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Fatty acid transport and transporters in muscle are critically regulated by Akt2

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ABSTRACT

Muscle contains various fatty acid transporters (CD36, FABPpm, FATP1, FATP4). Physiological stimuli (insulin, contraction) induce the translocation of all four transporters to the sarcolemma to enhance fatty acid uptake similarly to glucose uptake stimulation via glucose transporter-4 (GLUT4) translocation. Akt2 mediates insulin-induced, but not contraction-induced, GLUT4 translocation, but its role in muscle fatty acid transporter translocation is unknown. In muscle from Akt2-knockout mice, we observed that Akt2 is critically involved in both insulin-induced and contraction-induced fatty acid transport and translocation of fatty acid translocase/CD36 (CD36) and FATP1, but not of translocation of fatty acid-binding protein (FABPpm) and FATP4. Instead, Akt2 mediates intracellular retention of both latter transporters. Collectively, our observations reveal novel complexities in signaling mechanisms regulating the translocation of fatty acid transporters in muscle.
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1. Introduction

Fatty acids and glucose are essential fuels for skeletal muscle. The transport of these substrates into this tissue occurs via highly regulated, protein-mediated processes. Specifically, in skeletal muscle, glucose transport is facilitated by glucose transporter-4 (GLUT4) (cf. [1,2]), while fatty acid transport is facilitated by a number of fatty acid transporters, including fatty acid translocase/CD36 (CD36), plasma membrane associated fatty acid-binding protein (FABPpm), and selected fatty acid transport proteins (FATP1 and -4) (cf. [3]). Acute changes in glucose utilization induced by selected metabolic stimuli (insulin, 5′AMP-activated protein kinase (AMPK) activation, muscle contraction) are largely attributable to the translocation of GLUT4 from intracellular depots to the cell surface, thereby increasing the rate of glucose transport [2,4]. In recent years, similar metabolic flexibility has been shown for FAT/CD36, as this fatty acid transport protein is also induced to translocate to the cell surface by the same metabolic stimuli (cf. [3]).

The signaling mechanisms involved in GLUT4 translocation to the sarcolemma in response to selected metabolic perturbations are well characterized [2,4]. In contrast, the signaling pathways involved in the translocation of fatty acid transporters to the sarcolemma are largely unknown. Nevertheless, CD36 translocation appears to share, in part, a signaling pathway that may be similar to GLUT4. For example, both GLUT4 and CD36 are recruited from intracellular pools to the sarcolemma by insulin-stimulated activation of phosphatidylinositol-3-kinase (PI3K) [5,6]. Similarly, muscle contraction and the activation of muscle AMPK by 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR) induce the translocation of both GLUT4 and CD36 to the sarcolemma [3,5,7,8], as well as that of a number of other fatty acid transporters, namely FABPpm, FATP1, FATP4, but not FATP6 [7].

Despite the comparable responses of GLUT4 and fatty acid transporters to metabolic stimulation and the apparent sharing of PI3K signaling, there is evidence that the GLUT4 and the fatty acid transporter signaling pathways diverge at some point. For example, in insulin resistant, obese Zucker rats and in Zucker diabetic fatty acid rats GLUT4 is retained intracellularly [9], while CD36 is permanently relocated to the sarcolemma [10], resulting in an in an inverse relationship between sarcolemmal GLUT4 and

Abbreviations: AMPK, 5′AMP-activated protein kinase; CD36, fatty acid translocase/CD36; FABPpm, fatty acid-binding protein; FATP, fatty acid transport protein; GLUT4, glucose transporter-4

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CD36 ($r = -0.91$) [11] In addition, in muscle from obese Zucker rats insulin-stimulated GLUT4 translocation is impaired [9], while contraction-stimulated GLUT4 translocation is normal [1]. Moreover, both insulin and contraction-induced CD36 translocation are impaired in obese Zucker rat muscle [12]. Since FABPpm translocation is not impaired in these animals, it appears that CD36 and FABPpm may be regulated by different insulin and contraction-mediated signaling pathways [12]. Collectively, these observations indicate that the signaling pathways involved in the translocation of these transporters differ from those involved in GLUT4 translocation. In addition, their signaling pathways remain obscure, and there may be specific signaling pathways for the different fatty acid transporters.

