COMPARISON OF THE EXTRAPANCREATIC ACTION OF γ-LINOLENIC ACID AND n-3 PUFAs IN THE HIGH FAT DIET-INDUCED INSULIN RESISTANCE

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Objective. The effect of dietary borage oil (rich in the γ-linolenic acid [GLA]) on insulin sensitivity and lipid metabolism was compared with that of fish oil (rich in n-3 polyunsaturated fatty acids [PUFAs]) in high fat (HF) diet-induced insulin resistance (IR) of rats.

Methods. Male Wistar rats were fed *ad libitum* for 3 weeks a standard laboratory chow (Controls) or high fat diet consisting of 70-cal % fat. In addition, a group of rats was fed high fat (HF) diet where a part of saturated fat was replaced with fish oil as a source of n-3 PUFAs (HF+FO), or borage oil as a source of GLA (HF+GLA). *In vivo* insulin action was assessed by the euglycemic hyperinsulinemic clamp. Glucose, insulin, free fatty acids (FFA), triglycerides (Tg) and glycerol levels in blood and tissue depots were also measured.

Results. Increased levels of Tg, FFA and glycerol in circulation after HF diet were accompanied by their raised accumulation in insulin sensitive tissues. FO feeding lowered the concentration of all lipids in serum and prevented their accumulation in both tissues. On the other hand GLA supplementation into the high fat diet did not suppress increased levels of Tg, FFA and glycerol in circulation and tissue depots as well. FO feeding significantly reduced HF diet-induced in vivo IR, while GLA supplementation did not improve the *in vivo* insulin sensitivity in HF diet induced insulin resistance.

Conclusions: 1. Substitution of FO into the high fat diet led to an improvement of *in vivo* insulin action; 2. this insulin sensitizing effect of FO was accompanied by a decrease of circulating Tg, FFA and glycerol levels in the postprandial state and by a lower lipid content in liver and skeletal muscle. 3. on the opposite, GLA treatment failed to improve *in vivo* insulin action; and 4. was associated with an adverse effect on lipid levels both in circulation and tissue depots.

Key words: γ-linolenic acid – HF diet – lipids – insulin action

Insulin resistance is not only a substantial etiological factor in development of the metabolic syndrome X and a risk factor for cardiovascular complications (De Fronzo 1997; Haffner 1997), but also a target for new therapies. There is increasing evidence that raised lipid accumulation in muscle and liver, the major target tissues for insulin action, is associated with insulin resistance (Storlien et al 1987, 1991, 1997; Sebokova et al 1997; Kelley and Goodpaster 2001). On the other hand, reduction of obesity or lowering lipids generally improves insulin sensitivity (Storlien 1991; Klimes et al 1991, 1993, Sebokova et al 1996; Sebokova and Klimes 1997; Oakes et al 1997b).

An n-6 essential fatty acid and a prostaglandin precursor, i.e. γ-linolenic acid (GLA) has been recently shown to improve insulin-mediated glucose metabolism in animal models of insulin resistance and to

improve neurovascular function in diabetic patients and animals (Keen 1993; Certik 1993; Cameron and Cotter 1996).

Beneficial effects of dietary marine fish oil rich in long chain n-3 PUFAs have been demonstrated in prevention of insulin resistance and/or of abnormal lipid profile in rodents and humans as well (Klimes et al., 1993; Sebokova et al. 1993; Storlien et al., 1997; Roche and Gibney 2000). There is substantial body of evidence demonstrating that the favorable effect of marine fish oil (FO) may be at least partly explained by suppression of hepatic FA synthesis and secretion, an increase of FA oxidation, and by induction of enzymes involved in FA elongation and desaturation. These changes are associated with alterations in the genes involved in fatty acid metabolism already at the transcription level (Sebokova et al. 1996).

Consumption of high fat diet rich in saturated fat results into widespread insulin resistance with major effects on skeletal muscle (Storlien et al. 1987; Oakes et al. 1997a). This type of insulin resistance is usually accompanied by raised triglyceride availability, i.e. either by increased plasma triglyceride and/or by their accumulation in skeletal muscles (Storlien et al. 1993a; Klimes et al. 1998).

