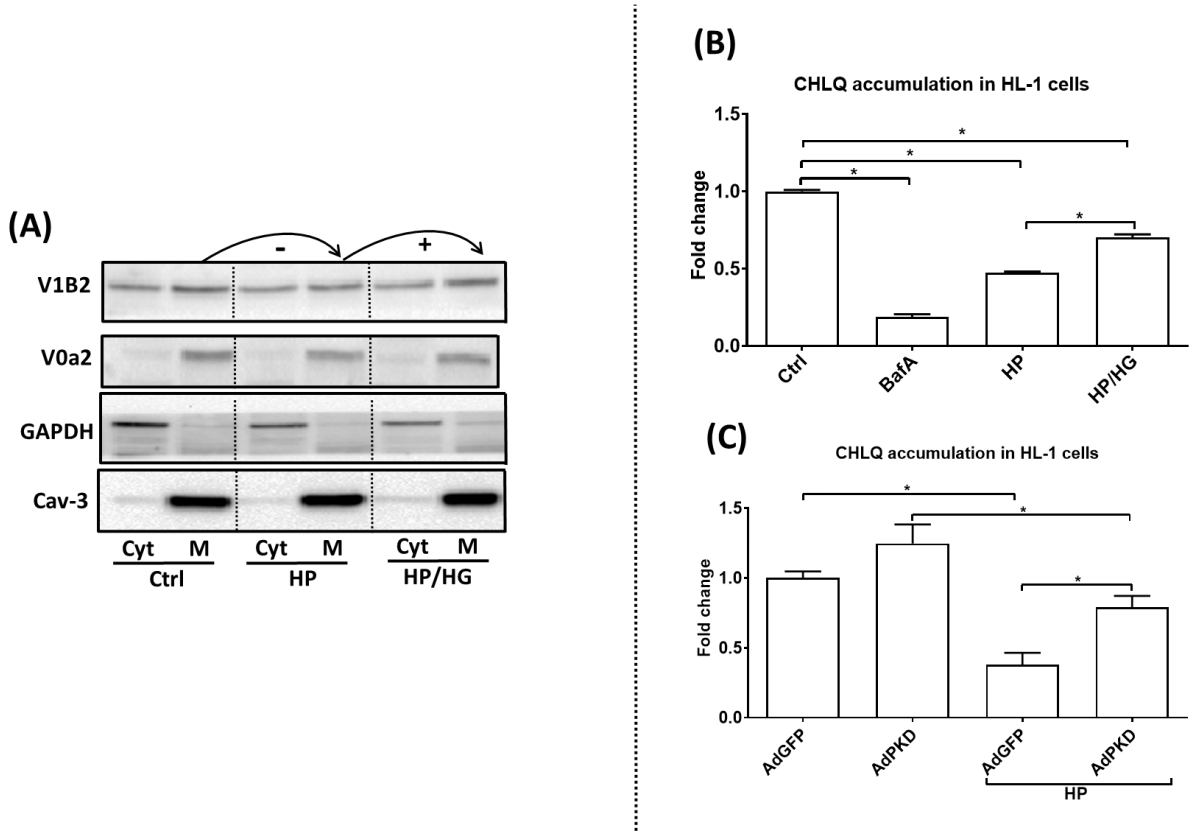
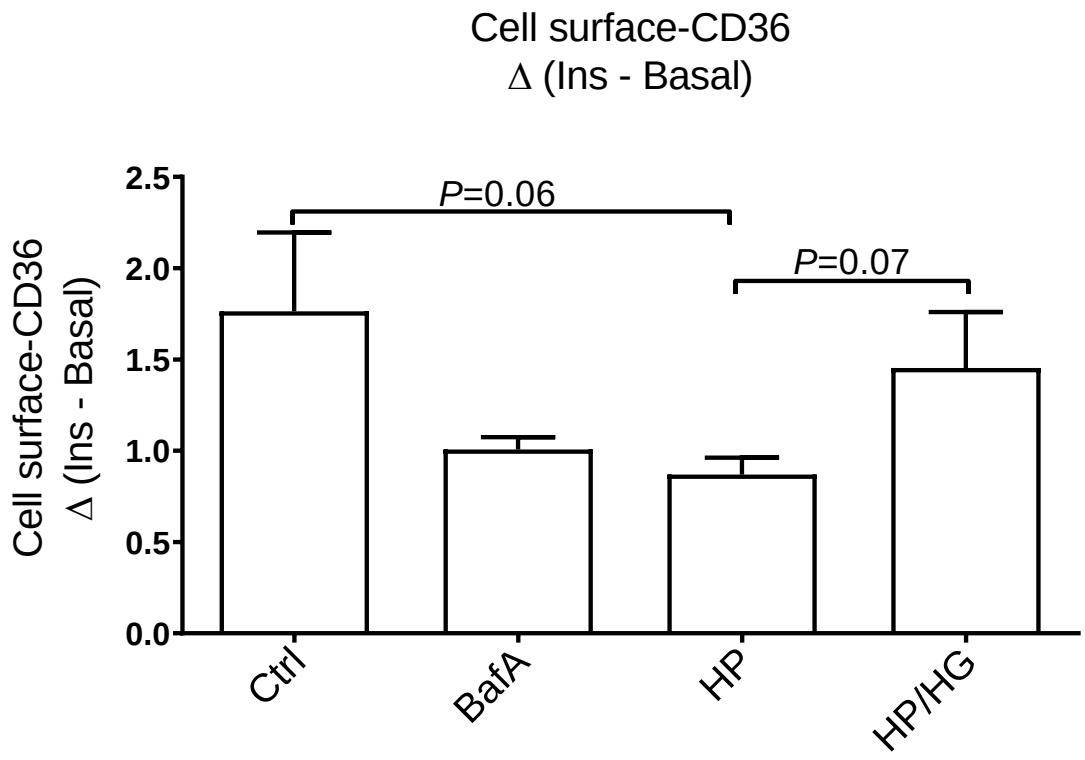
Supplemental Figures



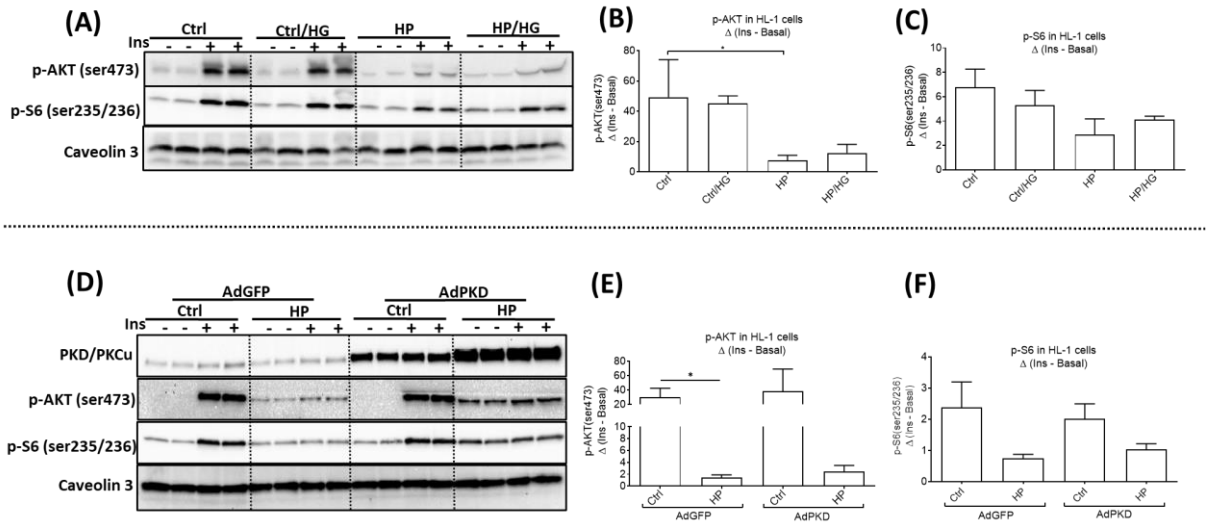
Supplemental Figure. 1 (Related to Figure 1). Status and activity of v-ATPase in lipid-overexposed cardiomyocytes.

(A) Fractionation in HL-1 cells: HL-1 cells were incubated for 24h with either control (Ctrl) medium, HP medium media containing 500µM palmitate and 100nM insulin, or HP medium with 25mM glucose addition (HP/HG). Contents of v-ATPase subunit a2 (V_0-a2) and v-ATPase subunit B2 (V_1-B2) were assessed by western blotting in the cytoplasmic fraction (Cyt) and the membrane fraction (M). Caveolin-3 and GAPDH were detected as the loading control of V_0-a2 and V_1-B2, respectively. Representative blots of three independent experiments are shown.

(B-C) Chloroquine (CQ) accumulation in lipid-overexposed HL-1 cells: **(B)** HL-1 cells were incubated for 24h with either Ctrl medium, Ctrl medium containing 100nM Bafilomycin-A (BafA), HP medium, or HP/HG medium (n=3). **(C)** HL-1 cells were incubated for 32h with either Ctrl medium containing 120µl Adenovirus Green Fluorescent Protein (AdGFP), Ctrl medium containing 120ul Adenovirus protein kinase D1 (AdPKD), HP medium containing 120µl AdGFP (AdGFP/HP), or HP medium containing 120µl AdPKD (AdPKD/HP). After the culturing of all conditions above, cells were ready for [^{3}H] CQ accumulation assay last 20 min. Values are displayed as mean ± SEM (n=3). $^*p<0.05$ were considered statistically significant.



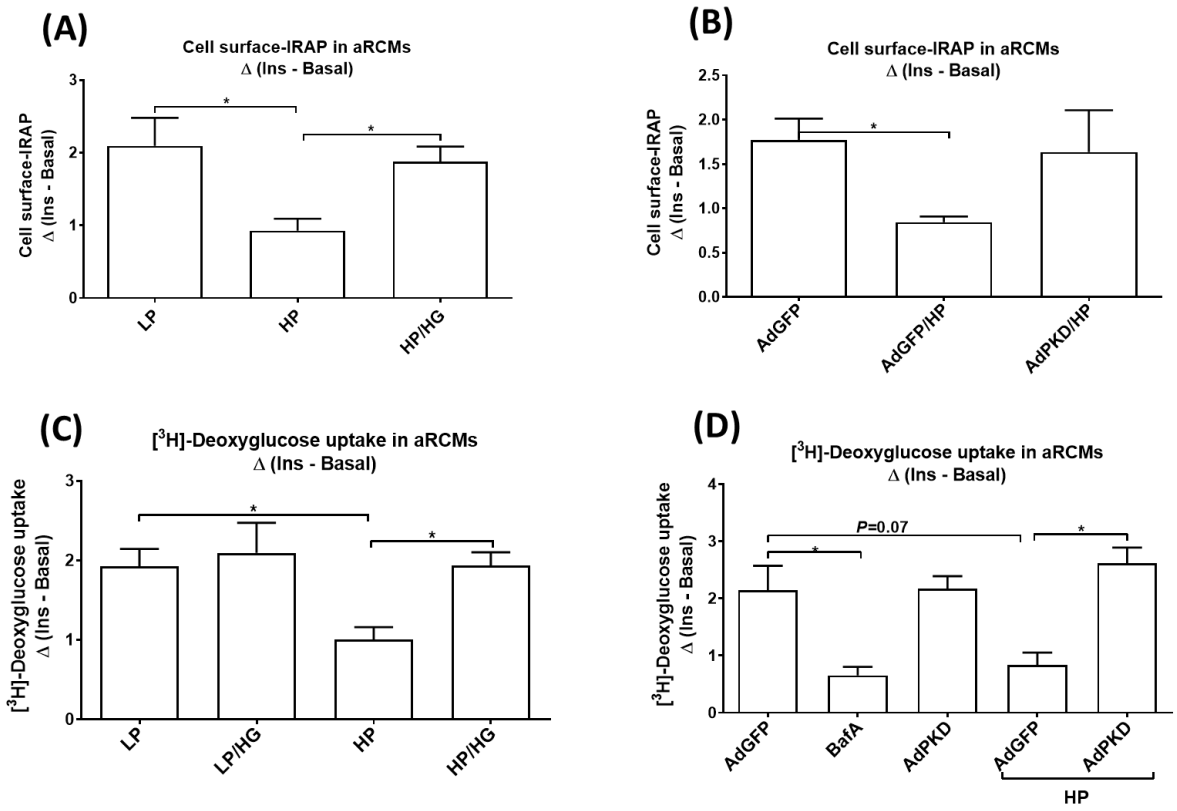
Supplemental Figure. 2 (Related to Figure 2A). The effect of insulin is expressed as difference between acute insulin-stimulated and basal for each condition (Δ Ins-Basal)**.** Values are displayed as mean ± SEM (n=3). $^{*}p<0.05$ were considered statistically significant.



Supplemental Figure. 3 (Related to Figure 3). Insulin sensitivity in lipid overloaded HL-1 cells.

(A-C) HL-1 cells were treated with control (Ctrl) medium, Ctrl medium containing 25mM glucose (Ctrl/HG), HP medium media containing 500µM palmitate and 100nM insulin, and HP medium with 25mM glucose addition (HP/HG) for 24h. Subsequently, cells were stimulated either with or without 200 nM insulin for 30 min and harvested for western blotting analysis of phosphorylation of Akt (pAkt) and of ribosomal protein S6 (pS6). **(A)** Representative blots. **(B-C)** Quantification of the level of pAKT and pS6 (n=3).

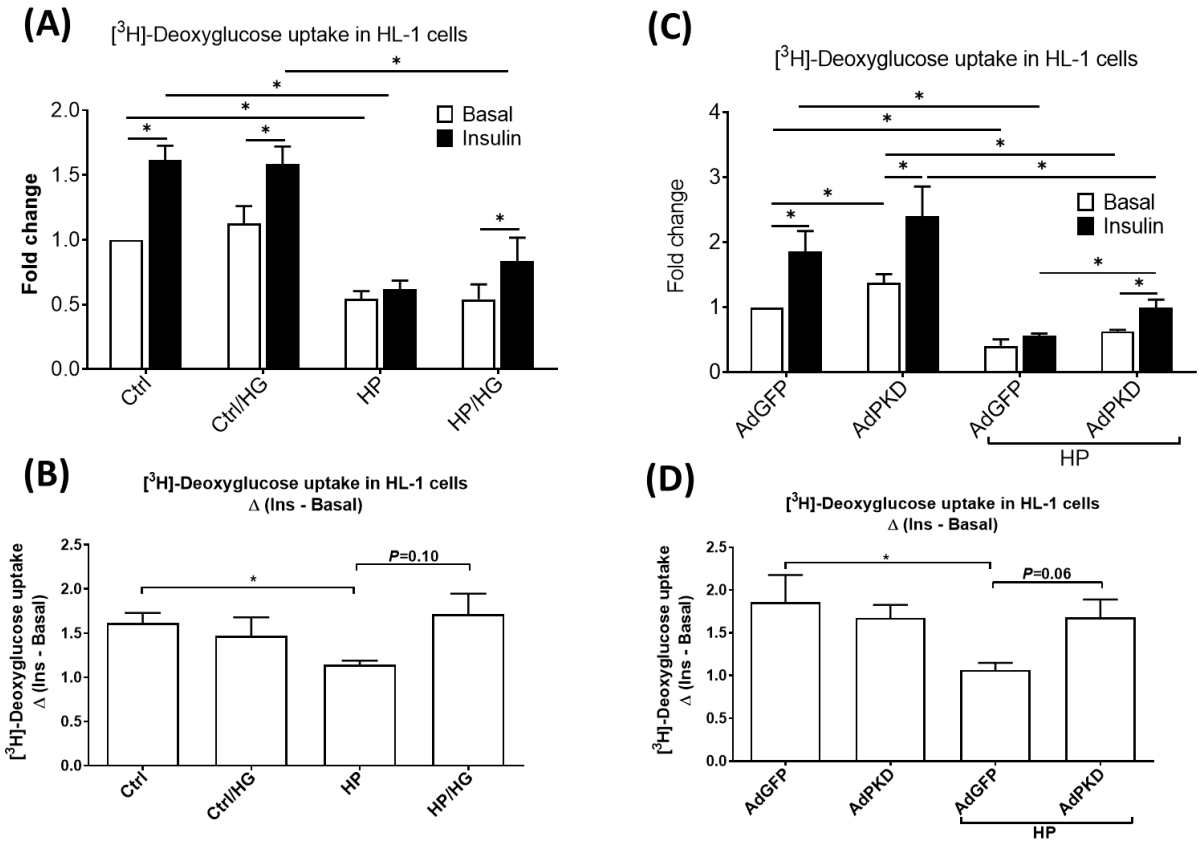
(D-F) HL-1 cells were treated with Ctrl medium containing 120µl Adenovirus Green Fluorescent Protein (AdGFP), HP medium containing 120µl AdGFP (AdGFP/HP), Ctrl medium containing 120ul Adenovirus protein kinase D1 (AdPKD), and HP medium containing 120µl AdPKD (AdPKD/HP) for 24h. Subsequently, cells were stimulated either with or without 200 nM insulin for 30 min and harvested for western blotting analysis of pAkt and pS6. **(D)** Representative blots. **(E-F)** Quantification of the level of pAKT and pS6. Values are displayed as mean ± SEM (n=3). $^{*}p<0.05$ were considered statistically significant.



Supplemental Figure. 4 (Related to Figure 4). The effect of insulin is expressed as difference between acute insulin-stimulated and basal for each condition (Δ Ins-Basal).

(A-B) These data are from the same set of experiments as the data from **Figure 4B and D** (n=4).

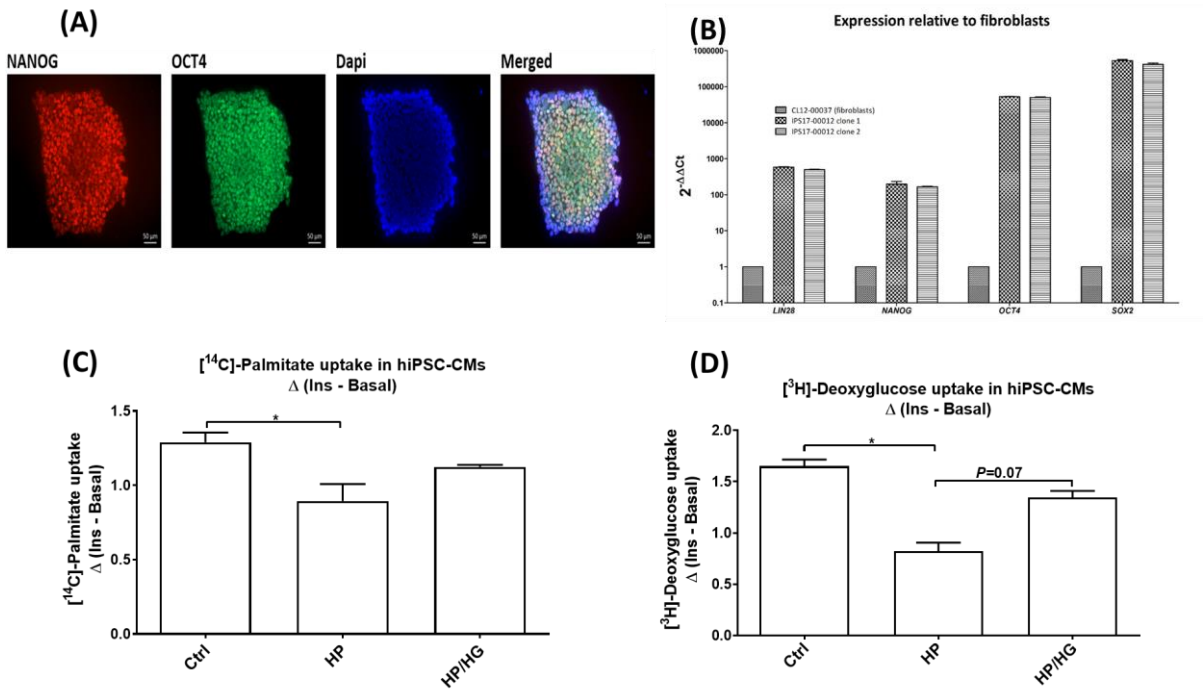
(C-D) These data are from the same set of experiments as the data from **Figure 4E-F**. Values are displayed as mean ± SEM (n=4). $^*p<0.05$ were considered statistically significant.



Supplemental Figure. 5 (Related to Figure 4). Insulin-stimulated glucose uptake in lipid-overexposed HL-1 cells.

(A) HL-1 cells were incubated for 24h with control (Ctrl) medium, Ctrl medium supplemented with either 25mM glucose (Ctrl/HG), HP medium media containing 500µM palmitate and 100nM insulin, or HP medium with 25mM glucose addition (HP/HG), followed by 30 min of (-/+) insulin (200 nM) incubation prior to [^{3}H]deoxyglucose labeling. **(B)** The effect of insulin is expressed as difference between acute insulin-stimulated and basal for each condition (Δ Ins-Basal). These data are from the same set of experiments as the data from **Supplemental Fig. 5A** (n=5).

(C) HL-1 cells were incubated for 48h with either Ctrl medium containing 120µl Adenovirus Green Fluorescent Protein (AdGFP), Ctrl medium containing 120ul Adenovirus protein kinase D1 (AdPKD), HP medium containing 120µl AdGFP (AdGFP/HP), or HP medium containing 120µl AdPKD (AdPKD/HP), followed by 30 min of (-/+) insulin (200 nM) incubation prior to [^{3}H] deoxyglucose labeling. **(D)** The effect of insulin is expressed as difference between acute insulin-stimulated and basal for each condition (Δ Ins-Basal). These data are from the same set of experiments as the data from **Supplemental Fig. 5C**. Values are displayed as mean ± SEM (n=4). $^*p<0.05$ were considered statistically significant.



Supplemental Figure. 6: Related to Figure 6.

(A) Expression of pluripotency markers (e.g., Nanog and Oct4) and Dapi in human induced pluripotent stem cells (hiPSCs) by using immunofluorescent staining. Scale bar is 50µm. **(B)** Gene expression of pluripotency markers in hiPSCs relative to fibroblasts.

(C-D) The effect of insulin is expressed as difference between acute insulin-stimulated and basal for each condition (Δ Ins-Basal). These data are from the same set of experiments as the data from **Figure 6B-C**. Values are displayed as mean ± SEM (n=3). $^*p<0.05$ were considered statistically significant.

Supplemental Figure. 7: Karyotyping results of hiPSCs from the Department of Clinical Genetics, Maastricht UMC+, demonstrating that there were no chromosomal abnormalities observed in these cells.

Chapter 3

Lipid overload-induced contractile dysfunction rescued by amino acid supplementation: role of vacuolar H^+-ATPase–mTOR axis

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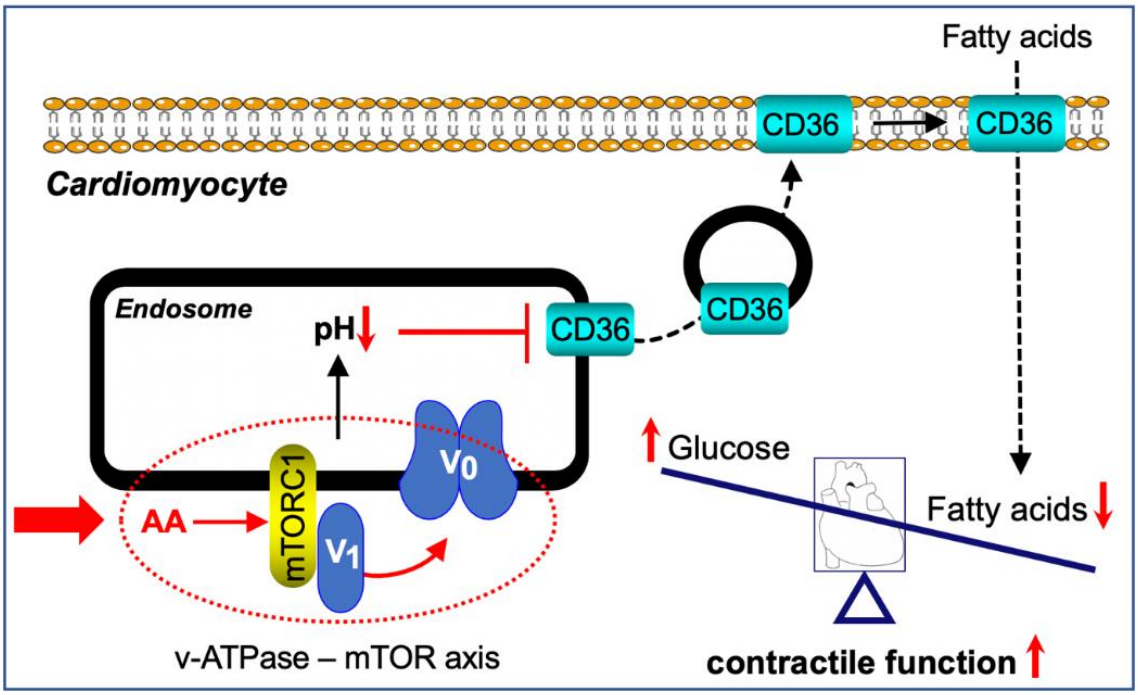
Synopsis



Abstract

In the lipid-overloaded diabetic heart, the shift in substrate utilization from glucose to lipids is facilitated by increased translocation of lipid transporter CD36 (SR-B2) to the sarcolemma. In the healthy heart, CD36 is retained in endosomes, and this endosomal CD36 retention is dependent on proper functioning of the endosomal proton pump, named vacuolar H^+-ATPase (v-ATPase). During lipid oversupply, v-ATPase disassembles and becomes dysfunctional, leading to increased CD36 translocation. V-ATPase is also required for binding of the anabolic master-regulator mTORC1 to the endosomes and its subsequent activation in response to amino acids (AAs). Here, we examined whether the relationship between v-ATPase and mTORC1 also operates reciprocally, specifically whether AAs induce v-ATPase re-assembly in an mTORC1-dependent manner and thus may reverse lipid-induced contractile dysfunction. Upon establishment of the most potent AA cocktail (Arginine/Leucine/Lysine combination) to activate/assemble v-ATPase, this cocktail was added to (rodent/human) lipid-overloaded cardiomyocytes, where it re-internalized CD36 to the endosomes, reversed lipid accumulation, and restored insulin-stimulated glucose uptake and contractile function. These beneficial effects were dependent on mTORC1 activation and binding to v-ATPase. In

conclusion, specific AAs acting through v-ATPase re-assembly may offer simple and effective treatment options against lipid-induced cardiomyopathy. (191 words)

Key words: Vacuolar H+-ATPase; endosomal CD36; mTORC1; lipid-induced Insulin resistance, Contractile function; Diabetic heart

Introduction

Lipid-induced heart failure is among the most common causes of mortality in type 2 diabetic (T2D) patients [1, 2], while, conversely, the diabetic heart is characterized by massive lipid accumulation [3, 4]. Notably, myocardial lipid accumulation is predominantly due to increased uptake of fatty acids via the lipid transporter CD36 (SR-B2) [5]. In the healthy heart, CD36 is localized for a large part in intracellular membrane compartments, specifically the endosomes, which are characterized by luminal acidification. Upon long-term overexposure of the heart to lipids, CD36 is expelled from the endosomes to translocate to the sarcolemma. This initiates a vicious cycle of increased fatty acid uptake and lipid accumulation, ultimately culminating into cardiac contractile dysfunction [6]. Therefore, pharmacological inhibition of CD36 translocation is expected to be a valuable treatment strategy to counteract impaired lipid accumulation and lipid-induced contractile dysfunction in the diabetic heart [7].

Recently, we unveiled part of the mechanism of lipid-induced CD36 translocation. Excess palmitate taken up by cardiomyocytes via CD36 is intracellularly sensed by v-ATPase. V-ATPase is commonly referred to as the endosomal/lysosomal proton pump, and responsible for acidifying the lumen of these organelles. This protein complex consists of >14 subunits divided over two subcomplexes. The membrane-inserted V_0 subcomplex mediates the transmembrane proton movement, and the cytoplasmic V_1 subcomplex contains an ATP hydrolysis-driven rotor, allowing to pump the protons into the endosomes/lysosomes against a gradient. Sensing of lipids (e.g., palmitate) by v-ATPase involves disassembly of V_1 from V_0 and disappearance of V_1 into the cytoplasm. This results in decreased proton pump activity and loss of endosomal acidification. The de-acidified endosomes can no longer serve as storage compartment for CD36, so that CD36 is forced to translocate to the sarcolemma [6]. Hence, induction of v-ATPase re-assembly would be an attractive strategy to combat lipid-induced contractile dysfunction. V-ATPase re-assembly can be induced by exposing cells to high glucose

conditions, as first established in yeast [8], and later on confirmed in cardiomyocytes [9]. Indeed, as expected, in cardiomyocytes this glucose-induced v-ATPase assembly was found to prevent lipid overload-induced contractile dysfunction [9]. However, glucose addition can never be part of a strategy to treat type-2 diabetic heart failure.

In our quest for alternative strategies to re-assemble v-ATPase, we considered its dynamic participation in the formation of lysosomal protein super-complexes, which recently received considerable attention. Data implicate v-ATPase as essential component in the docking of the anabolic master regulator mTORC1 to the endosomal membranes and its subsequent activation [10]. The activation of mTORC1 is initiated by amino acids (AA) [11, 12] and is dependent on other adaptor protein complexes such as Ragulator, which bridges mTORC1 to v-ATPase.

There is much controversy in the field of diabetes whether AA supplementation is detrimental or beneficial as treatment strategy. On the one hand, the potential use of AA has been discredited, however, merely based on association studies: in cross-sectional human studies, strong positive correlations exist between plasma branched-chain AA levels and the severity of insulin resistance/type-2 diabetes On the other hand, AA supplementation has been reported to protect against insulin resistance in rats fed a high-fat diet [13-15].

