Free Rad. Res. Comms., Vol. 14, Nos. 5–6, pp. 323–334
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ASSOCIATION OF ANTIOXIDANT SYSTEMS IN THE PROTECTION OF HUMAN FIBROBLASTS AGAINST OXYGEN DERIVED FREE RADICALS

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(Received October 2, 1990; in revised form November 23 1990)

The protection of human diploid fibroblasts against high oxygen tension was investigated using various combinations of the three major antioxidant enzymes: superoxide dismutase, catalase and gluthathione peroxidase. α-Tocopherol, a well-known hydrophobic antioxidant, was also tested in combination with the different enzymes. Microinjection of solutions containing different combinations of the three enzymes was compared with the injection of each single enzyme. We observed that the protections given by catalase or superoxide dismutase on the one hand, and by glutathione peroxidase on the other hand, were additive. Surprisingly, the combinations of catalase and superoxide dismutase were less effective than catalase alone and was even toxic at low SOD concentrations. Addition of α-tocopherol following the injection of any of the three enzymes was highly beneficial, but the strongest synergistic effect was obtained with glutathione peroxidase. These results stress the importance of membrane protection by α-tocopherol and indirectly by glutathione peroxidase. They also showed that any injection leading to the decrease in the $O_2^{\cdot -}$ or H_2O_2 concentration combined with one of these two protectors is very beneficial for the cells probably by decreasing the $OH^{\cdot}$ concentration. This is also proven by the very good protective effect obtained with desferrioxamine.

KEY WORDS: Hyperoxia, superoxide dismutase, catalase, glutathione peroxidase, α-tocopherol, microinjection.

INTRODUCTION

One condition for the survival of organisms in the presence of oxygen is their ability to keep at a low stationary phase the levels of oxygen free radicals through an equilibrium between their formation and inactivation.[1,2] These reactive molecules are produced in the cells by numerous processes such as autooxidation of some molecules, electron transfer, enzymatic reactions or metabolism of xenobiotics (for a review, see[3]). Their production leads to cellular damages including oxidation of thiols and inactivation of enzymes,[4] alterations of nucleic acids[5] and especially peroxidation of polyunsaturated lipids.[6,7]

Protection against these reactive oxygen species is provided by enzymatic systems such as catalase, superoxide dismutase (SOD) and glutathione peroxidase (GSH peroxidase)[8–10] and by antioxidant molecules like α-tocopherol and ascorbic acid.[11,12]

Many studies have demonstrated the protective effects of these defence systems in different models of free radical-induced toxicity (for a review, see[13,14]). However, since the concentrations of various free radical species are difficult to measure in the cells, it is difficult to know the exact role of these defence systems.

One approach to study the relative importance of these antioxidants is to use a microinjection technique in order to introduce increasing concentrations of each of the three above mentioned enzymes into cells and to observe their protective capacity on cells cultivated under high oxygen pressure.[15,16] It was effectively shown that catalase, superoxide dismutase and especially glutathione peroxidase were able to protect cells against the degeneration induced by hyperoxia.

In these previous investigations, the protection was observed with the injection of one enzyme at a time. However, *in situ*, the three enzymes are active but they are located in various cellular compartments and they act on different reactive oxygen species. The general consensus is that they are complementary to each other. It is this aspect of enzymatic cooperation that we wanted to test. For this purpose, we studied different combinations of the enzymes as well as the combination of α-tocopherol with each of the enzymes. These various combinations were compared for their protective ability on fibroblasts exposed to high oxygen tension.

METHODS

Chemicals

Enzymes and enzyme substrates came from Sigma Chemical Co. (St. Louis, MO, USA). The source of other chemicals was Merck A.G. (Darmstadt, Germany). Desferrioxamine came from Ciba Geigy (Grand Bigard, Belgium). Purified bovine erythrocyte CuZn superoxide dismutase (EC.1.15.1.1.), bovine liver catalase (EC.1.11.1.6.), and bovine erythrocyte glutathione peroxidase (EC.1.11.1.9.) were used for the microinjection. Injection of 6.10^5 U/ml SOD, 55 U/ml catalase or 6 U/ml GSH peroxidase corresponds to an increase respectively of 170 fold, 2.1 fold or 1/9 of the native content of these cells. The injected volume was 10^{-10} ml/cell. These enzymes injected at the same concentrations as used in this work did not affect the growth rate of human fibroblasts under normoxia.[17] α-Tocopherol. (Sigma, St. Louis, MO) was dissolved at 0.2 M in 100% ethanol and then diluted at 5×10^{-4} M in medium containing 10% fetal calf serum.

