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ACYLGLYCEROL SYNTHESIS IN LIVER OF TYPE II DIABETIC RATS FED A DIET SUPPLEMENTED WITH EITHER N-6 OR N-3 FATTY ACIDS

IRMA N. TACCONI DE GOMEZ DUMM, R. ARIEL IGAL

Instituto de Investigaciones Bioquímicas de La Plata (INIBIOLP), Facultad de Ciencias Médicas, Universidad Nacional de La Plata

Abstract Liver is one of the tissues most actively involved in triacylglycerol synthesis and secretion. Hypertriglyceridemia is commonly associated with the diabetic state which has been detected in very young rats after the induction of experimental diabetes. In the present work, acylglycerol synthesis in liver of streptozotocin-treated rats, fed a diet supplemented with n-3 and n-6 fatty acids, was studied. At the onset of the experiment, plasma triacylglycerol levels increased significantly in diabetic animals when compared to controls. Two weeks after the dietary treatment, the aforementioned parameter decreased in diabetic animals consuming either n-6 or n-3 fatty acids. In control rats, n-3 fatty acids depressed triacylglycerol synthesis in liver microsomes. In the diabetic group both diets increased diacylglycerol and triacylglycerol synthesis. The addition of liver cytosolic fraction from control rats to the incubation medium, stimulated the triacylglycerol synthesis in all the groups. Nevertheless, the radioactivity recovered in the neutral lipid fractions was lower in the samples from rats fed n-3 fatty acids compared to n-6. We conclude that dietary n-3 fatty acids decreased significantly triacylglycerol plasma levels in diabetic rats probably through the inhibition of liver triacylglycerol secretion. In addition, there probably is an n-3 fatty acid sensitive factor in the liver cytosolic fraction able to depress triglyceride synthesis.

Resumen ***Síntesis de acilglicerol en hígado de ratas diabéticas tipo II alimentadas con una dieta suplementada con ácidos grasos de las series n-6 y n-3.*** El hígado es uno de los tejidos más activamente involucrados en la síntesis y secreción de triacilglicerol. La hipertrigliceridemia se ha observado aun en ratas muy jóvenes después de la inducción experimental de la diabetes. En el presente trabajo se estudió la síntesis de acilglicéridos (a partir de ácido 1-C^{14}-palmítico), en ratas diabéticas alimentadas con una dieta suplementada con ácidos grasos de las series n-6 y n-3. Al inicio del experimento el contenido de triacilglicerol plasmático fue más elevado en los animales diabéticos que en los controles. Después de 2 semanas de tratamiento dietético esos valores descendieron en los animales diabéticos alimentados con ácidos grasos n-6 y n-3. En microsomas hepáticos la ingesta de ácidos grasos n-3 disminuyó la síntesis de triacilglicerol en las ratas normales. En las diabéticas se observó un aumento de la síntesis de diacilglicerol y de triacilglicerol con ambas dietas. El agregado de la fracción citosólica hepática de ratas controles al medio de incubación produjo un ascenso de la síntesis de triglicéridos en todos los grupos. No obstante, el incremento fue mucho menor en los animales alimentados con ácidos grasos n-3, respecto de los que ingieren n-6. Se concluye que la disminución de los niveles de triglicéridos plasmáticos en presencia de ácidos grasos de la serie n-3 se produciría a través de la inhibición de la secreción hepática de los mismos. En la fracción citosólica hepática existiría un componente sensible a los ácidos grasos n-3 capaz de deprimir la síntesis de triglicéridos.

Key words: acylglycerol, n-3 fatty acids, n-6 fatty acids, diabetes, triacylglycerol, glycerolipid synthesis

Liver is one of the tissues most actively involved in triacylglycerol (TAG) synthesis and secretion. Among the different routes generally recognized for TAG biosynthesis, the phosphatidic acid (PA) pathway is dominant in liver. This process takes place in the endoplasmic reticulum and involves a stepwise acylation of sn-glycerol-3-phosphate and/or dihydroxyacetone phosphate to yield PA. The PA is then hydrolyzed to sn-1,2-diacylglycerol (DAG), which is further acylated to TAG[1].

