Title: Phosphorylation of protein kinase B, the key enzyme in insulin signalling cascade, is enhanced in linoleic and arachidonic acid treated HT29 and HepG2 cells.

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Abstract (250)

Objective: Defects in insulin signalling pathway have been implicated in the pathogenesis of impaired glucose uptake, insulin resistance and type II diabetes. However, the specific defects that precipitate these abnormalities are yet to be fully elucidated. The plasma membrane embedded insulin receptor transmembrane protein, after binding to insulin, initiates a cascade of phosphorylation which leads to the activation of protein kinase B (AKT) and subsequently to initiation of some metabolic actions of insulin. The activities of this receptor, insulin binding and tyrosine kinase activation, is dependent on its plasma lipid environment. There are scarcity of published data on the influence of omega-3 and -6 polyunsaturated fatty acids on insulin response. Moreover, the findings of the published investigations, most of which have used omega-3 and -6 PUFA blend, have been inconclusive. Hence, a need for well- thought-out further research. The aim was to elucidate the effect of treatments with LNA, ARA, ALA, DHA, and EPA on cell membrane composition and consequently on insulin signalling pathway, specifically AKT phosphorylation.

Research Methods and Procedures: Human colon adenocarcinoma (HT29) and liver hepatocellular (HepG2) cells were treated with or without 40 µM of linoleic (LNA), arachidonic (ARA), alpha-linolenic (ALA), eicosapentaenoic (EPA) or docosahexaenoic (DHA) for 48 hours. Fatty acids composition of phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn) from the treated cells by capillary gas liquid chromatograph. Cells were incubated for 30 minutes with or without human insulin (50ng/ml) and the phosphorylation of AKT assessed with the use of western blotting.

Results: The fatty acids were incorporated in PtdCho and PtdEtn of both cell lines; but, the level of incorporation was higher in HT29. Phosphorylation of AKT increased when HT29 was treated with LNA (P<0.05) and ARA (P<0.01), but not with ALA, EPA or DHA. A

similar but non-significant increase in AKT phosphorylation was observed in LNA and ARA treated HepG2 cells. **Conclusion:** The finding of this investigation demonstrates, plasma membrane lipid bilayer enrichment with LNA or ARA treatment enhances insulin action by AKT activation.

Highlights

- Levels of linoleic (LNA), arachidonic (ARA), alpha-linolenic (ALA), eicosapentaenoic (EPA) and docoshexaenoic (DHA acids, and protein kinase B (AKT) phosphorylation were assessed in HepG2 and HT29 cells treated with the fatty acids.
- LNA and ARA were more efficiently incorporated in HT29 than in HepG2 cells.
- Phosphorylation of AKT increased significantly in HT29 cells treated with LNA and ARA, but not with ALA, EPA or DHA.
- A non-significant increase in AKT phosphorylation was observed in LNA and ARA treated HepG2 cells

Introduction

Alterations of polyunsaturated fatty acid (PUFA) membrane composition have been proposed to alter cellular functions including cellular response to insulin. Defects in insulin signalling pathway are responsible for insulin resistance and type 2 diabetes [1–3], thus the understanding of insulin transduction pathway is of clinical importance.

Human and animal studies have suggested that long chain PUFA, in particular of the omega-3 family, may be protective against type 2 diabetes and insulin resistance [4]. Epidemiological studies showed that consumption of diets high in omega-3 PUFA correlated with lower incidence type 2 diabetes compared with diets rich in saturated fatty acids [5–7]. Studies on fatty acid membrane composition showed that low levels of omega-3 PUFA were found in association with type 2 diabetes [8], whilst insulin sensitivity positively correlated with high levels of total omega-3 and -6 PUFA [9]. Similarly, an enhanced insulin sensitivity has been reported in experimental animal studies fed on omega-3 and -6 PUFA [10, 11].

Insulin is a pancreatic hormone involved in many functions. Mainly, it regulates the uptake of glucose (the entry of glucose into cells) in muscles, adipose tissue and liver [12]. In addition, it is involved in the regulation of several metabolic enzymes, as well as the promotion of storage of incoming glucose as glycogen, and synthesis of proteins and triglycerides from free fatty acids.

Insulin binds to the insulin receptor [13], a transmembrane heterotetrameric tyrosine kinase receptor, which consists of two extracellular ligand-binding α subunits and two transmembrane catalytic β subunits. After insulin binds to the IR, IR undergoes autophosphorylation of tyrosine residues in the β domains. The activation of the receptor

