Nutrition 57 (2019) 52–58

Contents lists available at ScienceDirect

Nutrition

journal homepage: www.nutritionjrnl.com

Basic nutritional investigation

Phosphorylation of protein kinase B, the key enzyme in insulin-signaling cascade, is enhanced in linoleic and arachidonic acid–treated HT29 and HepG2 cells

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ARTICLE INFO

Article History:
Received 10 November 2017
Received in revised form 24 May 2018
Accepted 29 May 2018

Keywords:
Membrane phospholipids
Omega 6 fatty acids
Omega 3 fatty acids
AKT phosphorylation

ABSTRACT

Objectives: Defects in the insulin-signaling pathway have been implicated in the pathogenesis of impaired glucose uptake, insulin resistance, and type 2 diabetes. However, the specific defects that precipitate these abnormalities are yet to be fully elucidated. After binding to insulin, the plasma membrane–embedded insulin receptor transmembrane protein initiates a cascade of phosphorylation that leads to the activation of protein kinase B (AKT) and subsequently to the 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. Published data on the influence of omega-3 and -6 polyunsaturated fatty acids on insulin response are scarce. Moreover, the findings of the published investigations, most of which used omega-3 and -6, polyunsaturated fatty-acid blends, have been inconclusive. Hence, further, well thought out research is needed. The aim of the current study was to elucidate the effect of treatments with linoleic acid (LNA), arachidonic acid (ARA), alpha-linolenic acid (ALA), docoshexaenoic acid (DHA), and eicosapentaenoic acid (EPA) on cell membrane composition and consequently on the insulin-signaling pathway and specifically AKT phosphorylation.
Methods: Human colon adenocarcinoma (HT29) and liver hepatocellular (HepG2) cells were treated with or without 40 μM of LNA, ARA, ALA, EPA, or DHA for 48 h, the fatty-acid composition of phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn) from the treated cells by capillary gas liquid chromatograph. Cells were incubated for 30 min with or without human insulin (50 ng/mL), and the phosphorylation of AKT was assessed with the use of Western blotting.
Results: The fatty acids were incorporated in the 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.
Conclusions: The finding of this investigation demonstrates that plasma membrane lipid bilayer enrichment with LNA or ARA treatment enhances insulin action by AKT activation.

Introduction

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

The results of human and animal studies suggest that long-chain PUFA, particularly of the omega-3 family, may protect against type 2 diabetes and insulin resistance [4]. Epidemiologic studies have shown that the consumption of diets high in omega-3 PUFA correlate with a lower incidence of type 2 diabetes compared with diets that are rich in saturated fatty acids [5–7]. Studies on fatty-acid membrane composition have shown that low levels of omega-3 PUFA were found in association with type 2 diabetes [8], but 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 studies of animals that were fed omega-3 and -6 PUFA [10,11].

Insulin is a pancreatic hormone that is involved in many functions. Mainly, insulin regulates the uptake of glucose (i.e., entry of

Sources of support: This study was supported by FP6 Marie Curie Actions–Transfer of Knowledge (MTKD-CT-2005-029914), The Mother and Child Foundation, and The Letten Foundation.
Conflicts of interest: The authors declare no conflicts of interest.
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https://doi.org/10.1016/j.nut.2018.05.033
0899-9007/

glucose into cells) in muscles, adipose tissue, and the liver [12]. In addition, insulin is involved in the regulation of several metabolic enzymes as well as the promotion of storage of incoming glucose as glycogen and the synthesis of proteins and triacylglycerols from free fatty acids.

Insulin binds to the insulin receptor (IR) [13], which is a transmembrane heterotetrameric tyrosine kinase receptor that 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 and insulin receptor substrates 1 and 2 (IRS-1 and 2). The main target of IRS is the phosphatidylinositol 3-kinase (PI3 K) [14]. After phosphorylation, PI3 K 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 (AKT). AKT has a pleckstrin homology domain that is located at the 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 phosphorylated first 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 that are 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 the subsequent uptake of glucose into cells.

