Importance of various antioxidant enzymes for cell stability

Confrontation between theoretical and experimental data

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A theoretical model was developed taking into account the production and destruction of oxygen-derived free radicals. The steady state of the system was derived by using the rate equations of these reactions, and the stability of the system was tested. In the simplified model, only one stable steady state was found. However, we know that glutathione peroxidase can be inhibited by hydroperoxides, and, when incorporated into the model, this effect led to a complex situation with the presence of some stable and some unstable domains according to the concentration of either the enzyme or the hydroperoxide. This qualitative description of the system was compared with experimental data on the protection given by three antioxidant enzymes, and concordance of data was found which allows some quantification of the system. A general view of the efficiency of the three antioxidant enzymes and of the stability of the system according to their concentrations could be produced.

INTRODUCTION

Free radicals are well-known reactive molecules mainly derived from univalent reduction of oxygen and giving rise to numerous by-products through reactions with almost all the unsaturated double bonds found in natural living cells. The detrimental effects of such reactive molecules have been well described, since they can destabilize cell membranes by reacting with unsaturated fatty acids (Mead, 1976; Freeman & Crapo, 1982; Slater *et al.*, 1987), they can inactivate, fragment or aggregate enzymes through reaction with double bonds on amino acids or with cysteine (Machlin & Bendich, 1987; Davies, 1987; Webster & Nunn, 1988), and finally they can introduce mutations within DNA by modification of bases and by DNA-strand scission (Freeman & Crapo, 1982; Machlin & Bendich, 1987; Webster & Nunn, 1988; Schulte-Frolinde & Von Sonntag, 1985).

The production of free radicals in cells is mainly attributed to the activity of oxidases such as xanthine or aldehyde oxidases, flavoprotein dehydrogenases or the mitochondrial electron-transport proteins and to cytochrome *P*-450 activity; autoxidation of molecules such as thiols, hydroquinones, flavins and catecholamines will also contribute to the formation of these radicals (Freeman & Crapo, 1982; Machlin & Bendich, 1987). The concentrations of superoxide ($O_2^{\cdot -}$) and H_2O_2 have been estimated to be respectively 10^{-11} M (Chance *et al.*, 1979) and 10^{-8} M (Oshino *et al.*, 1973). The reactivity of $OH^{\cdot}$ is so high [half-life 10^{-9} s (Pryor, 1976)] that it is difficult to estimate its intracellular concentration (Green & Hill, 1984).

The production of free radicals may drastically increase in unusual conditions, such as during hyperoxia (Jamieson *et al.*, 1986), in the presence of antineoplastic agents, under irradiation and in the presence of chemical pollutants (Freeman & Crapo, 1982). For this reason, free radicals are considered to be the main factors responsible for cell damage and cell death observed under these conditions of stress.

Free radical production in cells is relatively low in normal conditions, given the various and very active defence systems including chemical scavengers or antioxidant molecules and the three enzymes, superoxide dismutase (SOD), catalase and glutathione peroxidase (GPX) (Fridovich, 1976).

Various approaches have been tested to define the importance of such protective systems. Molecular genetics have been used to

study the regulation of SOD [for a review, see Hassan (1988) and Touati (1988)] and GPX (Saedi *et al.*, 1988; Mullenbach *et al.*, 1988). The effects of oxidative stress on different cell components or functions have been extensively studied. Examples are the effects of oxidative stress on mitosis (Brosemer & Rutter, 1961; Honda & Matsuo, 1983; Burdon & Rice-Evans, 1989; Michiels *et al.*, 1990a), energy metabolism (Corbisier *et al.*, 1990), the redox-state, the GSH/GSSG ratio (Remacle *et al.*, 1984) and the glutathione cycle (Andreoli *et al.*, 1986; Spector *et al.*, 1987; Honda & Matsuo, 1988; Hoener *et al.*, 1989).

