Acute elevation of circulating fatty acids impairs downstream insulin signalling in rat skeletal muscle in vivo independent of effects on stress signalling

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Abstract

The aim of this study was to examine the effect of an acute, physiological increase in plasma free fatty acid (FFA) on initial signalling events in rat red quadriceps muscle (RQ). Male Wistar rats received a 7% glycerol (GLYC) or 7% Intralipid/heparin (LIP) infusion for 3 h, after which they were either killed or infused with insulin at a rate of 0.5 U/kg per h for 5 min, before RQ collection. Plasma FFAs were elevated to ~2 mM in the LIP rats only. Insulin-stimulated insulin receptor (IR) Tyr1162/Tyr1163 phosphorylation and IR substrate (IRS)-1 Tyr612 phosphorylation were increased at least twofold over basal in GLYC rats with insulin and this increase was not significantly impaired in the LIP rats. However, there was no insulin-stimulated protein kinase B (PKB) Ser473 or glycogen synthase kinase (GSK)-3β Ser9 phosphorylation in the LIP rats, compared with at least a twofold increase over basal in GLYC rats for both proteins. c-Jun N-terminal kinase, inhibitor of κ kinase β and inhibitor of nuclear factor-κB phosphorylation and total protein expression, as well as Ser307-IRS-1 phosphorylation, were not altered by lipid infusion compared with GLYC infusion. These data indicate that acute, physiological elevation in FFA has a greater impact on insulin signalling downstream of IR and IRS-1, at the level of PKB and GSK-3β, and that under these conditions stress signalling pathways are not significantly stimulated. Decreased PKB and GSK-3β phosphorylation in RQ may therefore be primary determinants of the reduced insulin action observed in situations of acute FFA oversupply.


Introduction

The accretion of triglycerides (TGs) and various lipid intermediates in non-adipose tissue such as liver and muscle correlates strongly with reduced insulin action in these tissues (Storlien et al. 1991, Pan et al. 1997). We have recently shown that chronic feeding of a high saturated or polyunsaturated fat diet to rats impairs insulin action, and is associated with increased skeletal muscle TG content and reduced phosphorylation of insulin receptor substrate (IRS)-1 and protein kinase B (PKB) under conditions of physiological insulin elevation (Frangoudakis et al. 2005). Insulin resistance can also be induced by an acute infusion of lipid to elevate circulating fatty acid (FA; Chalkley et al. 1998, Ye et al. 2002). The degree of insulin resistance in acute lipid-infused rats can be similar to that observed in chronic high-fat-fed rats, and consequently it has been suggested that the lipid-induced insulin resistance in these models may share a similar mechanism. Studies assessing signalling after acute lipid infusion in rodents and humans have not always produced consistent results. For example, in skeletal muscle from rats, defective insulin action due to acute lipid infusion has been reported to occur in conjunction with reduced insulin-stimulated IRS-1-associated phosphatidylinositol-3-kinase (PI3-K) activity and reduced insulin-stimulated IRS-1 tyrosine phosphorylation in one study, although PKB phosphorylation was not measured (Griffin et al. 1999). In another study, PKB Ser473 phosphorylation was reduced but upstream signalling was not assessed (Ye et al. 2002). In humans, impaired skeletal muscle IRS-1 tyrosine phosphorylation and associated PI3-K activity has been described, but PKB phosphorylation was unchanged (Kruszynska et al. 2002).

Variable study conditions used to induce insulin resistance and provide an insulin stimulus may have influenced the reported differences in insulin signalling thought to represent the in vivo effect of elevated FA in insulin-resistant states. The levels of FA attained in the rodent studies described above (Griffin et al. 1999, Ye et al. 2002) were very high, in the range of 3–5 mM. FAs at this concentration are not physiological and could conceivably be directly deleterious to the normal cellular environment by destabilising membranes (Newsholme & Leech 1983). Furthermore, extended euglycaemic–hyperinsulinaemic clamps (Ye et al. 2002) and/or i.v. bolus insulin injections (Griffin et al. 1999) may not be ideal methods for examining the...
initial activation of the insulin signalling pathway that is thought to be critical for optimal insulin action.