Akt is a family of protein kinases regulating multiple anabolic pathways, and at present there are three isoforms described. In general, Akt1 mediates hypertrophic signaling, Akt2 is involved in insulin signaling, whereas much less is known about Akt3 [13]. In the past few years it has become evident that Akt2 contributes to the regulation of lipid metabolism. For example, Akt2 signaling promotes mammary gland [14] and hepatic triacylglycerol accumulation [15–17]. Yet, it is unknown whether the regulation of lipid metabolism by Akt2 signaling extends to the fatty acid transport process. Moreover, whether a signaling divergence between GLUT4 and selected fatty acid transporters, or among different fatty acid transporters, occurs at the level of Akt2 is also unknown. Therefore, in the present study, we investigated whether Akt2 is involved in the insulin-, and/or contraction-mediated signaling for fatty acid transport and fatty acid transporter translocation (CD36, FABPpm, FATP1 and -4), and compared this to glucose transport and GLUT4 translocation.

2. Materials and methods

2.1. Animals

Akt2-knockout (KO) mice were a gift from Pfizer Inc. (New York, NY [18]) and corresponding DBA/1 wildtype (WT) mice were purchased from Jackson Laboratories (Bar Harbor, Maine). WT and KO female mice were carefully matched for age (8 weeks). At this age, the body weights of WT mice (20.8 ± 0.7 g) were greater than in KO mice (16.3 ± 0.5 g) $P < 0.05$, as has been reported by others [18,19]. The animals were housed in controlled temperature and humidity conditions on a 12:12-h light–dark cycle and were given standard laboratory chow and water *ad libitum*. Mice were anesthetized with sodium pentobarbital (6 mg/100 g body wt ip; MTC Pharmaceuticals, Cambridge, ON), and all procedures were approved by the University of Guelph Animal Care Committee.

2.2. Genotyping

Genotypes of KO mice were confirmed using standard DNA isolation and PCR methods (Extract-N-Amp, Sigma–Aldrich, St. Louis, MO) using forward 5′-GCA-GGA-TCT-CCT-GTC-ATC-TCA-CC-3′ and reverse 3′-GAT-GCT-CTT-CGT-CCA-GAT-CAT-CC-5′ primer sets targeted towards the neo cassette.

2.3. Experimental treatment

Basal tail vein glucose concentrations were determined using a glucose meter (Ascensia Elite XL, Bayer Inc., Toronto, ON). Intraperitoneal glucose and insulin tolerance tests were conducted in separate groups of overnight fasted WT and KO animals. Mice were injected intraperitoneally with either glucose (0.75 g/kg body wt) or insulin (1.0 U/kg body wt) and blood glucose was recorded at 15, 30, 45, 90 and 120 min. To examine the effects of insulin and muscle contraction on glucose and fatty acid transport and transporters, fasted WT and KO mice were assigned to the following groups: (i) control (no treatment), (ii) insulin treatment for 15 min (Humulin, 1.0 U/kg body wt, ip; Eli Lilly, Toronto, ON), or (iii) muscle contraction via the sciatic nerve (train delivery 100 Hz/3 s at 5 V, train duration 200 ms, pulse duration 10 ms) applied for 3 repetitions of 5 min with 2 min of rest between stimulations [7]. Following treatment periods, hindlimb muscles were immediately harvested for isolation of giant sarcolemmal vesicles or were freeze-clamped in liquid N_2 for muscle homogenate preparation.

2.4. Isolation of giant sarcolemmal vesicles and substrate transport

Fatty acid and glucose transport as well as sarcolemmal presence of transport proteins were determined in giant sarcolemmal vesicles isolated from hindlimb muscles [7,20]. Vesicle protein yield was determined using the bicinchoninic acid assay. Vesicles were used immediately for substrate transport assays [7,20].

2.5. Western blotting

Protein expression was determined in giant sarcolemmal vesicles and muscle homogenates via Western blotting [7,20]. Blotting protocols with antibodies against total Akt2, AS160, AMPK, phosphorylated Akt-Thr^{308}, AS160-Thr^{642}, AMPK-Thr^{172} (Cell Signaling, Danvers, MA), CD36, FATP1, and -4 (Santa Cruz Biotechnology, Santa Cruz, CA, [21]) and GLUT4 (Millipore, Temecula, CA) were performed according to manufacturer's instructions. Antibodies against FABPpm were kindly donated by Dr. J. Calles-Escandon, Wake Forest University. Following incubation with secondary antibodies, membranes were detected and quantified using chemiluminescence (Perkin Elmer Life Science, Boston, MA) and ChemiGenius2 Bioimaging (SynGene, Cambridge, UK). Equal loading of protein was confirmed via Ponceau-S staining.

