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Mitochondrial Respiratory Chain Features After γ-Irradiation

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Radiation provokes damage to DNA but also to membrane and protein structure. Radiolysis is a tool used very often in the study of free radical biological effects and of scavenger molecules effectiveness. Nitroimidazoles have been demonstrated to enhance the radiation effects on biological structures. The studies we have performed on isolated mitochondria irradiated, with and without nitroimidazoles, at a radiation dose equal to LD_{90}, indicate that this treatment is not able to affect the structural and functional features investigated (ubiquinone-10, fatty acids, respiratory cytochrome levels or membrane fluidity and respiratory enzymatic activities), suggesting that an involvement of such externally produced radicals on membrane damage is unlikely. Moreover it was ascertained that the mitochondrial redox activities do not take part into the intracellular nitroimidazole reduction.

INTRODUCTION

Free radicals can be produced in the cell by various processes, including homolysis of molecules which possess weak bonds, radiolysis, photolysis, one electron transfer from transition metal ions to organic species, exposure to pollutants such as ozone, NO_2, singlet oxygen, and enzymatic processes. Ionizing radiations act on biochemical systems via free radicals production, mainly due to water radiolysis and their effectiveness in killing cells is mainly ascribed to double strand breaks in the DNA[1] but even the membrane damage should not be underestimated.[1–3] We also have recently obtained evidences that severe peroxidative damages could be provoked by gamma radiation in Coenzyme Q (CoQ) depleted mitochondria.[4–6] Similar peroxidative damages at membrane level have been described to occur when free radical production is provoked either physiologically or pseudophysiologically.[7,8] For this reason γ-irradiation could be an useful tool for producing free radical insult at controlled rates and extents. For example, it is possible to calculate that in an aqueous solution $2{\cdot}10^{-6}$ M of OH radicals are produced by a 8 Gray irradiation dose.

The role of chemical modifiers in cancer treatment is to combine with cell killing effects of radiation in order to obtain a therapeutic gain.[9]

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In particular the electron-affinic radiosensitizers are used to overcome the resistance of hypoxic regions to the radiation treatment, due to the poor content of oxygen: in these cells they can mimic the radiation damages induced by oxygen free radicals.

The radiobiology behaviours of a couple of electron-affinic nitroimidazole radiosensitizers such as Misonidazole (MISO) and Etanidazole (SR 2508) have been extensively studied both *in vitro* and *in vivo*[10,11,12] and some data are also available with regard to their subcellular compartmentalisation.[13] In hypoxic conditions these compounds are reduced by cellular enzymes, like Xantine Oxidase or NADPH-cyt.c reductase,[14] or by radiation-produced radicals.[15] The reduced forms, mainly the one-electron nitro radical anion, bind to the cell macromolecules leading to irreversible free radical damages.[16]

Many workers have reported damaging effects of ionizing radiation on biological membranes in both model systems and in whole cells. A wide variety of effects has been observed including peroxidation of unsaturated lipids, increased permeability of membranes to iones, changes in electrophoretic mobility and inactivation of membrane-bound enzymes.[17] However, the irradiation doses frequently used in these studies are several orders of magnitude higher than the dose lethal for living cells and the data available are, for this reason, unsuitable for the applications to physiological (or physiopathological) aims.

Electrochemical techniques as polarography and cyclic voltammetry are easy and precise methods to investigate the redox status of molecules and the determination of the electrochemical behaviour is basic in the studies involving radiosensitizers.

Since few data are still available on radiosensitizers' possible effects on organelles like mitochondria, responsible of intensive redox activity, we have undertaken systematic studies in order to evaluate if: a) they could be reduced by mitochondrial redox systems and then affect their structural or functional features; b) their radiation-stimulated reduction can modify the γ-rays effects on membranes. Moreover, a fundamental aim was to clarify the existence and the extent of peroxidative damages on lipids or proteins after an irradiation dose corresponding to a LD_{90}: such irradiation dose is greatly lower than that usually reached in studies for determining irradiation effects, but at the same time it is the maximum amount that could allow some probability of cell survival.

