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Chapter 4

Fatty Acids Stimulate Glucose Uptake by the PI3K/AMPK/Akt and PI3K/ERK1/2 Pathways

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Additional information is available at the end of the chapter

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1. Introduction

Obesity-driven type II diabetes mellitus has become a major crisis in modern societies. In the United States, over 80% of type II diabetic patients are obese [1]. In the case of Chinese adult diabetic patients, diabetes is also significantly associated with obesity [2]. Previous investigations have focused on looking for obesity-related factors that cause insulin resistance, the failure of the body to respond to insulin, which is the hallmark of type II diabetes. The abnormal plasma fatty acid metabolism associated with diabetes mellitus [3], and the high level of obesity-related plasma free fatty acids (FFAs, also known as non-esterified fatty acids, NEFA) have been identified since the 1950s as major risk factors for insulin resistance.

Natural fatty acids are carboxylic acids with saturated or unsaturated aliphatic tails which have an even number of carbon atoms from 4 to 28. When they are not incorporated into other compounds, like triglyceride or phospholipids, they are known as "free" fatty acids. When metabolized, fatty acids yield a large quantity of ATP, and thus represent an important fuel for the body, particularly for heart and skeletal muscle. They are not only essential dietary nutrients, but also function in many cellular events by activating nuclear receptors, such as the peroxisome proliferator-activated receptors (PPARs), and fatty acid binding proteins (FABPs).

How FFAs induce insulin resistance is not a novel topic in pathological studies on obesity-associated type II diabetes. Many efforts have been made to uncover the underlying molecular mechanisms, but they remain elusive. It seems that FFA-induced insulin resistance occurs not via a single pathway but rather via a complicated network of pathways in organs, tissues, and cells.

1.1. Acute cellular responses to Free Fatty Acids

Major investigatons of the mechanisms of extra FFA-induced insulin resistance have focused on the chronic effects of FFAs. However, plasma FFA concentrations are not consistent and

vary widely from hour to hour, displaying waves according to nutritional state and the presence of regulators including hormones (Figure 1). The normal level of postprandial plasma FFAs is about 0.1- 0.4 mmol/L, while in obese individuals this value can reach to 0.2 - 0.6 mmol/L [4]. In healthy people the level of plasma FFAs decreases during the 2 h after a meal until it drops to nearly 0.1 mmol/L, and then rises to a concentration of about 0.3-0.4 mmol/L before the next meal. Such plasma FFA fluctuations also occur in people with metabolic disorders, but display a different pattern. In mild essential hypertensive patients, the plasma FFA concentrations at 3 and 4 h after a meal are significantly higher than that in healthy people (Figure 1, lower panel) [5]. The response of the body to acute variation in plasma FFA concentration is probably associated with the energy balance of the whole body, and requires further investigation to obtain a more in-depth understanding of the pathology of obesity-related metabolic diseases.

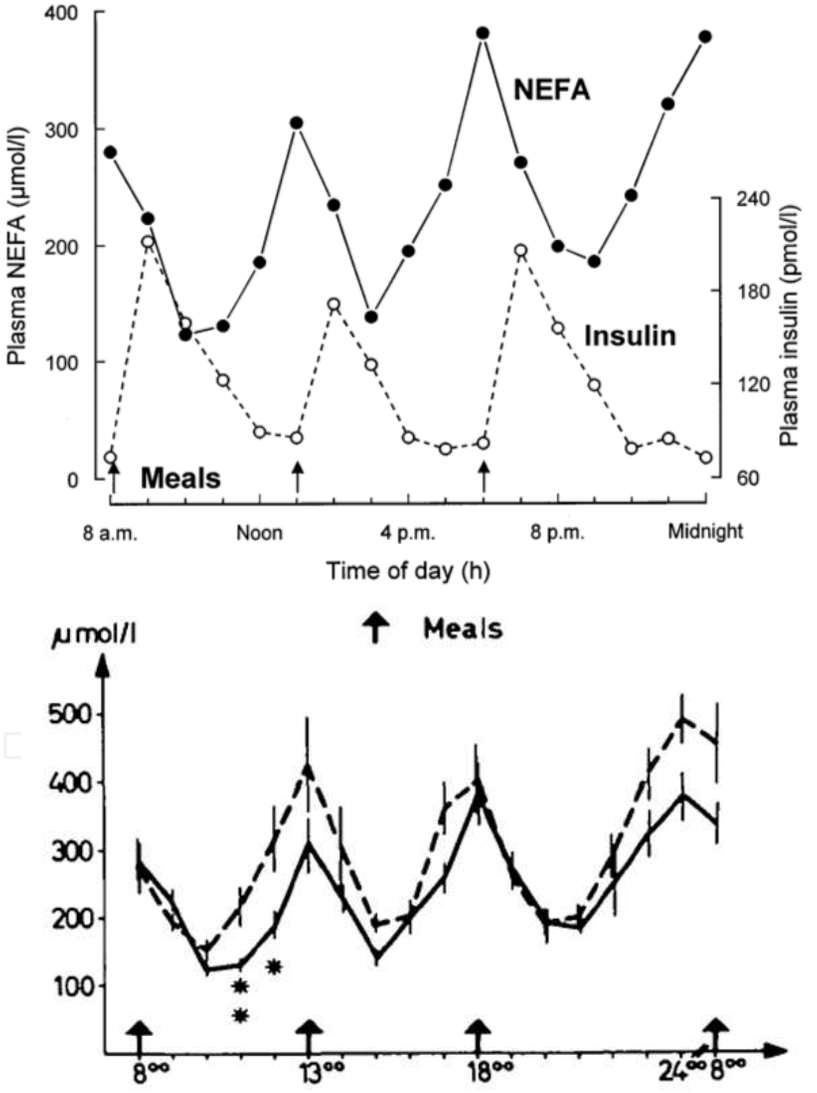

Figure 1. Variation in free fatty acids (●) and insulin (○)concentrations in response to meals in healthy people (upper panel, reprinted from Frayn KN, 1998) [6] and fatty acid levels in mild essential hypertensive patients (---) and normotensive control subjects (——) (lower panel, reprinted from Singer P et al. 1985) [5].

Previous works have reported that FFAs are able to acutely induce several cellular events in various tissues. For example, FFAs can stimulate insulin secretion in pancreatic β-cells [7, 8], leptin secretion in adipocytes [9], and glucose uptake in adipocytes and skeletal muscle cells [10, 11]. All these happen within a short interval after FFA treatment, implying that the FFAs may work as signaling molecules such as hormones, to trigger signal transduction and subsequent physiological events.