Defects in both upstream and downstream targets of AKT (PI3 K-AKT signaling pathway) have been implicated in insulin resistance [17,18]. Insulin receptors are proteins that are located in the plasma membrane, and the phosphorylation of PI3 K is dependent on inositol-containing membrane phospholipids. Therefore, changes in plasma membrane composition can be assumed to have an effect on the insulin-signaling pathway.

Cultured cells are widely used as a good model to investigate the relationships between membrane fatty acids and insulin response. In vitro, alterations in the lipid composition of erythroleukemia and ascites cells have shown a high percentage of linoleic acid (LNA)-rich oil (e.g., sunflower oil) versus oil that contains mainly saturated fatty acids (e.g., coconut oil), which was associated with an increase in the insulin receptor number and a decrease in binding receptor affinity [19,20]. Bruneau et al. [21] have demonstrated that lipid alterations (via treatments with LNA) of rat hepatoma cells led to an insulin-resistant state. Subsequently, the same research group reported also that feeding human hepatoma cells with LNA and eicosapentaenoic acid (EPA) modified the insulin receptor autophosphorylation and the phosphorylation of the major insulin receptor substrate, IRS-1 [22].

These studies, most of which used a heterogeneous blend of omega-3 and -6 PUFA, have highlighted a possible relationship between these fatty acids and insulin response. Therefore, a need exists 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], and although the gastrointestinal tract is the main target of insulin, this is where nutrients are processed and absorbed. Consequently, the insulin pathway is thought to have an indirect effect on insulin resistance [24]. The liver is widely recognized as the primary insulin target, and hepatocytes are often used to investigate insulin response [25,26].

Methods

Reagents

Alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), LNA, and arachidonic acid (ARA) were purchased from Sigma-Aldrich, dissolved in 100% ethanol, and stored at −20°C. Insulin human recombinant was obtained from Sigma-Aldrich as well.

Cell culture

HT29 and HepG2 cells were cultured respectively in McCoys or DMEM medium (Sigma), supplemented with 2 mM glutamine, 5 mM streptomycin, 5 mM penicillin, and 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Fatty acid treatments

HT29 and HepG2 cells (2×10^5 cells/mL) were seeded in 21 cm^2 plates and allowed to attach and divide for 24 h. Subsequently, the medium was siphoned and replaced with fresh medium that contained 10% FBS with or without 40 μM of different PUFAs (i.e., ALA, EPA, DHA, LNA, or ARA) [27,28]. After 48 h, treated and untreated cells were collected and used for analysis of fatty-acid composition. The final concentration of ethanol/medium, which was <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 1 X PBS. To extract cell-membrane total lipids, 1.5 mL of hexane/isopropanol (3:2, v/v; HIP) and 0.01% BHT were added to each well on ice. After 5 min of incubation on a shaker, the HIP mixture was transferred to a clean glass tube. The HIP mixture was completely evaporated under nitrogen, and 1.8 mL of chloroform/methanol (2:1, v/v) +BHT and 0.6 mL of water were added to the tubes. The tubes were vortexed and then centrifuged at 4000 rpm for 5 min 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). PtdCho and PtdEtn were separated by thin-layer chromatography on 20 × 20 cm silica gel plates (TLC silica gel 60, Merck kGaA, United Kingdom) by 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 with standards. Fatty-acid methyl esters were prepared by heating the lipid fractions with 4 mL of 15% acetyl chloride in methanol in a sealed vial at 70°C for 3 h under nitrogen. Fatty-acid methyl esters were separated by a capillary gas liquid chromatograph (HRGC MEGA 2 Series, Fisons Instruments, Italy) and identified by comparing the retention times of the authentic standards (Sigma-Aldrich, United Kingdom) and by calculating the equivalent chain-length values. Peak areas were quantified with a computer chromatography data system (Agilent EZChrom Elite 3.2, Scientific Software, Inc., Pleasanton, CA).