Therefore, in the present study the long-term effect of dietary GLA supplementation on *in vivo* insulin action was compared with that of the n-3 PUFAs in the high fat diet fed rats, in the well-established model of insulin resistance, glucose intolerance, hyperinsulinemia, and dyslipidemia. As triglycerides, free fatty acids and glycerol are known to negatively modulate insulin action in skeletal muscle the plasma and tissue levels of these variables were also assessed.

Materials and Methods

Animals and diets. The Institute of Experimental Endocrinology Animal House Ethics Committee approved all the experiments reported here.

Male Wistar Charles River rats (AnLab, Prague, Czech Republic) aged 15 weeks were housed in wire mesh cages in a temperature (22±2 °C) and light controlled room (12 h light: dark cycle; lights off at 18.00 h) throughout the study. They were divided into 4 groups fed *ad libitum* one of the following diets: 1. Control diet (C) (ST1 standard laboratory rat chow, Velaz, Prague, Czech Republic); 2. High fat diet (HF) (consisting of 70-cal% fat) (Storlien et al. 1987); 3. n-3 PUFA supplemented high fat diet (HF+FO) in which 10 wt % of saturated fat was replaced with n-3 PUFAs (EPAX 5500 TG, Pronova Biocare, Sandefjord, Norway) and 4. n-6 PUFAs supplemented high fat diet (HF+GLA) in which 18,5 wt % of saturated fat was replaced with GLA (Borage oil, Flaveko s.r.o, Pardubice, Czech Republic). The fatty acid composition (expressed as % in oil) of borage oil was as follows: 16:0 = 11.3 %, 16:1 = 0.1 %, 18:0 = 2.9 %, 18:1= 20.1 %, 18:2 = 41.1%, 18:3 γ= 23.9 %, 22:1 = 0.6 wt %.

The above feeding and housing conditions were maintained for 21 days before surgery for cannula implantations. Body weight (weekly) and food consumption (daily) were measured regularly. This approach created the following groups of animals: C, HF, HF+FO and HF+GLA.

Euglycemic clamp studies. After 3 weeks of feeding the above diets, rats were anesthetized by injection of xylazine hydrochloride (10 mg per kg B.W.) plus ketamine hydrochloride (75 mg per kg B.W.), and fitted with chronic artery and jugular cannula as described by Koopmans et al. (1992). After surgery feeding continued and studies were conducted 72 hours after catheter implantation in the unrestrained sedentary conscious state. Food was removed 16 hours before the study. Euglycemic hyperinsulinemic clamps were performed according to Kraegen et al. (1985) as described in details earlier (Klimes et al., 1998). Briefly, a continuous infusion of human insulin (Actrapid, Novo Nordisk, Denmark) was given at a dose of 6.4 $mU.kg^{-1}.min^{-1}$ to achieve plasma insulin concentrations in the mid upper physiological range. This infusion was maintained for 90 minutes. The arterial blood glucose concentration was clamped at the basal fasting level using a variable rate glucose infusion. Blood samples were obtained for glucose and insulin determination in all clamp studies at 15 minutes intervals.

Analytical methods. Serum glucose concentrations were measured with aid of the Beckman Glucose Analyzer (Fullerton, CA, USA). Tg levels in serum or in tissue lipid extracts were measured using a specific, commercially available enzymatic set (TG-DST-P, Australia). Lipids from liver and skele-

TABLE 1. **Animal characteristics and serum parameters**

	C	HF	HF + FO	HF + GLA
Body weight gain (g)	78±5[a]	89±9[a]	64±8[a]	81±5[a]
Food consumpt (g.kg.$^{-1}$day^{-1})	82±2[a]	59±3[b]	49±5[b]	52±3[b]
Serum glucose (mmol.l^{-1})	5.1±0.4[a]	5.5±0.3[a]	5.2±0.2[a]	6.1±0.2[a]
Serum insulin (μU.ml^{-1})	8.7±1.6[a]	23.4±5.0[b]	11.2±2.2[a]	18.6±4[b]
Serum Tg (mmol.l^{-1})	2.3±0.3[a]	4.2±0.1[b]	1.2±0.3[c]	3.2±0.4[ab]
Serum FFA (mmol.l^{-1})	0.5±0.01[a]	1.2±0.1[b]	0.7±0.2[a]	1.0±0.1[b]
Serum glycerol (μmol.l^{-1})	101±4[a]	268±23[b]	138±15[a]	343±26[b]

