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AMPK-mediated increase in myocardial long-chain fatty acid uptake critically depends on sarcolemmal CD36

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Abstract

CD36, also named fatty acid translocase, has been identified as a putative membrane transporter for long-chain fatty acids (LCFA). In the heart, contraction-induced 5′ AMP-activated protein kinase (AMPK) signaling regulates cellular LCFA uptake through translocation of CD36 and possibly of other LCFA transporters from intracellular storage compartments to the sarcolemma. In this study, isolated cardiomyocytes from $CD36^{+/+}$- and $CD36^{-/-}$ mice were used to investigate to what extent basal and AMPK-mediated LCFA uptake are CD36-dependent. Basal LCFA uptake was not altered in $CD36^{-/-}$ cardiomyocytes, most likely resulting from a (1.8-fold) compensatory upregulation of fatty acid-transport protein-1. The stimulatory effect of contraction-mimetic stimuli, oligomycin (2.5-fold) and dipyridamole (1.6-fold), on LCFA uptake into $CD36^{+/+}$ cardiomyocytes was almost completely lost in $CD36^{-/-}$ cardiomyocytes, despite that AMPK signaling was fully intact. CD36 is almost entirely responsible for AMPK-mediated stimulation of LCFA uptake in cardiomyocytes, indicating a pivotal role for CD36 in mediating changes in cardiac LCFA fluxes.
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Keywords: 5′ AMP-activated protein kinase; CD36; Dipyridamole; Fatty acid binding proteins; Fatty acid uptake; Oligomycin; Sulfo-*N*-succinimidyl-palmitate

Long-chain fatty acids (LCFA) and glucose are the predominant substrates for the heart, and contribute, under normal circumstances, for approximately 70% and 20%, respectively, to cardiac energy production [1,2]. Cardiac LCFA uptake in rodents is largely mediated by protein-mediated transport, with a minor (<20%) part attributable to passive diffusion [3]. Three proteins have been identified as functioning in transmembrane transport of LCFA and each is expressed in the cardiac muscle: fatty acid translocase/CD36, plasma membrane fatty acid binding protein (FABPpm), and fatty acid transport protein (FATP) [4]. Transgenic mice would present an ideal model to investigate the contribution of each individual transporter to the cardiac LCFA flux.

The first studies with CD36 transgenic mice were performed by Febbraio et al. in 1999 [5]. CD36 null ($CD36^{-/-}$) mice showed a significant increase in fasting

Abbreviations: ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AMPK, 5′ AMP-activated protein kinase; CD36, fatty acid translocase CD36; FABPpm, plasma membrane fatty acid binding protein; FATP, fatty acid transport protein; GLUT4, glucose transporter 4; LCFA, long-chain fatty acids; SSP, sulfo-*N*-succinimidylpalmitate.

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plasma levels of cholesterol, (nonesterified) LCFA, and triacylglycerol, suggesting a functionally important role for CD36 in whole body lipid metabolism [5]. Tail vein injections of $CD36^{-/-}$ mice with radio-iodinated LCFA derivatives (BMIPP, IPPA) revealed that LCFA uptake into adipose tissue, skeletal muscle, and heart was reduced, and was associated with lower LCFA esterification into triacylglycerols in each tissue [6]. In addition, experiments with perfused hearts indicated that cardiac LCFA oxidation was markedly decreased in $CD36^{-/-}$ mice. This depressed cardiac LCFA oxidation was accompanied by a compensatory increase in glucose oxidation [7], highlighting the metabolic flexibility of the heart. Conversely, muscle-specific overexpression of CD36 elicits higher LCFA oxidation rates in skeletal muscle in response to contraction, but not during rest [8]. Only during an extra demand for energy, increased CD36 expression results in increased utilization of LCFA. Altogether, these studies with transgenic mouse models suggest that CD36-mediated LCFA uptake into heart and muscle is closely linked to changes in LCFA metabolism [9]. However, possible compensatory alterations in the expression of other LCFA transporters in these mice have not yet been evaluated, so that the quantitative involvement of CD36 in cardiac LCFA uptake remains unknown.