Cell culture and microinjection

The human diploid cell strain WI-38 was purchased from the "American Type Culture Collection" and serially cultivated as described by Hayflick.[18]

One day before microinjection, cells between population doubling levels 30 and 40 were subcultivated in squared Petri Dishes (Falcon Plastics, CA, USA) at a density of 100 cells per cm^2 in Eagle's basal medium supplemented with 10% fetal calf serum (Eurobio, Paris, France) and incubated at 37°C under normal atmosphere. Microinjection was performed as described by Ansorge[19] according to the method developed by Graessmann *et al.*[20] Just after microinjection, cells were exposed to 2 atm of 95% O_2 and 5% CO_2 in the same medium. Injected and non-injected cells still attached to the flask were counted individually every day and considered as surviving cells. 30 to 40 cells were considered in each group. The low density of cell culture was needed in order to follow individually the injected cells.

The results are expressed as "percentage of protection": it is the ratio between the integration of the survival curve of enzyme injected cells and the integration of buffer

injected cells at days 1,2 and 3 after injection. The protection brought by the enzymes was maximum during these three days: indeed, during the first day, a few cells die due to the stress of the microinjection and after 4 days, the protection diminished probably because of degradation of the injected enzymes. The estimated half-lives of SOD and catalase are respectively 80 and 186 hours.[21] These half-lives were estimated for natural enzymes in human cells but the degradation rate of proteins seems to be dependent only on the enzyme properties and not on the cell species.[22] However, these data are not yet known for GSH peroxidase. Other controls like non-injected cells or cells injected with heat-inactivated enzymes were also performed. More details on the technique were also given in a previous article.[16]

Statistical analysis

The results are presented as mean $\pm$ 1 SD (standard deviation) and data were analysed by a Student's test.

RESULTS

Microinjection of two enzymes

Previous studies showed that the protection achieved by microinjection of catalase, superoxide dismutatse or glutathione peroxidase was concentration dependent and that GSH peroxidase was the most efficient enzyme.[16] In this work, we first investigated the possibility of enzymatic cooperation by injecting various combinations of these three enzymes.

An example of such an experiment is given in Figure 1A which illustrates the effect of combination of catalase and GSH peroxidase on the survival of injected cells. The survival curves for cells injected with a solution containing 55 U/ml catalase alone or a solution of 3 U/ml GSH peroxidase alone were slightly higher than for buffer-injected cells. However, the survival curve for cells injected with a solution containing catalase and GSH peroxidase was markedly higher than the two others. It shows that the protection afforded by the combination of the two enzymes is greater than with one of the two enzymes injected alone indicating an addition of the protective effects. Controls with heat-inactivated catalase were also performed (Figure 1B). The cells injected with a catalase solution containing 55 U/ml showed a higher survival than control cells injected with buffer. On the contrary, survival curve of cells injected with heat-inactivated catalase showed a similar evolution than the one of buffer-injected cells indicating that the protection was due to the presence of active enzyme and not the result of the injection of proteins.

In Figure 2A, different concentrations of SOD were injected with a constant concentration of GSH peroxidase (3 U/ml) which alone gave 20% protection. At concentrations lower than 0.45. 106 U/ml, SOD alone was uneffective in protecting these cells and its association with GSH peroxidase was nearly equal to the GSH peroxidase (18%). At higher concentrations, the protection was evident both for the enzyme alone and for the enzyme associated with GSH peroxidase, reaching 35% at 0.6. 106 U/ml SOD.

The same experiment was performed using different concentrations of catalase combined with a constant concentration of GSH peroxidase (3 U/ml) (Figure 2B).