Furthermore, mTORC1 action has a negative connotation in type-2 diabetes research, because its activation by insulin mediates a negative feedback loop in insulin signaling, likely contributing to peripheral insulin resistance. Specifically, activated mTORC1 reduces signaling through Insulin-Receptor-Substrate-1 via inhibitory Ser-phosphorylation [16].

In the present study, we speculated that the relationship between v-ATPase and mTORC1 can work in both ways, being that the AA-induced interaction between v-ATPase and mTORC1 does not only serve to induce the activation of mTORC1, but also to a mutual activation of v-ATPase. Hence, we hypothesized that, via addition of AA, endosomes will be re-acidified in an mTORC1-dependent manner, thereby allowing the re-internalization of CD36, which will limit excessive fatty acid uptake and resolve lipid-induced insulin resistance and contractile dysfunction.

To answer this research question, we first set out to identify the individual AA on their ability to activate v-ATPase. Subsequently, the three most effective AA were combined into a cocktail for studies in lipid-overexposed cardiomyocytes. Specifically, we added this AA cocktail to cardiomyocytes cultured in high palmitate-containing media, and tested v-ATPase

assembly/activity, CD36 localization, myocellular lipid accumulation, insulin stimulated phosphorylation of Akt and its downstream target S6, insulin stimulated GLUT4 translocation and glucose uptake, and ultimately contractile parameters. Further, we performed co-immunoprecipitation experiments of v-ATPase subunits from the two sub-complexes and of mTORC1. The data suggest that v-ATPase assembly is induced via AA supplementation, involving mTORC1 activation at the endosomal/lysosomal surface, to antagonize lipid overload-induced contractile dysfunction.

Results and discussion

AA treatment prevents v-ATPase disassembly and de-activation in lipid-overexposed cardiomyocytes

To select the most potent AA for v-ATPase activation, we tested each individual AA on its ability to increase cell-associated accumulation of the divalent weak base chloroquine (CHLQ) in a radio-activity assay. CHLQ becomes specifically trapped in acidic organelles, such as endosomes, and when added to cells in trace amounts, it provides quantitative information about luminal acidification [17]. For defining baseline v-ATPase activity, HL-1 cardiomyocytes were incubated in starvation medium containing no AAs. For assessing cell-associated CHLQ accumulation in the absence of v-ATPase activity, cells were incubated with Bafilomycin A (BafA), which inhibited CHLQ accumulation by 80% **(Fig 1A)**. When each of the AAs were added at similar (1x) concentrations as occurring in DMEM-F12 (reflecting their presence in the circulation of 24 h-starved rats [18], v-ATPase activity was partly inhibited **(Fig 1A)**, in agreement with earlier work [19].

When each AA was individually added at 4x its low-physiological concentration, four AAs appeared to stimulate v-ATPase activity significantly, of which arginine (Arg), leucine (Leu) and lysine (Lys) exerted the largest effects (**Fig 1A)**. It may not be coincidence that among these AA are two basic AA, which will accumulate into endosomes/lysosomes due to weak base trapping, and then can start signaling via the lysosome-centric inside-out-mechanism of AA sensing to support mTORC1 – v-ATPase interaction [20]. With respect to Leu, this AA has been generally regarded as one of the most potent mTORC1 activators, which effect is mediated via the adaptor protein Sestrin-2 with a high affinity for Leu [21]. We also confirmed the positive actions of Arg, Leu and Lys on v-ATPase activity in HEK293 cells **(Suppl Fig 1A)**.

For subsequent experiments, these three AA species were added in a combined manner at their 4x concentration (indicated as 4*AA).

As observed previously [6], v-ATPase activity decreased upon culturing of cardiomyocytes in high palmitate (HP) media **(aRCMs: Fig 1B; HL-1 cells: Suppl Fig 1B)**. When HP exposure was combined with the 4*AA cocktail, v-ATPase activity was largely preserved in HL-1 cells (**Suppl Fig 1B)** and completely preserved in isolated adult rat cardiomyocytes (aRCM) **(Fig. 1B)**. As expected, genetic (siRNA-mediated silencing of the B2 subunit) **(Fig 1C and Suppl Fig 1C)** or pharmacological (bafilomycin-A) (**Fig 1B)** inhibition of v-ATPase abolished this AA action. This effect was also sensitive to rapamycin addition **(Fig 1B)**, indicating that mTOR activation is necessary for AA-induced v-ATPase activation during lipid overload. Also in human induced pluripotent stem cells differentiated into cardiomyocytes (hiPSC-CMs) (**Suppl Fig 1D-E**), 4*AA treatment protected against lipid-induced loss of endosomal acidification in an mTORC1 dependent manner **(Fig 1D)**, indicating that this AA action is evolutionary conserved from rodents to humans.

Lipid-induced v-ATPase inhibition is due to v-ATPase disassembly into its two sub-complexes [6]. Using subcellular fractionation, we confirmed that in all the conditions the V_0-d1 subunit (as part of the membrane-bound V_0 sub-complex) was retained in the membrane fraction **(Fig 1E)**. Upon HP exposure, the V_1-B_2 subunit (as indicator of the soluble V_1 sub-complex) relocalized from the membrane fraction to the cytoplasmic fraction **(Fig 1E)**, indicating V_0/V_1 disassembly of v-ATPase. Notably, when cultures of cardiomyocytes exposed to HP media were treated with 4*AA, V_1-B2 was redistributed back to the membrane fraction in a rapamycin-sensitive manner **(Fig 1E).** In line with the data from the CHLQ uptake assay, treatment with 4*AA via mTORC1 activation preserves v-ATPase assembly, so that v-ATPase activity and endosomal acidification are maintained during lipid overload.

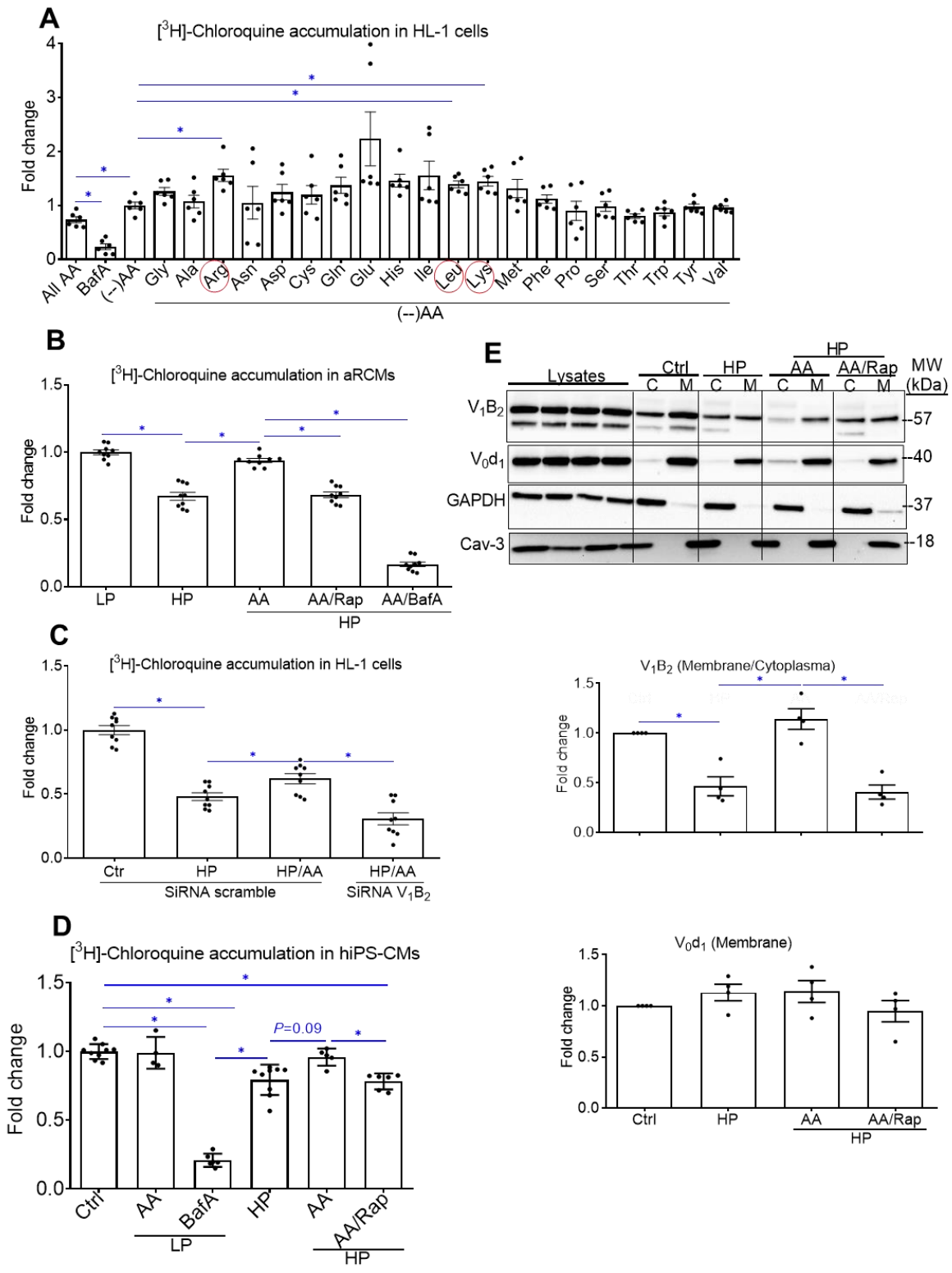


Figure 1. Amino acids (AA) treatment prevents v-ATPase disassembly and de-activation in lipid-overexposed cardiomyocytes.

(A) [3H] CHLQ accumulation in HL-1 cardiomyocytes: after AA starvation, re-addition of individual AA alters v-ATPase activity.

In panels of **B-E**, cells are incubated for 24 h with the same additions, such as Ctrl/LP, HP, HP/AA, and HP/AA supplemented with Rap or BafA.

(B-D) [^{3}H] CHLQ accumulation in lipid-overexposed cardiomyocytes: **(B)** 4*AA addition prevents the loss of v-ATPase activity in aRCMs; **(C)** HL-1 were transfected with negative control (Ctrl) scrambled siRNA or with siRNA targeting v-ATPase B2 subunit RNA (B2-kd). 32 h after transfection, cells were cultured under either Ctrl, HP, or HP/AA. **(D)** 4*AA addition prevents the loss of v-ATPase activity in hiPSC-CMs.

(E) Fractionation in lipid-overexposed HL-1 cells: representative blotting of v-ATPase subunit B2 (V_1-B2) and v-ATPase subunit d1 (V_0-d1) and of it's quantification. Content of V_1-B2 and V_0-d1 were assessed by Western blotting in the cytoplasmic fraction (C) and the membrane fraction (M). Caveolin-3 and GAPDH: loadings controls for membrane and cytoplasmic fraction, respectively.

Data information: bar values are means ± SEM of n > 3 independent experiments. *p<0.05 were considered statistically significant.

AA treatment prevents CD36-mediated lipid accumulation in lipid-overexposed cardiomyocytes

In lipid-overloaded cardiomyocytes, v-ATPase inhibition leads to increased CD36 translocation to the sarcolemma, and this contributes to an increase of fatty acid (FA) uptake and myocellular lipids [6]. Using a surface biotinylation assay, we confirmed the HP-induced CD36 translocation. Specifically, CD36 cell surface content was increased by 1.5-fold in absence of insulin **(Fig 2A)**. Moreover, insulin did not further stimulate CD36 translocation in the HP condition **(Fig 2A)**, indicating that lipid-overload induces CD36 translocation from insulin-responsive endosomal stores. 4*AA mixture co-incubation prevented the increased HP-induced basal CD36 translocation but preserved insulin-stimulated CD36 translocation **(Fig 2A)**. When fatty acid uptake was studied, similar trends were observed: an HP-induced increase with loss of insulin stimulation, which was reversed by 4*AA mixture **(HL-1 cells: Fig 2B; aRCMs: Fig 2C; hiPSC-CMs: Fig 2D)**. The AA-induced decrease in CD36 translocation and fatty acid uptake in lipid-overexposed cardiomyocytes is expected to impact on myocellular lipid accumulation. Indeed, myocellular triacylglycerol content was increased in cardiomyocytes upon HP culturing **(HL-1 cells 1.7-fold, Fig 2E; aRCMs 4-fold, Fig 2F)**. In addition, 4*AA mixture prevented this increase; totally in HL1 cells and partly (–25%) in aRCM, while in the presence of rapamycin this effect was lost **(Fig 2E-F)**. Altogether, in

cardiomyocytes exposed to excess lipid, AA treatment prevents CD36-mediated lipid accumulation in an mTORC1-dependent manner.

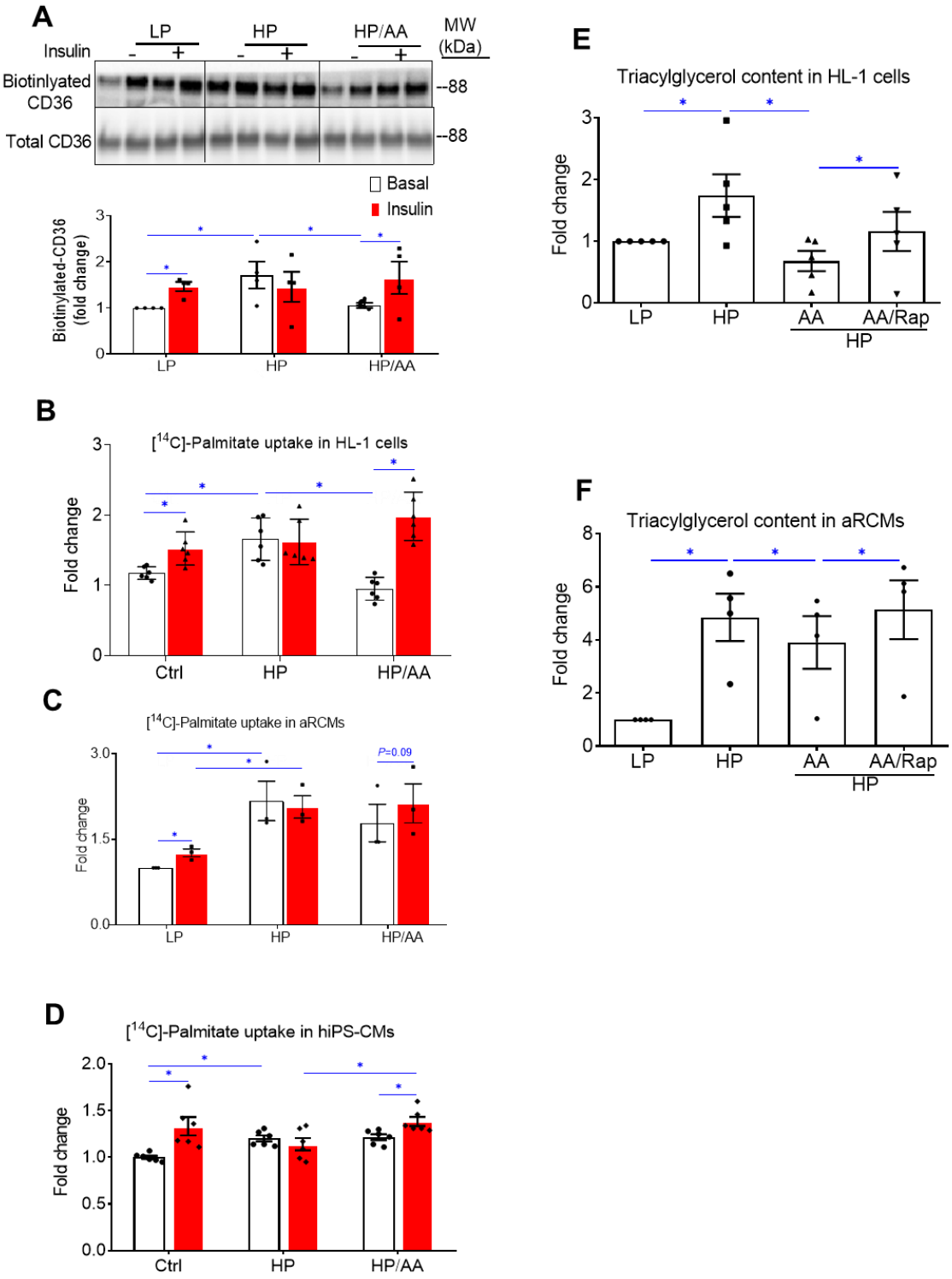


Figure 2. AA treatment prevents CD36-mediated lipid accumulation in lipid-overexposed cardiomyocytes.

In all panels, cells are incubated for 24 h with the same additions, such as Ctrl/LP, HP, HP/AA, and HP/AA supplemented with Rap.

(A) Assessment of cell-surface CD36 in aRCMs (representative blotting and its quantification of CD36 from biotin-labeled and total lysate fractions).

(B-D) [^{14}C] palmitate uptake in lipid-overexposed cardiomyocytes.

(E-F) Triacylglycerol contents in lipid-overexposed cardiomyocytes.

Data information: bar values are means ± SEM of n > 3 independent experiments. *p<0.05 were considered statistically significant.

AA treatment partially prevents insulin resistance in lipid-overexposed cardiomyocytes, but completely preserves insulin-stimulated glucose uptake

It is known that increased CD36-mediated fatty acid uptake and lipid accumulation precede the loss of insulin-stimulated glucose uptake and the associated development of insulin resistance [22]. Does the AA-induced preservation of lipid dynamics extend to preservation of insulin sensitivity in lipid-overloaded cardiomyocytes? For evaluation of insulin signaling, phosphorylation levels of Akt (pAkt Ser473) and of ribosomal protein S6 (pS6 Ser235/236) were assessed. As expected [6], HP exposure caused a loss of insulin-stimulated phosphorylation of Akt and of S6 in HL-1 cells **(Fig 3A and Suppl Fig 2A-B)**, aRCMs **(Fig 3B and Suppl Fig 2C-D)**, and hiPSC-CMs **(Fig 3C and Suppl Fig 2E-F).** 4*AA treatment prevented this loss of insulin-stimulated Akt phosphorylation and S6 phosphorylation in lipid-overloaded HL-1 cells **(Fig 3A and Suppl Fig 2A-B)** and hiPSC-CMs **(Fig 3C and Suppl Fig 2E-F)**, at least partially, but were ineffective in preserving insulin signaling in aRCM **(Fig 3B and Suppl Fig 2C-D)**. Also the effects of rapamycin on top of the 4*AA addition were not unequivocal. In HL-1 cells, rapamycin blocked the AA-induced re-phosphorylation of S6 upon HP exposure, but not of Akt **(Fig 3A-B and Suppl Fig 2A-D)**. In conclusion, AA treatment does not entirely alleviate the inhibitory effects of HP-treatment on insulin signaling in cardiomyocytes.

Next, a surface detection assay of insulin-responsive aminopeptidase (IRAP) was applied to study GLUT4 translocation. Insulin-stimulated GLUT4 translocation was largely decreased upon HP exposure, but was almost entirely maintained when HP exposure was combined with 4*AA addition **(Fig 3E).** This AA effect was blocked upon addition of rapamycin or

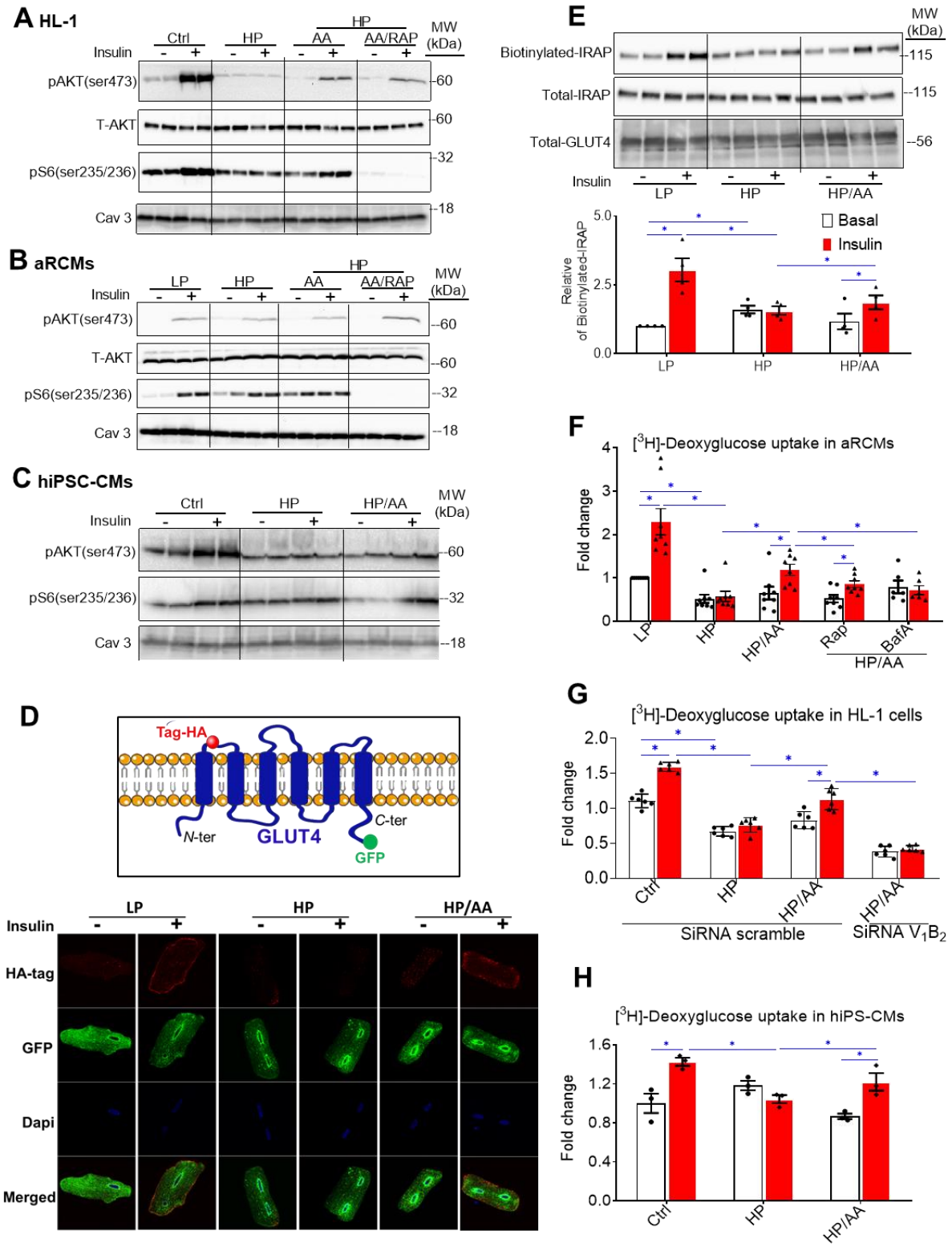
bafilomycin-A, indicating the involvement of mTORC1 and v-ATPase, respectively **(Fig 3E)**. A microscopic inspection of cell surface levels of HA-tagged GLUT4 in adenovirally transfected aRCM yielded the same results: AA protect against HP-induced loss of insulin-stimulated GLUT4 translocation **(Fig 3D)**.

GLUT4 translocation is a major regulatory event in cardiac glucose uptake [23]. We applied a radiometric assay to measure glucose uptake. As expected [6, 9] , HP exposure in HL-1 cells **(Fig 3F)**, aRCM **(Fig 3G)** and hiPSC-CM **(Fig 3H)** led to a loss of insulin-stimulated glucose uptake, which was again rescued by the 4*AA mixture **(Fig 3F-H)**. The mutual confirmation of the effects of HP exposure and of 4*AA treatment in the GLUT4 translocation and glucose uptake assays provide strong support to the notion that AA preserve insulin-induced glucose uptake in lipid-overloaded cardiomyocytes. Importantly, the involvement of v-ATPase and mTORC1 in the lipo-protective action of 4*AA on insulin-stimulated GLUT4 translocation and on insulin-stimulated glucose uptake likely extends from rodents to man, because siRNA silencing of the v-ATPase B_2 subunit, or treatment with BafA or rapamycin abrogated this beneficial AA action on insulin-stimulated glucose uptake in both HL-1 cells, aRCM and hiPSC-CM **(Fig 3F-H)**.

When combining the findings on insulin signaling and insulin-stimulated GLUT4 translocation/glucose uptake, there is an apparent discrepancy between the partial preservation of insulin signaling and the complete preservation of insulin-stimulated glucose uptake upon 4*AA treatment in lipid-overexposed cardiomyocytes, so that we may conclude that preservation of insulin-stimulated glucose uptake occurs, at least to a certain extent, independent of insulin signaling. As part of an explanation, we suggest that besides the decrease in insulin signaling, chronic lipid exposure also negatively affects other processes contributing to insulin-stimulated GLUT4 translocation. One such process might be the participation of SNARE proteins such as SNAP23 in fusion of the GLUT4 vesicle with the sarcolemma. Since SNAP23 is also involved in trafficking of lipid droplets, it has been suggested that increased lipid droplet formation during lipid overexposure highjacks SNAP23 from the GLUT4 translocation machinery [24]. Thus, the prevention of lipid accumulation by 4*AA treatment may serve to keep the SNARE proteins available for GLUT4 translocation.

We also tested whether these beneficial effects of AA at the level of prevention against lipid-induced loss of insulin-stimulated glucose uptake could be extended to treatment of cardiomyocytes in which insulin-stimulated glucose uptake already had been impaired by 10 h HP exposure. The 4*AA mixture did not restore insulin-stimulated glucose uptake until 5 h of

treatment, but from 10 h treatment onwards the beneficial effects became apparent **(Fig 3I)**. Hence, 4*AA treatment does not only prevent lipid-induced changes in glucose uptake but also reverses these changes **(Fig 3I)**, eluding to the perspective that the Arg/Leu/Lys mixture could be tested as treatment against lipid-induced cardiomyopathy in future studies.


A HL-1
Ctrl
HP
HP
AA
AA/RAP
MW (kDa)
Insulin
pAKT(ser473)
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Cav 3
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60
32
18
B aRCMs
LP
HP
HP
AA
AA/RAP
MW (kDa)
Insulin
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N-ter
GLUT4
C-ter
GFP
Insulin
LP
HP
HP/AA
HA-tag
GFP
Dapi
Merged
E
MW (kDa)
Biotinylated-IRAP
Total-IRAP
Total-GLUT4
115
115
56
Insulin
LP
HP
HP/AA
Relative of Biotinylated-IRAP
5.0
2.5
0.0
Basal
Insulin
LP
HP
HP/AA
F
[3H]-Deoxyglucose uptake in aRCMs
Fold change
4
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Rap
BafA
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[3H]-Deoxyglucose uptake in HL-1 cells
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2.0
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SiRNA scramble
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[3H]-Deoxyglucose uptake in hiPS-CMs
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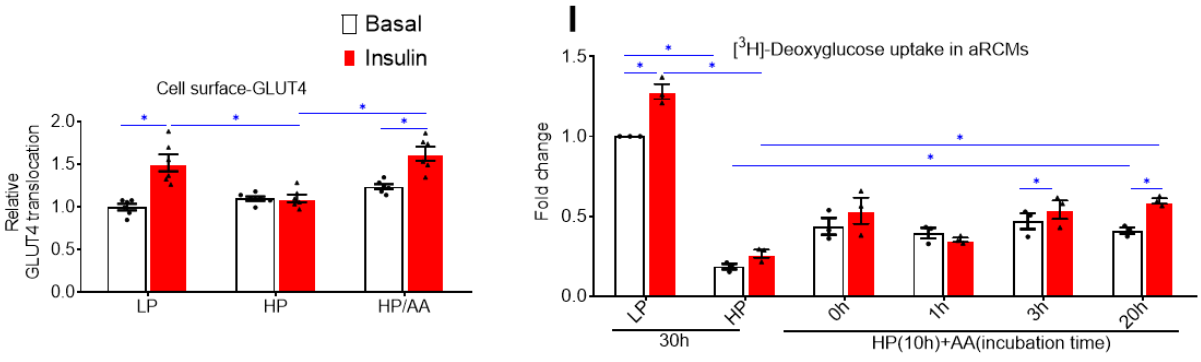

Figure 3. AA treatment partially prevents insulin resistance in lipid-overexposed cardiomyocytes, but completely preserves insulin-stimulated glucose uptake.

In all panels, cells are incubated for 24 h with the same additions, such as Ctrl/LP, HP, HP/AA, and HP/AA supplemented with Rap or BafA.

(A-C) Representative blots of pAKT (ser 473), T-AKT, pS6 (ser 235/236), and Cav-3 (loading control) in lipid-overexposed cardiomyocytes.

(D-E) Assessment of cell-surface GLUT4 in lipid-overexposed aRCMs**: (D)** Insulin-induced GLUT4 insertion/translocation into the plasma membrane; **(E)** Representative blotting and its quantification of insulin-regulated aminopeptidase (IRAP, which reflects GLUT4 trafficking) from biotin-labeled and total lysate fractions.

(F-H) [^{3}H] deoxyglucose uptake in lipid-overexposed cardiomyocytes.

(I) A time-course study of [^{3}H] deoxyglucose uptake in aRCMs: aRCMs were cultured with LP, or HP medium for 10h, and then HP medium supplemented with 4*AA for 0, 1, 3, 10, and 20 h.

Data information: bar values are means ± SEM of n > 3 independent experiments. *p<0.05 were considered statistically significant.

AA treatment prevents contractile dysfunction in lipid-overexposed cardiomyocytes

Lipid overexposure of the heart, as seen in high fat diet-fed rats, ultimately leads to contractile dysfunction via increased CD36-mediated myocellular lipid accumulation [25]. This mechanism of CD36-dependent contractile dysfunction was confirmed *in vitro* in HP exposed aRCM [26]. Also in the present study, lipid overexposure induced contractile dysfunction in aRCM. Specifically, HP exposure reduced sarcomere shortening by 50% **(Fig 4A)**, did not affect acceleration time **(Fig 4B)** and increased 90% relaxation time (RT90) by 15% **(Fig 4C-D)**. 4*AA treatment entirely prevented the negative effects of HP exposure on sarcomere

shortening and RT90, while upon rapamycin addition, the AA-induced protection was lost **(Fig 4A and D)**. Hence, AA preserve contractile function in lipid-overloaded cardiomyocytes in an mTORC1 dependent manner.