During signal transduction, many intracellular signaling proteins work as molecular switches and are activated by GTP binding or phosphorylation. That FFAs acutely stimulate protein phosphorylation suggests that FFAs are able to evoke signal transduction. One study reports that arachidonic acid is able to stimulate the phosphorylation of tyrosine-containing proteins in cultured vascular endothelial and smooth muscle cells [12]; arachidonic acid-induced phosphorylation was rapid and transient, reaching a peak 0.5 min after the addition of arachidonic acid and returning to baseline by 8 min. When cyclooxygenase, lipoxygenase, and epoxygenase pathways were inhibited, phosphorylation was still detected, suggesting it was fatty acid, not its metabolites that triggered the phosphorylation. In addition, increased protein tyrosine phosphorylation was also observed after treatment with oleic, linolenic and γ-linoleic acid. In another work it was reported that unsaturated fatty acids are able to stimulate protein phosphorylation by activating protein kinase C in intact hippocampal slices [13]. Oleic acid stimulated phosphorylation of several proteins of molecular weights 92,000, 58,000, 50,000, 47,000 and 44,000 Da. The 44,000 and 47,000 Da proteins were particularly sensitive to fatty acids and were phosphorylated in a dose- and time-dependent manner. Increased ^{32}P incorporation into the 44,000 Da protein was apparent after 1 min and reached a maximum at 5 min. Phosphorylation of the 47,000 Da protein followed a similar pattern. Studies on fatty acid-stimulated protein phosphorylation have shed light on the role of fatty acids as signal molecules.

1.2. Free Fatty Acid Receptors

During the last decade, a series of free fatty acid receptors (FFARs) has been identified, indicating that like other extracellular signal molecules, FFAs bind to their receptors on the plasma membrane to trigger signal transduction. The FFARs identified belong to a large protein family, the G protein-coupled receptors (GPCRs), which are integral membrane proteins with seven trans-membrane domains. The extracellular parts of the receptors sense external signals and activate heterotrimeric G proteins to transduce signals to downstream molecules. GPCRs are activated by various types of ligands, including ions, nucleotides, amino acids, lipids, peptides, and proteins. It is estimated that more than half of modern drugs target these receptors [14]. The known FFARs include FFAR1 (GPR40), FFAR2 (GPR43), FFAR3 (GPR41), GPR84, GPR119 and GPR120 (Table 1).

Protein	Tissue Expression	Ligand	Function	Synthetic Agonist	G protein-coupling
GPR 40 (FFAR1)	Pancreatic β -cell [15, 16], intestinal tract [17], muscle[16], brain, monocytes [18]	Medium- and long- C8-C22 [18, 15]	Insulin secretion [15]; incretin secretion [17]	Thiazolidinedione [16], GW9508, MEDICA16 [18]	$G_{q/11}$, G_i [15, 16]
GPR 41 (FFAR3)	Adipose tissue [9], sympathetic ganglia [19], enteroendocrine cells [20]	Short C2-C4 [9]	Leptin secretion [9]; PYY secretion [20]	/	$G_{i/o}$ [19, 21]
GPR 43 (FFAR2)	Leukocyte, spleen, bone marrow, adipose tissue [22]	Short C2-C4 [21]	5-HT secretion; PYY secretion [23]; inhibition of lipolysis [24]	/	$G_{q/11}$, $G_{i/o}$ [21]
GPR 84	Immune cell, bone marrow, leukocyte, lung, lymph node, spleen [25, 26]	Medium C9-14C [26]	Amplify IL-12 p40 [27]	/	$G_{i/o}$ [26]
GPR 119	Brain, gastrointestinal tract, pancreas [28]	Ethanolamide [28], Lysophosphatidyl choline [29]	Insulin secretion [29]; food intake; body weight [28]	PSN632408, PSN37569 [30], AR23145 [31]	G_s [29]
GPR 120	Intestinal tract, Macrophage, lung, adipose tissue [32, 33]	Medium- and long- C10-C22 [32]	GLP-1 secretion [32]	NCG21 [34], GW9508 [35]	$G_{q/11}$ [32]

Table 1. Characteristic of FFARs.

It has been reported that GPR40 is activated by medium- and long-chain FFAs [18, 15, 16]. GPR40 is abundantly expressed in the pancreas, and is especially enriched in pancreatic β-cells. When activated by FFAs, GPR40 activates G-protein, which transduces the signal leading to stimulation of insulin secretion. Using Chinese hamster ovary (CHO) cells in which GPR40 is stably expressed, Itoh et al. found that free fatty acids are able to stimulate the formation of inositol 1,4,5-trisphosphate, intracellular Ca^{2+} mobilization, and the phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 [15]. Furthermore, in 2006 Feng et al. reported that fatty acids, especially linoleic acid, are able to stimulate insulin secretion in rat β-cells by reducing the voltage-gated K^+ current via GPR40 and the cAMP-protein kinase A system [36].

Unlike GPR40, the physiological ligands of GPR41 and GPR43 are short chain fatty acids (SCFAs), including acetate (C2), propionate (C3), butyrate (C4), and valerate (C5). SCFAs are generated by bacterial fermentation of undigested carbohydrates from ingested dietary fiber

in the gut. Subsequently SCFAs are released in the bloodstream and accumulate to micromolar concentrations.

GPR41 is expressed abundantly in adipose tissue, enteroendocrine cells, and sympathetic ganglia. SCFAs activate GPR41 and stimulate leptin expression in mouse adipocytes and mouse primary-cultured adipocytes. Acute oral administration of propionate increases circulating leptin levels in mice [9]. Overexpression of exogenous GPR41 and knockdown of GPR41 by RNAi regulates leptin production positively and negatively. Given that leptin is a potent anorexigenic hormone that reduces food intake, propionate may inhibit food intake by increasing leptin release. The analysis of GPR41-deficient mice showed that GPR41 is expressed in enteroendocrine cells, and GPR41 deficiency is associated with reduced expression of PYY [20]. GPR41 is also abundantly expressed in sympathetic ganglia in mice and humans [19]. Studies using GPR41-/- mice and co-culturing of fetal-isolated cardiomyocytes with primary-cultured sympathetic neurons have shown that propionate promotes sympathetic outflow via GPR41, reduces intracellular cAMP concentrations and promotes ERK1/2 phosphorylation, phenomena which were not observed in sympathetic neurons from GPR41−/− mice. GPR41-mediated rise in beat rate was effectively blocked by Gallein ($G\beta_\gamma$ blocker) and pertussis toxin (PTX) treatments, whereas NF023 ($G_{\alpha(i/o)}$ blocker) had no inhibitory effects. Knockdown of PLCβ 2/3 or ERK1/2 by RNAi significantly inhibits the propionate-induced rise in the beat rate of cardiomyocytes. These results indicate that GPR41 activation of sympathetic neurons may involve $G\beta_\gamma$, PLCβ, and MAPK.

GPR43 is highly expressed in immune cells, spleen, and bone marrow, and is also detected at low levels in the placenta, lung, liver, and adipose tissues [22]. A study on adipocytes showed that acetate and propionate can reduce lipolytic activity and thus plasma FFA level in a mouse *in vivo* model. This inhibition of lipolysis is abolished in adipocytes isolated from GPR43 knockout animals [24]. Similar to GPR41, GPR43 activation is also coupled to intracellular Ca^{2+} release, ERK1/2 activation, and a reduction in cAMP accumulation. Unlike GPR41, however, which signals via the $G_{i/o}$ family, GPR43 signals via both the $G_{i/o}$ and G_q pathways [21].