METHODS

Misonidazole (2-nitro-1-imidazolyl)3-methoxy-2-propanol (MISO) and etanidazole N-(2-hydroxyethyl)-2-nitroimidazolyl acetamide (SR2508) were supplied by Hoffman-La Roche, Basel, Switzerland and A.G. Phama, Linz, Austria, respectively. Different ubiquinone (CoQ) homologues were supplied by Eisai Co., Tokyo, Japan and they were stored as solutions in absolute ethanol at –20°C at concentrations ranging between 1 and 10 mM as determined spectrophotometrically at 275 nm using extinction coefficients typical of each homolog.[18] Ubiquinone-2 was reduced using the method of Rieske.[19] 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.

The redox state of MISO and SR2508, before and after incubation with mitochondria, was determined by cyclic voltammetry using an AMEL multipolarograph Mod. 433 connected with a PC. The measurements were made in the voltage range –0.2/–0.8 V, scan speed 2 V/sec. A dropping mercury electrode was used as working electrode, a saturated calomel electrode (SCE) and a platinum electrode as reference and counter electrode, respectively. Nitroimidazoles were prepared both in 25mM K-phosphate buffer, pH 7.5 and 0,25 M Sucrose- 10 mM TRIS buffer, EDTA 1 mM, pH 7,4, using 0.1 M KCl as supporting electrolyte. The mitochondria, sus-

pended in the same Sucrose buffer, were incubated in presence of nitroimidazoles for different times, with a maximum of two hours, both in air and in N_2 atmosphere. Incubation in anoxic conditions was performed in modified flasks to allow a continuous flux of N_2. NADH at concentration 4-fold the nitroimidazoles was added for inducing mitochondrial electron transfer.

The irradiations of diluted mitochondria suspensions (0.5–1.0 mg/ml) were performed in a γ-cell (^{60}Co) (Canadian atomic energy, Ltd.) to reach an irradiation dose of 8 Gray with a dose rate of 4 Gray/min. The cobalt-60 rods were arranged in a cylindrical fashion around a cavity in which irradiation was carried out and samples could be moved up and down by means of a mechanical plug controlled by a digital timer.[6] Beef heart mitochondria (BHM) were prepared as described elsewhere.[20] When present the concentrations of drugs were 0.058 mM and 0.2 mM for MISO and SR 2508 respectively. The mitochondria were assayed for CoQ content by reversed-phase HPLC analysis as previously described[21] after extraction with methanol and light petroleum using the method of Kröger[22] in a WATERS Data Module M730-Model 721 Programmable System Controller equipped with LAMBDA-MAX Model 481 LC Spectrophotometer. The content in cytochromes 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.[21] All respiratory chain activities were determinated at 25°C in a 25 mM K-phosphate buffer, pH 7.5 under quasi-saturating substrate concentrations. NADPH-cyt. c reductase, succinate-cyt. c reductase and ubiquinol 2-cyt. c reductase activities were performed, in the above mentioned buffer with the addition of 1 mM KCN, by monitoring the absorbance increase of cyt. c (Sigma horse heart type III) upon reduction at 550 minus 540 nm in a Sigma ZWS dual wavelength spectrophotometer using an extinction coefficient of 19.1 mM^{-1} cm^{-1} for cytochrome c.[21] In the determination of ubiquinol 2-cyt. c reductase activity the final ethanol concentration never exceeded 0.2% and CoQ_2H_2 concentration never exceeded 15 μM according to Battino *et al.*[23] Cytochrome oxidase activity was assayed using cyt.c reduced by dithionite and purified on a Sephadex G-25 column as substrate, by monitoring the absorbance decrease of cyt.c upon oxidation at 417-409 nm.[21,24]

Succinate-CoQ reductase activity was measured indirectly by following the CoQ_2 dependent reduction of Dichloroindophenol (DCIP)[25] as the absorbance decrease at 600 nm utilizing a Perkin-Elmer 559 UV-VIS spectrophotometer and using an extinction coefficient (reduced minus oxidized) of 21 mM^{-1} cm^{-1}. Mitochondrial membrane suspensions were treated with the lipid-soluble fluorophore 1,6diphenyl-1,3,5-hexatriene (DPH) (obtained from Molecular Probes, Junction City, OR) and steady-state fluorescence polarization measurements were accomplished in a Jasco FP-777 Spectrofluorometer. The polarization of fluorescence was expressed in terms of the fluorescence anisotrophy, "r", that provides an estimate of the static components of "fluidity", i.e. lipid order.[21] Total lipids were extracted from mitochondria according to Folch et al.[26] Fatty acids were methylated and composition analysis was performed on a Varian mod. 3700 gas-chromatograph equipped with a glass column (2 m × in i.d.) packing with 15% DEGS on 80/100 mesh Gas-Chrom P at 200°C with N_2 as carrier gas at a flow rate of 30 ml/min. The gaschromatographic peaks were identified on the basis of their retention time ratio relative to methylstearate, predetermined on authentic sample. Gas-chromatographic traces and quantitatives evaluations were obtained using a Spectra Physic mod. 4100 computing integrator. Mitochondrial protein concentrations were determined with the method of Lowry.[27]