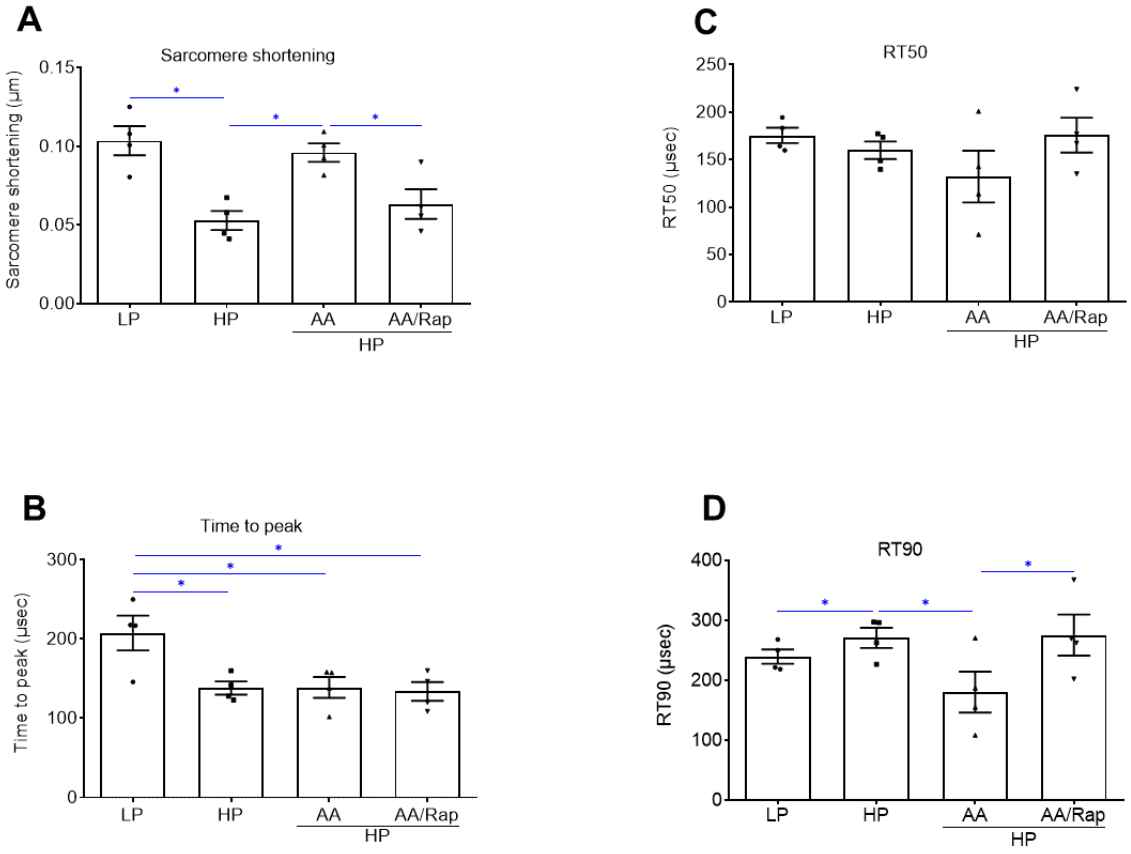

Figure 4. AA treatment prevents contractile dysfunction in lipid-overexposed cardiomyocytes.

Contractile function in lipid overloaded aRCMs incubated for 24 h in either LP, HP, HP/AA, or HP/AA/Rap. **(A)** sarcomere shortening; **(B)** time to peak; **(C)** decay time to 50% percent (RT50); **(D)** decay time to 90% percent (RT90). Bar values are displayed as mean ± SEM (n=5; imaging of 10 cells/condition). *p<0.05 were considered statistically significant.

Mechanism of AAs sensing involved in v-ATPase re-assembly in lipid-overloaded cardiomyocytes

What is the molecular mechanism by which 4*AA protect against lipid-induced contractile dysfunction? To answer this question we need to consider the first step in the maladaptive sequence of events initiated by lipid oversupply, being the lipid-induced v-ATPase disassembly, which is preserved by 4*AA treatment in mTORC1-dependent manner. We first examined the effect of 4*AA treatment on subcellular location of mTORC1, and whether this

treatment was able to activate mTOR. These issues were investigated by studying membrane-binding of mTORC1 and by assessing its Ser2448 phosphorylation state. Already under LP conditions, mTORC1 was found to be entirely associated with (endosomal) membranes with no alterations under HP condition and/or 4*AA treatment **(Fig 5A)**. With respect to the mTOR–Ser2448 site, HP exposure did not alter phosphorylation at this site, but when combined with 4*AA treatment there was an increase in this phosphorylation event (2.3-fold; **Fig 5A**; compare HP/- with HP/AA/-). Additionally, 4*AA treatment restored insulin-stimulated mTOR–Ser2448 phosphorylation in HP-treated HL-1 cells **(Fig 5A; compare +/- insulin)**, which is in line with the (partial) restoration by this treatment of insulin signaling upon lipid overload, as already discussed above **(Fig 3A-C)**.

Next, we investigated whether this AA-induced mTOR activation in lipid-overloaded cells was associated with mTOR binding to v-ATPase during v-ATPase re-assembly. Besides by fractionation **(Fig 1E and Fig 5B)**, the v-ATPase assembly state can be assessed by co-immunoprecipitation (co-IP) of subunits when applying antibodies that recognize specific subunits of V_0 and V_1 sub-complexes. For this, we checked the presence of the V_0 subunits a2 and d1 in an IP against the V_1 subunit B2 **(Fig 5C)**, as well as the presence of B2 in an IP against d1 **(Fig 5D)**. These IPs confirm the findings of the fractionation: HP exposure induces v-ATPase disassembly, which is prevented by 4*AA treatment. These IPs were also assessed on the presence of mTOR. In the LP condition when v-ATPase is assembled, mTOR appears to bind only to the V_1 sub-complex, and not to V_0 **(Fig 5C-D)**. These findings were confirmed in the reverse IP against mTOR, where in the LP condition the B2 subunit, but not a2 and d1, were found to be present **(Fig 5E)**. HP exposure decreased binding of mTOR to V_1, which was prevented by 4*AA treatment **(Fig 5C-E)**. Moreover, these events coincide with HP-induced v-ATPase disassembly and 4*AA-induced re-assembly **(Fig 5C-E)**.

Taken together, in lipid-overexposed cells AA treatment results in activation of mTOR, which attracts the V_1 subunit to the endosomal membrane, allowing V_1 to re-assemble with V_0 **(Fig 5F)**. Next, this re-assembly of an mTOR – v-ATPase super-complex will be the start of the beneficial series of events including preventio/restoration of CD36-mediated myocellular lipid accumulation **(Fig 2)** as well as preservation/restoration of insulin-stimulated glucose uptake **(Fig 3)** and of contractile function **(Fig 4)**.

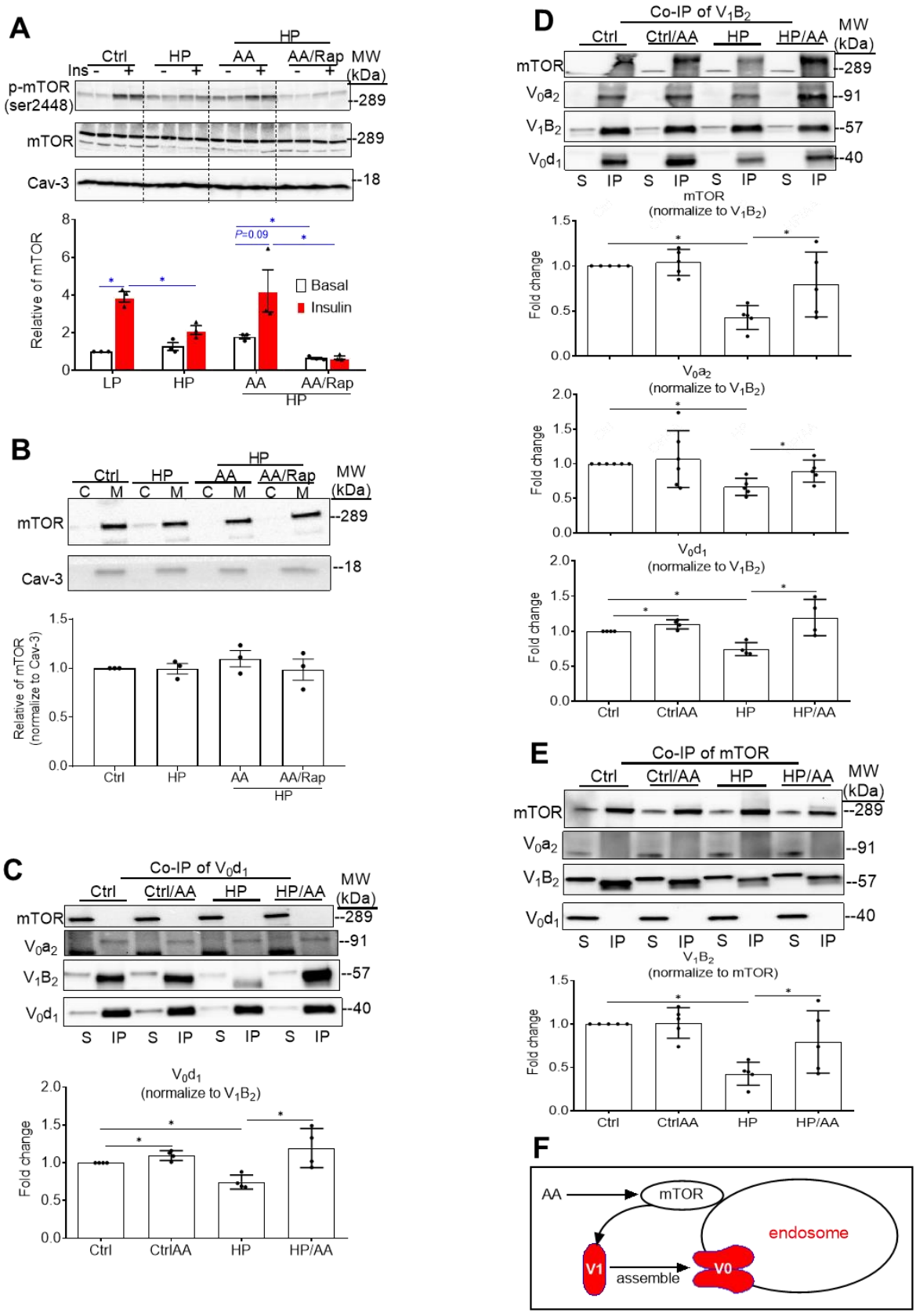

Figure 5. Mechanism of AAs sensing involved in v-ATPase re-assembly in lipid-overloaded cardiomyocytes.

In all panels, cells are incubated for 24 h with the same additions, such as Ctrl/LP, HP, HP/AA, and HP/AA supplemented with Rap.

(A) AA stimulates mTORC1 activation in lipid-overexposed aRCMs: representative blots of p-mTOR, T-mTOR, and Cav-3 and its quantification.

(B) Fractionation in HL-1 cells: representative blotting of mTOR and it's quantification. Contents of mTOR and were assessed by Western blotting in the cytoplasmic fraction (C) and the membrane fraction (M). Caveolin-3 was detected as the loading control.

(C-E) Co-Immunoprecipitation (Co-IP) of v-ATPase subunit d1 (V0-d1) (C), subunit B2 (V1-B2) (D), or mTOR (E) in HL-1 cells. Co-IP samples were immunoblotted with antibodies against v-ATPase subunits mTOR, V0-a2, V0-d1, and V1-B2. Representative Western blotting and its quantification. **(F)** Scheme illustrating that AAs sensing in the v-ATPase –mTOR axis in lipid-overexposed cardiomyocytes.

Data information: bar values are means ± SEM of n > 3 independent experiments. *p<0.05 were considered statistically significant.

The greater perspective

For a long time, v-ATPase was merely regarded as a proton pump mediating the luminal acidification of subcellular organelles, but in recent years it became evident that this protein complex is also an essential component in mTOR activation by AA [27]. However, in this AA sensing pathway, the established mechanism of v-ATPase regulation, being v-ATPase cycling between assembled and/disassembled states, has been disregarded. Moreover, the possibility of mutual activation of mTORC1 and v-ATPase has not yet been considered. In the present study we indeed found powerful evidence for this latter notion, i.e. upon treatment with 4*AA. This makes AA not only important in mTOR-dependent cell growth, but also in the regulation of cardiac substrate preference, especially in the situation of lipid overexposure. Since the applied 4*AA cocktail might have similar effects on mTORC1 – v-ATPase activation in skeletal muscle compared to the heart, the depreciative perception of AA supplementation in type-2 diabetes may be unjustified. This also might apply for the bad reputation of mTORC1, as initiator of the negative feedback loop in insulin signaling [28]. Yet, when activated by AA, and not by insulin, mTORC1 remains attached to (endosomal) membranes, which could restrict its access to cytoplasmically localized IRS1, so that the inhibitory phosphorylation of proximal insulin signaling might not occur.

Finally, the present findings as observed in rodents also extend to the human setting, given that v-ATPase assembly is a target to normalize contractile function in hiPSC-CMs upon over-exposure of these cells to lipids. For future perspectives, further studies on the AA-induced m-TORC1–v-ATPase action, which involves a great number of other proteins, including Rag-GTPases, adaptor proteins and lysosomal AA transporters (for review see [27, 29], may reveal that several of these other proteins may offer additional, even better druggable, targets to combat lipid-induced cardiomyopathy.

Materials and methods

Antibodies

Detailed antibodies that used Western blotting (WB) and immunofluorescence (IF) analysis are provided in Table S1.

Isolation and culturing of primary rat cardiomyocytes

Male Lewis rats, 250-300 grams, were purchased from Charles River laboratories, and were maintained at the Experimental Animal Facility of Maastricht University. Animals were housed in a controlled environment (21-22°C) on a 12:12h light dark cycle (light from 0700 to 1900h) and had free-access to food and tap water. All experiments were performed according to Dutch regulations and approved by the Maastricht University Committee for Animal Welfare.

Adult rat cardiomyocytes (aRCMs) were isolated by using a Langendorff perfusion system, as previously described [30]. Briefly, after a 2h adhesion period, aRCMs were incubated for 24 h in either low palmitate (LP, palmitate/BSA ratio 0.3:1), high palmitate (HP, palmitate/BSA ratio 3:1), HP supplemented with 4*AA (Arg-1.36mM, Leu-1.84mM and Lys-1.56mM, AA concentration is 4 times higher in M199 medium) [HP/AA], HP/AA supplemented with 100nM Rapamycin (Rap) [HP/AA/Rap], or HP/AA supplemented with 100nM Bafilomycin-A (BafA) [HP/AA/BafA]. aRCMs were cultured as previously described [9].

Culturing of HL-1 cardiomyocytes

HL-1 cells were kindly provided by Dr. W. Claycomb (Louisiana State University, New Orleans, LA, USA) and cultured as previously described [6]. To investigate whether AA can alter v-ATPase activity, HL-1 cells were first subjected to complete AA starvation for 1h

followed by AA-starvation medium [(--)AA], basal medium [all AA], all AA condition supplemented with 100nM Bafilomycin-A (BafA), or 4*AA re-addition (AA concentration is 4 times higher in M199 medium) for 1h. Furthermore, HL-1 cells were also cultured for 24h in either control (Ctrl, palmitate/BSA ratios of 0.3:1) medium, Ctrl supplemented with 4*Arg (1.36 mM) (Ctrl/Arg), Ctrl supplemented with 4*Leu (1.84 mM) (Ctrl/Leu), Ctrl supplemented with 4*Lys (1.56 mM) (Ctrl/Lys), Ctrl supplemented with 4*AA (Arg-1.36 mM, Leu-1.84 mM and Lys-1.56 mM, AA concentration is 4 times higher in M199 medium) [HP/AA], high palmitate medium (HP, palmitate/BSA ratios of 3:1), HP supplemented with 4*Arg (HP/Arg), HP supplemented with 4*Leu (HP/leu), HP supplemented with 4*Lys (HP/Lys), HP supplemented with 4*AA (HP/AA), or HP/AA supplemented with BafA.

Culturing of HEK-293T cells

HEK-293T cells were kindly provided by Department of Clinical Genetics (Maastricht University Medical Center+, Maastricht, The Netherlands) and cultured as previously described [19]. To further confirm whether readdition of these AAs (e.g., Arg, leu, Lys, Glu, and Ser) [19] affect on v-ATPase activity, HEK-293T cells were first subjected to complete AA starvation for 1h followed by AA-starvation medium [(--)AA], individual AA readdition (AA concentration is same as DMEM-F12) for 1h, or basal medium [all AA] for 1 h.

Human induced pluripotent stem cell (hiPSC) maintenance and differentiation into cardiomyocytes (hiPSC-CMs)

Skin fibroblasts from healthy adult male individuals were collected and hiPSCs were generated by episomal reprogramming at the Stem Cell Technology Centre, Radboudumc (Nijmegen, Netherlands). Protocols of cell culture and differentiation into cardiomyocytes were previously described [9]. All treatments on hiPSC-CMs were performed as indicated for HL-1 cells.

Measurement of v-ATPase disassembly/assembly

Two methods were applied to measure disassembly, namely, immunoprecipitation (IP) (i) and subcellular fractionation (ii).

(i) IP: the method of IP was conducted as previously described [9]. v-ATPase a2 (V_0-a2), v-ATPase d1 (V_0-d1, an indicator of the membrane-bound V_0 sub-complex), v-ATPase B2 (V_1-B2, an indicator of cytosolic V_1 sub-complex), and mTOR proteins were detected by Western blotting.

(ii) Subcellular fractionation: the method of subcellular fractionation was also conducted as previously described [6]. For subcellular fractionation, V_0-a2, V_0-d1, and V_1-B2, mTOR, GAPDH, and caveolin-3 (Cav-3) proteins were detected by Western blotting.

Measurement of cellular chloroquine (CHLQ) accumulation as readout of v-ATPase function

[^{3}H] CHLQ accumulation assay in HL-1 cells, aRCMs, and hiPSC-CMs was measured as previously described [9].

Surface-protein biotinylation

Surface-protein biotinylation was measured as previously described with the modifications described below [9]. After culturing with the medium, aRCMs were incubated for 30min (-/+) 100 nM insulin. Subsequently the cells were biotinylated with the cell-impermeable reagent sulfo-NHS-LC-biotin (0.5 mg/ml dissolved in M199 medium, Thermo Fisher Scientific, Fremont, CA, USA) for 10 min at 37°C. Other steps of surface-protein biotinylation assay were carried out as previously described [9]. Insulin-regulated aminopeptidase protein (IRAP, which reflects GLUT4 trafficking), and CD36 was detected by Western blotting.

Quantification of triacylglycerol contents

Quantification of triacylglycerol in HL-1 cells and aRCMs was performed using a Triglyceride Assay Kit (ab65336, Abcam, San Francisco, CA, USA) following the manufacturer's instructions.

Determination of insulin signaling

Insulin signaling in HL-1 cells and aRCMs were measured as previously described [9]. Briefly, after culturing HL-1 cells and aRCMs were exposed to insulin (HL-1 cells, 200 nM; aRCMs, 100nm) for 30 min to be able to compare basal-phosphorylation to insulin-stimulated phosphorylation. Afterwards, these cells were lysed in sample buffer and used for protein detection by SDS-polyacrylamide gel electrophoresis, followed by Western blotting. phospho-Ser2448-mTOR (p-mTOR), phospho-Ser473-Akt (pAKT), phospho-Ser235/236-S6 (pS6), total-mTOR, total-AKT, GAPDH, and caveolin-3 proteins were detected by Western blotting.

Measurement of substrate uptake

[^{3}H]Deoxyglucose- and [^{14}C]palmitate uptake assay in HL-1 cells, aRCMs, and hiPSC-CMs were measured as previously described [9].

Measurement of cardiomyocyte contraction dynamics

Contractile properties of aRCMs were assessed at 1 Hz field stimulation using a video-based cell geometry system to measure sarcomere dynamics (IonOptix, Milton, MA, USA) **(Supple Fig 3)**. From the digitized recordings acquired with IonWizard acquisition software, the following parameters were calculated: sarcomere shortening, time to peak, and decay time. This was conducted as previously described [6].

Evaluation of GLUT4 translocation in aRCMs

Adenoviral construction expressing hemagglutinin (HA)-GLUT4-green fluorescent protein (GFP) was kindly provided by Prof. Luc Bertrand's lab (Institute of Experimental and Clinical Research (IREC), Pole of Cardiovascular Research, UCLouvain, Brussels, Belgium). Evaluation of GLUT4 translocation was conducted as previously described with the modifications described below [31]. Briefly, GFP was fused to the carboxyl-terminus of GLUT4 to track all exogenous GLUT4. In contrast, HA epitope was inserted in the first exofacial loop of GLUT4, allowing the exclusive detection of GLUT4 inserted into the membrane of non-permeabilized cells. aRCMs were infected with HA-GLUT4-GFP adenoviruses at a multiplicity of infection of 5 for 45 h . After paraformaldehyde 4% (vol/vol) fixation and blocking with BSA 5% (wt/vol), non-permeabilized cells were incubated with a primary antibody (anti-HA), followed by secondary fluorescent antibody (Alexa Fluor 594). Nuclei were stained with DAPI. The cells were mounted on a glass slide and imaged at 63x objective with the confocal microscope (Leica SPE). Red fluorescent dots (HA staining) were quantified relative to GFP by ImageJ Fiji.

Immunofluorescent staining for pluripotency markers in hiPSC-CMs

iPSC1700012 (wild type) cells were stained with pluripotent markers SSEA4 and OCT4. iPSC 1700012 (wild type) cell line was fixed with fixative solution and incubated for 15 minutes at RT. The staining procedure was performed as per manufacturer's instructions using the Pluripotency Stem Cell 4-Market Immunocytochemistry kit (Thermofisher Scientific). Permeabilization solution was added to the cells and incubated for 15 minutes at RT. Blocking solution was added and incubated for 30 minutes. The cells were stained with primary antibody (1:100 SSEA4 with 1:200 OCT4 in blocking buffer) for 1 hour. Cells were washed with

washing buffer and stained with the secondary antibody (Alexa Fluor 488 and Alexa Fluor 555) for 1 hour at room temperature. Cells were washed with wash buffer and 1 drop of NucBlue Fixed Cell stain (DAPI) was incubated for 5 minutes. The cells were mounted on a glass slide and imaged at 63x objective with the confocal microscope (Leica SPE). Images were analyzed with ImageJ Fiji **(Suppl. Fig 1D)**.

QPCR analysis for pluripotency markers in hiPSC-CMs

RNA of the samples was isolated using the High pure RNA isolation kit (Roche). After RNA was extracted, cDNA synthesis was performed. The cDNA synthesis was carried out in a total volume of 20 µl containing 1x qScript cDNA supermix (Quantabio) and 500 ng RNA template. PCR program: 5 min at 25°C, 30 min at 42°C, 5 min at 80°C and hold at 4°C The qPCR was carried out in a total volume of 10 µl containing 2x Sensimix SYBR Hi-ROX (Bioline), 25 uM primers and 5 times diluted cDNA. Samples are run on the Lightcycler 480 (Roche) with the following program: 10 min at 95°C and 40 cycling with 15 sec at 95°C, 15 sec at 60°C and 15 sec at 72°C, with the relative quantification analysis **(Suppl. Fig 1E)**. **Table S2** displays primers for pluripotency genes in hiPSC-CMs.

Transfection with siRNA

HL-1 cells grown to 70% confluence were transfected with siRNAs against negative control (NC) scrambled siRNA or with siRNA targeting v-ATPase B2 subunit RNA (B2-kd) using Lipofectamine-RNAiMAX (Invitrogen, Carlsbad, CA, USA) in Opti-MEM® I Reduced-Serum Medium, according to the manufacturer's protocol. After transfection, cells were kept in complete medium (Claycomb medium) for 20 h, and then exposed to depletion medium (DMEM 31885) for 20 h. The transfection efficiency was evaluated by V_0a2, V_1B2, V_0d1 protein expression using Western blot analysis **(Suppl. Fig 1C)**.

Statistics

All data are presented as means ± SEM. Statistical analysis was performed by two-way ANOVA using SPSS (Version 25.0 for WINDOWS; SPSS Inc, Chicago, USA). *P*-values of less than 0.05 are considered statistically significant.

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Author's Contributions: SW and JL analyzed data, SW, LW, A Sun and A Strzelecka performed the experiments, JL, DM, MN and JG designed the experiments, SW, JL, DM, MN and JG wrote the manuscript. All authors discussed the data and commented on the manuscript before submission.

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Conflicts of Interest: The authors declare no conflict of interest.

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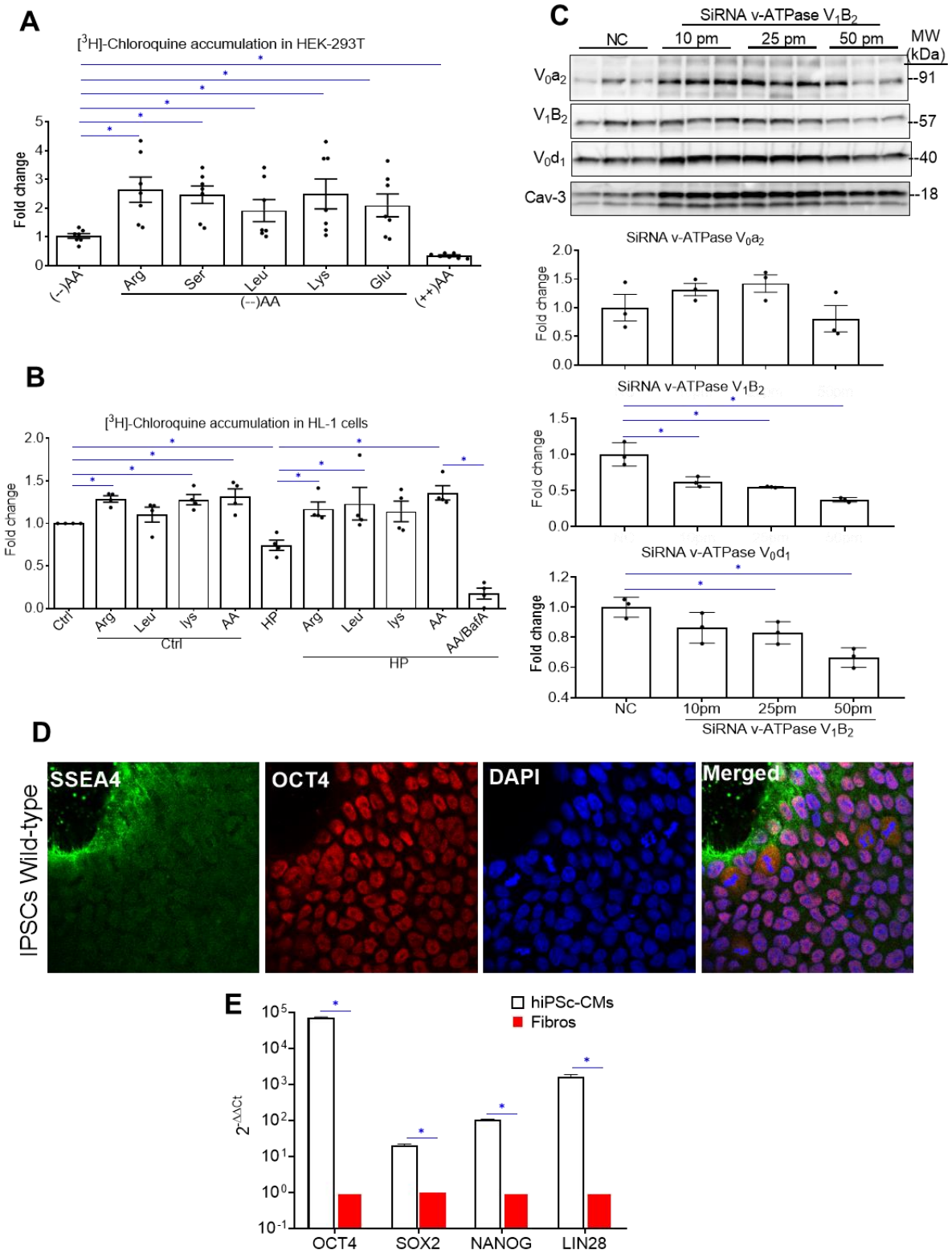
Supplementary Figures and Tables



Supplemental Figure 1

(A) **Related to Figure 1A.** AA alters v-ATPase activity in HEK-293T cells: after AA starvation, re-addition of individual AA alters v-ATPase activity (n=7).

