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Mitochondrial Responses under Chemical Stress

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Abstract

The science of toxicology today has reached a stage where the change from exploration of biological responses to xenobiotics in terms of phenomenology, and to mechanistic explanations based on molecular logistics is all quite apparent. A conceptual mechanism regarding unity and diversity of stress has been proposed by our laboratory. The generation of free radicals leading to membrane damage and alterations in calcium homeostasis seems to be the common unspecific mechanism in toxic responses which may lead to cell death. Changes in the mitochondrial structure and functions due to chemical or oxidative stress gives mechanistic clues and is an ideal system for studying such a phenomenon. Mitochondrial responses to some chemical agents with respect to its redox equilibrium are presented here.

Keywords: mitochondria, reactive oxygen species, calcium deregulation.

Introduction

There are many factors that decide a chemical's adverse effects. Tissue distribution of xenobiotics, compartmentation within a cell's microenvironments, localization of enzyme systems and the cellular distribution of antioxidant defense mechanisms all contribute to our inability to establish generalized mechanisms for all xenobiotics [1]. This may be the reason why a xenobiotic is toxic in one tissue and not highly toxic in another. Advancement in biochemical methodology and molecular biology has led to the development of a molecular approach in toxicology [2]. Introducing any chemical or other stress factor in any dose, by any route, or for any duration and reporting any change, as compared with placebo controls, is no longer the approach of biochemical toxicology. The accent now is on arriving at specific clues to the mechanism of initiation and propagation of the toxic processes and biological defenses against this, so that diagnostic and curative measures may emerge.

In this reductionist approach of toxicologists, the accent today is on knowing the phenomenon taking place inside a cell. It has been observed that the generation of free radicals leading to peroxidative decomposition of polyunsaturated fatty acids and ensuing membrane perturbations are the rule rather than the exception in chemical toxicity. These changes seem to be the effects of an early unspecific event in toxicity rather than specific causative mechanisms. Another aspect which has been much accepted is the role of the deregulation of Ca^{2+} and functions [3] and their far reaching implications in chemically induced cytotoxicity. To study the two phenomenon together mitochondria were found to be the ideal choice as they act as safety devices against toxic increases of cytosolic Ca^{2+} and are also a major source of intracellular active oxygen species generation.

Materials and Methods

Male albino Wistar rats weighing 100-150 g, fed on Lipton pellet diet and water ad libitum were used throughout the study.

All the chemicals used were either procured from Sigma Chemicals Co., St. Louis, MO, USA or E.Merck extrapure. Deionized HPLC-grade water was used throughout the study. Injectible diazepam was procured from Ranbaxy Laboratories Ltd., India.

Diazepam was administered to a group of 6 rats i.p. in a dose of 3 mg/kg body weight which were sacrificed after 1 hr. Another groups of six animals given i.p. saline served as control. The brains were immediately taken out and mitochondria prepared by differential centrifugation [4]. For *in vitro* studies liver was also taken out, mitochondria prepared as above and suspended in 0.02 M Tris-0.15 M KCl, pH 7.4. All the operations were carried out under cold conditions (0-4°C).

Swelling of freshly isolated rat liver mitochondria was carried out according to the spectrophotometric method of Lehninger [5]. It was recorded as a decrease in optical density at 520 nm at 1 min interval for a period of 5 minutes as reported earlier [6]. The influence of some biological response modifiers was tested by preincubating them with freshly isolated mitochondria.

Ca^{2+} stimulated Mg^{2+} dependent ATPase was assayed according to Stewart [7] and µmole Pi liberated was calculated. NADH cytochrome c reductase was measured by the method of Mahler [8]. Cytochrome c oxidase was spectrophotometrically assayed according to Cooperstein and Lazarow [9] and Monoamine oxidase by Tabor [10]. Nicotinamide adenine nucleotidase (NADase) was assayed according to Kaplan [11]. The activity was expressed in terms of cleavage of 1 µmole of NAD+ under the specified conditions. Glutathione reductase activity was measured by the method of Carlberg and Mannervik [12] and reduced glutathione using Ellman's reagent in the deproteinized extract of the subcellular organelle [13]. Protein was estimated according to the method of Lowry et al. [14], using bovine serum albumin as standard.

Each experiment was done in 4 replicates unless stated otherwise and statistical evaluation was done using Student's *t* test [15] considering $p < 0.05$ as significant.