CD36 is not only a facilitator for LCFA uptake but also appears to be involved in the acute regulation of LCFA uptake. This regulation occurs by stimulus-induced reversible CD36 translocation from intracellular storage compartments to the sarcolemma [10]. These stimuli include cellular contractions and the hormone insulin, both of which have been found to induce glucose uptake through translocation of the glucose transporter GLUT4 [11,12]. Just like insulin-induced GLUT4 translocation, insulin-induced CD36 translocation, but not contraction-induced CD36 translocation was found to be dependent on activation of phosphatidylinositol-3 kinase [11]. Recently, studies have started to disclose the contraction signaling cascade involved in translocation of CD36 and GLUT4 in rat cardiomyocytes. This cascade is elicited by elevation of intracellular levels of AMP, leading to activation of 5' AMP-activated protein kinase (AMPK) and, subsequently, downstream kinases, eventually resulting in the concomitant translocation of both CD36 and GLUT4 [13]. Interestingly, several pharmacological agents, among which oligomycin and dipyridamole, were also found to stimulate LCFA uptake through CD36 translocation [13,14]. Both oligomycin, a mitochondrial F_1F_0-ATPase inhibitor, and dipyridamole, a phosphodiesterase V/VI inhibitor, stimulated LCFA uptake in a non-additive manner to contractions, indicating that these agents activate the same signaling cascade as contractions. Indeed, oligomycin was found to potently activate AMPK. The stimulatory action of dipyridamole on LCFA uptake was unrelated to inhibition of phosphodiesterase activity, since zaprinast, another phosphodiesterase V/VI inhibitor, and isobutyl-methylxanthine, a general phosphodiesterase inhibitor, had no effect on LCFA uptake [15]. In fact, dipyridamole was able to translocate CD36 without influencing subcellular GLUT4 localization, indicating that it activates AMPK signaling downstream of AMPK [14].

Besides regulating LCFA uptake, AMPK signaling is also involved in regulation of LCFA oxidation via control of the phosphorylation state of acetyl-CoA carboxylase (ACC), intracellular malonyl-CoA levels and of inhibition of CPT-I [16]. The simultaneous recruitment of CD36 and activation of CPT-I by AMPK signaling serves to channel the extra LCFA taken up specifically into oxidative metabolism to increase ATP production needed for increased cardiac workload.

In order to properly assess the quantitative contribution of CD36 to initial LCFA uptake rate both in the absence and presence of AMPK stimulation, we chose freshly isolated cardiomyocytes as a model system for allowing to study LCFA uptake under carefully controlled conditions. Measurements of LCFA uptake and, for comparison, glucose uptake were performed in cardiomyocytes from both CD36 wild-type ($CD36^{+/+}$) mice and $CD36^{-/-}$ mice under quiescent conditions and upon addition of oligomycin or dipyridamole. Sulfo-*N*-succinimidyl-palmitate (SSP) which specifically inhibits CD36 [17] was used to determine the involvement of CD36 in LCFA uptake in $CD36^{+/+}$ mice.

Materials and methods

Animals. $CD36^{-/-}$ mice were generated by targeted homologous recombination and crossed back eight times to the C57Bl/6 background [5]. $CD36^{-/-}$- and $CD36^{+/+}$ mice were bred at TNO Quality of Life, Leiden, The Netherlands. The Experimental Animal Committee of Maastricht University gave approval for all experiments involving animals.

Isolation of mouse cardiomyocytes. Adult mouse cardiomyocytes (mice 11–14 months of age) were isolated using a Langendorff perfusion system according to the procedure described by Severson et al. [18]. The main differences in cardiomyocyte isolation between mouse and rat include the type of collagenase used (Liberase blendzyme 1 versus Worthington collagenase type II) and the flow rate (constantly at 2.5 ml/min versus progressively increasing from 6 ml/min at the beginning till 11 ml/min at the end of the perfusion period) [18,19]. For all the experiments the viability of cardiomyocytes was 60–80% (data not shown).