GPR84 mRNA is expressed mainly in bone marrow, leukocytes, the spleen and lung [25, 26]. GPR84 functions as a receptor for medium-chain FFAs with carbon chain lengths of 9–14. Capric acid (C10:0), undecanoic acid (C11:0), and lauric acid (C12:0) are the most potent agonists of GPR84. A functional study conducted in GPR84-/- mice revealed that primary stimulation of T cells with anti-CD3 results in increased IL-4, but not IL-2 or IFN-γ production, compared to wild-type mice [27]. Wang et al. reported that medium-chain FFAs act through GPR84 to amplify the stimulation of IL-12 p40 production by lipopolysaccharides in monocytes/macrophages [26]. Medium-chain FFAs induce Ca^{2+} mobilization and inhibit cAMP production. The activation of GPR84 by medium-chain FFAs is primarily coupled to a PTX-sensitive $G_{i/o}$ pathway [26].

GPR119 in humans and rodents is expressed predominantly in the pancreas and gastrointestinal tract and also in the rodent brain [28]. The lipid signaling agent oleoylethanolamide (OEA) is an endogenous ligand of GPR119. OEA is a peripherally acting

agent that reduces food intake and body weight gain in rat feeding models, suggesting that GPR119 might mediate the OEA-induced reduction of food intake [28]. Lysophosphatidyl choline (LPC) is another bioactive lipid mediator that activates GPR119 to stimulate insulin release from pancreatic islets, via G_s activation which leads to cAMP production [29].

GPR120 is highly expressed in the human and mouse intestinal tract, as well as in adipocytes, taste buds, and lungs [32, 33]. GRP120 activation by saturated FFAs with a carbon chain length of 14–18, and by unsaturated FFAs with a chain length of 16–22 has been detected [32]. Activated by medium- and long- chain fatty acids, GPR120 increases insulin secretion indirectly by stimulating the secretion of glucagon-like peptide-1 (GLP-1), the most potent insulinotropic incretin, which is coupled to the elevation of Ca^{2+} and activation of the ERK cascade [32]. In addition, GPR120 is also reported to function as an ω-3 FA receptor in proinflammatory macrophages and mature adipocytes that mediates the potent anti-inflammatory effects of DHA and EPA by inhibiting both the TLR and TNF-α inflammatory signaling pathways [37]. Chronic tissue inflammation is another important mechanism causing insulin resistance, so the effect of GPR120 on insulin sensitivity as well as on the stimulation of insulin-secretion will make it an attractive drug target for diabetes-therapeutic agents.

The discovery of FFARs developed our understanding of the role of FFAs as signal molecules. Cells expressing FFARs, such as pancreatic β-cells, adipocytes, and macrophages sense FFAs and make various corresponding responses to control metabolic homeostasis (Figure 2). FFARs have thus attracted considerable attention due to their potential as valuable drug targets.

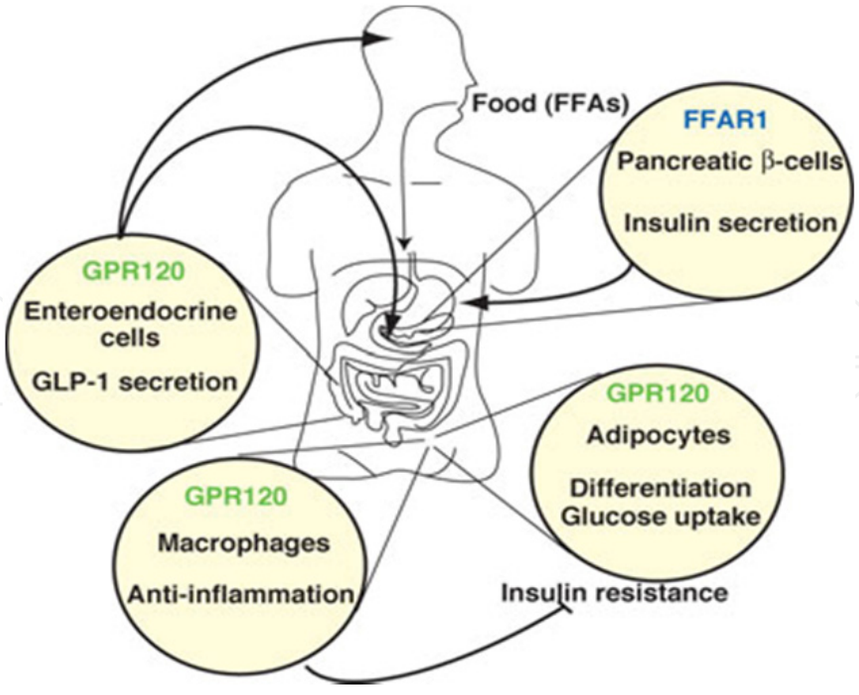

Figure 2. Roles of GPR40 and GPR120 in nutritional regulation. Free fatty acid receptors control metabolism through promoting the secretion or production of peptide hormones (Reprinted from Hara et al., 2011) [38].

In addition to the functions described above, FFAs are also able to acutely stimulate glucose uptake in adipocytes and skeletal muscle cells, which is directly associated with metabolic homeostasis. A few reports indicate that fatty acids have acute effects on glucose uptake, but conclusions have been inconsistent and the underlying molecular mechanisms controlling these responses are still elusive. For example, alpha-lipoic acid has been shown to enhance basal glucose uptake both in normal and ob/ob mice [10], while palmitic acid (PA) treatment was reported to inhibit insulin-stimulated but not basal glucose uptake [11].

Although both adipocytes and skeletal muscle cells are able to ingest glucose by stimulation of FFAs, skeletal muscle consumes more than 70% of the plasma glucose, suggesting that whole body plasma glucose concentration is tightly associated to the sensitivity of muscle tissue to insulin [39, 40]. We therefore focused on the molecular mechanism of fatty acid-induced glucose uptake in skeletal muscle cells [41].

2. Mechanism study on Free Fatty Acid acute stimulation of glucose uptake

2.1. Palmitate stimulates glucose uptake, GLUT4 translocation, and phosphorylation of Akt, AMPK, and ERK1/2 in L6 cells

A rat skeletal muscle cell line L6 with stable expression of myc-tagged GLUT4 (L6) was used to study the acute effects of fatty acids. When L6 cells were treated with palmitic acid (PA), the most abundant free fatty acid in the blood, glucose uptake increased rapidly in a time-dependent manner, beginning from 5 min, and reaching a peak at 20 min (Figure 3, lower panel). By incubating intact PA-treated L6-GLUT4myc cells with myc antibody to detect plasma membrane-located GLUT4, we found that PA stimulates GLUT4 translocation from the cytosol to the plasma membrane (Figure 3, upper panel). The stimulatory effects of PA on glucose uptake and GLUT4 translocation are similar to those of insulin.

Fluorescence imaging shows GLUT4 translocation to the cell surface after L6-GLUT4myc (L6) cells are treated with (upper right panel) or without (upper left panel) PA or insulin (upper middle panel). The lower panel shows that glucose uptake increases in a time-dependent manner when L6 cells are treated with PA.