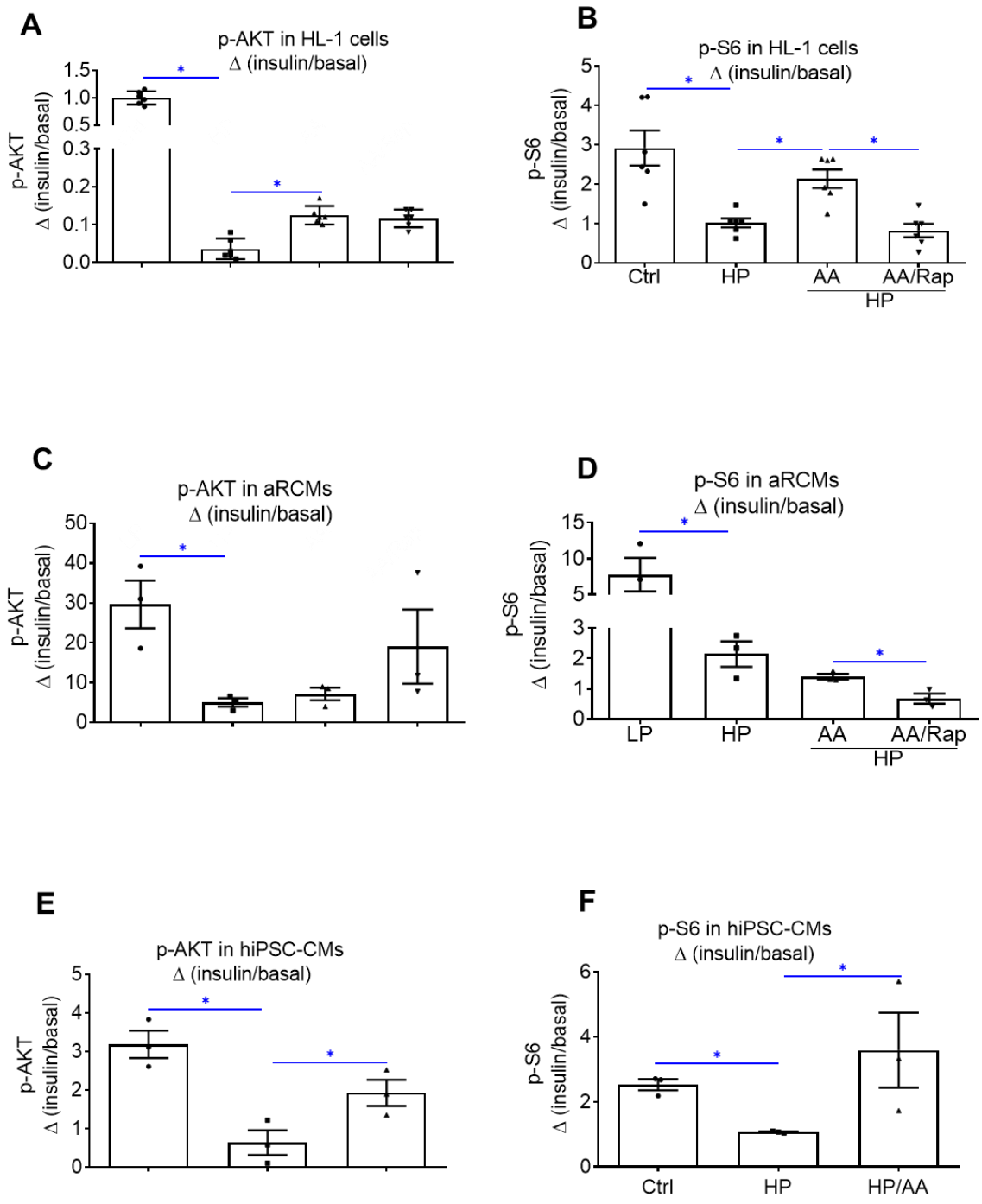
(B) **Related to Figure 1B:** HL-1 cells were first subjected to either Ctrl, HP, HP supplemented with arginine (HP/Arg), HP supplemented with leucine (HP/Leu), HP supplemented with lysine (HP/Lys), HP supplemented with a mixture of 4*AA (HP/AA, Arg-1.36mM, Leu-1.84mM and Lys-1.56mM), or HP/AA BafA] (n=4).

(C) **Related to Figure 1C:** HL-1 cardiomyocytes was transfected with negative control (Ctrl) scrambled siRNA or with 10pm, 25pm, 50pm siRNA targeting v-ATPase B2 subunit RNA (B2-kd). 32 h after transfection, cells were cultured under control (Ctrl) medium. For the transfection efficiency, it was evaluated by V_0a2, V_1B2, V_0d1 protein expression using Western blot analysis. **(C)** Representative Western blotting of V_0a2, V_1B2, V_0d1 and its quantification.

(D) **Related to Figure 1D**: Expression of pluripotency markers (e.g., SSEA4 and Oct4) and Dapi in human induced pluripotent stem cells (hiPSCs) by using immunofluorescent staining. Scale bar is 10μm.

(E) **Related to Figure 1D**: Gene expression of pluripotency markers in hiPSCs relative to fibroblasts.

Data information: bar values are means ± SEM of n > 3 independent experiments. *p<0.05 were considered statistically significant.



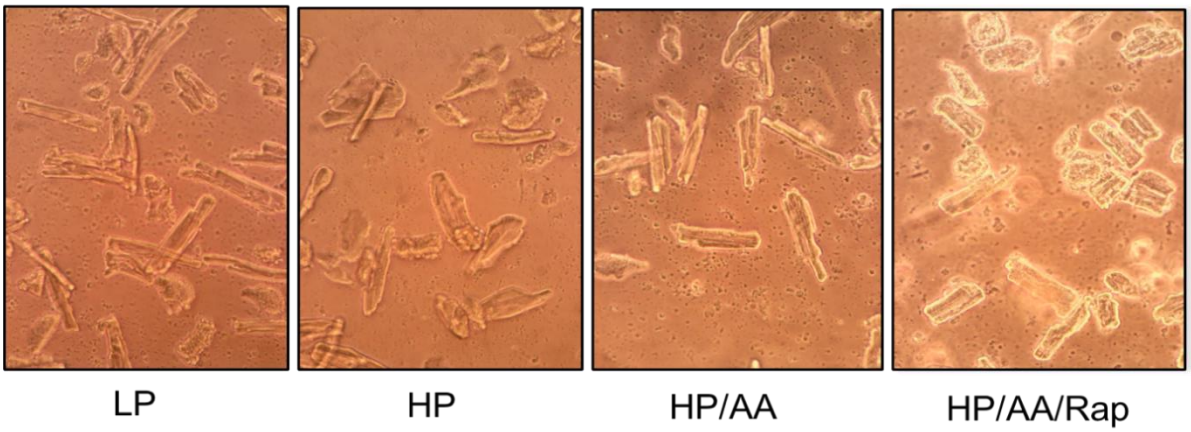
Supplemental Figure 2

(A-B) Related to Figure 3A: the quantification of pAKT (ser 473) and pS6 (ser 235/236) in HL-1 cells.

(C-D) Related to Figure 3B: the quantification of pAKT (ser 473) and pS6 (ser 235/236) in aRCMs.

(E-F) Related to Figure 3C: the quantification of pAKT (ser 473) and pS6 (ser 235/236) in hiPSC-CMs.

Data information: bar values are means ± SEM of n > 3 independent experiments. *p<0.05 were considered statistically significant.



Supplemental Figure 3

Related to Figure 4: Representative pictures of aRCMs, which were incubated for 24 h in either LP, HP, HP/AA, or HP/AA+Rap.

Table S1. Antibodies used in this study.

Antibodies	Source	Identifier
p-AKT (Ser473)	Cell signaling	#9271
Total-AKT	Cell signaling	#9272
p-ACC (Ser79)	Cell signaling	#07-303
Total-ACC	Cell signaling	#3662
p-mTOR (Ser2448)	Cell signaling	#2971
Total-mTOR	Cell signaling	#2972
pS6 (Ser235/236)	Cell signaling	#4856
Insulin-regulated aminopeptidase (IRAP)	Cell signaling	#MABN483
GAPDH	Cell signaling	#2118
Anti-ATP6V_0D1 (V0d1)	Abcam	#ab202899
Anti-ATP6V_1B2 (V1B2)	Abcam	#ab73404
Anti-ATP6V0A2 (V0-a2)	Abcam	#ab82638
HA-Tag (C29F4)	Abcam	#3724(S)
CD36 (MO25)	Gift from Dr.N.Tandon	No
GLUT4	Millipore	#07-1404
Caveolin-3	BD transduction Laboratories	#610421
SSEA4	Thermo Fisher Scientific	MA1-021
OCT4	Thermo Fisher Scientific	# MA1–104

Table S2. Primers for pluripotency genes in hiPSC-CMs.

Pluripotency genes	**Species**	**Primer sequence**	**Melting temperature (°C)**	**Fwd/Rev**
SOX2	Human	CCCAGCAGACTTCACATGT	60	fwd
SOX2	Human	CCTCCCATTTCCCTCGTTTT	60	rev
OCT4	Human	CCTCACTTCACTGCACTCTA	60	fwd
OCT4	Human	CAGGTTTTCTTTCCCTAGCT	60	rev
NANOG	Human	CTCCATGAACATGCAACCTG	60	fwd
NANOG	Human	GGCATCATGGAAACCAGAAC	60	rev
LIN28	Human	CACAGGGAAAGCCAACCTAC	60	fwd
LIN28	Human	TGCACCCTATTCCCACTTTC	60	rev

Chapter 4

Ketone body exposure of cardiomyocytes impairs insulin sensitivity and contractile function through vacuolar-type H^+-ATPase disassembly

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Abstract

The heart is a metabolically flexible organ that under normal conditions mainly uses lipids and glucose as substrates for energy provision. However, under specific conditions like uncontrolled diabetes, heart failure, fasting, and starvation, high plasma levels of ketone bodies occur and lead to their increased utilization as substrate for oxidation. It remains unclear whether increased cardiac ketone body utilization is beneficial or detrimental for cardiac functioning. Yet, it is known that predominant lipid utilization is detrimental for cardiac function. The mechanism of the latter includes disassembly of vacuolar-type H^+-ATPase (v-ATPase), followed by endosomal acidification and expulsion of CD36 from the endosomes to the sarcolemma to induce lipid accumulation and insulin resistance. Interestingly, lipid-induced v-ATPase disassembly can be prevented by supply of specific amino acids (Chapter 3). In the present study, we tested the effect of ketone bodies on isolated cardiomyocytes with respect to v-ATPase function, CD36 translocation, insulin signaling, and contractile function, and in comparison to lipids. The results showed that exposure to 3-β-hydroxybutyrate (3HB), but not acetoacetate, induced v-ATPase disassembly, loss of endosomal acidification, CD36 translocation to the sarcolemma, myocellular lipid accumulation, insulin resistance and contractile dysfunction, similarly to lipid exposure, while all these maladaptive changes were prevented by addition of specific amino acids. This data suggests that in sedentary/prediabetic subjects the use of ketone bodies should be limited because of possible aggravation of cardiac insulin resistance and further loss of cardiac function. With respect to therapy, specific amino acids may be promising nutraceuticals to combat lipid and ketone body-associated cardiomyopathies.

Key words: Ketone bodies; Vacuolar H+-ATPase; Endosomal CD36; lipid-induced insulin resistance, Contractile function; Diabetic heart

Introduction

The heart is a metabolically flexible organ that can use a variety of substrates for energy provision. To fulfil cardiac energy requirements the healthy adult heart mainly uses fatty acids (FA) and glucose, but it can switch its substrate preference to alternative substrates like ketone bodies and amino acids (AA) when being exposed to physiological or pathological stimuli [1, 2]. Ketone bodies are synthesized predominantly in the liver under conditions where there is limited carbohydrate and surplus fatty acid availability [3]. Ketone bodies are synthesized from FA oxidation (FAO)-derived acetyl-coenzyme A (CoA), and then are transported to key extrahepatic oxidative tissues like heart and brain for oxidation [4, 5]. Myocardial ketone body oxidation is particularly increased under conditions such as uncontrolled diabetes, fasting, starvation, post-exercise, the neonatal period, pregnancy, and during adherence to low-carbohydrate diets [6-10]. The circulating concentration of ketone bodies in healthy individuals normally exhibits circadian oscillations between around 100 μM and 250 μM, but can rise to ~1 mM after prolonged exercise or long-term fasting, and can increase to as high as 20 mM in pathological states (e.g., diabetic ketoacidosis) [9, 11, 12]. The increase in plasma ketone body levels is suggested to be an adaptive and compensatory response to energy deficit [9].

Additionally, supplementation of ketone bodies has been shown to have multiple beneficial effects in both health and disease. Thus, ketone bodies serve as an alternative fuel in trained athletes [10], and were beneficial in patients with heart failure with reduced ejection fraction and atrial fibrillation [4, 13]. Jensen *et al*. [14] demonstrated that ketone body infusion specifically improved cognitive parameters in patients with type 2 diabetes. Moreover, observations in diabetes therapy with sodium-glucose co-transporter-2 (SGLT2) inhibitor drugs suggested a positive link between beneficial heart failure outcomes and increased levels of ketone bodies [9]. It has been suggested that the beneficial effects of increased myocardial ketone body utilization are attributable to ketone bodies being used at the expense of FA, thereby leading to more oxygen-efficient oxidation [15-17], but evidence for this is lacking. Additionally, excessive levels of blood ketone bodies may lead to acidosis (ketoacidosis) [18, 19]. It currently remains unclear whether changes in myocardial ketone body utilization are beneficial or detrimental for cardiac metabolism and function. These various considerations prompted us to test the effects of ketone bodies on FA and glucose handling by isolated cardiomyocytes.

Recently we showed that the endosomal proton pump vacuolar H^+-ATPase (v-ATPase) functions as a master regulator of cardiac metabolism. V-ATPase is a multimeric protein complex, composed of an ATP-hydrolytic domain (V_1) and a proton translocation domain (V_0), and operates by a rotary mechanism [20] so as to maintain endosomal acidification [21]. We recently discovered that v-ATPase function in the heart is regulated via its assembly/disassembly status, which, in turn, can be modulated by various metabolic substrates such as FA [22], glucose [23], and amino acids (AA) (Chapter 3). For instance, excess lipid (e.g., palmitate) exposure of cardiomyocytes induces disassembly of v-ATPase, which impairs its function, results in loss of endosomal acidification, and subsequently triggers increased translocation of the membrane FA transporter CD36 from the endosomes to the sarcolemma. The latter initiates a vicious cycle of increased FA uptake and lipid-induced insulin resistance, eventually contributing to cardiac dysfunction [22, 24]. In contrast, re-assembly of v-ATPase, which was achieved through either increased glucose availability [23] or the addition of AA (in particular a mixture of arginine/leucine/lysine) (Chapter 3), restores lipid-induced insulin resistance and lipid-induced contractile dysfunction. Apart from the effect of palmitate, glucose and AA, a possible regulation of v-ATPase by other metabolic substrates like ketone bodies has not yet been studied. Hence, it would be of high interest to test whether ketone bodies have an impact on the functioning of v-ATPase, either directly or indirectly.

In this study, we first tested the effects of ketone bodies, in particular acetoacetate (AcAc) and 3-β-hydroxybutyrate (3HB), on v-ATPase assembly and function in both control and lipid-overloaded rodent cardiomyocytes. After we found that 3HB, not AcAc, induces v-ATPase disassembly, we subsequently studied whether 3HB-induced v-ATPase inhibition results in an increase of FA uptake and lipid accumulation, loss of insulin sensitivity (i.e., pAKT), and a decrease of insulin-stimulated glucose uptake and contractile function, and clarified the involvement of CD36. Additionally, we investigated functional alterations of v-ATPase in human stem cell-derived cardiomyocytes (hiPSC-CMs) subjected to excess ketone bodies. The collected data demonstrate that chronic 3HB exposure triggers CD36-mediated FA uptake, and then results in the loss of insulin sensitivity and of contractile function via the inhibition of v-ATPase function.

Results

Chronic 3HB exposure causes v-ATPase disassembly and de-activation

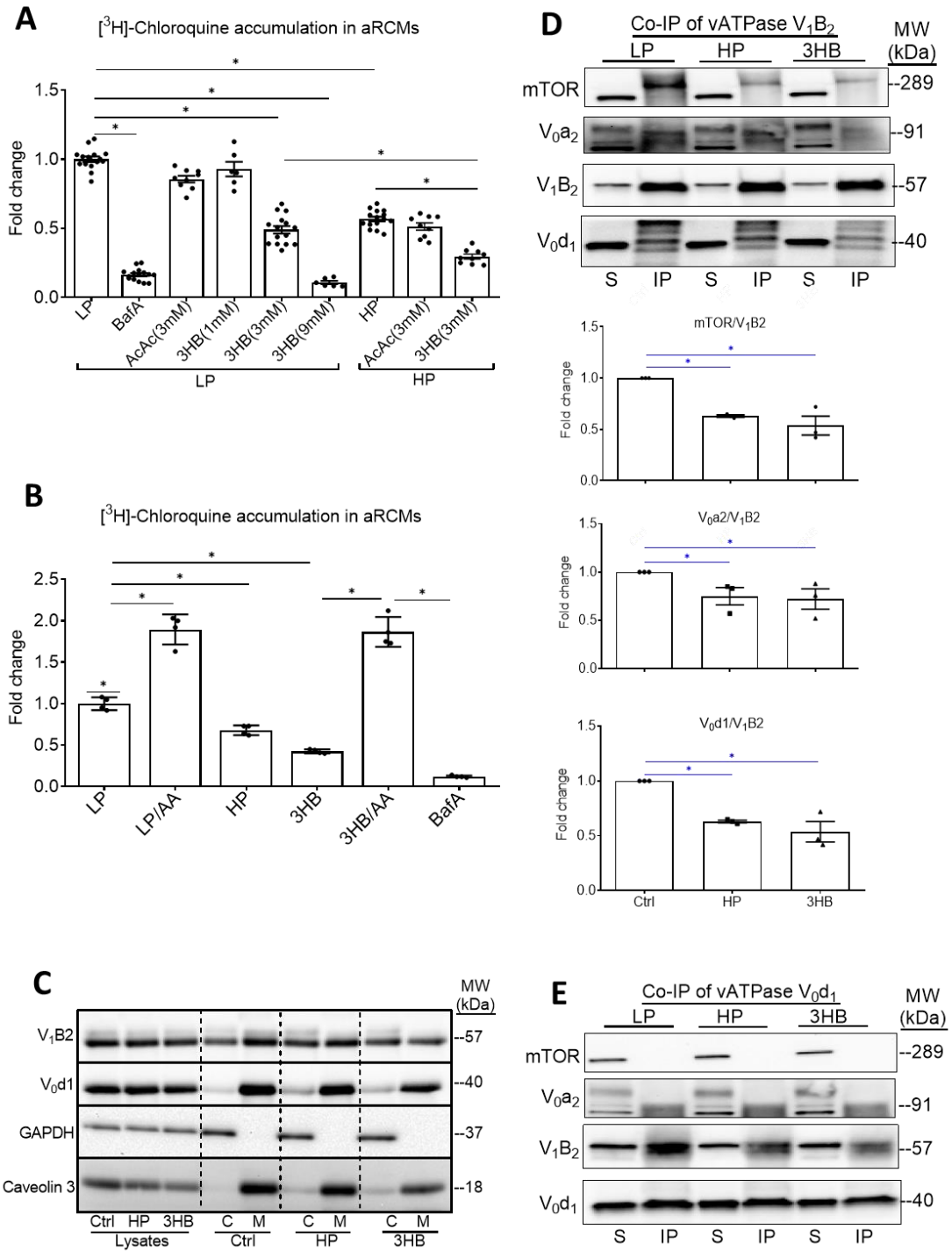
Firstly, we tested the effects of ketone bodies on cell-associated accumulation of the divalent weak base chloroquine (CHLQ) using a radioactive assay. CHLQ becomes specifically trapped in acidic organelles, such as endosomes, and when added to cells in tracer amounts, it provides quantitative information about luminal acidification [25]. Hence, the degree of cell-associated CHLQ accumulation can be used as readout of v-ATPase activity. Anticipating on a possible beneficial action of ketone bodies on regulation of cardiac substrate uptake at the level of v-ATPase, we first tested whether ketone bodies (AcAc and 3HB) could increase v-ATPase activity in both control and lipid-overloaded rodent adult cardiomyocytes (aRCMs). When compared with LP condition, v-ATPase activity was reduced by >50% in both HP condition (as expected) and 3HB condition (at concentrations of ≥3 mM) (Figure 1A). Interestingly, exposure with 3HB for 24 h, at concentrations of ≥3 mM caused an even bigger decrease in v-ATPase activity as compared with HP condition alone (Figure 1A). In contrast, 3 mM AcAc had no effect (Figure 1A). The inhibitory effect of 3HB (at 3 mM) was already for a large part present after 1 h (Suppl Figure 1). Furthermore, given that we previously showed that a mixture of AA (arginine, leucine and lysine) prevented lipid-induced v-ATPase impairment (Chapter 3), we also investigated whether this mixture (referred to as AA) could prevent 3HB-induced v-ATPase impairment to indeed confirm this hypothesis (Figure 1B).

Palmitate-induced v-ATPase inhibition is due to v-ATPase disassembly into its two sub-complexes [22]. Using subcellular fractionation, we verified that in all the conditions the V_0-d1 subunit (as part of the membrane-bound V_0 sub-complex) was detected within the membrane fraction (as was expected) (Figure 1C). Upon chronic HP and 3HB exposure, the V_1-B2 subunit (as indicator of the soluble V_1 sub-complex) was re-localized from the membrane fraction to the cytoplasmic fraction (Figure 1C), indicative of v-ATPase disassembly.

In a complementary approach, we studied the influence of 3HB on v-ATPase V_0/V_1 assembly by applying immunoprecipitation (IP) of v-ATPase sub-complexes (Figure 1D-F). Using antibodies against the d1 subunit of the V_0 super-complex (V_0-d1) or against the B2 subunit as part of the soluble V_1 super-complex of v-ATPase, we verified that 3HB-overexposure condition leads to a lower degree of co-IP with the other subunits as compared to control condition (Figure 1D-E). Hence, these IPs confirm the findings of the fractionation: chronic 3HB exposure induces v-ATPase disassembly (Figure 1D-E). Given that lipid exposure causes the dissociation between v-ATPase and mTORC1 (Chapter 3), we tested if chronic 3HB exposure would also lead to a similar dissociation of the mTOR – v-ATPase super-complex. Indeed, we found that in the control condition, when v-ATPase is assembled, mTOR binds only

to the V_1 sub-complex, and not to V_0 (Figure 1F). These findings could be confirmed in the reverse IP against mTOR, where in the control condition the B2 subunit, but not a2 nor d1, were found to be present (Figure 1D-F). Therefore, similar to lipid exposure [22], 3HB exposure decreased binding of mTOR to V_1 (Figure 1D and 1F).

Taken together, these results indicate that, similarly to lipid exposure, 3HB exposure of isolated cardiomyocytes induces v-ATPase disassembly and loss of v-ATPase function.


A
[3H]-Chloroquine accumulation in aRCMs
Fold change
LP
BafA
AcAc(3mM)
3HB(1mM)
3HB(3mM)
3HB(9mM)
HP
AcAc(3mM)
3HB(3mM)
B
[3H]-Chloroquine accumulation in aRCMs
Fold change
LP
LP/AA
HP
3HB
3HB/AA
BafA
C
MW (kDa)
V1B2
V0d1
GAPDH
Caveolin 3
--57
--40
--37
--18
Ctrl HP 3HB
C M C M C M
Lysates
Ctrl
HP
3HB
D
Co-IP of vATPase V1B2
LP HP 3HB
MW (kDa)
mTOR
V0a2
V1B2
V0d1
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mTOR/V1B2
V0a2/V1B2
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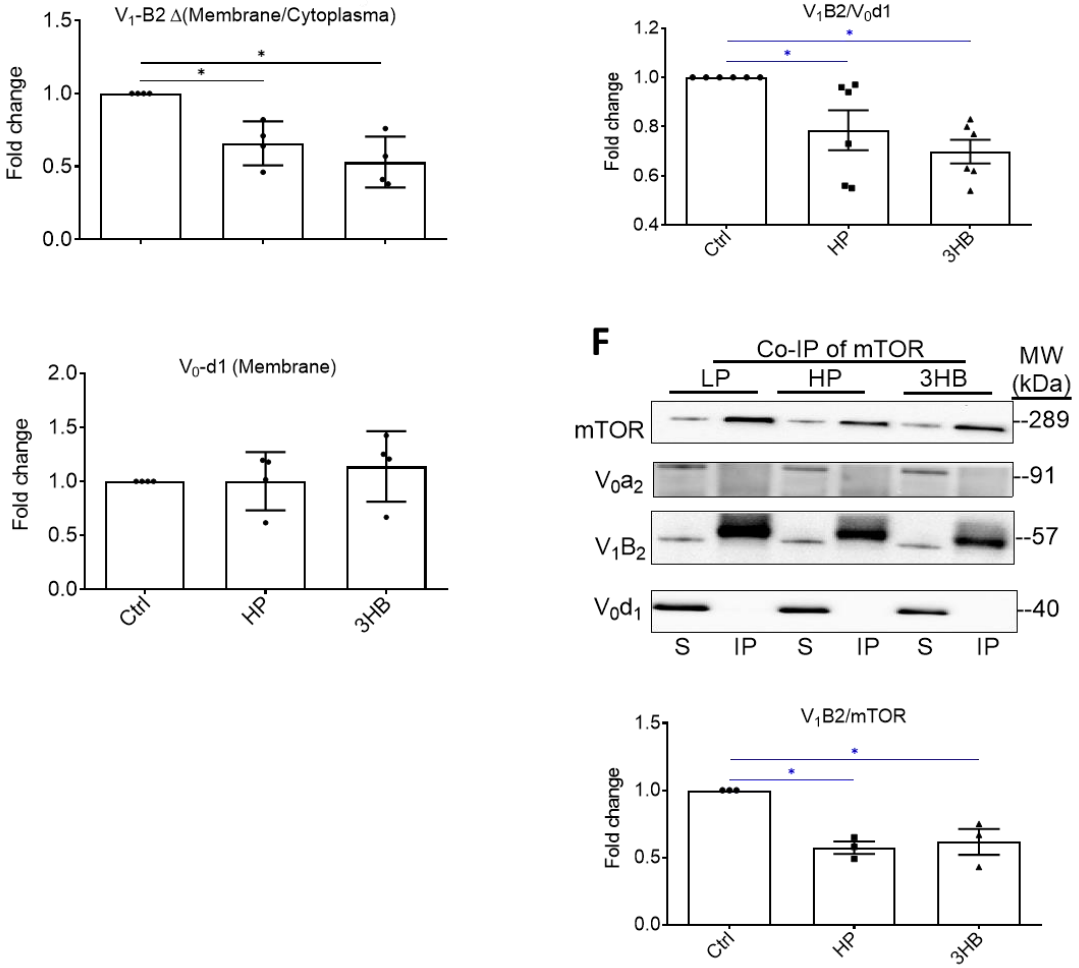


Figure 1. Chronic 3HB exposure causes v-ATPase disassembly and de-activation in cardiomyocytes.

(A) 3HB exposure results in the loss of v-ATPase activity in adult rat cardiomyocytes (aRCMs): aRCMs were incubated for 24h with either low palmitate (LP, palmitate/BSA ratio 0.3:1), LP supplemented with 100nM of bafilomycin-A (BafA), LP supplemented with 3mM of acetoacetate (AcAc), or LP supplemented with different concentrations of 3-β-hydroxybutyrate (3HB; 1mM, 3mM, and 9mM), high palmitate (HP, palmitate/BSA ratio 3:1), HP supplemented with 3mM of AcAc (HP/AcAc), or HP supplemented with 3mM of 3HB (HP/3HB). After the culturing of all conditions above, cells were used for [^{3}H] Chloroquine (CHLQ) accumulation assay last 20 min.

(B) Amino acids (AA) prevent 3HB-induced v-ATPase inhibition in aRCMs: aRCMs were incubated for 24h with either LP, HP, BafA, LP supplemented with the AA mixture (Arg-1.36mM, Leu-1.84mM and Lys-1.56mM) (LP/AA), 3HB (3mM), 3HB supplemented with the AA mixture. After the culturing of all conditions above, cells were used for [^{3}H] Chloroquine (CHLQ) accumulation assay last 20 min.

(C-F) 3HB exposure induces v-ATPase disassembly: **(C)** Representative Western blotting and it's quantification of fractionation in HL-1 cells: Cells were incubated for 24h with either control (Ctrl) medium, HP medium (500µM palmitate and 100nM insulin), or Ctrl medium supplemented with 3mM of 3HB medium (3HB). Contents of v-ATPase subunit B2 (V_1B2) and v-ATPase subunit d1 (V_0d1) and were assessed by Western blotting in the cytoplasmic fraction (C) and the membrane fraction (M). Caveolin-3 (Cav-3) and GAPDH were detected as the loading control of V_0d1 and V_1B2, respectively (n=4). **(D-F)** Representative Western blotting and it's quantification of co-Immunoprecipitation (Co-IP) of v-ATPase subunit B2 (V_1B2) **(D)**, subunit d1 (V_0d1) **(E)** or mTOR **(F),** from HL-1 cells after incubation for 24 h with either Ctrl, HP, or 3HB medium.

Data information: bar values are means ± SEM of n > 3 independent experiments. *p<0.05 were considered statistically significant.

Chronic 3HB exposure induces CD36-mediated lipid accumulation

In lipid-overloaded cardiomyocytes, v-ATPase inhibition leads to increased CD36 translocation to the sarcolemma, which contributes to an increase of FA uptake and myocellular lipid levels [22]. Therefore, we investigated whether the similarity between the effects of palmitate and 3HB exposure on v-ATPase dynamics can be extended to lipid parameters. First, using a surface biotinylation assay, we confirmed that chronic 3HB exposure induced the translocation of CD36 to the sarcolemma (Figure 2A). Specifically, CD36 cell surface content was increased by 1.4-fold (Figure 2A). Moreover, the 3HB-induced CD36 translocation was accompanied by a loss of short-term insulin stimulation (Figure 2A), indicating that 3HB exposure induces CD36 translocation from insulin-responsive endosomal stores. When 3HB exposure was combined with the AA mixture, 3HB-induced CD36 translocation was prevented, and insulin-stimulated CD36 translocation was largely preserved (Figure 2A). Hence, AA treatment prevents CD36-mediated lipid accumulation, not only during lipid overload, but also during 3HB exposure.

