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AGE-RELATED CHANGES IN LINOLEATE AND α-LINOLENATE DESATURATION BY RAT LIVER MICROSOMES

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The first and rate limiting step in the conversion of linoleic and α-linolenic acid is catalyzed by the delta - 6 - desaturase (D6D) enzyme. Rat liver microsomal D6D activity decreases on linoleic acid at a rate proportional to the animal age; on α-linolenic acid the decrease in D6D activity begins only later than on linoleic acid. The fatty acid composition of liver microsomes determined by gas chromatographic analysis confirms the impairment of the enzymatic activity directly measured. Our data indicate a correlation between aging and D6D activity impairment. The loss of D6D activity may be a key factor in aging through altering the eicosanoid balance.

The delta-6-desaturase enzyme (acyl-CoA, hydrogendonor : oxidoreductase, E.C. 1.14.99.5) (D6D) converts cis-linoleic acid to gamma-linolenic acid (GLA), the first and rate limiting step in the conversion of linoleate into arachidonate (1), and alfa-linolenic acid to octadecatetraenoic acid.

D6D activity is profoundly affected by various physiological conditions of the animal such as hormonal status (2-4), dietary conditions (5-7) and aging (8-10).

The main consequences of D6D loss are deficiencies of GLA, dihomogamma-linolenic acid (DGLA) and prostaglandin (PG) E1 (9). GLA gives rise also to arachidonic acid, and the prostaglandins of the series 2; it would be reasonable to expect that limitation in synthesis of arachidonate would reduce production of prostanoids.

The D6D activity on (n-3) fatty acid series was not studied as much as the desaturation reaction on (n-6) series. Information as to the ability of liver microsomes to desaturate (n-3) fatty acids is lacking. The D6D enzyme catalyzes the rate limiting step in the conversion of 18:3(n-3) to eicosapentaenoic and

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docosahexaenoic acids. It has been suggested that 20:5(n-3) competes with 20:4(n-6) at the level of cyclooxygenase, leading to decreased formation of thromboxane A_2 and increased synthesis of thromboxane A_3, which, unlike thromboxane A_2, is not pro-aggregatory (11,12).

Aging is characterized by a wide variety of defects, particularly in the immune and cardiovascular systems. Thus the study of D6D activity during aging is particularly important as some prostanoids derived from 18:2(n-6) are associated with thrombus formation (13,14).

The aim of the present experiments is to enlarge the understanding on the D6D loss during aging studying the enzyme activity both on (n-6) and (n-3) fatty acid series in liver microsomes of rats of different ages, evaluating also the correlation between D6D activity and microsomal lipid fatty acid composition.

MATERIALS AND METHODS

[1-^{14}C] linoleic acid (59 mCi/mmol) and [1-^{14}C] α-linolenic acid (56 mCi/mmol) were purchased from Amersham (Amersham, U.K.); NADH, Coezyme A (CoA) and ATP were purchased from Sigma Chemical Co. (St. Louis, MO). All unlabelled fatty acids were obtained from Nu. Check. (Elysian, MN); all chemicals and solvents were of analytical grade.

Normal male Wistar rats were used for experiments at 1 month (group 1), 6 months (group 2), 10 months (group 3), and 25 months (group 4) of age. They had free access to water and standard rat food (Dottori & Piccioni, Brescia) and were mantained under a 12 h dark / 12 h light schedule. The animals were sacrified by decapitation (between the times 9.00 and 10.00 a.m. to avoid circadian variations(15)) and livers were quickly excised, washed with ice-cold physiological saline, blotted and minced with scissors. Microsomes were isolated as previously described (10). All microsomal preparations were done at 0-4 °C and frozen at -70°C until use. The purity of microsomes was assessed by assaying for glucose-6-phosphatase (16). 5'-nucleotidase (17) and succinic dehydrogenase (18) activities were determined to check for plasma membrane and mitochondrial contamination, respectively. Proteins were assayed according to the method of Lowry et al. (19).

