EFFECT OF DIFFERENT CARBON SOURCES ON THE BIOSYNTHESIS OF POLYUNSATURATED FATTY ACIDS OF α-LINOLENIC ACID FAMILY IN CULTURE OF MINIMAL DEVIATION HEPATOMA 7288 C CELLS

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Summary

The effect of three different carbon sources on the biosynthesis of polyunsaturated fatty acids of the α-linolenic acid series was investigated in hepatoma tissue culture (HTC) cells. Alpha linolenic acid was converted to higher homologs by a desaturating route that synthetized mainly 18:4 (Δ6, 9, 12, 15), 20:4 (Δ8, 11, 14, 17) and 20:5 (Δ5, 8, 11, 14, 17) and an elongating route that produced 20:3 (Δ11, 14, 17) and 20:4 (Δ5, 11, 14, 17) acids. 'Fasting' decreased both biosynthetic routes whereas glucose reactivated only the elongating pathway. Lactalbumin hydrolysate enhanced significantly only the desaturating route whereas glycerol was inactive. Glucose and aminoacids increased similarly the incorporation of labeled α linolenic acid ir the cells. The results are independent of hormonal effects.

Introduction

The regulatory effect of nutritional factors on several enzymes involved in fatty acid oxidative desaturation is well established[1–3]. Evidence obtained with microsomal preparations from fasted and diabetic rats indicates a reduction in their fatty acid desaturation activity[1,4–6] compared to normal animals. The modifications of this activity produced by starvation and diabetes are reversed by glucose refeeding or by insulin administration respectively[1,4–6]. Nevertheless, we also demonstrated that long term administration of glucose to animals produced a significant decrease in the Δ6 desaturation of linoleic to γ-linolenic acid[2]. These results are consistent with the decrease of the Δ6 desaturation of α-linolenic acid found by Inkpen *et al.*[7] under similar experimental conditions. In addition, feeding of a high protein diet to rats evokes a very significant activation in liver of the Δ6-desaturation of linoleic to γ-linolenic acid or α-linolenic to octadeca-6, 9, 12, 15-tetraenoic acid[7,8]. However, it evokes a rather small increase of the Δ5 desaturation of eicosa-8,11-dienoic acid to eicosa-5,8,11-trienoic acid[9].

Since glucose and other metabolites in liver are intimately related to hormonal secretions it is difficult to elucidate clearly from the aforementioned studies, whether the effect of these metabolites are evoked per se or mediated through hormonal secretions. Therefore, we decided to choose a new approach to investigate the direct effect of fasting and glucose, glycerol and protein refeeding using HTC cultured cells. By this procedure we avoided the interference of physiological interactions with the rest of the body.

HTC (7288 C) cells were chosen because we have shown that these cells in culture preserved

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their ability to convert α-linolenic acid to eicosapentaenoic acid and other homologs of the α-linolenic acid family[10] and were sensitive to insulin and dibutyryl cyclic AMP[1].

Material and Methods

Radiochemicals
1-[^{14}C]α-linolenic acid (47.1 mC/mmole, 99% radiochemically pure) was obtained from The Radiochemical Centre, Amersham, England.

Culture conditions
HTC 7288C cells grown at 37° in confluent layer attached to glass were used. The cells were grown on Swim's 77 medium supplemented with 10% calf serum[11] using conventional sterile conditions as described previously[10].

Assay procedure
The cells were detached from the glass surface with the aid of 0.25% trypsin solution. They were suspended in culture medium and counted in a hemocytometer. Aliquots (2.5×10^6 cells per bottle) were cultured in 70 cm^2 flasks with 20 ml Swim's 77 medium supplemented with 10% calf serum as described elsewhere[10]. After 48 hr, when the bottles contained approximately 5×10^6 cells, the medium was changed for 10 ml of Krebs Ringer bicarbonate solution and the cells were maintained in this medium for 12 hr. After this period 1-[^{14}C]α-linolenic acid (0.5 nmole per million of cells) was added into the medium and the incubation continued for another 12 hr. These cells are denominated 'fasted' cells. Simultaneously with the addition of the labeled acid some groups of cells were 'refed' with different carbon sources. The carbon sources consisted of either glucose, glycerol or lactalbumin hydrolysate in a concentration of 3.6 g ‰. A control group of bottles maintained in a complete medium was cultured for 24 hr in Swim's 77 medium without serum. The labeled acid was added after 12 hr incubation.