Modification of the content of antioxidant enzymes has also provided relevant information about their physiological functions in the cells. The controlled decrease in antioxidant enzyme activity by antibodies (Michiels *et al.*, 1988) or chemicals (Michiels *et al.*, 1989) showed that GPX is the key enzyme of the antioxidant system under normal conditions and conditions of oxidative stress. Furthermore, the controlled increase in antioxidant enzyme activity by microinjection into fibroblasts (Raes *et al.*, 1987) or endothelial cells (Michiels *et al.*, 1990b) also showed that GPX is the crucial enzyme of the antioxidant system in cultured fibroblasts and cultured human endothelial cells. Indeed, a much lower amount of GPX than catalase or SOD had to be injected to afford the same protection during oxidative stress.

If experimental data on the relative importance of the three antioxidant enzymes have been numerous, a theoretical approach which would allow synthesis of all the data into a general model is, however, lacking. The production of such a model is the main purpose of this work. We first developed a theoretical model based on the fact that free-radical concentration is the result of a balance between their formation and elimination by enzymic and chemical scavengers. For this purpose we derived kinetic equations for the main cellular free-radical reactions. We then tested the evolution and stability of the system of equations given a fluctuation of the radical species. In the model, we also took into consideration the fact that antioxidant enzymes, especially SOD and GPX, can be inhibited by some free-radical species (Pigeolet *et al.*, 1990). The resolution of this theoretical model gave qualitative information on the stability and instability of the system according to the concentration of free radicals and antioxidant enzymes. These qualitative data were then compared with experimental values, and they were found to agree, which

Fig. 1. Model of oxygen free-radical formation and elimination taking into account the most important reactions

Each of the reactions can be characterized by one or two rate coefficients (k_i). The three main enzymic eliminations of free radicals are catalysed by SOD, catalase and GPX.

allows a general picture to be drawn which expresses the stability of the antioxidant system according to the evolution of these antioxidant enzymes and allows a comparison of efficiencies of the three antioxidant enzymes. This model is only a simplification of the situation *in vivo*, since we did not take into consideration intracellular compartmentalization of the three antioxidant enzymes and the presence of many enzymes with GPX activity (Chance *et al.*, 1979).

THEORETICAL MODELLING

The reactions leading to free-radical production and the various systems for their elimination or destruction are well known. We have incorporated the most important ones into the model presented in Fig. 1.

Since all these reactions are incorporated into the same model, a dynamic system is formed which represents the balance between the formation and removal of these intermediate free-radical species. One kinetic constant has been given for each reaction, so that the possibility of a reverse reaction has been neglected. Two constants have been attributed to the reaction catalysed by GPX, given its peculiar kinetic behaviour. The rate of each reaction can be written using these parameters. Since the concentrations of free radicals are much lower than the K_m for catalase and SOD, we assumed first-order rate kinetics. Because of the presence of glutathione, the complete kinetic equation has been used for GPX (Flohé *et al.*, 1972; Chaudière & Tappel, 1983). The variation in the concentration of each intermediate with time can then be written as the difference between its rate of formation and its rate of removal.

The model has to be completed by the observations that free radicals can themselves act on the three protective enzymes and lead to their inhibition (Pigeolet *et al.*, 1990). We consider here two main inhibitions. First, SOD is very sensitive to the presence of H_2O_2 . Secondly, GPX is also rapidly inactivated by hydroperoxides with, for example, a 50% inhibition by *t*-butyl

hydroperoxide at 50 μM (Pigeolet *et al.*, 1990). To take into account the possible inhibition of these two enzymes, their concentrations in the equations have been corrected by the term $1 - \alpha[H_2O_2]$ and $1 - \beta[ROOH]$, α and β being respectively the inhibitory constants of SOD by H_2O_2 and GPX by ROOH with the limits:

$$0 < \alpha \leq (\text{Max. } [H_2O_2])^{-1}$$

and

$$0 < \beta \leq (\text{Max. } [ROOH])^{-1}$$

For each free-radical species, the rate of variation of its concentration will be:

$$\frac{[O_2^-]}{dt} = \dot{X} = k_1[O_2][e^-] - k_2[O_2^-]^2 - \{k_3[SOD](1 - \alpha[H_2O_2]) + k_4[Fe^{3+}] + k_5[XH] + k_{10}[RH]\}[O_2^-] \quad (1)$$

$$\begin{aligned} \frac{[H_2O_2]}{dt} = \dot{Y} = & \{k_5[XH] + k_3[SOD](1 - \alpha[H_2O_2]) \\ & + k_6[SH_2][\text{oxidases}] - k_7[Fe^{2+}] + k_8[\text{catalase}]\}[H_2O_2] \\ & - k_9 k_9 [GSH](k_9[H_2O_2]) \\ & + k_9[GSH]^{-1}(1 - \beta[ROOH])[GPX][H_2O_2] \end{aligned} \quad (2)$$

$$\frac{[OH^-]}{dt} = \dot{Z} = k_7[Fe^{2+}][H_2O_2] - (k_{11}[RH] + k_{16}[XHH])[OH^-] \quad (3)$$

$$\begin{aligned} \frac{[ROOH]}{dt} = \dot{R} = & k_{13}[RH][ROOH^-] - k_{14}k_{14}[GSH](k_{14}[ROOH]) \\ & + (k_{14}[GSH])^{-1}(1 - \beta[ROOH])[GPX][ROOH] \end{aligned} \quad (4)$$

$$\begin{aligned} \frac{[R^\cdot]}{dt} = \dot{W} = & k_{10}[RH][O_2^-] + k_{11}[RH][OH^-] + k_{13}[RH][ROOH] \\ & - (k_{12}[O_2] + k_{15}[XHH])[R^\cdot] \end{aligned} \quad (5)$$

$$\frac{[ROO^\cdot]}{dt} = \dot{U} = k_{12}[O_2][R^\cdot] - k_{13}[RH][ROOH^\cdot] \quad (6)$$

where $\dot{X}$, $\dot{Y}$, $\dot{Z}$, $\dot{W}$, $\dot{R}$ and $\dot{U}$ are the derivatives of the free-radical variables with time.

The parameters, variables, α , β and kinetic constants are defined in Table 1. Fe^{2+} and Fe^{3+} have been treated as parameters in the model, since their concentrations are very well controlled by various proteins (Halliwell & Gutteridge, 1984).

Model without enzyme inhibition ($\alpha = \beta = 0$)

In the working system, the rate of formation of free radicals is quickly compensated by their rate of elimination, so that we can postulate that there is a stationary state where the variation in their concentration is nil: so that eqns. (1), (2), (3), (4), (5) and (6) are equal to zero. Given this condition, the overall dynamic system described by these six equations has one, and only one, stationary state characterized by the value (X_0 , Y_0 , Z_0 , W_0 , R_0 , U_0) for each free radical. These values are given by resolution of the six differential equations. For example, resolution (1) gives:

$$X_0 = \frac{1}{2k_2} ((k_3[\text{SOD}] + k_4[\text{Fe}^{3+}] + k_5[\text{XH}] + k_{10}[\text{RH}])^2 + 4k_1 k_2 [\text{e}^-] [\text{O}_2])^{1/2} - (k_3[\text{SOD}] + k_4[\text{Fe}^{3+}] + k_5[\text{XH}] + k_{10}[\text{RH}])$$

and the integration of eqn. (1) with time gives:

$$x(t) = \frac{1}{2k_2} [(T+P) - (T-P) \exp(t+a)] [\exp(t+a) - 1]^{-1}$$

with $a > 0$, with $T = k_3[\text{SOD}] + k_4[\text{Fe}^{3+}] + k_5[\text{XH}] + k_{10}[\text{RH}]$ and $P = (T^2 + 4k_1 k_2 [\text{O}_2] [\text{e}^-])^{1/2}$

Calculation of this equation shows that

$$\lim_{(t \rightarrow \infty)} x(t) = x_0$$

Let us first consider the stability of eqns. (1) and (2), which are only dependent on X and Y and can then be studied as an independent system. The stability of the system has been tested by a commonly used method, i.e. the determination of the eigenvalues of the Jacobian built from the system of equations. The sign of these eigenvalues was estimated by the criteria developed by Routh-Hurwitz (Murray, 1989).

