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Effects of dietary fats on red blood cell membrane insulin receptor in normo- and hypercholesterolemic miniature swine

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Abstract

It has been demonstrated that the type of dietary fat affects insulin receptors in various tissues in normal humans and animals by altering membrane fluidity. This study compares the effects of n-3 fatty acids from fish oil and n-6 fatty acids from corn oil on red blood cell membrane insulin receptors in normal and hypercholesterolemic minipigs. A group of minipigs were made hypercholesterolemic by feeding cholesterol and lard for 2 months; the other group served as controls and was fed stock diet. Both groups were then fed experimental diets containing either corn oil or menhaden oil or a mixture of the two for 23 additional weeks. Blood was collected at 0, 2, 12 and 23 weeks after the start of the experimental diets and membranes were prepared from the red blood cells. Insulin binding to red blood cell membranes was measured by radioreceptor assay. Plasma insulin was measured by radioimmunoassay. Insulin binding to red blood cell membrane was compared with the fluidity of the membrane measured and reported earlier. There was no significant effect of cholesterol feeding on plasma insulin concentrations. After 23 weeks on experimental diet plasma insulin was significantly higher in minipigs fed menhaden oil compared to those fed corn oil. No such effect was observed in hypercholesterolemic minipigs. No significant effect of either hypercholesterolemia or fish oil was observed on red blood cell insulin binding. A significant negative relationship was observed between insulin binding and anisotropy at 4°C for all probes but at 37°C significant negative relationship was observed only with polar probes. The data suggest that n-3 fatty acids from fish oil significantly increases plasma insulin in minipigs compared to n-6 fatty acids from corn oil. However, the unsaturation has no significant effect on insulin receptors on erythrocytes. Similarly, prior hypercholesterolemic state also has no effect on plasma insulin levels or the insulin binding to red blood cell membranes.

Keywords: Plasma insulin; insulin binding; cholesterol; n-3 fatty acids; n-6 fatty acids; miniature swine

1. Introduction

The importance of n-3 fatty acids from fish oils especially eicosapentaenoic and docosahexaenoic acids in human and animal nutrition is well recognized; especially in the prevention and treatment of heart disease, hypertension, cancer, skin disorders, immunological function, blood pressure regulation, decreasing insulin resistance and in the development of the brain and central nervous system [1–4]. Similar beneficial effects of n-6 polyunsaturated fatty acids have also been recognized [1,3] Polyunsaturated fatty acids of both series are readily incorporated into lipids of cell membranes in a variety of tissues in humans and animals. Depending on the dietary availability, they replace each other in plasma [5], heart [6], liver [7], adipose tissues [8], brain [9], erythrocytes [10] and in aortic plaque [5]. Thus, chemical composition and properties of the cell membrane are determined by the presence of specific fatty acids in the structure. Similarly, dietary cholesterol also induces changes in cell membrane composition and affects physical and chemical properties such as fluidity [11] and insulin receptors [12] thereby impacting on physiological processes.

Engelhard et al. [13] and Ghosh et al. [14] have shown that changes in membrane fluidity affect hormone receptor binding, release and uptake of neurotransmittters, transport of ions, activities of membrane bound enzymes and the phosphorylation of proteins. We and others have shown that

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Table 1
Experimental Protocol

Diet	Baseline: 0–2 months	Experimental Diet: 1–23 weeks
C/CO[1]	2 g C + 13 g lard/100 g feed	15 g CO/100 g feed
C/MO	2 g C + 13 g lard/100 g feed	15 g MO/100 g feed
S/CO	Stock diet	15 g CO/100 g feed
S/MO + CO	Stock diet	7.5 g CO + 7.5 g MO/100 g feed
S/MO	Stock diet	15 g MO/100 g feed

[1] C, cholesterol; CO, corn oil; MO, menhaden oil; S, stock diet.

fatty acids of different chain length and of different saturations have quantitatively different effects on membrane fluidity and on hormone receptor activity [7,11,15]. Insulin is intimately involved in lipid metabolism and promotes lipogenesis, and acts by binding to its receptors. Similarly, level and type of dietary fat affects plasma insulin level in humans [16–18] and animals [15,19,20] and also insulin receptors [12,19–22]. Since hormones act via their receptors, in the present study we examined the effect of n-3 fatty acids (predominantly 20:5 and 22:6) from menhaden oil and n-6 fatty acids (predominantly 18:2) from corn oil at two different dietary levels on the erythrocyte insulin receptor in normal and hypercholesterolemic miniature swine.