The sodium salt of the labeled acid bound to defatted albumin[12] according to Spector *et al.*[13] in a ratio of 2 moles fatty acid per mole of albumin was used in all the experiments. After 12 hr of incubation with 1-[^{14}C]-α-linolenic acid the attached cells were washed twice with 0.85% NaCl, removed from the container with a rubber policeman, collected into tubes and levelled to 5 ml with 0.85% NaCl. Viability of cells was determined with trypan blue. No significant staining was found, indicating that few dead cells were present. An aliquot of the suspension was used to determine the amount of cellular proteins by the method of Lowry *et al.*[14], and the rest was centrifuged at 2000 × g for 5 minutes. The saline solution was decanted and the cells were saponified at 85° with 2 ml of 10% KOH in ethanol for 45 min. The fatty acids were extracted from the acidified solution with light petroleum (b.p. 30–40 C) and esterified with methanolic 3 N HCl for 3 hr at 60°. The radioactivity of the recovered methyl esters was determined in a Packard Tri-Carb liquid scintillation counter with 86% efficiency for [^{14}C] using a scintillation solution prepared with 4 g of 2.5 diphenyl-oxazol (PPO) and 100 mg of 1.4 bis 2 (5 phenyloxazoil) benzene (POPOP) per liter in toluene. The distribution of radioactivity between the fatty acids was determined by gas liquid radiochromatography in an apparatus equipped with a Packard proportional counter[15]. The samples were analyzed using a column packed with 15% diethylene glycol succinate on chromosorb W (80–100 mesh) at 180 °C. The acids were identified by the procedure already described[18].

The specific activities for α-linolenic acid and 20:5 (Δ5, 8, 11, 14, 17) were calculated with those data after measuring the radioactivity in an aliquot of the final mixture in which the mass distribution of the fatty acids had previously been determined by gas liquid chromatography in the presence of an internal standard of eicosaenoic acid. The specific activities of other fatty acids was not determined due to the very low mass of these acids present.

Separation of lipids
In order to determine the incorporation of 1-[^{14}C]-α-linolenic acid into cellular lipids, the lipids were extracted with chloroform-methanol (2:1 V/V) by the procedure of Folch *et al.*[16]. Individual lipid classes were separated by thin layer chromatography (TLC) on plates of activated silica gel G (0.5 mm). The solvent mixture used was chloroform–methanol–water 65:25:4

v/v/v. The spots of the lipids were scraped off and suspended in 10 ml Bray's solution[17]. The radioactivity was measured as usual in the Packard Tricarb Scintillation counter.

Results

Figure 1 shows the effect of 'fasting' and 'refeeding' with different carbon sources in the absence of hormones upon the metabolism of α-linolenic acid. We had reported previously that[18] HTC cells convert 1-[^{14}C]α-linolenic acid into octadeca-6,9,12,15-tetraenoic acid, eicosa-11,14,17-trienoic acid, eicosa-5,11,14,17 and 8,11,14,17-tetraenoic acid, eicosa-5,8,11,14,17-pentaenoic acid and traces of higher polyunsaturated fatty acids (22:4 and 22:5). The same acids were synthetized in this experiment. In Figure 1 only the conversion of labeled α-linolenic acid to the quantitatively most important products is shown. Octadeca-6,9,12,15-tetraenoic acid was not included since it is always found in very small amounts and is