Results

The effect of some chemicals on the mitochondrial structure and function was tested. Since superoxide radial is generated by monovalent reduction of O_2 and under many conditions of chemical stress, its effect on some key enzymes of mitochondria was observed. Table 1 shows that in situ O^-_2 generation caused 5 fold lowering of Ca^{2+} stimulated Mg^{2+} dependent ATPase. When the assay system was preincubated with 70 units of SOD the activity was still 40% lower than control. NADase activity was also inhibited by 52 % in the presence of O^-_2 and SOD reduced this to half. Similar results were obtained with NADH cytochrome c reductase where O^-_2 caused 55% inhibition of the activity and presence of SOD in the incubation system gave some protection against the onslaught of O_2 but the value remained 65% of that of control. Thus, in situ generation of superoxide ions lowers the activity of some key enzymes of mitochondria which play an important role in maintaining the structural and functional integrity of mitochondria.

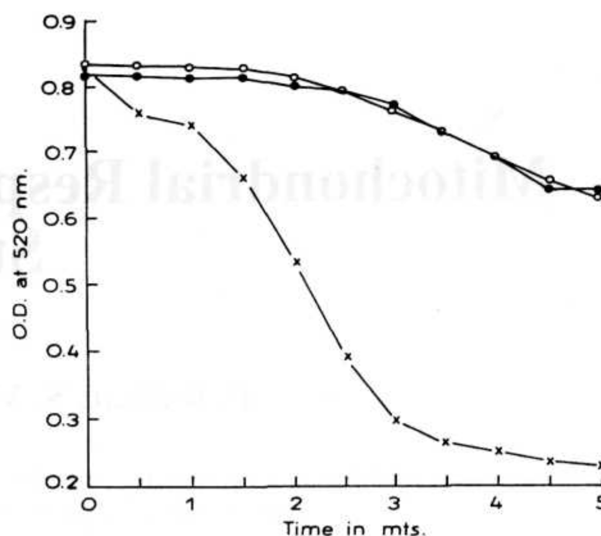

Fig. 1. Effect of 0.5 mM (0—0) and 1 mM (•—•) EDTA on t-BHP (75 µM x—x) induced swelling of mitochondria.

Mitochondria act as safety devices during the sustained increase in intracellular Ca^{2+} under toxic conditions. They often pass through a transient condensation phase, loose their metrical granules and eventually undergo swelling before the membrane ruptures and they undergo irreversible injury. During the course of our studies we have found that Ca^{2+} induced and oxidative swelling of mitochondria are good models to get indications for the toxicity of a chemical. Since under many chemical and oxidative stress conditions peroxides are formed, studies were conducted with isolated mitochondria using organic and inorganic hydroperoxides. Fig. 1 shows that t-BHP causes large amplitude swelling of mitochondria which was abolished to a great extent in the presence of EDTA a chelator of transition metal ions like Fe^{2+} Cu^{2+}. Diethyl dithiocarbamate, a Cu^{2+} chelator, prolonged the lag phase of swelling (Fig. 2) but did not abolish it completely and the effect was dose dependent. Thus during peroxide induced swelling of mitochondria,

Table 1 Changes in some mitochondrial enzymes due to O_2^- generation and its scavenging.

Enzymes units/mg protein	Enzyme activity units/mg protein		
	Control (a)	H.X + X.O. (b)	O_2^- generation + SOD (c)
Ca^{2+} – Mg^{2+} ATPase	1.040 ± 0.034	0.208 ± 0.013*	0.624 ± 0.029*
NADH cytochrome c reductase	8.085 ± 0.665	3.61 ± 0.259*	5.205 ± 0.375*
NADase	0.345 ± 0.012	0.165 ± 0.014*	0.260 ± 0.020*

Values are arithmetic mean of 4 observations ± S.D
* $p < 0.001$ calculated b vs (a) and (c) vs (b); H.X - Hypoxanthine $5x10^{-5}$ M; X.O. - Xanthine oxidase 0.025 units; SOD - superoxide dismutase 70 units.

Fe^{2+} and Cu^{2+} seem to play an important role in interconversion and the generation of free radicals and compromising membrane impermeability. During the manifestation of peroxide-induced changes in mitochondria, the level of reducing equivalents in the form of GSH plays an important role in protecting the mitochondria. Table 2 shows the decrease in GSH levels in the presence of H_2O_2 t-BHP and cumene hydroperoxide. Further, depletion of GSH by N-ethylmaleimide caused potentiation of t-BHP induced swelling as shown in Fig. 3. Thus, confirming the protective role of GSH during peroxidative processes.