2. Materials and methods

2.1. Animals and treatment

Thirty female miniature Hormel-Hanford swine, 4–11 years old and weighing 60–110 kg, were assigned by age and weight into five experimental groups. The entire experimental protocol was approved by the FDA Institutional Animal Care and Use Committee. Pigs were housed individually (in heated pens in winter) with indoor and outdoor runs with free access to water. Twelve pigs (two groups of six pigs each) were first made hypercholesterolemic by feeding them 13% lard, 2% cholesterol diet for two months at 20 g feed/kg body weight, while the other animals (three groups of six pigs each) were kept on a basal stock diet (basal period). At the end of the 2 months period the pigs were switched to experimental diet containing 15 g/100g corn oil (CO), menhaden oil (MO), or mixtures thereof for 23 additional weeks (experimental period). Group assignments and diets are described in Table 1. No litter mates were assigned to the same group. During the experimental period, pigs were fed daily at 13 g feed/kg body weight, corresponding to 1.95 g fat/kg daily. Pigs were weighed each week, and the total amount of feed for each animal was adjusted each week to maintain the same intake of 13 g/kg. Diets were prepared by combining the ingredients of a basal stock diet (Table 2) with corn and/or menhaden oil to provide test diets containing either 15% CO, 7.5% CO + 7.5% MO, or 15% MO. The total diet provided 15.34 g protein, 49.94 g carbohydrate, and 17.01 g fat per 100 g feed. The menhaden oil, containing 14% eicosapentaenoate (EPA) and 8% docosahexaenoate (DHA), was provided through the National Institutes of Health Fish Oil Test Material Program. The menhaden oil as supplied was fortified with vitamin E and TBHQ as antioxidants to prevent deterioration. EPA and DHA contents of the oils were verified as each drum was opened. Corn oil was provided by Best Foods (Best Foods, Union, NJ), and appropriate quantities of vitamin E and TBHQ were added to the corn oil to correspond to the amount present in the menhaden oil. Feed stability was verified by monitoring EPA and DHA levels and testing for peroxides during the course of the study.

2.2. Blood sampling and erythrocyte membrane isolation

Morning blood samples were taken from food-deprived pigs immediately before starting the CO and MO diets and subsequently at 2, 12, and 23 weeks. Blood was drawn into vaccutainers with Potassium EDTA used as the anticoagulant. After removal from plasma and platelets by differential

Table 2
Diet Composition

Ingredients	Stock diet USDA 1160	Experimental diets
Corn meal	739 g/kg	226
Sucrose	—	226
Soybean meal	179	256
Alfalfa leaf meal	50	50
Dicalcium phosphate	21	21
Cellulose	—	60
$CaCO_3$	3.5	3.5
Swine mineral mix[1]	1.0	1.0
NaCl	5.0	5.0
Selenium	0.5	0.5
Swine vitamin mix[2]	1.0	1.0
Added fat[3]	—	150

[1] Mineral mix composition: $CaCO_3$, $Ca(IO_3)_2$, $CoCO_3$, CuO, $FeCO_3$, $FeSO_4$, MnO, and ZnO.

[2] Vitamin mix composition (g/kg): retinyl acetate, 1.32; all-rac-a-tocopheryl acetate, 7.3; cholecalciferol, 22; vitamin B-12 44; riboflavin 8.81; D-pantothenic acid, 17.62; niacin, 38.84; choline chloride, 220; and choline, 191.

[3] Cholesterol diet contained 130 g lard and 20 g cholesterol; test diets contained corn oil, menhaden oil or a mixture. The menhaden oil contained 14% eicosapentaenoic acid, 8% docosahexaenoic acid and 8% other (n-3) fatty acids.