Insulin treatments

HT29 and HepG2 cells (2×10^5/mL) were seeded in 21 cm^2 plates and allowed to attach and divide for 24 h. Subsequently, the old medium was siphoned off and replaced with a fresh medium that contained 10% FBS, supplemented with 40 μM of different PUFAs (i.e., ALA, EPA, DHA, LNA, or ARA). Two plates were grown in 10% FBS medium without adding fatty acids.

After 48 h of incubation, the old medium was discarded and the cells incubated in a fresh, serum-free medium for 6 h. Subsequently, the medium was siphoned off, and the cells were incubated for 30 min with a fresh medium with and without 50 ng/mL of human insulin [29].

Western blot

HT29 and HepG2 cells were washed twice with ice-cold PBS 1 X. Subsequently, cells were lysed in an ice-cold, radioimmunoprecipitation assay buffer (1% NP-40, 0.1% sodium dodecyl sulphate [SDS], 0.5% deoxycholate, 20 mM Tris-hydrogen

chloride buffer (pH 7.4), 150 mM NaCl, 10 mM NaF, 1 mM Na3 VO4, 1 mM EDTA, 1 mM EGTA). To prevent protein degradation and dephosphorylation, a phosphatase and protease inhibitor cocktail (Sigma-Aldrich, UK) was added to the radioimmunoprecipitation assay 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.

A total of 40 μg of proteins was diluted in a 3 X Laemmi loading buffer (1.5% Tris-hydrogen chloride buffer [pH 6.8], 4% SDS, 1% glycerol, 0.2% bromophenol blue, 0.4% β-mercaptoethanol in water) and loaded on a 10% SDS-polyacrylamide mini gel (Biorad apparatus). The separated proteins were transferred from the gel onto a nitrocellulose membrane (Biorad) using a Mini Trans-Blot Cell apparatus at 0.09 mA overnight. The membranes were blocked for 1 h in 5% dried non-fat milk in TBS-T 1 X, and then washed three times in a TBS-T 1 X buffer. Subsequently, membranes were blotted with rabbit antiphospho-AKT (Ser473) (1:1000, Cell Signaling technology) in 5% BSA/TBST 1 X overnight at 4°C. After incubation, membranes were washed three times for 5 min in TBS-T 1 X and incubated with goat antirabbit IgG HRP conjugate secondary antibody in 5% non-fat dry milk in TBST 1 X for 1 h at room temperature.

After probing, secondary antibody membranes were washed three times for 5 min in TBS-T 1 X. 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 rabbit anti-AKT (1:1000, Cell Signaling technology) in 5% BSA/TBST 1 X overnight at 4°C. After incubation, membranes were washed three times for 5 min in TBST 1 X and incubated with goat antirabbit IgG conjugate secondary antibody in 5% dried non-fat milk in TBS-T 1 X for 1 h at room temperature.

After probing, secondary antibody membranes were washed three times for 5 min in TBS-T 1 X 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 phospho-AKT was normalized to the level of total AKT.

Statistical analysis

Data were calculated as mean percentage of total fatty acids (± standard error) 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) was employed to analyze the data.

Results

Omega-3 and -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 the 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 and increased from 2.44% to 20.43% in the PtdEtn and from 0.63% to 3.96% in the PtdCho ($P < 0.005$) compared with 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 <$0.05). Treatment with LNA did not increase the level of ARA in either the PtdCho or the PtdEtn. Incubation with ARA enhanced ARA to 19.62% in the PtdCho and to 37.09% in the PtdEtn from 0.89% and 5.74%, respectively ($P < 0.05$) compared with control cells. The percentage of adrenic acid (22:4 n-6) or docosapentaenoic acid (22:5 n-6) was not affected by ARA treatments (data not shown) in either the PtdCho or 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 with control cells.