Hepatic TAG are known to accumulate in experimental diabetes[2]. The excessive store may result from oversynthesis of TAG or its decreased output from liver (as very low density lipoproteins) or a combination of these factors. Murthy and Shipp[3], using diabetic rats, demonstrated that hepatic TAG accumulation was associated to an augmentation in the rate of TAG production. An increase in the *in vivo* hepatic TAG biosynthesis was also shown by Woods et al. after the administration of streptozotocin to rats[4].

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Postal address: Dr. R. Ariel Igal, INIBIOLP, Facultad de Ciencias Médicas, Universidad Nacional de La Plata, calles 60 y 120, 1900 La Plata, Argentina
Fax: (54-221)4258988 e-mail: aigal@atlas. med.unlp.edu.ar

The consumption of polyenoic fatty acids results in a reduction of blood lipid levels. The low incidence of coronary heart disease in populations consuming such oils has been attributed to a lowering effect of blood lipids[5]. However, opposite evidence concerning the effectiveness of polyunsaturated fatty acis in decreasing serum TAG levels has been published. Some authors described that although n-6 fatty acids from vegetable origin are effective hypolipemics, long chain n-3 fatty acids of marine oils are quantitatively more potent effectors[6-8]. Another group found that dietary sunflower oil reduced fasting serum TAG levels whereas salmon oil had no such property[9]. The mechanism by which polyenoic fatty acids, especially n-3, lowers blood TAG concentration was attributed to a suppression of hepatic TAG production caused by the inhibition of DAG acyltransferase[10]. In addition, eicosapentaenoic acid inhibits the synthesis and secretion of TAG in cultured rat hepatocytes[11].

Taking into account these considerations, the present study was undertaken to evaluate the effect of equivalent amounts of either n-6 or n-3 fatty acid supplementation on hepatic acylglycerol synthesis in chemically induced diabetic rats.

Material and Methods

Chemicals

Chemicals were purchased from the indicated suppliers in the United States: [1^{14}C] Palmitic acid [57 Ci/mol], New England Nuclear Corp. (Boston, MA); unlabeled palmitic acid and lipid standards, Nu-Chek Prep (Elysian MN), and Coenzyme A, Sigma Chemicals Co (St. Louis, MO). Streptozotocin (STZ) was kindly donated by Upjohn Laboratories (Kalamazoo, MI). All other chemicals were of analytical grade.

Animals and experimental design

Male Wistar rats, weighing 200-250 g, were maintained on standard purina chow and water ad libitum before being placed on the experimental diet. Diabetes was induced by the intravenous injection of STZ (70 mg/kg), dissolved in citrate buffer (pH 4.5). Control animals received only an injection of equivalent volume of buffer alone. Only those rats with blood glucose levels higher than 300 mg/dL were considered diabetic.

Ten control and 10 diabetic animals were divided into two groups of 5 animals each. One group was fed a basal diet consisting of (in cal) 70% starch, 22% casein plus vitamins and minerals, and supplemented with 2% (by weight) of free fatty acids extracted from corn oil. The same diet was also administered to the other group except that the corn oil component was replaced by fatty acids extracted and concentrated from cod liver oil. Cod liver oil concentrated was enriched with both eicosatrienoic and docosahexaenoic acids to reach the n-3 fatty acid levels which should be equivalent to that of the n-6 fatty acids in corn oil (52% n-6 vs 46% n-3)[12]. Control and diabetic rats were fed on each of these diets for a total of 2 weeks. All animals were kept in groups of two or three in stainless-steel cages with free access to food and water.

Isolation of liver microsomes and cytosolic fraction

At the end of the dietary treatment the animals were killed by decapitation. Blood was drained off and collected for plasma glucose and TAG determinations. Livers were quickly excised, weighed, and homogenized in an ice-cold buffer (1:3 wt/vol) containing 0.25 M sucrose, 62 mM phosphate buffer (pH 7.0), 0.15 M KCl, 5 mM $MgCl_2$ and 100 µM EDTA. The homogenate was centrifuged at 10 000 g for 20 min at 4 °C. The pellet was discarded and the supernatant was centrifuged again (100 000 g for 60 min) at 4 °C. The supernatans for the second centrifugation were considered as cytosolic fractions. The pellets (microsomes) were resuspended in cold homogenizing solution. Protein content in both microsomal and cytosolic fractions was determined by the method of Lowry et al.[13]. Plasma glucose and TAG levels were quantitated using commercial enzymatic kits (Wiener Lab. Test, Rosario, Argentina).

