BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **226,** 70–74 (1996)
ARTICLE NO. 1312

Pyruvate Enhances DNA Single-Strand Break Formation While Abolishing Cytotoxicity in U937 Cells Exposed to *tert*-Butylhydroperoxide

Andrea Guidarelli, Liliana Brambilla, Flaminio Cattabeni, and Orazio Cantoni[1]

Istituto di Farmacologia e Farmacognosia and Centro di Farmacologia Oncologica Sperimentale, Università di Urbino, Urbino I-61029, Italy

Received April 25, 1996

tert-Butylhydroperoxide (tB-OOH) induces killing and DNA single strand breaks (SSBs) in cultured U937 cells. Pyruvate while increasing the rate of oxygen consumption also increased the magnitude of the DNA scission produced by tB-OOH. Rotenone, an inhibitor of complex I, abolished both effects but did not, however, affect the DNA SSB-frequency observed after treatment with tB-OOH alone. These results collectively suggest that pyruvate potentiates the DNA-damaging activity of tB-OOH via stimulation of oxygen consumption. Importantly, under the same experimental conditions, pyruvate was found to abolish both the decline in nonprotein sulfhydryls (NPSH) and the cytotoxicity induced by tB-OOH. Thus, cells with energized mitochondria are more sensitive to the DNA-damaging effects of tB-OOH and display resistance against its cytotoxic effects. As a consequence, DNA SSBs promoted by tB-OOH do not appear to be toxic for the cell.

Inorganic and organic peroxides are generated within the cell as intermediates of a number of metabolic reactions. Although under normal conditions these peroxides are efficiently removed by the antioxidant defense system, oxidative stress can occur when the delicate redox balance is altered. Indeed, abnormal levels of these species can be generated in certain disease states (1-3) and as a consequence of the metabolism of specific drugs and xenobiotics (4). The organic peroxide tB-OOH has been widely utilized as a model hydroperoxide to investigate and characterize the toxic effects of these species. It has been shown that tB-OOH induces a number of cellular dysfunctions including peroxidation of membrane lipids (5), depletion of glutathione (6, 7), mobilization of mitochondrial calcium (8, 9) and mitochondrial damage (9-13). The effects of tB-OOH on DNA homeostasis have been investigated to a lesser degree, although it was demonstrated that this peroxide introduces damage at the DNA level by causing strand scission (14-17), an event which can be prevented by iron chelators (17). Removal of the peroxide results in rapid reversal of DNA strand breakage (14-17). It remains to be demonstrated what mechanism(s) and species are involved in these processes; in particular, whether or not mitochondria, which appear to be important targets for the toxicity of the hydroperoxide (11-13), also participate in the formation of DNA damage needs to be clarified.

Our results strongly suggest that mitochondrial functions may participate in the induction of DNA SSBs and toxicity in cells exposed to tB-OOH. In particular, we demonstrate that pyruvate causes an enhancement in oxygen consumption and a parallel increase in DNA single strand breakage, both of which can be prevented by an inhibitor of complex I. Pyruvate, however, does not increase but, rather, markedly decreases the decline in cellular NPSH and the toxicity induced by tB-OOH.

[1] Correspondence should be addressed to Orazio Cantoni. Fax +39-722-327670.

Abbreviations: SSBs, DNA single strand breaks; tB-OOH, *tert*-butylhydroperoxide; NPSH, nonprotein sulfhydryls.