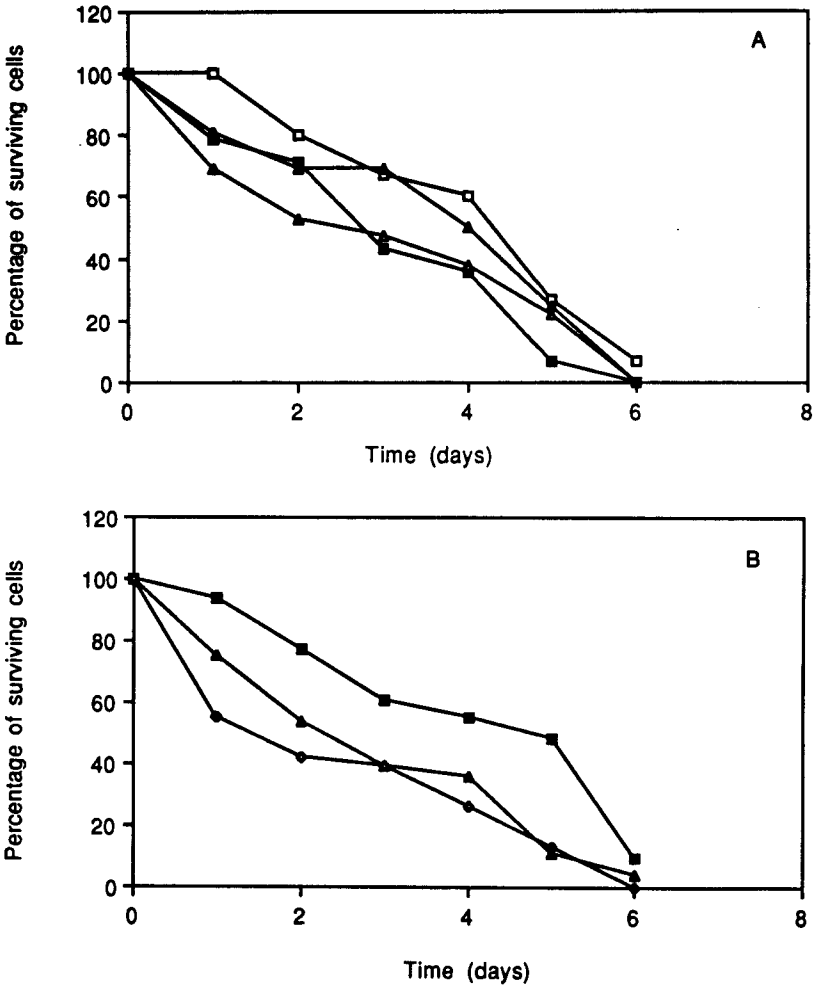
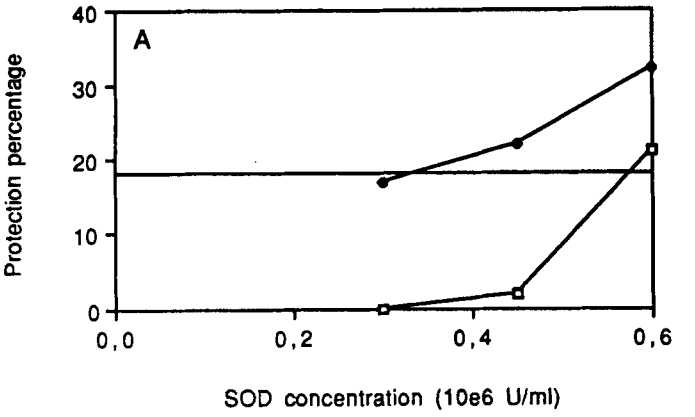
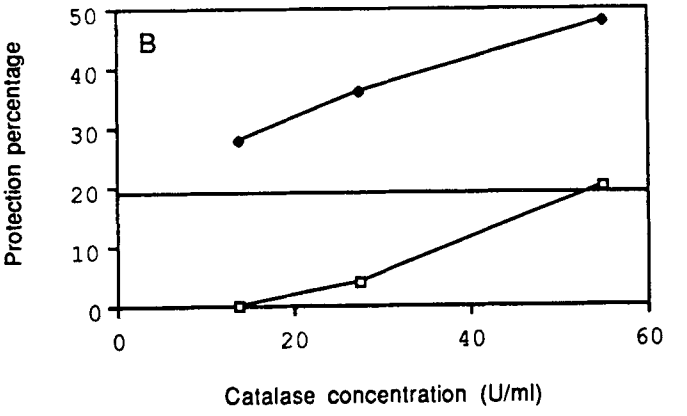
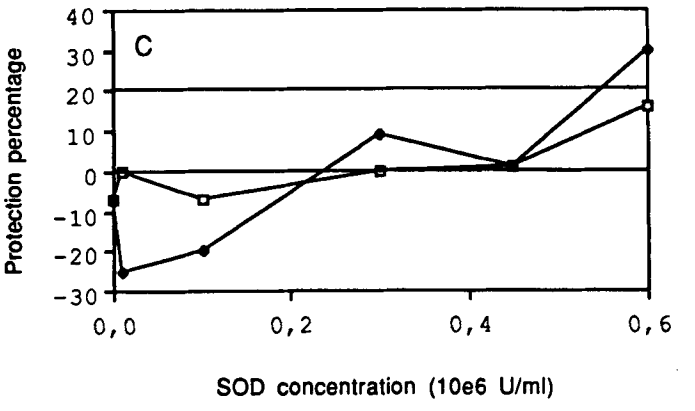


FIGURE 1 (A) Survival curves of fibroblasts exposed under 2 atm of oxygen and injected with buffer (△), a solution containing 55 U/ml catalase (■), with a solution of 3 U/ml GSH peroxidase (▲) or a solution containing 55 U/ml catalase + 3 U/ml GSH peroxidase (□). The results are expressed as percentage of surviving cells related to the number of cells considered at day 0 (± 40 cells). (B) Survival curves of fibroblasts exposed under 2 atm of oxygen and injected with buffer (△), 55 U/ml catalase (■) or 55 U/ml heat-inactivated catalase (1 h 30 at 85°C) (◇). The results are expressed as percentage of surviving cells related to the number of cells considered at day 0 (± 40 cells)

The protection curve obtained with both enzymes was parallel to the curve of catalase alone but shifted by 30% to higher values. The effect of both enzymes appears to be synergistic. These two data showed that cells submitted to oxidative stress react rather positively to combinations of, at the one hand, SOD or catalase and, on the other hand, GSH peroxidase.







