REVISTA ESPAÑOLA DE FISIOLOGIA, 30, 127-132. 1974

Control of Phospholipid Peroxidation and Enzyme Activity Losses in Mitochondria by Antioxidants *

J. L. Segovia,** N. López-Moratalla ** and E. Santiago

Department of Biochemistry
University of Navarra
Pamplona (Spain)

(Received on 15 March, 1974)

J. L. SEGOVIA, N. LOPEZ-MORATALLA and E. SANTIAGO. *Control of Phospholipid Peroxidation and Enzyme Activity Losses in Mitochondria by Antioxidants.* Rev. esp. Fisiol., 30, 127-132. 1974.

The antioxidant efficiency of BHT (2,6-tert-butyl-4-methylphenol) and of thyroxine in preventing phospholipid peroxidation, membrane disaggregation and losses of enzyme activity has been studied in suspensions of inner mitochondrial membranes from rat liver incubated in the presence of 1 mM ascorbate or 0.8 mM cysteine. The addition of BHT, at zero-time and at a concentration of 5×10^{-5} M in the case of ascorbate, or of 5×10^{-6} M in the case of cysteine, completely inhibited lipid peroxidation and related processes. If BHT was added at a later time during the incubation, different degrees of inhibition were obtained. Similar effects were observed with 10^{-5} M thyroxine. These results suggest the possibility of using a simple method in the study of associations between phospholipids and membrane-bound enzymes.

Lipid peroxidation in mitochondria, or in isolated mitochondrial membranes, can be induced by some redox agents such as glutathione, cysteine, ascorbate or ferrous ions. Lipid peroxidation is accompanied by swelling and structural disaggregation of mitochondrial membranes (5, 8-10, 19, 22). It has also been observed that different antioxidants such as α-tocopherol, BHT (2,6-tert-butyl-4-methylphenol), BHA (butylated hidroxyanisoles), thyroxine, etc., prevent lipid peroxidation (5, 8-10, 23) and consequently mitochondrial swelling and lysis caused by peroxidating agents. The mechanism of action of these antioxidants has been widely studied by WITTING (25) and by TAPPEL (23). Lipid peroxidation and structural disaggregation of mitochondrial membranes is also accompanied by a decrease in phospholipid content (20) and losses in activity of a variety of mitochondrial enzymes (2, 11,

* This work was supported by a grant from the Spanish Ministry of Education and Science.

** N. L.-M. and J. L. S. were recipients of scholarships from «Consejo Superior de Investigaciones Científicas» and from the Spanish Ministry of Education and Science respectively.

15). CHIO and TAPPEL (3, 4) using ribonuclease A as a model system have found that the enzyme reacts with peroxidating lipid causing an intermolecular cross-linking of the protein; the malonaldehyde produced in the peroxidation reaction would be responsible for this cross-linking through formation of a conjugated imine structure between two ε-amino groups in the protein molecules. Besides SANTIAGO *et al.* (20) have found that the peroxidized phospholipids can also form a stable bond with neighboring proteins. MINSSEN and MUNKRES (13) using antioxidants have prevented lipid peroxidation and protein damage in mitochondria. In the present report the use of antioxidants BHT and thyroxine has been studied as a way to control the peroxidation reaction induced by ascorbate or cysteine and its effects on the activity of membrane enzymes such as mitochondrial ATPase, cytochrome oxidase and succinate cytochrome c reductase.

Materials and Methods

Wistar rats weighing approximately 200 g were used in all the experiments. Livers were rapidly removed and homogenized in 0.25 M sucrose and mitochondria isolated according to the method of HOGEBOOM (7). Isolated mitochondria were subjected to osmotic rupture following the method of PARSONS *et al.* (16). Inner mitochondrial membrane were obtained using Parsons' «low speed pellet» as starting material. In order to remove the outer membranes still present in this fraction, it was thoroughly washed three times resuspending it in 0.02 M phosphate buffer, *p*H 7.4, centrifuging at 1,900 × g for 15 minutes, and once more resuspending it in 0.25 M sucrose and centrifuging at 8,500 × g for 10 minutes (20). Incubation of inner membranes was carried out in a medium 1 mM ascorbate, 0.25 M sucrose, at 30° C, during one hour. In other experiments cysteine replaced ascorbate with a final concentration of 8×10^{-4} M. In every experiment controls without ascorbate or cysteine, were incubated. The lysis of the membranes was followed by the changes of turbidity of the suspension in a 1 cm cuvet and setting the wavelength of the spectrophotometer at 520 nm.