0006-291X/96 $18.00

MATERIALS AND METHODS

Cell culture and treatments. U937 cells were grown in RPMI 1640 culture medium (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Seralab, Sussex, UK), penicillin (50 units/ml), and streptomycin (50 μg/ml), at 37°C in T-75 tissue culture flasks (Corning, Corning, NY, USA) gassed with an atmosphere of 95% air-5% CO_2. Stock solutions of tB-OOH and pyruvate were freshly prepared in Saline A (8.182 g/l NaCl, 0.372 g/l KCl, 0.336 g/l $NaHCO_3$ and 0.9 g/l glucose). Rotenone was dissolved in 95% ethanol. Cells (2.5×10^5 /ml) were treated for 30 min in Saline A (2 ml), washed and either analyzed immediately for DNA damage or NPSH content, or post-incubated for 6 hr in complete medium and then analyzed for cell viability. Pyruvate or rotenone was added to the cultures 5 min prior to addition of tB-OOH. When pyruvate and rotenone were used in conjunction, the complex I inhibitor was added 5 min prior to addition of the NADH-linked substrate.

Alkaline elution assay. Cells were labelled overnight with [^{14}C]-thymidine (0.05 μCi/ml) and incubated for a further 6 hr in a medium containing unlabelled thymidine (1 μg/ml). After treatments, the cells were analyzed for DNA damage by the alkaline elution assay, using a procedure virtually identical to that described by Kohn *et al.* (18) with minor modifications (19). Strand Scission Factor (SSF) values were calculated from the resulting elution profiles by determining the absolute log. of the ratio of the percentage of DNA retained in the filters of the drug-treated sample to that retained from the untreated control sample (both after 8 hr of elution).

Oxygen consumption. Cells were washed once in Saline A and then resuspended in the same medium at a density of 1×10^7 cells/ml. Oxygen consumption was measured using a Y.S.I. oxygraph equipped with a Clark electrode (model 5300, Yellow Springs Instruments Co., Yellow Springs, OH, USA). The cell suspension (3 ml) was transferred to the polarographic cell and basal respiration was measured under constant stirring for 3 min at 37° C. Oxygen consumption was also measured after addition of 5 mM pyruvate and after addition of 0.5 μM rotenone. The rate of oxygen utilization was calculated as described by Robinson and Cooper (20).

Cytotoxicity assay. Cytotoxicity was determined using the trypan blue exclusion assay.

NPSH assay. After treatments, the cells were washed twice with ice-cold Saline A and NPSH were extracted with 0.3 M metaphosphoric acid, 4.4 mM EDTA (tetrasodium salt) and 5 M NaCl (2×10^6 cells/100 μl) at ice temperature for 15 min. Cell extracts were then centrifuged at 12,000 rpm for 5 min and an aliquot of the supernatant was transferred to a cuvette containing 0.3 M Na_2HPO_4. To each cuvette 100 μl of 0.04% 5,5′-dithiobis(2-nitrobenzoic acid) in 1% Na-citrate were added, the contents were mixed and absorbance was measured at 412 nm. The concentration of NPSH in the samples was determined by comparing the optical density readings to a standard curve constructed using reduced glutathione.

Protein contents were assayed as described by Lowry *et al.* (21), using bovine serum albumin as standard.

RESULTS AND DISCUSSION

To elucidate the role of the mitochondrial respiratory chain in the deleterious effects generated by an organic hydroperoxide, we examined the effect of pyruvate on DNA strand breakage and cell killing induced by tB-OOH in U937 cells. Pyruvate has the advantages of being membrane-permeant and generating NADH within the mitochondria, which will in turn readily stimulate the electron transport. Indeed, as shown in Table I, pyruvate (5 mM) significantly increased oxygen consumption and this effect was abolished by rotenone (0.5 μM), an inhibitor of complex I.

Logarithmically growing U937 cells were treated for increasing time intervals with 200 μM tB-OOH in the absence or presence of 5 mM pyruvate in Saline A, and then analyzed for DNA single strand breakage by the filter elution assay. The results given in Fig. 1 indicate that a large proportion of the SSBs resulting from tB-OOH were generated within the first 15 min of exposure. Addition of pyruvate markedly enhanced the accumulation of these lesions throughout the entire period of exposure to the peroxide, and this effect was particularly pronounced at 30 min (approximately 50% increase). Importantly, pyruvate was not DNA-damaging when given alone to the cultures. These data are certainly surprising and lead to the intriguing conclusion that pyruvate, which enhances the electron transport, also augments the formation of DNA SSBs in U937 cells exposed to tB-OOH. This increased DNA damage could either be the result of an enhanced formation of DNA-damaging species or depend on a less specific event such as inhibition of repair. We investigated this second possibility by measuring the effect of pyruvate on the rate of DNA SSB-removal in pre-damaged cells and found that pyruvate did not affect the rate of repair of DNA SSBs induced by tB-OOH (not