2.6. Statistics

All data are reported as mean ± S.E.M. and were analyzed using analysis of variance and Fisher's LSD post hoc test when appropriate. Significance was accepted at $P < 0.05$.

3. Results

3.1. Akt2-KO phenotype

Pre-diabetic state: Basal blood glucose concentrations were lower in WT (4.2 ± 0.2 mM) than in Akt2-KO animals (5.4 ± 1.0 mM, $P < 0.05$), and Akt2-KO mice exhibited whole body glucose intolerance (Fig. 1A). These observations confirm previous results reported elsewhere for these animals [18,19,22].

3.2. Expression of insulin signaling proteins, AMPK and substrate transporters

As expected in Akt2-KO mice, Akt2 protein was not detected (Fig. 1B). The presence of insulin signaling proteins insulin receptor substrate-1, phosphatidylinositol-3 kinase and AS160 (the downstream target of Akt2) in skeletal muscle did not differ between WT and Akt2-KO mice (Fig. 1B). Neither the metabolic regulator AMPK (Fig. 1B), nor GLUT4 or fatty acid transporter (CD36, FABPpm, FATP1 and -4) protein contents differed between WT and Akt2-KO mice (Fig. 1C).

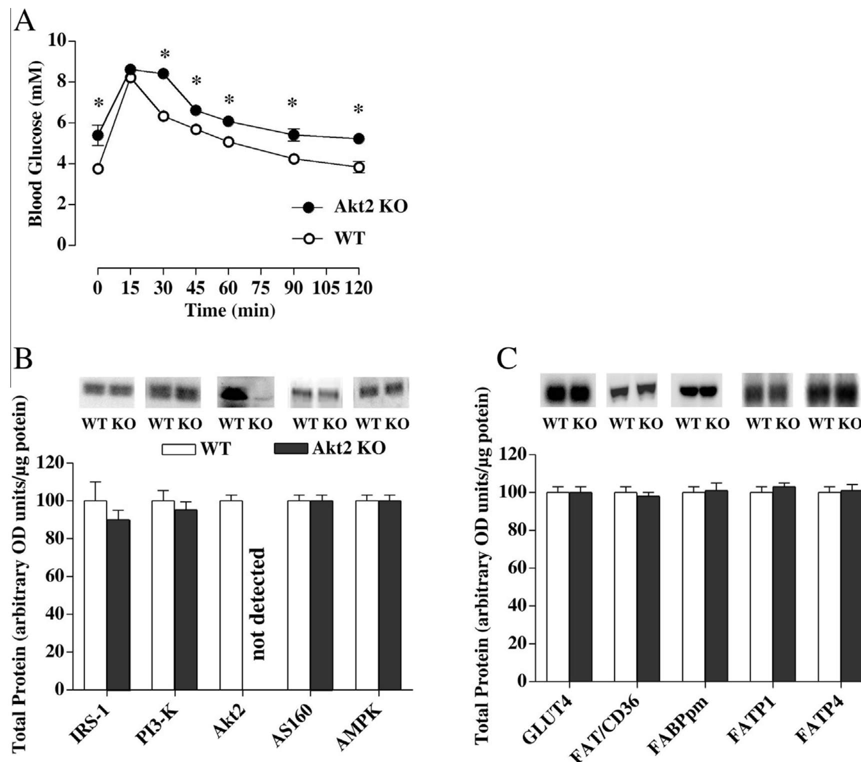

Fig. 1. Effects of Akt2 ablation on glucose tolerance (A), as well as expression of selected signaling proteins (B) and substrate transporters (C) in skeletal muscle. Representative Western blots are displayed. Mean ± S.E.M., $n = 4–6$ independent determinations for each measurement. $P < 0.05$, KO vs WT.