Desaturation of 18:2(n-6) and 18:3(n-3) by liver microsomes was estimated as previously described (10). The reaction medium contained, in a total volume of 1 ml, 5 mM $MgCl_2$, 50 μM CoA, 2 mM ATP, 1 mM NADH, 50 mM potassium phosphate buffer pH 7.4 and 40 μM [1-^{14}C] linoleate or 40 μM [1-^{14}C] α-linolenate. Incubations were carried out with approximately 2.5 mg of microsomal protein in a shaking water-bath at 37 °C for 20 min. The reactions were stopped by adding 4 ml of chloroform / methanol (1:1 v/v) and lipids extracted according to Folch et al. (20). Lipids were methyl esterified with methanol/hydrochloric acid (5% by vol.) as reported by Stoffel (21). Fatty acid methyl esters were separated

on thin layer chromatography plates coated with silica gel G, impregnated with 10% (w/v) $AgNO_3$. Carrier methyl esters were spotted along with the labelled esters. Plates were developed in hexane/diethyl ether (8:2 v/v), the spots were made visible under ultraviolet light by spraying with 2',7'-dichlorofluorescein (0.2% w/v in ethanol), scraped off into scintillation vials and counted in 10 ml of liquid scintillation mixture (Instagel, Packard) using a Packard Tri-Carb liquid scintillation spectrometer. Enzyme activity is expressed as pmol of the radioactive fatty acid converted into the product per min per mg of microsomal protein.
The fatty acid composition of microsomal total lipids was determined by gas chromatography (Carlo Erba mod. 4160) using a capillary column (SE 52 0.10 - 0.15 μm) at a programmed temperature (60 - 320 °C), as previously described (22).
All results are expressed as means ± S.D., statistical differences were assessed by using Student's t-test (23).

RESULTS

The conversion of [1-^{14}C] linoleic acid to more unsaturated homologues by D6D in rats of different ages is reported in Tab. 1. A 25% decrease in D6D activity is observed in 6 month-old rats, a 35% decrease in 10 month-old rats and a 60% decrease in 25 month-old rats as compared to 1 month-old rats. A linear correlation has been found between D6D activity and the animal age ($y = 108.46 - 2.55\ x$, $r^2 = 0.93$) using linoleic acid as substrate (data not shown).
The use of [1-^{14}C] α-linolenic acid as substrate leads to a less dramatic age-dependent decrease in D6D activity (Tab. 2). Only in a later period of the life of the animals a impairment of the enzyme activity on α-linolenic acid is observed (15% decrease in 10 month-old rats, 23% decrease in 25 month-old rats). No

Table 1. Liver microsomal D6D activity on linoleic acid, and arachidonic acid / linoleic acid ratio in rats of different ages

Group	D6D activity (pmol x min^{-1}x mg^{-1})		20:4 / 18:2	
1	114.37 ± 19.51	(8)	1.68 ± 0.07	(7)
2	88.27 ± 9.33	(5)°	1.57 ± 0.07	(8)°
3	75.67 ± 10.14	(5)*	1.42 ± 0.16	(8)*
4	48.56 ± 6.87	(6)§	1.35 ± 0.14	(8)§

D6D activity was measured using [1-^{14}C] linoleic acid as a substrate,as detailed in Materials and Methods. Values are means ± S.D.; the number of animals in each group is shown within the brackets . The 20:4 / 18:2 ratio was calculated from gas chromatographic data. Comparisons with group 1 (1 month-old rats) were made using Student's test: °p <0.05; *p<0.01; §p<0.001.

Table 2. Liver microsomal D6D activity on α-linolenic acid and PUFA (n-3) / 18:3 (n-3) ratio in rats of different ages

Group	D6D activity (pmol x min^{-1} x mg^{-1})		PUFA (n-3)/18:3(n-3)	
1	469.55 ± 52.01	(4)	26.56 ± 6.47	(7)
2	471.84 ± 29.92	(5)	13.72 ± 3.14	(8)*
3	403.29 ± 17.19	(5)°	12.60 ± 3.28	(8)§
4	365.11 ± 36.09	(6)*	11.91 ± 3.36	(8)§

D6D activity was measured using [1-^{14}C] α-linolenic acid as a substrate as detailed in Materials and Methods.Values are means ± S.D. ; the number of animals in each group is shown within the brackets . The PUFA (n-3) / 18:3 (n-3) ratio was calculated from gas chromatographic data . Comparisons with group 1 (1 month-old rats) were made using Student's test:° $p<0.05$; * $p<0.01$; § $p<0.001$.

differences are detected between 1 month-old and 6 month-old rats, and no linear correlation between D6D activity and the animal age is evident.