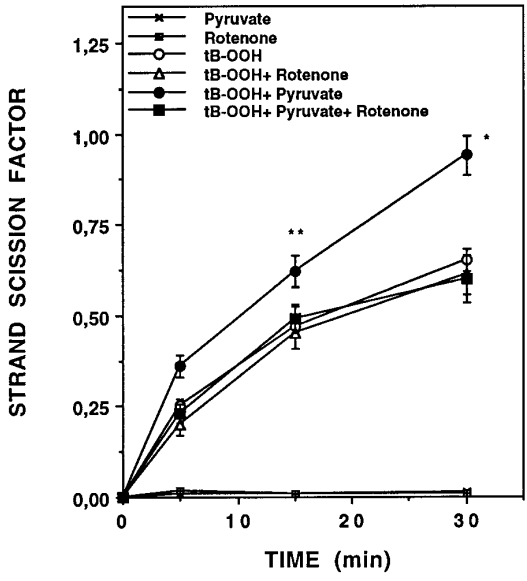

FIG. 1. Pyruvate enhances DNA single strand breakage induced by tB-OOH and this effect is prevented by rotenone. Cells were incubated for increasing time intervals in Saline A with 200 μM tB-OOH in the absence or presence of 5 mM pyruvate. The effect of 0.5 μM rotenone on these treatments was also investigated. Pyruvate or rotenone was not DNA damaging when given alone to the cultures. The level of DNA SSBs was measured immediately after the treatments using the alkaline elution technique. The SSF values were calculated from the resulting elution profiles, as detailed in the Methods section. Results represent the means ± S.E.M. calculated from three separate experiments and were significantly different from DNA damage generated by the oxidant alone at *$p<0.0001$ and **$p<0.05$ (unpaired *t* test).

shown). Thus, the increased accumulation of DNA SSBs observed in cells treated with tB-OOH in the presence of pyruvate appears to be the consequence of an increased production of DNA SSBs. As discussed above, pyruvate produced a large enhancement of the respiration rate as revealed by an increase of about 20% in oxygen consumption (Table I), and rotenone (0.5 μM) abolished this effect. The results illustrated in Fig. 1 demonstrate that rotenone also prevented the pyruvate-mediated enhancement of tB-OOH-induced DNA single strand breakage and did not influence the amount of DNA breaks triggered by the hydroperoxide alone.

Taken together, the above results demonstrate that an enhancement in oxygen consumption is paralleled by an increased formation of DNA SSBs in cultured cells exposed to the organic hydroperoxide tB-OOH and suggest the existence of a cause-effect relationship between these two parameters.

The cytotoxic effect of tB-OOH in the absence or presence of pyruvate was assessed by measuring the number of dead cells (using the trypan blue exclusion assay) following treatment for 30 min and a 6 hr growth in drug-free complete culture medium. Fig. 2 indicates that, under these experimental conditions, tB-OOH was toxic over a concentration-range of 300-1000 μM and that pyruvate virtually abolished this response. Thus pyruvate, under the same conditions resulting in an increased rate of oxygen consumption and formation of tB-OOH-induced DNA SSBs, inhibits the cell killing promoted by the hydroperoxide. Finally, we investigated the effect of pyruvate on the tB-OOH-induced decrease in cellular NPSH. The results illustrated in the inset to Fig. 2 indicate that a 30 min exposure to 1 mM tB-OOH induces a statistically significant decrease in cellular NPSH. This effect was prevented under conditions in which the exposure to tB-OOH was performed in the presence of 5 mM pyruvate.