The third association was obtained with increasing SOD concentrations added to a constant concentration of catalase (27.5 U/ml) which gives alone 20% protection (Figure 2C). Surprisingly, the presence of small amounts of SOD decreased the catalase protection and even speeded up the degeneration of the cells. The mortality of cells injected with combinations of 27.5 U/ml of catalase with 0.1. 10^6 or 0.01 10^6 U/ml of SOD was much higher than the mortality of cells injected with buffer. Only at the highest concentration of SOD, was it possible to obtain a positive effect of this enzyme. This experiment was repeated and the same conclusions were obtained.

Association of enzymes and α-tocopherol

In the experimental conditions described in Materials and Methods, α-tocopherol added at 5×10^{-4} M to the culture medium gave a 20% protection to human fibroblasts cultivated under high oxygen pressure when compared to its vehicle (ethanol 0.25%). In order to determinate if a synergistic effect could exist between vitamin E and each of the three antioxidant enzymes, the latter were injected separately in cells and the culture medium was supplemented with 5×10^{-4} α-tocopherol.

Figure 3A shows the protection produced by catalase with or without α-tocopherol. The catalase injection alone gave respectively 11.5 and 24.5% protection at 27.5 and 55 U/ml but the protection raised to 30.5 and 60.5% when α-tocopherol was added in the medium. The combination of both antioxidants seems to be additive or even slightly synergistic.

A similar but more marked protective effect could be observed with SOD (Figure 3B) but the most synergistic effect was observed with GSH peroxidase (Figure 3C): the protection was indeed much higher than the sum of the protections given by each of the antioxidant systems. For example, at 6 U/ml of GSH peroxidase, the protection of the enzyme alone was 25.5% when it reached 115.5% in the presence of α-tocopherol. Similarly, at 0.6×10^6 U/ml of SOD, the protection was 17% for the enzyme alone but 62.5% when α-tocopherol was also present in the culture medium. The statistical analysis shows that a highly significant difference is obtained in the comparison of fibroblasts injected with each enzyme concentration with or without α-tocopherol: the addition of α-tocopherol gave thus a significant increase of protection. α-tocopherol is seen as an effective complement to the protection given by each of the three enzymes.

Desferrioxamine protection

Since the observed toxic effect under oxygen could be mediated through hydroxyl

FIGURE 2 (A) Protection of fibroblasts incubated under 2 atm of oxygen by microinjection of increasing concentrations of SOD with (◆) or without (□) GSH peroxidase at a constant concentration (3 U/ml). The horizontal line represents the 20% protection obtained by the injection of GSH peroxidase alone. The protection is expressed as the ratio between the integration of the survival curve of cells injected with the active enzyme and cells injected with buffer. Protection is given as a function of the enzyme concentrations in microinjection solutions. (B) Protection against 2 atm of oxygen after microinjection of increasing concentrations of catalase with (◆) or without (□) GSH peroxidase at a constant concentration (3 U/ml). The horizontal line represents the 20% protection obtained by the injection of GSH peroxidase alone. (C) Protection against 2 atm of oxygen after microinjection of increasing concentrations of SOD with (◆) or without (□) catalase at a constant concentration (27.5 U/ml). The horizontal line represents the 20% protection obtained by the injection of catalase alone.