Next, FA uptake was studied in 3HB-exposed cells in the absence/presence of short-term insulin stimulation (Figure 2A). Given that FA uptake is predominantly regulated by CD36, indeed, as documented for lipid exposure a similar trend was observed upon 3HB exposure, i.e., an increase in basal FA uptake, and a loss of insulin-stimulated FA uptake (Figure 2B). Finally, the 3HB-induced increase in CD36 translocation and FA uptake in aRCMs is then expected to impact on myocellular lipid levels. Indeed, myocellular triacylglycerol content was increased

in cardiomyocytes upon both HP culturing (3.27-fold, Figure 2C) and 3HB culturing (1.70-fold, Figure 2C). Altogether, chronic 3HB exposure enhanced CD36-mediated lipid accumulation in aRCMs.

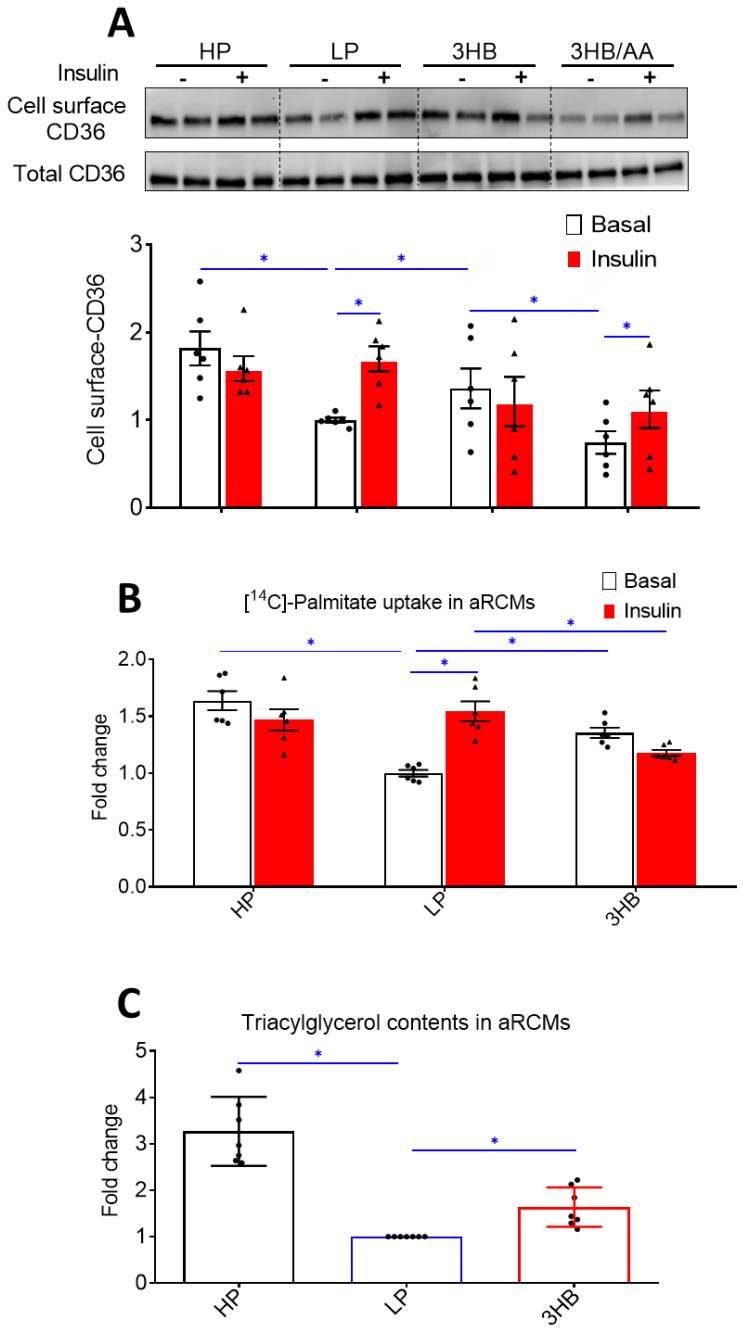


Figure 2. Chronic 3HB exposure induces CD36-mediated lipid accumulation in cardiomyocytes.

(A) Cell surface CD36 translocation in aRCMs: representative immunoblotting and its quantification from biotin-labeled and total lysate fractions of aRCMs, which are incubated for 24 h with high palmitate (HP, palmitate/BSA ratio 3:1), low palmitate (LP, palmitate/BSA ratio 0.3:1), LP supplemented with

3mM of 3-β-hydroxybutyrate (3HB), 3HB supplemented with the AA mixture (Arg-1.36mM, Leu-1.84mM and Lys-1.56mM, AA concentration is 4 times higher in M199 medium) [3HB/AA], prior to biotin labeling and lysis.

(B) [^{14}C] palmitate uptake in aRCMs: aRCMs were incubated with either LP, HP or 3mM of 3HB for 25 h, followed by 30 min of (-/+) insulin (100 nM) incubation prior to [^{14}C] palmitate labeling.

(C) Triacylglycerol contents in aRCMs: aRCMs were incubated for 24 h with LP, HP or 3HB medium.

Data information: bar values are means ± SEM of n > 3 independent experiments. *p<0.05 were considered statistically significant.

Chronic 3HB exposure interferes with insulin signaling and insulin-stimulated glucose uptake

It is well-known that increased CD36-mediated FA uptake and lipid accumulation precede the development of insulin resistance and the associated loss of insulin-stimulated glucose uptake [26]. Will the similarity of HP exposure with 3HB exposure further extend to insulin resistance? For evaluation of insulin signaling, phosphorylation levels of Akt (pAkt Ser473), AS160, mTOR (p-mTOR ser2448), and ribosomal protein S6 (pS6 Ser235/236), were assessed (Figure 3 A-D). As expected [22], HP exposure caused a loss of insulin-stimulated phosphorylation of Akt, mTOR, AS160 and S6 in aRCMs (Figure 3 A-D)**.** Similar to previous observations (Chapter 3), the addition of the AA mixture partially prevented the 3HB-induced loss of insulin-stimulated Akt phosphorylation, and of mTOR and S6 phosphorylation. Interestingly, when aRCMs were exposed to 3HB during variable periods in the absence/presence of insulin it was observed that 3HB culturing induced a relatively rapid ~40% decrease in insulin-stimulated Akt/AS160/S6 phosphorylation within the first 5h, which was followed by a slower rate of further decrease until a maximum level was reached at ~80% at 25 h (Suppl Figure 2).

Taken together, FA and 3HB exposure of cardiomyocytes similarly decrease insulin signaling (Figure 2 and Suppl Figure 2). In addition, AA supplementation of the medium partially rescues the inhibitory effect of 3HB on insulin-stimulated Akt/AS160/S6 phosphorylation (Figure 3).

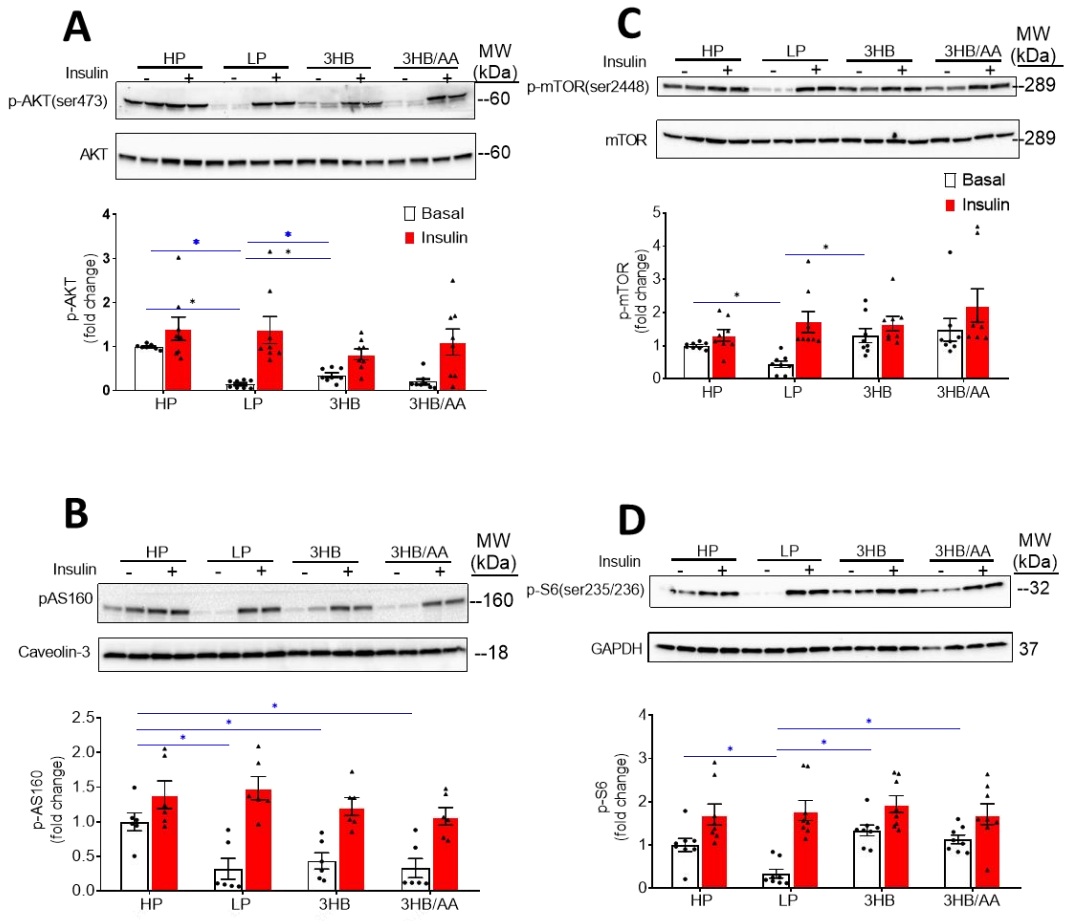


Figure 3. Chronic 3HB exposure interferes with insulin signaling in cardiomyocytes.

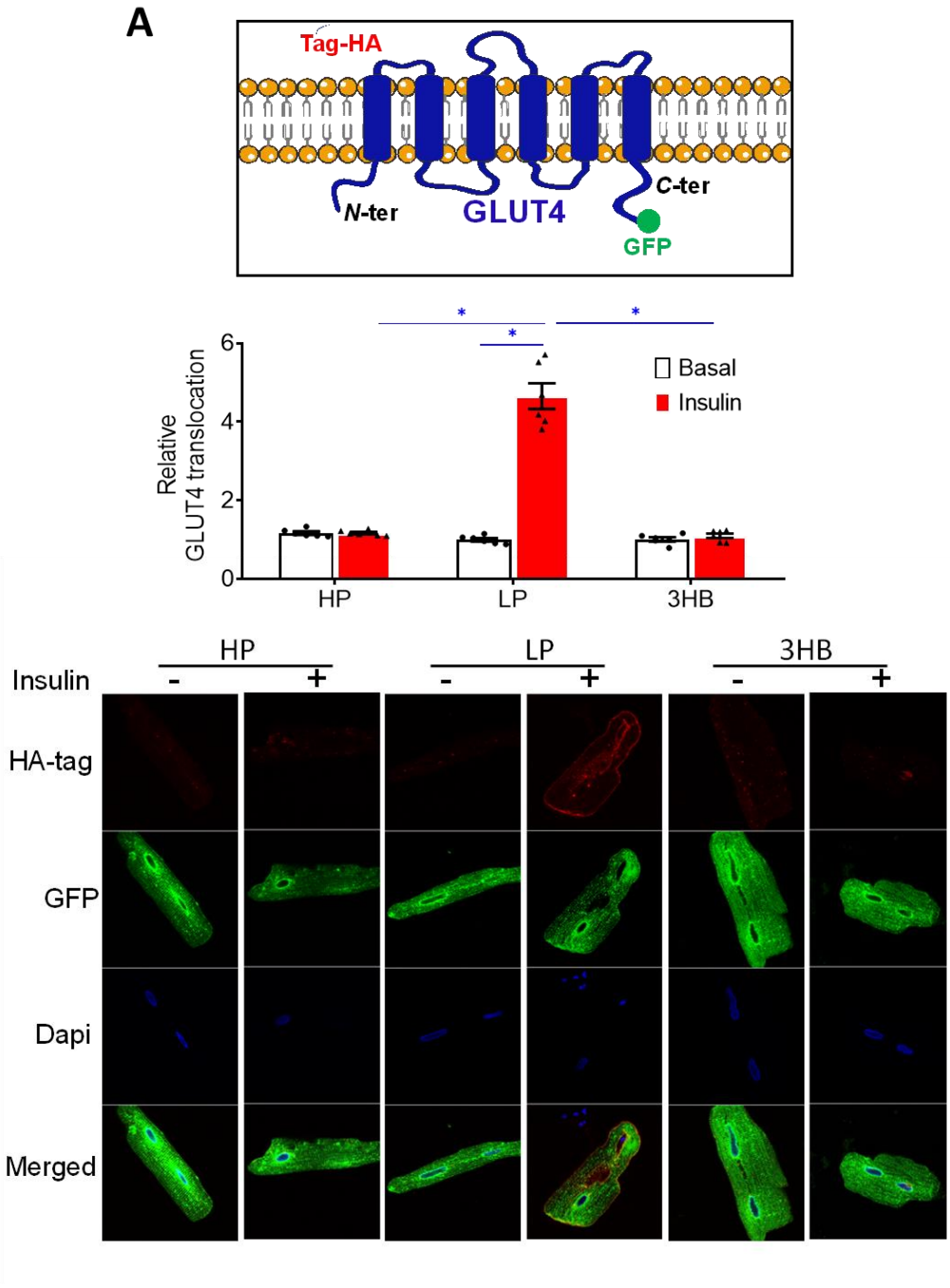
aRCMs were incubated for 24 h with high palmitate (HP, palmitate/BSA ratio 3:1), low palmitate (LP, palmitate/BSA ratio 0.3:1), LP supplemented with 3mM of 3-β-hydroxybutyrate (3HB), 3HB supplemented with the AA mixture (Arg-1.36mM, Leu-1.84mM and Lys-1.56mM) [3HB/AA]. Subsequently, cells were stimulated either with or without 100 nM insulin for 30 min. Representative blots and quantification of phospho-Ser473-Akt (pAKT) **(A)**, phospho-AS160 (pAS160) **(B)**, phospho-Ser2448-mTOR (p-mTOR) **(C)**, phospho-Ser235/236-S6 (pS6) **(D)**, and GAPDH/Cav-3 (loading control).

Data information: bar values are means ± SEM of n > 3 independent experiments. *p<0.05 were considered statistically significant.

Next, two techniques were employed to investigate the effects of ketone bodies on insulin-stimulated GLUT4 translocation in cardiomyocytes: (i) a microscopic inspection of cell surface levels of HA-tagged GLUT4 in adenovirally transfected aRCM; (ii) a surface detection assay of insulin-responsive aminopeptidase (IRAP) (Figure 4B). Both methods show that

insulin-stimulated GLUT4 translocation was largely decreased upon HP and 3HB exposure (Figure 4B), which is in line with the impairment of insulin signaling under both conditions.

Because GLUT4 translocation is a major regulatory event in cardiac glucose uptake [27], we also studied this parameter using a radioactivity assay. As expected, for both lipid and 3HB exposure this yielded a similar pattern of changes in GLUT4 translocation assay (Figure 4C).



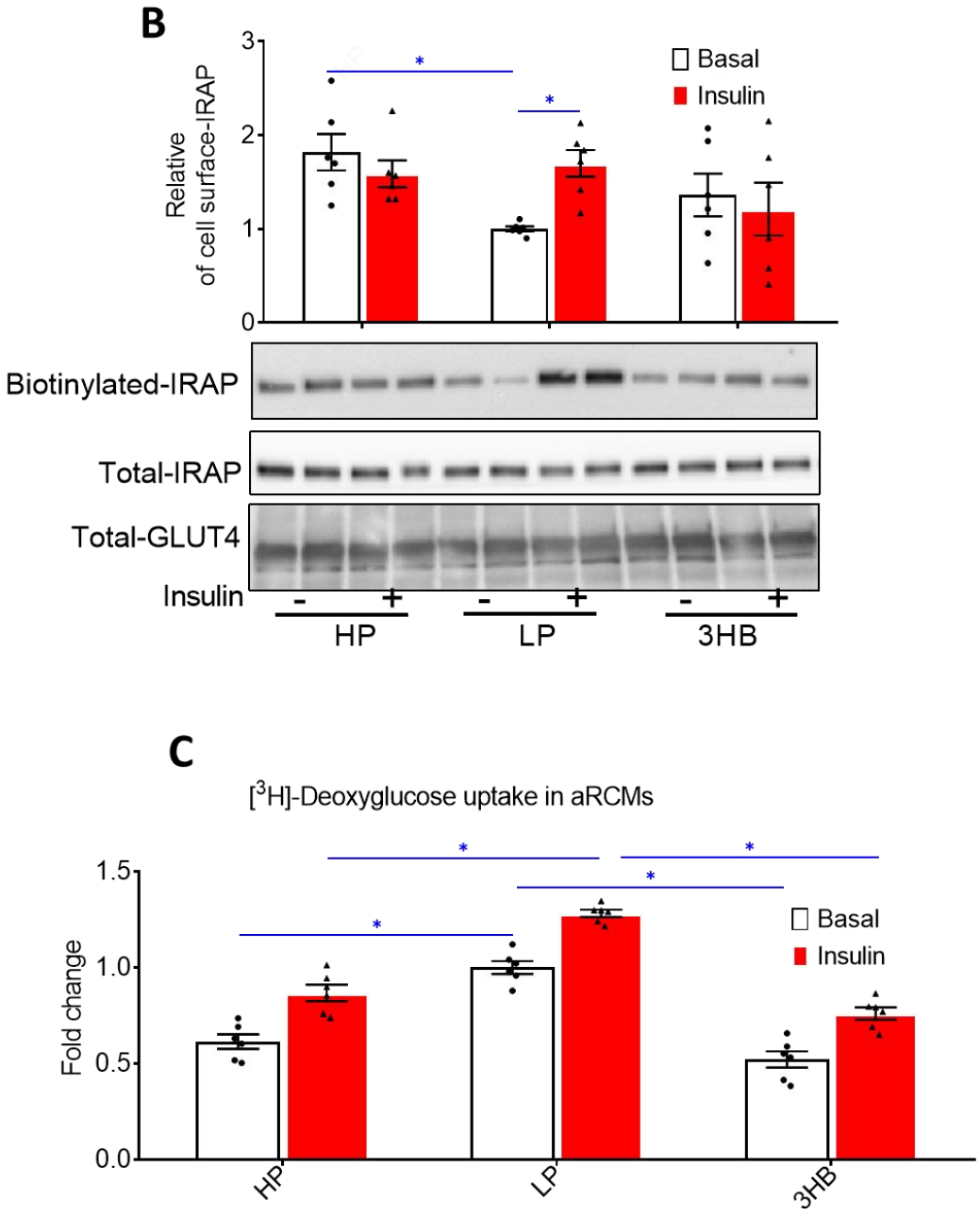


Figure 4. Chronic 3HB exposure prevents insulin-stimulated GLUT4 translocation and glucose uptake in cardiomyocytes.

(A) Insulin-stimulated glucose transporter 4 (GLUT4) insertion/translocation in aRCMs. **(A upper panel)**: representation of the fusion protein HA-GLUT4-GFP; **(A middle panel and a lower panel)**: the effects of 3HB on insulin-induced GLUT4 insertion/translocation into the plasma membrane. aRCMs were infected for 45 h and then incubated with 100 nM insulin for the last 30 min. Nonpermeabilized cells were anti-HA immunostained. Ratios of red (HA-tag) and green (GFP) intensity per pixel were quantified by Image J (n=3; imaging of 6 cells/measurement condition). DAPI, 4,6-diamidino-2-phenylindole.

(B) Biotinylated-GLUT4 translocation in aRCMs: Representative blots and it's quantification of Insulin-regulated aminopeptidase (IRAP, which reflects GLUT4 trafficking) from biotin-labeled and total lysate fractions of aRCMs, which are incubated for 24 h with LP, HP, or 3HB medium, followed by 30 min of (-/+) insulin (100 nM) incubation prior to biotin labeling and lysis.

(C) [^{3}H] Deoxyglucose in aRCMs: aRCMs were incubated with either LP, HP or 3mM of 3HB for 25 h, followed by 30 min of (-/+) insulin (100 nM) incubation prior to[^{3}H] deoxyglucose labeling.

Data information: bar values are means ± SEM of n > 3 independent experiments. *p<0.05 were considered statistically significant.

Chronic 3HB exposure induces contractile dysfunction

As previously reported, HP exposure of cultured aRCM leads to contractile dysfunction via increased CD36-mediated myocellular lipid accumulation [28]. In this study, the negative impact of HP on contractility was confirmed (64% decrease in sarcomere shortening; Figure 5A). The magnitude of the negative action of 3HB exposure was similar (79% decrease in sarcomere shortening; Figure 5A). The other contractile parameters [such as time to peak, relaxation duration (RT50 and RT90)] were not negatively influenced by HP nor 3HB (Figure 5B-D). In contrast, AcAc exposure did not affect sarcomere shortening (Figure 5A). Finally, AA addition entirely prevented the negative effect of chronic 3HB exposure on sarcomere shortening (Figure 5A).

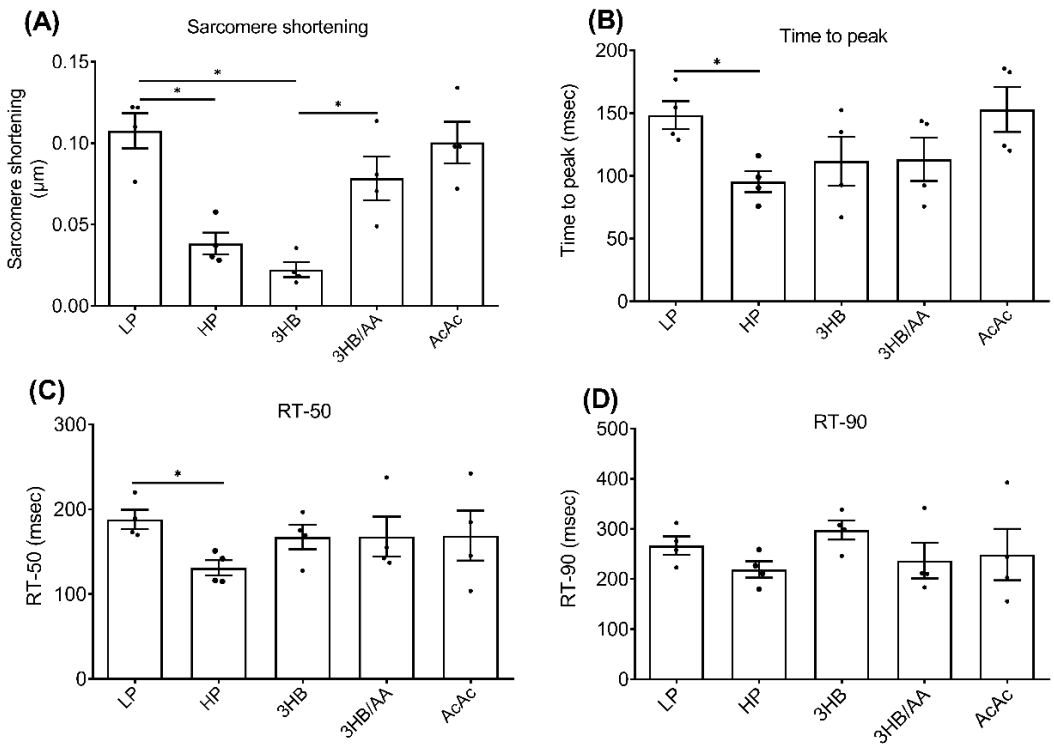


Figure 5. Chronic 3HB exposure contributes to contractile dysfunction in cardiomyocytes.

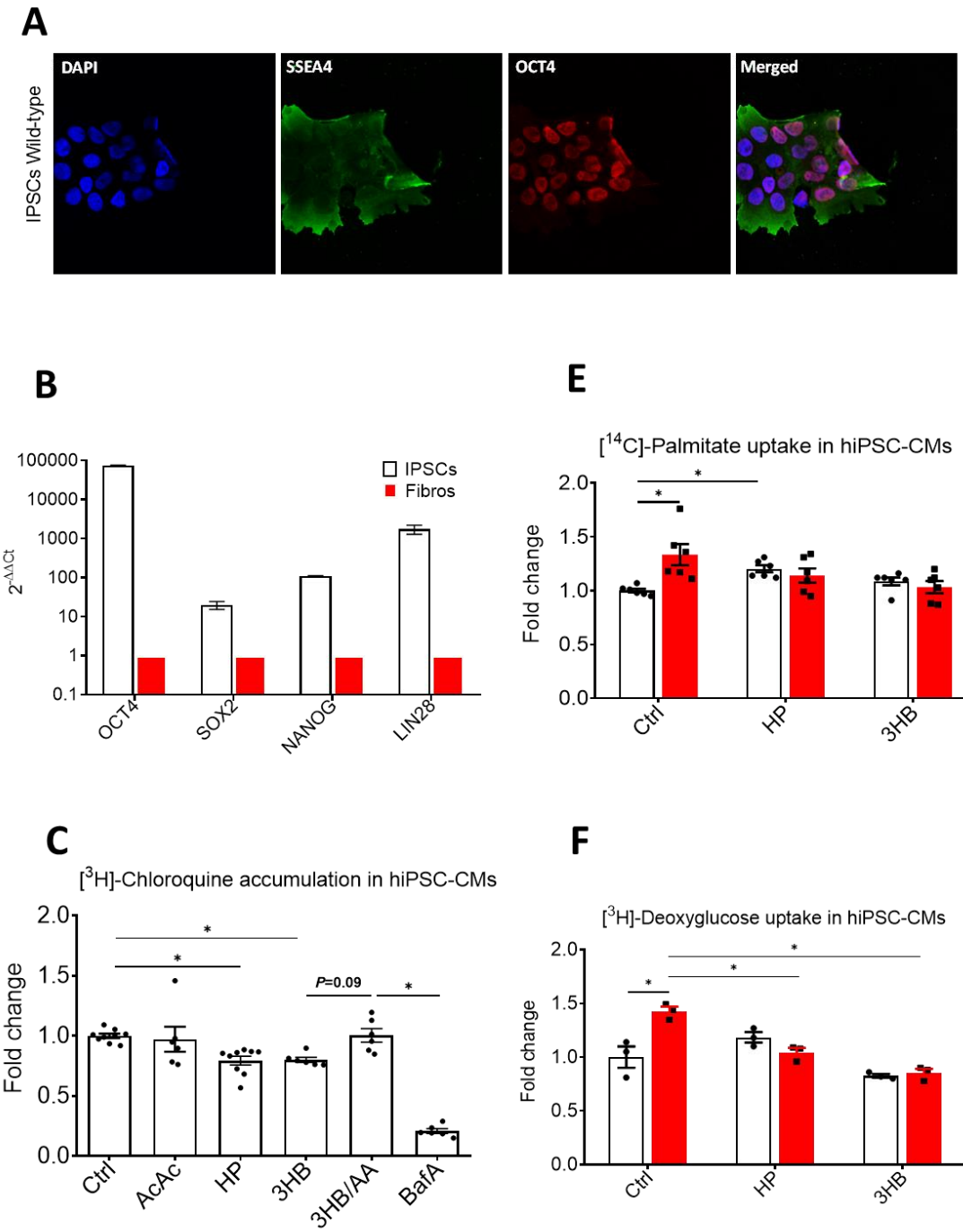
aRCMs incubated for 24 h in either low palmitate (LP, palmitate/BSA ratio 0.3:1), high palmitate (HP, palmitate/BSA ratio 3:1), LP supplemented with 3mM of 3-β-hydroxybutyrate (3HB), 3HB supplemented with the AA mixture (Arg-1.36mM, Leu-1.84mM and Lys-1.56mM) [3HB/AA], or LP supplemented with 3mM Acetoacetate (AcAc). Parameters of contractile function, including sarcomere shortening **(A)**, time to peak (TTP) **(B)**, decay time to 50% percent (RT50) **(C)**, and decay time to 90% percent (RT90) **(D)** of values are displayed as mean ± SEM (n=4; imaging of 10 cells/measurement condition). *p<0.05 were considered statistically significant.

Inhibition of v-ATPase function shifts energy substrate uptake away from glucose to FA in human iPSC-derived cardiomyocytes

To examine whether the HP-mimetic effect of chronic 3HB exposure on the induction of insulin resistance via v-ATPase disassembly also occurs in the human heart, hiPSC-CMs were employed. For this, hiPSCs first were characterized for their pluripotency prior to their differentiation. Figure 6A-B shows that the pluripotent markers (e.g., SSEA4 and OCT4) were highly expressed in hiPSCs (Figure 6A). Additionally, the mRNA expression levels of OCT4, SOX2, NANOG, and LIN28 were also found to be highly expressed in hiPSC when compared

to fibroblasts (Figure 6B). These findings strongly suggest that the hiPSC were pluripotent and had a strong differentiation potential at the start of their differentiation into cardiomyocytes (Figure 6A-B).

Similar to our observations in rodent cardiomyocytes (Figure 1-4), hiPSC-CMs exposed to HP or to 3HB displayed loss of v-ATPase function, while AcAc had no effect (Figure 6C). Moreover, hiPSC-CMs developed some key features of insulin resistance upon HP or 3HB culturing, i.e., increased basal FA uptake and loss of insulin-stimulated FA and glucose uptake (Figure 6D-E), as well as loss of insulin-stimulated Akt and S6 phosphorylation (Figure 6F). Notably, when 3HB exposure was combined with the AA mixture (arginine/leucine/lysine), v-ATPase function was partially restored (Figure 6C), further supporting our findings in aRCMs that the addition of AA preserves insulin sensitivity via v-ATPase assembly. Taken together, the molecular mechanism of 3HB exposure-driven v-ATPase disassembly to disturb substrate uptake in cardiomyocytes appears conserved between rodents and humans.


