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Published in:
PLoS ONE

DOI:
10.1371/journal.pone.0092255

Publication date:
2014

Document Version
Publisher's PDF, also known as Version of record

Link to publication in Discovery Research Portal

Citation for published version (APA):

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Enhanced Insulin Sensitivity Associated with Provision of Mono and Polyunsaturated Fatty Acids in Skeletal Muscle Cells Involves Counter Modulation of PP2A

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Abstract

Aims/Hypothesis: Reduced skeletal muscle insulin sensitivity is a feature associated with sustained exposure to excess saturated fatty acids (SFA), whereas mono and polyunsaturated fatty acids (MUFA and PUFA) not only improve insulin sensitivity but blunt SFA-induced insulin resistance. The mechanisms by which MUFAs and PUFAs institute these favourable changes remain unclear, but may involve stimulating insulin signalling by counter-modulation/repression of protein phosphatase 2A (PP2A). This study investigated the effects of oleic acid (OA; a MUFA), linoleic acid (LOA; a PUFA) and palmitate (PA; a SFA) in cultured myotubes and determined whether changes in insulin signalling can be attributed to PP2A regulation.

Principal Findings: We treated cultured skeletal myotubes with unsaturated and saturated fatty acids and evaluated insulin signalling, phosphorylation and methylation status of the catalytic subunit of PP2A. Unlike PA, sustained incubation of rat or human myotubes with OA or LOA significantly enhanced Akt- and ERK1/2-directed insulin signalling. This was not due to heightened upstream IRS1 or PI3K signalling nor to changes in expression of proteins involved in proximal insulin signalling, but was associated with reduced dephosphorylation/inactivation of Akt and ERK1/2. Consistent with this, PA reduced PP2Ac demethylation and tyrosine307 phosphorylation - events associated with PP2A activation. In contrast, OA and LOA strongly opposed these PA-induced changes in PP2Ac thus exerting a repressive effect on PP2A.

Conclusions/Interpretation: Beneficial gains in insulin sensitivity and the ability of unsaturated fatty acids to oppose palmitate-induced insulin resistance in muscle cells may partly be accounted for by counter-modulation of PP2A.


Editor: Juergen Eckel, GDC, Germany
Received December 13, 2013; Accepted February 19, 2014; Published March 14, 2014

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Funding: This work was supported by grants from BBSRC and Diabetes UK. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Sustained elevation in circulating non-esterified free fatty acids (NEFA) as seen during obesity and Type 2 diabetes is associated with impaired insulin signalling in tissues such as skeletal muscle thus potentially contributing to disturbances in whole body glucose metabolism in obese or diabetic individuals [1–3]. In particular, over-provision of saturated fatty acids (SFA, e.g. palmitate) result in tissue accumulation of lipotoxic fatty acid derivatives such as diacylglycerol (DAG) and ceramide that promote activation of (i) DAG-sensitive PKC, (ii) atypical PKCs and (iii) PP2A that, in turn, impair IRS- and Akt-directed insulin signalling by mechanisms involving IRS serine phosphorylation or repression of Akt activation/phosphorylation [4–9]. Strikingly, dietary substitution of SFAs or co-supplementation with unsaturated fatty acids antagonise the insulin desensitising effects of SFAs, enhance energy expenditure and improve muscle lipid composition and serum acylcarnitine profiles in humans [10–14]. Such observations are in line with cell-based studies demonstrating that monounsaturated fatty acids (MUFAs), such as palmitoleate and oleate, not only attenuate the SFA-induced loss in mitochondrial integrity and function but also repress their proinflammatory and ER stress-inducing potential [15–18]. Moreover, the observation that increases in serum palmitoleate induce a strong insulin potentiating effect in mouse liver and skeletal muscle implies that this MUFA possesses unique metabolic attributes not held by its saturated counter-part, palmitate [19]. However, despite the observed gain in insulin sensitivity brought about by palmitoleate in these peripheral tissues, our understanding of the underlying mechanism remains poor [19].