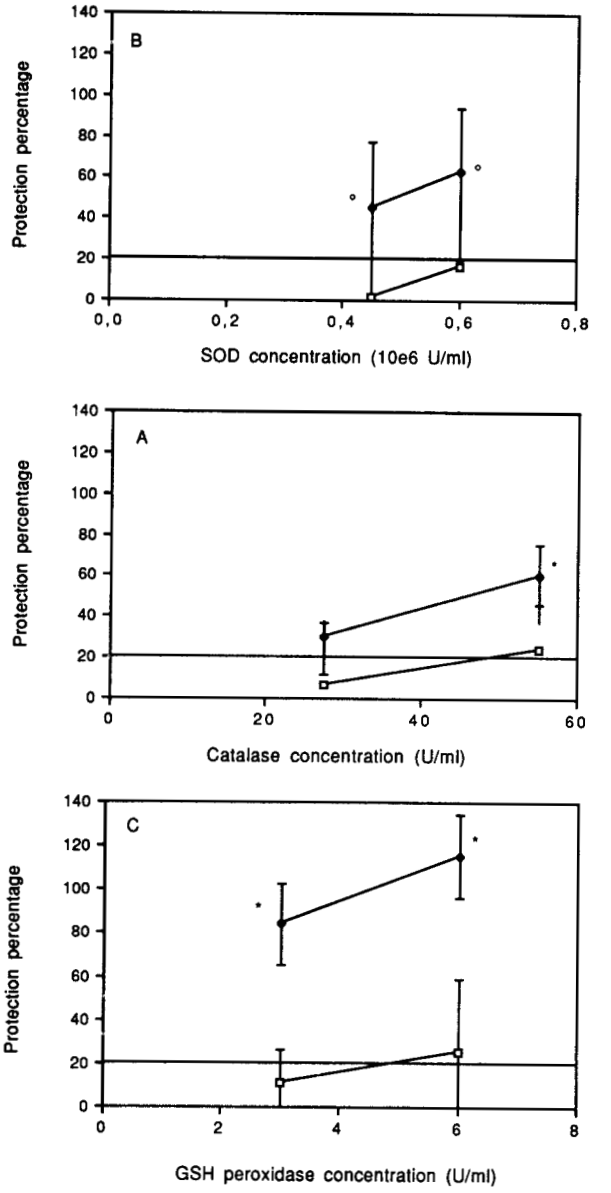


FIGURE 3 Protection of fibroblasts incubated under 2 atm of oxygen by microinjection of catalase (A), SOD (B) or GSH peroxidase (C). The cells were incubated in medium containing (◆) or not (□) α-tocopherol at a constant concentration ($5\,10^{-4}$ M). The continuous horizontal line represents the 20% protection obtained by the addition of α-tocopherol alone. The protection is the ratio between the integration of the survival curve of enzyme-injected cells in the presence of α-tocopherol and the curve of cells buffer-injected and cultivated without α-tocopherol in the medium. Results are expressed as means of two experiments ± 1 SD. *significantly different from the corresponding enzyme-injected cells cultivated without α-tocopherol ($\alpha = 0.05$) °significantly different from the corresponding enzyme-injected cells cultivated without α-tocopherol ($\alpha = 0.10$)

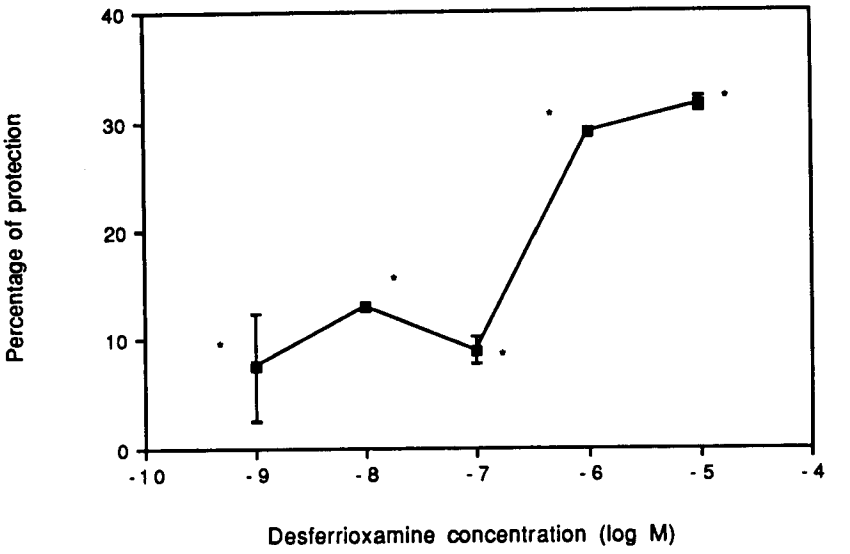


FIGURE 4 Protection of fibroblasts incubated under 2 atm of oxygen by desferrioxamine added in the culture medium. The protection is expressed as the ratio between the integration of the survival curve of cells incubated with desferrioxamine and control cells incubated without it. Results are expressed as means of two experiments ± 1 SD. *significantly different from fibroblasts incubated under 2 atm of oxygen without desferrioxamine ($\alpha = 0.05$)

radical production, we tested the protective effect of desferrioxamine. It is a well known chelator of iron preventing its further reaction with H_2O_2.[23] When added in the culture medium, desferrioxamine very markedly protected fibroblasts against hyperoxia toxicity (Figure 4): 32% protection was obtained at 10^{-5} M and 8% at 10^{-9} M. All these protections were significant (Student's test, $P > 0.95$).