Akt plays an important role in insulin-stimulated GLUT4 translocation and glucose uptake. Akt, also known as protein kinase B (PKB), is a serine/threonine-specific protein kinase. Akt possesses a protein domain known as the PH domain, which binds to phosphoinositides. Binding to PIP3, and phosphorylated from PIP2 by PI3 Kinase (PI3K) via its PH domain, Akt can be phosphorylated by phosphoinositide dependent kinase 1 (PDK1) at threonine 308 and/or the mammalian target of rapamycin complex 2 (mTORC2) at serine 473. In the insulin signaling pathway, the insulin receptor (IR) is activated and tyrosine is phosphorylated after binding to insulin, subsequently activating hte IRS-1/PI3K/PDK/Akt cascade, and finally increasing the level of GLUT4 in the plasma membrane (Figure 4).

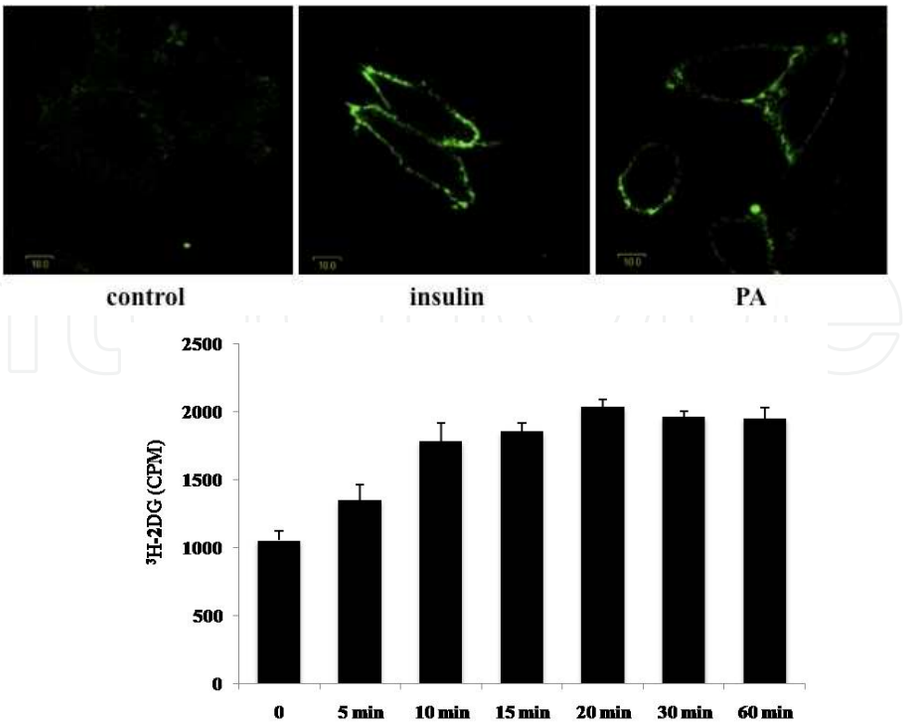

Figure 3. Palmitate stimulates GLUT4 translocation and glucose uptake [41].

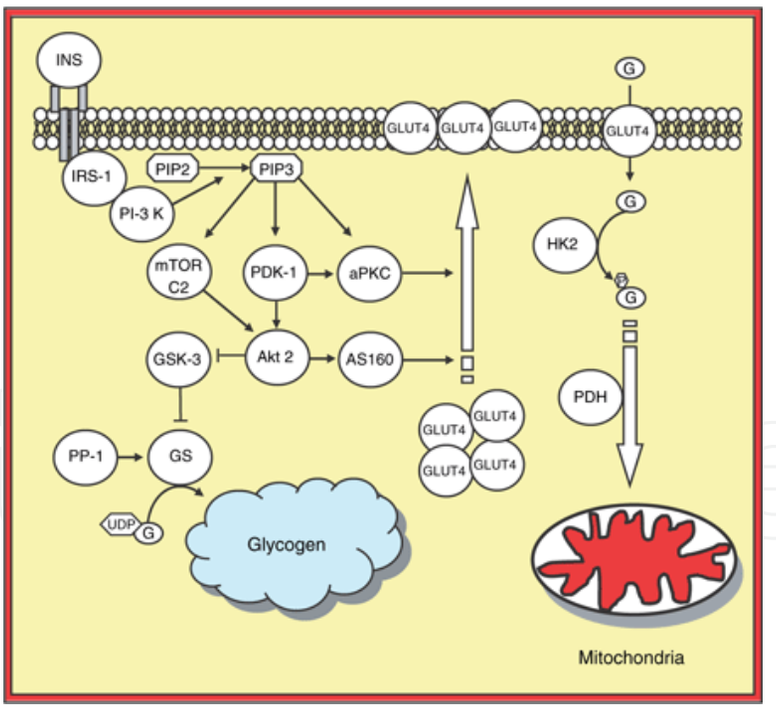

Figure 4. Insulin signaling pathway for glucose uptake stimulation (Reprinted from Frøsig and Richter, 2009) [42].

In our study, PA stimulated Akt phosphorylation at serine 473 in a time- and dose-dependent manner (Figure 5). During PA treatment, Akt phosphorylation was detected after 10 min, peaked at 45 min, then decreased dramatically after 1 h, and became nondetectable

after 3 h. When treated with different concentrations of PA, Akt phosphorylation increased with PA concentration, beginning from 0.2 mM. Such time- and dose-dependent responses to PA treatment in cells match the characteristics of signal transduction, and so it is possible that a signal transduction cascade initiated by PA leads to the activation of Akt. To further verify the stimulatory effect of PA on Akt activation, we treated rat skeletal muscle tissue with PA. Rats were anesthetized and perfused with 2 mM PA. Skeletal muscle strips were collected and then incubated *in vitro* with PA. Similar to the results from cells, Akt phosphorylation also increased in PA-treated skeletal muscle tissue, suggesting that this acute response of PA may be physiologically relevant.

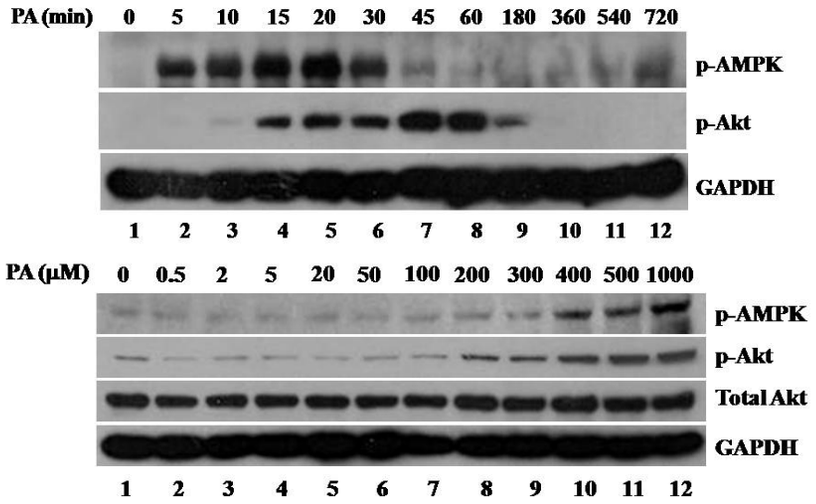

Figure 5. Palmitate acutely stimulates AMPK and Akt phosphorylation in a time- and dose-dependent manner [41].