TABLE I
The Effect of Pyruvate on U937 Cell Oxygen Consumption

Treatment[a]	QO_2[b]
—	12.7 ± 0.39
5 mM pyruvate	15.05 ± 0.64**
0.5 μM rotenone	0.46 ± 0.15*

[a] Cells were rinsed with Saline A and then analyzed for oxygen consumption as detailed in the Methods section. Oxygen consumption was monitored for 3 min at the beginning of the experiment, after addition of 5 mM pyruvate and, finally, following addition of 0.5 μM rotenone.

[b] Respiration rates are expressed as $nmolO_2/min/10^7$ cells. Results represent the mean ± S.E.M. calculated from at least 3 separate experiments.

** $p < 0.01$, oxygen consumption stimulated by pyruvate was significantly different from basal respiration (unpaired *t* test).

* $p < 0.0001$, oxygen consumption after addition of pyruvate was significantly reduced by rotenone (unpaired *t* test).

In conclusion, the results presented in this paper demonstrate that pyruvate stimulates electron transport and enhances DNA SSB-formation in intact cells exposed to tB-OOH. These two events appear to be causally related since inhibiting respiration with rotenone also prevents

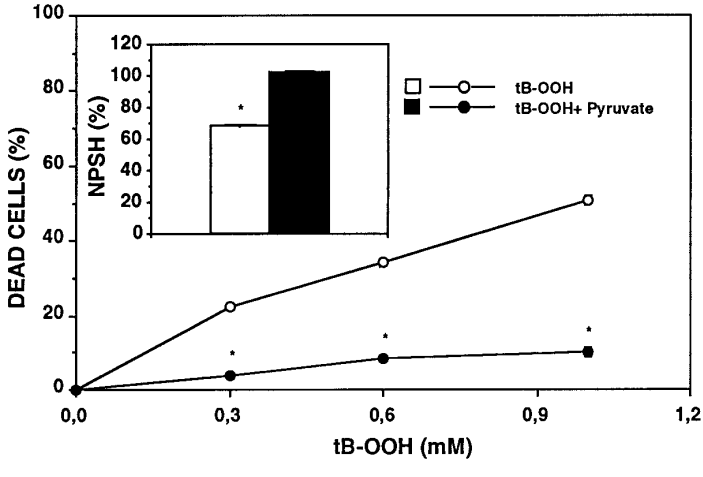

FIG. 2. Pyruvate prevents cell killing and the decline in cellular NPSH promoted by tB-OOH. Cells were exposed for 30 min to increasing concentrations of tB-OOH in the absence or presence of 5 mM pyruvate. The relative number of dead cells was measured after 6 hr of post-challenge growth using the trypan blue exclusion assay. Results represent the mean ± S.E.M. calculated from 3–5 separate experiments and were significantly different from cell killing generated by the oxidant alone at *$p<0.0001$ (unpaired *t* test). The inset shows the effect of 5 mM pyruvate on the decline of cellular NPSH induced by treatment with 1 mM tB-OOH for 30 min. NPSH levels in cells exposed to tB-OOH were significantly different from those measured in untreated cells at *$p<0.0001$ (unpaired *t* test). Untreated U937 cells had 15.47 ± 0.54 NPSH/mg of protein. Treatment with pyruvate alone did not significantly alter the NPSH pool (15.31 ± 1.20).

