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DIETARY LIPID MODULATION OF RAT LIVER MITOCHONDRIAL SUCCINATE: CYTOCHROME *c* REDUCTASE

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Diets supplemented with high levels of either saturated fatty acids or unsaturated fatty acids were fed to adult rats for a period of 9 weeks and changes in the liver mitochondrial membrane phospholipid fatty acid composition and thermal behaviour of succinate: cytochrome *c* reductase were determined. The dietary treatment induced a change in the ω6 to ω3 unsaturated fatty acid ratio in the membrane lipids, with the ratio being highest with the unsaturated fatty acid and lowest with the saturated fatty acid diet. Arrhenius plots of succinate: cytochrome *c* reductase activity exhibited differences in both critical temperature (T_f) and Arrhenius activation energy (E_a) depending on the type of dietary treatment. The T_f was elevated from 23°C in control to 32°C in the saturated fatty acid-supplemented group. No significant effect on the T_f was observed in the unsaturated fatty acid-supplemented group however higher E_a values were observed due to the unsaturated fatty acid diet. The changes in succinate: cytochrome *c* reductase are probably due to changes in the lipid-protein interactions in the membrane, induced by the dietary lipid supplementation.

Introduction

Changes in the physical properties of biological membranes have been shown to influence the behaviour of certain membrane-associated enzymes [1–3] and to affect many physiological processes [4–6]. In liver mitochondria from homeothermic animals, the activation energy (E_a) for mitochondrial succinate oxidation changes dramatically at a critical temperature [1,4,5,7,8]. This phenomenon is believed to be due to a temperature-induced change in the physical properties of the mitochondrial membrane lipids which can be detected by such biophysical techniques as spin labelling [9,11–13] and fluorescent probes [14–16]. This physical change may be the result of a phase transition occurring in a relatively small domain of higher melting point lipids in which the membrane lipids undergo a change from a liquid-crystalline or fluidus phase to a crystalline or solidus phase on lowering the temperature. Such phase transitions which occur well above 0°C have been observed for bovine heart submitochondrial membranes [17] and rat liver mitochondria [18]. The temperature at which changes in the E_a of various membrane-associated enzyme occurs is influenced by the membrane lipid composition [2,3]. This is evident when comparing membranes from different types of animals, e.g. homeotherms and poikilotherms [5,7], in altered physiological states such as hibernation or torpor [8,10,19–21], or when the membrane lipid composition has been altered experimentally such as by dietary treatment [12,21–23].

Dietary lipid supplementation has been used

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for altering the fatty acid composition and physical properties of biological membranes with resulting effects on membrane-associated enzyme function. Modulation of erythrocyte membrane-associated enzyme functions [22], platelet lipid fluidity [23] and a variety of respiratory parameters associated with rat liver mitochondria [24,25], have been observed after dietary lipid treatment. Dietary treatment of adult sheep with a formaldehyde-treated, protein-coated sunflower-oil diet [27] for four weeks, elevates the content of $\omega 6$ unsaturated fatty acids in liver mitochondrial membranes and alters the Arrhenius (temperature-activity) profile of the associated succinate oxidase system [22]. These changes in thermal behaviour are characterized by a reduction in the critical temperature and an increase in the E_a in the physiological temperature range. Although similar changes in the liver mitochondrial succinate oxidase system were observed in young rats maintained on a high dietary level of unsaturated fatty acids since birth, the effect was lost after 180 days [22], indicating that some form of compensatory mechanism similar to the mechanism of homeoviscous adaptation initially reported for prokaryote membranes [28], may have been operative.

Dietary manipulation of liver mitochondrial membrane lipid composition of adult rats and its concomitant effects on the membrane-associated succinate : cytochrome *c* reductase system have been investigated in this study, particularly with regard to possible opposing effects of diets rich in either saturated or unsaturated fatty acids. We show that the changes in the liver mitochondrial phospholipid fatty acid composition, which can be induced by dietary lipid supplementation, have effects on the thermal behaviour of succinate : cytochrome *c* reductase which are consistent with changes in the fluidity of the membrane lipids.