To investigate the putative PA-mediated signaling pathway, we tested the activity of other molecules and found that AMP-activated protein kinase (AMPK) and extracellular signal-related kinase (ERK1/2) can also be activated by acute PA treatment. AMPK is a heterotrimeric complex composed of a catalytic α subunit and regulatory β and γ subunits, which together make a functional enzyme that plays a role in cellular energy homeostasis [43]. AMPK is activated by an elevated AMP/ATP ratio and undergoes a conformational change of its γ subunit to expose the active site (Thr172) on the catalytic subunit α that is phosphorylated by the upstream kinase AMPK kinase (AMPKK) [44]. Upon activation, AMPK decreases energy consumption by inhibiting fatty acid and protein synthesis and enhances energy production by stimulating fatty acid oxidation and glucose transport to increase cellular energy levels. While it is known that AMPKα is phosphorylated at Thr258 and Ser485, its upstream kinases still need further study [45]. ERK1/2 belongs to the mitogen-activated protein kinase (MAPK) family, a widely conserved family of serine/threonine protein kinases. The ERK1/2 (p44/42 MAPK) signaling pathway responds to various extracellular stimuli including mitogens, growth factors, and cytokines [46]. Upon activation by MEK1 and MEK2 by phosphorylation of its Thr202 and Tyr204 residues, respectively, ERK1/2 phosphorylates downstream targets, forming a signal cascade.

Similar to Akt, AMPK in L6 cells is also activated acutely by PA. AMPK phosphorylation (Thr172) starts as early as 5 min after PA treatment and reaches a peak at 20 min. After 1 h, the signal cannot be detected (Figure 5, upper panel). In addition, PA-induced phosphorylation of AMPK is also dose-dependent. Unlike Akt and AMPK, ERK1/2 is phosphorylated for a shorter duration, increasing after 5 min and returning to basal level after 15 min (Figure 6).

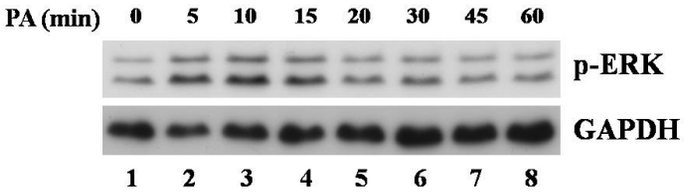

Figure 6. Palmitate acutely stimulates ERK1/2 phosphorylation in a time-dependent manner [41].

2.2. Akt, AMPK and ERK1/2 are involved in PA-stimulated glucose uptake

To test if Akt, AMPK, and ERK1/2 are involved in PA-stimulated glucose uptake, tools such as inhibitors, dominant negative constructs, and short interference RNA (siRNA) were used to inhibit their protein activity or expression levels. Western blotting and glucose uptake assay results showed that all of these proteins participate in signal transduction.

We applied API-2, an Akt selective inhibitor, to block Akt activity. As a result, API-2 abolished Akt phosphorylation as well as significantly decreasing PA-induced glucose uptake (Figure 7, left panel). In addition, siRNA duplexes were nucleofected into L6 cells; compared to the negative control (N.C.), total Akt expression level was efficiently down-regulated. Glucose uptake assays showed that PA-induced glucose uptake decreased when Akt expression decreased due to RNAi (Figure 7, right panel). Together, these data suggest that PA induces glucose uptake in skeletal muscle cells via Akt activation.

To study the role of AMPK in PA-induced glucose uptake, we used AMPK inhibitor Compound C, an myc-tagged AMPK dominant negative (AMPK-DN) plasmid, and siRNA targeting AMPK catalytic subunits α1 and α2. Since AMPK and Akt activation was observed sequentially in PA-treated cells, we also examined the relationship between AMPK and Akt. AMPK inhibitor Compound C suppressed AMPK activity and decreased PA-induced Akt phosphorylation and glucose uptake (Figure 8, left panel). Similar results were obtained when AMPK-DN was nucleofected into L6 cells. Furthermore, an siRNA duplex mixture targeting AMPK α decreases PA-stimulated Akt phosphorylation in L6 cells (Figure 8, right panel), consistent with the inhibitor and AMPK-DN experiments.

In contrast, when AMPK agonist AICAR was used to stimulate AMPK phosphorylation, Akt was also stimulated rapidly in a time-dependent manner (Figure 9), suggesting that it is possible to stimulate Akt via AMPK activation in L6 cells. These data suggest that PA-stimulated AMPK phosphorylation may contribute to regulating Akt activity and is involved in PA-induced glucose uptake.

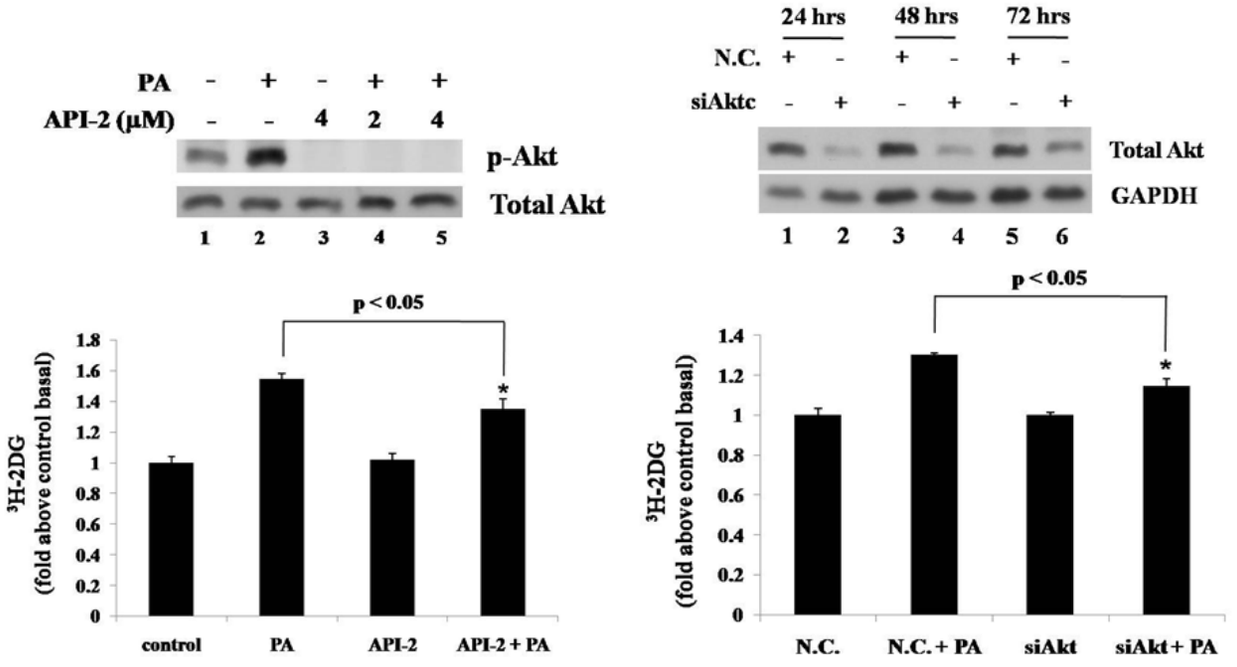

Figure 7. PA-induced glucose uptake is decreased when Akt activity is blocked by an Akt inhibitor (left panel) or RNAi (right panel) [41].