Determination of glycerolipid synthesis

Glycerolipid synthesis was measured by estimating the incorporation of labeled palmitic acid into phosphatidate, diacylglyceride and triglyceride molecules, according to Lloyd-Davies et al.[14]. Five nmol of labeled acid plus 40 nmol of unlabeled acid were incubated with 250 µg of liver microsomal protein in a metabolic shaker at 37 °C for 60 min. The incubation medium contained 50 mM phosphate buffer, pH 7.4; 2 mM $MgCl_2$; 5 mM N-acetylcysteine; 0.2 mM Coenzyme A and 20 mM dl-α-glycerophosphate. In some experiments the hepatic cytosolic fractions obtained from the corresponding control group were added to the incubation medium (0.8 mg protein/tube). In all cases the final volume of the incubation medium was 350 µl. The reaction was stopped by the addition of 3 ml of chloroform-methanol mixture (2:1 by vol.). Lipids were extracted according to Folch method[15] and separated by high performance thin layer chromatography (TLC) on silica G plates developed in hexane: diethyl ether: acetic acid (80:20:1 by vol.). The bands of glycerolipids were identified using iodine vapor. The spots were cut off from the plates and quantitated by liquid scintillation counting.

Statistical analyses

Results were tested statistically by a one-way analysis of variance (ANOVA).

Results

After one week of STZ injection, the rats became severely diabetic. The two-week dietary treatment did not alter the hyperglycemic state (data not shown). The values for plasma TAG levels determined both at the beginning of the dietary treatment and at the time of sacrifice (two weeks later) are shown in Fig. 1. At the onset of the experiment, diabetic rats showed a significant increase in the TAG content. The values obtained at the start of the experiment in control rats were not modified by the dietary treatment. In diabetic animals consuming either n-6 or n-3 polyunsaturated fatty acids a significant decrease in circulating TAG levels was found. In both cases the values obtained were even significantly below the non diabetic group.

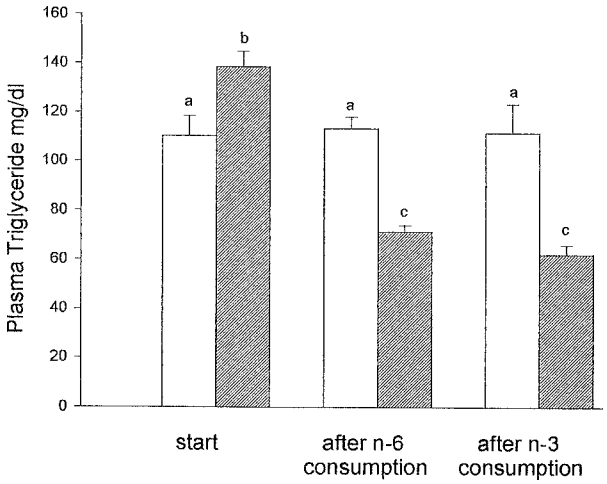

Fig. 1.– Plasma TAG levels in control (open bars) and diabetic (stripped bars) rats at the beginning of the experiment (start) and after 2 weeks on either n-6 or n-3 fatty acid supplemented diets. Data are the mean ± SEM from 5 animals. Values not bearing the same superscript letter are significantly different at P < 0.05 or less.