Materials and Methods

Rats (Hooded Wistar) weighing between 230 to 260 g were maintained on various lipid supplemented diets ad libitum, for a period of nine weeks. One group of animals was fed standard laboratory pellets (Milling Industries Australia Ltd.) containing 5% (w/w) total fat, derived from a variety of sources. A second group of rats received pellets soaked in sunflower oil (Nuttelex Pty. Ltd., Victoria) to give a pellet containing 15% total fat (designated unsaturated fatty acid diet). A third group of rats received pellets soaked in sheep perirenal fat which was heated, strained free of solid matter and maintained at 60°C with stirring for 30 min before being allowed to drain and cool, to give a pellet containing 15% total fat, (designated saturated fatty acid diet). After nine weeks the animals were killed by decapitation and liver mitochondria were isolated.

Liver mitochondria were prepared in media containing 250 mM sucrose, 30 mM L-histidine, 20 mM Tris, 1 mM EDTA, pH 6.8. After removal of the liver, it was chopped and rinsed in the above media and then homogenized using a Polytron tissue disintegrator (PT 35; Kinematica GmbH, Switzerland) at setting 3.5 for three bursts of 6 seconds. After differential centrifugation, the mitochondrial fraction sedimenting between $1000 \times g$ for 10 min and $15000 \times g$ for 15 min, was resuspended in wash media consisting of 250 mM sucrose, 20 mM Tris, 1 mM EDTA, pH 7.6 and centrifuged at $15000 \times g$ for 15 min. The final pellet was resuspended in wash media to a concentration of approx. 12–15 mg protein per ml and used immediately. All operations were carried out at 4°C.

Succinate : cytochrome *c* reductase activity was assayed spectrophotometrically by following the reduction of cytochrome *c* at 550 nm in a Gilford model 250 spectrophotometer equipped with a Gilford Thermoset Temperature controller (Part No. 25036 × 129). Freshly isolated liver mitochondria were first added to buffer containing 5 mM potassium phosphate, 1.25 mM potassium cyanide, pH 7.2 in the proportion of approx. 50 μg mitochondrial protein per ml of buffer. This mix was maintained at 37°C for 10 min in order to lyse the mitochondria. Thereafter the mix was kept on ice. Sufficient mix was prepared so that duplicate assays at all the required temperatures could be performed for each mitochondrial sample from the one stock of diluted, lysed mitochondria. For each assay, 450 μl of the above mix in a 1 ml cuvette was incubated for 2 min at the required assay temperature, then the reaction was started by the addition of succinate and cytochrome *c* at a final concentration of 10 mM and 1.1 mg/ml, respec-

tively, in a final assay volume of 520 μl.

Fatty acid analysis was performed on the phospholipid fraction isolated from mitochondrial lipid extracts. Prior to lipid extraction, the mitochondrial fraction was washed free of sucrose by dilution in 1 mM $CaCl_2$, followed by centrifugation at 25 000 $\times g$ for 20 min. The resulting pellet was washed three times as above and finally resuspended in double-distilled water. Lipids were extracted by shaking first with 4 vol. isopropanol followed by 8 vol. chloroform in the presence of the antioxidant butylatedhyroxyanisole (0.1% of the lipid weight). Upon addition of 1 vol. of water, the bottom layer containing the lipid, was collected and evaporated to dryness. Phospholipids were separated from non-polar lipids by thin-layer chromatography on Silica gel H plates which were developed in petroleum ether/diethyl ether/acetic acid (90 : 15 : 1, v/v). The material at the origin was removed and methylated in 1% H_2SO_4 in methanol by heating at 70°C for 3 h. Fatty acid methyl esters were extracted and analysed by gas chromatography using the method of Gibson and Kneebone [29]. Columns were packed with 5% SP-2310 on 100/120 chromosorb WAW (Supelco Inc. Bellafonte, PA, U.S.A.) with the column temperature programmed from 125 to 225°C at 4 K per min.

The method for determining changes in slope of the Arrhenius plot for succinate : cytochrome *c* reductase activity was as follows. The coefficient of determination r^2 and the residual sum of squares was calculated for all possible combinations of points fitted to two straight lines from the upper to the lower temperature extremes. A change in slope was considered to occur at the first minimum for the sum of the residual sum of squares of the two straight lines. The calculations were continued using all the points, from the calculated point of a change in slope (as determined above), to the lower temperature extreme. If another minimum for the sum of the residual sum of squares occurred, this was taken as the point of a second change in slope. The difference in slope (m) and intercept (b) for the straight line defined by $y = mx + b$ about a designated point of slope change, were tested for statisical significance as described by McMurchie and Raison [8] and were adopted as actual points of slope change if the level of significance was $P < 0.005$.