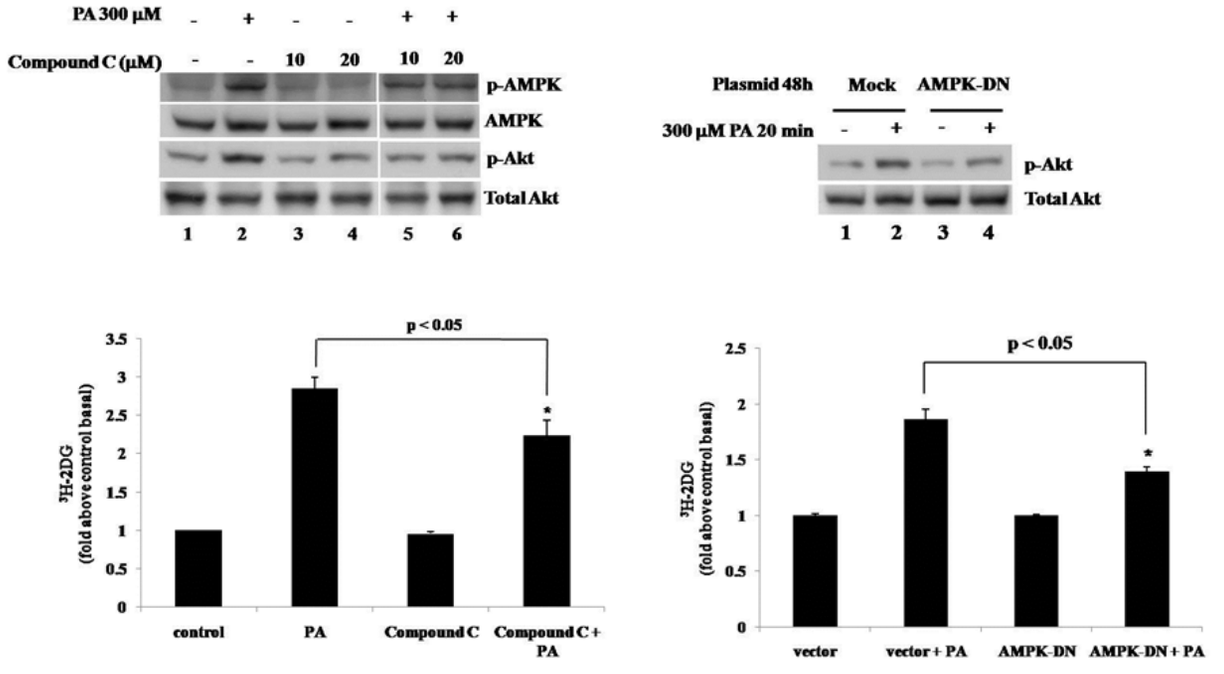

Figure 8. PA-induced glucose uptake and Akt phosphorylation is decreased when AMPK activity is reduced by an AMPK inhibitor (left panel) or AMPK dominant-negative construct (right panel) [41].

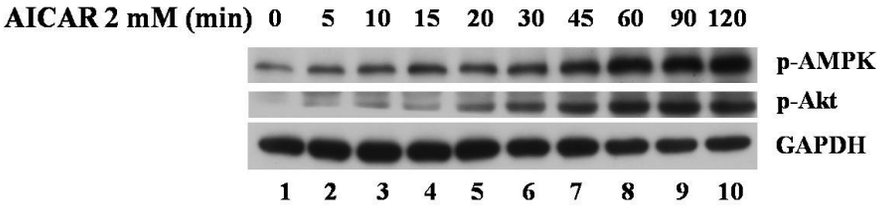

Figure 9. AMPK agonist AICAR stimulates Akt phosphorylation in a time-dependent manner [41].

The role of ERK1/2 in PA-stimulated signal transduction was examined by using the MEK1/2 inhibitors PD98056 and U0126. While both inhibitors decreased basal and PA-induced ERK1/2 phosphorylation, U0126 was more potent (Figure 10, upper left). When ERK1/2 activity was inhibited by U0126, PA-induced glucose uptake was reduced significantly (Figure 10, upper right). These data suggest that PA-stimulated ERK1/2 phosphorylation may contribute to PA-induced glucose uptake. To determine the relationship between ERK1/2 and the AMPK/Akt pathway, AMPK α1/α2 siRNA transfected cells were used to test ERK1/2 activity; ERK1/2 phosphorylation increased at the same rate as in N.C. cells after PA treatment (Figure 10, lower left). In addition, the Akt inhibitor API-2 did not affect PA-induced ERK1/2 phosphorylation, and MEK1/2 inhibitors PD98056 and U0126 did not affect PA-induced Akt phosphorylation (Figure 10, lower right). These data suggest that ERK1/2 contributes to PA-stimulated signal transduction independently from the AMPK/Akt pathway, consistent with the partial decrease in PA-induced glucose uptake by inhibition of either Akt, AMPK, or ERK1/2.

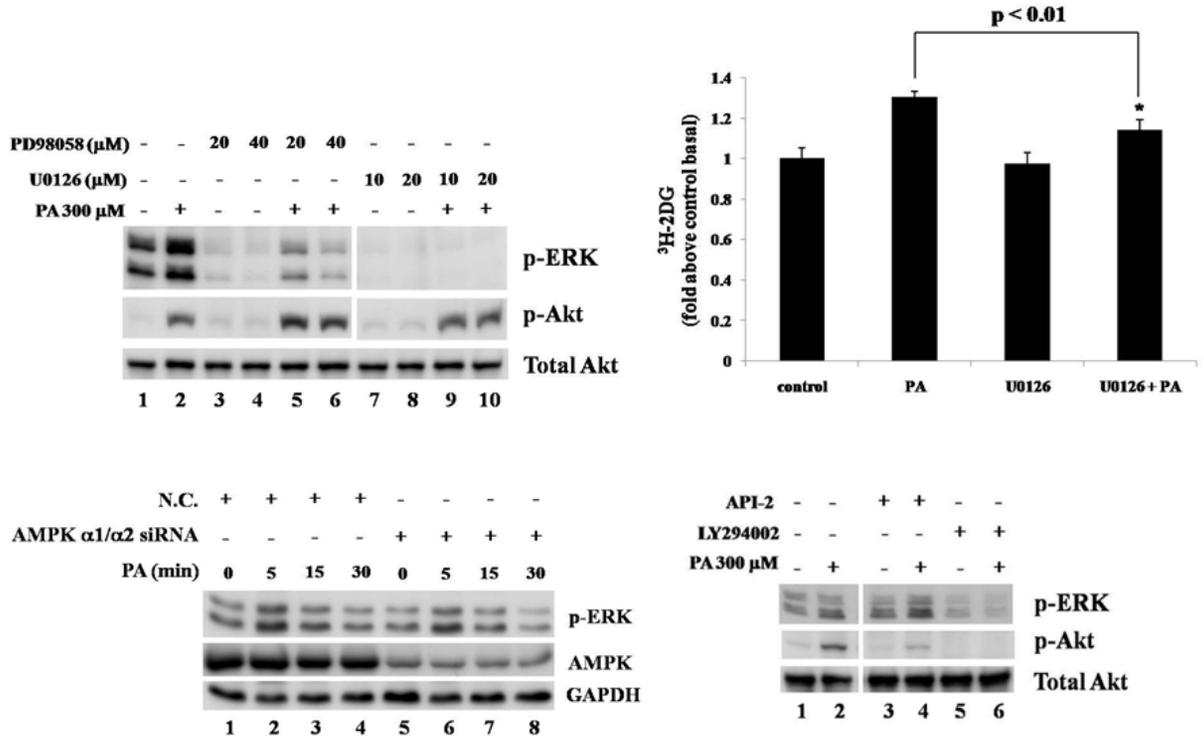

Figure 10. MEK1/2 inhibitors decrease PA-induced ERK1/2 phosphorylation and glucose uptake, but do not affect Akt activity; AMPK and Akt activity inhibition does not affect ERK1/2, while PI3K inhibitor does [41].