3.3. Phosphorylation of insulin signaling proteins and AMPK

Bolus administration of insulin (15 min) was used to examine insulin's effects on skeletal muscle Akt2, AS160 and AMPK phosphorylation. In addition, phosphorylation of these proteins was examined in response to electrically stimulated muscle contraction (15 min). We confirmed the expected effects of Akt2 ablation in skeletal muscle, namely impaired insulin-stimulated Akt phosphorylation Akt in Akt2-KO mice ($P < 0.05$, Fig. 2A, and [23]). Residual Akt phosphorylation in Akt2-KO muscle is likely to be attributable to phosphorylation of the other Akt isoforms [19]. Furthermore, insulin-stimulated phosphorylation of AS160, the downstream target of Akt2, was completely abolished in Akt2-KO mice (Fig. 2B). Hence, other Akt isoforms do not contribute to insulin-stimulated AS160 phosphorylation.

Muscle contraction had a small but significant effect on Akt phosphorylation in WT muscle (Fig. 2A). Furthermore, muscle contraction markedly stimulated AS160 phosphorylation in WT muscle (Fig. 2B), in agreement with the notion that AS160 is a convergence point of insulin and contraction signaling. Both contraction-induced AS160 and AMPK phosphorylations were preserved in Akt2-KO muscle (Fig. 2B and C), thereby confirming that Akt2 is not involved in contraction signaling. Finally, Akt2 is not involved in basal AMPK phosphorylation, and as expected, insulin stimulation did not alter AMPK phosphorylation (Fig. 2C).

3.4. Glucose transport and GLUT4 translocation in Akt2-KO muscle

Under basal conditions, glucose transport into giant vesicles and sarcolemmal GLUT4 content were not different in WT and Akt2-KO mice (Fig. 3A and B). As expected, in Akt2-KO muscle insulin-stimulated glucose transport and GLUT4 translocation were markedly blunted (Fig. 3A and B). In contrast, contraction-stimulated glucose transport and sarcolemmal GLUT4 content were each increased comparably ($P < 0.05$) in WT and Akt2-KO mice (Fig. 3A and B), in agreement with other observations in Akt2-KO mice [24].

3.5. Fatty acid transport and transporters in Akt2-KO muscle

In giant vesicles from WT muscle, fatty acid transport was markedly increased, either with insulin stimulation (+117%) or with muscle contraction (+107%) (Fig. 4A). However in Akt2-KO muscle, insulin and contraction were unable to stimulated fatty acid transport (Fig. 4A).

We next examined whether the impairments in fatty acid transport in Akt2-KO muscle were associated with concurrent alterations in insulin-, and contraction-stimulated fatty acid transporter translocation. Basal contents of sarcolemmal CD36 and FATP1 were not altered in Akt2-KO mice (Fig. 4B and C). In contrast, basal levels of sarcolemmal FABPpm (+12%) and FATP4 (+38%) were increased in Akt2-KO mice (Fig. 4D and E).

In WT mice, insulin stimulation was associated with increased sarcolemmal presence of all fatty acid transporters (CD36 (+77%), FABPpm (+13%), FATP1 (+64%), and FATP4 (+54%)) (Fig. 4B–E), confirming previous observations [7]. In contrast in Akt2-KO mice, insulin failed to stimulate CD36 and FATP1 translocation (Fig. 4B and C). Moreover, insulin stimulation did not increase sarcolemmal contents of FABPpm and FATP4 in Akt2-KO mice beyond the increases observed under basal conditions (Fig. 4D and E).

Muscle contraction increased the sarcolemmal presence of all fatty acid transporters in WT mice (CD36 (+101%), FABPpm (+21%), FATP1 (+70%), and FATP4 (+50%)) (Fig. 4B–E), confirming previous observations [7]. However, as with insulin stimulation, muscle contraction in the Akt2-KO mice failed to induce CD36 and FATP1 translocation (Fig. 4B and C). In addition, muscle con-