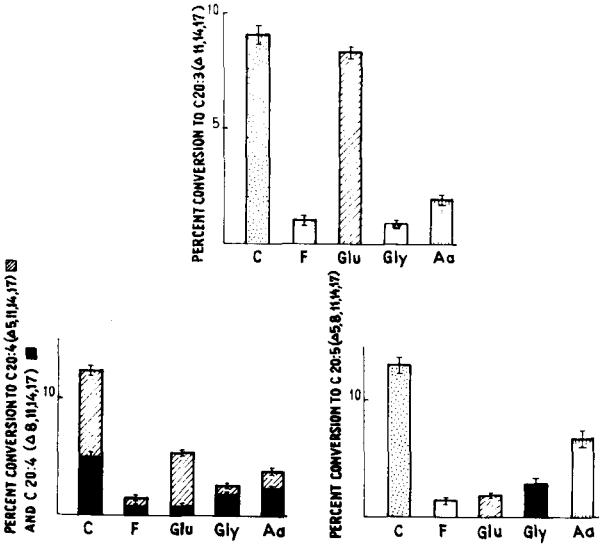

Fig. 1. Effect of 'fasting' and different carbon sources on the conversion of 1-[^{14}C]α-linolenic acid into higher polyunsaturated fatty acids. Cells incubated in Swim's 77 medium without serum for 24 hr (C). Cells incubated in Krebs Ringer bicarbonate solution for 24 hr (F). After 12 hr incubation in Krebs Ringer bicarbonate solution, cells were 'refed' with 3.6 g‰ of either glucose (GLU), glycerol (GLY) or lactalbumin hydrolysate (Aa) for another 12 hr. 1-[^{14}C]α-linolenic acid was added after 12 hr incubation. Results are the mean of 3 incubations. The vertical lines represent SEM.

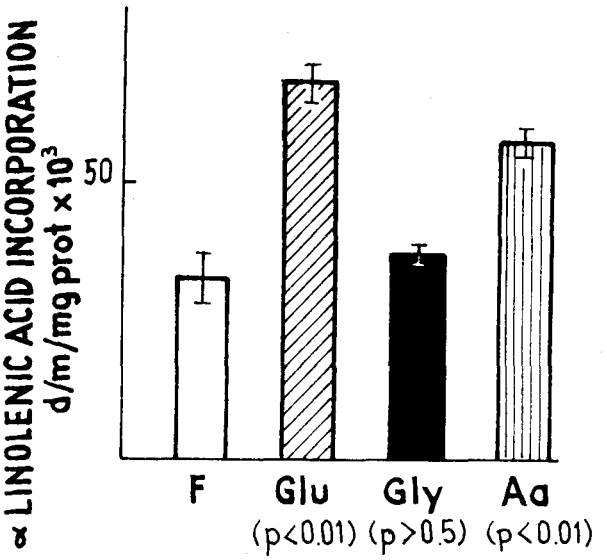

Fig. 2. Effect of 'fasting' and different carbon sources on α-linolenic acid incorporation in HTC cells. Experimental conditions are as in Fig. 1.

rapidly converted into higher homologs. 'Fasting' caused a decrease in the conversion of α-linolenic acid to higher homologs compared to the complete medium. 'Refeeding' with glucose enhanced the transformation of some acids only. It increased 20:3 (Δ11, 14, 17) and 20:4 (Δ5, 11, 14, 17) but no significant changes were found in the conversion to 20:4 (Δ8, 11, 14, 17) and 20:5 (Δ5, 8, 11, 14, 17).

In an opposite way, it is apparent that lactalbumin hydrolysate 'refeeding' evoked significant increases of 20:4 (Δ8, 11, 14, 17) acid and 20:5 (Δ5, 8, 11, 14, 17) whereas very low and no significant increases were found for 20:3 (Δ11, 14, 17) and 20:4 (Δ5, 11, 14, 17) acids. Glycerol did not modify greatly the biosynthetic pattern of the 'fasted' cells (Fig. 1). The small increases found for 20:4 (Δ8, 11, 14, 17) and 20:5 (Δ5, 8, 11, 14, 17) acids are not significant. Therefore, of the three carbon sources studied, two modified the biosynthesis of polyunsaturated fatty acids in different ways, whereas glycerol was practically inactive.

