Mitochondrial respiratory chain deficiency leads to overexpression of antioxidant enzymes

Liliana Brambilla[a], Gaetano Cairo[b], Piero Sestili[a], Valery O'Donnel[c], Angelo Azzi[c], Orazio Cantoni[a,*]

[a] *Istituto di Farmacologia e Farmacognosia and Centro di Farmacologia Oncologica Sperimentale, Università di Urbino, Via S. Chiara 27, 61029 Urbino, Italy*

[b] *Centro di Studio sulla Patologia Cellulare, Consiglio Nazionale delle Ricerche and Istituto di Patologia Generale, Università di Milano, Milan, Italy*

[c] *Institut für Biochemie und Molekularbiologie der Universität Bern, Bern, Switzerland*

Received 15 October 1997

Abstract U937 cell growth in the presence of either chloramphenicol or ethidium bromide rapidly leads to respiratory deficiency. The novel finding of this report is that this response is paralleled by a specific increase in Se-dependent and -independent glutathione peroxidase activities as well as of glutathione peroxidase and heme oxygenase mRNAs. Under the same experimental conditions, catalase activity and catalase mRNA do not show appreciable changes. These results can be explained by an increased formation of H_2O_2 at the early times of development of respiratory deficiency followed by induction of antioxidant enzymes.

Key words: Respiratory deficiency; Antioxidant enzyme; Glutathione peroxidase; Heme oxygenase; Oxidative stress

1. Introduction

Chronic exposure of cultured mammalian cells to ethidium bromide (EtBr) leads to depletion of mitochondrial DNA and consequent respiratory deficiency since a number of polypeptides encoded by mitochondrial DNA are essential components of the respiratory chain [1]. Supplementation with chloramphenicol (CP) promotes similar effects, which are however mediated by inhibition of the synthesis of protein encoded by mitochondrial DNA [2]. These systems are being widely utilized to investigate an array of biochemical responses associated with the respiratory-deficient phenotype.

In the present study we report a novel response occurring at the early times of exposure to EtBr or CP. A selective stress aimed at the mitochondria by either of these two agents promoted a rapid inhibition of oxygen consumption paralleled by a specific increase in Se-dependent and -independent glutathione peroxidase (GPx) activities as well as GPx mRNA accumulation. Under the same experimental conditions, catalase activity and the amount of catalase mRNA did not show appreciable variations. The fact that addition of EtBr was also followed by a rapid and persistent induction of heme oxygenase 1 (HO-1) strongly suggests that upregulation of GPx was the consequence of enhanced formation of hydrogen peroxide.

2. Materials and methods

2.1. Cell culture and treatments

Human myeloid leukemia U937 cells were grown in RPMI 1640 culture medium (Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum, penicillin (50 U/ml), and streptomycin (50 μg/ml) (Sera-lab, Ltd., Crawley Down, UK), at 37°C in T-75 tissue culture flasks (Corning, Corning, NY, USA) in an atmosphere of 95% air-5% CO_2.

Respiration-deficient U937 cells were obtained by culturing the cells in RPMI medium containing 110 μg/ml pyruvate, 5 μg/ml uridine and 400 ng/ml ethidium bromide or 50 μg/ml chloramphenicol for 6 days with medium changes every 2 days.

2.2. Oxygen consumption

Cells were washed once in glucose-free saline A (8.182 g/l NaCl, 0.372 g/l KCl and 0.336 g/l $NaHCO_3$) and suspended in the same buffer at a density of 1×10^7 cells/ml. Oxygen consumption was measured using a YSI oxygraph equipped with a Clark-type electrode (model 5300, Yellow Springs Instruments Co., Yellow Springs, OH, USA) at 37°C. The cell suspension (3 ml) was transferred to the polarographic cell and, under constant stirring, basal respiration was measured immediately after addition of 5 mM glucose over a 3 min period. 5 mM pyruvate was then added and the rate of oxygen utilization was monitored for a further 3 min. The rate of oxygen utilization was calculated as described by Robinson and Cooper [3].

2.3. Mitochondrial respiratory enzyme assays

Mitochondrial respiratory enzyme activities were determined in digitonin-permeabilized cells using cytochrome *c* as an electron donor/acceptor, as previously described [4].