A
IPSCs Wild-type
DAPI
SSEA4
OCT4
Merged
B
IPSCs
Fibros
$2^{-\Delta\Delta Ct}$
OCT4
SOX2
NANOG
LIN28
E
[14C]-Palmitate uptake in hiPSC-CMs
Fold change
Ctrl
HP
3HB
C
[3H]-Chloroquine accumulation in hiPSC-CMs
P=0.09
Ctrl
AcAc
HP
3HB
3HB/AA
BafA
F
[3H]-Deoxyglucose uptake in hiPSC-CMs
Ctrl
HP
3HB

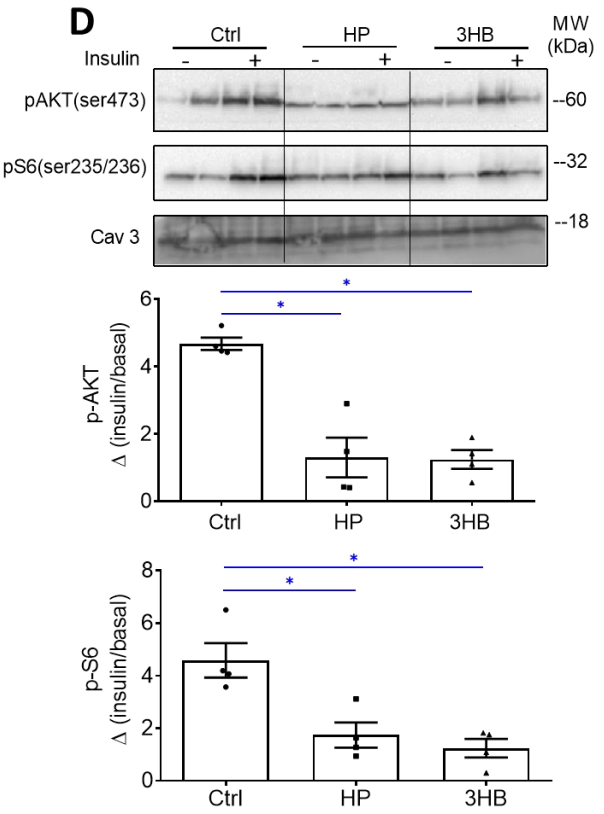


Figure 6. Chronic 3HB exposure reduces v-ATPase activity and insulin sensitivity in human cardiomyocytes

(A) Expression of pluripotency markers (e.g., SSEA4 and Oct4) and Dapi in human induced pluripotent stem cells (hiPSCs) by using immunofluorescent staining. Scale bar is 10µm. **(B)** Gene expression of pluripotency markers in hiPSCs relative to fibroblasts.

(C) V-ATPase function in hiPSC-CMs: Cells were cultured for 24 h in either control medium (Ctrl), Ctrl with addition of 100nM Bafilomycin-A (BafA), high palmitate medium (HP), Ctrl with addition of 3mM Acetoacetate (AcAc), Ctrl with addition of 3mM 3-β-hydroxybutyrate (3HB), 3HB with addition of the AA mixture (Arg-1.36mM, Leu-1.84mM and Lys-1.56mM) [3HB/AA]. After the culturing of all conditions above, cells were sub for [^{3}H] CHLQ accumulation assay last 20 min.

(D) 3Insulin signaling pathway in hiPSC-CMs: Cells were incubated for 24h with either Ctrl, HP, or 3HB medium. Subsequently, cells were stimulated (-/+) 200nM insulin for 30 min and harvested for Western blotting analysis. **(D)** Representative blots of pAKT and pS6, and it's quantification. Caveolin 3 were detected as the loading control.

(E) [^{14}C] palmitate uptake in hiPSC-CMs: Cells were incubated for 24h with either Ctrl, HP, or 3HB, followed by 30 min of (-/+) insulin (200 nM) incubation prior to [^{14}C] palmitate labeling.

(F) [^{3}H] deoxyglucose uptake in hiPSC-CMs: Cells were incubated for 24h with either Ctrl, HP, or 3HB, followed by 30 min of (-/+) insulin (200 nM) incubation prior to[^{3}H] deoxyglucose labeling.

Data information: bar values are means ± SEM of n > 3 independent experiments. *p<0.05 were considered statistically significant.

Discussion

The major findings of the present study are that **1)** upon chronic exposure of rodent/human cardiomyocytes to 3HB, CD36-mediated FA uptake is triggered due to endosomal alkalinization, resulting in an impairment of both insulin signaling and GLUT4-mediated glucose uptake, and a loss of contractile function. **2)** This 3HB-induced endosomal alkalinization is caused by v-ATPase disassembly.

As established earlier, high palmitate exposure increases CD36-mediated lipid metabolism progressively, thereby contributing to the loss of insulin resistance, and of contractile function [22]. In this study, we show that similarly to lipids, 3HB exposure accelerates CD36-mediated FA uptake, which then also culminates into insulin resistance and contractile dysfunction. Notably, the results from this study are consistent with several prior studies showing that prolonged exposure to ketone bodies (i.e., 3HB) diminished insulin-stimulated glucose uptake in rat adult cardiomyocytes [29] and isolated mouse soleus [30] which was associated with inhibition of insulin signaling (e.g., reduced phosphorylation of Akt and AS160). Our findings suggest that cardiac insulin resistance by chronic 3HB exposure may be lipid-mediated. Deciphering the mechanism behind ketone body-induced insulin resistance could prove useful to further our understanding of insulin resistance and associated diseases (e.g., T2D). As earlier reported, the molecular mechanism involved is palmitate-induced v-ATPase impairment which triggers a vicious cycle of CD36-mediated lipid accumulation, setting the heart on a road to insulin resistance and contractile dysfunction [22, 23]. Importantly, high palmitate and 3HB appear to trigger the same sequence of events. Yet, at present, the upstream mechanism by which 3HB induces v-ATPase disassembly remains unknown. To this end, it is interesting to note that the other abundant ketone body, acetoacetate, does not affect v-ATPase function, indicating the necessity of further studies to clarify this difference.

Another important finding of the present study is that an AA mixture (arginine/leucine/lysine) significantly counteracted the negative effects of 3HB on insulin action. Also this finding is consistent with our previous observations on lipid-overexposed

cardiomyocytes (Chapter 3), in which this AA mixture was employed to induce v-ATPase re-assembly in an mTORC1-dependent manner and found to protect the cardiomyocytes from lipid-induced insulin resistance and contractile dysfunction. The underlying molecular mechanism is not known but may involve activation/deactivation of the protein kinase mTOR. As earlier established, v-ATPase is a scaffold for mTOR activation [31], and mTOR activation can also induce v-ATPase activation (Chapter 3), suggesting that the association between mTOR activation and v-ATPase activation operates in both directions. In Chapter 3, we found that mTORC1 activation by the AA mixture re-assembles v-ATPase in the lipid-overloaded cardiomyocytes. In the present study, chronic 3HB exposure was found to result in loss of v-ATPase activity (Figure 1A-B), whereas the addition of the AA mixture could prevent this 3HB-induced v-ATPase deactivation (Figure 1B-C) through stimulating mTOR phosphorylation (Figure 3C). Consistent with the findings presented in Chapter 3, we further confirmed that AA-induced v-ATPase activation of the mTOR–v-ATPase axis also works in the opposite direction to activate mTOR. Likely, this explains why AA can rescue the 3HB-induced deactivation of mTOR and of v-ATPase. However, the link between v-ATPase activity and mTOR activation might be more complex than currently appreciated because this complex involves a great number of additional proteins (e.g., Rag-GTPases, adaptor proteins and lysosomal AA transporters). Hence, further studies are needed to clarify the molecular mechanism by which ketone bodies mediate the assembly/disassembly of v-ATPase in the mTOR–v-ATPase axis.

Emerging evidence suggests that ketone bodies are efficient and rapid fuels of cardiac muscle so that increased cardiac uptake of ketone bodies may be an adaptive and compensatory response to the impaired energy metabolism of the diabetic heart [5, 13, 32, 33]. In the present study, chronic exposure of cardiomyocytes to ketone bodies was observed to result in the development of insulin resistance and contractile dysfunction, suggesting that ketones bodies (at least 3HB) should not be provided as neutraceuticals, at least not during prolonged periods. The current finding seems inconsistent with previous findings that ketone bodies, as an alternative fuel, are beneficial for conditions of energy deficit. Thus, the present finding prompts us to consider why 3HB can be simultaneously beneficial (as an energy substrate) and detrimental (as the onset of insulin resistance and contractile dysfunction). Park et al. [34] reported that mild ketosis by central infusion of ketones improves energy and glucose metabolism through the potentiation of leptin and insulin signaling in the hypothalamus of diabetic rats. The concentration of plasma ketone bodies and possible fluctuations therein may

also be important parameters determining whether these alternative substrates are beneficial or not. Ketone bodies have also been shown as especially beneficial for improving oxidative energy transduction in elite athletes during extreme energy consumption [10]. In general, athletes are extremely insulin sensitive and 3HB may thus not be capable of reducing insulin signaling to a worrying extent, especially if compensated by immediate exercise. However, in sedentary/prediabetic subjects, the use of ketones may not be warranted, because of aggravation of insulin resistance in the heart.

Materials and methods

Antibodies

Details on antibodies that are used for Western blotting and immunofluorescence (IF) analysis are provided in Table S1.

Isolation and culturing of primary rat cardiomyocytes

Male Lewis rats, 250-300 grams, were purchased from Charles River laboratories, and were maintained at the Experimental Animal Facility of Maastricht University. Animals were housed in a controlled environment (21-22°C) on a 12:12h light dark cycle (light from 0700 to 1900h) and had free-access to food and tap water. All experiments were performed according to Dutch regulations and approved by the Maastricht University Committee for Animal Welfare.

Adult rat cardiomyocytes (aRCMs) were isolated by using a Langendorff perfusion system, as previously described [35]. Briefly, after a 2h adhesion period, aRCMs were incubated for 24 h in either low palmitate (LP, palmitate/BSA ratio 0.3:1), LP supplemented with 100nM Bafilomycin-A (BafA), LP supplemented with 3mM AcAc, LP supplemented with different concentrations of 3HB (1mM, 3mM, and 9mM), 3HB supplemented with the mixture of AA (Arg-1.36mM, Leu-1.84mM and Lys-1.56mM, AA concentration is 4 times higher in M199 medium) [3HB/AA], high palmitate (HP, palmitate/BSA ratio 3:1), HP supplemented with 3mM AcAc (HP/AcAc), or HP supplemented with 3mM 3HB (HP/3HB). aRCMs were cultured as previously described [23].

Culturing of HL-1 cardiomyocytes

HL-1 cells were kindly provided by Dr. W. Claycomb (Louisiana State University, New Orleans, LA, USA) and cultured as previously described [22]. Briefly, HL-1 cells were cultured

for 24h in either control medium (Ctrl, palmitate/BSA ratios of 0.3:1), Ctrl supplemented with 3mM 3HB, or high palmitate medium containing 500µM palmitate and 100nM insulin (HP).

Human induced pluripotent stem cell (hiPSC) maintenance and differentiation into cardiomyocytes (hiPSC-CMs)

Skin fibroblasts from healthy adult male individuals were collected and hiPSC-CMs were generated by episomal reprogramming at the Stem Cell Technology Centre, Radboudumc (Nijmegen, The Netherlands). Protocols of cell culture and differentiation into cardiomyocytes were previously described [23]. All treatments on hiPSC-CMs were performed as indicated for HL-1 cells.

Measurement of v-ATPase disassembly/assembly

Two methods were applied to measure disassembly, namely, immunoprecipitation (IP) (i) and subcellular fractionation (ii).

(i) IP: the method of IP was conducted as previously described [23]. v-ATPase a2 (V_0-a2), v-ATPase d1 (V_0-d1, an indicator of the membrane-bound V_0 sub-complex), v-ATPase B2 (V_1-B2, an indicator of cytosolic V_1 sub-complex), and mTOR proteins were detected by Western blotting.

(ii) Subcellular fractionation: the method of subcellular fractionation was also conducted as previously described [22]. For subcellular fractionation, V_0-d1, and V_1-B2, GAPDH, and caveolin-3 (Cav-3) proteins were detected by Western blotting.

Measurement of cellular chloroquine (CHLQ) accumulation as readout of v-ATPase function

[^{3}H] CHLQ accumulation assay in HL-1 cells, aRCMs, and hiPSC-CMs was measured as previously described [23].

Surface-protein biotinylation for GLUT4 and CD36

Surface-protein biotinylation was measured as previously described with the modifications described below [23]. After culturing with the medium, aRCMs were incubated for 30min (-/+) 100 nM insulin. Subsequently the cells were biotinylated with the cell-impermeable reagent sulfo-NHS-LC-biotin (0.5 mg/ml dissolved in M199 medium, Thermo Fisher Scientific, Fremont, CA, USA) for 10 min at 37°C. The rest steps of surface-protein

biotinylation assay were carried out as previously described [23]. Insulin-regulated aminopeptidase protein (IRAP, which reflects GLUT4 trafficking), and CD36 was detected by Western blotting.

Microscopic assessment of GLUT4 translocation

To further assess GLUT4 translocation, adenoviral constructs expressing hemagglutinin (HA)-GLUT4-green fluorescent protein (GFP) [a gift from Prof. Luc Bertrand's lab, Institute of Experimental and Clinical Research (IREC), Pole of Cardiovascular Research, UCLouvain, Brussels, Belgium] were employed in this study. Evaluation of GLUT4 translocation was conducted as previously described [36].

Quantification of triacylglycerol contents

Quantification of triacylglycerol in aRCMs was performed using a Triglyceride Assay Kit (ab65336, Abcam, San Francisco, CA, USA) following the manufacturer's instructions.

Determination of insulin signaling

Insulin signaling in aRCMs were measured as previously described [23]. Briefly, after culturing, aRCMs were exposed to 100nM insulin for 30 min to be able to compare basal-phosphorylation to insulin-stimulated phosphorylation. Afterwards, these cells were lysed in sample buffer and used for protein detection by SDS-polyacrylamide gel electrophoresis, followed by Western blotting. Phospho-Ser2448-mTOR (p-mTOR), phospho-Ser473-Akt (pAKT), phospho-AS160, phospho-Ser235/236-S6 (pS6), total-mTOR, total-AKT, GAPDH, and caveolin-3 proteins were detected by Western blotting.

Measurement of substrate uptake

[^{3}H]deoxyglucose and [^{14}C]palmitate uptake in aRCMs and hiPSC-CMs were measured as previously described [23].

Measurement of cardiomyocyte contraction dynamics

Contractile properties of aRCMs were assessed at 1 Hz field stimulation using a video-based cell geometry system to measure sarcomere dynamics (IonOptix, Milton, MA, USA). From the digitized recordings acquired with IonWizard acquisition software, the following parameters were calculated: sarcomere shortening, time to peak, and decay time. This was conducted as previously described [22].

Immunofluorescent staining for pluripotency markers in hiPSC-CMs

Immunofluorescent staining for pluripotency markers (e.g., SSEA4 and OCT4) in hiPS-CMs was conducted as previously described (Chapter 3).

QPCR analysis for pluripotency markers in hiPSC-CMs

QPCR analysis for pluripotency markers (OCT4, SOX2, NANOG, and LIN28) in hiPS-CMs was conducted as previously described (Chapter 3). **Table S2** displays primers for pluripotency genes in hiPSC-CMs.

Statistics

All data are presented as means ± SEM. Statistical analysis was performed by one-way ANOVA using SPSS (Version 25.0 for WINDOWS; SPSS Inc, Chicago, USA). *P*-values of less than 0.05 are considered statistically significant.

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Author's Contributions: SW analyzed data, SW, LW, A Sun and A Strzelecka performed the experiments, JL, DM, MN and JG designed the experiments, SW, JL, DM, MN and JG wrote the manuscript. All authors discussed the data and commented on the manuscript before submission.

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Conflicts of Interest: The authors declare no conflict of interest.

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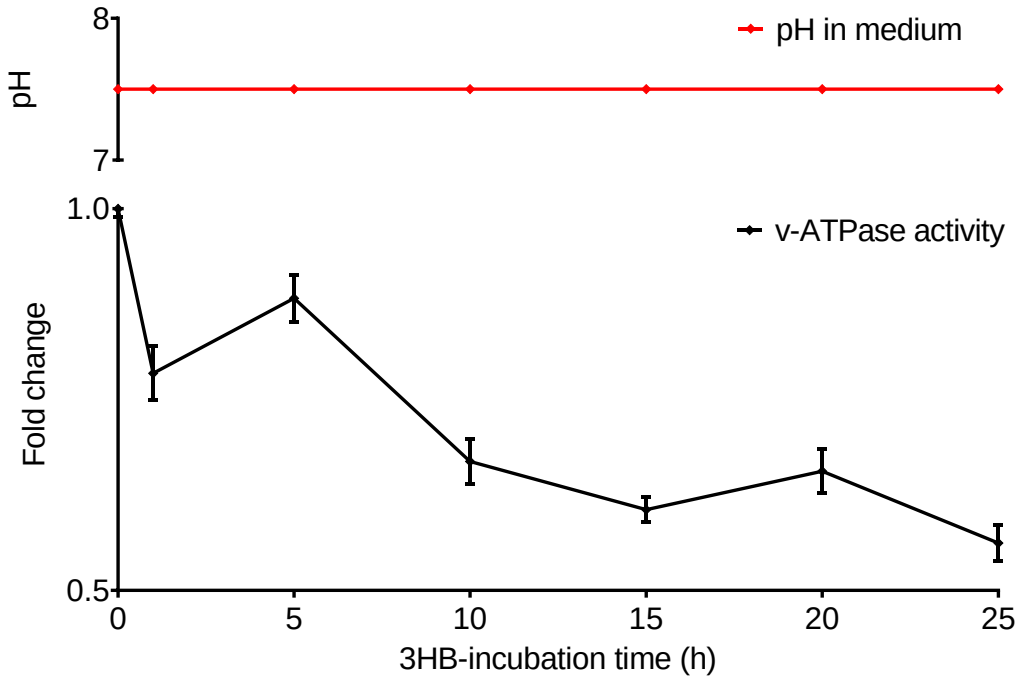
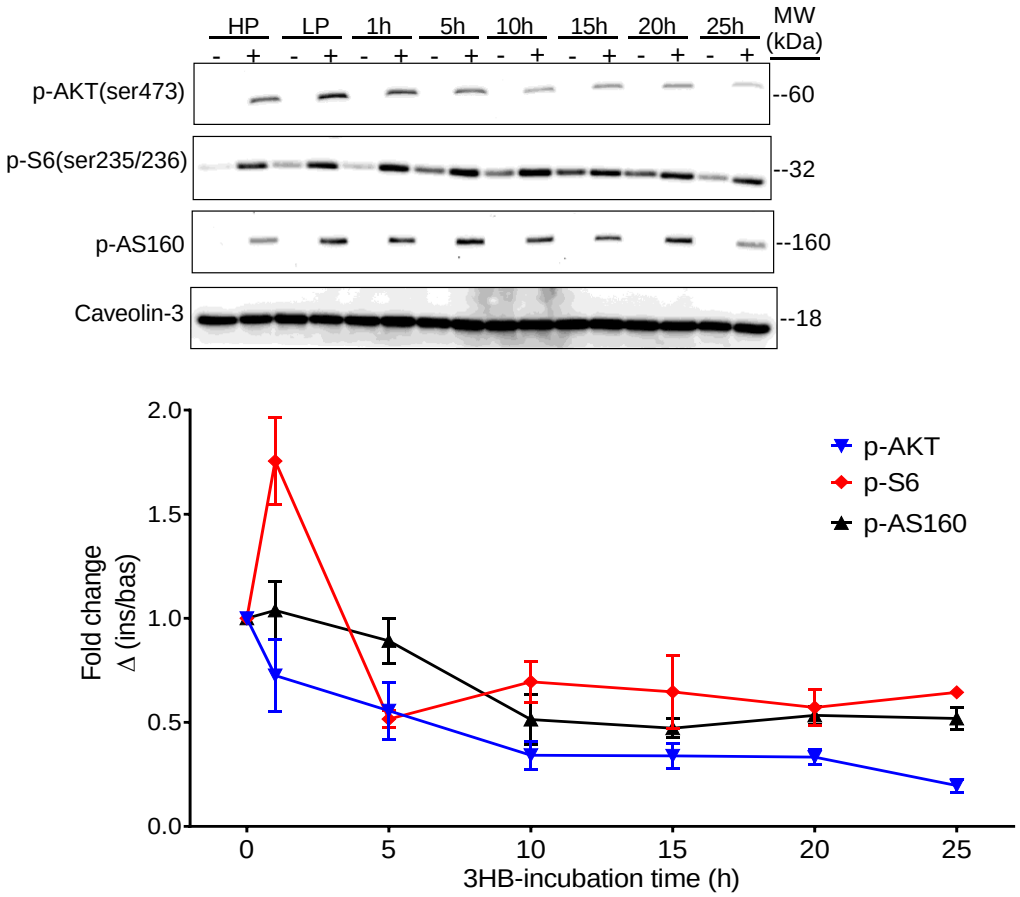
Supplementary data



Supplemental Figure 1 (related to Figure 1A).

The time course of 3HB induced-effect on v-ATPase activity in adult rat cardiomyocytes (aRCMs)**:** aRCMs were incubated with low palmitate (LP, palmitate/BSA ratio 0.3:1) and high palmitate (HP, palmitate/BSA ratio 3:1) for 25 h, and 3mM of 3-β-hydroxybutyrate (3HB) was added at the start of the LP culturing for 0, 1, 5, 10, 15, 20 and maximum 25 h. After the culturing, cells were used for a [3H] chloroquine (CHLQ) accumulation assay which lasted 20 min (n>4). Values are displayed as mean ± SEM.



Supplemental figure 2 (Related to Figure 3).

The time course of 3HB exposure interferes with insulin signaling in adult rat cardiomyocytes (aRCMs)**:** aRCMs were incubated with low palmitate (LP, palmitate/BSA ratio 0.3:1) and high palmitate (HP, palmitate/BSA ratio 3:1) for 25 h, and 3mM of 3-β-hydroxybutyrate (3HB) was added at the start of the LP culturing for 0, 1, 5, 10, 15, 20 and maximum 25 h. After the culturing, aRCMs were followed by 30 min of (-/+) insulin (100 nM) incubation. Representative blots and its quantification of phospho-Ser473-Akt (pAKT), phospho-AS160, phospho-Ser235/236-S6 (pS6), and Cav-3 (loading control) (n=3). Values are displayed as mean ± SEM. *p<0.05 were considered statistically significant.

Table S1. Antibodies used in this study.

Antibodies	Source	Identifier
p-AKT (Ser473)	Cell signaling	#9271
Total-AKT	Cell signaling	#9272
p-mTOR (Ser2448)	Cell signaling	#2971
Total-mTOR	Cell signaling	#2972
pS6 (Ser235/236)	Cell signaling	#4856
Insulin-regulated aminopeptidase (IRAP)	Cell signaling	#MABN483
GAPDH	Cell signaling	#2118
Anti-ATP6V_0D1 (V0d1)	Abcam	#ab202899
Anti-ATP6V_1B2 (V1B2)	Abcam	#ab73404
Anti-ATP6V0A2 (V0-a2)	Abcam	#ab82638
HA-Tag (C29F4)	Abcam	#3724(S)
CD36 (MO25)	Gift from Dr.N.Tandon	No
GLUT4	Millipore	#07-1404
Caveolin-3	BD transduction Laboratories	#610421
SSEA4	Thermo Fisher Scientific	MA1-021
OCT4	Thermo Fisher Scientific	# MA1–104

Table S2. Primers for pluripotency genes in human induced pluripotent stem cells.

Pluripotency genes	Species	Primer sequence	Melting temperature (°C)	Fwd/Rev
SOX2	Human	CCCAGCAGACTTCACATGT	60	fwd
SOX2	Human	CCTCCCATTTCCCTCGTTTT	60	rev
OCT4	Human	CCTCACTTCACTGCACTCTA	60	fwd
OCT4	Human	CAGGTTTTCTTTCCCTAGCT	60	rev
NANOG	Human	CTCCATGAACATGCAACCTG	60	fwd
NANOG	Human	GGCATCATGGAAACCAGAAC	60	rev
LIN28	Human	CACAGGGAAAGCCAACCTAC	60	fwd
LIN28	Human	TGCACCCTATTCCCACTTTC	60	rev

Chapter 5

General discussion

Main findings of the thesis

In this thesis, we investigated the possible role of v-ATPase reassembly in lipid-induced insulin resistance and contractile dysfunction, with focus on the underlying molecular mechanism. Meanwhile, we also examined v-ATPase as a potential target against cardiac metabolic diseases such as diabetic cardiomyopathy. We aimed to find nutritional strategies to reassemble v-ATPase in the lipid-overloaded heart. The main findings of the thesis are:

1. Forced glucose availability, as achieved through high glucose addition or adenoviral overexpression of PKD1, reverses lipid-induced disassembly and de-activation of v-ATPase in cardiomyocytes **(Chapter 2)**.
2. Re-activating v-ATPase via increased glucose availability triggers endosomal acidification in lipid-overloaded (rodent/human) cardiomyocytes, thus leading to endosomal CD36 retention and decreased lipid accumulation which, in turn, improves insulin-stimulated glucose uptake and contractile function **(Chapter 2)**.
3. In (rodent/human) cardiomyocytes, addition of amino acids (AA), i.e., a mixture of arginine, leucine, and lysine, results in activation of mTOR, which then binds to the V_1 part of v-ATPase, thereby facilitating V_1 to re-assemble with V_0. The subsequent re-assembly with mTOR into an mTOR – v-ATPase super-complex will lead to v-ATPase activation and to increased endosomal acidification **(Chapter 3)**.
4. The increased endosomal acidification elicited by the AA cocktail (arginine/leucine/lysine combination) subsequently inhibits CD36-mediated lipid uptake and metabolism, thus preventing cardiomyocytes from lipid overload-induced insulin resistance and contractile dysfunction **(Chapter 3)**.
5. In (rodent/human) cardiomyocytes, chronic exposure to 3-β-hydroxybutyrate (3HB) results in v-ATPase disassembly, and subsequently to decreased v-ATPase activity and thus loss of endosomal acidification **(Chapter 4)**.
6. Chronic 3HB exposure stimulates CD36-mediated lipid uptake and metabolism via the inhibition of v-ATPase function, resulting in a loss of insulin sensitivity and contractile function in (rodent/human) cardiomyocytes **(Chapter 4)**.

In summary, besides lipid (e.g., palmitate), other nutrients including glucose **(Chapter 2)**, AA (e.g., arginine, leucine, and lysine) **(Chapter 3)**, and ketone bodies (e.g., 3HB) **(Chapter 4)** can be added to the list of metabolites/nutrients regulating v-ATPase function. Together, this suggests that in the heart v-ATPase integrates nutritional information (Figure 1).

Accordingly, v-ATPase re-assembly by increased glucose availability and/or AA supplementation is employed as a target to restore lipid-induced insulin resistance and cardiac contractile dysfunction in the diabetic heart **(Chapter 2 and 3)**. Our novel findings will be discussed in a broader perspective, in the context of the crosstalk between v-ATPase reassembly, impaired energy metabolism, and cardiac dysfunction. In addition, possible molecular mechanisms by which the assembly/disassembly cycle of v-ATPase responses to changes in nutrient availability (e.g., palmitate, glucose, amino acids, and ketone bodies) will be further discussed. A growing of evidence indicate that ketone bodies have multiple beneficial effects on cardiac energy metabolism in elite athletes [1] as well as patients with diabetes, heart failure and atrial fibrillation [2-4], it currently remains unclear whether changes in their use by the diseased heart are beneficial or detrimental for cardiac functioning. In **Chapter 4**, we tested the potential effects of ketone bodies on cardiac fatty acids (FA)/glucose handling by cardiomyocytes. The results suggest that chronic exposure to ketone bodies (e.g., 3HB) increase CD36-mediated FA uptake, and then results in a loss of insulin sensitivity, and of contractile function in cardiomyocytes. Hence, it is questioned whether ketone bodies are act as a double-edged sword for the diabetic heart?

Finally, based on our current results, future perspectives are given as to how the presented work could be followed up.

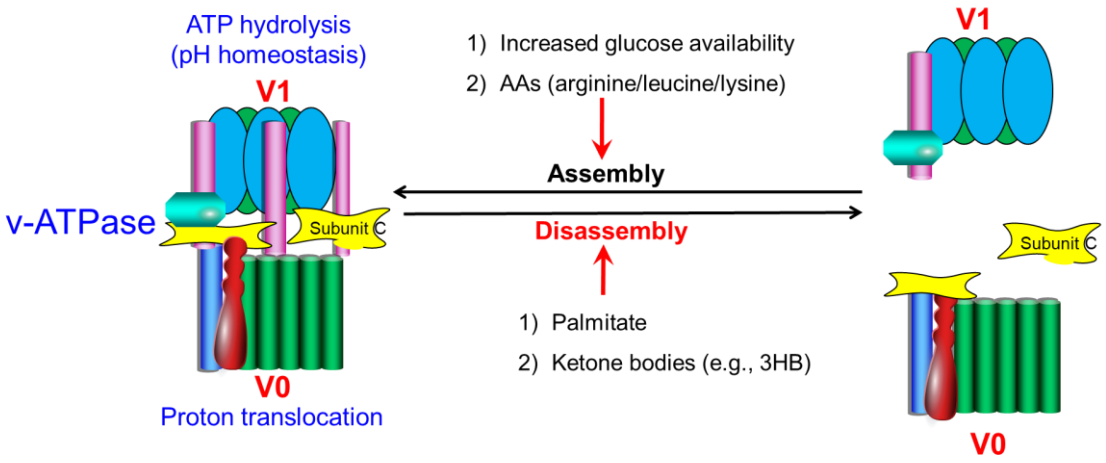


Figure 1. Assembly/disassembly cycles of v-ATPase in response to nutrient availability in the heart.