Substrate uptake by cardiomyocytes. Cell suspensions were incubated with 1% DMSO or 500 μmol/l SSP in bicarbonate medium (120 mM NaCl, 5.4 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 20 mM $NaHCO_3$, pH 7.5) supplemented with 100 μM $CaCl_2$ and 0.2% bovine serum albumin (BSA) during the recovery period of 30 min at room temperature, and subsequently washed and resuspended in bicarbonate medium supplemented with 100 μM $CaCl_2$ and 2.0% BSA. Thereafter, cell suspensions (2.0 ml; 5–9 mg wet mass/ml) were pre-incubated in capped 20-ml incubation vials with or without 15 μmol/l oligomycin or 100 μmol/l dipyridamole for 20 min at 37 °C under continuous shaking. At the applied concentrations of oligomycin and dipyridamole, LCFA uptake rates were found to be maximally stimulated (data not shown). Oligomycin (15 μmol/l) increased the cellular AMP/ATP ratio by >2-fold, thereby increasing AMPK activity without inhibiting oxygen consumption [13]. To study palmitate and deoxyglucose uptake, 0.5 ml of a mixture of [1-^{14}C]palmitate/BSA complex and [1-^{3}H]deoxyglucose was added at the start of the incubations so that the final concentration of both palmitate and deoxyglucose amounted to 100 μmol/l with a corresponding

palmitate/BSA ratio of 0.3 [20]. Cellular uptake of palmitate (3-min incubation) and deoxyglucose (3-min incubation) was determined upon washing the cells three times for 2 min at 150*g* in an ice-cold stop solution containing 0.2 mmol/l phloretin.

Western blotting. The relative amounts of the substrate transporters CD36, FABPpm, FATP1 and -4, and GLUT4 were determined in cardiomyocyte pellets obtained after a pulse spin in a microcentrifuge, and subsequently dissolved in sample buffer containing 62.5 mmol/l Tris–HCl (pH 6.8), 2 mmol/l EDTA, 20 mmol/l dithiothreitol, 7.5% (wt/vol) SDS, and 50% sucrose. Upon SDS–ploy acrylamide gel electrophoresis, we applied a mouse monoclonal anti-CD36 antibody 1258, a rabbit polyclonal anti-FABPpm antiserum, a goat polyclonal anti-FATP1 and -4 antiserum, and a sheep polyclonal anti-GLUT4 antiserum, respectively for the detection of these transporters according to manufacturer's instructions.

Upon pulse centrifugation of cardiomyocytes and dissolution of the pellet in sample buffer, activation of AMPK and ACC was measured with antibodies against phospho-AMPKα (Thr172) and phospho-ACC (Ser79), according to the manufacturer's instructions.

Materials. [1-^{14}C]palmitic acid and 2-deoxy-D-[1-^{3}H]glucose were obtained from Amersham Life Science (Little Chalfont, UK). BSA (fraction V, essentially fatty acid free), phloretin, oligomycin, and dipyridamole were obtained from Sigma (St. Louis, MO). Liberase blendzyme 1 was purchased from Roche Diagnostics (Indianapolis, IN). SSP is routinely synthesized in our laboratory, as has been previously described [21]. The antibody directed against phosphorylated ACC was from Upstate (Dundee, UK), and anti-phospho-AMPK from Cell Signalling (Danvers, MA). Antibodies directed against CD36 and GLUT4 were obtained from Chemicon International Inc. (Temecula, USA), and FATP1 and FATP4 from Santa Cruz Biotechnology Inc. (Santa Cruz, USA). Anti-FABPpm was a gift from Dr. J. Calles-Escandon, Wake Forest University School of Medicine and Baptist Medical Center, Winston-Salem NC.

Data presentation and statistics. All data are present as means ± SEM for the indicated number of cardiomyocyte preparations. Statistical difference between means was analyzed by the paired Student's *t* test. *P* values $\leqslant 0.05$ were considered significant.

Results and discussion

Prior studies addressing cardiac LCFA uptake in CD36 transgenic mice have only used semi-quantitative methods in assessing a role for CD36 in this process [6,8]. Our approach with freshly isolated cardiomyocytes in suspension provides accurate quantitative information about the initial LCFA uptake rate under controlled conditions. In addition with single-cell suspension of cardiomyocytes pharmacological agents such as the contraction-mimicking agents oligomycin and dipyridamole have easy access to their intracellular target allowing to study the dynamics of LCFA uptake, particularly in response to changes in energy demand that occur during alterations in myocytes contraction.

