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CYTOCHROME OXIDASE INDUCTION AFTER OXIDATIVE STRESS INDUCED BY ADRIAMYCIN IN LIVER OF RATS FED WITH DIETARY OLIVE OIL

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Summary: The influence of different kinds of dietary fat (8%) and of endogenous lipid peroxidation with regard to cytochrome c oxidase activity and cytochrome $a+a_3$ concentrations in mitochondria from rat liver has been investigated. It was possible to confirm that the dietary fat induced higher phospholipid degradation in mitochondrial membranes; moreover an endogenous oxidative stress induced by adriamycin was able to increase the peroxidative effects. We have found that the peroxidative effects could sometimes induce an apparent enhancement of cytochrome oxidase activity due to a significant increase of cytochrome $a+a_3$ content. This finding lets us suppose that both changes in the lipid environment and some peroxidation damage could occur in the membrane as a consequence of the fat assumed. Furthermore we should suggest that an induction of the synthesis of cytochrome $a+a_3$ might be related to an enhanced production of peroxides at membrane level.

Cytochrome oxidase is the terminal enzyme of the mitochondrial respiratory chain and it is a vital component of cellular energy transduction, responsible for virtually all oxygen consumption in mammals (1). It is composed of 13 subunits; 3 of these are transcribed and translated within mitochondria, and the remaining subunits are transcribed in the nucleus, traslated in the cytoplasm, and imported into mitochondria prior to assembly (2,3). Cytochrome c oxidase is highly dependent of cardiolipin for maximal activity (4). Yamaoka et al. (5) have observed that the fatty acid composition of rat mitochondrial cardiolipin and cytochrome c oxidase activity were drastically changed when the dietary lipids were modified. Furthermore, the O_2 consumption of rat mitochondria decreases as the quantity of 18:2(n-6)/18:2(n-6) cardiolipin species decreases (5).

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On the other hand, fatty acids in animal tissues reflect the fatty acids in the animal's diet, suggesting that dietary lipids affect biomembrane functions (6,7). The effect is most pronounced when the diet is high in a dietary fat consisting primarily of polyunsaturated fatty acids (8). The administration of diets particularly rich in linoleic and oleic acids also induces a clear enhancement of linoleic and oleic content in the phospholipids of microsomes (9,10) and mitochondria respectively (3). Also polyunsaturated side chains of membrane lipids are especially susceptible to free radicals initiated oxidation, which can be generated either by metabolism of xenobiotics or by normal aerobic cellular metabolism (11,12).

We have previously shown that in mitochondrial membranes of rat liver, the dietary fats differently influenced the mitochondrial levels of Coenzymes Q_9 and Q_{10} and the rate of endogenous lipid peroxidation (13). Furthermore, an oxidative stress induced by adriamycin produced a sharp decrease in mitochondrial CoQ_9 level in rats fed with polyunsaturated but no changes in rats fed with monounsaturated fats (13). Therefore, it is interesting to examine if different fatty acid contributions in the diet, with the same degree of unsaturation but distinct diene/monoene ratios (olive oil and corn oil) and of unsaponifiable material content (virgin olive oil and refined olive oil) and a subsequent peroxidative induction of membrane phospholipids, could affect cytochrome oxidase activity and cytochrome $a+a_3$ content in mitochondria of rat liver that have been fed for 16 weeks with controlled diets.

MATERIALS AND METHODS

All chemicals used were purchased from Sigma Chemical Co. Ltd., St Louis, MO 63178, U.S.A. and all solvents were pure reagents of Merck and Carlo Erba.

Male Wistar rats weighing 50-55 g were randomly assigned to six groups each containing 12 animals. During 16 weeks each group was fed a basal diet containing 8% fat (Dottori Piccioni, Brescia, Italy), but in each of two groups the diet contained different fats: "virgin" olive oil (groups VO and VO+A), "refined" olive oil (RO and RO+A) and corn oil (CO and CO+A). In addition to fats, diets contained 58.3% starch+sucrose, 27.1% vitamin free-casein, 2.2% cellulose, 3.7% mineral mix, 0.1% vitamin, 0.4% DL-methionine and 0.2% choline chloride (14). Adriamycin (ADR) (a kind gift from Farmitalia, Milano, Italy) at a dose 20 mg/kg/day was administered intraperitoneally to the animals of VO+A, RO+A and CO+A groups during the last four days of the treatment period. The intraperitoneal administration was always performed at 9 a.m. The fatty acid composition of the three diets is shown in Table 1.