The influence of glucose, glycerol and proteins on the incorporation of linolenic acid radioactivity into the cell is shown in Figure 2. It is evident that the incorporation of labeled acids is increased very significantly by the presence of glucose and aminoacids in the medium, when compared to the 'fasting' cells. The increase of the incorporation was also

similar for both substances. Glycerol, in spite of being a potential substrate for glycerides synthesis did not modify greatly the incorporation capacity of the 'fasting' cells.

The modification of the fatty acid incorporation capacity of the cell by three different carbon sources is shown in Figure 3. In this case, the specific radioactivities of α-linolenic acid and 20:5 (Δ5, 8, 11, 14, 17) are compared. It is apparent that α-linolenic acid specific radioactivity reflects the incorporation of the labeled acid in the HTC cells. Comparison of Figure 2 with Figure 3 shows a very good correlation between both data. The specific activity of α-linolenic acid is higher in media containing glucose or aminoacids than in Krebs Ringer Bicarbonate-glycerol. However, the specific radioactivity pattern of 20:5 (Δ5, 8, 11, 14, 17) in the different media is different. It is very high in an amino acid containing medium and very low and similar in Krebs Ringer-Bicarbonate and Krebs-Ringer-Bicarbonate glycerol. With glucose it was only very little higher than in the Krebs-Ringer-Bicarbonate medium. Therefore, it is evident from these results that aminoacids have increased the conversion of α-linolenic acid to 20:5 (Δ5, 8, 11, 14, 17) acid.

The distribution of the labeling among the different lipid fractions after incubation of the cells with 1-[^{14}C]α-linolenic acid and different kinds of media is shown in Table 1. Aminoacids and the complete media evoked a similar distribution in the different lipid classes. A similar distribution was also found in the 'fasted' cells, and in the cells incubated with glucose. The most important difference between the two groups was the enhancement of labeling

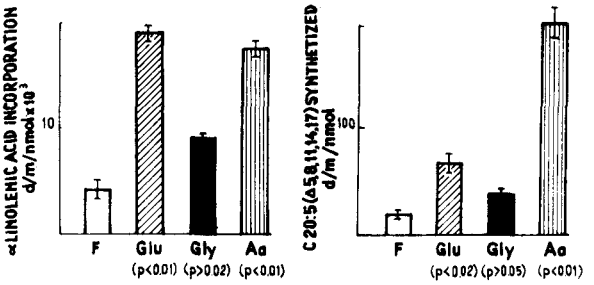

Fig. 3. Effect of fasting and different carbon sources on the specific activity of α-linolenic acid and C20:5 (Δ5, 8, 11, 14, 17) in HTC cells. Experimental conditions are as in Figure 1.

Table 1
Distribution of radioactivity among the lipids of HTC cells with different carbon sources

Lipid fractions	Label distribution				
	Complete medium	24 hr "Fasted"	Cells refed with		
			Glucose	Glycerol	Lactalbumin hydrolysate
	%	%	%	%	%
Phosphatidyl choline	43.9	35.6	38.2	40.1	36.3
Phosphatidyl ethanolamine	24.7	20.8	22.9	21.6	23.5
Free acids	3.8	16.9	17.8	12.9	3.8
Neutral lipids	21.8	10.1	9.9	17.1	22.9

Complete medium: Cells incubated in Swim's 77 medium without serum for 24 hr.
'Fasted' cells: Cells incubated in Krebs Ringer bicarbonate solution for 24 hr.
After 12 hr incubation in Krebs Ringer bicarbonate solution cells were 'refed' with 3.6 g‰ of either glucose or glycerol, or lactalbumin hydrolysate for another 12 hr. 1-[^{14}C]α-linolenic acid was added after 12 hr incubation. The results are expressed as percentage of total radioactivity on the TLC plate. Radioactivity in the rest of the plate makes up 100%.

in free fatty acids and the decrease in the triglycerides in 'fasting' and in glucose 'refeeding' cells. However, glycerol evoked a typical pattern of its own, with high labeling in free fatty acids and triglycerides.