Involvement of CD36 in basal cardiac LCFA uptake

Palmitate uptake into cardiomyocytes was studied at defined conditions at which the uptake rate is linear with time and linear with the exogenous concentration so as to allow the measurement of unidirectional influx in the absence of saturation of transporters [19]. Under basal conditions there is no difference in LCFA uptake between CD36$^{+/+}$- and CD36$^{-/-}$ cardiomyocytes (Fig. 1A). Remarkably, LCFA uptake into CD36$^{+/+}$ cardiomyocytes was decreased by 39% in the presence of SSP, while LCFA uptake into CD36$^{-/-}$ cardiomyocytes was unaffected by SSP (Fig. 1A). Several conclusions can be drawn from these observations. First, the lack of an effect of SSP on LCFA uptake into CD36$^{-/-}$ cardiomyocytes strongly confirms that SSP solely inhibits the CD36 component in the LCFA uptake process, and does not affect the activity of other transporters and/or passive diffusional uptake. Second, CD36 mediates 39% of basal LCFA uptake into mouse cardiomyocytes, which is comparable to the CD36 component in basal LCFA uptake into cardiomyocytes from rat (50%) [20]. Third, in CD36$^{-/-}$ cardiomyocytes there must be full compensation at the level of sarcolemmal transporters to maintain LCFA uptake. Therefore, we have measured in CD36$^{+/+}$- and CD36$^{-/-}$ cardiomyocytes expression levels of other LCFA transporters (Fig. 2). It appears that expression levels of FABPpm and FAPT4 were not different in CD36$^{+/+}$- and CD36$^{-/-}$ cardiomyocytes. However, FATP1 was 1.8-fold ($P < 0.05$) upregulated in CD36$^{-/-}$ cardiomyocytes, which could adequately explain the unaltered rate of basal cardiomyocytic LCFA uptake in the absence of CD36.

Involvement of CD36 in AMPK-mediated cardiac LCFA uptake

Two contraction-like pharmacological stimuli (oligomycin and dipyridamole) were used to study contraction-induced LCFA uptake into CD36$^{-/-}$ cardiomyocytes. We established previously that both agents stimulate AMPK signaling: oligomycin acts upstream of AMPK [13], and dipyridamole acts downstream of AMPK [14]. Accordingly, oligomycin potently stimulated phosphorylation of both AMPK and the AMPK target ACC in CD36$^{+/+}$ cardiomyocytes (Fig. 3A and B). Oligomycin had a similar effect on phosphorylation of both proteins in CD36$^{-/-}$ cardiomyocytes, indicating that AMPK-mediated signaling is fully intact in CD36$^{-/-}$ cardiomyocytes. Dipyridamole, which appears to act on a still unidentified protein kinase downstream of AMPK, did not significantly alter the phosphorylation states of AMPK and ACC either in CD36$^{+/+}$- or CD36$^{-/-}$ cardiomyocytes (Fig. 3).

Just like in rat cardiomyocytes [13,14], oligomycin (2.5-fold) and dipyridamole (1.6-fold) markedly stimulated LCFA uptake into CD36$^{+/+}$ cardiomyocytes (Fig. 1B). However, in CD36$^{-/-}$ cardiomyocytes, oligomycin-induced LCFA uptake was reduced by 79% to a residual (but significant) level of 1.3-fold above basal. Interestingly, we have recently shown in rat cardiomyocytes that the AMPK activation by the adenosine analog 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) results in translocation of both CD36 and FABPpm from an intracellular compartment to the sarcolemma [22]. Therefore, this small 1.3-fold increase in oligomycin-stimulated LCFA uptake into CD36$^{-/-}$ cardiomyocytes is most likely due to