2.4. Catalase and GPx activity assays

Cell pellets obtained after centrifugation at 1200 rpm for 5 min were washed twice with ice-cold PBS (0.121 M NaCl, 10 mM NaH_2PO_4, 1.5 mM KH_2PO_4, 3 mM KCl), resuspended in distilled water at a final density of 2×10^6 cells/100 μl and sonicated three times for 15 s at 20 W with a Heat Systems sonifier model XL (Heat Systems, Farmingdale, NY, USA). Catalase and GPx (total and Se-dependent) activities were assayed spectrophotometrically in the supernatant by the methods of Aebi [5] and Lawrence et al. [6], respectively.

2.5. Northern blot analysis

Total RNA was extracted, run in 30 μg aliquots in denaturing agarose gels and blotted to nylon filters (Amersham). To confirm that each sample contained equal amounts of total RNA, the ribosomal RNA content was estimated in the EtBr-stained gels. Filters were sequentially hybridized as previously detailed [7] with ^{32}P-labeled, gel-purified DNA fragments excised from a human GPx cDNA (obtained from ATCC), the rat catalase pMJ512 clone [8] (obtained from Dr. S. Furuta, Nagano, Japan) and human HO 2/10 cDNA [9] (obtained from Dr. R. Tyrrell, Bath, UK). Hybridization signals were quantified by densitometry and the values were calculated after normalization to the amount of ribosomal RNA.

*Corresponding author. Fax: (39) (722) 327670.
E-mail: cantoni@fis.uniurb.it

Abbreviations: GPx, glutathione peroxidase; EtBr, ethidium bromide; CP, chloramphenicol; HO-1, heme oxygenase

PII S0014-5793(97)01393-8

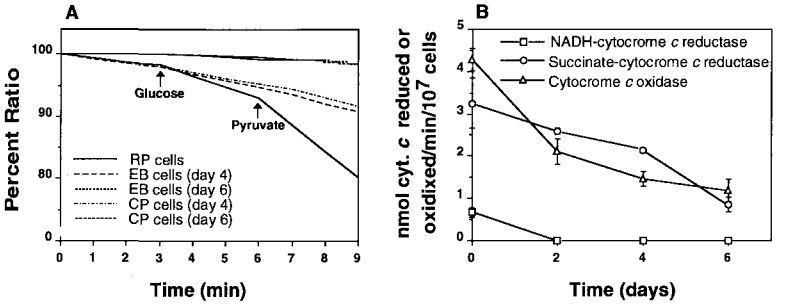

Fig. 1. Time dependence for inhibition of U937 cell oxygen consumption and mitochondrial respiratory enzyme activities. A: U937 cell oxygen consumption was assessed 4 and 6 days after addition of vehicle (respiration-proficient, RP, cells), 400 ng/ml EtBr (respiration-deficient, EB, cells) or 50 μg/ml CP (respiration-deficient, CP, cells), as detailed in Section 2. Basal respiration (12.7 ± 0.39 nmol O_2/min/10^7 cells) was recorded for 3 min in the presence of 5 mM glucose. Oxygen consumption was then monitored for a further 3 min after addition of 5 mM pyruvate. Data are expressed as the percent ratios between the oxygen concentration measured at each time point and at time 0. Traces are representative of three consistent experiments. B: U937 cells respiratory enzyme activities were measured as described in Section 2. Results represent the mean ± S.E.M. calculated from three separate experiments, each performed in duplicate.

3. Results

Continuous exposure of actively growing cells to either CP or EtBr leads to respiratory deficiency [1]. The experimental system utilized in this study involved growth of U937 cells in RPMI medium containing either 400 ng/ml EtBr or 50 μg/ml CP for 6 days with medium changes every 2 days. 110 μg/ml pyruvate and 5 μg/ml uridine were added to these cultures to allow maintenance of cell viability and ability to proliferate [1]. The results illustrated in Fig. 1 indicate that U937 cells were extremely sensitive to these treatments, since a severe decline in the ability of these cells to consume oxygen as well as in the activity of their respiratory complexes was observed after only 2–6 days. Indeed, the extent of oxygen utilization in response to glucose (5 mM) or pyruvate (5 mM) was markedly reduced or abolished after 4 or 6 days, respectively (Fig. 1A). Supplementation with EtBr or CP led to superimposable results. Consistent with the results reported in Fig. 1A, the activities of NADH-cytochrome *c* reductase (complexes I–III), succinate-cytochrome *c* reductase (complexes II–III) and cytochrome *c* oxidase (complex IV) diminished significantly after 2 days of growth in the presence of EtBr and disappeared thereafter (Fig. 1B).