RESULTS AND DISCUSSION

Nitroimidazoles, used either as antiparasitic drug or as tumor radiosensitizers undergo metabolic or

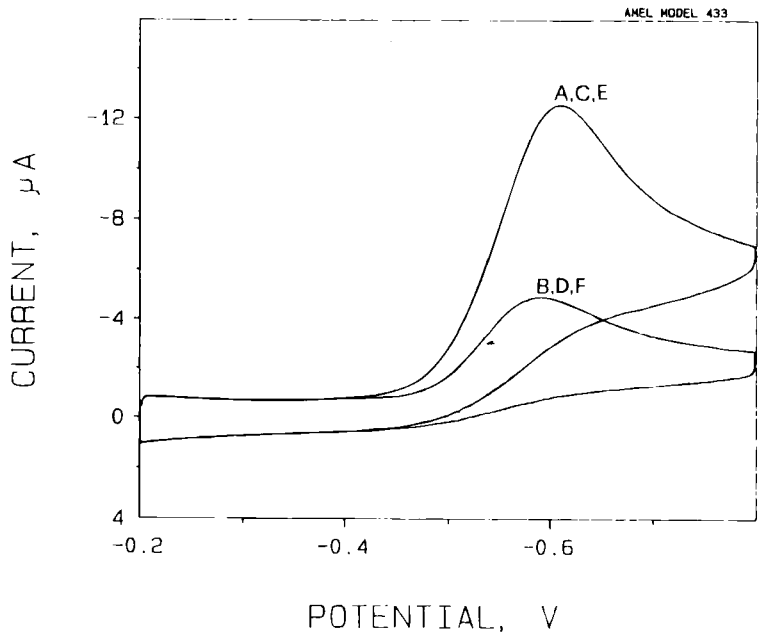

FIGURE 1 Pattern of voltammetric waves of SR2508 (A, C, E) and MISO (B, D, F) in the following conditions: alone in a sucrose buffer (A) and (B), respectively; after two hours incubation with mitochondria in anoxic (C) and (D) and oxygenated conditions (E) and (F), respectively.

radiolytic reduction, and have damaging effects via the involvement of a radical anion. Both nitroimidazoles, in anoxic conditions and in presence of reductive substrates to promote their reduction, did not show any change in their redox state, even after 2 hours of incubation with mitochondrial membranes. The presence of Misonidazole or Etanidazole in the unchanged (i.e. oxidized form) was revealed by the appearance, in the voltammetric graph, of the reduction peak at the voltage value typical for each drug in Sucrose buffer. Fig. 1 shows the peaks obtained for MISO and SR, respectively, after two hours incubation with mitochondria. They are identical to the graphs obtained from Sucrose buffer solution of the drugs. As expected no changes occurred in the case of incubation in oxygenated conditions. These results clearly show that the nitroimidazoles are not a suitable alternative substrate for the redox activities of mitochondrial respiratory chain, as it was also confirmed spectrophotometrically.

Ionizing radiations are known to produce their damaging effects on biological structures via a typical free radical process, with subsequent modifications of molecules structure and function. The experiments we have performed indicate that γ-irradiation at LD_{90} dose both in presence and in absence of drugs capable to amplify free radical amount and damage, do not affect the parameters investigated. In fact the HPLC and GC analysis (Table I and Table II, respectively) point out how both CoQ and fatty acids levels are unchanged either in the solely

TABLE I Coenzyme Q_{10} levels detected by HPLC measurements. Data are expressed as nmoles/mg mitochondrial protein.