EPA increased to 0.61% and 1.64% in the PtdCho and PtdEtn fractions, respectively ($P < 0.05$ compared with control cells). DHA was preferentially incorporated into the PtdEtn fraction with an increase from 4.21% to 38.83% ($P < 0.05$) compared with the PtdCho fraction, which was enhanced from 1.76% to 12.76% ($P < 0.01$) in comparison with 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% ($P < 0.01$) but was enhanced from 4.46% to 18.92% ($P < 0.01$) in the PtdEtn compared with untreated cells.

Table 1
Change in omega-3 and -6 fatty acid levels in PtdEtn and PtdCho after treatment in human colon adenocarcinoma cells

		Human colon adenocarcinoma cells 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***

ALA, alpha-linolenic acid; ARA, arachidonic acid; DHA, docoshexaenoic acid; EPA, eicosapentaenoic acid; LNA, linoleic acid; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine.
Human colon adenocarcinoma cells were treated for 48 h with 40 μM ALA, EPA, DHA, LNA, or ARA. Each experiment was repeated twice at minimum and the data were reported as mean percentage of total fatty acids ± standard error.
†Significant difference from control at $P < 0.01$.
‡Significant difference from control at $P < 0.005$.
*Significant difference from control at $P < 0.05$.

Table 2
Change of omega-3 and -6 fatty acid levels in PtdEtn and PtdCho after treatment in liver hepatocellular cells

		Liver hepatocellular cells Fatty acids %														
		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****

ALA, alpha-linolenic acid; ARA, arachidonic acid; DHA, docoshexaenoic acid; EPA, eicosapentaenoic acid; LNA, linoleic acid; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine.
HepG2 cells were treated for 48 h with 40 μM ALA, EPA, DHA, LNA, or ARA. Each experiment was repeated minimum twice and the data were reported as mean percentage of total fatty acids ± standard error.
†Significant different from $P < 0.01$.
‡$P < 0.005$
*Significant difference from control at $P < 0.05$.

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 5.93% to 13.59% in the PtdEtn ($P < 0.01$) compared with 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 that were treated with insulin only or pretreated with fatty acids and then incubated with insulin showed enhanced phosphorylation of the AKT protein compared with control cells with no insulin ($P < 0.05$; Fig. 1A).

Pretreatment with ALA, EPA, and DHA before insulin stimulation did not alter the phosphorylation level of AKT compared with insulin-only stimulated cells (Fig. 1A). Pretreatment 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 $P < 0.01$, respectively; Fig. 1A). In HepG2 cells, LNA and ARA showed a slight but not significant increase in AKT phosphorylation (Fig. 1A).

Further research was conducted to elucidate whether the enhancement of AKT phosphorylation in LNA- and ARA-treated cells was dependent on insulin and not merely due to ARA and LNA treatments. Cells that were treated with LNA and ARA but not