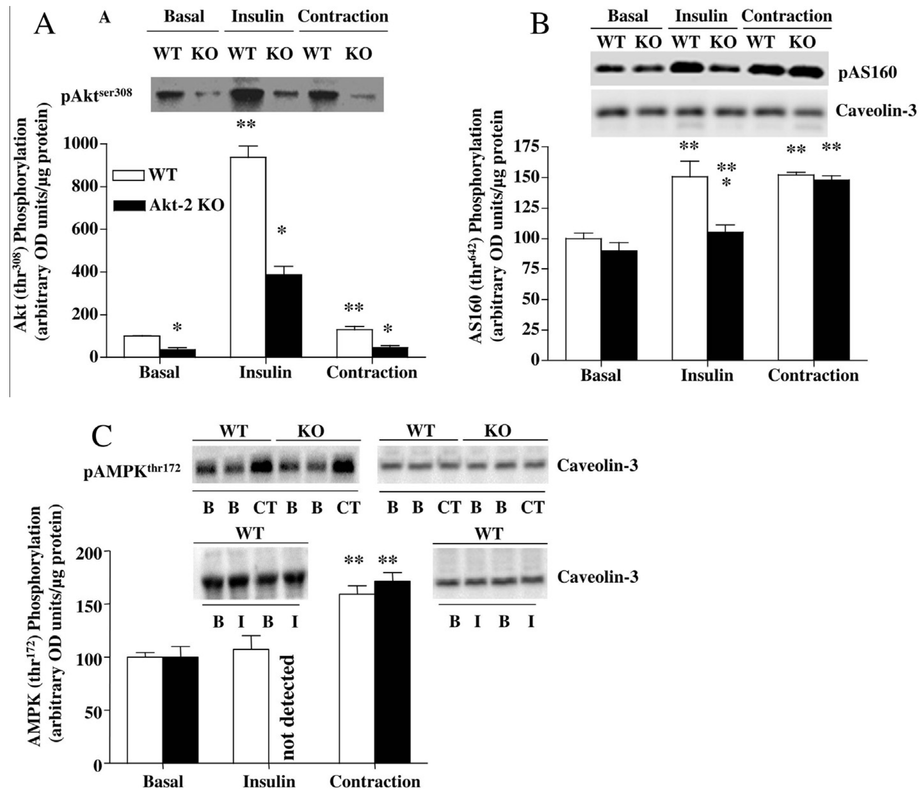

Fig. 2. Effects of Akt2 ablation on insulin and contraction-stimulated phosphorylation of Akt at Thr^{308} (A), AS160 at Thr^{642} (B) and AMPK at Thr^{172} (C) in muscle. B: basal; I: insulin; CT: contraction. Representative Western blots are displayed. Caveolin-3 was used as loading control for both the Akt-phospho-Thr^{308} and AS160-phospho-Thr^{642} blots. Mean ± S.E.M., n = 4–6 independent determinations for each measurement. $^{*}P < 0.05$, KO vs WT; $^{**}P < 0.05$, insulin vs basal or contraction vs basal within WT or within Akt2-KO.

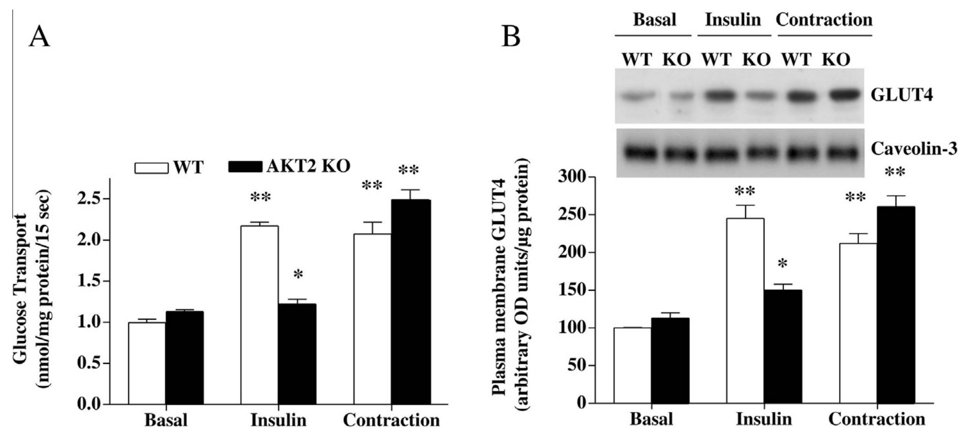

Fig. 3. Effects of Akt2 ablation on glucose transport into giant sarcolemmal vesicles (A) and sarcolemmal GLUT4 content (B) under basal conditions and after stimulation with insulin or with muscle contraction. A representative Western blot is displayed with caveolin-3 as loading control. Mean ± S.E.M., n = 4–6 independent determinations for each measurement. $^{*}P < 0.05$, KO vs WT; $^{**}P < 0.05$, insulin vs basal, or contraction vs basal within WT or within Akt2-KO.

traction also failed to increase sarcolemmal contents of FABPpm and FATP4 in Akt2-KO mice beyond the increases observed under basal conditions (Fig. 4D and E).