leads to the recruitment of an adaptor protein, the insulin receptor substrate 1 and 2 (IRS-1 and 2). The main target of IRS is the phosphatidylinositol 3-kinase (PI3K) [14]. After phosphorylation, PI3K converts the phosphatidylinositol 4,5-bisphosphate (PtdIns-4,5-P_2) to phosphatidylinositol 3,4,5-triphosphate (PtdIns-3,4,5-P_3). A key effector of PtdIns-3,4,5-P_3 is the protein kinase B (PKB), also known as AKT. AKT has a pleckstrin homology domain located at its amino terminus, which binds to PtdIns-3,4,5-P_3, and permits the recruitment of AKT to the plasma membrane [15] . Once located at the membrane, AKT is firstly phosphorylated at 308-Thr residue and subsequently at 473-Ser residue. The phosphorylation of the 308-Thr residue is due to the PDK1 protein, which as AKT presents a PtdIns-3,4,5-P_3 binding pleckstrin homology domain [16]. The mechanism of 473-Ser phosphorylation is still controversial. The phosphorylation of both the 308-Thr and 473-Ser residues is necessary for AKT full activation. Activated AKT dissociates from the plasma membrane and phosphorylates several substrates involved in insulin dependent responses. A major effect of activated AKT is the promotion of the translocation of the insulin dependent glucose transporter 4 (GLUT4) to plasma membrane and subsequent uptake of glucose into cells.

Defects in both upstream and downstream targets of Akt, PI3K-Akt signalling pathway, have been implicated in insulin resistance [17, 18]. Insulin receptors are proteins localised in plasma membrane, and phosphorylation of PI3K is dependent on inositol containing membrane phospholipids. Therefore, it is tenable to assume that changes in plasma membrane composition would have an effect on insulin-signalling pathway. Cultured cells are widely used good model for investigating relationships between membrane fatty acids and insulin response. *In vitro*, alterations in lipid composition of erythroleukemia cells and ascites cells showed a high percentage of linoleic acid (LNA) rich oil (sunflower oil) versus oil containing mainly saturated fatty acids (coconut oil) was associated with an increase in

insulin receptor number and a decrease in binding receptor affinity[19, 20]. Bruneau et al. [21] demonstrated that lipid alterations (*via* treatments with LNA) of rat hepatoma cells led to an insulin-resistant state. Subsequently, the same group reported that feeding human hepatoma cells with LNA and eicosapentaenoic acid (EPA) modified insulin receptor autophosphorylation and the phosphorylation of the major insulin receptor substrate, IRS-1[22] .

The aforementioned studies, most of which have used a heterogeneous blend of omega-3 and -6 PUFA, have highlighted a possible relationship between these fatty acids and insulin response. Therefore, there is need for further investigations to help elucidate the effect of the individual fatty acid members of the two PUFA families.

In the present study, human colon adenocarcinoma (HT29) and hepatocellular carcinoma (HepG2) cell lines were used as *in vitro* systems to study whether alterations of phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn) fatty acid composition alter insulin-induced AKT activation/phosphorylation. Both cell lines express a functional insulin pathway [22, 23]. Although the gastrointestinal tract is the main target of insulin, it is where nutrients are processed and absorbed. Consequently, it is thought to have an indirect effect on insulin resistance [24]. The liver is widely-recognised primary insulin target and hepatocytes are often used to investigate insulin response [25, 26].

Material and Methods

Reagents

α-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), linoleic acid (LNA) and arachidonic acid (ARA) were purchased from Sigma-Aldrich, dissolved in 100% ethanol, and stored at -20 ^{0}C. Insulin Human recombinant was obtained from Sigma-Aldrich.

Cell culture

The human colonic adeno carcinoma cells (HT29) (ECACC) and Human hepatocellular liver carcinoma cell line (HepG2) were cultured respectively in Mc`Coys or DMEM medium (Sigma) supplemented with 2 mM glutamine, 5 mM streptomycin, 5 mM penicillin and 10% foetal bovine serum (FBS) (PAA) at 37 ^{0}C in a humidified atmosphere of 95 % air and 5% CO_2.

Fatty acid treatments

HT29 and HepG2 cells (2X10^{5}cells/ml) were seeded in 21 cm^{2} plates and allowed to attach and divided for 24 hours. Subsequently, medium was siphoned and replaced with fresh medium containing 10% FBS with or without 40 µM of different polyunsaturated fatty acids (ALA, EPA, DHA, LNA or ARA) [27, 28]. After 48 hours, treated and untreated cells were collected and used for analysis of fatty acids composition. The final concentration of ethanol/medium, which was less than 0.1% (v/v%), did not affect cell proliferation.