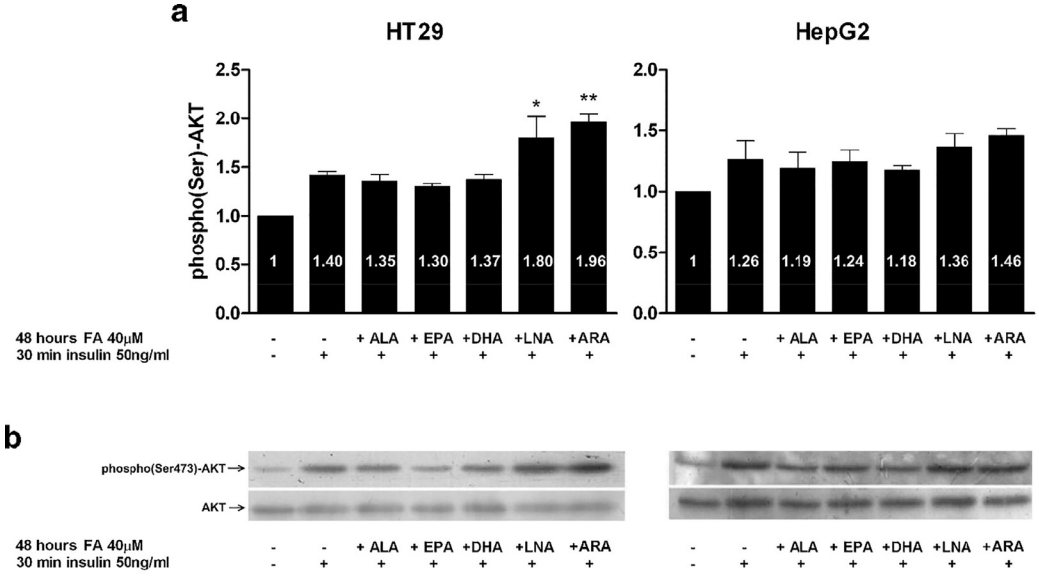

Fig. 1. Expression of phospho-protein kinase B (AKT; Ser473) after 30 min of stimulation with 50 ng/mL insulin in human colon adenocarcinoma and liver hepatocellular cells. (A) Level of AKT phosphorylation was normalized to the expression of AKT total protein and expressed as ratio-to-control untreated cells. Data are shown as mean (± standard error) of two independent experiments. *Significant difference from insulin stimulated only at $P < 0.05$. **Significant difference from insulin stimulated only at $P < 0.01$. (B) Western blot.

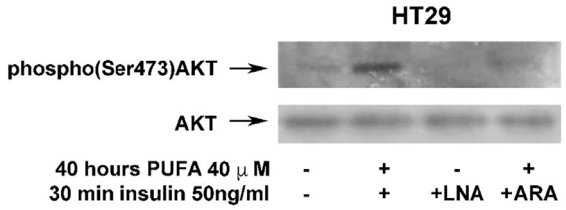

Fig. 2. Expression of phospho-protein kinase B (AKT; Ser473) after linoleic and arachidonic acid treatments in human colon adenocarcinoma cells. Western blot analysis of human colon adenocarcinoma cell lysates probed with anti AKT and anti phoshpo-AKT. Cells were untreated, stimulated with insulin or treated with linoleic acid and arachidonic acid.

insulin were compared with untreated cells that were stimulated with insulin (Fig. 2). As expected, insulin-stimulated cells responded to insulin by enhancing AKT phosphorylation. In contrast, LNA and ARA did not enhance the level of phospho-AKT, which shows that AKT phosphorylation is insulin-stimulation dependent.

Discussion

In this study, we demonstrated that both HT29 and HepG2 cells incorporate fatty acids in PtdCho and PtdEtn, which are the most abundant cell-membrane phospholipids. Moreover, we showed that the incorporation and resultant changes in membrane phospholipid fatty-acid composition influences insulin-induced AKT activation. The incorporation of LNA and ARA significantly increased 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 either of the two cell lines. These findings are inconsistent with previously published reports [1,3]. The 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, the observed effect on inulin sensitivity cannot be categorically attributed to EPA and DHA.

The increase in AKT phosphorylation was more remarkable in HT29 than HepG2 cells that were 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 cells and a 6-fold increase 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 cells and 9.5-fold in HepG2 cells. ARA increased 7.4-fold in HT29 cells and 2.6-fold in HepG2 cells in PtdEtn. The observed difference in response to insulin and fatty-acid incorporation between HT29 and HepG2 cells is most likely a reflection of their intrinsic characteristics. Indeed, the impact of insulin on cell types is postulated to be 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 that were 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 reports from previous studies. Field et al. [10] demonstrated that rats that were fed diets containing increasing ratios of LNA compared with rats fed saturated fatty acids (palmitic and stearic) had enhanced insulin-binding capacity. A higher insulin-stimulated glucose uptake in adipocytes of rats that were fed an LNA-rich diet compared with rats fed a high EPA/DHA diet was demonstrated by Fickova et al. [31]. Similarly, Lee et al. [13] reported increased insulin sensitivity in experimentally given n-6 polyunsaturated fatty acids. The in vitro studies by Meuillet et al. [22], showed increased IR and IRS1 phosphorylation in HepG2 cells that were 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 levels in healthy men. Likewise, Villegas-Comonfort [32] demonstrated that ARA induces AKT activation in MDA-MB-231 cells. In contrast, Talukdar et al. [33] reported that ARA inhibits insulin-induced AKT phosphorylation but promotes IRS-1 phosphorylation in rat hepatocytes.