Diets were stored at 4°C, and rats were fed fresh food daily. Food and water were provided *ad libitum*. A 12 h dark/12 h light cycle was maintained in the room, and the room temperature was 22 $\pm$ 1 °C. After consuming the experimental diet for 16 weeks, the animals of each group were decapitated and the livers were rapidly removed. The animals were sacrificed at 9 a.m. in order to avoid any circadian influence.

Rat liver mitochondria were isolated by the method of Fleischer et al. (15). The cytochrome content was evaluated by the differential spectra (dithionite reduced minus ferricyanide oxidized in the presence of 1% deoxycholate) in a Jasco (Uvidec-610) double-beam spectrophotometer according to Vanneste (16) and Nicholls (17). Cytochrome oxidase activity was assayed using reduced cytochrome c (reduced by dithionite and purified on a Sephadex G-25 column (18,19) as substrate by monitoring the absorbance decrease of

TABLE 1

Fatty acid composition (%) of experimental diets

	Virgin olive oil	Olive oil	Corn oil
16:0	8.92	11.32	12.6
16:1(n-7)	1.06	1.28	0.2
18:0	1.97	2.17	1.9
18:1(n-9)	78.73	75.82	24.1
18:2(n-6)	8.36	9.17	60.1
18:3(n-3)	0.96	0.34	1.0
Saturated	10.89	13.49	14.6
Unsaturated	89.11	86.61	85.4
Monoene	79.79	77.10	24.3
Diene	9.32	9.51	60.1
Diene/monoene	0.10	0.12	2.4

Fatty acids are designed by the number of carbons followed by the number of double bonds. The positions of the first double bond relative to the methyl (n) end of the molecule is also indicated.

cytochrome c upon oxidation at 417-409 nm; the extinction coefficient used for cytochrome c was 40.7 $mM^{-1}.cm^{-1}$ (19). Peroxidation of mitochondrial and microsomal phospholipids was monitored measuring malondialdehyde (MDA) by the thiobarbituric acid assay (20). The protein concentration of all samples was determined with the method of Lowry et al. (21) with bovine serum albumin as standard.

RESULTS

The measurement of MDA, one of the end-products of lipid peroxidation, confirmed that the dietary fat induced higher phospholipid degradation in mitochondrial membranes of CO and RO groups (Table 2). Furthermore, when during the last four days of experimental treatment a daily intraperitoneal dose of ADR [an anthracycline antibiotic that enhances lipid

TABLE 2

Effect of dietary fat and endogenous lipid peroxidation (induced by adriamycin) on Malondialdehyde (MDA) content in rat liver mitochondria

GROUP	MDA nmol/mg
Virgin olive oil	2.75 ± 0.13[a]
Virgin olive oil+ADR	3.61 ± 0.32[b]
Corn oil	3.64 ± 0.33[b]
Corn oil+ADR	4.69 ± 0.31[c]
Refined olive oil	3.98 ± 0.12[b]
Refined olive oil+ADR	4.92 ± 0.27[c]

Values are means ± SEM, n=8. Means within a column not followed by the same superscript letter are significantly different at $p < 0.05$ or less, by a least significant difference analysis.