The Jacobian $J(X, Y)$ of the system of these two equations $\delta(\dot{X}, \dot{Y})/\delta(X, Y)$, has determinant $\det J(X_0, Y_0) > 0$ and trace $J(X_0, Y_0) < 0$, so (X_0, Y_0) is a stable stationary state for the system of eqns. (1) and (2). This state can be a stable node or a stable focus according respectively to whether $4 \det J(X_0, Y_0) - [\text{trace } J(X_0, Y_0)]^2$ is negative or positive.

Now let us consider the general system $(X_0, \dots, U_0)$ with all six equations (1, ..., 6). The Jacobian of the system is $J(X, \dots, U) = \delta(\dot{X}, \dots, \dot{U})/\delta(X, \dots, U)$.

$(X_0, \dots, U_0)$ is a stable stationary state of the system ((1), ... (6)) only if the roots of the characteristic equation:

$$\det [J(X_0, \dots, U_0) - \lambda I_6] = 0 \quad (7)$$

have strictly negative real parts. Eqn. (7) can be written:

$$(a_1 + \lambda)(a_2 + \lambda)(a_3 + \lambda)[\lambda^3 + (a_4 + a_5 + a_6)\lambda^2 + (a_4 a_5 - a_4 a_6 + a_5 a_6 - a_7 a_8)\lambda + (a_4 a_5 a_6 - a_7 a_8 a_9)] = 0$$

where $a_j (j = 1, \dots, 9)$ are strictly positive constants and where $a_4 > a_8$.

Table 1. Variables, parameters, inhibitory terms and kinetic constants for Fig. 1 and eqns. (1)–(6)

Variables	
$[\text{O}_2^-]$	Superoxide anion concentration
$[\text{H}_2\text{O}_2]$	Hydrogen peroxide concentration
$[\text{OH}^\cdot]$	Hydroxyl radical concentration
$[\text{ROOH}]$	Lipid peroxide concentration
$[\text{ROO}^\cdot]$	Peroxy radical concentration
$[\text{R}^\cdot]$	Acyl radical concentration
Parameters	
[Oxidases]	Oxidase concentration
[GSH]	GSH concentration
$[\text{Fe}^{3+}]$	Fe^{3+} concentration
$[\text{Fe}^{2+}]$	Fe^{2+} concentration
$[\text{O}_2]$	Molecular oxygen concentration
$[\text{e}^-]$	Electron concentration
[XH]	Single reducing molecule concentration
[SH ₂]	Double reducing molecule concentration
[RH]	Acyl chain concentration
[catalase]	Catalase concentration
[SOD]	Superoxide dismutase concentration
[GPX]	Glutathione peroxidase concentration
Inhibitory terms to [SOD] and [GPX] parameters	
$(1 - \alpha[\text{H}_2\text{O}_2])$	Inhibitory term of SOD by H_2O_2 with $0 < \alpha \leq (\text{Max. } [\text{H}_2\text{O}_2])^{-1}$
$(1 - \beta[\text{ROOH}])$	Inhibitory term of GPX by ROOH with $0 < \beta \leq (\text{Max. } [\text{ROOH}])^{-1}$
Kinetic constants	
k_1	Kinetic constant for the univalent reduction of O_2 to O_2^- which is proportional to O_2 and to the electron donor concentration
k_2	Kinetic constant for the spontaneous dismutation of O_2^- into H_2O_2 . Spontaneous dismutation is low compared with enzymic dismutation
k_3	Kinetic constant for enzymic dismutation of O_2^- . The rate of dismutation is directly proportional to its substrate, at least at low cell concentration (Chance <i>et al.</i> , 1979)
k_4	Kinetic constant for one of the Fenton reactions
k_5	Kinetic constant for the reduction of O_2^- by hydrogen donors
k_6	Kinetic constant for the direct reduction of molecular oxygen to H_2O_2 , for example by oxidases such as xanthine oxidase
k_7	Kinetic constant for the other of the Fenton reaction
k_8	Dismutation of H_2O_2 by catalase. The rate of dismutation is directly proportional to its substrate, at least at low cell concentration (Chance <i>et al.</i> , 1979)
k_9, k_9' and k_{14}, k_{14}'	Kinetic constant for the peculiar kinetics of GPX (Flohé <i>et al.</i> , 1972) when reducing H_2O_2 (k_9, k_9') or ROOH (k_{14}, k_{14}')
k_{10}	Kinetic constant for the direct transfer of a hydrogen atom from RH to O_2^- , for example, in the lipid-peroxidation processes
k_{11}	Kinetic constant for the direct transfer of a hydrogen atom from RH to $\text{OH}^\cdot$, for example, in the lipid-peroxidation processes
k_{12}	Kinetic constant for peroxy radical formation by oxygen addition to a free radical $\text{R}^\cdot$, for example, in lipid peroxidation
k_{13}	Kinetic constant for the propagation reaction observed in lipid peroxidation
k_{15}	Kinetic constant for the removal of free radicals by scavengers such as vitamin E
k_{16}	Kinetic constant for removal of $\text{OH}^\cdot$ by scavengers such as vitamin E