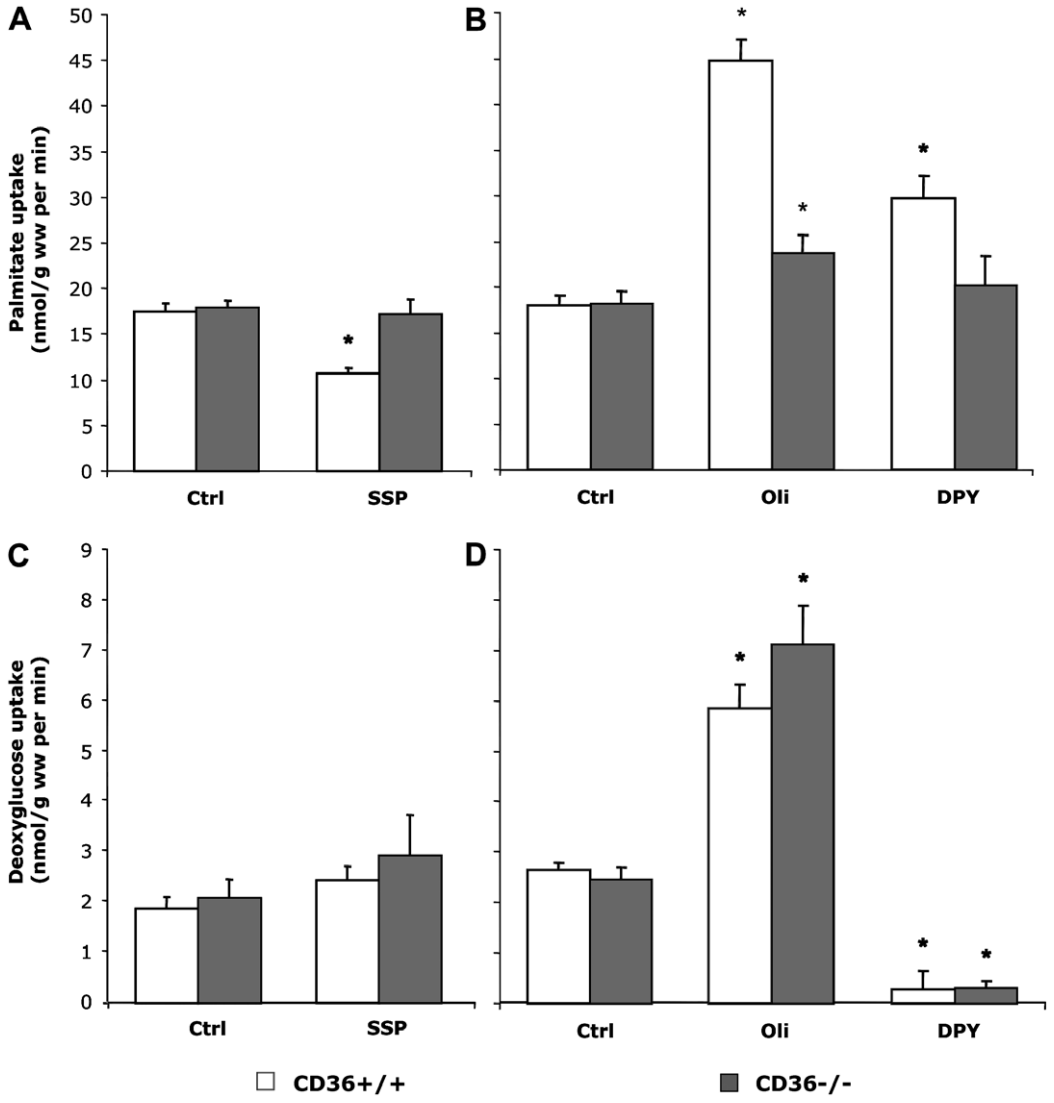

Fig. 1. Effects of inhibition of CD36 and AMPK-mediated stimulation on substrate uptake by cardiomyocytes from $CD36^{+/+}$- and $CD36^{-/-}$ mice. $CD36^{+/+}$ (□) and $CD36^{-/-}$ cardiomyocytes (■) were incubated in the absence (Ctrl) or presence of 500 μmol/l SSP (preincubation during the 30 min recovery period), centrifuged, washed and resuspended, whereafter uptake rates of palmitate (A) and deoxyglucose (C) were measured. To mimic contraction signaling, cardiomyocytes from $CD36^{+/+}$ mice (□) and $CD36^{-/-}$ mice (■) were incubated for 20 min in the absence (Ctrl) or presence of 15 μmol/l oligomycin (Oli) or 100 μmol/l dipyridamole (DPY) which was again followed by measurement of palmitate (B) and deoxyglucose (D) uptake. Values are means ± SEM for 4–7 animals per group. *Significantly different from Ctrl, $P < 0.05$.

translocation of FABPpm. Just like genetic ablation of CD36, blocking CD36 with SSP in $CD36^{+/+}$ cardiomyocytes completely inhibited oligomycin-stimulated LCFA uptake (oligomycin: 44.7 ± 2.4 i.e. 2.5-fold basal versus oligomycin + SSP: 14.2 ± 0.2 i.e. 0.8-fold basal; $n = 4$, $P < 0.05$), allowing us to estimate the contribution of CD36 to LCFA uptake into fully stimulated cardiomyocytes (i.e., optimal AMPK activation) to amount to 68%.

