Synthesis of chain elongation–desaturation products of linoleic acid by liver and brain microsomes during development of the pig

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Microsomes isolated from liver and brain tissue were assayed to examine transitions in metabolic capability to synthesize chain elongation–desaturation products of $C_{18:2(9,12)}$ during the perinatal development of the pig. Microsomal synthesis of trienes, tetraenes and pentaenes was compared for fetal, neonatal and postnatal piglets. Rates of synthesis of tetraenes and pentaenes by chain elongation–desaturation of $C_{18:2(9,12)}$ were greatest in liver. During the later half of gestation, the capability to synthesize tetraenes increased 2–3-fold on a per mg of microsomal protein basis. Increase in the capacity to synthesize tetraenes suggests a significant transition in the activity of Δ^5 desaturase during the last half of gestation. For brain, synthesis of $C_{22:5(4,7,10,13,16)}$ from $C_{18:2(9,12)}$ was greatest at term. These observations indicate that in liver and brain the capability to chain elongate–desaturate $C_{18:2(9,12)}$ to longer chain homologues increases significantly during early development. It is suggested that during gestation the activity of the Δ^5 desaturase limits synthesis of C_{20} and C_{22} homologues of $C_{18:2(9,12)}$. The metabolic conversion of C_{20} and C_{22} fatty acids by chain elongation of $C_{20:4(5,8,11,14)}$ does not appear to limit the synthesis of very long chain homologues of linoleic acid in fetal liver or brain.

Accretion of polyunsaturated long chain metabolites of $C_{18:2(9,12)}$ and $C_{18:3(9,12,15)}$ in human brain has been documented (Friedman, 1980; Clandinin *et al.*, 1980*a,b*) and the analogy to an appropriate animal model has been drawn (Purvis *et al.*, 1982). $C_{20:4(5,8,11,14)}$, $C_{22:4(7,10,13,16)}$, $C_{22:5(4,7,10,13,16)}$, $C_{22:5(7,10,13,16,19)}$ and $C_{22:6(4,7,10,13,16,19)}$ are significant constituents of synaptosomal and other subcellular membranes in brain (Bersohn & Cohen, 1972; Cotman *et al.*, 1969; Galli *et al.*, 1970; Foot *et al.*, 1982), imparting specific structural characteristics on the membrane, influencing membrane functions and the activity of membrane-bound enzymes (Foot *et al.*, 1983; Sun & Sun, 1974; Cotman *et al.*, 1969; Mead & Dhopeswarkar, 1972). Biosynthesis of these fatty acids during brain development is not well established and it is not known whether fetal

accretion of long chain metabolites of the essential fatty acids occurs as a result of fetal synthesis or of mechanisms involving maternal–placental synthesis and transfer. There is evidence for a maternal source (Naughton, 1981); however, recent studies in an appropriate animal model, the piglet, (reviewed by Purvis *et al.*, 1982) indicate some fetal metabolic capability to chain elongate–desaturate $C_{18:2(9,12)}$ (Purvis *et al.*, 1983).

During brain development, understanding of the activity of Δ^6 , Δ^5 and Δ^4 desaturases forming the metabolic pathway for chain elongation–desaturation of essential fatty acids (Scheme 1) is lacking. Thus, little is known of basic and potentially rate-limiting biosynthetic steps responsible for synthesis of the building blocks of membranes integral to the ‘brain growth spurt’ known to occur during early brain development (Dickerson & Dobbing, 1966; Dobbing & Sands, 1979). In addition, understanding of this area of brain lipid metabolism is of significance to the premature human infant because the maternal supply of long chain essential fatty acid homologues is terminated at a developmental period

Abbreviations used: $C_{18:2(9,12)}$, linoleic acid; other fatty acids are represented in a similar way.

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Scheme 1. Chain elongation-desaturation of linoleic acid

when these constituents would normally accrue in brain *in utero*. Thus, the present study was designed to determine microsomal synthesis of specific chain elongation-desaturation products of $C_{18:2(9,12)}$ in liver and brain tissue during the perinatal period by examination of an appropriate animal model, the piglet.