Fatty acid analysis

HT29 and HepG2 cells were washed twice with 2 ml per well of 1X PBS. In order to extract cell membrane total lipids, 1.5 ml of hexane/isopropanol (3:2, v/v) (HIP) and 0.01% BHT

was added to each well on ice. After 5 minutes of incubation on a shaker, the HIP mixture was transferred to a clean glass tubes. The HIP mixture was completely evaporated under nitrogen, and 1.8 ml of chloroform/methanol (2:1, v/v) + BHT, plus 0.6 ml of water were added to the tubes. The tubes were vortexed and then centrifuged at 4000 rpm for 5 minutes at 4 °C. After centrifugation, the lipid enriched organic phase was collected, the solvent was completely evaporated under nitrogen and the lipids were dissolved in 1 ml chloroform/methanol (2:1, v/v). Phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn) were separated by thin-layer chromatography on 20x20 cm silica gel plates (TLC silica gel 60, Merck kGaA, UK) by the use of the developing solvents: chloroform/methanol/methylamine (65:30:15, v/v) and 0.01% BHT. The phospholipid bands were detected by spraying the plates with a 2,7-dichlorofluorescein/methanol solution (0.01 % w/v) and identified by the use of standards. Fatty acid methyl esters (FAMEs) were prepared by heating the lipid fractions with a 4 ml of 15% acetyl chloride in methanol in a sealed vial at 70 ^{0}C for 3 hours under nitrogen. FAMEs were separated by a capillary gas liquid chromatograph (HRGC MEGA 2 Series, Fisons Instruments, Italy) and identified by comparison of retention times of authentic standards (Sigma-Aldrich, United Kingdom) and by calculation of equivalent chain length values. Peak areas were quantified by a computer chromatography data system (Agilent EZChrom Elite 3.2, Scientific Software, Inc., Pleasanton, CA).

Insulin treatments

HT29 and HepG2 cells (2X10^{5}/ml) were seeded in 21 cm^{2} plates and allowed to attach and divide for 24 hours. Subsequently, the old medium was siphoned off and replaced with fresh medium containing 10 % FBS supplemented with 40 μM of different polyunsaturated fatty acids (ALA, EPA, DHA, LNA or ARA). Two plates were grown in 10 % FBS medium

without adding fatty acids. After 48 hours of incubation, the old medium was discarded and the cells incubated in fresh serum free medium for 6 hours. Subsequently, the medium was siphoned off, and the cells incubated for 30 minutes with fresh medium with and without 50ng/ml of human insulin [29].

Western blot

HT29 and HepG2 cells were washed twice with ice cold PBS 1X. Subsequently, cells were lysed in ice-cold RIPA buffer (1% NP-40, 0.1% SDS, 0.5% deoxycholate, 20mM Tris-HCl buffer (pH 7.4), 150 mM NaCl, 10 mM NaF, 1 mM Na3VO4, 1 mM EDTA, 1 mM EGTA). To prevent protein degradation and dephosphorylation, phosphatase and protease inhibitor cocktail (Sigma-Aldrich, UK) were added to the RIPA buffer. The lysed cells were transferred to a clean 1.5 ml tube and the insoluble material removed by centrifugation (13,000×g at 4°C for 20 min). The supernatant was transferred to a new chilled tube and its protein content quantified by Bradford Protein Assay. 40 µg of proteins were diluted in 3X Laemmi loading buffer (1.5% Tris-HCl buffer (pH 6.8), 4% SDS, 1% glycerol, 0.2 % bromophenol blue, 0.4% β-mercaptoethanol in water) and loaded on a 10% Sodium Dodecyl Sulphate (SDS) –polyacrylamide mini gel (Biorad apparatus). The separated proteins were transferred from the gel on to a nitrocellulose membrane (Biorad) using a Mini Trans-Blot Cell apparatus at 0.09 mA overnight. The membranes were blocked for 1 hour in 5% dried non-fat milk in TBS-T 1X then washed three times in TBS-T 1X buffer. Subsequently, membranes were blotted with Rabbit Anti-phospho-Akt (Ser473) (1:1000, Cell Signaling technology) in 5% BSA/TBST 1X overnight at 4°C. After incubation, membranes were washed 3 times for 5 minutes in TBS-T 1X and incubated with Goat anti-rabbit IgG HRP conjugate secondary antibody in 5% nonfat dry milk in TBST 1X for one hour at room temperature. After probing with secondary antibody membranes were washed 3 times for 5

minutes in TBS-T 1X. Proteins signals were revealed by enhanced chemiluminescence (Immobilon western HRP substrate, Millipore) in the dark with the use of X-Ray films, developing and fixing reagents. After detection, the membranes were stripped and re-probed with and Rabbit anti-AKT (1:1000, Cell Signaling technology) in 5% BSA/TBST 1X overnight at 4 °C. After incubation, membranes were washed 3 times for 5 minutes in TBST 1X and incubated with Goat anti-rabbit IgG conjugate secondary antibody in 5% dried non-fat milk in TBS-T 1X for one hour at room temperature. After probing with secondary antibody membranes were washed 3 times for 5 minutes in TBS-T 1X and Akt signal was detected by enhanced chemiluminescence (Immobilon western HRP substrate, Millipore) with the use of X-Ray films, developing and fixing reagents. The density of the bands was quantified by the use of the UVP Vision Works software and the level of the phospho-Akt was normalised to the level of the total Akt.

Statistical analysis

Data were calculated as mean percentage of total fatty acids (± S.E.) and statistical significance was set at $P<0.05$. Unpaired t-test was used to compare the difference in fatty acid composition between the different treatments. The statistical software SPSS for Windows (version 23, SPSS Inc., Chicago, IL, USA) was employed to analyse the data.