In this study we have investigated the effects of oleic acid (OA; a cis-C18:1, n-9 MUFA) and linoleic acid (LOA; a cis-C18:2, n-6 PUFA) upon insulin signalling events in cultured rat and human skeletal myotubes. Our work reveals that both oleic acid and...
linoleic acid induce an increase in insulin sensitivity based on enhanced Akt- and Erk-directed insulin signalling. These effects cannot be attributed to an elevation in upstream IRS/Pi3K signalling but were associated with increased tyrosine phosphorylation (Tyr^308) and demethylation of the PP2A catalytic subunit (PP2Ac) – covalent changes that help retain the phosphatase in a repressed state [20,21]. In contrast, palmatine (PA) attenuated tyrosine phosphorylation and demethylation of PP2Ac by insulin in line with the known stimulatory effect that this SFA has upon PP2A [22]. These PA-induced changes in PP2A were antagonised by both OA and LOA. Our data indicate that increases in insulin sensitivity brought about by exposure of muscle cells to OA and LOA may, in part, be brought about by covalent modification and cellular repression of PP2A.

**Materials and Methods**

**Materials**

3-minimal essential medium, fetal bovine serum and antibiotic/antimycotic solution were from Life Technologies. All other reagent-grade chemicals, including BSA and fatty acids, were obtained from Sigma–Aldrich unless otherwise stated. Wortmannin and okadaic acid were from Calbiochem-Merck. Fraction V fatty acid-free BSA was from Roche. Insulin was from R&D systems. Complete protein phosphatase inhibitor tablets were purchased from Roche Diagnostics. Phospho-Akt^Thr308, phospho-Akt^Ser473, phospho-ERK1/2^Thr202/204, phospho-GSK3^β^Ser21/9, phospho-CREB^Ser133, phospho-Src^Tyro1166, anti-Akt and anti-ERK1/2 antibodies were from Cell Signaling Technology. Anti-AktSer473, phospho-ERK1/2Thr202/Tyr204, phospho-GSK3Ser21/9, anti-PP2A subunit C and supernatant frozen in liquid nitrogen prior to storage at -20°C.

**Cell culture, fatty acid treatment and cell lysis**

Human skeletal muscle cells derived from biopsies (quadriceps muscle) from healthy adult lean volunteers obtained in the context of approved preclinical and clinical trials [23], and the Tissue Bank for Research (Myobank) of the French Association against Myopathies (AFM), in agreement with the French bioethical law considered statistically significant at P<0.05.

**Results**

**Effects of OA and LOA on insulin signalling**

To characterise any insulin potentiating effect that OA and LOA may have on intracellular signalling we performed time and dose studies for both NEFAs but in the presence of a sub-maximal (20 nM) insulin concentration [Fig 1A]. At this concentration, insulin induced a modest increase in Akt phosphorylation, which was barely detectable in untreated cells (Fig 1B,1C). However, Akt phosphorylation was enhanced significantly when myotubes were preincubated with OA or LOA for 16h in a dose-dependent manner. This enhancement was significant at fatty acid concentrations as low as 200 μM (i.e. within the physiological range) and was ~2-fold greater than that elicited by insulin treatment alone when myotubes were pre-exposed to maximally effective doses (700 μM) of OA or LOA (Fig 1B, 1C). The increase in Akt phosphorylation was detectable after 9h but was not maximal until 16h of fatty acid treatment (Fig 1D,1E). Extended fatty acid incubation periods up to 24h did not induce further increases in Akt phosphorylation over and above that seen after 16h (data not shown). Fig 1D and 1E show that the increase in signalling was not restricted to Akt and that ERK1/2 phosphorylation was also enhanced. In the absence of insulin, neither fatty acid had any effect on Akt or ERK1/2 phosphorylation (Fig 1D, 1E). It is also important to stress that treatment of myotubes with the vehicle (i.e. BSA) alone did not enhance insulin-dependent phosphorylation of Akt or ERK when compared with cells not exposed to the vehicle (data not shown).