V-ATPase is a therapeutic target to rebalance cardiac substrate utilization in diabetic cardiomyopathy

As earlier established, chronic v-ATPase V_0/V_1 disassembly, induced by excess lipid exposure, sets the heart on a road to the development of insulin resistance and eventually diabetic cardiomyopathy [5, 6]. In view of the putative mechanism of nutrients affecting v-ATPase assembly outlined above, v-ATPase might be a promising therapeutic target to rebalance cardiac substrate utilization in diabetic cardiomyopathy. Intriguingly, several studies in yeast and mammalian kidney cells revealed that assembly/disassembly cycles of v-ATPase were found to be regulated by glucose availability, as glucose deprivation caused v-ATPase disassembly, whereas glucose-enriched conditions favored assembly and hence restoration of organellar acidification [7-9]. According to this, in **Chapter 2** we first confirmed that increased glucose availability, which was achieved through high glucose addition or adenoviral overexpression of PKD1, reverses disassembly and de-activation of v-ATPase in lipid-overloaded (rodent/human) cardiomyocytes. Next, this increased v-ATPase function leads to endosomal acidification, then induces endosomal CD36 retention and decreases lipid accumulation to improve insulin-stimulated glucose uptake and contractile function **(Chapter 2)**. Of course, high glucose addition by itself in a diabetic setting could be a truly counter-intuitive strategy to restore lipid-induced insulin resistance and lipid-induced contractile dysfunction. High glucose addition was therefore employed to provide our first proof-of-principle for assessing strategies to restore lipid-induced insulin resistance and lipid-induced contractile dysfunction via v-ATPase re-assembly. Other strategies to employ glucose-induced v-ATPase re-assembly uptake, i.e., adenovirus-mediated overexpression of wild-type PKD1, would be more favorable in the diabetic setting because it stimulates glucose entry into cardiomyocytes via stimulation of contraction-responsive GLUT4 translocation (without affecting CD36 translocation), and it would even lead to a decrease in circulating glucose [10]. In this context, also the development of specific PKD1 activators would hold promise for future therapy in the diabetic heart.

In addition to glucose being able to regulate v-ATPase function, v-ATPase was shown to be involved in AA regulatory pathways. AA are well known to initiate the activation of mTOR, a master regulator of anabolic pathways. V-ATPase was shown to provide an essential docking platform that is required for AA-induced activation of mTOR [11]. We therefore wanted to investigate whether AA addition induces v-ATPase re-assembly in an mTORC1-dependent manner and whether AA could thus reverse lipid-induced insulin resistance and lipid-induced

contractile dysfunction. The data presented in **Chapter 3** indicates that long-term (24h) addition of a mixture of AA [4*AA (arginine-1.36mM, leucine-1.84mM and lysine-1.56mM), 4x its low-physiological concentration)] in lipid-overloaded cardiomyocytes leads to mTOR activation, which then can bind to the V_1 subcomplex of v-ATPase, thereby allowing the V_1 subcomplex to re-assemble with the V_0 subcomplex. Reciprocal to the previous observation that v-ATPase is a scaffold for mTOR activation, the data presented in **Chapter 3** proposes that mTOR activation can induce v-ATPase activation, suggesting that the relationship between v-ATPase and mTORC1 operates in both directions. Thereafter, this reactivated mTOR–v-ATPase super-complex results in endosomal acidification, and thus inhibits CD36-mediated lipid uptake and metabolism to prevent cardiomyocytes from lipid overload-induced insulin resistance and contractile dysfunction. Hence, long-term addition of 4*AA prevents the loss of insulin sensitivity, eluding to the perspective that the AA cocktail (arginine, leucine, and lysine) could be tested as prevention of against lipid-induced cardiomyopathy in future studies. Interestingly, when each of the AAs were added long-term at (1x) concentrations, similar as occurring in DMEM-F12, v-ATPase activity was partly inhibited (Chapter 3, Fig 1A). This was in agreement with earlier work [12]. As part of an explanation, it is possible that under AA-deprivation (defining the control condition), high autophagic proteolytic flux would increase lysosomal AA contents, which would support mTORC1 – v-ATPase interaction via the lysosome-centric inside-out model of AA sensing [11]. This would then explain the relatively high level of luminal acidification under these control conditions. Low concentrations of added AA would be sufficient to inhibit autophagy and production of intra-lysosomal AA and thereby prohibit this inside-out signaling, while at the same time their cytoplasmic concentrations do not reach sufficient levels to activate mTOR via cytoplasmic AA sensing. This lack of mTOR activation at 1xAA then leads to low v-ATPase activity and low endosomal acidification. In contrast, 4xAA addition (leucine) would lead to such cytoplasmic AA concentrations that exceed the threshold level for induction of cytoplasmic AA sensing, while at the same time the cytoplasmic AA concentrations of basic AA (arginine/leucine) would be sufficiently high to result in lysosomal weak base trapping, so to simultaneously increase the AA sensing via the endo/lysosomes. We also asked ourselves the question whether short-term addition (1h) of 4*AA also induces v-ATPase reassembly and restores lipid-induced insulin resistance and lipid-induced contractile dysfunction. Unfortunately, in this respect, short-term addition (1h) of the 4*AA mixture did not have such effects (Figure 2A).

Next, we tested whether short-term addition (1h) of our cocktail of the three AA, but then at higher concentrations [HAA: 10mM-arginine, 12mM-leucine, and 7mM-lysine] restored lipid-induced insulin resistance in cardiomyocytes via v-ATPase re-assembly. Regarding a chosen concentration of HAA (10mM-arginine, 12mM-leucine, and 7Mm-lysine), they were employed to this thesis according to previous studies that found these AAs have beneficial effects on insulin sensitivity in pre-diabetic/diabetic states [13-16]. Indeed, short-term addition (1h) of the HAA cocktail restores lipid-induced v-ATPase disassembly (Figure 2B-C), and subsequently preserved insulin-stimulated p-AKT (Figure 2D) and insulin-stimulated glucose uptake (Figure 2E-G) in rodent/human cardiomyocytes. Although these specific AA that act through v-ATPase re-assembly offer a simple and effective treatment to cure lipid-induced cardiomyopathy *in vitro*, the putative beneficial effects of these AA on rats fed a high-fat diet still needs to be tested.

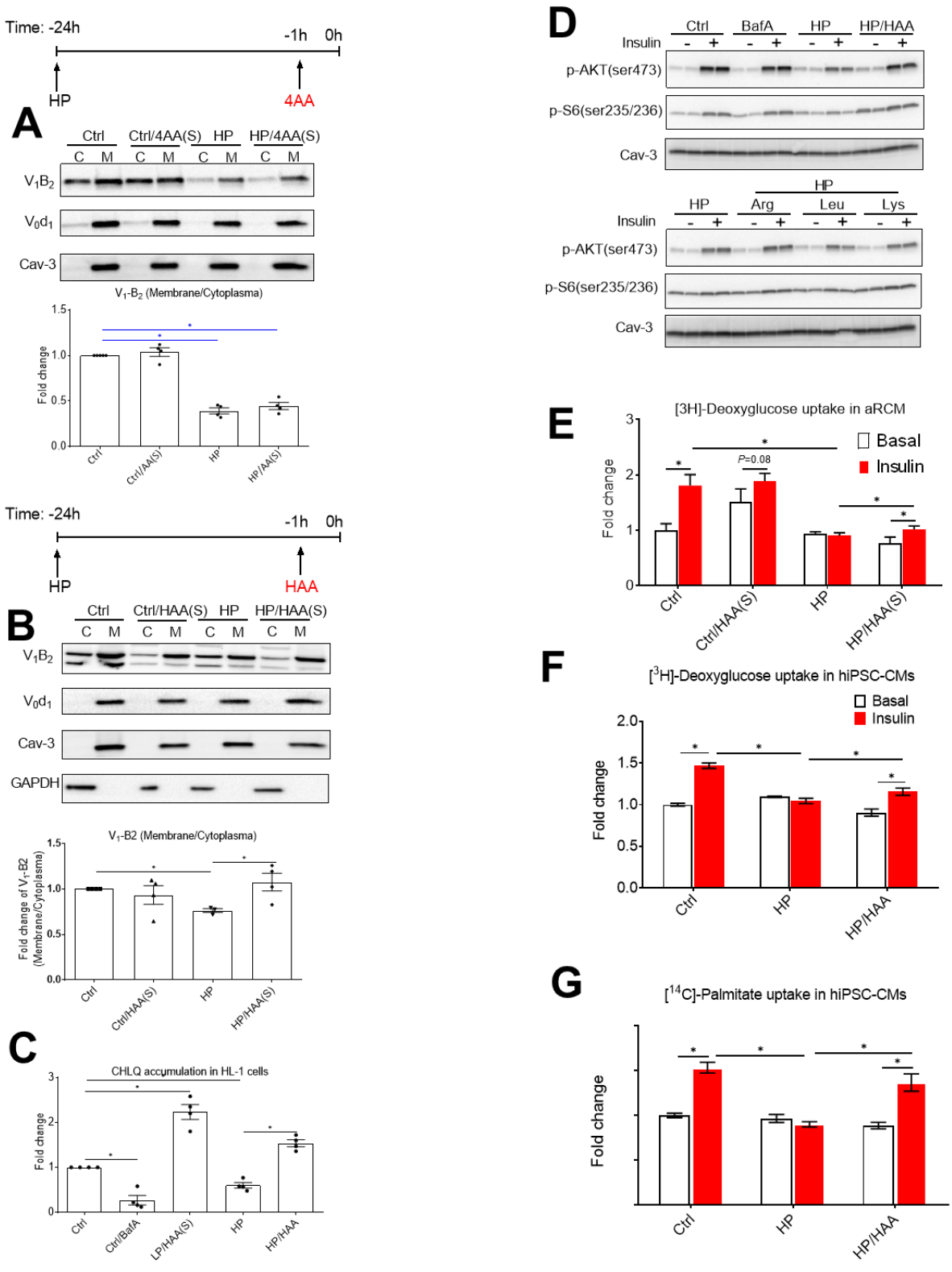


Figure 2. HAA (a short-term addition)-induced v-ATPase re-assembly partially restores lipid-induced insulin resistance in cardiomyocytes.

(A-B) Fractionation in HL-1 cells: (A) HL-1 cells were incubated for 24h with either Ctrl, or HP medium, followed by (-/+) 4*AA addition (4AA: Arg/Leu/Lys combination, Arg-1.36mM, Leu-1.

84mM and Lys-1.56mM, AA concentration is 4 times higher in DMEM-F12 medium) for 1h. **(B)** HL-1 cells were incubated for 24h with either Ctrl, or HP medium, followed by (-/+) short-term addition (1h) of H*AA cocktail (HAA: the mixture of HAA containing 10mM-Arg, 12mM-Leu, and 7mM-Lys). Representative blotting and its quantification of V_0d1 and V_1B2 is shown. Caveolin-3 and GAPDH were detected as the loading control of V_0d1 and V_1B2, respectively (n=4).

(C) HAA (a short-term addition) increases v-ATPase activity in lipid-overloaded cardiomyocytes: HL-1 cells were first subjected to Ctrl medium, or HP medium (500μM palmitate and 100nM insulin) for 20h, followed by (-/+) HAA for 1h. After the culturing of all conditions above, cells were sub for [^{3}H] CHLQ accumulation assay last 20 min (n=4).

(D) Insulin sensitivity in lipid overloaded cardiomyocytes: HL-1 cells were incubated for 20h with either Ctrl, or HP medium, followed by (-/+) BafA (100nM), H*Arg (10mM), H*Leu (12mM), H*Lys (7mM), and H*AA addition for 1h. Subsequently, cells were stimulated (-/+) insulin (200 nM) for 30 min and harvested for Western blotting analysis. Representative blots of pAKT and pS6, Caveolin-3 was detected as the loading control (n=3).

(E-G) [^{3}H]deoxyglucose and [^{14}C] palmitate uptake in lipid-overexposed (rodent/human) cardiomyocytes: Cells were incubated for 20h with either Ctrl, or HP medium, followed by (-/+) HAA addition for 1h prior to 30 min of (-/+) insulin (200 nM) stimulation. After the culturing of all conditions above, cells were sub for [^{3}H] deoxyglucose uptake assay last 10 min (n=3). Values are displayed as mean ± SEM. *p<0.05 were considered statistically significant.

Currently, we are testing whether *in vivo* dietary AA supplementation is an effective approach to stimulate v-ATPase assembly thereby decreasing lipid accumulation and restoring cardiac function in high-fat diet fed rats **(Figure 3)**. The primary outcome parameters from this ongoing study include: *in viv*o: 1) cardiac function and structure (e.g., fractional shortening, left ventricular posterior wall thickness via echocardiography); ex vivo: 2) AA signaling pathway [e.g., phosphorylated mammalian target of rapamycin (p-mTOR), and phospho-S6 ribosomal protein (p-S6) via Western blotting]; 3) cardiac lipid accumulation (e.g., myocardial triglyceride content via lipid assay kit); 4) v-ATPase function (e.g., the protein contents of super-complex v-ATPase V_0 and v-ATPase V_1 via co-immunoprecipitation).

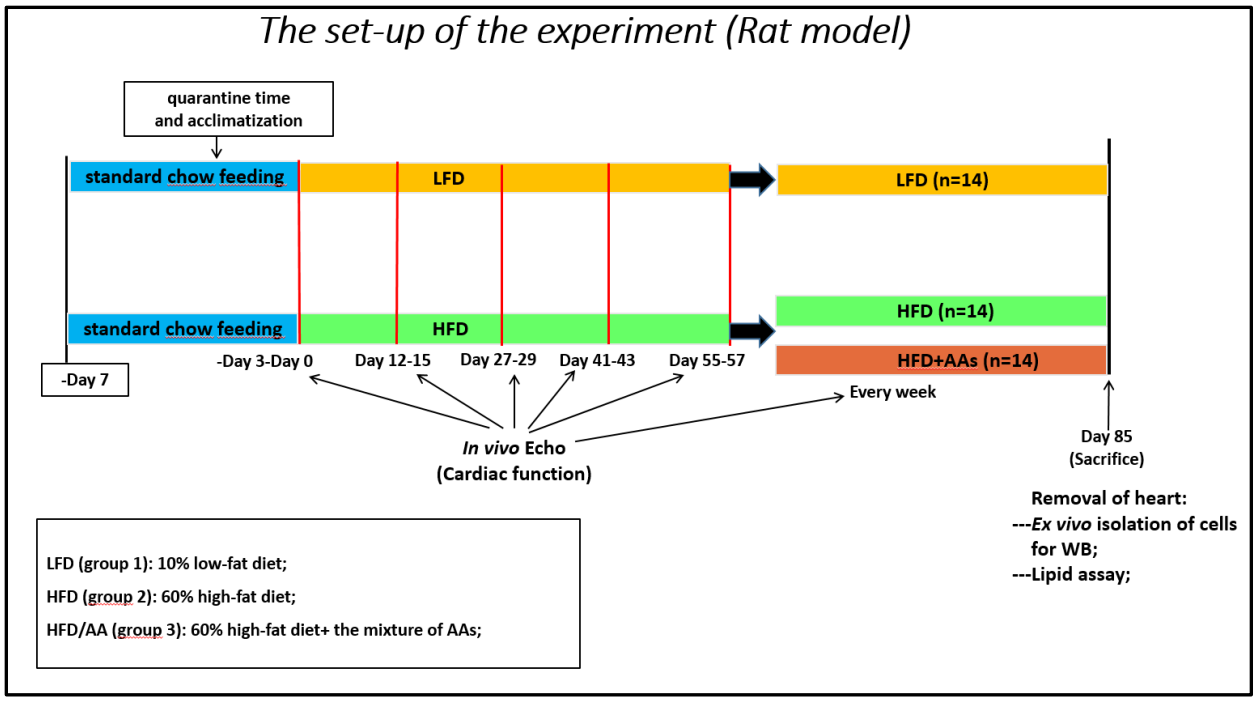


Figure 3. Experimental design of *in vivo* dietary amino acid supplementation experiment.

Possible mechanisms regulating v-ATPase function in response to nutrients in the heart

The results of **Chapter 2–4** suggest that v-ATPase integrates nutritional information, including lipid (e.g., palmitate), glucose, AA (e.g., arginine, leucine, and lysine), and ketone bodies (e.g., 3HB) in the heart. Therefore, this finding raises the question of how these nutrients are involved in the disassembly/assembly cycle of v-ATPase in the heart, and what constitutes the underlying molecular mechanism.

Consistent with our previous findings [5], the results of this thesis **(Chapters 2, 3, and 4)** further confirm that high palmitate exposure inhibits v-ATPase function via its disassembly into the V_0 and V_1 sub-complexes. In this respect, we searched for a feasible answer to explain how lipids cause v-ATPase disassembly. A possible explanation might include the involvement of AMPK in the control of v-ATPase assembly. As earlier reported, AMPK activation precedes increased v-ATPase activity, and this is prevented by the AMPK inhibitor dorsomorphin [17]. Specifically, AMPK, together with the scaffolding protein Axin, is recruited to the same lysosomal v-ATPase-Ragulator complex under low energy conditions, where it is activated by phosphorylation of liver kinase B1 [18, 19]. When intramyocellular fatty acids (FA) levels are high in obesity and type 2 diabetes (T2D), there may be lipotoxic effects that either suppress maximal AMPK activity or prevent this pathway from exerting its antidiabetic effects [20],

allowing lipid-induced v-ATPase disassembly. Given the role of v-ATPase in controlling AMPK activation, further studies are warranted to clarify the exact regulation of v-ATPase disassembly by lipid-mediated AMPK pathway in cardiomyocytes.

Interestingly, like fatty acids, ketone bodies (e.g., 3HB) also are sensed by v-ATPase through disassembly of V_1 from V_0 and disappearance of V_1 into the cytoplasm, and this change also results in a decreased proton pump activity of v-ATPase and loss of endosomal acidification **(Chapter 4)**. With respect to this link between v-ATPase and ketone bodies, virtually no information is available in the scientific literature. Interestingly, several studies reported that ketone body exposure inhibits mTOR pathway signaling in the brain and liver of healthy rats, and then prevents late hippocampal mTOR activation [21, 22]. In chapter 4 we revealed that chronic 3HB exposure results in deactivation of mTOR. As discussed earlier, an association between mTOR activation and v-ATPase activation operates in both directions, it suggests that the 3HB-induced deactivation of mTOR on the mTOR–v-ATPase axis also works in the opposite direction to deactivate v-ATPase. Further studies are needed to clarify whether ketone bodies regulate the assembly/disassembly of v-ATPase in an mTORC1-dependent manner.

As described In **Chapter 2** and mentioned before in the general discussion, increased glucose availability reversed lipid-induced v-ATPase disassembly and de-activation in cardiomyocytes. In a search for potential protein partners that interact with v-ATPase, the glycolytic enzyme aldolase has been shown to bind directly to v-ATPase [23-25]. V-ATPase disassembly observed in aldolase deletion mutant cells can be restored to normal levels by aldolase complementation, indicating direct coupling of glycolysis to the proton pump [23]. Accordingly, the binding of aldolase to the v-ATPase provides the cell with a means for localized ATP generation by glycolysis [25]. The interaction between aldolase and v-ATPase increased dramatically in the presence of glucose, thereby indicating that aldolase acts as a glucose sensor for v-ATPase regulation [23, 24]. Future studies should investigate that the possible roles of the glycolysis in mediating v-ATPase assembly and activity in the heart.

The data presented in **Chapter 3** indicated that addition of AA (e.g., arginine, leucine, and lysine) leads to v-ATPase assembly in the heart. This finding raises the question of how these AA regulate the assembly of v-ATPase? As discussed above (page 6) v-ATPase is a scaffold for mTOR activation [11], and mTOR activation can also induce v-ATPase activation (**Chapter 3**). In more detail, V-ATPase has been shown to directly associate with the Ragulator complex on the lysosomal membrane. The Ragulator serves as a platform for the Rag-GTPases,

which, when activated by the presence of AA, recruit mTORC1 to lysosomes, allowing it to be activated by lysosomally localized Ras homolog enriched in brain (Rheb) [11, 26]. Although this mechanism by which AA signaling to mTORC1 begins within the lysosomal lumen communicating to v-ATPase was clearly identified, the precise AA sensors (e.g., arginine, leucine, and lysine) that stimulates v-ATPase are unknown yet. First, not mTORC1 localisation but Ragulator localisation at the lysosomal surface is triggered by arginine via SLC38A9 (the lysosomal AA transporter) [27, 28]. Lysine mimicked the effect of arginine on the interaction of SLC38A9 with Ragulator [28]. Leucine independently activates mTOR in the cytoplasm through the leucine sensor Sestrin2, and then mTOR anchors to Ragulator at the lysosomal surface. Accordingly, Ragulator must first localize to the lysosome before leucine can stimulate mTORC1 via Ragulator. Therefore, lysine and arginine on one hand and leucine on the other hand independently and synergistically stimulate mTOR activation. It also follows logically that leucine does not stimulate mTORC1 if applied in absence of arginine and lysine. Finally, mTORC1 activation by the AA, including arginine, leucine, and lysine, re-assembles v-ATPase in the lipid-overloaded cardiomyocytes **(Chapter 5)**. In this respect, genetics strategies, i.e., knockdown of v-ATPase subunits and AA transporters, are needed to further under the effects of the AA cocktail on v-ATPase function. For future perspectives, further studies on the AA-induced m-TORC1–v-ATPase action, which involves a great number of other proteins, including Rag-GTPases, adaptor proteins and lysosomal AA transporters (for review see [29, 30], may reveal that several of these other proteins may offer additional, even better druggable, targets to combat lipid-induced cardiomyopathy.

Ketone bodies: a double-edged sword for the diabetic heart?

The fourth type of nutritional substrate, after lipids, glucose and AA, of which we tested the effect on v-ATPase function were the ketone bodies. In healthy individuals, the circulating concentration of ketone bodies (e.g., 3HB) is around 0.2 mM, whereas during prolonged starvation and uncontrolled diabetes it increases to above 10 mM [31, 32]. The latter has to do with increased oxidation of FA in T2D patients, which results in accelerated ketone body (AcAc, 3HB, and acetone) production by the liver [2]. Ketone bodies are suggested to be efficient and rapid fuels for key extrahepatic oxidative tissues like the heart. Increased cardiac uptake of ketone bodies can be seen as an adaptive and compensatory response to energy deficit (e.g., starvation and prolonged exercise), calorie deprivation, and to the impaired FA/glucose

metabolism as seen in as part of the clinical manifestations of diseases (e.g., heart failure and uncontrolled diabetes) [3, 4, 33, 34]. Administration of 3-hydroxybutyrate (3-HB) to patients with heart failure and reduced ejection fraction increased cardiac output and improved left ventricular ejection fraction [3]. Additionally, observations in diabetes therapy, with SGLT2 inhibitor drugs, suggested a positive link between beneficial heart failure outcomes and increased levels of ketone bodies [35]. The effects of SGLT2 inhibitors on the body are however multifactorial and the precise underlying mechanisms explaining the positive cardiac effects remain unclear. Additionally, excessive levels of blood ketone bodies can lead to acidosis (ketoacidosis) [36, 37]. The data presented in **Chapter 4** indicated that chronic 3HB exposure impairs CD36-mediated lipid metabolism, insulin sensitivity and contractile function in cardiomyocytes, suggesting that ketones bodies (at least 3HB) should not be provided as neutraceuticals to everybody. This finding raises a question of why 3HB can be simultaneously beneficial (as an energy substrate) and detrimental (as the onset of insulin resistance).

Cox et al. (2016) reported that ketone drinks are especially beneficial for improving oxidative energy transduction in elite athletes during extreme energy consumption [1], when they are extremely insulin sensitive. Therefore, it would be impossible for 3HB to push the impact of insulin on signaling/metabolism to the complete opposite, especially during extreme exercise. Due to the aggravation of insulin resistance, the use of ketones in sedentary/prediabetic subjects is not warranted. When viewed in this way, sedentary/prediabetic subjects on a ketogenic diet should be under strict medical supervision because the ketones can significantly result in insulin resistance. Thus deciphering the mechanism behind ketone bodies-induced insulin resistance could prove useful in furthering our understanding of insulin resistance and associated diseases (e.g., T2D) [31].

Concluding remarks and future perspectives

According to the data of **Chapters 2** and **3**, v-ATPase assembly by both increased glucose availability and AA addition might form the basis of novel therapies to combat lipid-induced cardiomyopathy. At present, v-ATPase is a currently underexplored protein, which is composed of >14 subunits, several of which exist as more than one isoform, thus its complexity results in the lack of insights into the regulatory mechanisms in v-ATPase function. In addition, endosomal acidification not only depends on v-ATPase-mediated proton pumping, but also on restriction of proton leakage. Although such proton leak in endosomes could be demonstrated

upon pharmacological inhibition of v-ATPase [38], little is known about the identity of the proteins responsible for this leak. Furthermore, still emerging is the importance of the v-ATPase in controlling the activity of various signaling pathways, including Wnt, Notch, mTOR, and AMPK. As we come to better understand the mechanisms of regulating v-ATPase assembly and trafficking, new possibilities for therapeutic intervention in these signaling pathways arise.

Moreover, the potential roles of v-ATPase reassembly in lipid-induced insulin resistance and contractile dysfunction also should be investigated in other tissues (e.g., brain, skeletal muscle, and liver) in addition to the heart. Skeletal muscle and heart show a simliar mechanism of CD36-mediated lipid metabolism. In this respect, further work is still needed to verify whether v-ATPase trafficking, assembly, and function in the control of lipid metabolism operates similarly in skeletal muscle and other tissues .

Additionally, the data presented in Chapter 4 indicate that 3HB exposure disturbs cardiac FA /glucose metabolism by v-ATPase disassembly. In the heart, the specific receptor/sensors of 3HB remain unclear. Most recently, 3HB acts as a signaling molecule on FA metabolism via G protein-coupled receptors (i.e., GPR109A and GPR41) in the brain [39], further studies on the 3HB-mediated FA/glucose metabolism via v-ATPase is still needed to knock down these G protein-coupled receptors in the heart.

On the basis of *in vitro* data in this thesis, v-ATPase is confirmed to integrate nutritional information, including glucose, palmitate, AA, and ketone bodies, however, we still need to test the potential effects of these nutrients, especially for AA and ketone bodies, on v-ATPase function in the diabetic heart *in vivo*. Indeed, there is a great need for detailed studies in diabetic cardiomyopathy in the clinical setting to extrapolate the current findings in experiment animals, isolated heart and isolated cardiomyocytes to the diabetic patient. The collected data in this thesis will lead to an increased understanding of the processes underlying diabetes and will eventually lead to better possibilities for treating patients suffering, among others, from diabetic cardiomyopathy.

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Appendices

Summary

Samenvatting

总结

Valorization

Acknowledgments

Curriculum vitae

Summary

Under normal physiological circumstances, the heart is a metabolically flexible organ that can utilize a wide range of substrates for energy provision, and therefore can be regarded as an omnivore. To meet it's immediate energy needs, the healthy adult heart mainly uses long-chain fatty acids (FA) and glucose, whereas it also may shift its energy substrate preference to alternative substrates like amino acids (AA) and ketone bodies when being exposed to physiological or pathological stimuli. Recent studies from our lab have shown that vacuolar-type H^+-ATPase (v-ATPase) is closely linked to cardiac energy metabolism. V-ATPase, a multimeric protein complex (>14 subunits), is structurally divided into a cytosolic V_1 sub-complex and a transmembrane V_0 sub-complex, encompassing the ATP catalyzing activity and a rotary mechanism so as to maintain endosomal acidification, respectively. Notably, assembly/disassembly cycles of the V_1 and V_0 sub-complexes is rapid and reversible, so that it leads to rapid modulation of v-ATPase function. As earlier established, upon disassembly of the two sub-complexes, such as when cells are subjected to lipid oversupply or glucose withdrawal, v-ATPase function declines. Accordingly, the general aim of this thesis was to test the potential roles of cardiac energy substrates, i.e., lipid (palmitate), glucose, amino acids (AA), and ketone bodies, on the functioning of v-ATPase in the heart, either directly or indirectly. Moreover, we also investigated whether the possible roles of v-ATPase re-assembly are regulated by these energy substrates in lipid-induced insulin resistance and contractile dysfunction, with focus on unraveling the underlying molecular mechanism.

Chapter 1 provides a general introduction on the supply and utilization of the energy substrates lipid (palmitate), glucose, AA, and ketone bodies by (rodent/human) cardiomyocytes. Additionally, this chapter also describes the roles of FA transporter CD36 and glucose transporter GLUT4 in cardiac lipid accumulation, and then explains the possible mechanism by which lipids impair the function of v-ATPase thereby setting the heart on a road towards diabetic cardiomyopathy. At the end of this chapter, the aim and the outline of this thesis has been further discussed in greater detail.

Chapter 2 highlights that increased cellular glucose availability rescues lipid-induced v-ATPase impairment in rodent/human cardiomyocytes. Specifically, this restoration of v-ATPase function leads to endosomal acidification in lipid-overloaded (rodent/human) cardiomyocytes, thus resulting in a decrease of CD36-mediated lipid accumulation which, in turn, improves insulin-stimulated glucose uptake as well as contractile function. As a result,

this chapter provides the first proof-of-principle for testing potential strategies to counteract lipid-induced insulin resistance and contractile dysfunction via v-ATPase re-assembly.