We next examined the activities of the two enzymes involved in hydrogen peroxide metabolism, namely catalase and GPx. It was interesting to find that whereas the activity of the former was not affected by CP or EtBr exposure (inset in Fig. 2A), the total (Fig. 2A) as well as the Se-dependent (Fig. 2B) GPx activities progressively increased in a time-dependent fashion. These responses were remarkably similar in CP- or EtBr-supplemented cells. In addition, the total and the Se-dependent GPx activities appeared to be stimulated to a similar extent. As reported above, the culture medium of the cells incubated with EtBr or CP was supplemented with 110 μg/ml pyruvate and 5 μg/ml uridine. We therefore asked the question whether the observed changes in enzyme activities were promoted by these supplements. As illustrated in Fig. 2, the levels of both the total (A) and Se-dependent (B) GPx activities in pyruvate/uridine-supplemented cells were identical to those of mock-treated cells.

To understand the molecular mechanisms underlying the changes in enzyme activity, we assessed the relative amounts of the corresponding mRNAs by Northern blot analysis (Fig. 3). The amount of GPx mRNA increased progressively from the low level present in untreated cells, reaching a maximum (3-fold greater than control levels) 48 h after the addition of EtBr. Under the same conditions, the levels of catalase mRNA did not show appreciable variations. It is important to note that each sample contained equal amounts of total RNA, as estimated by UV shadowing of EtBr-stained ribosomal RNA (not shown). Interestingly, treatment with EtBr also evoked a dramatic stimulation of HO-1 mRNA expression; the response was maximal after 6 h (12-fold stimulation) and the HO-1 mRNA content remained remarkably elevated up to

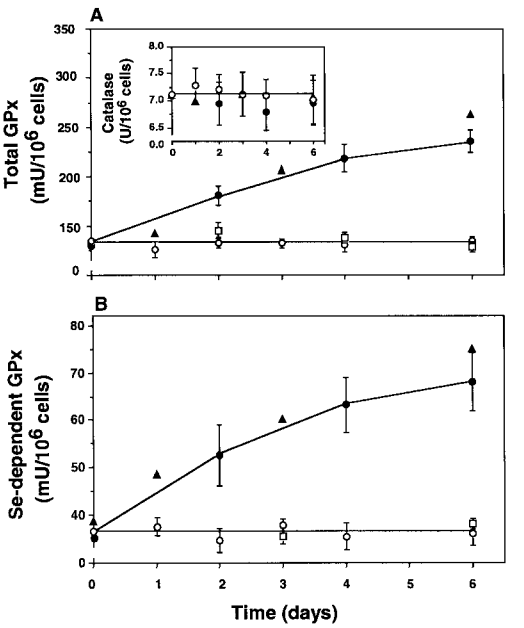

Fig. 2. GPx and catalase activities during the development of experimentally induced respiratory deficiency. U937 cells were grown in RPMI medium containing 110 μg/ml pyruvate, 5 μg/ml uridine and either 400 ng/ml EtBr (closed circles) or 50 μg/ml CP (closed triangles) for 6 days with medium changes every 2 days. Sister cultures were grown in RPMI medium with no supplements (open circles) or with the cocktail pyruvate/uridine (open squares). Total GPx (main graph, panel A), Se-dependent (panel B) and catalase (inset in panel A) activities were determined as described in Section 2. Results represent the mean ± S.E.M. calculated from at least three separate experiments, each performed in duplicate. The experimental points indicated by the closed triangles represent the mean of two separate experiments.