DISCUSSION

Cultivated cells are widely used for basic research on oxidative stress because of the facilities given by the experimental conditions.[25-27] For instance, microinjection of individual cells with antioxidant enzymes allowed a quantitative comparison of the efficiency of each of the three antioxidant enzymes and it was found that GSH peroxidase was far and away the most efficient one.[16,27] In this work, the same powerful technique was used in order to investigate the complementarity and cooperation existing between these antioxidant enzymes and α-tocopherol, which has also been shown to be protective under high oxygen tension. Controls performed with heat-inactivated enzymes did not show any protection; so it is indeed the enzymatic activity which is responsible for the protective effect.[16]

Cooperation between catalase and superoxide dismutase has been described by Freeman *et al.*[28] They observed a significant increase in the protection of endothelial cells against oxygen via liposomes loaded with both enzymes in comparison with liposomes containing one enzyme. However, Bagley *et al.*[29] did not observe any protecting effect when they introduced catalase and SOD, by using the "scrape-load-

ing" method, into Chinese Hamster ovary cells challenged with paraquat. Moreover, Finazzi-Agro *et al.*[30] investigating erythrocyte hemolysis, showed that the addition of SOD to liposomes loaded with catalase, decreased the protection observed with catalase alone. In this paper, we observed that for the combination of SOD and catalase, the effect was very much concentration dependent with a negative and even toxic effect at low SOD concentrations but a protection at very high SOD concentrations. As an example, the highest concentration which gave 16% protection, represents an injection of 170 fold the native content while the lowest one which gave 7% toxic effect compared to the buffer-injected cells, represents 1/3.5 of the native content.

One explanation for this strong concentration dependency could be that addition of low SOD concentrations sufficiently increases the production of H_2O_2 as to promote the Haber Weiss reaction which is responsible for the formation of highly toxic OH· radicals. Allen *et al.*[31] effectively found an increase of H_2O_2 when the intracellular SOD activity of slime mold cells is increased via liposomes. The possibility of OH· involvement in the oxygen toxicity was well demonstrated by the protective effect of desferrioxamine. Desferrioxamine prevents by chelation the interaction of iron with H_2O_2 thus preventing OH· formation (Figure 4). If this is true, the interpretation of the results could be that when either the SOD or/and the catalase concentrations are high enough, the Haber Weiss reaction is inhibited since at least one of the reagents is missing and a protection can then be observed. If it is not the case, H_2O_2 and $O_2^{\cdot -}$ can react together with iron to generate OH· which is highly toxic and can explain the observed toxicity. We propose that some contradictory results of the literature could be probably explained by the differences in the relative concentrations of both enzymes used in the experiments.

The importance of catalase and superoxide dismutase in protecting cells against oxygen species has been recognized for a long time and they are even used as therapeutic medicine.[32] But more and more researches are performed on glutathione peroxidase given its crucial role. GSH peroxidase is an essential enzyme for the destruction of H_2O_2 acting even better than catalase under physiological conditions.[33,34] Therefore, if GSH peroxidase detoxifies H_2O_2 efficiently at low concentrations, it is not surprising to observe a better complementarity of SOD with GSH peroxidase (Figure 2A) than with catalase (Figure 2B).

The importance of H_2O_2 rather than $O_2^{\cdot -}$ for cell death has already been stressed by different authors.[35,36] Glutathione peroxidase reduces H_2O_2 and organic hyperoxides, protecting cytosol and cellular membranes by preventing lipid peroxidation.[37] On the other hand, catalase and superoxide dismutase principally act in hydrophilic regions of the cell. Consequently, the protections of the hydrophilic and hydrophobic levels are complementary to keep the cellular integrity against the oxygen free radical attacks. An experimental argument for this principle is the additive protection obtained when catalase or SOD are combined with GSH peroxidase.

The combination of α-tocopherol with the three enzymes was also very effective especially with GSH peroxidase since, in this case, the resulting protection was higher than the sum of the protections of the individual systems. Vitamin E gets into the hydrophobic core of biomembranes where it terminates the peroxidative chain reaction. On the other side McCay *et al.*[37] showed that GSH peroxidase protected membranes from peroxidation by preventing initiation of such a reaction by destroying H_2O_2 and by removing hydroperoxides released by the phospholipase A_2.[38]

Therefore, the combination of GSH peroxidase and α-tocopherol should be a very efficient system for the protection against any free radical attack on the membranes.