Materials and methods

Materials

cis,cis-[1- 14 C]-Linoleic acid (54.7 μ Ci/ μ mol) was obtained from New England Nuclear, Montreal, Quebec, Canada. Radiochemical purity determined by argentation t.l.c. was >95%. Non-radioactive fatty acid standards were purchased from Supelco Inc., Bellefonte, PA, U.S.A. and were checked by g.l.c. for purity. Solvents were glass redistilled before use. Incubation cofactors were obtained from Sigma Chemical Co.

Emulsions of fatty acid substrate were prepared with twice the molar amount of 0.1 M- NH_4OH and then the mixture was suspended in a 1% (w/v) aqueous solution of Triton WR 1339 (Ruger Chemical Co., Irvington, NJ, U.S.A.).

Animals

Male Yorkshire piglets at 60 days of gestation, full-term (116 days of gestation), and 12 weeks postnatal age were obtained from the University of Guelph, Department of Animal and Poultry Science. All animals were killed by decapitation; postnatal piglets were electrocuted first. Brains and livers were immediately removed, placed in ice-cold iso-osmotic saline and then frozen at -80°C and used for isolation of microsomal membranes.

Microsomal isolation

Isolation procedures were carried out at $0-4^\circ\text{C}$ (Kurata & Privett, 1980) as previously described (Purvis *et al.*, 1983). Microsomal purity was assessed (Purvis *et al.*, 1983) and the absence of mitochondrial membrane verified. Microsomal protein was measured by a colorimetric method (Lowry *et al.*, 1951).

Synthesis of chain elongation-desaturation products of $C_{18:2(9,12)}$

Incubations were carried out at 37°C for 20 min in a metabolic shaker. These assay conditions have been examined in a previous report (Purvis *et al.*, 1983). [1- 14 C] $C_{18:2(9,12)}$ (50 nmol; 200000 d.p.m.) in 100 μ l of NH_4OH solution was added to each incubation. The assay was initiated by adding microsomal protein (0.7–1.4 mg) to a reaction mixture containing 100 μM -[1- 14 C]- $C_{18:2(9,12)}$, 5 mM- MgCl_2 , 150 mM-KCl, 1.5 mM-glutathione, 50 mM-potassium phosphate (pH 7.0), 5 mM-ATP, 1.25 mM-NADH, 1.25 mM-NADPH, 0.3 mM-CoA and 0.3 mM-malonyl-CoA in a final volume of 0.5 ml. Controls contained equivalent amounts of boiled microsomal protein and were incubated under identical assay conditions at the same time. Enzyme assays were terminated by addition of 0.5 ml of 20% (w/v) KOH, followed by saponification at 80°C for 30 min. Each tube was then acidified with 0.5 ml of 5 M-HCl. Lipids were extracted twice with chloroform/methanol (2:1, v/v) containing 0.005% butylated hydroxytoluene as antioxidant. Solvent was evaporated under N_2 and fatty acids were methylated with 10% (w/v) methanolic HCl at 95°C for 45 min.

Lipid analysis

Fatty acid methyl esters were separated according to degree of unsaturation on recently prepared silica gel G t.l.c. plates containing 6% (w/w) AgNO_3 using toluene (1 h) then toluene/acetone (19:1, v/v; 1.5 h) as sequential solvent systems. Fatty acids were visualized under u.v. light after spraying the plates with 0.1% 2',7'-dichlorofluorescein in 95% (v/v) ethanol. Bands containing saturated, monoenoic, dienoic, trienoic, tetraenoic, pentaenoic and hexaenoic fatty acids could be identified by comparison with reference standards.

The identity of labelled fatty acid products was confirmed by collecting labelled fatty acid peaks separated by g.l.c. Chromatography was conducted essentially as described earlier (Innis & Clandinin, 1981) with the injection splitter switched off. Labelled fatty acid products formed were collected at the appropriate retention time by placing a removable glass wool plugged tube over the detector tip. Fatty acids collected co-chromato-

graphed with all-*cis* homologues of $C_{18:2(9,12)}$. Both sample and control reaction mixtures were carried through the entire assay procedure before the sample minus control value was determined.