a_j are algebraic functions of the kinetic constants and of the stationary concentrations. The computation of these functions is straightforward, but the explicit expressions are very long.

Therefore we only have to analyse the roots of the equation:

$$\lambda^3 + (a_4 + a_5 + a_6)\lambda^2 + (a_4 a_5 + a_4 a_6 + a_5 a_6 - a_7 a_8)\lambda + (a_4 a_5 a_6 - a_7 a_8 a_9) = 0$$

Routh-Hurwitz criteria ensure that the roots have strictly negative real parts only if

$$a_4 + a_5 + a_6 > 0 \quad (8)$$

and

$$[(a_4 + a_5 + a_6)(a_4 a_5 + a_4 a_6 + a_5 a_6 - a_7 a_8) - (a_4 a_5 a_6 - a_7 a_8 a_9)] > 0 \quad (9)$$

The explicit computation of the a_j shows that condition (8) and condition (9) are true since $a_4 > a_8$. The stated characteristics of the constants a_j are true for all positive values of the kinetic constants.

In conclusion, if we do not take into account the enzymic inhibitions, the system of production and elimination of free radicals is always stable towards an arbitrary in the parameters.

We will now consider the effect of enzymic inhibition on the system.

Fig. 2. Phase plane of the stationary values X_0 (superoxide anion concentration) versus Y_0 (H_2O_2 concentration) as obtained from the solution of eqns. (1 and 2) for which an inhibition of SOD was considered ($\alpha \neq 0$)

Only one stationary state (X_0, Y_0) is observed when X_0 and Y_0 have positive values. The two straight lines indicate the limits of the two curves.

Fig. 3. Relationship between GPX activity and hydroperoxide concentration [ROOH] in the model of free-radical production and elimination shown in Fig. 1, but taking into account the fact that GPX concentration is above a critical level $[GPX_c]$

When the activity of the enzyme is low, the concentration of ROOH necessary to reach the unstable state decreases and a lower fluctuation can bring the system to an irreversible unstable process.

Inhibition of antioxidant enzymes by free radicals

As explained in the presentation of the model, we had to take into account the inhibition of SOD by H_2O_2 and GPX by hydroperoxides (Pigeolet *et al.*, 1990). To take into account the possible inhibition of these two enzymes, their concentrations in the equations have been corrected by a fraction depending on α and β (Table 1).