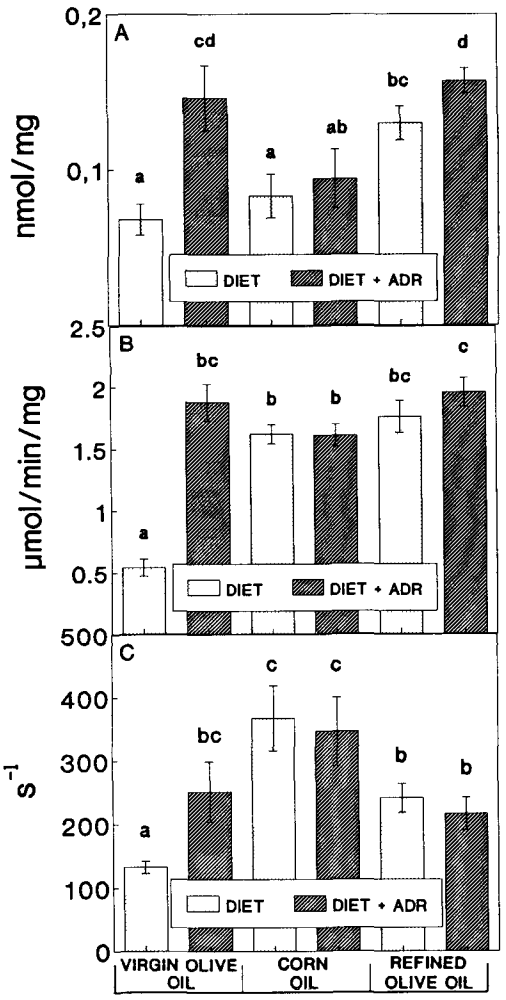

FIGURE 1. Effect of dietary fat and endogenous lipid peroxidation (induced by adriamycin) on cytochrome c oxidase activity (A), cytochrome $a+a_3$ content (B) and cytochrome c oxidase's turnover number (C) in rat liver mitochondria. Values are means ± SEM, n=8. Means of values not followed by the same letter are significantly different at $p < 0.05$ or less, by a least significant difference analysis.

peroxidation both in microsomes (22) and mitochondria (23) and inactivates respiratory chain enzymes (24,25)] was administered, a statistically significant increase of MDA mitochondrial content in all studied groups was found. However the MDA amount reached in VO+A group is lower than that produced by the fat-diet alone in CO and RO groups.

Figure 1A illustrates the mitochondrial cytochrome $a+a_3$ content. The experimental groups VO and CO display the same cytochrome $a+a_3$ content (0.069 ± 0.009 and 0.085 ± 0.011 nmol/mg respectively) and only in RO group it was found to increase significantly (0.130 ± 0.011 nmol/mg, $p < 0.05$). Adriamycin induced a statistically significant increase of cytochrome $a+a_3$ in the VO+A and RO+A groups (0.137 ± 0.018 and 0.157 ± 0.007

nmol/mg respectively), but not in the CO+A group, with respect to the same experimental groups that were not treated with ADR.

The specific activities of mitochondrial cytochrome c oxidase are represented in the Fig. 1B. As it is shown, ingestion for 16 weeks of a diet with 8% virgin olive oil, refined olive oil and corn oil as sole fat source, induced higher statistically significant specific activity in the groups CO (1.62 $\pm$ 0.08 μmol/min. mg) and RO (1.76 $\pm$ 0.13 μmol/min. mg) with regard to VO group (0.54 $\pm$ 0.07 μmol/min. mg). The intraperitoneal administration of adriamycin induced a statistically significant increase of cytochrome c oxidase specific activity in VO+A (1.88 $\pm$ 0.15 μmol/min. mg), with regard to the same experimental group that was not treated with ADR. On the contrary, in CO+A (1.61 $\pm$ 0.09 μmol/min. mg) and RO+A (1.96 $\pm$ 0.12 μmol/min. mg), the ADR did not induce significant changes.

The reaction velocities of the oxidase as defined by their turnover numbers (Fig. 1C) was completely different in each experimental group, with the highest value in CO group (367.3 $\pm$ 51.4 s^{-1}) and the lowest in VO group (132.5 $\pm$ 9.3 s^{-1}). The endogenous stress induced by adriamycin was able to produce a sharp increase of oxidase's turnover number in VO+ADR group (251.1 $\pm$ 47.6 s^{-1}, $p < 0.01$) but not in CO+ADR and RO+ADR groups with respect to the same experimental groups that were not treated with ADR.

DISCUSSION

The results of these experiments indicate that there was indeed a control on the cytochrome oxidase activity that was determined by its lipid environment induced by the diet. In fact in the case of the experimental diets VO and CO, that in our studies induced the same cytochrome $a+a_3$ content, cytochrome oxidase was much more active in the CO group rather than in the VO one since the former displayed turnover and specific activity values 3 times higher. Early studies indicated that cytochrome oxidase activity reaches its maximun when at least 75% of the cardiolipin in the mitochondria is of the 18:2(n-6)/18:2(n-6) species, and this situation happening in the case of rats fed with corn oil (5). In fact, when this fat is substituted by sardin oil or hydrogenated corn oil (5), the activity decreases together with the lowering of this species of cardiolipin. Also Trivedi et al. (26) have demostrated that in a double fatty acid yeast mutant culture the cytochrome oxidase turnover was two-fold in mutants grown under continuous supplementation with C16:0 and C18:2 rather than in mutants grown in presence of C16:0 and C18:1.

We have shown, in previous investigations, that diet with olive oil induced a decrease of 18:2(n-6)/18:1(n-9) ratio in cell membranes in comparison with corn oil (9) and this could

be the reason for the higher cytochrome oxidase activity in CO group than in VO group that we have found.