It also remains unclear whether FAs themselves have a direct effect on insulin signalling to impair insulin action or whether the observed defects are a consequence of a systemic adaptation to excess lipid availability. Altered adipocytokine secretion (Matsuzawa et al. 1999), for example, can lead to the activation of inflammatory stress signalling pathways that have recently been suggested to be mediators of the insulin resistance seen in diabetes and obesity. Specifically, it has been postulated that excess FA can activate the c-Jun N-terminal kinase (JNK; Hirosumi et al. 2002) and inhibitor of κB kinase (IKK)/nuclear factor-κB (NF-κB; Kim et al. 2001, Yuan et al. 2001) pathways leading to inhibitory phosphorylation of insulin signalling intermediates (Aguirre et al. 2000, Gao et al. 2002, Le Marchand-Brustel et al. 2003). The extent to which this happens in muscle with lipid infusion has not been determined.

The aim of this study was to examine the consequences of an acute, physiological elevation of plasma FA on components of the insulin and stress signalling pathways in vivo in skeletal muscle, with or without an insulin infusion similar to the beginning of a euglycaemic–hyperinsulinaemic clamp. Our hypotheses were as follows: 1) changes in insulin signalling following acute lipid infusion may differ to those reported following chronic high-fat feeding and 2) stimulation of stress signalling may play a role in any observed impairments. We show that defective insulin signalling in this model originated downstream of the IR and IRS–1, at the level of PKB and its downstream target, glycogen synthase kinase (GSK)–3β. This was not associated with stimulation of stress signalling intermediates.

Materials and Methods

Experimental animals and dietary treatment

All procedures were approved by the Garvan Institute/St Vincent’s Hospital Animal Experimentation Ethics Committee and were in accordance with the National Health and Medical Research Council of Australia Guidelines on Animal Experimentation. Male Wistar rats (~300 g or 10 weeks of age) supplied from the Animal Resources Centre (Perth, Australia) were acclimatised in communal cages at 22 ± 1 °C with a 12 h light:12 h darkness cycle (lights on 0600 h) for 1 week and had access to a standard chow diet (Gordon’s Specialty Stock Feed, Sydney, Australia) and water ad libitum.

Animal preparation

After acclimatisation, rats were chronically cannulated via the right jugular vein and the left carotid artery under halothane (Fluothane; Cenvet Pty Ltd, NSW, Australia) inhalation anaesthesia (5% induction, 2% maintenance) and the cannulae were exteriorised via a small incision at the back of the neck. Post-surgery recovery over a 7-day period was closely monitored with the measurement of food intake and body weight gain. The rats were handled daily to minimise stress and only those with fully recovered body weight were used for the study.

Lipid infusion and insulin stimulation

Rats were fasted 5 h prior to study and were randomly assigned to be infused with either a 7% Intralipid/heparin infusion (LIP) for 3 h to induce insulin resistance or a 7% glycerol infusion (GLYC) for 3 h as control. The protocol used in this study was based on Ye et al. (2004), with an adjustment in the amount of TG emulsion being infused to limit the elevation in free FAs (FFAs) to within a range that may be seen clinically in type 2 diabetic patients (Fraze et al. 1985, Chen et al. 1987). The rate of infusion was 2 ml/h, with a concomitant heparin infusion of 40 U/h to aid in the lipolysis of the TG emulsion. Blood samples (100 μl) were taken every 30 min during the 3 h LIP or GLYC infusions and every 2 min during the insulin infusion for the analysis of FFA and insulin concentrations. After the LIP or GLYC infusion, the rats were either killed with an i.v. overdose of sodium pentobarbitone (Nembutal; Cenvet Pty Ltd) and tissues rapidly collected (BASAL), or a subset from each group was stimulated with insulin at a rate of 0.5 U/kg per h for 5 min (INSULIN) before tissue collection. Red quadriceps skeletal muscle (RQ) was rapidly dissected, freeze-clamped with aluminium tongs pre-cooled in liquid nitrogen and stored at −80 °C for subsequent analysis. Stress signalling results are from basal samples only; insulin signalling results are from both basal and insulin-stimulated samples.

Metabolite measurements

Plasma FFAs were determined spectrophotometrically using an enzymatic colorimetric method (NEFA–C kit; Wako Pure Chemical Industries, Osaka, Japan). Plasma insulin was determined by RIA using a rat-specific kit (Linco Research, St Charles, MO, USA). RQ skeletal muscle TGs were extracted using the method of Folch et al. (1957) and quantified using an enzymatic colorimetric method (GPO-PAP reagent; Roche Diagnostics).