Results

The fatty acid composition of the various diets is shown in Table I. Soaking the commercial rat pellets in sunflower oil increased the unsaturated fatty acid content about 85% over control diets by substantially increasing the linoleic acid (C18 : 2, ω6) content and decreasing the palmitic acid (C16 : 0) content. Soaking the pellets in sheep perirenal fat increased the proportion of saturated fatty acids to about 52% by increasing the proportions of myristic (C14 : 0), palmitic (C16 : 0) and oleic (18 : 1, ω9) acids and decreasing the linoleic acid (C18 : 2, ω6) content. All diets contained at least 1% linolenic acid (C18 : 3, ω3) but no diet contained any measurable quantity of arachidonic (C20 : 4, ω6) or docosapentaenoic (C22 : 5, ω3) acids. Only trace amounts of docosahexaenoic acid (C22 : 6, ω3) were present in the unsaturated fatty acid and saturated fatty acid diets.

The fatty acid composition of rat liver mitochondrial phospholipids after 9 weeks dietary lipid supplementation, is shown in Table II. Although both the level of fat and the extent of lipid unsaturation varied considerably between diets, the change in the absolute level of saturated and

TABLE I

FATTY ACID ANALYSIS OF LIPID-SUPPLEMENTED RAT DIETS

Fatty acids are designated by the number of carbon atoms followed by the number of double bonds. The fatty acid composition is expressed in % (w/w).

Fatty acid	Control diet	Fatty acid diet	
		Unsat.	Sat.
14:0	1.1	0.2	4.1
16:0	16.7	8.0	20.5
16:1 ω7	1:8	0.3	2.0
17:0	0.4	0.1	1.6
18:0	4.6	5.2	24.4
18:1 ω9	21.8	23.3	31.8
18:2 ω6	42.1	58.5	6.2
18:3 ω3	3.4	1.2	1.2
20:0	0.4	0.4	1.4
20:1	1.3	0.5	0.5
22:1	1.7	0.4	0.2
22:6 ω3	2.2	0.3	0.2
Unsat.	74.3	84.5	42.1
Sat.	23.2	13.9	52.0

TABLE II

RAT LIVER MITOCHONDRIAL PHOSPHOLIPID FATTY ACIDS 9 WEEKS ON LIPID-SUPPLEMENTED DIETS

Data are presented as mean relative % (w/w)±S.E. for single determinations of liver mitochondrial phospholipid fatty acid composition from $n = 4$ animals in each dietary group. tr, present at less than 0.5%.

Fatty acid	Control diet	Fatty acid diet	
		Unsat.	Sat.
16:0	16.7±0.2	11.9±0.6	12.3±0.1
16:1 ω7	1.2±0.3	0.5±0.1	1.0±0
17:0	0.6±0.1	0.5±0	0.6±0
18:0	22.6±1.4	26.5±0.5	26.3±0.2
18:1 ω9	7.7±0.5	6.6±0.2	11.1±0.3
18:2 ω6	12.8±0.2	12.7±0.5	10.7±0.2
18:3 ω6	tr	tr	tr
18:3 ω3	0	0	tr
20:0	tr	tr	tr
20:1	tr	0.6±0.1	tr
20:3 ω9	tr	1.3±0	1.2±0.1
20:3 ω6	1.2±0.1	0.5±0	1.7±0
20:4 ω6	26.0±0.9	30.8±0.1	20.3±0.2
22:1	1.2±0.3	tr	1.6±0.1
22:4 ω6	tr	0.5±0	tr
24:0	tr	0.7±0	0.6±0.1
22:5 ω3	0.9±0.1	tr	0.8±0
22:6 ω3	7.3±0.4	6.0±0.4	10.5±0.3
Σ Unsat.	59.6	60.3	59.8
Σ Sat.	40.4	39.7	40.0
Σ ω6	40.6	44.8	33.1
Σ ω3	8.2	6.3	11.4
Σ ω6/Σω3	4.95	7.11	2.90
U.I. [a]	195	201	193