The fatty acid composition of liver microsomes shows a significant increase in the proportion of 18:2(n-6), 18:3(n-3) and 20:4(n-6) fatty acids, and a significant decrease in the proportion of 22:6(n-3) fatty acid in 6, 10 and 25 month-old rats

Table 3. Fatty acid composition (%,w/w) of liver microsomes of rats of different ages

Fatty acid	Group 1 (n=7)	Group 2 (n=8)	Group 3 (n=8)	Group 4 (n=8)
12:0	0.14 ± 0.14	0.19 ± 0.23	0.05 ± 0.01	0.07 ± 0.05
14:0	0.60 ± 0.14	0.63 ± 0.43	0.29 ± 0.18	0.34 ± 0.16
15:0	1.02 ± 0.40	0.71 ± 0.29	0.54 ± 0.15	0.43 ± 0.10
16:0	18.86 ± 1.11	17.07 ± 0.74*	17.33 ± 0.55*	17.68 ± 1.66
16:1	1.76 ± 0.43	1.62 ± 0.17	1.76 ± 0.19	1.76 ± 0.56
17:0	1.05 ± 0.62	1.04 ± 0.12	1.01 ± 0.04	0.94 ± 0.10
(x)	0.93 ± 0.21	0.90 ± 0.15	0.86 ± 0.08	0.80 ± 0.17
18:0	19.99 ± 1.26	20.81 ± 1.43	19.32 ± 1.01	19.22 ± 2.32
18:1	10.88 ± 3.20	9.15 ± 0.53	9.45 ± 0.53	9.52 ± 1.32
18:2(n-6)	11.53 ± 1.55	13.53 ± 1.25°	15.43 ± 1.38§	15.83 ± 1.07§
18:3(n-6)	0.52 ± 0.28	0.53 ± 0.07	0.49 ± 0.14	0.54 ± 0.32
18:3(n-3)	0.42 ± 0.22	0.73 ± 0.15*	0.76 ± 0.10*	0.83 ± 0.29*
20:3(n-9)	0.49 ± 0.36	0.76 ± 0.18	0.72 ± 0.18	0.51 ± 0.32
20:3(n-6)	0.91 ± 0.88	0.93 ± 0.33	0.79 ± 0.19	0.82 ± 0.31
20:3(n-3)	1.82 ± 0.58	1.78 ± 0.38	1.48 ± 0.17	1.62 ± 0.41
20:4(n-6)	19.28 ± 2.35	21.22 ± 0.80°	21.88 ± 1.34°	21.28 ± 1.47
20:5(n-3)	0.40 ± 0.04	0.30 ± 0.10	0.38 ± 0.22	0.35 ± 0.22
22:4(n-6)	0.31 ± 0.26	0.34 ± 0.06	0.23 ± 0.10	0.35 ± 0.10
22:5(n-6)	0.24 ± 0.19	0.28 ± 0.08	0.26 ± 0.10	0.35 ± 0.10
22:5(n-3)	1.13 ± 0.43	0.99 ± 0.23	0.94 ± 0.36	1.38 ± 0.34
22:6(n-3)	7.71 ± 0.77	6.43 ± 0.82*	6.01 ± 0.43*	5.69 ± 0.72§

Gas chromatographic analysis was performed on methyl esters in the conditions reported in Materials and Methods. Values are means ± S.D.; the number of animals in each group is shown within the brackets. Comparisons with group 1 (1 month-old rats) were made using the Student's test: °$p < 0.05$; *$p < 0.01$; §$p < 0.001$.

(Tab. 3). Notwithstanding the increase of arachidonic content, the 20:4 / 18:2 ratio is significantly lower in 6 , 10 and 25 month-old rats (Tab. 1).
Even the PUFA(n-3) / 18:3(n-3) ratio is lower in 6, 10 and 25 month-old rats than in 1 month-old rats (Tab. 2), these results confirming the directly observed decrease in D6D activity.