Protein extraction

RQ muscle was homogenised in an ice-cold solubilisation buffer containing 65 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Nonidet P40, 0.5% Na-deoxycholate, 0.1% SDS, 10% GLYC, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 10 mM NaF, 1 mM Na3VO4 and 1 mM phenylmethylsulphonyl fluoride. The homogenate was solubilised for 2 h at 4 °C and then centrifuged at 12 000 g for 15 min to remove insoluble material. Protein concentration of the supernatants was determined using the Bio–Rad protein assay (Bio–Rad Laboratories).
Immunoblot analyses

Protein homogenates were prepared in Laemmli buffer (Laemmli 1970) and subjected to SDS-PAGE. To allow for quantification between blots, an in-house standard was also run on each gel. Proteins were separated on 6 or 7.5% gels, transferred to polyvinylidenedifluoride (PVDF) membranes (Hybond-P; Amersham Biosciences) and blocked in 1% BSA or 5% skim milk–Tris-buffered saline containing 0.025% Tween 20. The membranes were probed with the following primary antibodies that were mainly purchased from Cell Signaling Technology (Beverly, MA, USA), unless otherwise stated: anti-IR/insulin-like growth factor–1-pYpY1162/1163 (Biosource, Camarillo, CA, USA), anti-IRβ (BD Transduction Laboratories, San Diego, CA, USA), anti-IRS-1-pY612 (Biosource), anti-phospho (Ser307)-IRS-1 (Upstate Cell Signaling Solutions, Lake Placid, NY, USA), anti-IRS-1 (Upstate Cell Signaling Solutions), anti-phospho (Ser473)-Akt, anti-Akt, anti-phospho (Ser21/9)-GSK-3β, anti-GSK-3β, anti-phospho (Thr183/Tyr185)-JNK, anti-JNK, anti-phospho (Ser176/180)-IKKa/b, anti-IKKα, anti-phospho (Ser32)-IkBα or anti-IkBβ. The membranes were then incubated with donkey anti-rabbit-horseradish peroxidase (HRP) or protein A-HRP secondary antibody and bands detected by chemiluminescence (Perkin–Elmer Life Sciences, Boston, MA, USA) after exposure to film (Fuji Photo Film Co, Tokyo, Japan). The membranes were probed with phosphorylated antibodies first, then stripped and re-probed with the corresponding total antibodies. Films were subsequently scanned and the densities of bands of interest were quantified using IPLab Gel software (Signal Analytics Corporation, Vienna, VA, USA).

Statistical analysis

Results are presented as mean ± S.E.M. Data were analysed by factorial ANOVA followed by Fisher’s PLSD post hoc tests. Statistical calculations were performed using a commercial software package (StatView 5.0; SAS Institute Inc., Cary, NC, USA). Differences were considered significant at P<0.05.

Results

General metabolic parameters

In order to examine the effect of an acute elevation of plasma FAs on components of the insulin and stress signalling pathways, rats were infused with LIP or GLYC as a control for 3 h, with or without an extra 5-min insulin infusion at the end. The body weight of all rats used in this study was not significantly different (GLYC, 338 ± 6.4 g; LIP, 337 ± 8.6 g; n = 12–13 rats/group). Plasma insulin levels during the LIP or GLYC infusions did not vary from basal levels and were not statistically different from each other (Fig. 1A). With exogenous insulin infusion at 0.5 U/kg per h for 5 min,

Figure 1 Time course of plasma insulin and FFA concentrations in glycerol and lipid-infused rats. Plasma insulin levels during infusion with Intralipid/heparin or glycerol for (A) 3 h and (B) after exogenous insulin at 0.5 U/kg per h for 5 min. (C) Plasma FFA concentrations in the glycerol and lipid-infused rats. Values are mean ± S.E.M. of n = 6–7 rats/group. ***P<0.001 versus basal.
circulating insulin was increased to a similar degree in both groups of rats, approximately threefold over basal (Fig. 1B). Plasma FFAs were significantly elevated in the LIP rats compared with the GLYC-infused rats ($P<0.0001$). Circulating FFAs increased to $\sim2\text{mM}$ in the lipid-infused rats by 60 min after the start of infusion, whereas the levels remained constant (Fig. 1C). Plasma FFAs were not significantly changed with the GLYC infusion (Fig. 1C).