Results

Omega-3 and omega-6 PUFA induced changes in membrane fatty acid composition of HT29 and HepG2 cells.

HT29 cells

Incubation with ALA, EPA, DHA, LNA or ARA altered membrane composition of HT29 cells (**Table 1**).

Treatment with ALA increased its level to 26.35% in PtdCho and 32.33% in PtdEtn ($P<0.05$ to untreated cells). EPA treatments enhanced the level of EPA from 0.08% to 3.43% in the PtdCho ($P<0.05$ to control cells), and from 2.63% to 8.10% in the PtdEtn ($P<0.05$ to control cells). DHA was mainly incorporated in the PtdEtn fraction. It increased from 2.44% to 20.43% in the PtdEtn, and from 0.63% to 3.96% in the PtdCho ($P<0.005$), compared to control cells. Incubation with ALA or EPA did not increase the level of DHA in the PtdCho or PtdEtn fractions.

In comparison with control cells, LNA treatments increased LNA percentage from 3.47% to 40.70% in the PtdCho ($P<0.05$) and from 3.14% to 28.96% in the PtdEtn ($P <0.05$). Treatment with LNA did not increase the level of ARA in neither the PtdCho nor the PtdEtn.

Incubation with ARA enhanced ARA to 19.62% in the PtdCho and to 37.09% in the PtdEtn from 0.89% and 5.74%, respectively ($P <0.05$) compared to control cells. The percentage of adrenic acid (ADA, 22:4n-6) or docosapentaenoic acid (DPA-6, 22:5n-6) was not affected by ARA treatments (data not shown) in neither the PtdCho nor the PtdEtn.

HepG2 cells

The fatty acid profile of HepG2 cells after supplementation with ALA, EPA, DHA, LNA and ARA is shown in **Table 2**.

After incubation with ALA, the level of ALA increased from 0.15% to 11.31% ($P<0.01$) in the PtdCho, and from 0.07% to 9.15% ($P<0.05$) in the PtdEtn, compared to control cells. EPA increased to 0.61% and 1.64 % in the PtdCho and PtdEtn fractions ($P<0.05$ to control cells). DHA was preferentially incorporated into the PtdEtn fraction with an increased from

4.21% to 38.83% (P<0.05), rather than to the PtdCho fraction where it was enhanced from 1.76% to 12.76% (P<0.01), compared to control cells. There was no increase in DHA in the PtdCho or PtdEtn as a result of incubation with ALA. Incubation with EPA slightly increased the level of DHA.

LNA was mainly incorporated in the PtdCho where it increased from 5.76% to 29.98% (P<0.01), whilst it was enhanced from 4.46% to 18.92% (P<0.01) in the PtdEtn, compared to untreated cells. Incubation with LNA did not enhance the level of ARA in the PtdCho or PtdEtn. ARA supplements significantly enhanced ARA level from 2.49% to 19.76 % in the PtdCho (P<0.01) and from 5.93% to 13.59 % in the PtdEtn (P<0.01), compared to untreated cells. After ARA incubation, the percentage of ADA increased from 0.86% to 19.89% in the PtdCho (P<0.005) and 0.89% to 20.88% in the PtdEtn (P<0.005) (data not shown).

Changes in membrane PUFA composition alter AKT phosphorylation in HT29 and HepG2 cells.

Cells treated with insulin only or pre-treated with fatty acids and then incubated with insulin showed enhanced phosphorylation of the Akt protein compared to control cells with no insulin (P<0.05) (**Figure 1a**).

Pre-treatment with ALA, EPA and DHA before insulin stimulation did not alter the phosphorylation level of Akt compared to insulin only stimulated cells (**Figure 1a**).

Pre-treatment with LNA and ARA before insulin stimulation increased the amount of Akt phosphorylation compared with insulin only treated cells. This increase was statistically significant in HT29 cells (P<0.05 and P<0.01) (**Figure 1a**). In HepG2 cells, LNA and ARA showed a slight but not significant increase in Akt phosphorylation (**Figure 1a**).

Further investigation was conducted to elucidate whether the enhancement of Akt phosphorylation seen in LNA and ARA treated cells, was dependent on insulin and not

merely due to ARA and LNA treatments. Cells treated with LNA and ARA but not insulin were compared to untreated cells stimulated with insulin (**Figure 2**). As expected, insulin stimulated-cells responded to insulin by enhancement of Akt phosphorylation. In contrast, LNA and ARA did not enhance the level of phospho-Akt, showing that Akt phosphorylation is insulin-stimulation dependent.

Discussion and Conclusions

In this study, we have demonstrated that both HT29 and HepG2 cells incorporate fatty acids in PtdCho and PtdEtn - the most abundant cell membrane phospholipids. Moreover, we have shown that the incorporation and resultant changes in membrane phospholipid fatty acid composition influences insulin-induced Akt activation. The incorporation of LNA and ARA increased significantly insulin-induced Akt phosphorylation in HT29 cells and slightly but not significantly in HepG2 cells.