However the lipid environment altered by dietary fats affects also the cytochrome $a+a_3$ content. The experimental group fed with refined olive oil (RO) displayed a cytochrome $a+a_3$ content significantly higher ($p < 0.01$) than in VO and CO groups. Also Trivedi et al (26) utilizing a double fatty acids yeast culture observed that the expression of heme $a+a_3$ varied fourfold in this mutant strain as a consequence of the lipid growth media supplementation; mitochondria whose membranes were characterized by their elevated levels of C18:1 had the highest heme $a+a_3$ content, while those membranes which contained the increased levels of linoleic acid had the lowest (26). In spite of the findings of Trivedi et al. (26) we have found that with VO and RO diets, containing the same fatty acid profile, a minimum and a maximum cytochrome $a+a_3$ content respectively has been produced. This finding let us suppose that some other factors, in addition to the lipid environment changed by the dietary fats, should probably occur. One of these factors might be the peroxidative degree of the mitochondrial phospholipid produced by each experimental diet. In fact in our studies, as the dietary fat increased the mitochondrial MDA production [either because it was more polyunsaturated (CO) or because it contained less unsaponifiable material (RO)] at the same time it increased also the cytochrome $a+a_3$ content.

When during the last four experimental days, all the groups were treated with a strong oxidative stress produced by intraperitoneal administration of ADR, cytochrome oxidase specific activity and turnover values did not change in CO+ADR and RO+ADR groups while they were significantly enhanced ($p<0.001$) in VO+ADR group. This strong enhancement of the specific activity in VO+ADR group was parallel to a concomitant clear increase of cytochrome $a+a_3$ content in VO+ADR group. This finding could be related to an isoenzymic form much more active but at the same time less influenced by C18:2n6/C18:2n6 cardiolipin. However it was possible to observe such an enhancement in cytochrome $a+a_3$ content also in RO+ADR group even if there was not significatively increase in the enzymatic activity.

The recent evidence for isoforms of cytochrome c oxidase has focused attention on tissue specificity and developmental regulation of the enzyme (3,27). Regulation of cytochrome c oxidase activity by altering the amount of enzyme present in the cell may be particularly important in multicellular organisms in which a tissue could have different energy requirements. This control could be modulated differentially by transcriptional or translational control of different isoforms in heart or in liver, two tissues with very different metabolic needs for ATP (28). The suggested cytochrome $a+a_3$ genetic induction in VO+ADR and

RO+ADR groups agree well with previous data (29) that indicate how during lipid membrane peroxidation there is also an increased demand of cellular energy.

In the CO+ADR group no enhancement was produced and this could be related to a damage of mitochondrial DNA. It must be borne in mind that, in mitochondria, a high lipid/DNA ratio exists allowing the possibility that the extent of covalent modification of mitochondrial DNA is 40 to 50 times greater than that of nuclear DNA (30). This situation should be much more clear in CO group mitochondria after ADR administration because, as we have shown in previous papers (9,10), dietary corn oil produced much more amount of C18:2(n-6) in the subcellular membranes than olive oil. Moreover, it was confirmed in studies "in vitro", that for each C18:1(n-9) molecule that underwent oxidation, the same had happened before for 40 molecules of C18:2(n-6) (29).

In this study we have focused our attention on the cytochrome $a+a_3$ induction in VO+ADR and RO+ADR groups. Induction of membrane enzymes has been observed by several authors especially in case of chemicals and xenobiotics action (31) and a clear example is the induction of mitochondrial cytochrome P-450 that increases two orders of magnitude (31,32,33).

Nevertheless, the mechanisms that lead to an enzymatic induction are still not clear and require much more investigations. We might hypothize that in the same tissue it could be possible to obtain an induction of different isoenzymatic forms of cytochrome oxidase during an increase of cell energy demand, as a consequence of the enhancement of membrane phospholipid peroxidation. It was observed that cytochrome oxidase activity increased together with the increase of MDA (i.e. mitochondrial lipid peroxidation). However it is well known that mitochondrial activities were increased when diets were enriched in polyunsaturated fatty acids; this was explained as a consequence of the lower lifetime of mitochondrial phospholipid fatty acid because increasing the degree of unsaturation also the degree of peroxidation increases (29).

Recently it has been reported that oxygen could be a strong inducer of the synthesis of cytochrome $a+a_3$ (34). We should suggest that another mechanism of induction might be an enhanced production of peroxides at membrane level.