**IR activation**

Basal levels of phosphorylation of the IR at Tyr1162 and Tyr1163 residues (autophosphorylation sites required for complete kinase activation; Tavare & Siddle 1993) were not significantly different between the GLYC and lipid-infused rats (Fig. 2A and B). Insulin stimulation at 0.5 U/kg per h for 5 min resulted in a similar level of Tyr1162/Tyr1163 phosphorylation of the IR in both the GLYC and lipid-infused rats, approximately twofold higher than basal (Fig. 2A and B). Total IR$\beta$ protein expression was not significantly affected by infusion with GLYC, lipid or insulin (Fig. 2B).

**Activation of IRS-1**

IRS-1 Tyr612 phosphorylation (a PI3-K-binding site; Sun et al. 1993, Esposito et al. 2001) was unchanged with lipid infusion, compared with GLYC infusion, in the basal state (Fig. 3A and B). Insulin infusion caused a two- to threefold increase in skeletal muscle IRS-1 Tyr612 phosphorylation in both the GLYC and lipid-infused rats (Fig. 3A and B). There was no significant difference in the effect of insulin stimulation on IRS-1 at Tyr612 between the GLYC and lipid-infused rat muscle (Fig. 3A and B). Total IRS-1 protein expression was not significantly altered by infusion with GLYC, lipid or insulin (Fig. 3B). Phosphorylation of IRS-1 at the inhibitory Ser307 site was not altered with the 3 h GLYC or lipid infusion or the additional 5-min insulin infusion (Fig. 3C and D).

**PKB phosphorylation**

Phosphorylation of PKB at the Ser473 residue is required for complete activation of this protein (Alessi et al. 1996, Kandel & Hay 1999). Basal PKB Ser473 phosphorylation was not statistically different between the GLYC and lipid-infused rats (Fig. 4A and B). Furthermore, while there was a significant 2.4-fold insulin stimulation effect on the GLYC-infused rats, insulin did not elicit a significant effect over basal in the lipid-infused rats (Fig. 4A and B). Therefore, the level of phosphorylation in the insulin-stimulated, lipid-infused rats was significantly lower than that of the insulin-stimulated, GLYC-infused rats. Total PKB protein expression was not significantly modified by the various interventions (Fig. 4B).

**GSK3$\beta$ phosphorylation**

The inactivation of GSK-3$\beta$ by phosphorylation at the Ser9 residue alleviates its inhibition on the enzyme glycogen synthase, thereby promoting glycogen synthesis as an end point of the insulin signalling pathway (Whiteman et al. 2002). Only the $\beta$-isoform of phosphorylated GSK-3 was detected in our model (Fig. 5B). Basal Ser9-GSK3$\beta$ phosphorylation was similar in the GLYC and lipid-infused rats (Fig. 5A and B). In line with what was observed at the level of PKB, there was a significant 2.1-fold increase in Ser9-GSK3$\beta$ phosphorylation in the insulin-stimulated GLYC-infused rats, compared with only a 44% non-significant increase in the insulin-stimulated lipid-infused rats, i.e. insulin-stimulated phosphorylation in the lipid-infused rats was significantly lower than that of the GLYC-infused rats (Fig. 5A and B). Total GSK3$\beta$ protein expression was not significantly changed by the various interventions (Fig. 5B).

**Stress signalling**

Activation of the JNK and IKK/NF-κB stress signalling pathways have recently been implicated in insulin resistance by negatively regulating insulin signal transduction. Thr183/Tyr185-JNK and total JNK (Fig. 6A and B), as well as

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**Figure 2** Activation of the insulin receptor by phosphorylation at Tyr1162/1163 in glycerol and lipid-infused rats. Rats were infused with 7% glycerol or 7% Intralipid/heparin for 3 h, after which they were either killed (BASAL) or a subset from each group further stimulated with insulin at 0.5 U/kg per h for 5 min (INSULIN). Red quadriceps skeletal muscle was collected and western blotting subsequently performed. (A) Quantification of all results. (B) Representative blots of phosphorylated and total IR$\beta$. Values are mean ± s.e.m. of $n=6–7$ rats/group. ***$P<0.001$ versus basal.
Ser32-IkBa and total IkBa (Fig. 7A and B), were not significantly different between the GLYC and lipid-infused rats. Furthermore, Ser176/180-IKKb phosphorylation and IKKb expression was also not significantly different between groups (data not shown). Consistent with these results, inhibitory Ser307-IRS-1 phosphorylation was not increased in the lipid-infused animals, compared with the GLYC-infused animals (Fig. 3C and D).