Liquid-scintillation counting

^{14}C -labelled fatty acid methyl esters separated by argentation t.l.c. were scraped directly from plates into scintillation vials containing 10 ml of toluene-based fluor containing 4 g of 2,5-diphenyl-oxazole and 30 mg of 1,4-bis-(5-phenyloxazol-2-yl)-benzene per litre. Radioactivity was counted in a Beckman model LS-7800 liquid scintillation spectrometer (Beckman Instruments, Palo Alto, CA, U.S.A.). Radioactivity was expressed as d.p.m. before molar equivalents were calculated. Corrections for quenching were made by using ^{14}C standards and the external standard ratios method to express radioactivity as d.p.m.

Results and discussion

For the piglets examined, it is apparent that liver and brain microsomes were capable of chain

elongating-desaturating $C_{18:2(9,12)}$ to synthesize homologous trienes, tetraenes and pentaenes (Figs. 1 and 2). Liver synthesis of Δ^6 , Δ^5 and Δ^4 desaturase products was lower at the midpoint of gestation versus term or post-term periods (Fig. 1). No significant increase in rate of synthesis was observed from term to post-term periods. Liver synthesis of tetraenes increased approx. 3-fold over rates observed midway through gestation ($P < 0.01$). For brain, product synthesis by the Δ^6 desaturase was greatest during the preterm period ($P < 0.01$) with no significant change occurring after term (Fig. 2). Synthesis of tetraenes by brain microsomes increased from preterm to term ($P < 0.01$) and from term to post-term ages. Significant change in the synthesis rate of $C_{22:5(4,7,10,13,16)}$ produced by the Δ^4 desaturase was also observed from preterm to term ages ($P < 0.01$) but was not observed after term (Fig. 2).

Comparison of the relative desaturase activities during this fetal-neonatal period suggests that chain elongation-desaturation of trienes to tetraenes to pentaenes occurs at similar rates for term and post-term liver, whereas conversion of trienes

Fig. 1. Synthesis of trienes, tetraenes and pentaenes from $C_{18:2(9,12)}$ by isolated liver microsomes

All assays were conducted in duplicate and corrected for corresponding control assays. Values for each age group represent the mean $\pm$ S.E.M. for four individual animals. The proportional distribution of label present in fatty acids found in each separate argentation t.l.c. band is indicated in Table 2. Values observed for the tetraene fraction are significantly different for preterm versus term ages ($P < 0.01$).

Fig. 2. Synthesis of trienes, tetraenes and pentaenes from $C_{18:2(9,12)}$ by isolated brain microsomes

All assays were conducted in duplicate and corrected for corresponding control assays. Values for each age group represent the mean $\pm$ S.E.M. for four individual animals. The proportional distribution of label present in fatty acids found in each separate argentation t.l.c. band is indicated in Table 2. Values observed for each fraction are significantly different for preterm versus term ages ($P < 0.01$).

to tetraenes is reduced at mid-gestational period. For brain, conversion of trienes to tetraenes is apparently greater than the rate of triene synthesis of term and post-term periods, whereas the rate of conversion of trienes to tetraenes is reduced for the mid-gestational period ($P < 0.05$; Table 1).

Analysis of the distribution of label present in triene and tetraene fractions (Table 2) indicates that for liver the amount of $C_{20:3(8,11,14)}$ synthesized is similar to the amount of $C_{18:3(6,9,12)}$ produced. In brain, microsomes isolated from term piglets have a relatively greater capacity to elongate the $C_{18:3(6,9,12)}$ to $C_{20:3(8,11,14)}$ than at other developmental periods examined. Analysis of the proportional distribution of label present in the tetraene fraction indicates that at the early stage of development examined most of the $C_{20:4(5,8,11,14)}$ synthesized by the Δ^5 desaturase is converted to $C_{22:4(7,10,13,16)}$. The proportion of label found in $C_{22:4(7,10,13,16)}$ is lower (Table 2) at

later stages of development when increased synthesis of $C_{20:4(5,8,11,14)}$ occurs (Figs. 1 and 2). Similarly, the proportion of chain elongated trienes formed implies that elongation of the fatty acid does not limit synthesis of $C_{20:4(5,8,11,14)}$ observed for the mid-gestational period in liver and brain tissue (Figs. 1 and 2). From these observations, it is likely that during the last half of gestation there is a significant increase in Δ^5 desaturase activity resulting in increased synthesis of C_{20} and C_{22} homologues of essential fatty acids.