The stimulatory effect of dipyridamole (1.6-fold) on LCFA uptake into $CD36^{+/+}$ cardiomyocytes was completely absent in $CD36^{-/-}$ cardiomyocytes (Fig. 1B). Together, these findings strongly indicate that AMPK-mediated increase in LCFA uptake into the heart is critically dependent on the presence of CD36.

Regulation of glucose uptake in $CD36^{-/-}$ hearts

Similar to LCFA uptake, the rate of glucose uptake is not different between $CD36^{+/+}$- and $CD36^{-/-}$ cardiomyocytes under basal conditions (Fig. 1C). In line with this, the expression of GLUT4 is also not altered in $CD36^{-/-}$ cardiomyocytes (Fig. 2). SSP has no effect on glucose uptake (Fig. 1C), underlining that its inhibitory action is restricted to the LCFA uptake process. Oligomycin stimulated glucose uptake into $CD36^{-/-}$-(2.9-fold) and $CD36^{+/+}$ cardiomyocytes (2.2-fold) to a similar extent, while dipyridamole similarly inhibited glucose uptake into both $CD36^{+/+}$- (−89%) and $CD36^{-/-}$ cardiomyocytes (−87%) (Fig. 1D). The inhibition of glucose uptake by dipyridamole is likely due to a

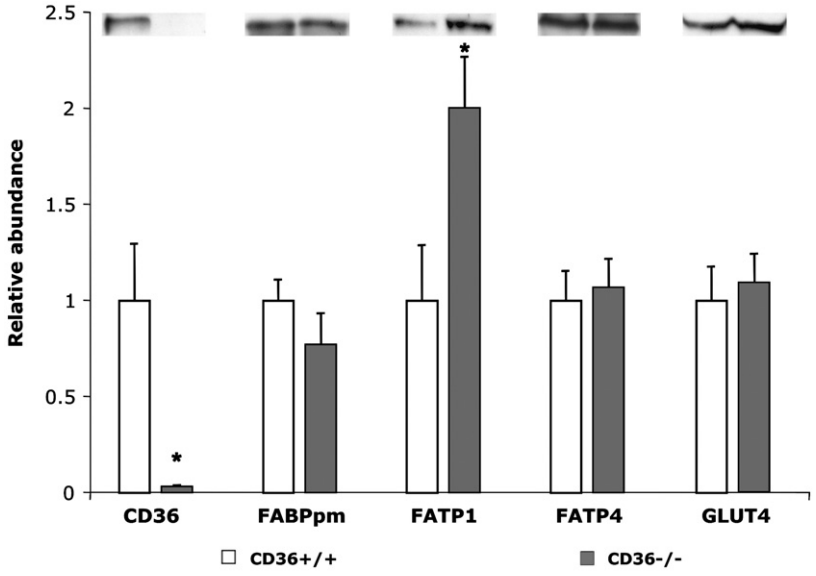

Fig. 2. Expression of substrate transporters in cardiomyocytes from $CD36^{+/+}$- and $CD36^{-/-}$ mice. Protein expression of CD36 (88 kDa), FABPpm (43 kDa), FATP1 (63 kDa), FATP4 (70 kDa), and GLUT4 (45 kDa) in cardiomyocytes from $CD36^{+/+}$ mice (□) and $CD36^{-/-}$ mice (■) was determined by Western blotting followed by densitometry (relative abundance $CD36^{+/+}$ set at 1). From each sample, 20 μg of myocytes wet mass was taken for electrophoresis. Western blotting with antibodies against caveolin-3 and β-actin further confirmed no differences in protein amount per lane (data not shown). Values are means ± SEM for 6–10 animals per group. Representative blots are shown. *Significantly different from $CD36^{+/+}$, $P < 0.05$.