Having shown that the two pathways work independently in PA-induced glucose uptake in L6 cells, we investigated signaling molecules upstream of the intersection. When we used PI3 Kinase (PI3K) -specific inhibitor LY294002 to treat L6 cells, PA-induced glucose uptake was totally abolished (Figure 11, upper panel), suggesting that PI3K may control these two pathways. Indeed, LY294002 could abolish PA-stimulated AMPK, Akt, and ERK1/2 phosphorylation (Figure 10, lower right and Figure 11, lower panel). Results from the above experiments indicate that, in skeletal muscle cell lines and tissues, acute PA-stimulated glucose uptake occurs via activation of the PI3K/AMPK/Akt and PI3K/ERK1/2 pathways leading to GLUT4 translocation (Figure 12).

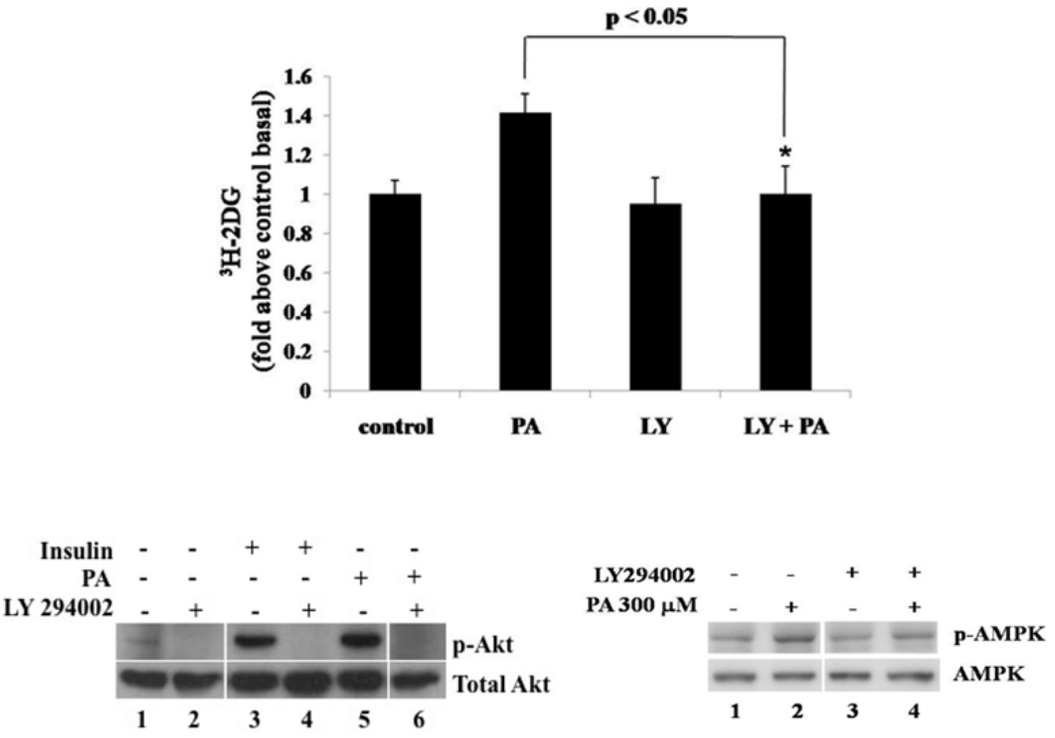

Figure 11. PI3K-specific inhibition abolishes PA-induced glucose uptake and phosphorylation of Akt and AMPK [41].

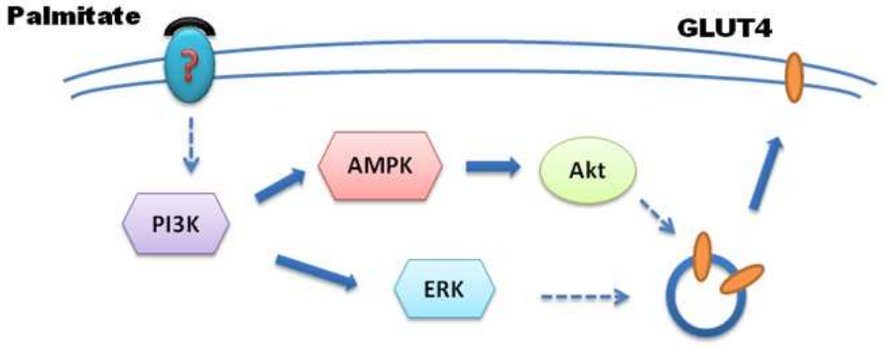

Figure 12. Schematic diagram of PA-induced signal pathway stimulation of acute glucose uptake in skeletal muscle cells [41].

2.3. Palmitate stimulates Akt phosphorylation via binding to the plasma membrane

As shown in Figure 12, how PA activates PI3K is still unknown. According to our current understanding of signal transduction, we speculate that PA may bind to a protein on the cell plasma membrane to trigger signal transduction. We performed fatty acid binding assays to test this hypothesis.

L6 cells were incubated with PA at low temperature (4°C) to facilitate PA binding to the cell surface while preventing its internalization, then washed with buffer to remove unbound PA, or with BSA solution to remove not only unbound but also some membrane-bound PA by competitive binding, Cells were then transferred to 37°C to recover cellular activity. The amount of PA which binds to the cell surface was measured by adding trace amounts of ^{3}H-labeled PA to the solution. Results showed that after washing, with either buffer or BSA solution, very little PA remained (Figure 13, upper panel). When these cells were transferred to 37°C, Western blot results showed that Akt phosphorylation took place at a similar level to that in cells kept at 37°C, and increased in a time-dependent manner in buffer-washed cells, while p-Akt was not detectable in BSA solution-washed cells (Figure 13, lower panel). These results suggest that the amount of cell surface-bound PA was sufficient to activate Akt; intracellular PA accumulation was not required. Moreover, based on lipid analysis by TLC, fatty acids were the main component of total lipids during cell treatment with fatty acid and the 10 min incubation at 37°C. These results indicate that it is fatty acids rather than their metabolites that trigger signal transduction .