Inhibition of SOD by H_2O_2 ($\alpha \neq 0$, $\beta = 0$)

Let us first consider the inhibition of SOD by H_2O_2 ($\alpha \neq 0$, $\beta = 0$). By a graphical solution of the equation

$$X(X, Y, \alpha) = Y(X, Y, \alpha) = 0 \quad (10)$$

one shows quite easily that the system does admit one and only one stationary state. Indeed using the same procedure as before, by calculating the Jacobian and using the Routh-Hurwitz criteria, we can show that the stationary point $(X_0, Y_0, W_0, R_0, U_0)$ is stable only if

$$-2b_1 X_0 - b_2 - b_4 - [b_5 / (1 + b_6 Y_0^2)] + b_7 (Y_0 - X_0) < 0 \quad (11)$$

and

$$(2b_1 X_0 + b_2 - b_7 Y_0) [b_4 + b_5 / (1 + b_6 Y_0^2)] + b_7 X_0 (2b_1 X_0 + b_2 - b_3) > 0 \quad (12)$$

where b_j ($j = 1, \dots, 7$) are strictly positive constants where $b_2 > b_3$.

Comparing the solutions of eqn. (10) with conditions (11) and (12), it can be shown that under biological conditions where X_0 and Y_0 are positive, these two conditions are fulfilled and there is only one stable state (Fig. 2). Therefore the incorporation of a possible inhibition of SOD by H_2O_2 does not lead to any instability of the system. This is not surprising; indeed, the terms added to the system when $\alpha \neq 0$ are not 'linear enough' to modify the stability of the system. The effect of an SOD inhibition is the limitation of H_2O_2 production.

We can in fact show that $Y_0 < (k_5 [XH] + k_3 [SOD]) / (k_3 \alpha [SOD])$.

Inhibition of GPX ($\alpha = 0$, $\beta \neq 0$)

In this case, the situation completely changes. There is a critical point that can be observed when the hydroperoxide concentration ([ROOH]) is expressed for various concentrations of GPX (Fig. 3). This critical point has the co-ordinate of

$$GPX_c = \{k_{13} [RH] U_0 (1 + n R_0)\} / [k_{14} R_0 (1 - \beta R_0)]^{-1}$$

for the abscissa and

$$R_0 = [(\beta^2 + \beta)^{1/2} - \beta n] / (\beta n)^{-1}$$

for the ordinate where $n = k_{14} / k_{13} [GPX]$. The general behaviour of the system will depend on the [GPX] compared with this critical point ($[GPX_c]$). Three situations can occur depending on whether $[GPX] < [GPX_c]$, $[GPX] = [GPX_c]$ or $[GPX] > [GPX_c]$. If $[GPX] < [GPX_c]$, no stationary state is possible; if $[GPX] = [GPX_c]$, there is one and only one stationary state (it is the bifurcation point); if $[GPX] > [GPX_c]$, two stationary states exist: a stable and an unstable one.

The presence of a stable and unstable state is well exemplified in Fig. 3. The inferior branch of this bifurcation represents the minimal concentration of lipid peroxide existing in the system; it is the stable stationary state. The superior branch of the bifurcation represents the maximal concentration of lipid peroxide that can exist in the system; the concentration of lipid peroxide cannot increase further without destroying irrevocably the balance between production and elimination of free radicals,

Fig. 4. Phase plane of $[ROOH]$ versus $[ROO']$, which clearly exemplifies the critical concentration for both peroxide derivatives above which the system no longer converges to the stable stationary state

therefore the system cannot adjust further against a further increase in free-radical production.

We note also that the distance between both branches decreases when $[GPX]$ decreases and leads to $[GPX]_c$. Biologically, this means that if $[GPX]$ decreases without being below $[GPX]_c$, the fluctuation in peroxide concentration necessary to shift from a stable to an unstable stationary state will be smaller, so that the system will be more easily destabilized.