The increased sensitisation towards insulin elicited by both NEFAs was most apparent and significant when monitored using a sub-maximal insulin dose as no additional increase in Akt phosphorylation was observed when OA- and LOA-treated myotubes were treated with a maximally effective (100 nM) insulin concentration (Fig 2A, 2B). Fig 2C shows that we observe no major changes in the expression of the insulin receptor β-subunit, IRS-1, PI3K-p85 subunit or of 3′-phosphoinositide lipid phosphatase (PTEN), all of which lie upstream of Akt and ERK1/2 in response OA or LOA treatment. Furthermore, neither OA nor LOA had any effect on total Akt or ERK1/2 abundance thus excluding this as a possible explanation for the net increase in their phosphorylation. The data highlights that in addition to promoting Ser^173 phosphorylation, both OA and LOA induce an equivalent gain in Akt-Thr^308 phosphorylation (Fig 2C, 2D). Equimolar phosphorylation of both sites is required for full
activation of Akt and consistent with this we observed elevated phosphorylation of GSK3; a physiological downstream Akt target (Fig 2C). Likewise, enhanced ERK1/2 activation promotes greater phosphorylation/activation of CREB, a transcription factor regulated downstream of the ERK pathway. The increase in insulin-stimulated Akt phosphorylation induced by OA and LOA was not restricted to L6 myotubes. Fig 2E shows that incubation of human skeletal myotubes with OA or LOA caused a similar enhancement in Akt and ERK1/2 phosphorylation in response to insulin and that of GSK3 and CREB, which respectively lie downstream of Akt and ERK signalling.

To understand how OA and LOA might enhance insulin-signalling we monitored the effect of both fatty acids on p85-PI3-kinase association with IRS1 and cellular PIP3 synthesis. As anticipated, based on coprecipitation analysis, Fig 3A shows that insulin induced p85-PI3K interaction with IRS1. This interaction
was not influenced by OA or LOA. Since Akt activation relies upon insulin-dependent PIP₃ synthesis it is possible that OA and LOA may have enhanced production of this lipid. However, Fig 3B shows that, irrespective of whether muscle cells were incubated in the absence or presence of OA or LOA, insulin stimulated PIP₃ synthesis by ~2-fold.

OA and LOA help sustain Akt and ERK1/2 activation

Given that signalling events at the level of IRS1/PI3K were unaffected by OA or LOA we investigated the possibility that increased phosphorylation/activation of Akt and ERK1/2 by insulin may be a consequence of their reduced dephosphorylation. L6 myotubes were incubated in the absence or presence of these fatty acids for 16h and subjected to an acute insulin challenge in the penultimate 10 min period of this fatty acid incubation. Myotubes were subsequently immunoblotted using antibodies against the proteins indicated with quantification of phospho-Akt (Ser473 and Thr308) and phospho-ERK1/2 shown in panel A,B and D expressed as mean ± SEM of at least three experiments. The immunoblots shown in panel E are representative of two separate experiments. The asterisks signify a significant change (p<0.05) between the indicated bars (A,B) or the insulin-treatment alone (D).

doi:10.1371/journal.pone.0092255.g002

Figure 2. Oleic acid (OA) and linoleic acid (LOA) enhance insulin sensitivity of rat L6 and primary human myotubes. (A) L6 myotubes were incubated with serum-free media containing 2% (w/v) BSA (vehicle) ± 700 μM (A) oleic acid (OA) or (B) linoleic acid (LOA) for 16h prior to stimulation with insulin at concentrations indicated (10min). (C, D) L6 myotubes were incubated as in (A) prior to stimulation with insulin (20 nM, 10min). (E) Human myotubes were incubated with 200 μM OA or LOA for 24h prior to stimulation with insulin (20 nM, 10min). Cell lysates from A-E were subsequently immunoblotted using antibodies against the proteins indicated with quantification of phospho-Akt (Ser473 and Thr308) and phospho-ERK1/2 shown in panel A,B and D expressed as mean ± SEM of at least three experiments. The immunoblots shown in panel E are representative of two separate experiments. The asterisks signify a significant change (p<0.05) between the indicated bars (A,B) or the insulin-treatment alone (D).
and sustained phosphorylation of both kinases (Fig 5B and 5C) in a manner comparable to that seen following fatty acid treatment (Fig 4B-E).