Table 2. Effect of peroxides on GSH content of mitochondria.

Supplements	nmoles GSH/mg protein
M + None (Control)	8.350 ± 0.682
M. + 750 μM hydrogen peroxide	7.385 ± 0.416*
M. + 500 μM tert-butyl peroxide	5.419 ± 0.319**
M. + 500 μM cumene hydroperoxide	5.323 ± 0.398**

Values arc arithmetic mean of 4 ± S.D. observations
* $p<0.1$, ** $p<0.05$.
H_2O_2: hydrogen peroxide; t-BHP: tert-butyl peroxide; CHP: cumene hydroperoxide.

Effect of some commonly used chemical agents on $CaCl_2$ induced swelling of mitochondria was also observed. Anaesthetic ether extensively used on experimental animals caused potentiation of Ca^{2+} induced swelling (Fig. 4). It was found that histamine causes swelling of mitochondria whereas benadryl (Fig. 5), an antihistaminic agent widely used for therapeutic purposes, caused reduction in swelling, thus according protection.

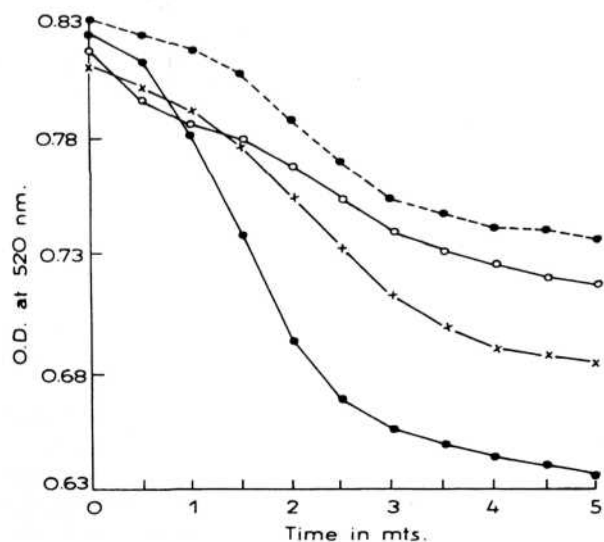

Fig. 2. Protective effect of 250 μM (x—x), 500 μM (0—0) and 1 mM (•--•) diethyl dithiocarbamate on t-BHP (75 μM •—•) induced swelling of mitochondria.

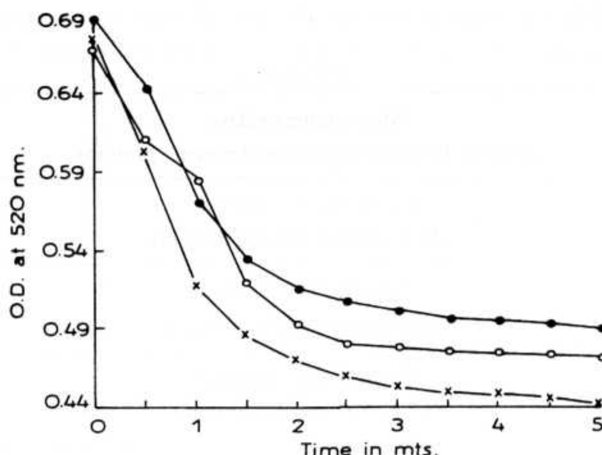

Fig. 3. Potentiation of t-BHP (75 μM •—•) induced swelling of mitochondria by 500 μM (0—0) and 1 mM (x—x) thiol depleting agent, A'-ethylmaleimide.

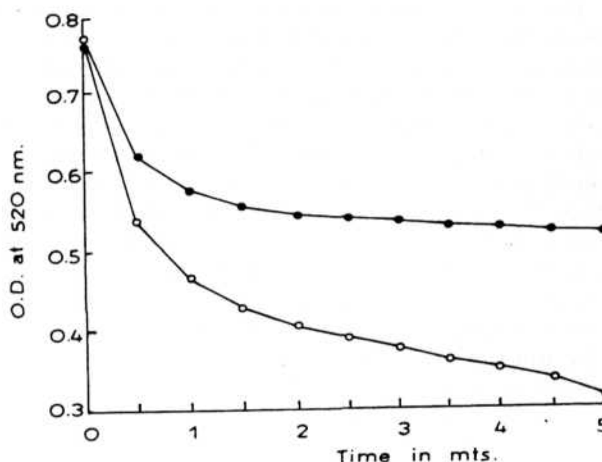

Fig. 4. Effect of in vivo anaesthetic ether exposure (0—0) on 2 mM $CaCl_2$ induced *in vitro* (•—•) swelling of isolated rat liver mitochondria.