The incorporation of ALA, EPA or DHA did not have any effect on insulin-induced Akt phosphorylation in neither of the two cell lines. These findings are inconsistent with previous published reports [1, 3]. Supplementation of rats with fish oil improved insulin sensitivity by increasing insulin binding and receptor number [11], and enhanced insulin-stimulated glucose transport [30] . These contradicting observations could be a reflection of the difference between *in vivo* and *in vitro* investigations. Moreover, because fish oil contains appreciable amounts of LNA and ARA it is not possible to categorically attribute the observed effect on inulin sensitivity to EPA and DHA.

The increase in Akt phosphorylation was more remarkable in HT29 than Hep2 cells treated with LNA and ARA. In addition, these fatty acids were more efficiently incorporated by

HT29 than HepG2 cells. In the PtdCho, incubation with LNA resulted in a 13-fold increase in HT29 and a 6-fold in HepG2 cells. LNA increased 10 and 4.5 fold in HT29 and HepG2 cell PtdCho, respectively. Similarly, in PtdCho, ARA incubation enhanced the level of ARA 18 fold in HT29 and 9.5 in HepG2 cells. ARA increased 7.4 fold HT29 and 2.6 in HepG2 cells in PtdEtn. The observed difference in response to insulin and in fatty acid incorporation between HT29 and HepG2 cells is most likely a reflection of their intrinsic characteristics. Indeed, it is postulated that the impact of insulin on cell types is different depending on relative IR-B: IR-A expression levels [31]. A difference in growth inhibition and apoptosis have been observed between HepG2 and HT29 cells treated with dehydroepiandrosterone [32]. IR-A isoform is more predominant in HT-29 [31] and IR-B in HepG2 [33].

Our findings of the effect of LNA on Akt phosphorylation is consistent with the reports of previous studies. Field et al. [10] demonstrated that rats fed diets containing increasing ratios of linoleic acid compared with those fed saturated fatty acids (palmitic and stearic) had enhanced insulin binding capacity. A higher insulin-stimulated glucose uptake in adipocytes of rats fed LNA-rich diet compared with those fed a high EPA/DHA diets was demonstrated by Fickova et al. [31]. Similarly, Lee et al. [13] have reported increased insulin sensitivity in experimental given n-6 polyunsaturated fatty acids. Meuillet et al. [22], *in vitro studies*, have observed increased IR and IRS1 phosphorylation in HepG2 cells incubated with LNA.

The findings of the limited studies that investigated the effect of ARA supplementation on insulin response have been inconclusive. Borkam et al. [9] found a positive relationship between insulin-sensitivity and muscle-ARA level in healthy men. Likewise, Villegas-Comonfort [32] demonstrated that ARA induces AKT activation in MDA-MB-231 cells. In

contrast, Talukdar and colleagues [33] have reported that ARA inhibits insulin induced Akt phosphorylation but promotes IRS-1 phosphorylation in rat hepatocytes.

It is not evident how the uptake of LNA and AA influences positively Akt phosphorylation. Phosphorylation of Akt is a complex signalling cascade that involves insulin binding to IR and stepwise phosphorylation of IR, IRS-1 and PI3K. Akt phosphorylation could be influence positively or negatively from changes in the aforementioned steps of the signalling cascade.

Modification of IR response to insulin by altered membrane composition might be linked to changes of cell membrane organisation, such as alterations of rafts and caveolae microdomains. Caveolae are thought to act as a platform for the recruitment of several signal transduction molecules, including the IR[34, 35] . It appears these membrane microdomains are critical for proper compartmentalization of the insulin transduction pathway and their modification leads to alterations of insulin transduction cascade. Indeed, there is evidence that the disruption of caveolae by depletion of cholesterol inhibits the ability of insulin to enhance glucose uptake, and IRS-1 and Akt phosphorylation in cell lines [36]. Similarly, impaired IRS-1 and Akt phosphorylation, insulin resistance condition, was observed in rats fed a high cholesterol diet [37]. Chapkin et al. [38] have proposed that the aversion of DHA and LNA to cholesterol would enhance segregation of cholesterol into lipid microdomains. The authors have demonstrated that the above fatty acids increased clustering of proteins in cholesterol-dependent microdomains in HeLa cells. The finding has highlighted a differential effect of LNA versus DHA indicating that only LNA treatment reduced clustering of proteins in non-raft regions. Consistent with the afore cited investigation, pretreatment of endothelial cells with LNA increased caveolin-1 expression and p38 MAPK activation [39]. The authors

postulated that certain fatty acids may either stabilize or perturb caveolae function, thus leading to modification of caveolae-dependent pathways [40]. Similarly, Schley et al. [41] demonstrated that LNA treatment of breast cancer cells resulted in higher content of cholesterol and sphingomyelin in rafts as compared with DHA and EPA. In addition, EPA and DHA decreased the expression of the epidermal growth factor receptor (EGFR) selectively in rafts.