How the uptake of LNA and AA positively influences AKT phosphorylation is unclear. The phosphorylation of AKT is a complex signaling cascade that involves insulin binding to IR and stepwise phosphorylation of IR, IRS-1, and PI3 K. AKT phosphorylation could be influenced positively or negatively by changes in the aforementioned steps of the signaling cascade.

The modification of IR response to insulin by altered membrane composition might be linked to changes of cell membrane organization 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]. These membrane microdomains appear to be critical for proper compartmentalization of the insulin transduction pathway and their modification leads to alterations of the insulin transduction cascade.

Indeed, there is evidence that the disruption of caveolae by depleting 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 conditions) were observed in rats that were fed a high-cholesterol diet [37]. Chapkin et al. [38] have proposed that the aversion of DHA and LNA to cholesterol would enhance the segregation of cholesterol into lipid microdomains. The authors demonstrated that fatty acids increased the clustering of proteins in cholesterol-dependent microdomains in HeLa cells. This finding has highlighted a differential effect of LNA versus DHA and indicates that only LNA treatment reduced the clustering of proteins in non-raft regions.

Consistent with the findings of these investigations, the 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 and thus lead to the modification of caveolae-dependent pathways [40]. Similarly, Schley et al. [41] demonstrated that the LNA treatment of breast cancer cells resulted in a higher content of cholesterol and sphingomyelin in rafts compared with DHA and EPA. In addition, EPA and DHA decreased the expression of the epidermal growth factor receptor selectively in rafts.

These studies highlight the importance of membrane organization in cell signaling and the link between cell transduction and membrane structure. Therefore, the enhancement of AKT phosphorylation after treatment with LNA might be due to the formation and compartmentalization of caveolae, lipid-rafts, and their interactions with IRs.

After activation, PI3 K phosphorylates PtdIns-4,5-P2 yielded to 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 fatty acid in PtdIns-4-*P* and PtdIns-4,5-P2 [39,43]. A question arises whether an increased ARA level in the cell membrane alters membrane phospholipid metabolism (i.e. accumulation, turnover), and particularly of inositol-containing phospholipids. ARA had an inhibitory effect on PtdIns-1,4-P2/PtdIns-1,4,5-P3 metabolism under the condition of cycle activation in submandibular acinar cells [44]. In contrast, the accumulation of inositol mono-bi-and tri-phosphate was simulated, but not inositol phosphoglycerides in astrocytes [45]. Also, ARA PtdCho was reported as suppressed by AKT membrane translocation but left the concentration of the anchor lipid phosphatidylinositol-3,4,5-trisphosphate unchanged in fibroblasts [46]. The question that has 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 of AKT phosphorylation in HT29 cells.

Previous studies have investigated the effect of n-6 and n-3 PUFAs on the AKT signaling pathway. However, most of these studies did not investigate whether 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. This study established a link between 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 (i.e., 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.

Conclusions

The findings of the present investigation demonstrate that the modification of membrane lipids with LNA or ARA treatment enhances insulin action by activation of AKT. Further work is needed to establish whether the increased AKT phosphorylation is due to altered IR, IRS1, and/or PI3 K activation. The expression and membrane localization 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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