Table 3
Plasma cholesterol concentration of minipigs fed corn oil (CO) or menhaden oil (MO) or a mixture of CO and MO at various time intervals[1]

Diet treatment	Weeks on experimental diet			
	0	2	12	23
		(mmol/L)		
C/CO	$8.96 \pm 2.41^{a,x}$	$1.99 \pm 0.33^{a,y}$	1.61 ± 0.29^{y}	$1.52 \pm 0.18^{a,y}$
C/MO	$9.02 \pm 1.98^{a,x}$	$1.80 \pm 0.42^{a,y}$	$1.13 \pm 0.14^{a,y,z}$	$0.91 \pm 0.11^{b,z}$
S/CO	$2.25 \pm 0.23^{b,x}$	$1.22 \pm 0.16^{b,y}$	1.51 ± 0.29	1.32 ± 0.17
S/CO + MO	$2.08 \pm 0.18^{b,x}$	$1.06 \pm 0.13^{b,y}$	1.82 ± 0.23^{b}	1.41 ± 0.21^{a}
S/MO	$2.24 \pm 0.19^{b,x}$	$1.04 \pm 0.09^{b,y}$	1.46 ± 0.11	1.02 ± 0.14^{y}

[1] Values are mean ± SEM, n = 6. a,b values in a column with different superscripts are significantly different, $p < 0.05$. x-z values in a row with different superscripts are significantly different, $p < 0.05$.

centrifugation, the erythrocytes were dispersed in isotonic phosphate buffer (310 mOsm, pH 7.4) and washed by repeated centrifugation (20 min 1,000 x *g*). Erythrocyte ghosts were prepared by hypotonic lysis in 20 mOsm phosphate buffer (pH 7.4) according to the procedure of Dodge et al. [23] Ghosts were washed repeatedly in the 20 mOsm phosphate buffer to remove hemoglobin and other cytoplasmic components. Aliquots were removed for fluidity measurements and the remainder of the ghost preparations was stored at -85°C for later assay of insulin binding. Plasma insulin levels were measured by radioimmunoassay [18]. Plasma cholesterol [24] and triacylglycerol [25] were measured enzymatically.

2.3. Insulin binding

Erythrocyte membrane insulin receptors were measured as described by Gambhir et al.[26] Ghosts (100 μg protein/tube) were incubated in 0.5 ml TRIS-HEPES buffer with 0.1 ng ^{125}I-insulin (specific activity 81.4 TBq/nmol) and 0–100 μg native porcine insulin/ml. ^{125}I-insulin was purchased from New England Nuclear (Boston, MA) and native porcine insulin was a gift from Eli Lilly & Co. (Indianapolis, IN). After the incubation at 4°C for twenty-four hours, or at 37°C for 2 hr, 0.2 ml aliquots were layered over 0.2 ml chilled TRIS-HEPES buffer and centrifuged (60 s 7500 x *g*). The ghost pellet was washed once with 100g/L sucrose and radioactivity was determined in a gamma counter (Model Minaxi γ 5000, Packard Instrument, Downers Grove, IL). Insulin binding measurements were made at 4°C and 37°C to correlate with anisotropy measurements at the same temperatures, and to assess the relation between insulin binding and membrane fluidity. Binding data obtained at 4°C were analyzed by Scatchard plots to measure the number of receptors and by competition-inhibition plots to assess the affinity of the receptors [22].

2.4. Statistical analysis

Data were subjected to ANOVA and linear regression analysis for the various measurements using SAS programs (Statistical Analysis System, 1988, SAS/STAT, Version 6.03, Cary, NC, USA). The model tested included variation in measurements due to changes in type of dietary fats and the duration of feeding. Duncan's multiple range test was used to determine differences in model classified composition data.

3. Results

Table 3 shows the effect of feeding corn oil and menhaden oil for 23 weeks on plasma cholesterol of minipigs fed either an atherogenic diet containing 13% lard + 2% cholesterol (C/CO and C/MO) or a basal stock diet (S/CO, S/CO+MO and S/MO) for 8 weeks. Plasma cholesterol concentrations were significantly higher in minipigs that were fed atherogenic diet for 8 weeks compared to those fed stock diet. It fell significantly within 2 weeks of switching minipigs from stock and atherogenic diets to either corn oil, menhaden oil or the mixture of the two, but was still significantly higher than those fed stock diet. The difference disappeared after 12 weeks and 23 weeks on experimental diet. Plasma cholesterol levels were lower in minipigs fed menhaden oil than those fed corn oil.