Data represent the mean ± SEM of 6-8 rats. Values without a common superscript are significantly different ($p<0.05$). C = Controls = Wistar rats fed a standard lab chow; HF = high fat diet, HF+FO = n-3 PUFAs supplemented high fat diet, HF+GLA = borage oil supplemented high fat diet.

TABLE 2. **Efect of fish oil and borage oil supplementation on tissue triglycerides, free fatty acids and glycerol in HF diet induced insulin resistance and hypertriglyceridemia**

	C	HF	HF + FO	HF + GLA
Liver Tg (μmol.g^{-1})	5±0.5[a]	20±4.2[b]	11.4±2.4[c]	19.5.±2[b]
Liver FFA (μmol.mg^{-1})	720±80[a]	1030±60[b]	825±94[a]	1490±120[c]
Liver glycerol (μmol.mg^{-1})	70±10[a]	300±30[b]	156±22[a]	770±80[c]
Muscle Tg (μmol.g^{-1})	1.6±0.2[a]	2.8±0.3[b]	1.9±0.3[a]	2.7±0.3[ab]
Muscle FFA (μmol.mg^{-1})	530±40[ab]	380±30[a]	420±60[ab]	650±40[b]
Muscle glycerol (μmol.mg^{-1})	60±10[a]	170±10[b]	88±15[a]	150±10[b]

Data represent the mean ± SEM of 6-8 rats. Values without a common superscript are significantly different ($p<0.05$). C = Controls = Wistar rats fed a standard lab chow; HF = high fat diet, HF+FO = n-3 PUFAs supplemented high fat diet, HF+GLA = borage oil supplemented high fat diet.

tal muscle were extracted by modified method of Blight and Dyer (1959) with chloroform – methanol – water (1:1:0.9) mixture. For measurement of FFA and glycerol in serum the enzymatic kit from Randox Laboratories Ltd. (Ardmore, UK) was used. Serum insulin levels were measured using the rat insulin RIA kit from Linco (USA).

Statistical evaluation. Results are expressed as mean ± SEM. Differences between groups were analyzed using analysis of variance (ANOVA) with the appropriate post hoc test (Afifi 1972) at the overall significance threshold of α=0.05.

Results

Animal characteristics and serum parameters. The initial body weights among the individual groups of rats did not differ. The body weight increased in a similar way during the 3 weeks feeding period irrespective of the dietary regime used (Table 1). The food consumption was lower in all HF diet fed groups. This was expected due to the higher caloric content of this diet in comparison to the rats fed control diet (Table 1). Feeding rats the HF diet led to an increase in fasting insulinemia with no change in fasting plasma glucose levels, which indicates a decrease of insulin action (Table 1). Supplementation of FO into the high fat diet led to normalization of insulinemia while GLA treatment failed to normalize fasting serum insulin levels (Table 1).

The HF feeding led also to a statistically significant increase in fed Tg, FFA and glycerol levels in circulation. Treatment of rats with FO lowered significantly serum Tg, FFA and glycerol concentrations. On the other hand the supplementation of GLA into HF diet did not show any significant positive effect on lipid parameters in circulation (Table1).