4. Discussion

We have examined the role of Akt2 in skeletal muscle on (a) insulin- and contraction-stimulated fatty acid transport, and (b) the translocation of fatty acid transport proteins to the sarcolemma. For comparison we studied GLUT4-mediated glucose uptake and confirm previous observations in Akt2-KO mice [18,19,22,25] that insulin-stimulated glucose transport and GLUT4 translocation are impaired, while contraction-stimulated glucose transport and GLUT4 translocation are comparably up-regulated in both WT and Akt2-KO mice. With respect to the fatty acid transporters, we made several novel observations, which indicate that their Akt2-dependent subcellular localization is regulated differently from that of GLUT4. Specifically, we demonstrate for the first time the critical role of Akt2 signaling in both insulin-stimulated and contraction-stimulated translocation of CD36 and FATP1. In contrast, under basal conditions, Akt2 ablation releases the brake on the subcellular retention of FABPpm and FATP4, allowing these

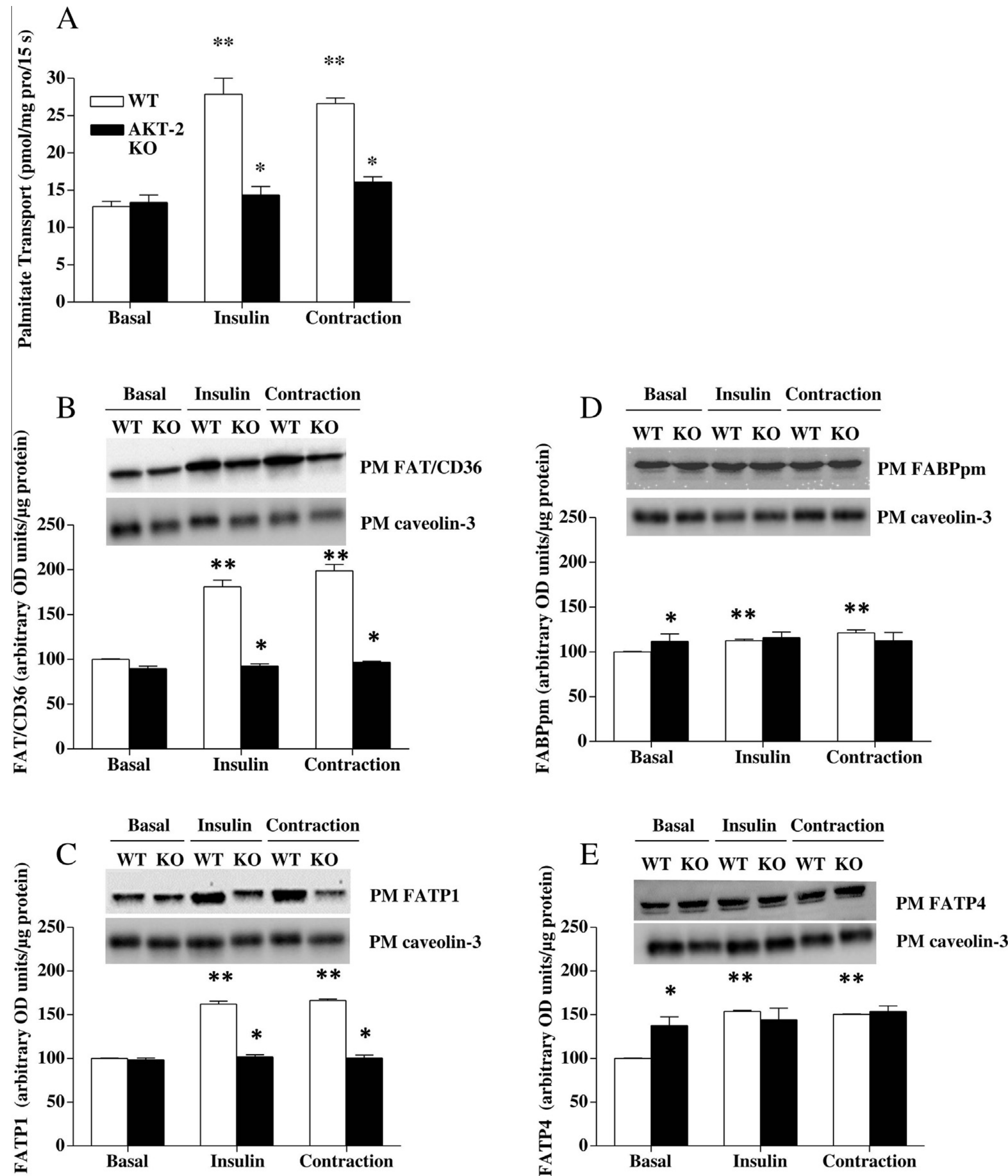

Fig. 4. Effects of Akt2 ablation on palmitate transport into giant sarcolemmal vesicles (A) and sarcolemmal contents of FAT/CD36 (B), FATP1 (C), FABPpm (D) and FATP4 (E) under basal conditions and after stimulation with insulin or with muscle contraction. Representative Western blots and corresponding caveolin-3 blots as loading controls are displayed. Mean ± S.E.M., n = 4–6 independent determinations for each measurement. $^{*}P < 0.05$, KO vs WT; $^{**}P < 0.05$, insulin vs basal, or contraction vs basal within WT or within Akt2-KO.

transporters to relocate to the sarcolemma, and rendering them insensitive to further stimulation.

4.1. Basal, insulin and contraction-stimulated signaling, glucose transport and GLUT4 translocation

As expected, Akt2-KO muscle exhibited impaired insulin signaling as shown by the abolishment of insulin-induced AS160 phosphorylation. Consequently, in Akt2-KO mice whole body glucose tolerance as well as insulin-stimulated glucose transport and GLUT4 translocation in muscle were impaired. In contrast, contraction-stimulated AMPK phosphorylation, AS160 phosphorylation, glucose transport and GLUT4 translocation were normal in Akt2-KO muscle. Hence, insulin