Another presentation of the mathematical findings is the phase plane ($ROOH$, ROO') (Fig. 4). In this Figure, the evolution of the ROO' concentration (peroxyl radicals) is compared with the $ROOH$ (hydroperoxide) concentration. Two characteristic points can be observed: a stable node and a saddle point.

First, the stable node of this phase plane takes into account the constant existence of a low concentration of lipid peroxide and peroxyl radicals in the stationary state. Any fluctuation given to the system will increase the $ROOH$ and/or the ROO' concentration, but as long as these changes are not too distant from the stable node, the system will come back to its stationary state. However, if a large fluctuation occurs and if $ROOH$ and/or ROO' concentration passes beyond a critical value, then the system will be unable to lower these concentrations and will run to infinity, i.e. the system of production and elimination of free radicals will be unbalanced towards free-radical overproduction. The limit between the stable and unstable domains is given by the saddle point.

It is also noteworthy that the incorporation of both α and β inhibitory terms (α and $\beta \neq 0$) involves a similar topological behaviour of the system.

The concentrations of the other antioxidant molecules termed RH or XH or GSH were considered to be constant like the Fe^{2+} concentration. The same mathematical development could be performed with changes in their concentrations, but we have focused in the present work on the importance of the three antioxidant enzymes, considering other parameters as constant.

We only incorporated inhibition terms for the enzymes SOD and GPX. Pigeolet *et al.* (1990) showed that free radicals do not affect catalase activity very much, and therefore no inhibition term was incorporated for it. However, inactivation of catalase by specific inhibitors such as 3-amino-1, 2, 3-triazole leads to cell death when 55% of its activity is lost (Michiels & Remacle, 1988). The model also leads to the same conclusion if catalase inhibition is taken into account in the equations. Since it had no

immediate physiological counterpart, it was not developed in this paper.

DISCUSSION

The mathematical processing of the model of free-radical production and elimination as presented in Fig. 1 leads to the presence of one and only one stable steady state. If inhibition of SOD by H_2O_2 is incorporated into the model, the same conclusion is drawn. However, inhibition of GPX by hydroperoxides ($ROOH$) gives rise to a more complex situation with a stable steady state within the limits of $ROOH$, ROO' and GPX. However, if the concentration of the peroxide derivatives is too high or if the GPX content decreases, an unstable situation can be obtained where the system can no longer play its regulatory role and the system diverges from its stable state. In fact, all these conclusions are exemplified by experimental data which give support to this theoretical model.

What is the relevance of such a model to the experimental results? Let us first examine the data on the variations of the GPX concentration. Experimentally, GPX was micro-injected into human fibroblasts and the beneficial effect of such an injection was evaluated for cells under various conditions of oxidative stress. In all the situations, injection of GPX gave protection against cell death which was concentration-dependent (Raes *et al.*, 1987; Michiels & Remacle, 1988; Michiels *et al.*, 1989).

The relationship between the critical free-radical concentration and the activity of GPX as discussed in Fig. 3 has been well exemplified in experiments of cell recovery after short periods of incubation under 2 atm of O_2 (Michiels *et al.*, 1990a). The incubation time under O_2 that would alter cells so that they would no longer be able to divide was estimated. Most of the cells could withstand only 5 h of exposure to O_2 before they lost their proliferative capacity. However, if GPX was injected into the cells before the incubation, they could withstand longer periods of incubation in O_2 , the higher the amount of GPX injected, the longer the period under O_2 . In this case, 5, 10, 15 and 20 h of exposure to 2 atm of O_2 could be reached with cells injected respectively with a solution containing 1, 5, 3 and 12 units of GPX/ml. If we estimate the injection volume to be 10^{-10} ml/cell, an increase of 1.2×10^{-9} units of GPX per cell would allow the cell to increase its incubation period from 5 to 20 h under 2 atm of O_2 . Since the native GPX activity of these cells was 5.5×10^{-9} units per cell, the injected GPX represents only an increase of 22% compared with the native GPX activity.