[a] The unsaturation index (U.I.) is Σ [(*a*)(*b*)] where *a* is the % (w/w) of each unsaturated fatty acid and *b* is the number of double bonds for that particular fatty acid.

unsaturated fatty acids was not significantly altered and little change was apparent in the unsaturation index. Despite the lack of change in these parameters, there were however significant changes in the level of certain individual fatty acids both saturated and unsaturated. In relation to the control diet, the unsaturated fatty acid dietary animals exhibited a decreased palmitic acid (C16:0) and an increased stearic acid (C18:0) content. In addition there was an elevation in the level of the ω6-series unsaturated fatty acids most notably arachidonic acid (C20:4, ω6), and a concomitant decrease in the level of the ω3-series unsaturated fatty acids. The saturated fatty acid dietary animals exhibited similar changes in the levels of palmitic (C16:0) and stearic (C18:0) acids as was apparent for the unsaturated fatty acid dietary animals. However, an opposite response with regard to the proportion of the ω6 and ω3 series unsaturated fatty acids was observed in this dietary group. Thus the saturated fatty acid diet significantly reduced the level of the ω6 unsaturated fatty acids by a reduction in both linoleic (C18:2, ω6) and arachidonic (C20:4, ω6) acid levels, and elevated the ω3 unsaturated fatty acid level by increasing the docosahexaenoic acid (C22:6, ω3) level. The Σω6/Σω3 ratio ranged from 2.90 in the saturated fatty acid-diet animals to 7.11 in the unsaturated fatty acid-diet animals compared to a control value of 4.95.

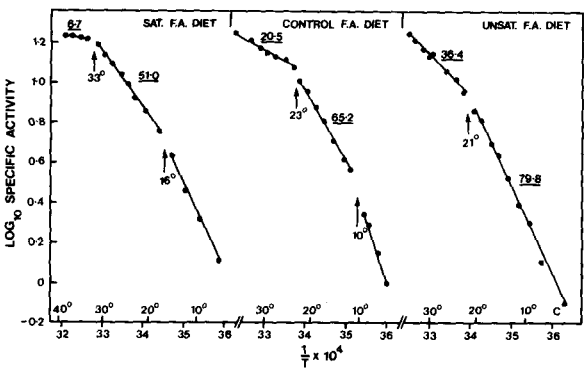

Fig. 1. Arrhenius plots of succinate:cytochrome *c* reductase activity of liver mitochondria from rats fed various lipid supplemented diets for a period of 9 weeks. Numbers underlined are the values for the activation energy (E_a) in kJ·mol^{-1} for each linear region of the plot. The critical temperatures (arrowed) were determined by the method of least squares as described in Methods.

Arrhenius plots of rat liver mitochondrial succinate:cytochrome *c* reductase activity for rats fed either saturated fatty acid, unsaturated fatty acid or control diets for 9 weeks are shown in Fig. 1. The Arrhenius plot for liver mitochondrial succinate:cytochrome *c* reductase activity from control rats is triphasic with critical temperatures (T_f) at about 23°C and 10°C. The activation energy (E_a) increases from 20.5 to 65.2 kJ·mol^{-1} as the temperature is reduced to 10°C. The mean value for the E_a in the temperature region above T_f

TABLE III

RAT LIVER MITOCHONDRIA-SHORT TERM DIETARY LIPID SUPPLEMENTATION

Numbers in parentheses refer to the actual number of animals from each dietary group which were used for the particular determination. Data are presented as the mean ± S.E.

Dietary lipid supplement	Specific activity [a]	Succinate: cytochrome *c* reductase		
		T_f (°C)	E_{a_I} (kJ·mol^{-1})	$E_{a_{II}}$ (kJ·mol^{-1})
Sat. F.A.	17.2 ± 2.0 (4)	31.7° ± 1.0 (4)	14.2 ± 7.5 (2)	56.4 ± 5.4 (2)
Control	17.4 ± 3.2 (4)	23.0° ± 0.6 (8)	18.8 ± 5.0 (4)	69.8 ± 5.0 (4)
Unsat. F.A.	17.1 ± 0.7 (4)	23.3° ± 0.6 (10)	31.8 ± 6.7 (5)	75.7 ± 3.3 (5)

[a] Specific activity: μmol·h^{-1}·mg^{-1} protein at 38°C.