Tissue parameters. Feeding the rats with HF diet for 3 weeks led to a marked increase in tissue Tg, FFA and glycerol both in liver and skeletal muscle. Application of FO to rats fed the HF diet prevented the increased accumulation of Tg in both tissues. On

Figure 1

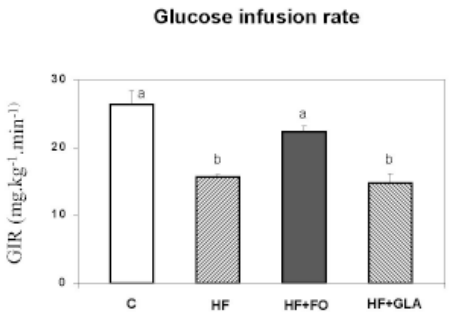

Fig. 1. Effects of fish oil (FO) and GLA on *in vivo* insulin sensitivity. Bars represent mean ± SEM of 6-8 rats. Values without a common superscript (a,b) are significantly different at p<0.05. C = control lab chow diet; HF = high fat diet, HF+FO = high fat diet supplemented with fish oil, HF+GLA = high fat diet supplemented with borage oil.

the contrary, GLA treatment was not able to suppress the accumulation of Tg either in liver or skeletal muscle. In addition, consumption of GLA enriched diet was accompanied by an increase of FFA and glycerol content both in liver and skeletal muscle up to the levels even higher than in the HF diet per se fed rats (Table 2).

Euglycemic hyperinsulinemic clamp data. The HF diet fed control rats had a low exogenous glucose infusion rate (GIR) (15.7±0.5 mg/kg/min) required to maintain euglycemia during hyperinsulinemia (Figure1). In fact, their *in vivo* insulin-induced glucose utilization was reduced by close to 60 % when compared to GIR of the control rats with normal insulin action at a comparable steady-state plasma insulin level. A three weeks treatment of rats with the FO enriched HF diet was accompanied by a clear improvement of insulin action as reflected by a higher GIR (22.8±0.4 mg/kg/min) required to maintain euglycemia. In contrast, GLA was not able to advance *in vivo* insulin action under the dose and experimental setting used (Figure 1).

Discussion

In the present study, we have shown that in comparison to the beneficial effect of marine fish oil (rich in n-3 polyunsaturated fatty acids) on *in vivo* insulin action, the n-6 essential fatty acid supplement rich in GLA is not able to improve insulin sensitivity in the high fat diet induced insulin resistance. Moreover, our data have shown that consumption of GLA supplemented HF diet is not able to prevent the raised levels of Tg, FFA and glycerol in circulation and/or tissue depots as induced by the HF feeding. It is to be said also that in some cases the GLA treatment led to increased accumulation of lipids in tissue depots suggesting a negative effect of this supplementation on lipid metabolism. Although body weight alterations are generally important for changes in the *in vivo* insulin action, we did not observe any significant difference in body weight increments in the HF diet fed rats whether supplemented or not with the FO or GLA compounds.

In contrast to our findings, data from literature suggest that GLA has the ability to increase insulin-mediated skeletal muscle glucose transport in obese Zucker rat when applied alone or as a conjugate with antioxidant alfa lipoic acid (Peth et al. 2000). These authors also suggest that the positive effect of the GLA-LPA conjugate on whole body and skeletal muscle glucose disposal is dose dependent with higher efficiency at the lower doses e.c. 10 mg/kg B.W. However, it is noteworthy that at the dose of 10 mg/kg, neither LPA nor GLA individually, induced a reduction in plasma insulin or free fatty acid levels in the obese Zucker rats. Only the LPA-GLA conjugate elicited a significant lowering of both variables. This suggests an interaction between LPA and GLA on these particular variables. These findings also imply that whereas GLA can elicit metabolic improvements at lower doses, these effects are lost at higher doses. This is perhaps due to development of an unfavorable lipid composition – whether in circulation or in the tissues - having deleterious effect on insulin action, as in situations with a high availability of free fatty acids.