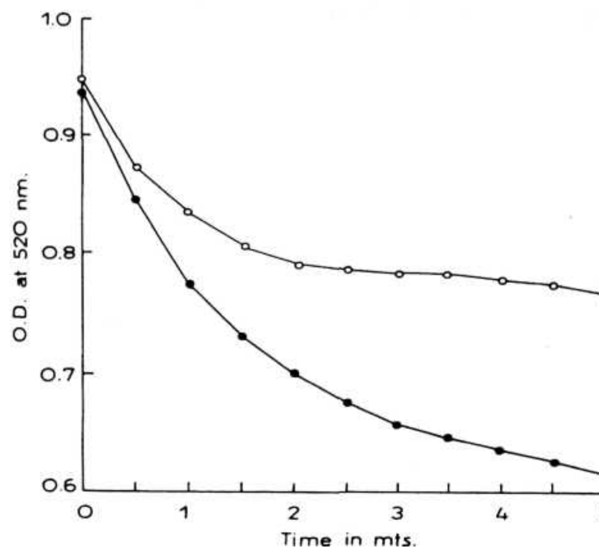

Fig. 5. Effect of 500 μM Benadryl (0—0) on 2 mM $CaCl_2$ induced (•—•) swelling of mitochondria.

Table 3. Changes in some mitochondrial enzymes due to diazepam treatment.

Enzymes	Control	Treated
Monoamine oxidase (nmoles benzaldehyde formed/min/mg protein)	1.424 +0.090	1.013+0.095*
Ca^{2+} – Mg^{2+} – ATPase (μM Pi liberated/hr/mg protein)	8.102 ± 0.114	9.182 ± 0.239*
Cytochrome c oxidase (μM activity/min/mg protein)	1.361 ± 0.063	1.972 ± 0.098*
Glutathione reductase (unit x 10^{-3}/min/mg protein)	18.743 ± 0.986	11.076 ± 0.610*

Values are arithmetic mean ± S.D. of six determinations in each case.

* $p < 0.001$

Diazepam, a benzodiazepine extensively used in anxiety disorders, was found to reduce O_2^- induced swelling of liver (Fig. 6) as well as brain mitochondria, showing free radical scavenging properties. Diazepam induces early oxidative changes at the subcellular level in rat brain [16]. Whereas TBARS formation was enhanced in mitochondria, as it has benzodiazepine receptors, Mn-SOD level was reduced significantly in cerebrum and brain stem regions. The activity of some key mitochondrial enzymes was estimated in brain of rats treated with 3 mg/kg diazepam and sacrificed after one hour. Table 3 shows significant reduction in monoamine oxidase activity whereas inner mitochondrial membrane enzyme cytochrome c reductase showed 44% enhanced activity. This could be due to binding of diazepam on the outer membrane of mitochondria. Ca^{2+} - Mg^{2+} - ATPase activity was also enhanced indicating changes in Ca^{2+} fluxes due to diazepam binding to mitochondria. The matrix enzyme glutathione reductase showed a significant decrease in activity being 59% of that of control. This supports our earlier observation of depletion of GSH in diazepam treated rat brain [16].

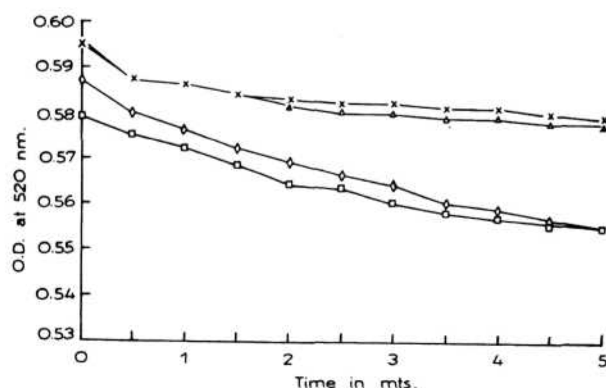

Fig. 6: Effect of 0.05 mg (□—□), 0.1 mg (Δ—Δ) and 0.15 mg diazepam (x—x) on O_2" induced (◊—◊) swelling of rat liver mitochondria.