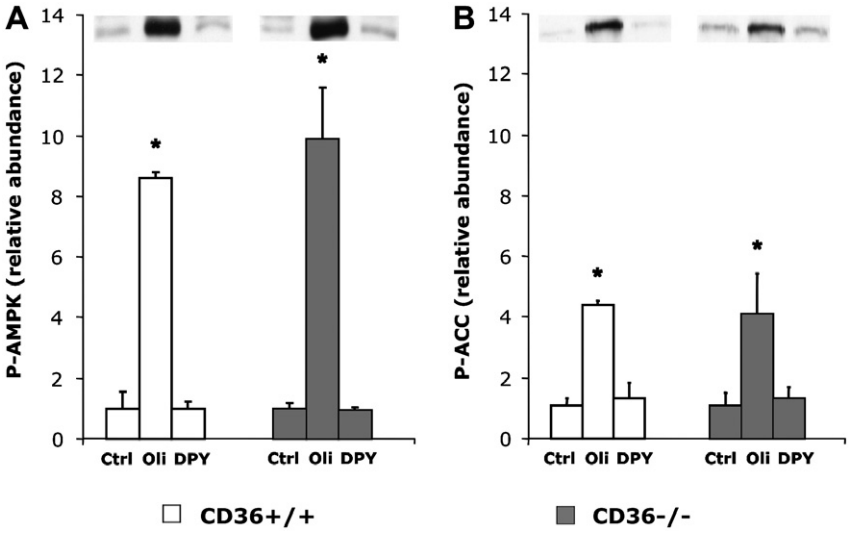

Fig. 3. Effects of AMPK-mediated stimulation on the contraction signaling cascade in cardiomyocytes from $CD36^{+/+}$- and $CD36^{-/-}$ mice. Cardiomyocytes from $CD36^{+/+}$ mice (□) and $CD36^{-/-}$ mice (■) were incubated for 20 min in the absence (Ctrl) or presence of 15 μmol/l oligomycin (Oli) or 100 μmol/l dipyridamole (DPY). Phosphorylation of AMPK (A) and of ACC (B) was determined by Western blotting followed by densitometry (relative abundance Ctrl set at 1). From each sample, 20 μg of myocytes wet mass was taken for electrophoresis. Western blotting with antibodies against caveolin-3 and β-actin further confirmed no differences in protein amount per lane (data not shown). Values are means ± SEM for 5–7 animals per group. Representative blots are shown. *Significantly different from Ctrl, $P < 0.05$.

direct interaction of dipyridamole with GLUT4 [23]. The selective inhibition of oligomycin-stimulated LCFA uptake, but not glucose uptake, into $CD36^{-/-}$ cardiomyocytes clearly illustrates that trafficking processes are not affected by CD36 ablation, and that AMPK-mediated GLUT4 translocation remains unaltered in $CD36^{-/-}$ cardiomyocytes. However, this study may seem in contrast to results obtained in a working heart model (continuous AMPK activation) [7]. In working hearts from $CD36^{-/-}$ mice, glucose oxidation was 3-fold upregulated relative to $CD36^{+/+}$ hearts to compensate for the drop in palmitate oxidation. In fact, we do not think that there is a conflict between our study and the study using the working heart model, because the present study only excludes that the compensatory increase in glucose oxidation observed in $CD36^{-/-}$ working hearts can be explained at the level of glucose transporters and their recycling. This leaves

open other compensatory mechanisms such as improved channeling of glucose metabolites into mitochondria and/or decreased storage of glucose into glycogen.

Concluding remarks

The major conclusions that can be drawn from these observations on cardiac substrate uptake in $CD36^{-/-}$ mice are: (i) CD36 contributes significantly to facilitating basal LCFA uptake, and its ablation results in compensatory upregulation of the myocardial expression of FATP1; (ii) CD36 is almost entirely responsible for the AMPK-mediated increase in LCFA uptake which increase is not compensated in cardiomyocytes from $CD36^{-/-}$ mice at the level of other LCFA transporters. The low rate of LCFA uptake in the working $CD36^{-/-}$ heart will likely contribute to the development of cardiac hypertrophy, as is seen in aged $CD36^{-/-}$ mice (M. Febbraio, unpublished observation) and human subjects lacking this major cardiac LCFA transporter [24].

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