	Not irradiated	Irradiated
Control	2.55 ± 0.06	2.56 ± 0.04
+MISO	2.54 ± 0.09	2.56 ± 0.02
+SR	2.52 ± 0.04	2.55 ± 0.10

Results are means ± S.D. for no. of samples ≥ 5.
+MISO = sample with MISO 58 μM, +SR = sample with SR 2508 200 μM.
Data of "Not irradiated" samples were obtained after two hours of incubation; "Irradiated" samples underwent irradiation (as specified in the text) after the same kind of incubation.

TABLE II Fatty acid levels detected by means of GC analysis

	C	C*	MISO	MISO*	SR	SR*
16:0	13.5 ± 3.1	13.6 ± 2.9	11.9 ± 4.0	12.3 ± 3.8	13.9 ± 3.2	12.7 ± 3.4
16:1	3.1 ± 0.6	3.1 ± 0.3	3.2 ± 0.5	3.1 ± 0.4	3.8 ± 0.5	4.0 ± 0.5
18:0	14.2 ± 1.4	14.6 ± 1.5	12.7 ± 2.9	14.1 ± 2.2	13.8 ± 2.1	14.1 ± 2.5
18:1	15.1 ± 2.1	15.2 ± 1.4	14.0 ± 3.8	14.1 ± 3.1	15.8 ± 2.5	14.7 ± 1.9
18:2	27.4 ± 3.3	27.4 ± 2.1	29.1 ± 4.0	29.9 ± 3.8	25.9 ± 3.4	26.6 ± 3.6
20:4	8.9 ± 1.1	8.8 ± 0.8	7.7 ± 1.5	7.9 ± 1.6	9.0 ± 1.4	8.5 ± 1.2

Data are expressed as per cent of total amount of fatty acid. Only the most indicative fatty acids are reported. Results are means ± S.D. for a no. of samples ≥ 5.
*Irradiated samples. C = control; other abbreviations and sample treatment as in Table I.

presence of gamma-irradiation or in drug enriched samples. This is of deep importance in the light of the mechanism that leads to the cell damage and death provoked by free radicals attack, since both CoQ and PUFA are critical markers of peroxidative injuries suffered by biological structures.[4,6,28,29] The finding of their unchanged levels might suggest that free radicals produced mainly in hydrophylic cell compartments have DNA as main biological target, as previously widely suggested and partially demonstrated.[1] Even if DPH fluorescence polarization investigations are not unambiguous,[21] because changes in depolarization of DPH fluorescence are unable to undoubtfully indicate changes in microviscosity of membrane lipids,[30] the DPH polarization values are thought to be indicators of changes in local lipid packing that might result from changes in bilayer structure or composition. However, the values obtained (Table III) are strictly similar to each other indicating that, in any case, probably no modifications occur in the different conditions tested.

Further investigations concerned the study of some mitochondrial respiratory activities and the detections of respiratory cytochrome amounts. All activities were tested in order to evaluate the specific sensitivity to their own respective inhibitors: NADH-cyt. c reductase activity was generally about 88% rotenone sensitive (0.1 μg/ml), Succinate- cyt.c reductase and Succinate-DCIP reductase activities about 95% malonate sensitive (10 mM), CoQ_2H_2-cyt. c reductase activity over 95% antimycin A sensitive (0.25 μg/ml) and Cytochrome oxidase activity 100% KCN sensitive (1 μM). Again, the data show (Table IV and Table V) that no significant alterations have been found. As far as the CoQ_2H_2-cyt.c activity is concerned, irradiation, with or without the sensitizer, even produced a slight stimulation of the activity itself, which is not significant. Moreover, the result that cytochrome levels too did not change in the different situations indicates that the unaltered values of enzyme activities were really due to unchanged properties of the different respiratory complexes and they were not due, for example, to less numerous but more active respiratory complexes. This kind of approach could be suitable for evaluating and discriminating the effect of free radicals spreading out in the acqueous phase on the membrane phase, too. The role played by the radicals produced in the environment surrounding the membranes as well as the damages they provoke are not univocally clarified in the framework of the overall process that lead to membrane damages and cell death.