Discussion

Fatty acids of the α-linolenic acid family are minor components of HTC cells lipids incubated in Swim's medium supplemented with calf serum[10]. Alpha linolenic acid and 20:5 (Δ5, 8, 11, 14, 17) are found only in traces, but the addition of α-linolenic acid to the medium increases the cellular content of α-linolenic acid and eicosa-5,8,11,14,17-pentaenoic acid[10].

In a previous work[18] we reported the existence of different pathways in HTC cells for the synthesis of polyunsaturate fatty acids of α-linolenic acid family. In the first place, α-linolenic acid was a substrate for a Δ6 desaturase which converted α-linolenic acid to octadeca-6,9,12,15-tetraenoic acid. Octadeca-6,9,12,15-tetraenoic acid was elongated to

eicosa-8,11,14,17-tetraenoic acid and converted by a Δ5-desaturase to eicosa-5,8,11,14,17-pentaenoic acid. This pathway 18:3 (Δ9, 12, 15) → 18:4 (Δ6, 9, 12, 15) → 20:4 (Δ8, 11, 14, 17) → 20:5 (Δ5, 8, 11, 14, 17) was termed the desaturating route since the first reaction was a Δ6 desaturation. Another pathway was the elongation of α-linolenic acid to eicosa-11,14,17-trienoic acid. This acid was a substrate for Δ5 desaturase which transformed it to eicosa-5,11,14,17-tetraenoic acid. This route: 18:3 (Δ9, 12, 15) → 20:3 (Δ11, 14, 17) → 20:4 (Δ5, 11, 14, 17) was termed the elongating pathway since the first reaction was the elongation of α-linolenic acid. Besides, SPRECHER and LEE[19] have shown that rat liver microsomes do not possess a Δ8 desaturase. The absence or very low activity of this enzyme unables the introduction of a double bond between 8 and 9 carbons on the fatty acids that already have bonds in 5 and 11. Therefore, it is very probable that the synthesis of eicosapentaenoic acid and other higher polyunsaturated fatty acids in HTC cells is only produced from octadeca-6,9,12,15-tetraenoic acid via eicosa-8,11,14,17-tetraenoic acid, while eicosa 5,11,14,17-tetraenoic acid would not be a precursor for further polyunsaturated fatty acid biosynthesis.

In the present work it is shown that the incubation of the cells with different carbon sources evokes different perturbations in the two biosynthetic routes. In the first place 'fasting' evokes a general decrease in the biosynthesis of all the acids (Fig. 1). These results are consistent with data obtained in experiments *in vivo* in which fasting depressed Δ6 desaturation[2,7] and the elongation of fatty acids[20]. Since hormonal effects are absent in this culture of HTC cells, it must be admitted that these inhibitions are a direct response of the cells to the absence of carbon sources in the medium. The absence of carbon sources in the medium undoubtedly switches off the anabolic pathways of the cell, and catabolic conditions prevail.

Unfortunately, neither the ATP/ADP and $NAD^+NADH+H^+$ ratios nor acetyl CoA and malonyl CoA concentration are known in either condition. Little acetyl-CoA is formed from glucose during fasting, and although acetyl-CoA is generated from fatty acid β oxidation, NUMA *et al.*[21] and KORCHAK and MASORO[22] have shown that acetyl carboxylase activity is greatly reduced in these nutritional conditions. Therefore, since malonyl-CoA synthetized by the carboxylase is a cofactor of elongation reactions, malonyl-CoA depression decreases the elongation route activity. GUYNN *et al.*[23] showed a depression of malonyl-CoA concentration in starved animals. This effect would be similar to the lipogenesis depression evoked by fasting[23]. However, it is difficult to suggest in a similar way which may be the cause of the inhibition of the desaturation route during fasting.