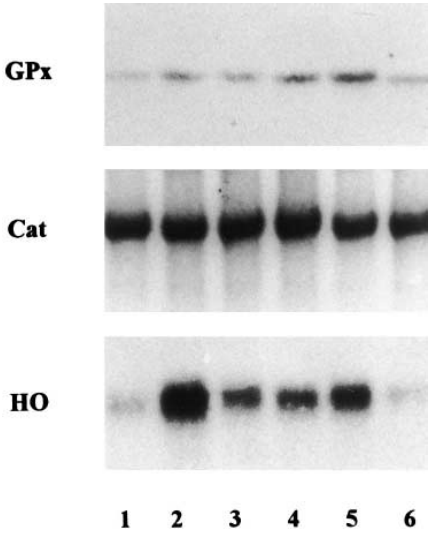

Fig. 3. Northern blot analysis of GPx, HO-1 and catalase mRNAs during the development of experimentally induced respiratory deficiency. A filter with equal amounts of total RNA extracted from U937 cells untreated (lane 1), treated with 110 μg/ml pyruvate and 5 μg/ml uridine for 24 h (lane 6) or treated in pyruvate/uridine-supplemented medium with 400 ng/ml EtBr for 6, 12, 24 and 48 h (lanes 2–5) was hybridized with probes for GPx, catalase (Cat) and HO-1 (HO) as indicated in Section 2. Each sample contained equal amounts of total RNA, as estimated by EtBr staining of the ribosomal RNA content (not shown). The autoradiograms shown are representative of three independent experiments.

48 h. Finally, the effects on GPx and HO-1 mRNAs were specifically triggered by EtBr, since the cocktail uridine/pyruvate was not active in the absence of EtBr (Fig. 3, lane 6).

4. Discussion

The results presented in this study indicate that two different agents, namely EtBr and CP, cause inhibition of U937 cell oxygen consumption in a relatively short time. The similarities in the kinetics and extent of these responses are striking since the mechanisms whereby EtBr and CP promote respiratory deficiency are remarkably different. EtBr inhibits mitochondrial DNA and RNA synthesis [10], and CP inhibits the synthesis of proteins encoded by mitochondrial DNA [2]. In the present report we also provide experimental evidence indicating that inhibition of oxygen utilization is paralleled by the selective induction of Se-dependent and -independent GPxs. This inference is supported by the observation that the activities of these enzymes, as well as the amount of GPx mRNA, increased in a time-dependent fashion. Although the question of whether the upregulation of GPx was due to activation of transcription or to post-transcriptional mechanisms was not directly addressed in this study, the long-lasting increase in GPx mRNA content suggests a continuous elevation of GPx transcription rate. However, the possibility of a stabilization of mRNA levels similar to those demonstrated for GPx mRNA in Se-supplemented cells [11] and for nuclear-encoded mitochondrial transcripts in respiratory-deficient Hep G2 cells [12] cannot be ruled out. Furthermore, the upregulation of GPx was not due to a general upregulation in the synthesis of H_2O_2-metabolizing enzymes since treatment with EtBr or CP did not affect catalase activity.

Taken together, the results presented in this study are consistent with the possibility that treatment with EtBr or CP promotes a pro-oxidant state triggering an adaptive response represented by overexpression of GPx. This conclusion is supported by the observation that, under these experimental conditions, we also found a dramatic increase in HO-1 mRNA, a response that is commonly considered to be a reliable marker of oxidative stress [13].

It is known that mitochondria possess sites in which reactive oxygen species are generated, and it is therefore possible that alterations in the respiratory chain resulting from exposure to EtBr or CP lead to enhanced formation of these species. Increased superoxide production has been reported in an array of pathologies characterized by genetically determined respiratory chain defects [14]. Thus, it may be hypothesized that formation of superoxides is one of the early events resulting from impairment of the respiratory chain in response to EtBr or CP. Conceivably, the superoxide anion readily dismutates to the diffusible and more stable hydrogen peroxide, which may then exit the mitochondria and trigger gene expression. The ability of hydrogen peroxide to stimulate gene expression is well documented [15] and the effects of the oxidant on transcriptional activator proteins NF-κB [16] and AP-1 [17] are also well characterized. In particular, hydrogen peroxide has been shown to upregulate both HO-1 [9,18] and GPx [19].