TABLE III DPH fluorescence polarization values at 25°C of the treated and untreated samples

	Not irradiated	Irradiated
Control	0.238 ± 0.008	0.239 ± 0.007
+MISO	0.235 ± 0.004	0.234 ± 0.006
+SR	0.236 ± 0.003	0.239 ± 0.005

Results are means ± S.D. for a no. of samples ≥ 5.
Abbreviations and sample treatment as in Table I.

The previous findings that only extremely high ionizing radiation doses severely interfere

TABLE IV Mitochondrial respiratory activities. Data are expressed as μmol/min/mg mitochondrial protein.

	C	C*	MISO	MISO*	SR	SR*
NADH-cyt.c	0.90 ± 0.02	0.88 ± 0.04	0.86 ± 0.04	0.84 ± 0.04	0.85 ± 0.05	0.87 ± 0.05
Succ-cyt.c	0.36 ± 0.02	0.35 ± 0.01	0.36 ± 0.01	0.36 ± 0.02	0.39 ± 0.02	0.39 ± 0.02
Succ-DCIP	0.17 ± 0.02	0.18 ± 0.01	0.19 ± 0.01	0.19 ± 0.01	0.18 ± 0.02	0.18 ± 0.01
Q_2H_2-cyt.c	2.29 ± 0.27	2.88 ± 0.21	2.80 ± 0.24	2.80 ± 0.19	3.01 ± 0.28	2.92 ± 0.26
Cyt.Ox.	4.70 ± 0.38	4.82 ± 0.43	4.67 ± 0.40	4.76 ± 0.38	4.91 ± 0.45	4.80 ± 0.41

Results are means ± S.D. for a no. of samples > 5. Abbreviations and sample treatment as in Table I.

with biomembrane structure and function are confirmed by our investigations. Inactivation of mitochondrial enzymes by γ-irradiation in vitro was deeply investigated in the past.[31–36] A study of the phosphorylative capacity of mitochondria prepared from livers of rats exposed 4 hours previously to 1,000 rads of total body X-irradiation showed no effects of exposure on oxygen consumption or phosphate esterification under a variety of conditions.[31] When mitochondria, from spleen and livers, were isolated 24 hours after radiation P:O ratio was somewhat depressed, but this effect was shown to be indirect, through activation of the pituitary and subsequently of the thyroid and the adrenal cortex.[32,36] In general, in experiments conducted by direct irradiation of isolated mitochondria, the oxidative system has proved to be more resistant to radiation than has the phosphorylative system, the first phosphorylating site being more readily uncoupled than the other two sites.[33] When a wide range of radiation dose was used (0–100 Krads), the respiratory control of mitochondria oxidizing different substrates fell by 20–42%, but only after 100 Krads of irradiation.[34]

Our data unequivocally show that, even in the presence of sensitizers, lower doses, administered to isolated mitochondria, do not produce any functional derangement. In fact irradiation dose that is lethal for the 90% of a cell culture was uncapable of producing any changes. Such results perfectly agree with the hypotesis that the location of free radicals source is one of the most decisive factor for the effectiveness of the free radicals themselves. In other words, when free radicals are produced far from their main targets (i.e. in this case outside of membrane environment) they could be more easily inactivated by the same water matrix. Otherwise it would be difficult to explain by which mechanism, a very limited, physiological, free radical productions yet taking place at critical membrane sites, could induce severe damages to membrane structure itself. Similarly, it must be underlined that the potential scavenging capability of antioxidant molecules can be displayed only when such molecules are sufficiently closed to the specific free radical targets, i.e. located in the same environment of both the radical source and the target structure.

TABLE V Respiratory cytochrome levels

	Before Irradiation			After Irradiation		
	$a + a_3$	b	$c + c_1$	$a + a_3$	b	$c + c_1$
C	1.08 ± 0.10	0.58 ± 0.07	1.21 ± 0.12	1.01 ± 0.11	0.62 ± 0.09	1.24 ± 0.13
MISO	1.12 ± 0.13	0.59 ± 0.05	1.25 ± 0.10	1.08 ± 0.09	0.60 ± 0.08	1.29 ± 0.11
SR	1.05 ± 0.10	0.56 ± 0.07	1.27 ± 0.14	1.09 ± 0.12	0.61 ± 0.09	1.21 ± 0.11

Data are expressed in nmol/mg mitochondrial protein. Results are means ± S.D., no. of samples >5. Abbreviations and sample treatment as in Table I.

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