**PP2Ac phosphorylation and demethylation**

The above findings support the idea that OA and LOA may enhance insulin signalling via repression of PP2A. To assess this possibility we investigated the effect of OA, LOA and the SFA, palmitate, on PP2A. Akt-directed insulin signalling is suppressed by PA and this effect is partly reliant upon the known stimulatory effect that PA exerts upon PP2A [32]. Fig 6A shows that, unlike OA or LOA, sustained exposure of L6 myotubes to PA reduced insulin-stimulated AktS473 phosphorylation (compare lane 2 with 4 and also lanes 6 and 8 with lane 4). This reduction appears to be antagonised when PA-treated myotubes were co-incubated with OA or LOA (compare Lane 4 with lanes 10 and 12). However, the overall gain in phosphorylation remains significantly lower when compared to myotubes exposed to OA or LOA alone (compare lanes 6 and 8 with 10 and 12, respectively).

To assess whether PP2A may be regulated in a manner that accounts for changes in Akt phosphorylation seen in response to insulin, OA, PA, LOA and the SFA, palmitate [32]. Fig 6A shows that, unlike OA or LOA, sustained exposure of L6 myotubes to PA reduced insulin-stimulated AktS473 phosphorylation (compare lane 2 with 4 and also lanes 6 and 8 with lane 4). This reduction appears to be antagonised when PA-treated myotubes were co-incubated with OA or LOA (compare Lane 4 with lanes 10 and 12). However, the overall gain in phosphorylation remains significantly lower when compared to myotubes exposed to OA or LOA alone (compare lanes 6 and 8 with 10 and 12, respectively).

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**Discussion**

Numerous studies have demonstrated that increased availability of SFAs negatively regulate skeletal muscle insulin sensitivity with important knock-on consequences for processes such as gene expression and cell metabolism that are normally regulated by the hormone [8,33–35]. In contrast, considerable evidence supports the view that insulin sensitivity is positively correlated with supply of MUFAs and PUFAs and that, in many instances, their provision not only promotes beneficial metabolic changes but negate some of the adverse effects associated with SFA oversupply [18,35,36].
Indeed, previous work from our group has demonstrated that, unlike PA, provision of MUFAs (e.g. palmitoleate and oleate) can induce insulin-like effects upon distal cellular responses, such as glucose uptake by inducing plasma membrane recruitment of glucose transporters [36]. Intriguingly, this effect does not involve activation of molecules involved in proximal insulin signalling (e.g. PI3K, Akt) and, moreover, no additional enhancement in glucose uptake is observed when muscle cells are simultaneously challenged with the MUFA and a maximally effective concentration of insulin [36]. This latter finding implies that signals initiated

Figure 4. Oleic acid (OA) and linoleic acid (LOA) enhance and prolong insulin-dependent phosphorylation of Akt and ERK1/2 in L6 myotubes. The experimental approach used to test the effect of OA and LOA provision on Akt and ERK1/2 phosphorylation is depicted in (A). L6 myotubes were incubated with serum-free media containing 2% (w/v) BSA (vehicle) ± 700 μM (B and C) oleic acid (OA) or (D and E) linoleic acid (LOA) for 16h prior to stimulation with insulin (20 nM, 10min). Cells were washed to remove insulin and subsequently incubated with 100 nM wortmannin (WM) and lysed at times indicated. Immunoblots and relevant quantification of phospho-Akt and phospho-ERK1/2 in L6 myotubes incubated with OA (B,C) or LOA (D,E). Data in the bar graphs is presented as mean ± SEM (n = 3) with asterisks indicating a significant change (p<0.05) between the respective filled (fatty acid treatment) and unfilled (control) bar at each time point.

doi:10.1371/journal.pone.0092255.g004
by palmitoleate and insulin ultimately converge upon a common end-point that promotes translocation and/or activation of glucose transporters. The current study indicates that, in addition to such effects, incubating myotubes with insulin at a sub-maximal concentration unveils an insulin-sensitising effect of unsaturated fatty acids, such as OA and LOA, which is likely to involve suppression of PP2A action.