Chapter 3 describes observations indicating that in (rodent/human) cardiomyocytes, addition of AA, in particular a mixture of arginine/leucine/lysine, stimulates mTOR activation, which then binds to the V_1 subcomplex of v-ATPase, thereby facilitating V_1 part to re-assemble with V_0. Given that v-ATPase is a scaffold for mTOR activation, this chapter proposes that mTOR activation also induces v-ATPase activation, suggesting that the relationship between v-ATPase and mTOR operates in both directions. Moreover, this AA-activated mTOR–v-ATPase axis leads to endosomal acidification, and thus inhibits CD36-mediated FA uptake to protect cardiomyocytes from lipid overload-induced insulin resistance and contractile dysfunction. Hence, the data presented in this chapter suggest that this specific AA-induced mechanism through v-ATPase re-assembly may offer simple and effective treatment options to combat lipid-induced cardiomyopathy.

Chapter 4 describes that chronic exposure of (rodent/human) cardiomyocytes to the ketone body 3-β-hydroxybutyrate (3HB) results in v-ATPase disassembly, and subsequently into loss of v-ATPase activity. Similarly to lipid exposure, 3HB-induced v-ATPase impairment results in the loss of endosomal acidification, followed by CD36 translocation to the sarcolemma, myocellular lipid accumulation, insulin resistance and finally contractile dysfunction, whereas all these maladaptive alterations were completely prevented by addition of the specific AA (a mixture of arginine/leucine/lysine). Hence, ketone bodies (at least 3HB) should not be provided as nutraceuticals to everybody. Regarding therapy, this chapter further confirms that this specific AA mixture offers a promising treatment option to combat lipid and ketone body-associated cardiomyopathies.

Chapter 5 provides an overview and general discussion of our main findings. In particular, our novel findings that v-ATPase reassembly appears a suitable therapeutic target to rebalance cardiac substrate utilization in diabetic cardiomyopathy were discussed in more detail. In addition, an elaborated insight into strategies [i.e., short-term addition (1h) of this specific AA mixture at high concentration] to induce v-ATPase re-assembly in the diabetic heart was given, and possible promising targets [i.e., the identification of the proteins responsible for v-ATPase-mediated proton pumping, and the investigation of various signaling pathways, including Wnt, Notch, and AMPK, involved in v-ATPase function] to restore disturbances in energy metabolism were highlighted. Finally, given that v-ATPase integrates

nutritional information in the heart, we also discuss possible upstream mechanisms regulating v-ATPase function in response to nutrients in the heart.

In conclusion, the current thesis shows that v-ATPase integrates nutritional information in the heart. The main findings of this thesis lead to a deeper understanding of fundamental processes underlying diabetes, and will eventually lead to better possibilities for treating diabetic cardiomyopathy.

Samenvatting

Het hart is een voortdurend actief orgaan dat voor zijn energievoorziening afhankelijk is van de continue aanvoer van brandstoffen. Onder normale, fysiologische omstandigheden is het hart een zogenoemde omnivoor, dat wil zeggen dat het allerlei brandstoffen als substraat voor de energievoorziening kan gebruiken. Dit wordt ook wel omschreven met de term 'metabool flexibel'. Kwantitatief zijn langketenige vetzuren en glucose de belangrijkste substraten. Daarnaast worden ook alternatieve substraten gebruikt, zoals aminozuren en ketonlichamen. Afhankelijk van fysiologische of pathofysiologische invloeden kan het aandeel van elk van deze substraten in de totale energievoorziening van het hart sterk wisselen.

In een serie recente studies is in ons lab aangetoond dat een enzym dat aanwezig is in bepaalde organellen van de hartspiercel (de endosomen) en daar verantwoordelijk is voor de zuurgraad van dit organel, een belangrijke rol speelt bij de keuze welk(e) substra(a)t(en) het hart gebruikt. Dit enzym is het *vacuolar H^+-ATPase* of kortweg v-ATPase. Dit v-ATPase is een eiwitcomplex bestaande uit 14 eenheden, en is opgebouwd uit twee deel-complexen, namelijk subcomplex V_0 dat zich in de membraan van het endosoom bevindt en het transport van H^+ ionen katalyseert, en subcomplex V_1 dat in het cytosol aanwezig is en het enzymatische deel (ATPase) bevat dat zorgt voor de aandrijving van dit ionentransport. Het enzymcomplex is actief als beide subcomplexen zijn verbonden (*assembly state*) maar wordt inactief als het V_1 subcomplex los laat en weg van het membraan het cytosol in migreert (*disassembly state*). Cycli van *assembly* en *disassembly* zijn snel en reversibel en maken het mogelijk dat de activiteit van het v-ATPase minutieus kan worden gereguleerd.

Als hartspiercellen worden blootgesteld aan bovenmatige hoeveelheden vetten (lipiden) of wanneer glucose als substraat wordt weggenomen blijkt het v-ATPase minder actief te worden (*disassembly*). Dit leidt dan tot een sterk verhoogde opname van vetzuren, zodanig dat vetzuren verreweg het belangrijkste substraat worden (>90% van de energievoorziening). Deze bevinding ligt aan de basis van dit proefschrift. Het doel van de in dit proefschrift beschreven studies was te onderzoeken hoe v-ATPase reageert, direct of indirect, op veranderingen in het aanbod van de verschillende energierijke substraten (langketenige vetzuren, glucose, aminozuren en ketonlichamen). Meer specifiek is daarbij onderzocht of deze nieuwe kennis ingezet kan worden om lipide-geïnduceerde insuline resistentie en contractiele disfunctie van hartspiercellen te voorkomen danwel te behandelen.

In **hoofdstuk 1** wordt een inleiding gegeven over de opname en het gebruik van energierijke substraten door de hartspiercel, namelijk langketenige vetzuren (zoals palmitaat), glucose, aminozuren en ketonlichamen. Speciale aandacht is er voor de vetzuurtransporter CD36 en de glucosetransporter GLUT4, omdat die een belangrijke rol spelen bij het ontstaan van diabete cardiomyopathie. Dit hoofdstuk wordt afgesloten met een overzicht van de uitgevoerde studies.

Hoofdstuk 2 beschrijft een studie waarin bij zowel rattehartspiercellen als hartspiercellen van de mens is aangetoond dat een lipide-geïnduceerde remming van de v-ATPase activiteit kan worden tegengegaan door vergroting van de intracellulaire beschikbaarheid van glucose. Het onderliggend moleculaire mechanisme is uitgezocht en is als volgt: glucose herstelt de proton transport activiteit van het v-ATPase (toename *assembly*), waardoor de endosomen zuurder worden, er minder CD36 migreert van endosomen naar het sarcolemma. Vervolgens vermindert de vetzuuropname en daarmee de intracellulaire vetzuuropslag, waardoor de GLUT4-gemedieerde glucose opname niet meer wordt geblokkeerd (opheffen insuline resistentie) en uiteindelijk de contractie van de hartspiercellen normaliseert. Deze studie verschaft *proof-of-principle* dat beïnvloeding van de v-ATPase activiteit (via verandering in de *assembly/disassembly* status) een bruikbare aanpak is voor behandeling van lipide-geïnduceerde insuline resistentie en contractiele disfunctie van het hart en dus mogelijk voor diabete cardiomyopathie.

De opzet van de studie beschreven in **Hoofdstuk 3** is analoog aan die van het voorgaande hoofdstuk maar is gericht op het effect van aminozuren, met name een mengsel van de aminozuren arginine, leucine en lysine. Dit aminozuurmengsel verhoogt de activiteit van het signaaleiwit mTOR, waarna dit bindt aan het V_1 subcomplex van v-ATPase dat daardoor gemakkelijker bindt aan het V_0 subcomplex en dan leidt tot een hogere v-ATPase activiteit. Het was al bekend dat v-ATPase nodig is voor activatie van mTOR; het feit dat het omgekeerde ook opgaat, d.w.z. mTOR activatie leidt tot v-ATPase activatie, is een nieuwe bevinding. Aangetoond is dus dat mTOR en v-ATPase elkaar wederzijds activeren.

De toepasbaarheid van deze resultaten is groot. Immers, het hart zou op eenvoudige wijze beschermd kunnen worden tegen lipide-geïnduceerde insuline resistentie en contractiele disfunctie door het toedienen van een mengsel van bepaalde aminozuren. Omdat aminozuren een natuurlijk bestanddeel zijn van onze voeding, wordt een dergelijke toepassing wel aangeduid met de term ‘nutraceutical’.

Ook de studie in **Hoofdstuk 4** is analoog opgezet aan die van de andere experimentele hoofdstukken. Hier is de blootstelling van hartspiercellen aan ketonlichamen bestudeerd. Het ketonlichaam 3- -hydroxyboterzuur (3HB) induceert *disassembly* van het v-ATPase, dus verlies van v-ATPase activiteit, uiteindelijk resulterend in vetstapeling en insuline resistentie. Deze nadelige effecten van 3HB, die geheel vergelijkbaar zijn met die van hoge concentraties lipiden, treden niet op bij blootstelling aan het ketonlichaam acetoacetaat. Ook bij deze studie kon het nadelige effect van 3HB op de v-ATPase activiteit worden tenietgedaan door toevoeging van het bovenbeschreven aminozuurmengsel arginine/leucine/lysine, en kon zodoende het ontstaan van insuline resistentie worden voorkomen.

De resultaten van de verschillende studies worden in **Hoofdstuk 5** samengevat, geïntegreerd en in detail bediscussieerd. Het endosomale enzym v-ATPase blijkt een sleutelrol te vervullen bij het ontstaan van voedingsgerelateerde insuline resistentie van hartspiercellen en diabete cardiomyopathie. Op moleculair niveau ligt de cyclus van *assembly* en *disassembly* van de subcomplexen V_0 en V_1 hieraan ten grondslag. Tevens is aangetoond dat de activiteit van v-ATPase kan worden gemanipuleerd door variatie van de aanwezigheid van de vier onderzochte energierijke substraten (langketenige vetzuren, glucose, ketonlichamen en aminozuren). Een dergelijke manipulatie van de v-ATPase activiteit kan naar verwachting worden ingezet als metabole therapie ter voorkoming en behandeling van diabete cardiomyopathie, en wellicht ook van andere metabole hartziekten zoals pathologische hypertrofie (dat gepaard gaat met een preferentieel glucoseverbruik door het hart). De ontdekking dat v-ATPase reageert op veranderingen in de aanvoer van energierijke substraten, dus in feite op veranderingen in voedingssamenstelling, zou ook in bredere zin moeten worden bestudeerd. Te denken valt aan het effect op het functioneren van andere weefsels, met name de skeletspier.

Tot besluit kan gesteld worden dat de in dit proefschift beschreven experimentele studies hebben geleid tot de identificatie in de hartspier van het endosomale enzym v-ATPase als centraal punt waar invloeden van verschillende voedingscomponenten samenkomen. Omdat tevens de betrokken moleculaire mechanismen grotendeels zijn opgehelderd, kan gerichte therapie worden opgezet ter bestrijding van cardiale insulineresistentie en uiteindelijk diabete cardiomyopathie.

总结

在正常生理情况下，心脏作为一个灵活性的代谢器官，可以利用多种能源底物为机体提供能量补给，因此心脏被视为杂食性器官。为了满足机体的能量需求，健康成年人的心脏主要以长链脂肪酸（FA）和葡萄糖能量补给为主，然而，在其他生理或者病理条件下，心脏的能量底物也来自其他物质，如氨基酸（AA）和酮体。我们实验室最新研究表明，vacuolar H^+-ATPase （v-ATPase）复合型蛋白与心脏能量代谢调控密切相关。V-ATPase 是一种多聚体蛋白质复合物（> 14 个亚基），在结构上分为胞质 V_1 区域和跨膜 V_0 区域物，它们分别具有催化 ATP 活性和维持胞内体酸化的旋转机制。值得我们注意的是，V_1 和 V_0 亚复合物的组装/分解周期是快速且可逆的过程，因此可以快速调节 v-ATPase 功能。如我们研究发现，当心肌细胞培养于高脂或无葡萄糖的培养环境中，v-ATPase 的两个亚复合（V_1/V_0）将会分解，进一步导致 v-ATPase 功能丢失。基于这些发现，该论文旨在研究心脏能量底物［如，脂质（棕榈酸），葡萄糖，氨基酸（AA）和酮体］对 v-ATPase 功能的直接/间接影响。此外，我们也探究是否 v-ATPase 功能的增加会改善高脂诱导的胰岛素拮抗及心肌收缩功能，且揭示潜在的分子机制。

第 1 章简约概述了（啮齿动物/人）心肌细胞对能量底物，如脂质（棕榈酸），葡萄糖，氨基酸（AA）和酮体的代谢及利用。此外，本章也阐述了脂肪酸转运蛋白（CD36）和葡萄糖转运蛋白（GLUT4）在心脏的脂代谢中的主要作用，且探讨了导致糖尿病性心肌病发生的潜在机制，尤其是高脂介导 v-ATPase 功能异常的代谢通路。在本章的最后部分，我们也详细讨论了该论文的主要目的和概述。

第 2 章揭示，在啮齿动物/人心肌细胞中，葡萄糖代谢利用率增加可以改善高脂介导 v-ATPase 的功能异常。具体而言，v-ATPase 功能的恢复会促进脂质超载（啮齿动物/人）心肌细胞的胞内体酸化，进一步抑制 CD36 参与调节的脂质蓄积，进而改善胰岛素刺激的葡萄糖代谢以及心肌收缩功能。基于这些研究发现，本章确立了这一潜在策略/方案：增加 v-ATPase 功能能够改善高脂介导的胰岛素拮抗和心肌收缩功能障碍。

第 3 章研究结果揭示，在（啮齿动物/人）心肌细胞中，补充氨基酸混合物（AA，精氨酸/亮氨酸/赖氨酸）能够激活 mTOR，然后激活的 mTOR 与 v-ATPase 的 V_1 亚复合物结合，从而促进 V_1/V_0 区域的重新组装。鉴于 v-ATPase 是 mTOR 激活的主要支架之一，本章也首次提出 mTOR 的激活也能调节 v-ATPase 功能，这证实 v-ATPase 和 mTOR 的互作是双向的。此外，该氨基酸（AA）激活的 mTOR－v-ATPase 轴也能够参与胞内体酸化的

调节，从而抑制 CD36 介导的脂肪酸吸收，进一步保护心肌细胞免于高脂所引起的胰岛素抵抗以及收缩功能障碍。因此，本章中的研究结果表明，氨基酸调控的 v-ATPase 重组可能为高脂诱导的心肌疾病提供了简单有效的治疗方案。

第 4 章阐述了，啮齿动物/人的心肌细胞长期暴露于高剂量酮体，如 3-β-羟基丁酸酯（3HB），会导致 v-ATPase 结构分解，进而引起 v-ATPase 功能丢失。与高脂介导的 v-ATPase 分子机制类似，3-β-羟基丁酸酯（3HB）诱导的 v-ATPase 功能降低也会引起胞内体碱性化，随后刺激脂肪酸转运蛋白（CD36）易位至细胞肌膜，肌细胞脂质进一步蓄积，最后引起胰岛素拮抗以及心肌收缩功能障碍。值得注意的是，该章也进一步证实补充氨基酸混合物（AA，精氨酸/亮氨酸/赖氨酸）能够有效改善由高酮/高脂所引起的胰岛素拮抗以及心肌收缩功能障碍。因此，基于该章节的研究发现，酮体日粮/饮料，3-β-羟基丁酸酯（3HB），不应作为保健食品提供给所有人群。此外，关于这方面的治疗方案，本章也进一步证实氨基酸混合物（AA，精氨酸/亮氨酸/赖氨酸）为高脂/高酮的相关心脏疾病提供了有效的治疗选择。

第 5 章详细地讨论了当前的新发现，即重组 v-ATPase 是一个调节糖尿病性心肌疾病能量代谢的治疗靶标。此外，该章也介绍了其他调控糖尿病性心肌细胞中 v-ATPase 重组的策略，例如，高浓度短期补充（1h）氨基酸混合物（AA，精氨酸/亮氨酸/赖氨酸），同时，也提出了其他的潜在靶标［如，v-ATPase 介导的质子泵蛋白，以及涉及 v-ATPase 功能的各种信号通路（包括 Wnt，Notch 和 AMPK）］参与调控改善心肌能量代谢的紊乱。最后，鉴于 v-ATPase 能够整合了心脏中营养信息的研究发现，我们也讨论了 v-ATPase 功能的调节以及以响应心脏细胞营养代谢的上游机制。

总之，当前研究结果表明，v-ATPase 整合了心脏中的营养信息。本论文的主要发现使人们对糖尿病预防及治疗的基本分子机制有了较为深入的理解，最终将为治疗糖尿病性心肌疾病提供更好的方案及靶点。

Valorization

Social and clinical relevance

Heart failure is the leading cause of morbidity and mortality worldwide, currently affecting at least 28 million humans. It raises global health concerns because of increasing economic and social impact given it's high incidence and prevalence. Over the past decades, despite the significant development of treatment options and prevention strategies of heart failure, the rates of morbidity and mortality remain very high. Therefore, more effective treatment and prevention against the development of heart failure are urgently needed. Diabetes mellitus (DM), obesity, pulmonary hypertension, chronic obstructive pulmonary disease, atrial fibrillation, anemia, and chronic kidney disease are thought to increase risk for the progression of heart failure. In this thesis, we mainly focus on diabetes mellitus-induced cardiomyopathy (DCM).

Diabetes mellitus (DM), a chronic and progressive disease, is an increasing problem worldwide with 451 million cases in 2017 and an estimated amount of 5 million deaths each year. Part of the diabetes cases are related to type 1 diabetes, where β-cells in the pancreas are not able to produce (sufficient) insulin, whereas the largest contribution (> 90%) to the diabetes problem arises from type 2 diabetes mellitus (T2DM). The main cause of morbidity and mortality among patients with T2DM is diabetic cardiomyopathy (DCM). In its early stages, DCM includes a hidden subclinical period characterized by structural and functional abnormalities, including left ventricular hypertrophy, fibrosis, and cell signaling abnormalities. These pathophysiological changes of cardiac fibrosis and stiffness and associated subclinical diastolic dysfunction often evolve into heart failure with normal (preserved) ejection fraction (HFpEF) and eventual systolic dysfunction accompanied by heart failure with reduced ejection fraction (HFrEF). Additionally, as obesity increases the risk for both T2D and cardiovascular disease, it has been postulated that obesity-mediated alterations in myocardial lipid metabolism are critical to the pathophysiology of DCM. When there is a mismatch between myocardial FA uptake and subsequent oxidation in mitochondria, this alteration in myocardial lipid metabolism results in the pathological accumulation of lipid intermediates to cause myocardial lipotoxicity and insulin resistance. Both lipotoxicity and insulin resistance are usually well-documented as the major contributors to the development of DCM in many rodent models and T2D patients, and was linked to an increased CD36-mediated FA uptake in the heart. Therefore, strategies to counteract lipid-induced insulin resistance and contractile dysfunction by

decreasing CD36-mediated FA uptake have been suggested to be very beneficial against the development of DCM.

The studies in this thesis reveal that v-ATPase reassembly, which is achieved through a) increased glucose availability and b) addition of the specific AA (a mixture of arginine/leucine/lysine), is an attractive target process to protect the heart from lipid-induced insulin resistance and contractile dysfunction, and to possibly prevent the development of DCM (**Chapters 2, 3, and 4**). Our findings hold great promise for the clinical application in the future and thereby eventually may reduce the economic costs on the healthcare of these cardiac metabolic diseases.

Potential target groups

The current thesis mainly focus on preventing and treating the pathophysiology of insulin resistance and contractile dysfunction as a consequence of the diabetic heart. The contents of this thesis offer relevant knowledge as well as understandings for: (1) academic researchers working on a wide range of research field encompassing human/animal nutrition, cellular biochemistry and signaling, cardiac energy metabolism, as well as cardiac (patho)physiology, (2) the pharmaceutical industry working on the anti-diabetic drug development, (3) clinical cardiologist, (4) and diabetic patients. More importantly, many scientists working on chronic metabolic diseases (i.e., obesity, diabetes, and cardiovascular and liver/kidney diseases) also could benefit from our current novel findings.

Innovation and potential application

In this thesis, we identify that in the heart lipids (long-chain fatty acids), glucose, AA, and ketone bodies are added to the list of metabolites/nutrients regulating v-ATPase function, suggesting that v-ATPase integrates nutritional information. Yet, at present, the underlying upstream mechanism by which these nutrients (palmitate and ketone bodies) contributes to v-ATPase disassembly remains unknown. Continuing and expanding this study is highly essential if we aim to understand and further disclosure the underlying downstream mechanisms by which these nutrients regulate the assembly/disassembly cycles of v-ATPase in the heart. In this thesis, we provide strong evidence for the potential roles of v-ATPase on regulating energy metabolism in the control heart and the lipid-overloaded heart. The data presented in **Chapter 4** demonstrate that, similarly to lipid exposure, chronic exposure to ketone bodies also increases CD36-mediated FA uptake via v-ATPase disassembly, and then

results in a loss of insulin sensitivity, and finally of contractile function in cardiomyocytes. Therefore, deciphering the mechanism behind ketone bodies-induced insulin resistance could prove useful in furthering our understanding of insulin resistance and associated diseases (e.g., T2D) and identify other targets for therapeutic intervention. On the other hand, v-ATPase re-assembly by increased glucose availability and/or AA supplementation by itself already can be regarded as a promising target to restore lipid-induced insulin resistance and contractile dysfunction in the diabetic heart **(Chapter 2** and **Chapter 3)**. Therefore, the information obtained in **Chapter 2** and **Chapter 3** reveals v-ATPase function as a key regulator of cardiac substrate preference and as a novel treatment approach for the diabetic heart.

In summary, v-ATPase is a promising and innovative target for treatment of T2D and DCM, even though a long road still awaits its final validation, drug development and clinical testing, before introducing a new medication into the medical market. It should be noted that v-ATPase being a therapeutic target to rebalance cardiac substrate utilization in T2D and DCM has been established in an *in vitro* model, so that the immediate next step should involve *in vivo* investigations in rodents subjected to high fat diets before clinical studies should be undertaken. The last chapter of this thesis **(Chapter 5)** describes that we have already initiated these *in vivo* studies.

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我同行的马城小伙伴们，**念慈/辛莹/天宇/张明**，很开心你们都已经顺利毕业了，也找到合适的工作了。过去几年，谢谢你们的帮助和分享，祝你们一切顺利！保持联系。此外，**张辛真/小邹**，**乔奇**，很开心能和你们成为邻居，在马城的生活中，谢谢你们给我带了快乐和分享，我觉得很温馨，我会保留这些美好，在离开荷兰之前，我也希望可以多聚聚。回国之后，我们也可以继续聊天喝酒。祝你们博士学业一起顺利。**马老师，战老师，郭院长**，我也很开心能够这认识你们，回顾我们的每一次聊天，聚餐，总是那么喜剧，真的很美好，我会珍藏这些记忆，保持联系。这里，我也要感谢我马城的篮球伙伴，**陈锐，老顾，小罗，罗鸿辛，万俊，丁思杰，吴光耀，石头，吴晶.....陈锐/老顾**，过去几年真的很开心（全荷赛/平时篮球馆/露天篮球场）认识你们，无篮球无兄弟。享受和你们的每一次对抗，也感受到你们的进步，望回国之前多打几次，回国后也可以聚聚，保持联系。

最后，致我的家人(我就不一一列举了)，有时我觉得自己很自私，过去几年我一直按我自己的计划去执行，感激你们的理解和支持，我不想在这里表达太多，因为我觉得文字和言语无法完全表达我的内心那份感动和愧疚。不管怎么样，我也即将踏上回国的路程，未来的人生还很长，让我们一起去呵护这些美好。再次，对您们的支持和理解，我表示感谢，也希望自己能够用余生去弥补我们的那些错过。从幼儿园，小学，初中，高中，大学，硕士，博士，甚至博士后，您们见证了我的成长，我希望这份成长也能够填在你们的记忆中。

Curriculum Vitae

Shujin Wang (王蜀金) was born on June 22th, 1988, in Chongqing city, China. He started his bachelor education in Animal Science (Sichuan Agriculture University, China, 2008). After obtaining his bachelor's degree, he started his master in Animal Nutrition and Feed Science at Southwest University for Nationalities, China (2012). On May of 2015, he was granted a scholarship from the Chinese Scholarship Council, which supported for the successful application for a PhD position in Wageningen Institute of Animal Sciences, Wageningen University, The Netherlands. Afterwards, he started his scientific career as a PhD student at Cardiac Energy Metabolism Group (Maastricht University, The Netherlands, 2017-2020) under supervision of Prof. J.F.C. Glatz, Dr. J. J.F.P. Luiken, Dr. M. Nabben, and Dr. D. Neumann. In his PhD project, he investigates the molecular mechanisms that trigger heart failure development in the diabetic heart and he aims at finding new metabolic strategies to prevent this.

List of Publications

During the PhD period

- **Wang, S**., Wong, L. Y., Neumann, D., Liu, Y., Sun, A., Antoons, G., ... & Luiken, J. J. (2020). Augmenting Vacuolar H+-ATPase Function Prevents Cardiomyocytes from Lipid-Overload Induced Dysfunction. ***International Journal of Molecular Sciences***, *21*(4), 1520.
- Liu, Y., Rodriguez-Calvo, R., **Wang, S**., Zhu, X., Broers, J. L., Glatz, J. F., ... & Neumann, D. (2019). Fluorescent labelling of membrane fatty acid transporter CD36 (SR-B2) in the extracellular loop. ***PloS One***, *14*(1), e0210704.
- **S Wang**, D Neumann, LY Wong, A Sun1, A Strzelecka, J Glatz, M Nabben, J. Luiken. Lipid overload-induced contractile dysfunction rescued by amino acid supplementation: role of vacuolar H+-ATPase–mTOR axis. **In preparation for *Nature Metabolism***.
- **S Wang**, D Neumann, LY Wong, A Sun1, A Strzelecka, J Glatz, J Luiken, M Nabben. Ketone body exposure impairs insulin sensitivity and contractile function in cardiomyocytes by vacuolar-type H+-ATPase disassembly. **In preparation for *Cell Reports***.
- Sun A, Simsek O, Dirkx E, Wong L, **Wang S**, Strzelecka A, Nabben M, Glatz JFC, Neumann D, and Luiken JJFP. Phosphatidylinositol 4-kinase IIIβ mediates contraction-induced GLUT4 translocation and revealing its anti-diabetic action in cardiomyocytes.

Submitted to *Cellular and Molecular Life Sciences* (Minor Revision).

During the Master period

- Zhong, X., Zhang, Z., **Wang, S (Corresponding author)**., Cao, L., Zhou, L., Sun, A., ... & Nabben, M. (2019). Microbial-driven butyrate regulates jejunal homeostasis in piglets during the weaning stage. ***Frontiers in Microbiology***, *9*, 3335.
- **Wang, S**., Wang, B., He, H., Sun, A., & Guo, C. (2018). A new set of reference housekeeping genes for the normalization RT-qPCR data from the intestine of piglets during weaning. ***PloS One***, *13*(9), e0204583.
- Zhou, L., Evan, Y. W., **Wang, S (Corresponding author)**., & Sun, C. (2019). African swine fever epidemic in China. ***The Veterinary Record***, *184*(23), 713.
- Zhang, Z., Cao, L., Zhou, Y., **Wang, S (Corresponding author)**., & Zhou, L. (2016). Analysis of the duodenal microbiotas of weaned piglet fed with epidermal growth factor-expressed *Saccharomyces cerevisiae*. ***BMC Microbiology***, *16*(1), 166.
- **Wang, S**., Guo, C., Zhou, L., Zhong, Z., Zhu, W., Huang, Y., ... & Berendschot, T. T. (2016). Effects of dietary supplementation with epidermal growth factor-expressing *Saccharomyces cerevisiae* on duodenal development in weaned piglets. ***British Journal of Nutrition***, *115*(9), 1509-1520.
- **Wang, S**., Guo, C., Zhou, L., Zhang, Z., Huang, Y., Yang, J., ... & Yang, K. (2015). Comparison of the biological activities of *Saccharomyces cerevisia*e-expressed intracellular EGF, extracellular EGF, and tagged EGF in early-weaned pigs. ***Applied Microbiology and Biotechnology***, *99*(17), 7125-7135.
- **Wang, S**., Zhou, L., Chen, H., Cao, Y., Zhang, Z., Yang, J., ... & Guo, C. (2015). Analysis of the biological activities of *Saccharomyces cerevisiae* expressing intracellular EGF, extracellular EGF, and tagged EGF in early-weaned rats. ***Applied Microbiology and Biotechnology***, *99*(5), 2179-2189.

Overview of completed training activities

Lists of Courses	**Time**	**Locations**
Advanced Statistics Course Design of Experiments	Dec, 2016	Wageningen
Essential Skills	Jan, 2017	Wageningen
The Intestinal Microbiome and Diet in Human and animal health	Feb, 2017	Wageningen
Research integrity &Ethics in Anima Sciences	March,2017	Wageningen
Summer Course English for IELTS	Sep, 2016	Wageningen
WIAS Introduction Day	Nov, 2016	Wageningen
Lab Animal Science Article 9	July, 2016	Maastricht
Effective Communication	Jan, 2016	Maastricht
From Human Neuroanatomy to Psychopathology	Feb, 2016	Maastricht
Radiation Protection level 5B	Oct, 2017	Maastricht
Advanced Optic Microscopy	May,2018	Maastricht
Non-invasive Imaging	June,2018	Maastricht
Teachings		
Daily supervision of junior master students during their internship	Sep.2018-Jun.2019	Maastricht
Daily supervision of senior master students during their internship	Nov.2019-Jun.2020	Maastricht
Presentations		
Genetics and Cell Biology Seminar+ Meeting (Oral presentation)	May, 2019	Maastricht

The society for Heart and Vascular Metabolism, SHVM (Post presentation)	June, 2019	Amsterdam
CARIM Symposium	May, 2018	Maastricht
Maastricht Microscopy Meeting (M3) on Advanced Optical Microscopy	May, 2018	Maastricht
Lab Chat on MolGen (Oral presentation)	Every half year	Maastricht