Phospholipids were extracted as previously described (18) from 10 ml aliquots of the membrane suspensions after precipitation with enough concentrated $HClO_4$ to give a final concentration of 0.3 N; the pellets obtained after centrifugation were resuspended in 5 ml of a solvent described by FOLCH (6) which consisted of chloroform-methanol-concentrated HCl (200: 100:1). After standing at room temperature for 30 minutes the extracts were washed with 5 ml of cold 0.1 N HCl centrifuged and the bottom chloroform layer carefully removed. The protein interface was extracted once more and the chloroform layers pooled. Phospholipids were separated by thin layer chromatography according to the technique of NESKOVIC *et al.* (14). Lipid phosphorus was determined by the method of BARTLETT (1). Proteins were determined by the method of LOWRY *et al.* (12) and the enzymatic activities, succinate cytochrome c reductase, cytochrome oxidase and ATPase were determined by the methods of TISDALE (24), SOTTOCASA (21) and PULLMAN *et al.* (17) respectively.

The interference of ascorbate in the determination of cytochrome oxidase activity and succinate cytochrome c reductase activity was eliminated with ascorbate oxidase as previously described (11).

Results and Discussion

Figure 1 shows the efficiency of different concentrations of BHT in the prevention of structural disaggregation of inner mitochondrial membranes, reflected by changes in turbidity, induced by the addition of either ascorbate or cysteine. It may be observed that the lowest concentration of BHT capable of inhibiting turbidity

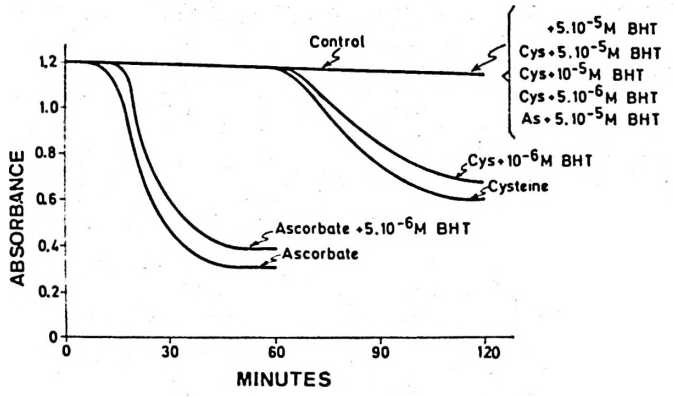

Fig. 1. *Changes in turbidity of a suspension of inner mitochondrial membranes in the presence of ascorbate, 1 mM or cysteine, 0.8 mM with or without BHT.*
At zero-time, BHT, dissolved in 95 % ethanol, was added using 0.1 ml/10 ml of membrane suspension to give the indicated concentrations. Ethanol was also added in the same proportion in the vessels incubated without BHT. See text for other experimental details.

Table I. *Efficiency of BHT in the prevention of phospholipid peroxidation and enzyme activity losses.*

Additions	BHT added at (min)	Turbidity (—Δ A)	Lipid P (μg/mg protein)	ATPase activity *
None	—	0.0	5.3	1.10
Cysteine	—	0.6	3.9	0.57
Ascorbate	—	0.9	2.1	0.12
BHT (5×10^{-5} M)	0	0.0	5.3	1.11
Cysteine+BHT (5×10^{-6} M)	0	0.0	0.0	1.10
Cysteine+BHT (5×10^{-6} M)	40	0.4	4.2	0.91
Ascorbate+BHT (5×10^{-5} M)	0	0.0	5.3	1.10
Ascorbate+BHT (5×10^{-5} M)	7	0.5	3.4	0.85

* μmol Pi × mg^{-1} protein × min^{-1}.

Table II. *Efficiency of thyroxine in the prevention of phospholipid peroxidation and enzyme activities losses.*
Thyroxine concentration was 10^{-5} M.

Additions	Thyroxine added at (min)	Turbidity (—Δ A)	μg P lipidic/mg Protein	Succinate cyt. c reductase activity *	Cytochrome oxidase activity **
None	—	0.00	5.3	0.38	0.24
Cysteine	—	0.55	3.9	0.15	0.16
Cysteine+Thyroxine	0	0.00	5.3	0.38	0.24
Cysteine+Thyroxine	30	0.00	5.3	0.38	0.24
Cysteine+Thyroxine	40	0.18	4.9	0.29	0.21
Cysteine+Thyroxine	50	0.33	4.5	0.20	0.19
Cysteine+Thyroxine	60	0.55	3.9	0.15	0.16

* μmol cyt. red × mg^{-1} prot × $minute^{-1}$.
** μmol cyt. c ox × mg prot × $minute^{-1}$.