Several groups have reported hyperactivation of PP2A in response to sustained oversupply of glucose and SFAs (i.e. glucolipotoxicity), which may be important in the pathogenesis of insulin resistance [22,37–39]. The heightened PP2A activity that is observed under such circumstances may not only involve an increase in PP2Ac expression but also that of the many regulatory PP2A subunits [40], a proposition based on the finding that Ptpa, B55β and B56α are all elevated in muscle and liver of insulin resistant (ZDF) rats [22]. Whilst we cannot discount the possibility that OA and LOA may repress expression of the catalytic and certain regulatory PP2A subunits, our finding that they restrain PA-induced changes in PP2Ac phosphorylation/methylation is likely to be of significance in helping to counter PP2A hyperactivation by SFAs. The differential effect that unsaturated and saturated fatty acids have upon PP2A may be explained, in part, by their contrasting effects on Src, which affects PP2A activity via direct phosphorylation of PP2Ac at Tyr307 [39,41]. In line with previous studies [42], we show that PA inhibits Src activation based on a reduction in SrcY416 phosphorylation and a concomitant reduction in the downstream phosphorylation of PP2Ac on Tyr307 (Fig 6B). As a consequence, PP2Ac would be expected to be more active under these circumstances leading to counter-modulation of signalling molecules activated by insulin. In sharp contrast, as reported in other cell types [43,44], both OA and LOA induce ScrY416 phosphorylation to a level comparable if not greater than that seen in response to insulin - thus promoting a concomitant increase in PP2Ac tyrosine phosphorylation (Fig 6C, 6D). Unlike PA, the modulation of Src by OA and LOA in our model would suppress PP2A action leading, as we show, to greater and more sustained activation of some of its targets (i.e. Akt and ERK1/2) by insulin. The molecular basis for this differential fatty acid effect on Src is currently unclear but may depend upon their ability to activate membrane bound fatty acid receptors (e.g. GPR40 and GPR120) that couple to signalling molecules influencing the activation status of Src. Activation of such

Figure 5. Okadaic acid emulates the insulin-sensitising effect of oleic acid (OA) and linoleic acid (LOA) upon Akt and ERK phosphorylation in L6 myotubes. L6 myotubes were incubated with serum-free media containing okadaic acid (100 nM) or vehicle (dH2O) for 15 min and with insulin (20 nM) during the penultimate 10 min period of this incubation. Cells were washed and then maintained in serum-free media containing wortmannin (WM, 100 nM) and lysed at times indicated. Cell lysates were subsequently immunoblotted using antibodies against the proteins indicated. Data in the bar graphs is presented as mean ± SEM (n = 3) with asterisks indicating a significant change (p<0.05) between the respective filled (okadaic acid treatment) and unfilled (control) bar at each time point. doi:10.1371/journal.pone.0092255.g005
receptors has been implicated in OA-induced activation of ERK1/2 and AP-1 DNA binding activity through a mechanism involving Src in MCF-7 breast cancer cells [43]. Whether such a mechanism explains activation of Src by OA and LOA in skeletal muscle cells and the rather gradual tyrosine phosphorylation and demethylation of PP2Ac is currently unknown, although, we do have evidence for GPR40 expression in L6 myotubes (data not shown). Defining whether GPR40 is a component of the mechanism by which OA and LOA enhance skeletal muscle insulin-sensitivity represents an important investigative goal of future work.

Our results indicate that both OA and LOA promote an increase in proximal insulin signalling and can attenuate the effect that PA has upon Src and PP2Ac phosphorylation. However, it is apparent, from comparing the net changes, that the ability with which both OA and LOA enhance insulin-dependent Akt phosphorylation is somewhat compromised when cells are also incubated with PA (Fig 6A). We recently demonstrated that PA uptake into myotubes is not affected by co-provision of MUFAs and that under these circumstances there is no reduction in PA-derived ceramide synthesis [18]. Consequently, sustained ceramide generation will activate atypical PKCs that physically associate with and negatively regulate Akt activation [7,8].
However, despite the inability to repress ceramide synthesis from the SFA it is apparent that MUFA and PUFA provision mitigate some of the deleterious effects that PA has upon insulin-action 

In summary, the enhanced insulin sensitivity associated with OA and LOA provision is likely to reflect the ability of these unsaturated fatty acids to modulate methylation and phosphorylation of PP2Ac in a manner that represses phosphatase action. Our observations support the view that diets enriched with OA for example, its ability to activate PP2A.

Acknowledgments

We are grateful to the tissue bank of the Association Française des Myopathies (AFM) for human biopsy for control human biopsy samples.

Author Contributions

Conceived and designed the experiments: FN EH HSH. Performed the experiments: FN DL RHH AG. Analyzed the data: FN CL AG EH HSH. Wrote the paper: FN EH HSH.

References