Inhibition of GPX has dramatic effects not only under typical conditions of oxidative stress, but also under normal conditions. When anti-GPX antibodies were injected into the cytoplasm, cells stopped dividing under physiological conditions and a significant increase in cell mortality was observed under 1 atm of O_2 (Michiels *et al.*, 1989). A more significant result was obtained with mercaptosuccinate, an inhibitor of GPX. Viability of the cells fell to 50% with 1 μM -mercaptosuccinate, which gives 21% inhibition of GPX (Michiels & Remacle, 1988). So even under normal conditions, inhibition of GPX by 21% is sufficient in these human fibroblasts to alter cell survival.

In Fig. 5, we summarize all these experimental results which confirm and give precision to the evolution of the free-radical critical concentration compared with GPX activity. This Figure gives precision to the theoretical development of Fig. 3.

In Fig. 5, we have also incorporated data on catalase and SOD. The behaviour of the model for catalase was similar to that for GPX. Experimental results also showed the same behaviour but with catalase being less efficient than GPX. For example, under oxidative stress, a solution of 41 units of catalase/ml gave

Fig. 5. Evolution of the critical concentration of free radicals which would lead to instability and cell death with variation of the activity of the three antioxidant enzymes

N, native enzyme activity; *P*, free-radical concentration under physiological conditions. Adapted from Fig. 3 and from Michiels *et al.* (1989) and Michiels & Remacle (1988).

the same protection as a solution of 5 units of GPX/ml (Raes *et al.*, 1987), the native concentration of catalase in the same cell being 2.6×10^{-9} units per cell. The ratio between the concentrations of catalase and GPX which give the same protection varies from 3 to 9 according to the severity of the stress (Michiels *et al.*, 1990a), catalase being relatively more efficient for milder stress which mainly affects the capacity for cell division and GPX being more efficient for protection against lethal effects. When the effect of catalase on cell viability was tested, 55% inhibition was required to obtain a 50% cell decrease compared with the control (Michiels & Remacle, 1988). These data have been taken into account in Fig. 5.

The situation for SOD is qualitatively very different from the other two enzymes. First, the theoretical approach shows that inhibition of SOD does not lead to an unstable system. The same conclusion is also obtained experimentally. Cytoplasmic SOD was inhibited by the injection of anti-SOD antibodies into the cytoplasm of the cells. Under normal physiological conditions, this injection did not have any toxic effect as the anti-GPX antibodies did, for example. However, under conditions of stress, inhibition of SOD was detrimental for the cells and injection of SOD gave protection. However, large amounts had to be injected to obtain protection comparable with that of GPX or catalase (Raes *et al.*, 1987). The ratio between the concentrations of SOD and GPX which give the same protection varies from 246 to 1008 according to the severity of the stress (Michiels *et al.*, 1990a), the efficiency of SOD being relatively higher for short periods of stress which mainly affect cell division rather than cell degeneration. This behaviour is exemplified in Fig. 5 with a small protection produced with increasing concentration, but with no toxic effect when inhibition of SOD is achieved under physiological conditions. Qualitative interpretations of the efficiency of catalase and particularly GPX for cell protection compared with SOD have been extensively reviewed and will not be discussed here (Raes *et al.*, 1987; Michiels & Remacle, 1988; Michiels *et al.*, 1989).

The theoretical model developed here allows a synthesis of experimental data by giving the evolution of the toxic free-radical concentrations related to the three antioxidant enzymes. It clearly emphasizes the concept of a critical level of errors resulting from the balance between free-radical production and the defence systems, mainly GPX and catalase.

Further calculations and experiments could be performed in the future using variations of other parameters of the model such as, for example, the stability of the system with the variation in the concentrations of other antioxidants. If one could know the concentration of all the parameters in one particular cell type and the kinetic constants of all these reactions in the cellular

environment, then the exact situation of these complex dynamics of free-radical production and elimination could be quantitatively determined by using the same mathematical model.

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