the enhanced production of DNA strand breaks. Rotenone, however, did not reduce the extent of DNA damage generated by the hydroperoxide in the absence of pyruvate, thus suggesting that mitochondrial respiration modulates the formation of DNA SSBs only in cells with energized mitochondria. The molecular basis for this effect is still to be determined and at present this does not appear to be an easy task, since the mechanisms and species involved in the formation of DNA SSBs induced by tB-OOH have not yet been indentified. To our best knowledge, these results represent the first demonstration of a potential role of the mitochondrial respiratory chain in the genotoxic response to an organic peroxide. Although at this stage the implications of these findings are unclear, we speculate that the data presented here should give insight into the biological impact of short-chain organic peroxides that are actively generated under diverse pathological conditions. It is intriguing that pyruvate, under the same experimental conditions in which it increases DNA damage via a respiration-based mechanism (Fig. 1), fully protects the cells against the toxicity promoted by tB-OOH (Fig. 2), an effect that may be ascribed to restoration of tB-OOH-depleted NPSH (inset to Fig. 2). Thus, pyruvate produces opposite effects on DNA damage and cytotoxicity induced by tB-OOH and these effects appear to be mediated by different mechanisms. As a corollary, DNA SSBs generated by tB-OOH are not cytotoxic. Maximizing the effects at the DNA level, while promoting complete viability, would seem to be a most favourable situation for the occurrence of neoplastic transformation.

ACKNOWLEDGMENT

This study was supported by a grant from the CNR.

REFERENCES

1. Fridovich, I. (1978) *Science* **201,** 875–880.
2. McCord, J. (1987) *Fed. Proc.* **46,** 2402–2406.
3. Clark, I., Cowden, W., and Hunt, N. (1985) *in* Medicinal Research Reviews, Vol. 5, pp. 297–332, Wiley, New York.
4. Cerutti, P. (1985) *Science* **227,** 375–381.
5. Masaki, N., Kyle, M. E., and Farber, J. L. (1989) *Arch. Biochem. Biophys.* **269,** 390–399.
6. Geiger, P. G., Lin, F., and Girotti, A. W. (1993) *Free Radical Biol. Med.* **14,** 251–266.
7. Korytowski, W., Bachowski, G. J., Geiger, P. G., Lin, F., Zhao, G., and Girotti, A. W. (1995) *Biochim. Biophys. Acta* **1267,** 31–40.
8. Livingston, F. R., Lui, E. M. K., Loeb, G. A., and Forman, H. J. (1992) *Arch. Biochem. Biophys.* **299,** 83–91.
9. Castilho, R. F., Kowaltowski, A. J., Meinicke, A. R., Bechara, E. J. H., and Vercesi, A. E. (1995) *Free Radical Biol. Med.* **18,** 479–486.
10. Imberti, R., Nieminen, A. L., Herman, B., and Lemasters, J. J. (1993) *J. Pharmacol. Exp. Ther.* **265,** 392–400.
11. Masaki, N., Kyle, M. E., Serroni, A., and Farber, J. L. (1989) *Arch. Biochem. Biophys.* **270,** 672–680.
12. Nieminen, A. L., Saylor, A. K., Tesfai, S. A., Herman, B., and Lemasters, J. J. (1995) *Biochem. J.* **307,** 99–106.
13. Wu, E. Y., Smith, M. T., Bellomo, G., and Di Monte, D. (1990) *Arch. Biochem. Biophys.* **282,** 358–362.
14. Sandstrom, B. E. (1991) *Free Radical Res. Commun.* **15,** 79–89.
15. Baker, M. A., and He, S. (1991) *Free Radical Biol. Med.* **11,** 563–572.
16. Sandstrom, B. E., and Marklund, S. L. (1990) *Biochem. J.* **271,** 17–23.
17. Guidarelli, A., Sestili, P., Cossarizza, A., Franceschi, C., Cattabeni, F., and Cantoni, O. (1995) *J. Pharmacol. Exp. Ther.* **275,** 1575–1582.
18. Kohn, K. W., Ewig, R. A. G., Erickson, L. C., and Zwelling, L. A. (1981) *in* DNA Repair: A Laboratory Manual of Research Procedures, Vol. 1, Part B, pp. 379–401, Dekker, New York.
19. Cantoni, O., Murray, D., and Meyn, R. E. (1986) *Biochim. Biophys. Acta* **867,** 135–143.
20. Robinson, J., and Cooper, J. M. (1970) *Anal. Biochem.* **33,** 390–399.
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.