Discussion

Mitochondria play an important role in maintaining a cell's viability. It has been observed that mitochondria are foci of events that initiate or effect cell death [17]. Thus, for the past few years our group and other international research groups have focussed their attention towards unravelling the mitochondrial responses to stress in general. In an earlier study it was observed that O_2^- and other free radicals formed due to it, cause significant ultrastructural changes such as break in inner and outer membrane, loss of matrix material, changes in cristae folding and clumping of mitochondria. Supplementation with external SOD accorded protection against it [18].

In the present study a decrease in NADH cytochrome c reductase indicates structural damage and possible impairment of biological oxidation. In addition to this, the decrease in Ca^{2+} stimulated Mg^{2+} dependent ATPase could indicate that Ca^{2+} fluxes are also affected. Similarly, nicotinamide adenine nucleotidase (NADase) is a key enzyme involved in the glutathione cascade of hydroperoxide metabolism. It catalyzes the hydrolysis of oxidized niacinamide nucleotide (NAD+), resulting in the formation of ADP-ribose and free nicotinamide. Earlier studies in our lab showed inhibition of cytochrome c oxidase, the inner membrane enzyme; and glutathione peroxidase (matrix enzyme) whereas monoamine oxidase (the outer membrane) enzyme was found to be activated [6] under the onslaught of O_2^-.Inhibition of glutathione peroxidase and NADase by ROS may prevent the metabolism and detoxification of hydroperoxides, thereby weakening the inbuilt defense mechanisms along with functional impairment of mitochondria.

Calcium-induced and oxidative swelling of mitochondria is a good model for studying changes in mitochondrial functions. It was found that chelating agents (Figs. 1 and 2) act as antioxidants and reduce oxidative swelling of mitochondria. Tert-butyl hydroperoxide and other peroxides depleted GSH from mitochondria (Table 2) and the protective role of GSH was further confirmed by enhancement of t-BHP induced swelling of mitochondria by N-ethyl maleimide, the GSH depleting agent (Fig. 3).

In a recent report it was observed that reduced thiols support the action of H_2O_2 to enhance the efficacy of Ca^{2+} induced Ca^{2+} release in cardiac myocytes [19]. Benadryl, an antihistaminic agent and diazepam (Fig. 5 and 6) were found to reduce Ca^{2+} induced swelling, whereas anaesthetic ether was found to enhance it (Fig. 4). Our earlier studies also showed generation of free radicals and modulation of antioxidant defenses of rat brain due to anaesthetic ether [20, 21]. Early biochemical changes in rat brain mitochondria due to diazepam also includes oxidative changes [16], whereas long term treatment with diazepam gives protection against ROS mediated processes (unpublished results). The changes in outer mitochondrial membrane enzyme MAO after in vivo diazepam treatment shows lowering of activity which could be due to binding of diazepam to benzodiazepine receptors and causing some membrane perturbations. Ca^{2+} - Mg^{2+} ATPase activity enhanced significantly indicating alterations in Ca^{2+} fluxes due to diazepam. Cytochrome c oxidase, the inner mitochondrial membrane enzyme showed enhanced activity which was 44 % more than in unexposed rat brain mitochondria. Glutathione reductase, the key enzyme responsible for converting GSSG to GSH showed significantly lowered activity ($p < 0.001$), thus confirming our earlier observations that GSH depletion is involved amongst the immediate effects of diazepam treatment.

A variety of chemically different prooxidants cause Ca^{2+} release from the mitochondria via a route which is physiologically relevant. When the released Ca^{2+} is excessively cycled by mitochondria, they are damaged. This leads to uncoupling, a decreased ATP supply and a decreased ability of mitochondria to retain Ca^{2+} [22, 23]. Calcium being the "toxicological second messenger" mitochondrial permeability transition plays a very important role in apoptotic and necrotic cell death [24, 25]. Our laboratory has been conducting studies in search of an explanation of the correlation between oxygen radicals, membrane integrity and calcium functions in toxicity [26]. Inside the cells microenvironment, mitochondria seems to be the organelle of choice for studying such a correlation as it has a well denned compartmentation by membranes, has a low affinity and high capacity for storing Ca^{2+} and leakage of electrons during oxidative phosphorylation can give rise to reactive oxygen species. Thus, it plays an important role in maintaining a cells redox equilibrium [27] and changes in its structure and function can give mechanistic clues regarding the toxicity/safety of a chemical.

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