Glucose addition to the Krebs Ringer-bicarbonate medium enhanced selectively the elongation route of the cells increasing the conversion to 20:3 (Δ11, 14, 17) and 20:4 (Δ5, 11, 14, 17) acids (Fig. 1). However, it did not modify the conversion to fatty acids of the desaturating route (20:4 Δ8, 11, 14, 17 and 20:5 Δ5, 8, 11, 14, 17). Addition of glucose to the medium also enhanced labeled fatty acid incorporation (Fig. 2) and α-linolenic specific radioactivity (Fig. 3). However, the increased incorporation cannot explain an increased conversion to 20:3 (Δ11, 14, 17) and 20:4 (Δ5, 11, 14, 17) acids, since this stimulation was specific and was much higher than the incorporation. Therefore, we are observing a stimulatory effect only on the elongating route. This may offer good support to the hypothesis that low malonyl CoA production during fasting depresses the elongating pathway since the addition of glucose reactivates malonyl CoA biosynthesis[23] as well as the elongation. However, it is very possible that some enzymes of the elongating reaction may also be directly stimulated by glucose metabolism in a way similar to that proposed for the fatty acid synthetase[23].

The effect of lactalbumin hydrolysate is different from that of glucose (Fig. 1). It activates preferentially the desaturating route, since the conversion to 20:4 (Δ8, 11, 14, 17) and 20:5 (Δ5, 8, 11, 14, 17) is enhanced significantly, whereas the conversion to 20:3 (Δ11, 14, 17) and 20:4 (Δ5, 11, 14, 17) does not change significantly. As aminoacids and glucose increase the incorporation of labeled acids into the cells in a similar way (Fig. 2), the differential effect of the two carbon sources is clearly demonstrated. Besides, Fig. 3 shows that the specific activity of 20:5 (Δ5, 8, 11, 14, 17) acid is also significantly increased by lactalbumin

aminoacids. These results confirm our previous experiments *in vivo*[2,8,24–26] in which aminoacids or a hiperproteic diet were shown to increase the Δ6 desaturation of linoleic or α-linolenic acids modifying the Vm but not the Km, nor the elongation reaction. However, the present experiment shows conclusively that the increase is independent of hormonal effects. It is very probably related to a specific increase of protein synthesis.

Glycerol is incorporated in HTC cells but it does not support the growth of the cells[27] and it does not modify significantly the incorporation of labeled acids in the cell, the specific activity of 20:5 (Δ5, 8, 11, 14, 17) and the fatty acid conversions of the two metabolic pathways (Figs. 1, 2, and 3). Glycerol is converted to α-glycerophosphate in the normal animal and metabolized by the glycolytic system thereby increasing fatty acid synthesis[28] and depressing Δ6 desaturase[29]. However, the absence of response of HTC cells to the glycerol, although in disagreement with these results, would confirm our hypothesis on the glucose effects on unsaturated fatty acid biosynthetic reactions, since numerous reports demonstrate the absence of α-glycerol phosphate dehydrogenase in malignant cells[30,31]. Therefore it is very possible that glycerol would not be metabolized in HTC cells by the glycolytic system and no acetyl CoA and malonyl CoA would be synthetized.

The data in Table 1 are also compatible with this suggestion, since glycerol, in contrast to glucose, increases the distribution of labeled acids in triglycerides. A decrease or absence of glycerol metabolization to acetyl CoA would increase the conversion to glycerol phosphate and incorporation of fatty acids in triglycerides. Unfortunately the concentration of α-glycerol phosphate was not measured. In conclusion, glucose and aminoacids independent of hormonal effects modify specifically the elongating and desaturating routes in the biosynthesis of unsaturated fatty acids. These specific effects may have implications in human beings. KINGSBURY *et al.*[32] have shown that in certain cases of atherosclerosis there is a correlation between coronary thrombosis and a decrease of 18:2 (Δ9, 12) and an increase of the product of elongation 20:2 (Δ11, 14). The relationship between our results and of KINGSBURY's observation is open to further investigation.

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