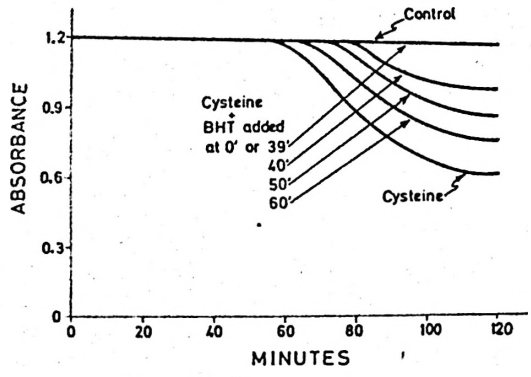

Fig. 2. *Efficiency of BHT in the prevention of inner mitochondrial membrane disaggregation induced by cysteine.*
At the indicated times after the beginning of the incubation in the presence of 0.8 mM cysteine, BHT, dissolved in 95 % ethanol, was added using 0.1 ml/10 ml of membrane suspension to give a concentration of 5×10^{-6} M. Ethanol was also added in the same proportion in the vessels incubated without BHT. See text for other experimental details.

changes of the suspension was 5×10^{-5} M in the case of ascorbate and 5×10^{-6} M in the case of cysteine. Lower concentrations gave intermediate degrees of disaggregation between the values of the control and those obtained with ascorbate or cysteine in the absence of BHT.

If BHT, at those lowest optimal concentrations, was added at a later time after the beginning of the incubation, intermediate values of turbidity were obtained, both for cysteine (Fig. 2) and for ascorbate (Fig. 3). The later the addition of BHT the greater was the decrease in turbidity gradually approaching the values of those obtained with either ascorbate or cysteine in the absence of antioxidant.

The protective effect of BHT against phospholipid degradation as well as against activity losses of mitochondrial ATPase has also been studied. Table I collects the results obtained when inner mitochondrial membranes were incubated in the presence of ascorbate or cysteine without or with BHT added at the optimal lowest concentrations at zero-time, or after the beginning of the incubation. It may be seen that the amount of extractable phospholipid diminished with respect to the controls when mitochondrial membranes were incubated with ascorbate or cysteine, and the same happened with the ATPase activity. No effect on either total phospholipid or on ATPase activity was observed when BHT was added at zero-time. This protective effect was more and more limited if BHT was added at later times during the incubation.

The efficiency of thyroxine in preventing the losses of some enzyme activities such as cytochrome oxidase and succinate cytochrome c redcutase has also been studied. Table II shows that thyroxine at a 10^{-5} M concentration inhibited not only the phos-

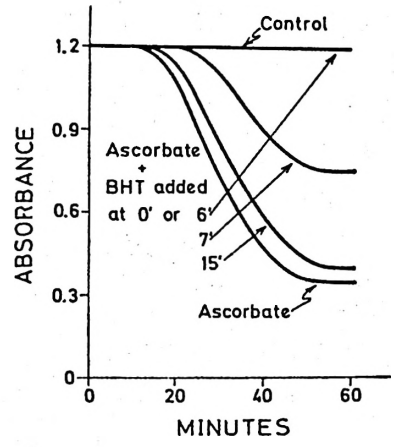

Fig. 3. *Efficiency of BHT in the prevention of inner mitochondrial membrane disaggregation induced by ascorbate.*
At the indicated times after the beginning of the incubation in the presence of 0.1 mM ascorbate, BHT, dissolved in 95 % ethanol, was added using 0.1 ml/10 ml of membrane suspension to give a concentration of 5×10^{-5} M. Ethanol was also added in the same proportion in the vessels incubated without BHT. See text for other experimental details.

pholipid degradation induced by cysteine, but also protected against losses or enzyme activity. This protection was complete when thyroxine was added not later than 30 minutes after the beginning of the incubation; if added at a later time only a partial protection was obtained.

These data confirm the relationship between the alteration of the phospholipids and losses in enzyme activities. They also suggest the possibility of using a rather simple approach to study specific associations between phospholipids and membrane enzymes.

ACKNOWLEDGEMENTS

The expert technical assistance of Mrs. Mercedes Preciado and Mr. J. J. González is gratefully acknowledged.