The above studies have highlighted the importance of membrane organisation in cell signaling and the link between cell transduction and membrane structure. Therefore, it is tenable to speculate that the enhancement of AKT phosphorylation after treatment with LNA might be due to the formation and compartmentalisation of caveolae, lipid-rafts, and their interactions with insulin receptors (IR).

After activation, PI3K phosphorylates PtdIns-4,5-P2 to yield PtdIns-3,4,5-P3. The generation of PtdIns-3,4,5-P3 is crucial for the recruitment and phosphorylation of AKT and activation of PDK1. There is evidence of selective incorporation of ARA in inositol phosphoglycerides [42] and elevated levels of the fatty acid in PtdIns-4-P and PtdIns-4,5-P2 [39, 43] . A question arises whether an increased ARA level in cell membrane alters membrane phospholipid metabolism (i.e. accumulation, turnover), particularly of inositol containing phospholipids. ARA had an inhibitory effect on PtdIns-1,4-P2/PtdIns-1,4,5-P3 metabolism under condition of cycle activation in submandibular acinar cells [44]. In contrast, it stimulated the accumulation of inositol mono-bi-and tri-phosphate, but not inositol phosphoglycerides in astrocytes [45]. Also, it is reported that ARA PtdCho suppressed Akt membrane translocation but left the concentration of the anchor lipid phosphatidylinositol-3,4,5-trisphosphate unchanged in fibroblasts [46]. The question yet to be answered is whether

increased ARA might alter the level of PtdIns-4,5-P_2 available for phosphorylation and in turn the level of PtdIns-3,4,5-P3 and effect AKT phosphorylation in HT29 cells.

Previous studies have investigated the effect of n-6 and n-3 polyunsaturated fatty acids on Akt signaling pathway. However, most of these studies did not investigate whether or not the supplemented fatty acids were incorporated into membrane phospholipids. In addition, some of these studies have used a mixture of fatty acids from fish oil or vegetable seed oils instead of individual fatty acids. The present study is original in that the cells were treated with individual fatty acids, and it has established a link between the treatment, specific changes in fatty acid composition of the major membrane phospholipids and the subsequent effect on Akt phosphorylation."

The small simple size was a limitation of this study. However, because transformed cells have a consistent feature (very low random variations) and a significance difference is established, the findings provide new valuable insights into the role of n-6 fatty acids in insulin signaling.

"In conclusion, the findings of the present investigation demonstrates that modification of membrane lipids with LNA or ARA treatment enhances insulin action by activation of AKT. Further work is required to establish whether the increased AKT phosphorylation is due to altered IR, IRS1 and/or PI3K activation. The expression and membrane localisation of IR and its association with caveolae should be elucidated. Furthermore, it is important to clarify if phospholipid levels and PtdIns metabolism are altered as a consequence of changes in membrane fatty acid composition."

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KM carried out all experiments, analysed the data and drafted the manuscript. KG and YM designed the study, interpreted the data and edited manuscript.