We therefore propose that our results are best explained by a sequence of events in which EtBr (or CP) promotes early alterations in the respiratory chain with a parallel enforced formation of superoxides, which promptly dismutate to hydrogen peroxide. Hydrogen peroxide would then be the final effector promoting changes at the level of gene expression.

A recent study [20] reported that the mitochondrial stress caused by EtBr was signalled to the nucleus and resulted in the induction of selected mitochondrial chaperons. The nature of the primary stress signal was not determined. Thus, the possibility exists that a common signal, possibly hydrogen peroxide, promotes overexpression of a number of proteins which on the one hand augment the antioxidant defence (GPx, this paper) and on the other increases the formation of chaperons implicated in the folding of mitochondrial proteins [20].

In conclusion, the results presented in this study demonstrate that the upregulation of GPx and HO-1 are early responses to mitochondrial stress caused by agents promoting respiratory deficiency. Under this perspective, overexpression of GPx may confer an adaptational improvement in the ability of the cells to survive oxidative stress. The results presented in this study provide novel information which may be of great importance in furthering our understanding of pathologies characterized by mitochondrial respiratory chain mutations. These mutations may result in a primary respiratory insufficiency followed by a secondary cytotoxic event mediated by H_2O_2.

References

[1] King, M.P. and Attardi, G. (1989) Science 246, 500–503.
[2] Morais, O. (1980) J. Cell. Physiol. 103, 455–466.
[3] Robinson, J. and Cooper, J.M. (1970) Anal. Biochem. 33, 390–399.
[4] Hatefi, Y. and Stiggal, D.L. (1978) Methods Enzymol. 53, 5–41.
[5] Aebi, H. (1984) Methods Enzymol. 105, 121–126.

[6] Lawrence, R.O. and Burk, R.F. (1976) Biochem. Biophys. Res. Commun. 71, 952–958.
[7] Cairo, G., Tacchini, L., Pogliaghi, G., Anzon, E., Tomasi, A. and Bernelli-Zazzera, A. (1995) J. Biol. Chem. 270, 700–703.
[8] Furuta, S., Hayashi, H., Hijikata, M., Miyazawa, S., Osumi, T. and Hashimoto, T. (1986) Proc. Natl. Acad. Sci. USA 83, 313–317.
[9] Keyse, S.M. and Tyrrell, R.M. (1989) Proc. Natl. Acad. Sci. USA 86, 99–103.
[10] Perlman, R. and Penman, S. (1970) Nature 227, 133–137.
[11] Bermano, G., Arthur, J.R. and Hesketh, J.E. (1996) FEBS Lett. 387, 157–160.
[12] Chrzanowska-Lightowlers, Z.M.A., Preiss, T. and Lightowlers, R.N. (1994) J. Biol. Chem. 269, 27322–27328.
[13] Tyrrell, R.M. and Basu-Modak, S. (1994) Methods Enzymol. 234, 224–235.
[14] Pitkanen, S. and Robinson, B.H. (1996) J. Clin. Invest. 98, 345–351.
[15] Azzi, A., Cantoni, O., Özer, N., Boscoboinik, D. and Spycher, S. (1996) J. Clin. Invest. 3, 79–90.
[16] Schreck, R., Albermann, K. and Baeuerle, P.A. (1992) Free Radical Res. Commun. 17, 221–237.
[17] Staüble, B., Boscoboinik, D., Tasinato, A. and Azzi, A. (1994) Eur. J. Biochem. 226, 393–402.
[18] Keyse, S.M. and Tyrrell, R.M. (1987) J. Biol. Chem. 262, 14821–14825.
[19] Shull, S., Heintz, N.H., Periasamy, M., Manohar, M., Janssen, Y.M.W., Marsh, J.P. and Mossman, B.T. (1991) J. Biol. Chem. 266, 24398–24403.
[20] Martinus, R.D., Garth, G.P., Webster, T.L., Cartwright, P., Naylor, D.J., Høj, P.B. and Hoogenraad, N.J. (1996) Eur. J. Biochem. 240, 98–103.