DISCUSSION

The level of D6D activity in relation to aging has been especially studied in rat testis (8). In the testis the enzyme activity begins to decline as early as 3-4 weeks and falls to approximately 10% of its peak by 26 weeks.
In the present study we have focused on liver microsome D6D activity both on linoleic and α-linolenic acid, investigating not only on its modifications during aging, but also on its ability to regulate the acyl group composition of liver microsomes. To date, no comparative experiments have been carried out on the rate of decline in D6D activity on (n-3) fatty acid series neither in rats nor in species which age at different rates.
Using linoleic acid as substrate, D6D activity linearly decreases from 1 month to 25 month-old rats, so then the enzyme function declines with age at a rate proportional to the aging of the specie. In the liver the decline begins later than in the testis (we observe a 25% decrease after 6 months), but activity is significantly reduced by the end of one year and strongly depressed by the end of two years.
Liver microsomal fatty acid composition shows an increase in 18:2 content during aging, this is consistent with an accumulation of the fatty acid that cannot be desaturated. The unexpected increase in 20:4 content in liver microsomes during aging could be accounted for by a higher conversion of DGLA to arachidonic acid by the delta-5-desaturase. Only limited amounts of DGLA are being formed because of the age-related decrease in D6D activity, then the activity of delta-5-desaturase could become critical in determining the rate at which arachidonic acid is formed. There are interesting species differences in the activity of the liver enzyme (24). This activity is high in rats or mice but low or absent in guinea pigs and rabbits. The high activity of the enzyme could be a factor in the rapid aging of rats and mice.
The decrease in GLA and consequently in DGLA with aging gives rise to a decreased synthesis of PGE_1; as postulated by Horrobin

(9) it may be that loss of PGE_1 is a crucial factor in aging.
On the other hand, an increased conversion of DGLA to arachidonic acid might be expected to be associated with high levels of PGs of the series 2 and related compounds which exert a pro-aggregatory function (13,14).
Notwithstanding the arachidonate increase in liver microsomes during aging, the 20:4 / 18:2 ratio decreases together with D6D activity. The arachidonic acid / linoleic acid ratio is often used as an indicator for D6D activity, so our results are consistent with a reduced conversion of 18:2 by D6D.
The deficiency of PGE_1 combined with an excess of PGs of the series 2 may be a critical problem in aging.
The impairment of D6D activity on α-linolenic acid does not begin until much later than on linoleic acid. The activity is significantly reduced only by the end of one year and a 25% decrease was observed only by the end of two years.
On the other hand, the gas chromatographic analysis of microsomal fatty acids shows a significant decrease in PUFA (n-3) / 18:3(n-3) ratio also in 6 month-old rats. Analysis of PUFA by gas chromatography shows the status of D6D activity on (n-3) fatty acids apparently more precisely than did its enzymatic assay. The S.D. of D6D activity on different microsome preparations is relatively large and real but small differences in rate of reaction may not be appreciated. Undetectable small differences in rate of reaction over extended time periods may lead to larger more easily detectable differences in the products incorporated and accumulated in structural and functional lipids.
Many of the changes in PUFA are <1% of total fatty acids and thus the differences in total amount are small, but they represent significant changes in proportions of available substrates for formation of prostaglandins. In particular, although we did not measure levels of prostaglandins, prostacyclins and thromboxanes in the animals, the decrease in PUFA (n-3) with age could reflect a decreased synthesis of eicosanoids of the 3-series which have anti - aggregatory properties (25,26). Thus the net result of impaired D6D activity would be to alter the eicosanoid balance. Diet and fatty acid desaturation are probably the major factors that might alter the pool of fatty acids available for incorporation into phosphoglycerides. Thus the major implication of altered fatty acid desaturation would be that this may alter lipid membrane composition and, hence, function. It is possible

that aging changes the structural physicochemical properties of the membrane which then modulate the D6D activity. Alternatively, D6D may respond first to aging and may alter lipid fluidity. Whether age-related changes in physicochemical properties are a consequence or a cause of changes in desaturase activity remains to be determined.

Our data evidence that there is a correlation between aging and the inhibition of D6D activity; this does not necessarily mean that the loss of the enzyme activity is a cause of any of the features of aging, but it may be a key factor which is lost during aging.

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