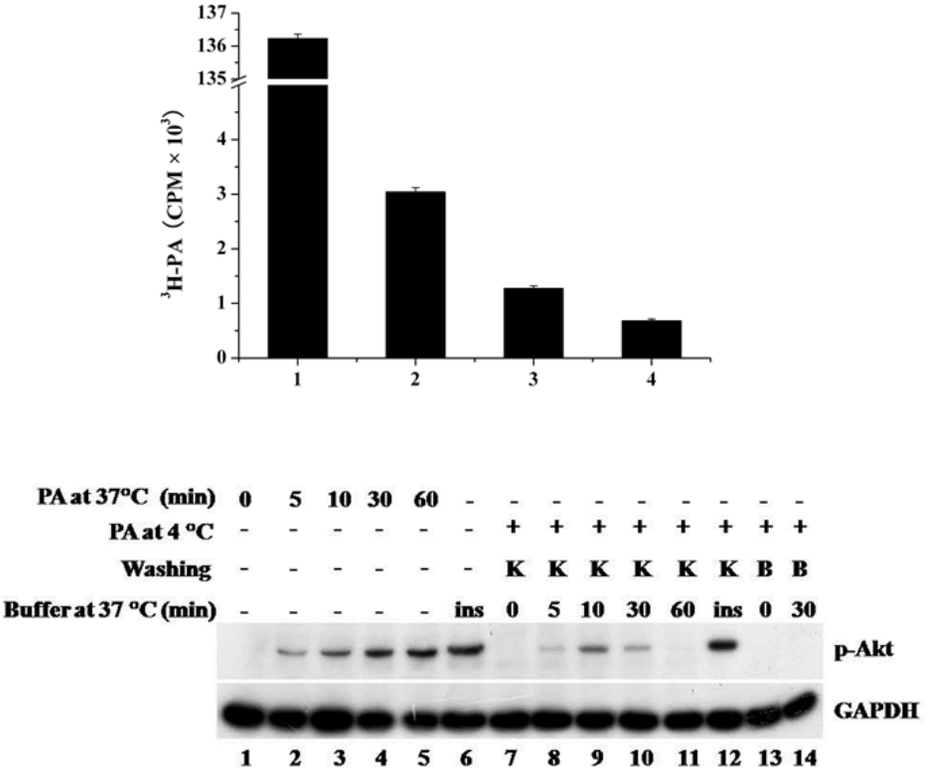

Figure 13. Cell plasma-bound PA stimulates Akt phosphorylation [41].

3. Future work

Could the postulated cell membrane protein which binds to FFAs and triggers signal transduction to stimulate glucose uptake in skeletal muscle cells be a G-protein coupled receptor? FFA binding assays suggest that PA initiates signal transduction via a protein(s) on the cell surface, and meanwhile in the FFA-stimulated signal cascade ERK1/2 pathways were involved, which also appeared in some known FFAR signal pathways. Therefore, a GPCR on the plasma membrane of skeletal muscle cells may be the FFA receptor we have postulated.

PA is a long-chain fatty acid with 16 carbons. We tested other long-chain fatty acids such as C18:1 (oleic acid), C18:2 (Linoleic acid), and C18:0 (Stearic acid) in addition to PA to examine their effects of stimulating AMPK and Akt phosphorylation. All of these fatty acids activated AMPK and Akt in a time- and dose-dependent manner. It is thus likely that our postulated FFAR may function using long-chain FFAs as its ligands.

The known long-chain FFARs include GPR40 and GPR120, both of which are related to insulin secretion. Oh et al. found that GPR120 agonists DHA and GW9508 enhance glucose uptake by activating the PI3K-Akt pathway and GLUT4 translocation in 3T3-L1 adipocytes. The stimulatory effect of DHA and GW9508 was blocked when GPR120 or $G_{\alpha q/11}$ was depleted by siRNA knockdown [37], indicating that FFAs stimulate glucose uptake in adipocytes via the FFA receptor GPR120. However, neither GPR120 nor GPR40 is expressed in muscles. In the Oh *et al*. study, DHA and GW9508 did not enhance glucose uptake in L6 skeletal muscle cells. We therefore conclude that known long-chain FFARs GPR40 and GPR120 are not our postulated FFA receptor. In 2005, Gaël Jean-Baptiste *et al*. described the GPCRs expressed in skeletal muscle tissue, but none of them are FFA receptors [47]. Our postulated receptor might therefore be a novel FFAR whose function is to stimulate glucose uptake in skeletal muscle tissue. Like GPR40 and GPR120, our postulated FFAR is also related to metabolic homeostasis, and so it is likely to be a potential drug target for the treatment of diabetes. Identification of this FFAR is one of our goals.

Another significant implication of our results is that PA plays two opposing roles in skeletal muscle. Under chronic treatment it inhibits insulin-stimulated glucose uptake by blocking Akt phosphorylation, while it enhances glucose uptake by activating Akt when cells are exposed to PA for a short time. What is the relationship between the long-term and short-term effects of FFAs on glucose uptake? Our results show that phosphorylation of Akt is only stimulated when the concentration of PA reached a certain level (at or above 0.2 mmol/L in C2C12 cells). We thus conclude that phosphorylation of Akt may require high concentrations of fatty acids under physiological situations. Akt phosphorylation is not detectable 3 h after fatty acid treatment (Figure 5, upper panel). In addition, when fatty acids are withdrawn, the Akt phosphorylation signal disappears after 3 h in C2C12 cells (data not shown), suggesting that fatty acid-induced phosphorylation and dephosphorylation of Akt can be completed within one cycle of a postprandial FFA wave. It is therefore possible that

Akt phosphorylation and dephosphorylation occur again and again as the concentration of FFA increases and decreases. Plasma FFA concentration starts to rise from 2 h after a meal (Figure 1) and continues to rise until the next meal due to the release of FFA from adipocytes during fasting. Based on our findings, when increasing plasma FFA reaches a certain level it stimulates Akt phosphorylation and glucose uptake. In obesity patients, elevated plasma FFA probably reaches the FFA level triggering Akt phosphorylation earlier during a plasma FFA wave, leading to abnormal glucose uptake. Many abnormal fatty acid cycles may contribute to the development of insulin resistance by disturbing glucose homeostasis. This Yin-Yang balance of PA in skeletal muscle is likely to be physiologically significant, and the possibility of its involvement in the development of insulin resistance needs to be investigated further.

4. Conclusion

Free fatty acids (FFAs) function as signal molecules by activating their receptors in the cell plasma membrane to evoke signal transduction by a series of protein phosphorylation events, eventually leading to physiological events. Some of the known cell responses to FFAs are directly or indirectly related to metabolic homeostasis, so the study of FFA-triggered signal transduction will help us to understand the development of metabolic disorders and to design strategies for therapy. FFA receptors have become attractive drug targets for metabolic diseases. We have investigated the mechanism of long-chain fatty acid palmitate-induced glucose uptake in skeletal muscle cells and found that the two independent PI3K/AMPK/Akt and PI3K/ERK1/2 pathways are responsible for this process. Our results also provide supporting evidence that palmitate triggers signal transduction via a cell surface protein(s) that is probably a novel FFA receptor whose identity still remains to be determined.

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