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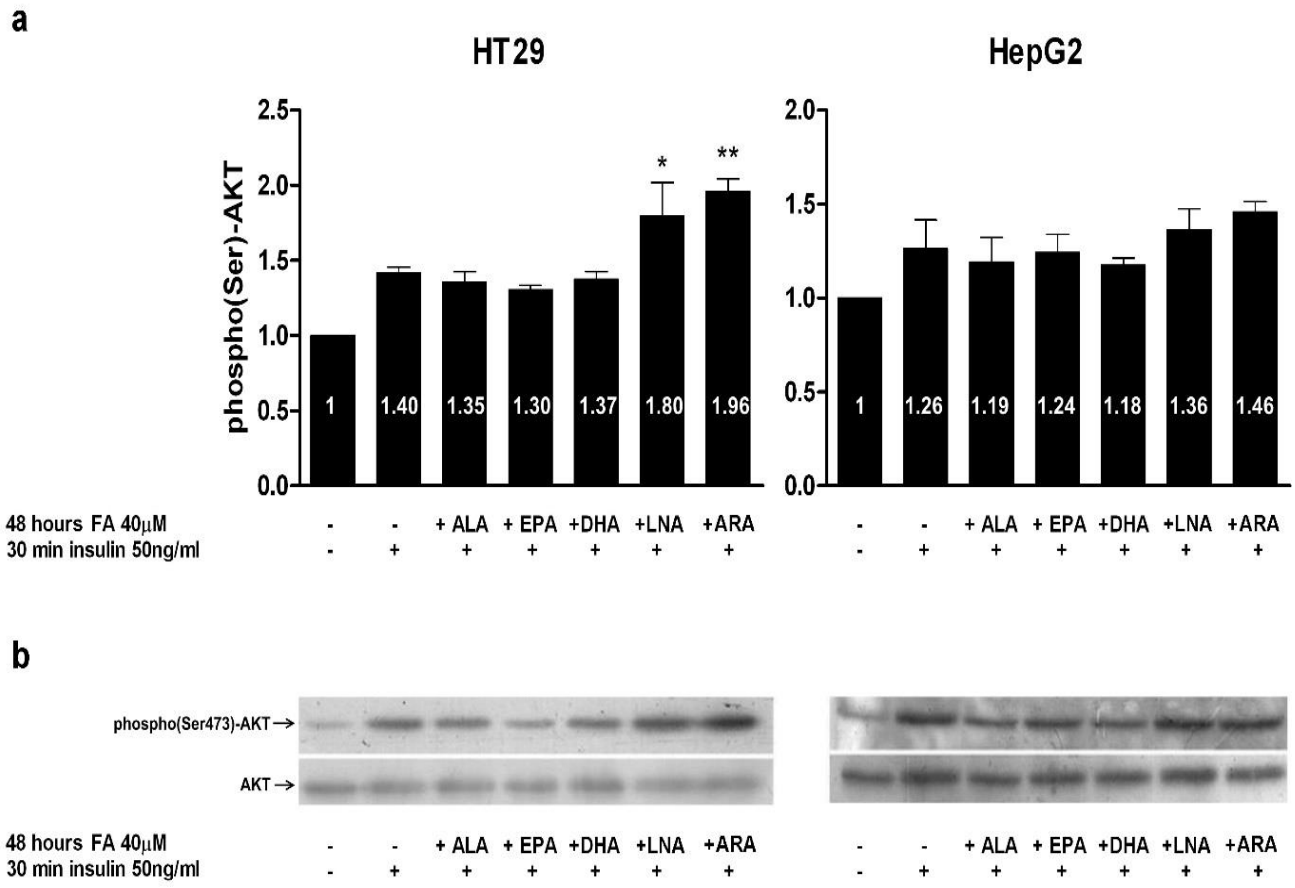
Figure 1. Expression of phosphor-AKT (Ser473) after 30 minutes of stimulation with 50ng/ml insulin in HT29 and HepG2 cells.



a – Graph

- Level of AKT phosphorylation was normalised to the expression of the AKT total protein and expressed as ratio to control untreated cells.
- Data are expressed mean (± SE) of two independent experiments.
- Significant difference from insulin stimulated only at $p<0.05$(*) and $p<0.01$ (**).

b - Western blot.

Figure 2. Expression of phosphor-AKT (Ser473) after LNA and ARA treatment in HT29 cells.

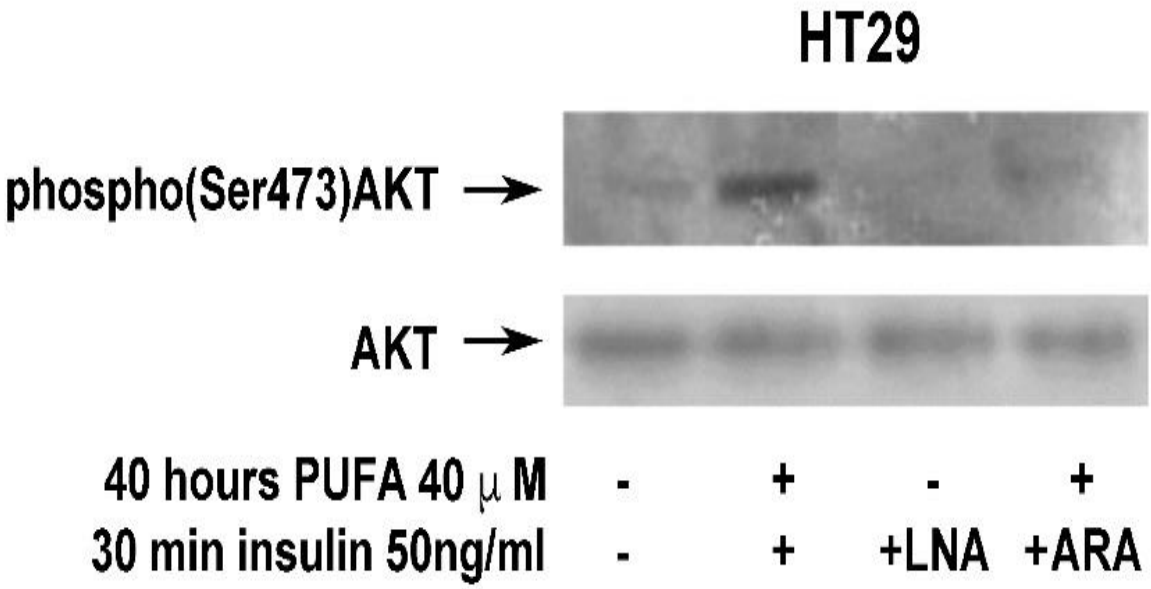


Table 1. Change of omega-3 and omega-6 fatty acid levels in phosphatidylethanolamine (PtdEtn) and phosphatidylcholine (PtdCho) after treatment in HT29 cells