Addition of catalase or SOD with α-tocopherol also improved the protection of the cells: the initiation of polyunsaturated fatty acids peroxidation involves the generation of hydroxyl radicals resulting from the interaction of H_2O_2 and $O_2^{\cdot -}$ catalyzed by iron or another transition metal.[39] If any of these two molecules is removed by one of the enzymes, the production of $OH^{\cdot}$ will be lowered and the lipids of the cell membranes protected. The additive protecting effects when combining either SOD or catalase with α-tocopherol therefore favour the hypothesis of the important role of lipid peroxidation in cell damage. The importance of $OH^{\cdot}$ was confirmed by the marked protection obtained when desferrioxamine was added in the culture medium.

In conclusion, various antioxidant protecting systems were combined. These combinations in general improved the protection of cells under oxidative stress. However, according to the combinations tested, the protecting effects were either slightly increased or additive or even synergistic, as observed with α-tocopherol associated with GSH peroxidase. Unexpectedly, when various concentrations of SOD were combined with catalase, the protecting effect due to catalase alone was decreased by SOD at least at the lower concentrations of the latter enzyme.

Acknowledgements

C. Michiels is a Research Assistant and M. Raes a Research Associate at the F.N.R.S. (Fonds National de la Recherche Scientifique, Brussels)

References

1. I. Fridovich (1985) Superoxide dismutases: regularities and irregularities *The Harvey Lectures*, **79**, 51–75.
2. W.A. Pryor (1976) The role of free radical reactions in biological systems in *Free Radicals in Biology*, vol I, ed by W. Pryor, pp. 1–49, Academic Press, New York.
3. J.M. McCord and I. Fridovich (1978) The biology and pathology of oxygen radicals *Annals of Internal Medicine*, **89**, 122–127.
4. L. Fucci, C.N. Oliver, M.J. Coon and E.R. Stadtman (1983) Inactivation of key metabolic enzymes by mixed-function oxidation reactions: possible implication in protein turnover and aging *Proceedings of the Natural Sciences*, **80**, 1521–1525.
5. P. Sestili, G. Piedmonte, F. Cattabeni and O. Cantoni (1986) Induction of DNA breakage and suppression of DNA synthesis by the OH radical generated in a Fenton-like reaction *Biochemistry International*, **12**, 493–501.
6. A. Tappel (1973) Lipid peroxidation damage to cell components *Federation Proceedings*, **32**, 1870–1874.
7. M. Comporti (1985) Biology of disease. Lipid peroxidation and cellular damage in toxic liver injury *Laboratory Investigation*, **53**, 599–623.
8. B.E. Leibovitz and M.S. Siegel (1980) Aspects of free radical reactions in biological systems: aging *Journal of Gerontology*, **35**, 45–56.
9. B. Halliwell (1978) Biochemical mechanisms accounting for the toxic action of oxygen on living organisms: the key role of superoxide dismutase *Cell Biology of International Reports*, **2**, 113–124.
10. I. Fridovich (1976) Oxygen radicals, hydrogen peroxide and oxygen toxicity in *Free Radicals in Biology*, vol I, ed by W. Pryor, pp. 239–277, Academic Press, New York.
11. G.W. Burton, K.H. Cheeseman, T. Doba, K.V. Ingold and T.F. Slater (1983) Vitamin E as an oxidant in vitro and in vivo in *Ciba Foundation Symposium*, vol 101, pp 4–14, ed Pitman, London.
12. T. Doba, G.W. Burtona, and K.V. Ingold (1985) Antioxidant and coantioxidant activity of vitamin C. The effect of vitamin C either alone or in the presence of vitamin E or a water soluble vitamin E analogue upon the peroxidation of aqueous multilamellar phospholipid liposomes *Biochimica et Biophysica Acta*, **835**, 298–303.