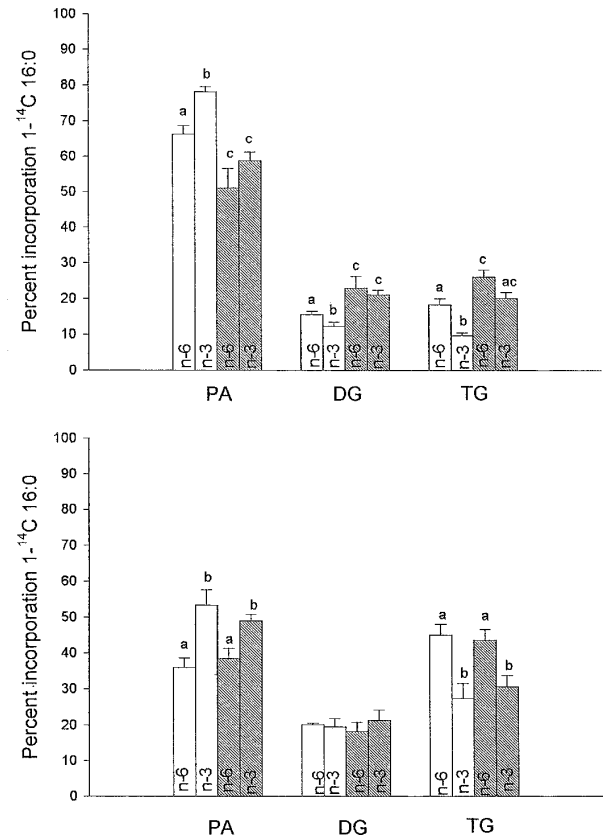

Fig. 2.– Incorporation of [1-^{14}C] palmitic acid (16:0) into various acylglycerols of control (open bars) and diabetic (stripped bars) rats, in liver microsomal membranes (upper panel) and liver cytosolic fraction (lower panel). Fatty acid families supplemented to the diets are indicated inside the bars. Data are the mean ± SEM from 5 animals. Values not bearing the same superscript letter are significantly different at p < 0.05 or less. PA: phosphatidic acid, DG: diacylglycerol, TG: triacylglycerol.

Figure 2, upper panel, illustrates the incorporation of palmitic acid into glycerolipids in liver microsomes. The fatty acid incorporated primarily in the PA fraction (50-75% of total radioactivity) in both control and diabetic animals. Comparing the effect of n-6 with n-3 diets, in control rats, it was observed that the acids belonging to marine oil enhanced the incorporation of palmitic acid into PA by 20%. The same diet decreased significantly the formation of DAG ant TAG by 20% and 50%, respectively. STZ-induced diabetes altered the synthesis of the three main lipid fractions studied. Thus, the formation of PA was reduced by around 25%, whereas the labeling of both DAG and TAG increased significantly. However, the changes of the latter fractions induced by diabetes were more profound when the rats were fed fish oil derivatives.

The effect of the addition of liver cytosolic fraction obtained from control rats on the incorporation of palmitic acid into PA, DAG and TAG in microsomes is shown in Fig. 2, lower panel. The incorporation of palmitic acid into PA was lower than that observed in the microsomes with no addition of cytosol. Nevertheless, the radioactivity recovered in PA was higher in those samples belonging to animals fed n-3 fatty acids than in those treated with n-6. No differences between control and diabetic samples were detected. An opposite behavior was evidenced when the incorporation of palmitic acid into TAG was measured. In this case, n-3 fatty acids produced a significant reduction in the radioactivity recovered either in control or diabetic samples, compared to n-6 fatty acids. No changes were observed in DAG synthesis among all the groups studied.

Discussion

A dietary mixture supplementation of a moderate amount of free fatty acids belonging to either n-6 or n-3 family, produced no changes in TAG plasma levels in normal rats (Fig. 1). These findings contrasted with those published in animal experimental trials, where a great proportion of fish oils in the diet decreased that parameter[16, 17]. However, TAG levels were not significantly modified by reducing the dietary amount of marine fatty acis[18], while in rats fed moderate amounts of fats, dietary sunflower oil efficiently reduced fasting serum TAG levels[9]. On the other hand, concerning marine oils it has been assumed that eicosapentaenoic (EPA) or docosapentaenoic acid (DHA) or both, are responsible for the TAG lowering effect. In most studies fish oil was used with various contents of EPA and DHA, and only in a few studies the effect of these acids, was examined separately. Recently, it was reported[19, 20] that EPA and not DHA, is the fatty acid primarily responsible for the TAG-lowering effect of fish oil in rats. This study is

consistent with observations in normolipidemic rats[21-23] but it is in contrast to other publications[24]. We assume that the differences on plasma TAG concentration informed by several authors, are the consequence of either the amount or grade of purification of the fatty acids given in the diet, as well as, the length of the feeding period.