Plasma triacylglycerol concentrations were less affected by dietary lipids (Table 4). After 12 and 23 weeks on experimental diets, the concentrations were significantly lower in hypercholesterolemic minipigs fed menhaden oil compared to those fed corn oil. Though basal levels of plasma triacylglycerol (0 week on experimental diet) are different between minipigs fed hypercholesterolemic and stock diets, none of the values are statistically significantly different. The apparent differences may be due to variation between individual animals (intragroup) as apparent from large standard errors of mean.

Comparing the data at time zero, no significant differences in plasma insulin (Table 5) were observed between minipigs fed the atherogenic diet and those fed the basal stock diet. There was a rise in plasma insulin with time and at the end of 12 and 23 weeks the levels were higher compared to zero time in all groups except in pigs previously fed cholesterol and switched to MO (C/MO).

Insulin binding to erythrocyte membrane is shown in

Table 4
Plasma triacylglycerol concentration of minipigs fed corn oil (CO) or menhaden oil (MO) or a mixture of CO and MO at various time intervals[1]

Diet treatment	Weeks on Experimental Diet			
	0	2	12	23
		(mmol/L)		
C/CO	0.98 ± 0.12	0.64 ± 0.09	1.04 ± 0.10[a]	0.76 ± 0.08[a]
C/MO	0.70 ± 0.08	0.56 ± 0.08	0.45 ± 0.07[b]	0.47 ± 0.03[b]
S/CO	0.96 ± 0.15[x]	0.52 ± 0.11[y]	0.62 ± 0.05[b]	0.74 ± 0.09[a]
S/CO + MO	0.46 ± 0.06	0.61 ± 0.09	0.78 ± 0.11	0.58 ± 0.04
S/MO	0.54 ± 0.11	0.47 ± 0.06	0.53 ± 0.07[b]	0.61 ± 0.08

[1] Values are mean ± SEM, n = 6. a,b values in a column with different superscripts are significantly different, $p < 0.05$. x,y values in a row with different superscripts are significantly different, $p < 0.05$.

Table 6. No significant differences were observed at zero time between minipigs fed stock diet and the atherogenic diet. Though all animals had higher insulin binding after two weeks on experimental diets, the levels were not statistically significantly different from the base line. After 2 weeks on experimental diets, insulin binding tended to decrease and at the end of 23 weeks there were no significant differences in insulin binding to erythrocytes between the minipigs fed either corn oil or menhaden oil with or without cholesterol. No significant effect of length of feeding CO or MO was observed in insulin binding. Similarly, no significant changes were observed either in the number of receptors or in the affinity of the receptors (data not shown).

We have previously reported the data on the effects of dietary fats on red blood cell fluidity in these pigs using both non-polar probe, diphenylhexatriene (DPH) and its polar trimethylammonium (DPH-TMA) and propionic acid (DPH-PA) derivatives [11]. The regression analyses were performed to examine the relationships between red blood cell fluidity using different flourescent probes and the insulin binding. At 4°C, significant negative relationships were observed between insulin binding and anisotropy using all three probes, according to the following equations:

$$IB = 11.38 - 28.10 \times (DPH) \; (p < 0.008)$$

$$IB = 19.07 - 53.42 \times (DPH\text{-}TMA) \; (p < 0.0001)$$

$$IB = 28.80 - 81.93 \times (DPH\text{-}PA) \; (p < 0.0001)$$

At 37°C significant negative relationships were observed only when polar probes (DPH-PA and TMA-DPH) were used but not when the non-polar probe (DPH) was used. The following equations represent the relationships at 37°C:

$$IB = 5.52 - 12.12 \times (DPH) \; (NS)$$

$$IB = 18.25 - 59.45 \times (TMA\text{-}DPH) \; (p < 0.0001)$$

$$IB = 22.13 - 70.86 \times (DPH\text{-}PA) \; (p < 0.0001)$$

4. Discussion

In the present study feeding cholesterol with lard for 8 weeks significantly increased plasma cholesterol levels however the levels decreased significantly within 2 weeks of feeding either corn oil or menhaden oil without cholesterol. Thus, in minipigs unlike in humans, hypercholesterolemia does not persist once the cholesterol is removed from the diet. The results also indicate that n-6 and n-3 fatty acids are equally effective in lowering the plasma cholesterol. Hypolipidemic effect of n-3 fatty acids from cod liver oil has been reported in normal rats [27]. There was a decrease in plasma total cholesterol, HDL-cholesterol and liver triacylglycerol [27]. Similarly fish containing n-3 fatty acids and fatty acid esters of n-3 also decrease triacylglycerol in normal as well as non-insulin-dependent diabetic humans [28,29]. In addition to lipid metabolism, insulin