Feeding rats for several weeks a diet containing 70 cal % of fat (about 40 wt % of saturated fatty acids) results into widespread insulin resistance with major effects in skeletal muscle (Storlien et al. 1987; Oakes et al. 1997a) as assessed by measurement of the glucose metabolic index Rg' in insulin target tissues after a single bolus of ^{3}H-deoxyglucose during the insulin clamp (Kraegen 2001). This type of insulin resistance is usually accompanied by raised triglyceride availability, i.e. either by increased plasma triglycer-

ide and/or by their accumulation in skeletal muscles (Storlien et al. 1993a, b; Klimes et al. 1998). Moreover, the increased concentration of FFA in circulation is also present. In contrast, insulin action improves by lowering plasma and/or intramuscular Tg and FFA in circulation by pharmacological treatment (Storlien et al. 1993b; Kraegen et al. 2001; Folley et al. 1997; Sebokova et al. 2002) or by dietary suplemetation with n-9 or n-3 PUFAs (Klimes et al. 1991, 1993; Sebokova et al. 1996; Sebokova and Klimes 1997).

Interestingly, FO supplementation into the HF diet resulted in a significant reduction in serum Tg, free fatty acids and glycerol, an effect not seen following treatment with GLA alone. More striking was the observation that free fatty acids and glycerol were even increased not only in circulation but also in tissue depots when the GLA was used as a supplement into the high fat diet fed rats. Free fatty acids can negatively influence the whole body insulin action. Thus, it is possible that the inability to reduce this variable was linked to a GLA failure to induce an improvement in insulin sensitivity. This was certainly not the cause in the improvement in insulin action after fish oil supplementation.

Our findings, i.e. that beneficial effects of fish oil is associated with a significant decrease of Tg, FFA and glycerol in circulation, and with a decrease in the accumulation of those lipids in skeletal muscle and liver are in harmony with our previous data and published information as obtained in various models of IR (Kraegen et al. 2001) and humans with Type 2 diabetes (Kelley and Goodpaster 2001). These data also provide another evidence for the important role of these lipids for the development of IR.

On the contrary, the effect of GLA on lipid metabolism seems to be negative, as we have found higher concentration of FFA in liver and skeletal muscle after the consumption of HF diet enriched with borage oil. Nevertheless, mechanism by which GLA might affect insulin sensitivity and lipid metabolism in a negative manner remains unclear. While marine FO rich in n-3 PUFAs performing as a potent PPARs activating agent is able to influence the key enzymes of fatty acid synthesis and metabolism already at the gene level, the absence of the insulin sensitizing effect of GLA compound may come from its inability to suppress the accumulation of Tg and FFA in muscle and liver.

Moreover, it was observed that a decline in essential fatty acids in individuals with diabetes, hypertension, and coronary heart disease might be an important contributing factor in the pathobiology of insulin resistance (De Fronzo and Ferrannini 1991; Reaven 1993). Treatment with the n-6 essential fatty acid GLA has been also demonstrated to improve the impaired nerve function in diabetic humans (Keen et al. 1993) and in animal models of diabetes (Cameron and Cotter 1996, Cameron et al. 1998). Also, administration of the essential fatty acid conjugated linoleic acid to Zucker diabetic fatty rats has been shown recently to improve peripheral insulin sensitivity, possibly via activation of the peroxisome proliferator-activated receptor- (PPAR-) (Houseknecht et al. 1998). Takahashi et al. (1993) demonstrated that treatment with evening primrose oil, rich in GLA, reduced fasting plasma glucose and increased levels of prostaglandin E_1 in type 2 diabetic subjects.

However, in the present study, the GLA supplement as prepared from the borage oil was not able to improve the whole body insulin action in the HF diet model of dietary induced insulin resistance in rat. This was also accompanied with negative effects on lipid metabolism.

Mechanism(s) for these deleterious effects are currently not known. However, a caution should be taken in account when drawing conclusions about the biological and clinical effects of GLA based on studies of one natural source of GLA. The effect of one source may well be specific to that oil. If one variety of GLA-oil has a particular effect, this does not mean that the other GLA-oil will have the same or similar qualitative or quantitative actions. Moreover, the biological effect of essential fatty acid also depends on its position in the glycerol backbone as these positions are certainly handled differently during the digestion and absorption process in the gut (Horrobin 1995). To date, almost no attention has been paid to this phenomenon. There are several sources of GLA in both the plant and microbial kingdom (Certik and Shimizu 1999). Therefore, it is clear that further studies with various forms of GLA in different concentrations are needed to shed more light on the mechanism of action of this potentially interesting compound.

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