(E_{a_I}), was 18.8 kJ · mol^{-1} and that for the E_a below the T_f ($E_{a_{II}}$) was 69.8 kJ · mol^{-1} (Table III).

Although supplementation of the diet with either saturated or unsaturated fatty acids has no significant effect on the specific activity of succinate: cytochrome *c* reductase in comparison to the Control diet (Table III), differences are observed in the Arrhenius profile which are clearly dependent on the nature of the dietary lipid supplementation. Thus, for rats fed the saturated fatty acid diet, there was an elevation in the critical temperatures in comparison to the control (Fig. 1). The mean value of T_f was elevated from 23.0°C in the control to 31.7°C in the saturated fatty acid dietary animals (Table III). Only a slight lowering of the mean E_{a_I} value was apparent, although some values as low as 6.7 kJ · mol^{-1} were observed (Fig. 1). Supplementation with the unsaturated fatty acid diet resulted in a significant elevation in the mean value of E_{a_I} from 18.8 kJ · mol^{-1} in control to 31.8 kJ · mol^{-1} in the experimental group. No significant effect on the T_f was observed; 23.0°C in control compared with 23.3°C in unsaturated fatty acid-dietary animals (Table III). The lower critical temperature was difficult to detect in this dietary group within the experimental temperature range (Fig. 1). This was also the case for succinate: cytochrome *c* reductase from rat heart mitochondria (unpublished results), indicating that the effect of lipid compositional changes on the thermal behaviour of succinate: cytochrome *c* reductase, are not solely confined to liver mitochondria.

Discussion

Significant changes were observed in the fatty acid profile of rat liver mitochondrial phospholipids after 9 weeks dietary lipid supplementation. These changes influenced the temperature-activity (Arrhenius) profile of the membrane-associated succinate: cytochrome *c* reductase system, but not the specific activity of this particular enzyme system. These effects are probably the result of changes in the fluidity or molecular ordering of the membrane lipids induced by alterations in the membrane fatty acid composition. Such fluidity changes would be expected to influence the phase transition temperature of both the bulk membrane lipids and those domains of lipid which may contribute in some manner to the 'breaks' in the Arrhenius plot. Changes in membrane lipid fluidity would also be expected to influence the E_a of succinate: cytochrome *c* reductase by changes in the lipid-protein interactions within the membrane. Furthermore, the nature and the extent of the change in membrane composition and function is dependent on whether the diet is supplemented with saturated or unsaturated fatty acids.

Although the experimental diets differed in their levels of lipid saturation, no significant effect was observed in the overall level of membrane lipid saturation. The decrease in palmitic (C16 : 0) and the increase in stearic (C18 : 0) acids which occurred with both lipid-supplemented diets, was not consistent with the level of these fatty acids in the respective diets. This may relate to the need to maintain a relatively constant level of saturated

fatty acids in the membrane despite large variations in the dietary intake of saturated and unsaturated fatty acids and thus may represent a form of homeoviscous adaptation [28].

The changes observed in the $\omega6/\omega3$ ratio as a result of the dietary lipid treatment are consistent with changes in the flux of the various unsaturated fatty acids through their particular conversion pathways. Thus the unsaturated fatty acid diet favoured the conversion of dietary linoleic acid (C18 : 2, $\omega6$) to arachidonic acid (C20 : 4, $\omega6$), whilst the saturated fatty acid diet resulted in an elevation of docosahexaenoic acid (C22 : 6, $\omega3$) presumably originating from linolenic acid (C18 : 3, $\omega3$), although levels of this fatty acid in the diet were extremely low. The elevation of various unsaturated fatty acids within any one particular unsaturated fatty acid series was also accompanied by a reduction of some of the unsaturated fatty acids of the alternate unsaturated fatty acid series, suggesting some form of regulation of desaturase activity was operative, as suggested by Caster et al. [30]. In addition to the changes in the $\omega6/\omega3$ ratio there was an increase in C18 : 1, $\omega9$ in the saturated fatty acid-supplemented rats consistent with the relatively high level in the diet. Thus the major effect of the two dietary lipid treatments employed in this study was on the type, rather than the overall amount of polyunsaturated fatty acids present in the membrane. We have also observed this phenomenon in mitochondrial, sarcolemmal and sarcoplasmic reticulum membranes from rat heart in response to saturated and unsaturated fatty acid diets (unpublished results), and in sheep liver and kidney mitochondria in response to unsaturated fatty acid diets [22].