Discussion

Several studies have suggested that the insulin resistance observed in obese or type 2 diabetic humans and diet-induced animal models of insulin resistance can be mimicked by an acute infusion of lipid to produce elevated levels of circulating FA (Boden et al. 1991, Roden et al. 1996, Chalkley et al. 1998). The aim of this study was to assess the effect of an acute, physiological elevation of circulating FA on the initial activation of insulin signal transduction. Insulin and stress signalling was assessed in RQ skeletal muscle, the major target tissue of insulin action. Other studies have demonstrated that similar elevation of FA produces insulin resistance but none have examined the initial signalling events under the same conditions (e.g. Griffin et al. 1999).

The major findings of this study were that altered RQ insulin signalling in this model was observed downstream of the IR and IRS-1, at the level of PKB and GSK-3β. Insulin-stimulated PKB Ser473 and Ser9-GSK3β phosphorylation was reduced in the lipid-infused rats (Figs 4 and 5 respectively), whereas there was no significant impact on insulin-stimulated IR Tyr1162/1163 (Fig. 2) or IRS-1 Tyr612 (Fig. 3A and B) phosphorylation. The reductions seen at the level of insulin-stimulated PKB and GSK3β phosphorylation may have been due to aberrant kinase activities of the proteins themselves, and/or impaired kinase activity of their respective upstream regulatory kinases (e.g. PDK1 for PKB, PKB for GSK3β) and/or increased serine–threonine phosphatase activity (e.g. PP2A). Examining the regulation of PKB and GSK3β in more detail could form the basis of a follow-up study, to further analyse the roles of PKB and GSK3β in acute lipid-induced insulin resistance.

Unlike other studies, we examined the onset of insulin signalling (with a short and physiological insulin infusion protocol), as this may be more indicative of associated downstream alterations in insulin action. Prolonged insulin infusion during clamps or bolus insulin injections is common, but not necessarily ideal for assessing insulin signalling. This is because down-regulation of the activation state of proteins as a consequence of proteolysis (Takano et al. 2001, Zhande et al. 2002) or secondary changes caused by normal feedback regulation mechanisms (Cohen 1989) can occur within the 1.5–2 h time frame of a clamp and only the maximal capacity of a tissue to respond to insulin can be assessed with bolus injections. It follows then that it is difficult to measure insulin signalling and insulin action in the same animal because the
time frame for measuring insulin action (1–2 h) and insulin signalling (1–10 min) are inherently different.

The differences in insulin signalling reported here, compared with other lipid infusion studies, may also be due to technical differences such as the use of phospho-specific antibodies. Specifically, the lack of effect of lipid infusion on IRS-1 tyrosine phosphorylation reported in this study (Fig. 3A and B) may be because we examined one phospho-tyrosine (pY) site on IRS-1, as opposed to immunoprecipitating IRS-1 and blotting for total pY as in other studies (Griffin et al. 1999, Kim et al. 2001, Hevener et al. 2002, Yu et al. 2002). However, the pY site that was examined in this study (Tyr612) is a known PI3-K-binding site (Myers et al. 1996) and so the effect seen was pertinent to signalling events specifically related to glucose metabolism. The reduced total IRS-1 tyrosine phosphorylation reported in other studies is not necessarily only related to glucose metabolism, because IRS-1 interacts with a number of other signalling intermediates via various pY residues and with putative functions in other cellular metabolic events (Sun et al. 1995). There are up to 30 potential pY sites on IRS-1 and only six of these are known PI3-K-binding sites (Myers et al. 1996). Results obtained using immunoprecipitation could therefore be confounded by the effect of lipid infusion on pY residues not necessarily involved in glucose metabolism.