and contraction stimulate GLUT4 translocation via distinct signaling pathways: insulin-stimulated, but not contraction-stimulated, GLUT4 translocation is dependent on Akt2 [2,4,26,27]. The confirmation of these GLUT4 dynamics also indicates that giant vesicles are well-suited to study translocation processes in vitro [20]. We have previously shown that increases in substrate transporter contents in these giant vesicles are due to their translocation from intracellular depots [20].

4.2. Basal, insulin- and contraction-stimulated fatty acid transport and sarcolemmal fatty acid transporters

In Akt2-KO muscle, total tissue expression of all four fatty acid transporters was unaltered. Furthermore, in Akt2-KO muscle, insulin or contraction failed to stimulate fatty acid transport and translocation of CD36 and FATP1. Conversely, Akt2 ablation caused an increase in sarcolemmal FABPpm and FATP4 in non-stimulated muscle with no further increases in response to metabolic stimuli. Hence, we propose the following novel roles of Akt2 in regulation of muscle substrate transporter dynamics: Akt2 mediates translocation of CD36 and FATP1 in response to both (i) insulin and (ii)

muscle contraction, as well as (iii) imposing intracellular retention of FABPpm and FATP4 under basal conditions. Moreover, the close correlation between fatty acid transport dynamics and alterations in sarcolemmal CD36 and FATP1, but not FABPpm and FATP4, indicates that CD36 and/or FATP1 are the main fatty acid transporters responsible for Akt2 regulation of muscle fatty acid transport. Although prior work has shown that FATP1 is insulin-sensitive [7,28], its fatty acid transport capacity is low [21] and it is generally poorly correlated with indices of fatty acid transport and metabolism among metabolic heterogeneous muscle tissues [21,29]. Moreover, in muscle from CD36-KO mice, insulin, contraction/exercise or the contraction-mimetic AMPK activator AICAR fail to stimulate fatty acid transport [30,31], despite the presence of all other fatty acid transporters. Together, these observations indicate that muscle metabolic flexibility is largely rendered by CD36. Below, we will further speculate on the three novel roles of Akt2 in regulation of muscle substrate transporter dynamics:

(i) Akt2 and insulin-induced CD36 and FATP1 translocation: Akt is known to regulate GLUT4 translocation via phosphorylation of AS160 [32]. In the non-phosphorylated state, AS160 blocks GLUT4 translocation by means of its Rab-GTPase activity, thereby keeping the Rab proteins mediating GLUT4 translocation in an inactive state. AS160 phosphorylation by insulin or contraction stimulation acts to relieve this blockade. Recently, AS160 was also shown to regulate CD36 translocation in HL1-cardiomyocytes via inhibition of specific Rabs [23]. FATP1 was not investigated in that study. In the current work we observed a loss of insulin-induced AS160 phosphorylation in Akt2-KO muscles (Fig. 2B), which correlated with a loss of insulin-induced GLUT4 (Fig. 3B), CD36 (Fig. 4A) and FATP1 (Fig. 4C) translocation. This may point to a critical involvement of Akt2-mediated AS160 phosphorylation in insulin-stimulated GLUT4 and CD36 (and perhaps FATP1) translocation in muscle.

(ii) Akt2 and contraction-induced CD36 and FATP1 translocation: In contrast to the decrease in AS160 phosphorylation in insulin-exposed muscle, AS160 phosphorylation is not altered in contracting muscle upon Akt2 ablation. Hence, the loss of contraction-induced CD36 (and FATP1) translocation cannot be explained at the level of AS160 regulation.
Notably, our work demonstrates that contraction-stimulation of fatty acid transport and transporters is distinct from contraction-induced glucose transport and GLUT4 translocation. Specifically, contraction-induced glucose transport and GLUT4 translocation are not impaired by Akt2 ablation in muscle, whereas Akt2 is required for contraction-stimulated fatty acid transport and FAT/CD36 and FATP1 translocation. Also previously we have reported differences between the regulation of GLUT4 and fatty acid transporter trafficking to the sarcolemma. These differences appear to occur at the level of SNARE complex formation, which is necessary for the proper fusion of transport vesicles with their target membrane. For instance, Munc18c, a t-SNARE-interacting protein, was established to be involved in insulin-stimulated GLUT4 translocation to the sarcolemma, but appeared not required for the trafficking of any of the fatty acid transporters (FAT/CD36, FABPpm, FATP1, -4 and -6), either in insulin-, or contraction-stimulated skeletal muscle [33]. Furthermore, VAMP proteins, a family of v-SNAREs, have been found to discriminate between GLUT4 translocation versus FAT/CD36 translocation as well as between insulin-stimulated transporter translocation versus contraction-induced transporter translocation, indicating that specific VAMP isoforms are involved in each of these processes [34]. Therefore, Akt2 binding to a specific VAMP isoform might explain the differences in dependency of contraction-induced FAT/CD36 (and possibly FATP1) translocation versus contraction-induced GLUT4 translocation.

(iii) Akt2 and intracellular retention of FABPpm and FATP4: another unexpected finding of the present study was that the translocation of the various fatty acid transporters to the sarcolemma is regulated differently by Akt2, as only FABPpm and FATP4 sarcolemmal contents in Akt2-KO muscles were increased under basal conditions with no further increase when metabolic stimuli are applied. The molecular mechanisms behind these differential migration patterns of FABPpm and FATP4 versus CD36 and FATP1 in Akt2-KO muscle are completely unknown. Furthermore, it remains to be established whether the Akt2-mediated retention of FABPpm and FATP4 is due to inhibition of their translocation or to induction of their endocytosis.

4.3. Summary

The present study demonstrated that in muscle, both insulin and contraction-stimulated fatty acid transport are critically dependent on Akt2-mediated translocation of FAT/CD36 and FATP1 to the sarcolemma. This differs from GLUT4 translocation, which is only Akt2-dependent when stimulated with insulin but not with muscle contraction. Akt2 regulates subcellular localization of FABPpm and FATP4 in quite a different manner, namely by retaining these fatty acid transporters within their subcellular depots. Collectively, our observations reveal a previously unknown complexity in signaling mechanisms regulating the intracellular translocation of fatty acid transporters in skeletal muscle. Finally, it remains to be investigated whether this Akt2-mediated short-term regulation of fatty acid transport would impact on long-term regulation of myocellular triglycerides and other fatty acid metabolites.

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