Results of the present study indicate that liver and brain microsomes from fetal pigs, a model analogous to the human with respect to fatty acid accretion in brain (Purvis *et al.*, 1982), are capable of synthesizing trienes, tetraenes and pentaenes from $C_{18:2(9,12)}$. However, synthesis of C_{20} and C_{22} homologues of $C_{18:2(9,12)}$ is apparently limited during development by Δ^5 desaturase activity. Mechanisms for stimulation of this function, thus increasing the availability of 'building blocks' required in membrane biosynthesis of the brain growth spurt and growth of other tissues, remain to be determined.

Accretion of $C_{20:4(5,8,11,14)}$ in pig brain has been estimated at approx. $20 \mu\text{g}$ (or $0.06 \mu\text{mol}$)/day during the last 50 days of gestation (Purvis *et al.*, 1982). Given errors inherent in estimating recovery it is difficult to compare directly total enzyme activity, rates of synthesis *in vitro* and estimated accretion *in vivo*. It is, however, of interest to note that total rates of synthesis of tetraenes from $C_{18:2(9,12)}$ based on those observed for brain in the present study can be estimated at greater than $0.06 \mu\text{mol}/\text{day}$ per brain for term ages. For the preterm neonate, whether or not this metabolic capacity to synthesize C_{20} and C_{22} essential fatty acid homologues exists in man or is only present in animal models reflective of human brain development remains conjecture (Clandinin *et al.*, 1982).

Table 1. Relative rates of conversion of trienes to products of the Δ^5 desaturase reaction

Values expressed are the mean ($n = 4$) for the absolute amount of trienes synthesized (Figs. 1 and 2) versus the total amount of products synthesized from the Δ^5 desaturase reaction *in vitro*. Absolute values for product synthesis are illustrated in Figs. 1 and 2. * Significantly different from preterm, $P < 0.05$.

Age	Trienes/ Δ^5 desaturase products	
	Liver	Brain
Preterm	0.6	0.7
Term	0.5	0.1*
Post-term	0.6	0.1*
Pooled $S/\bar{x}$	0.19	0.21

Table 2. Proportional distribution of label in fatty acids present in triene and tetraene fractions, respectively. Triene or tetraene fractions obtained from argentation t.l.c. plates were pooled for separation of individual labelled fatty acids by g.l.c. Labelled fatty acids were collected and counted. Based on the recovery of labelled $C_{18:2(9,12)}$, recovery of other labelled fatty acids was estimated to be $> 90\%$. Absolute molar values for each fraction are indicated in Figs. 1 and 2.

		Amount (%) of label in:			
Tissue	Age	Triene fraction		Tetraene fraction	
		$C_{18:3(6,9,12)}$	$C_{20:3(8,11,14)}$	$C_{20:4(5,8,11,14)}$	$C_{22:4(7,10,13,16)}$
Liver	Preterm	51.4	48.6	7.5	92.5
	Term	52.6	47.4	97.1	2.9
	Post-term	52.6	47.5	41.8	58.2
Brain	Preterm	54.4	45.6	0.1	99.9
	Term	12.6	87.4	93.8	6.2
	Post-term	55.9	44.1	25.6	74.4

Whether or not relative rates of formation of individual chain-elongated fatty acids supports the developing neonate's entire whole body utilization of these membrane components or only a portion thereof, with the balance supplied by the placenta during development *in utero*, and whether longer chain essential fatty acid homologues can be transported into brain for tissue synthesis, remain questions to be examined from both qualitative and quantitative perspectives.

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