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REFERENCES

1. Lenaz, G. (1988) J. Membrane Biol. **104**: 193-209.
2. Hare, J.F., Ching, E. and Attardi, G. (1980) Biochemistry **19**: 2023-2030.
3. Kadenbach, B., Jarausch, J., Hartmann, R. and Merle, P. (1983) Anal. Biochem. **129**: 517-521.
4. Von Jagow, G., Link, T.A. Schagger, H. (1990) in: "Highlights in Ubiquinone Research" (Lenaz, G., Barnabei, O., Rabbi, A. and Battino, M., eds.) Taylor & Francis, London, pp. 92-97.
5. Yamaoka, S., Urane, R., Kito, M., (1990) J. Nutr. **120**: 415-421.
6. Conroy, D.M., Stubbs, C.D., Belin, J., Pryor, C.L. and Smith, A.D. (1986) Biochim. Biophys. Acta, **861**: 457-462.
7. Holmes, R.P. and Kummerow, F.A. (1985) in: Structure and Properties of Cell Membranes (Benga, G., ed.), vol. 3, pp. 261-280, CRC Press, Boca Raton, FL.
8. Abuirmeileh, N.M. and Elson, C.E. (1980) Lipids, **15**: 925-931.
9. Periago, J.L., De-Lucchi, C., Gil, A., Suarez, M.D. and Pita, M.L. (1988) Biochim. Biophys. Acta, **962**: 66-72.
10. Giron, M.D., Mataix, F.J. and Suarez, M.D. (1990) Biochim. Biophys. Acta, **1045**: 69-73.
11. Freeman, B.A. and Crapo, J.D. (1982) Lab. Invest., **47**: 412-426.
12. Slater, T.F. (1984) Biochem. J., **222**: 1-15.
13. Huertas, J.R., Battino, M., Lenaz, G., Mataix, F.J. (1991) FEBS Lett. **287**: 89-92.
14. Report of ILAR's Committee on Laboratory Animal Diets. Nutr. Abstr. Rev. **49**: 413-419 (1979).
15. Fleischer, S., McIntyre, I.O. and Vidal, J.C. (1979) Methods Enzymol. **55**: 32-39.
16. Vanneste, W.H. (1966) Biochim. Biophys. Acta, **113**: 175-178.
17. Nicholls, P. (1976) Biochim. Biophys. Acat, **430**: 30-45.
18. Degli Esposti, M., Lenaz, G. (1982) Biochim. Biophys. Acta, **682**: 189-200.
19. Battino, M., Rugolo, M., Romeo, G, Lenaz, G (1986) FEBS Lett. **199**: 155-158.
20. Orrenius, S., Moldeus, P., Thor, H. and Hogberg, J. (1977) in: Microsomes and Drug Oxidations (Ullrich, V., Roots, I., Hilderbrant, A., Estabrook, R.W. and Conney, A., eds.) pp. 292-306, Pergamon Press, New York.
21. Lowry, O.H., Rosebrough, H.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. **193**: 265-275.
22. Sugioka, K., Nakano, H., Noguchi, T., Tsuchiya, J. and Nakano, M. (1981) Biochem. Biophys. Res. Commun., **3**: 1251-1258.
23. Solaini, G., Landi, L., Pasquali, P. and Rossi, C.A. (1987) Biochem. Biophys. Res. Commun., **147**: 573-580.
24. Goormaghtigh, E., Pollakis, G. and Ruysschaert, J.M. (1983) Biochem. Pharmacol. **32**: 889-893.
25. Demant, E.J.F. and Jensen, P.K. (1984) Eur. J. Biochem. **132**: 551-556.
26. Trivedi, A., Fantin, D.J. and Reno Tustanoff, E. (1986) Biochem. Cell. Biol. **64**: 1195-1210.
27. Kuhn-Nentwig, L. and Kadenbach, B. (1985) Eur. J. Biochem. **149**: 147-158.
28. Capaldi, R.A. (1990) Annu. Rev. Biochem. **59**: 569-596.
29. Sevanian, A., Hochestein, P. (1985) Ann. Rev. Nutr., **5**: 365-390.
30. Backer, J.M., Weinstein, I.B., (1980) Science, **209**: 297-299.
31. Dannan, G.A., Guengerich, F.P., Kaminsky, L.S., Aust, S.D. (1983) J. Biol. Chem. **258**: 1282-1288.
32. Waterman, M.R. (1982) Xenobiotica, **12**: 773-786.
33. Miller, M.S., Jones, A.B., Chauhan, D.P., Park, S.S., Anderson, L.M. (1991) Biochem. Biophys. Res. Commun. **176**: 280-287.
34. Asson-Batres, M.A. and Hare, J.F. (1991) J. Biol. Chem. **266**(15): 9932-9938.