Few studies showing the most potent reducing effect of diets rich in fish oil compared to diets rich in vegetable oil on plasma TAG levels, report the responses on liver TAG levels. Yeo and Holub[25] found a lowering effect of dietary fish oil on liver TAG synthesis compared to sunflower oil. Rustan et al.[26] noted that EPA acid reduces both the synthesis and secretion of TAG in rat hepatocytes. More recently, Flémont and Gozzelino reported that diets with salmon oil were more effective in reducing liver TAG concentration compared to those with sunflower oil[27]. This observation agrees with the data obtained here since TAG and DAG synthesis in liver was significantly decreased in non diabetic rats fed marine oil compared to those fed on vegetable oil (Fig. 2).

Hypertriglyceridemia is commonly associated with the diabetic state; this fact was even observed in rats a few days after the induction of chemical diabetes[4]. In the present work high levels of plasma TAG were clearly shown a week after the administration of STZ to rats (Fig. 1). This parameter was reduced to more than 50% when the animals were studied for 2 weeks after being on both diets, according to previous observations of other authors[8, 16, 17]. Moreover, the values obtained were significantly lower than those of control rats on the same diet. As published by Phillipson et al.[6], abnormally higher plasma TAG levels seem to react better to dietary lipid supplementation than those in normal concentration.

The higher rate of hepatic TAG biosynthesis is one of the factors that has been suggested to contribute to the diabetic-related change in serum TAG levels[4]. The results of our study on TAG synthesis are chiefly independent of the regimen, and they demonstrated an increased recovery of radioactivity by the neutral lipid (DAG, TAG) fractions in diabetes, while the incorporation of palmitic acid in PA decreased significantly. It was previously shown that the over-synthesis of TAG in diabetes was associated with higher activities of phosphatidate phosphohydrolase and DAG acyltransferase[3]. These observations could explain the flow of PA into the TAG biosynthetic pathway and hence the lower values of this substrate.

The ingestion of fish oils containing n-3 polyunsaturated fatty acid by normal rats produced a reduction in liver TAG synthesis[10, 11, 25, 26]. The incorporation of palmitic acid into TAG and DAG was lowered by 20% and 47%, respectively, in the fish oil group (Fig. 2, upper panel) suggesting an inhibitory effect on the enzymes involved in this metabolic pathway. According to these results, an inhibitory effect of EPA acid on the liver DAG acyltransferase has been reported[10, 26]. *In vivo* studies also demonstrated that DAG acyltransferase activity was significantly lower in fish-oil fed animals than in animals fed vegetable oils[28]. Moreover, Marsh et al.[29] suggested that feeding fish oil to rats suppresses the activity of phosphatidate phosphohydrolase.

In spite of the results obtained in control rats, n-3 dietary intake was not capable to depress high levels of acylglycerol biosynthesis observed in the diabetic state. However, TAG plasma levels decreased significantly under both polyunsaturated fatty acid dietary treatments. These results can be explained through the inhibition of liver TAG secretion produced by dietary polyunsaturated fatty acids in diabetic rats[30, 31].

The addition of the cytosolic fraction to the incubation medium increased the microsomal TAG synthesis in all groups of rats. This observation can be attributed to the stimulation of *in vitro* TAG synthesis in microsomes produced by a number of cytosolic proteins, in particular acyl CoA and fatty acid binding proteins[32]. Under these conditions, the enhancement of TAG synthesis was more limited in rats fed n-3 fatty acids than in those treated with n-6 series, indicating the presence of a stimulating factor sensitive to inhibition by n-3 fatty acids, probably acting at the last step on TAG biosynthesis.

In conclusion, dietary n-3 fatty acids decreased significantly TAG plasma levels in diabetic rats probably through the inhibition of liver TAG secretion, since the incorporation of labeled plamitic acid into TAG was clearly stimulated in the liver microsomal fraction. There is an n-3 fatty acid sensitive component in the liver cytosolic fraction able to depress TAG synthesis. Further studies are necessary in order to identify the aforementioned factor.

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