Table 5
Plasma insulin concentration of minipigs fed corn oil (CO) or menhaden oil (MO) or a mixture of CO and MO at various time intervals[1]

Diet treatment	Weeks on Experimental Diet			
	0	2	12	23
		(mmol/L)		
C/CO	70.2 ± 8.0	147.9 ± 42.6	141.9 ± 30.2	131.6 ± 15.6
C/MO	82.0 ± 24.7	85.9 ± 21.1	89.9 ± 21.0	99.6 ± 23.3[b]
S/CO	64.6 ± 10.1[x]	71.9 ± 11.6	118.2 ± 15.9[y]	107.8 ± 24.5[b]
S/CO + MO	64.6 ± 9.1[x]	105.6 ± 14.8	129.3 ± 36.5	183.7 ± 49.5[y]
S/MO	62.3 ± 6.9[x]	102.5 ± 11.4[x,y]	183.7 ± 39.8[y,z]	214.2 ± 34.9[a,z]

[1] Values are mean ± SEM, n = 6. a,b values in a column with different superscripts are significantly different, $p < 0.05$. x-z values in a row with different superscripts are significantly different, $p < 0.05$.

Table 6
Erythrocyte membrane insulin binding of minipigs fed corn oil (CO) or menhaden oil (MO) or a mixture of CO and MO at various time intervals[1]

Diet treatment	Weeks on Experimental Diet			
	0	2	12	23
	Percent specific binding/100 μg erythrocyte ghost protein			
C/CO	2.90 ± 0.23	3.52 ± 0.45	2.25 ± 0.13[b]	2.40 ± 0.32
C/MO	2.80 ± 0.21	3.90 ± 0.56[a]	3.83 ± 0.67[a]	3.51 ± 0.43
S/CO	3.43 ± 0.17	3.75 ± 0.16	3.59 ± 0.33	3.68 ± 0.30
S/MO + CO	3.09 ± 0.54	3.72 ± 0.72	3.00 ± 0.23	2.85 ± 0.29
S/MO	2.31 ± 0.54	2.65 ± 0.49[b]	3.21 ± 0.53	2.81 ± 0.55

[1] Values are mean ± SEM, n = 6. Values in a column with different superscripts are significantly different, $p < 0.05$.

also controls glucose homeostasis. Glucose metabolism in minipigs is similar to that observed in humans [30,31] and minipigs have been used as a model to study the aberration in carbohydrate and lipid metabolism [32,33].

The nutritional status of humans and animals alters plasma insulin levels and inversely alters insulin binding to target and non target tissues. Level of dietary fat affects plasma insulin level [34,35] in that high fat diet decreases plasma insulin and appears to be due to the effect on insulin secretion [34,36]. In humans fish oil feeding altered plasma insulin concentrations [28] but dietary trans fatty acids had no significant effect on plasma insulin concentration [37]. However in normal rats, n-3 fatty acids from cod liver oil significantly lowered plasma insulin without changing plasma glucose level indicating increased peripheral insulin sensitivity [27] and also improved insulin receptor tyrosine kinase activity [19]. Similarly in hypertensive rats [38], insulin-resistant rats [39] and spontaneously diabatic rats [40] ethyl esters of EPA prevented the development of insulin resistance. In generically diabetic mice [41] DHA has been shown to increase insulin sensitivity. In the present study we observed significant increase in the level of insulin in fish oil fed minipigs previously fed stock diet but not in those fed hypercholesterolemic diet where the level was lower in minipigs fed fish oil than those fed corn oil. However, the decrease was not significant. Behme [42] observed lower plasma insulin in minipigs fed MaxEPA after an overnight fast and during intravenous glucose tolerance test indicating enhanced insulin sensitivity.