Arrhenius plots of various membrane-associated enzyme activities have been shown to be triphasic, as has the molecular motion of spin label probes infused into the host membrane lipids [11–13,31]. The Arrhenius profile of succinate oxidation by liver mitochondria from homeothermic animals is also triphasic [11], and it has been shown that unsaturated fatty acid-dietary supplementation results in a change in the Arrhenius profile [22]. In the present study, succinate : cytochrome *c* reductase rather than succinate oxidase has been employed to determine the effect of changes in the membrane fatty acid composition on membrane-associated respiratory activity. The activity of rat liver mitochondrial succinate : cytochrome *c* reductase has been shown to be dependent on phospholipids [32], and Arrhenius plots of membrane-associated succinate : cytochrome *c* reductase activity of mitochondria isolated from a variety of sources, have been shown to be non-linear [31,33,34]. The Arrhenius profile of succinate : cytochrome *c* reductase activity from control rat liver mitochondria is virtually identical to that previously reported for rat liver mitochondrial succinate oxidase with regard to both the triphasic nature of the plot and the values of T_c and E_a [11,22]. In these previous studies it was shown that T_f (the upper critical temperature derived from the Arrhenius plot) was a good indicator of the relative fluidity of the membrane lipids, with lower values of T_f indicating relatively greater fluidity and vice versa [22]. The membrane lipid fluidity was also shown to be related to the value of the E_a, there being an inverse relationship between T_f and E_a. The inference from these observations is that the particular physical parameter of the lipids measured by T_f is in effect a measure of the capacity of the lipids to interact with membrane proteins and influence the conformation of the active site [22].

Considered in terms of the T_f, it is apparent that rat liver mitochondrial membrane lipid fluidity is significantly reduced due to the dietary saturated fatty acid supplementation; i.e. there is an increase in T_f accompanying the decrease in the $\omega6/\omega3$ unsaturated fatty acid ratio. In contrast, elevation of the $\omega6/\omega3$ ratio due to the unsaturated fatty acid-dietary supplementation did not affect the T_f, but did result in a significant increase in E_{a_1}. Thus the response of the membrane-associated succinate : cytochrome *c* reductase to changes in the membrane lipid fluidity differs markedly depending on the nature of the dietary lipid intake. There was no significant effect on the specific activity of succinate : cytochrome *c* reductase.

The activity of succinate : cytochrome *c* reductase has been shown to be reduced upon enriching the mitochondrial inner membrane with exogenous phospholipids [35]. This effect is believed to be due to an increase in the average distance between the participating integral mem-

brane proteins which influence the diffusion time and ultimately the reaction rate [35]. Although changes in the membrane lipid fluidity would have been expected as a result of the changes in the membrane lipid composition, in this study no effect was observed on the specific activity of succinate : cytochrome *c* reductase; however the parameters of T_f and E_a were affected depending on the nature of the diet. This would indicate that succinate : cytochrome *c* reductase was under the influence of both lipid-lipid and lipid-protein interactions, respectively. Changes in these interactions due to dietary-induced alterations in membrane phospholipid fatty acid composition were probably the primary factors responsible for the altered thermal behaviour of the enzyme. In this instance, the changes in the E_a and T_f of succinate : cytochrome *c* reductase are the result of changes in the ratio of the $\omega 6/\omega 3$ unsaturated fatty acids rather than in the overall level of lipid unsaturation. Many examples have been reported for the role of phospholipids in modulating the behaviour of membrane-associated enzyme systems [3,6,12–14,16,36]. At the molecular level these effects are most likely mediated by changes in membrane lipid fluidity and they may provide a mechanism for regulating reactions which involve membrane-associated enzymes. Situations which induce changes in membrane lipid fluidity such as the nature of the dietary lipid intake could, via such a mechanism, regulate cellular functions.

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