13. B. Halliwell and J.M.C. Gutteridge (1984) Oxygen toxicity, oxygen radicals transition metals and disease *Biochemical Journal,* **219**, 1–14.
14. B. Chance, H. Sies and A. Boveris (1979) Hydroperoxide metabolism in mammalian organs *Physiological Reviews,* **59**, 527–605.
15. C. Michiels, M. Raes and J. Remacle (1986) A new experiment model to study oxygen toxicity *Archives Internationales de Physiologie et Biochimie* **94**, S13-S17.
16. M. Raes, C. Michiels and J. Remacle (1987) Comparative study of the enzymatic defence systems against oxygen-derived free radicals: the key role of glutathione peroxidase *Free Radical in Biology and Medicine,* **3**, 3–7.
17. C. Michiels, M. Raes, E. Pigeolet, P. Corbisier, D. Lambert and J. Remacle (1990) Importance of a threshold level for error accumulation in cell degenerative processes. I. Modulation of the threshold in a model of free radical-induced cell degeneration *Mechanisms of Ageing and development,* **51**, 41–54.
18. L. Hayflick, and P.S. Moorhead (1961) The serial cultivation of human diploid cell strains *Experimental Cell Research,* **25**, 585–621.
19. W. Ansorge (1982) Improved system for capillary microinjection in living cells *Experimental Cell Research,* **140**, 31–37.
20. A. Graessmann M. Graessmann and C. Mueller, (1980) Microinjection of tissue culture using capillaries: methods in *Methods in Enzymology*, vol 65, pp 4831–4834, Academic Press, New York.
21. S. Rogers, R. Wells and M. Rechsteiner (1986) Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis *Science,* **234**, 364–368.
22. M. Rechsteiner, D. Chin, R. Hough, T. McGarry, S. Rogers, K. Rote and L. Wu (1984) What determines the degradation rate of an injected protein? in Ciba Foundation Symposium, vol 103, pp 181–195, ed Pitman, London.
23. B. Halliwell and J.M.C. Gutteridge (1986) Oxygen free radicals and iron in relation to biology and medicine some problems and concepts *Archives. Biochemistry and Biophysics,* **246**, 501–514.
24. A.K. Balin, D.B.P. Goodman, H. Hasmussen and V.J. Cristofalo. (1976) The effect of oxygen tension on the growth and metabolism of WI-38 cells *Journal of Cellular Physiology,* **89**, 235–250.
25. S. Honda and M. Matsuo (1983) Shortening of the in vitro lifespan of human diploid fibroblasts exposed to hyperbaric oxygen *Experimental Gerontology,* **18**, 339–345.
26. R.W. Brosomer and W.J. Rutter (1961) The effect of oxygen tension on the growth and metabolism of a mammalian cell *Experimental Cell Research,* **25**, 103–113.
27. C. Michiels and J. Remacle (1988) Quantitative study of natural antioxidant systems for cellular nitrofurantoin toxicity *Biochimica Biophysica Acta,* **967**, 341–347.
28. B.A. Freeman, J.F. Turrens, Z. Mirza, J.D. Crapo and S.L. Young, (1985) Modulation of oxidant lung injury by using liposome-entrapped superoxide dismutase and catalase *Federation Proceedings,* **44**, 2591–2595.
29. A.C. Ragley, J. Krall and R.E. Lynch (1986) Superoxide mediated the toxicity of paraquat for chinese hamster ovary cells *Proceedings of the National Academy of Sciences,* **83**, 3189–3193.
30. A. Finazzi-Agro, A. DiGuilo, G. Arnicosante and C. Crifo (1986) Photohemolysis of erythrocytes enriched with superoxide dismutase, catalase and glutathione peroxidase *Photochemistry and Photobiology,* **43**, 409–412.
31. R.G. Allen, A.K. Balin, R.J. Reimer, R.S. Sohal and C. Nations (1988) Superoxide dismutase induces differentiation in microplasmodia of the slime mold Physarum polycephalum *Archives in Biochemistry Biophysics,* **261**, 205–211.
32. A. Petkau (1986) Scientific basis for the clinical use of superoxide dismutase *Cancer Treatment Review* **13**, 17–44.
33. L. Flohé (1982) Glutathione peroxidase brought into focus in *Free Radicals in Biology*, vol V, ed by W. Pryor, pp 223–254, Academic Press, New York.
34. P. Hochstein and H. Ustley (1968) Hydrogen peroxide detoxification by glutathione peroxidase and catalase in rat liver homogenates *Molecular Pharmacology,* **4**, 574–579.
35. R.H. Simon, C.H. Scoggin and D. Paterson (1981) Hydrogen peroxide causes the fatal injury to human fibroblasts exposed to oxygen radicals *Journal of Biological Chemistry,* **256**, 7181–7186.
36. S.T. Sacks, C.F. Moldow, P.R. Craddock, T.K. Bowers and M.S. Jacob (1978) Oxygen radicals mediate endothelial cell damage by complement-stimulated granulocytes *Journal of Clinical Investigation,* **61**, 1161–1167.
37. P.B. McCay, D.D. Gibson, K.L. Fong and K.R. Hornbrook (1976) Effect of glutathione peroxidase activity on lipid peroxidation in biological membranes *Biochimica et Biophysica Acta,* **431**, 459–468.
38. F.J.G.M. Van Kuijk, G.J. Handelmann and E.A. Pratz (1985) Consecutive action of phospholipase A2 and glutathione peroxidase is required for reduction of phospholipid hydroperoxides and provides

a convenient method to determine peroxide values in membranes *Journal of Free Radicals in Biological Medicine*, **1**, 421–427.
39. K.L. Fong, P.B. McCay, J.L. Poyer, B.B. Keele and M. Misra (1973) Evidence that peroxidation of lysomal membranes is initiated by hydroxyl free radicals produced during flavin enzyme activity *Journal of Biological Chemistry*, **248**, 7792–7797.

Accepted by Prof Barry Halliwell