HT29

	Fatty Acids %																			
		ALA				EPA				DHA				LNA				ARA			
PdtEtn	CONTROL	0.15	±	0.15		2.63	±	1.64		2.44	±	1.18		3.14	±	0.06		5.74	±	3.07	
	+ ALA	32.33	±	4.59	*	2.56	±	0.75		3.07	±	0.64		4.79	±	0.03		6.92	±	0.78	
	+ EPA	0.38	±	0.06		8.10	±	0.80	*	1.91	±	0.23		5.19	±	0.98		6.32	±	0.59	
	+ DHA	0.11	±	0.11		13.66	±	1.74	*	20.43	±	1.11	**	2.79	±	0.18		6.70	±	0.01	
	+ LNA	0.00	±	0.00		1.88	±	0.16		2.34	±	0.31		28.96	±	4.21	*	6.52	±	0.13	
	+ ARA	1.13	±	1.13		0.53	±	0.21		3.18	±	0.59		2.93	±	0.60		37.09	±	1.78	*
PdtCho	CONTROL	0.10	±	0.10		0.08	±	0.08		0.63	±	0.25		3.47	±	0.46		0.89	±	0.32	
	+ ALA	26.35	±	3.45	*	0.11	±	0.11		0.50	±	0.14		4.72	±	0.60		0.85	±	0.31	
	+ EPA	0.31	±	0.31		3.43	±	0.34	*	1.40	±	0.07		10.19	±	0.97		3.40	±	0.03	
	+ DHA	0.09	±	0.09		3.63	±	2.07		3.96	±	0.88	*	3.47	±	0.95		1.08	±	0.43	
	+ LNA	0.06	±	0.06		0.10	±	0.10		0.69	±	0.20		40.70	±	6.20	*	1.12	±	0.27	
	+ ARA	0.05	±	0.05		0.14	±	0.14		0.90	±	0.28		3.88	±	0.66		19.62	±	6.22	*

- HT29 cells were treated for 48 hours with 40 μM of α-linolenic (ALA), Eicosapentaenoic (EPA), docosahexaenoic (DHA), linoleic (LNA) acids or arachidonic acid (ARA).
- Each experiment was repeated two times or more and the data are expressed as mean (±SE) percent total fatty acids.
- Significant difference from control at $p<0.05$ (*), $p<0.01$ (**), $p<0.005$ (***)

Table 2. Change of omega-3 and omega-6 fatty acid levels in phosphatidylethanolamine (PtdEtn) and phosphatidylcholine (PtdCho) after treatment in HepG2 cells

		HepG2																							
		ALA				EPA				DHA				LNA				ARA			
PdtEtn	CONTROL	0.07	±	0.07		0.00	±	0.00		4.21	±	0.00		4.46	±	0.72		5.93	±	0.55	
	+ ALA	9.15	±	0.21	*	0.40	±	0.40		3.04	±	1.46		5.49	±	0.12		5.53	±	0.14	
	+ EPA	0.13	±	0.13		1.64	±	0.10	*	6.63	±	1.93		4.60	±	0.51		6.85	±	0.40	
	+ DHA	0.05	±	0.05		1.50	±	0.48		38.83	±	6.16	*	2.63	±	1.21		2.24	±	0.04	
	+ LNA	0.00	±	0.00		0.17	±	0.17		7.17	±	0.81		18.92	±	1.00	**	4.88	±	0.11	
	+ ARA	0.04	±	0.04		0.06	±	0.06		4.13	±	0.51		2.89	±	1.28		13.59	±	1.67	**
PdtCho	CONTROL	0.15	±	0.08		0.15	±	0.08		1.76	±	0.85		5.76	±	0.93		2.49	±	1.05	
	+ ALA	11.31	±	0.42	*	0.10	±	0.10		0.68	±	0.09		4.23	±	0.62		1.29	±	0.20	
	+ EPA	0.10	±	0.10		0.61	±	0.28	*	2.00	±	1.03		5.90	±	0.90		3.31	±	1.87	
	+ DHA	0.09	±	0.09		2.33	±	0.60	*	12.76	±	0.80	**	4.06	±	0.09		2.43	±	0.18	
	+ LNA	0.05	±	0.05		0.00	±	0.00		0.66	±	0.01		29.98	±	4.66	**	0.86	±	0.02	
	+ ARA	0.00	±	0.00		0.00	±	0.00		2.24	±	0.29		4.09	±	0.51		19.76	±	2.01	**

HepG2 cells were treated for 48 hours with 40 μM of α-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), linoleic acid (LNA) or arachidonic acid (ARA); each experiment was repeated two times or more the data is expressed mean (±SD) percent total fatty acids. Significant difference from control at $p<0.05$ (*), $p<0.01$ (**), $p<0.005$ (***)