Resumen

Se ha estudiado la capacidad antioxidante del BHT (2,6-terbutil-4-metilfenol) y de la tiroxina para inhibir la peroxidación de fosfolípidos, desagregación de las membranas y pérdidas de actividades enzimáticas en suspensiones de membranas internas mitocondriales de hígado de rata incubadas en presencia de ascorbato 1 mM o de cisteína 0,8 mM. Si el BHT se añadía a tiempo cero a una concentración de 5×10^{-5} M en el caso del ascorbato, y de 5×10^{-6} M en el caso de la cisteína, inhibía completamente la peroxidación lipídica y procesos relacionados. Si el BHT se añadía más tarde durante la incubación se obtenían diferentes grados de inhibición. Efectos semejantes se observaron con tiroxina 10^{-5} M. Estos resultados sugieren la posibilidad de usar un método sencillo en el estudio de las asociaciones entre fosfolípidos y enzimas de membrana.

References

1. BARTLETT, G. R.: *J. Biol. Chem.*, 234, 466, 1959.
2. BERNHEIM, F., WILBUR, K. M. and KENASTON, C. B.: *Arch. Biochem. Biophys.*, 38, 177, 1952.
3. CHIO, K. S. and TAPPEL, A. L.: *Biochemistry*, 8, 2821, 1969.
4. CHIO, K. S. and TAPPEL, A. L.: *Biochemistry*, 8, 2827, 1969.
5. EUGUI, J. and SANTIAGO, E.: *Rev. Med. Univ. Navarra*, 15, 285, 1971.
6. FOLCH, J.: *J. Biol. Chem.*, 177, 497, 1949.
7. HOGEBOOM, G. M.: In «Methods in Enzymology» (S. P. Colowick and N. O. Kaplan, eds.), Vol. I, Academic Press, New York, 1955, p. 16.
8. HUNTER, F. E., JR., GEBICKI, J. M., HOFFSTEN, P. E., WEINSTEIN, J. and SCOTT, A.: *J. Biol. Chem.*, 238, 828, 1963.
9. HUNTER, F. E., SCOTT, A., HOFFSTEN, P. E., GEBICKI, J. M., WEINSTEIN, J. and SCHNEIDER, A.: *J. Biol. Chem.*, 239, 614, 1964.
10. LEHNINGER, A. L. and SCHNEIDER, M.: *J. Biophysic. Biochem. Cytol.*, 5, 109, 1959.
11. LÓPEZ-MORATALLA, N. and SANTIAGO, E.: *Rev. Med. Univ. Navarra*, 16, 127, 1972.
12. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J.: *J. Biol. Chem.*, 193, 265, 1951.
13. MINSSEN, M. and MUNKRES, K. D.: *Biochem. Biophys. Acta*, 291, 398, 1973.
14. NESKOVIC, N. M. and KOSTIC, D. M.: *J. Chromatog.*, 35, 297, 1968.
15. OTTOLENGHI, A., BERNHEIM, F. and WILBUR, K. M.: *Arch. Biochem. Biophys.*, 56, 157, 1955.
16. PARSONS, D. F., WILLIAMS, G. R. and CHANCE, B.: *Ann. N.Y. Acad. Sci.*, 137, 643, 1966.
17. PULLMAN, M. E., PENEFSKY, H. S., DATTA, A. and RACKER, E.: *J. Biol. Chem.*, 235, 3322, 1960.
18. SANTIAGO, E., MULE, S. J., REDMAN, C. M., HOKIN, M. R. and HOKIN, L. E.: *Biochim. Biophys. Acta*, 84, 550, 1964.
19. SANTIAGO, E., GUERRA, F. and MACARULLA, J. M.: *Rev. esp. Fisiol.*, 24, 25, 1968.
20. SANTIAGO, E., VÁZQUEZ, J. J., EUGUI, J., MACARULLA, J. M. and GUERRA, F.: *FEBS Symposium*, 20, 17, 1970.
21. SOTTOCASA, G. L., KUYLENSTIERNA, B., ERNSTER, L. and BERGSTRAND, A.: *J. Cell Biol.*, 32, 415, 1967.

22. TAPPEL, A. L. and ZALKIN, M.: *Arch. Biochem. Biophys.*, 80, 326, 1959.
23. TAPPEL, A. L.: *Ann. N.Y. Acad. Sci.*, 203, 12, 1972.
24. TISDALE, H. D.: In «Methods in Enzymology» (S. P. Colowick and N. O. Kaplan, eds.), Vol. X. Academic Press, New York, 1967, p. 213.
25. WITTING, L. A.: *Federation Proc.*, 24, 912, 1965.