We have recently shown that in RQ of rats fed high-fat diets enriched in either polyunsaturated or saturated fat, both IRS-1 Tyr612 and PKB Ser473 phosphorylation were reduced (Frangioudakis et al. 2005). The effects on insulin signalling with acute lipid infusion differ from those observed with chronic high-fat feeding. This suggests that the underlying mechanisms of impairment in insulin action could be different under situations of acute versus chronic lipid oversupply. These differences may be a consequence of a time-dependent effect of lipid oversupply on insulin signalling, such that elevated circulating FA alone, as was the case in this study, has a different impact compared with the...
longer-term effect of tissue TG accumulation, as seen with chronic high-fat feeding (Frangioudakis et al. 2005). The defect in IRS-1 tyrosine phosphorylation observed in chronic lipid oversupply may therefore be a consequence of a metabolic adaptation of the whole body to surplus lipids, resulting in an enhanced ability to clear FA from the circulation (Hegarty et al. 2002) and increased intracellular metabolites of FA, such as long chain acyl-CoA, diacylglyceride or ceramide, all of which contribute to additional signalling perturbations (Schmitz-Peiffer 2000). The model used in the present study may more appropriately reflect the acute impact of changes in the diurnal FA profile observed in high-fat-fed rats, where circulating FAs are markedly increased during the night-time feeding phase (Stavinoha et al. 2004).

In this study, it appears that stimulation of the JNK and IKK/NF-κB stress signalling pathways did not occur after 3 h of increased FA levels. Phosphorylation of major players in these pathways (JNK, IKKβ and IκBα) was not affected by lipid infusion. Furthermore, degradation of the inhibitor of NF-κB, IκBα, which occurs upon phosphorylation by the IKK complex (and is a commonly used surrogate measure of activation of IKK/NF-κB signalling in various systems, e.g. in palmitate-incubated L6 myotubes (Sinha et al. 2004) or tumour necrosis factor-α-treated HepG2 cells (Gao et al. 2002), was not observed in our in vivo model. This is consistent with no change in phosphorylation at the level of IRS-1 – either reduced activating tyrosine phosphorylation or increased inhibitory serine phosphorylation (Fig. 3). The results from the present study suggest that the activation of stress signalling pathways may not be a significant factor in the initial stages of insulin signalling defects induced by excess FA. In terms of JNK signalling, literature reports suggest that more severe physical insults such as atrophy or eccentric contraction of skeletal muscle result in robust phosphorylation of JNK (e.g. Boppart et al. 1999, Carlson et al. 2001, Martineau & Gardiner 2001, Hilder et al. 2003). Our comparatively mild intervention of acute lipid infusion may not be sufficiently ‘stressful’ to lead to activation of such pathways and therefore JNK may not necessarily have a role in the initial stages of FA-induced impairments in insulin signalling. In relation to IKK/NF-κB signalling, in human muscle biopsies obtained following a lipid infusion that raised circulating FA to 1-2 mM, IκBα protein abundance was only
found to be reduced after 6 h, but not after 2 h, of lipid infusion (Itani et al. 2002), suggesting that the time frame of lipid oversupply may be an important factor. Furthermore, whether this pathway is relevant in skeletal muscle remains controversial, with conditional disruption of IKKβ in mouse skeletal muscle failing to prevent obesity-induced insulin resistance (Rohli et al. 2004). The impact of IKK/NF-κB signalling on insulin action may also be tissue specific, since hepatic (Boden et al. 2005, Cai et al. 2005) and adipocyte (Nguyen et al. 2005) activation of this stress signalling pathway has recently been detected in insulin-resistant conditions.

In conclusion, the results obtained from these studies indicate that acute elevation of FA impacts on insulin-stimulated PKB and GSK-3β phosphorylation, before any obvious decrease in tyrosine phosphorylation of IR and IRS-1 at sites specifically involved in insulin signal transduction, or increase in inhibitory serine phosphorylation of IRS-1. There was also no significant activation of JNK and IKK/NF-κB stress signalling. Although defects in IRS-1 tyrosine phosphorylation may occur with higher FA levels than those used in this study or longer duration of lipid infusion, the present results suggest that defects in insulin-stimulated PKB and GSK-3β phosphorylation may be functionally more important in the acute effect of excess FA on insulin action.

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