Several studies have shown that type and amount of dietary fat have significant effect on insulin receptors on tissues and cells in culture [12,15,19–22,43–47]. Lowering the amount of dietary fat lowers insulin binding in humans and increases plasma insulin levels [43]. Feeding excess energy from fat increases insulin binding to monocytes but has no effect on plasma insulin [44]. In humans, fish oil had no significant effect on insulin binding to erythrocytes but significantly increased receptor affinity compared to placebo fat [48]. In rats, feeding a high fat diet for 5–10 days decreased insulin binding to soleus muscle [21] and adipocytes [20]. However, there was no consistent change in plasma insulin levels shown in either study. Dietary n-3 fatty acids have been reported to increase insulin binding to skeletal muscle sarcolema in rats [47] by increasing receptor number and that increased insulin binding may be responsible for improving insulin action.

In the present study we observed increase in plasma insulin with time on experimental diets. The increase was significant in minipigs previously fed stock diet but not in hypercholesterolemic minipigs. It is not clear why menhaden oil increased plasma insulin more than corn oil in minipigs fed stock diet. There was a concomitant decrease in insulin binding in minipigs fed stock diet when switched to menhaden oil compared to those fed corn oil, but the decrease was not significant. Similarly, receptor number and affinity were also not significantly altered.

We have previously shown that dietary cholesterol also affect insulin binding in rabbits [22] but not in minipigs [12]. In humans, membrane cholesterol:phospholipid ratio affects insulin receptors on erythrocyte membranes [49]. In the present study, we did not observe any significant changes in insulin binding in minipigs fed either the stock diet or those fed lard plus cholesterol. This is in line with our previous observation [12].

Dietary changes alter cell membrane lipid composition and thus modulate membrane fluidity and in turn affect some membrane physiological processes. One of the factors controlling insulin binding to membranes is the fluidity; but the matter of dietary control of membrane fluidity with resultant modulation of receptor activity is controversial. [15,19,22,25,50–52] We have studied insulin binding and erythrocyte membrane fluidity in several species fed different diets and observed different effects. In miniature swine feeding lard in addition to a stock diet lowered intact red blood cell insulin binding [12] but fluidity was not measured in that study. In rabbits feeding polyunsaturated fatty acids increased insulin binding but the results were inconclusive for the relationship between membrane fluidity and insulin binding [22]. In humans, high levels of PUFA increased erythrocyte ghost membrane fluidity as measured by the non-polar fluorescence probe DPH [50]. Thus, insulin binding to erythrocyte ghosts and fluidity show a direct linear correlation in humans [50]. In the present study in minipigs, we did not observe a significant relationship between membrane fluidity at 37°C, measured as anisotropy using the non-polar probe, DPH, and insulin binding. However, a

significant relationship was observed in the present study between fluidity measured by cationic and anionic polar probes and insulin binding.

It is important to note that the relationship between fluidity and insulin binding is dependent on the temperature at which they are measured. Insulin receptors exist in different conformation state at low temperature than at physiologic temperature [53]. Also at low temperature (below 15°C) lower membrane cholesterol concentration results in less insulin binding than at higher temperature and this effect appears to be due changes in membrane fluidity. This partly explains significant correlation between insulin binding and fluidity at 4°C but not at 37°C using non-polar probe, DPH.

Effects of both in vivo and in vitro alteration of membrane fatty acyl unsaturation and fluidity on receptor accessibility and activity have been reviewed by Shinitzky [54, 55]. Oleate and linoleate rich diets increase fluidity of human erythrocyte ghosts [50] and rabbit platelet membranes [56]. Thus, the erythrocyte ghosts of minipigs fed corn oil should be more fluid than those fed menhaden oil and hence should have higher insulin binding. However, in the present study we did not observe any significant differences in insulin binding to erythrocytes from minipigs fed either corn oil or menhaden oil. It is possible that total unsaturation rather than the type of unsaturation of fatty acids may be a more important modulator of insulin binding.

In the present study, though dietary fat had no significant effect on insulin receptors including number and affinity in minipigs, significant correlation was observed between percent insulin binding and fluidity measured using polar probes but not nonpolar probe. Thus, the use of polar probes is desirable to study the relationship between fluidity and hormone receptor function on the cell membrane.

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