Free Radical Research, February 2006; 40(02): 111–119

REVIEW

Part of the Series: From Dietary Antioxidants to Regulators in Cellular Signalling and Gene Expression

Role of reactive oxygen species and (phyto)oestrogens in the modulation of adaptive response to stress*

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Accepted by Professor R. Brigelius-Flohé

(Received 20 June 2005; in revised form 27 September 2005)

Abstract
There is increasing evidence that reactive oxygen species (ROS) are not only toxic but play an important role in cellular signalling and in the regulation of gene expression. We, here, discuss two examples of improved adaptive response to an altered cellular redox state. First, differences in longevity between males and females may be explained by a higher expression of antioxidant enzymes in females resulting in a lower yield of mitochondrial ROS. Oestrogens are made responsible for these phenomena. Oestradiol induces glutathione peroxidase-1 and MnSOD by processes requiring the cell surface oestrogen receptor (ER) and the activation of pathways usually involved in oxidative stress response. Second, oxygen radicals produced during moderate exercise as performed during training up-regulate the expression of antioxidant enzymes in muscle cells. An increased level of these enzymes might prevent oxidative damage during exhaustive exercise and should, therefore, not be prevented by antioxidants. The relevance of these findings is discussed in the context with observations made in transgenic animals overexpressing MnSOD or catalase.

Keywords: *Ageing, oxidative stress, exercise, oestrogens, glutathione, transgenics*

Introduction

The idea of the pervasive nature of free radicals has been firmly entrenched in the minds of scientists ever since the group of Britton Chance [1] developed the basic biochemical techniques to show that in the resting state 2% of all oxygen consumed by cells is converted into reactive oxygen species (ROS) rather than water. McCord and Fridovich first described superoxide dismutase thus suggesting a physiological role of superoxide [2]. Although, there is now an appreciation that the physiological generation of ROS is likely to be an order of magnitude less, their impact on biomolecules has been amply documented.

In response to this assault, the cell has developed a number of antioxidant defence systems such as superoxide dismutase, the peroxidases, the glutathione redox cycle with its associated constitutive enzymes as well as glutathione itself, whose concentration is higher in the cell than that of glucose [3]. Therefore, the cell has become well equipped to cope with the normal production of reactive species.

There is growing evidence that the continuous presence of a small stimulus such as low concentrations

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2006 Taylor & Francis
DOI: 10.1080/10715760500405778

of ROS is in fact able to induce the expression of antioxidant enzymes and other defence mechanisms. The basis for this phenomenon may be encompassed by the concept of hormesis [4], which can be characterized as a particular dose–response relationship in which a low dose of a substance is stimulatory and a high dose is inhibitory. In this context, radicals may be considered to be beneficial since they act as signals to enhance defences rather than deleterious as they are when cells are exposed to high levels of ROS.

A major aim of this review is to emphasize the role of radicals as signals in physiological and genetic models, with particular emphasis on aging and physical exercise.

Physiological models

ROS modulate the expression of a number of genes that are relevant to important physiological processes [5]. Recent studies reveal the role of ROS in various cellular phenomena, which in turn affect the physiology of the entire body. In this section of the review we will deal with two aspects of animal and human physiology in which ROS play an important role: longevity and exercise.

Longevity

Different production of ROS may explain the difference in longevity between males and females. Females live longer than males. This is true in several species including rats and humans. Table I shows that the average life span of male Wistar rats in our laboratory is 24 months whereas for female rats it is 29, i.e. 21% longer. Studying possible mechanisms underlying this important difference may provide insights into the process of aging and suggest interventions to correct the male-specific deficit in longevity.

Work from our laboratory has shown that mitochondrial H_2O_2 production in livers of female rats is 70% that of males [6]. This lower rate of mitochondrial peroxide production may result in a lower level of damage and also in an up-regulation of a number of longevity-associated genes. An apparent consequence is that the levels of reduced glutathione (GSH) are 50% higher in females than in males (Table I). Thus, not only do males produce more peroxides than females, they are also less protected by the key cellular thiol glutathione. If males are indeed less protected than females, one would expect an increased level of damage to critical molecules. One of such parameters is oxidative damage to mitochondrial DNA, which has been related to longevity [7,8]. We found oxidatively damaged mitochondrial DNA to be approximately four-fold higher in males than in females, and oxidation of mitochondrial DNA has indeed been shown to be related to the decline of cellular functions with age [9].

The amount of 16S ribosomal RNA has been proposed as suitable biological marker of ageing. In *Drosophila* and other animals, levels of 16S rRNA were shown to decrease as a function of age [10,11]. In young (3 months old) rats, we determined a level of 16S rRNA in males that was approximately 25% that of females [6]. Thus males, although they have the same chronological age, behave as if they were biologically older than females.

Gender and antioxidant enzyme activities. Possible explanations for the reduced levels of mitochondrial peroxide production in females include higher antioxidant enzyme activities or a higher activity of the respiratory chain complexes. Consequently, we determined the expression and activity of critical mitochondrial antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase (GPx), and the

Table I. Inverse relationship between longevity and oxidative stress in male and in female rats.

	Males	Females	Females/males (%)
Average life span (months)	24 ± 0.5	29 ± 0.6	121%
Mitochondrial H_2O_2 production(nmol/min mg prot)			
Liver	0.10 ± 0.03**	0.07 ± 0.02	70%
Reduced glutathione (GSH) (nmol/mg prot)	6.4 ± 0.9*	9.8 ± 1.8	153%
Mitochondrial DNA damage (8oxodG/100000 dG)	55 ± 5**	15 ± 8	27%
Cytochrome *c* oxidase			
Activity (IU/mg prot)	0.74 ± 0.12	1.12 ± 0.23	151%
Mn-Superoxide dismutase			
Expression (arbitary units)	3.1 ± 0.9*	6.5 ± 1.8	210%
Activity (IU/mg prot)	34 ± 8**	74 ± 18	218%
Glutathione peroxidase			
Expression (arbitary units)	2.6 ± 0.3**	5.4 ± 0.3	208%
Activity (IU/mg prot)	0.18 ± 0.06**	0.51 ± 0.01	283%
16S ribosomal RNA			
Expression (arbitrary units)	1.7 ± 0.4**	6.4 ± 0.3	376%
Estradiol prevents oxidative stress in vivo			
	Females	Ovaricetomized	Ovariectomized + Estrogens
Mitochondrial H_2O_2 production(nmol/min mg prot)			
Liver	0.07 ± 0.02	0.116 ± 0.043**	0.065 ± 0.024**

activity of a crucial respiratory chain component, cytochrome c oxidase in male and female rats.

RNA and activity of mitochondrial superoxide dismutase in females is approximately two times of that in males [6]. Similarly, RNA of GPx-1 and total mitochondrial GPx activity were higher in females than in males by a factor ranging from two to three (Table I). The fact that GPx activity is higher in females than in males had been known since the 60s [12], but so far this had not been related to longevity.

Cytochrome c oxidase can be considered as an antioxidant enzyme, as it drives electrons through the respiratory chain to oxygen. Therefore, a higher cytochrome c oxidase activity is likely to result in a decreased production of ROS by mitochondria. Notably, cytochrome c oxidase activity in mitochondria from females is 50% higher than in that from males (Table I). Mitochondria from females, thus, produce less ROS, because they exhibit higher cytochrome c oxidase. In addition, they have an increased capacity to detoxify ROS due to their higher superoxide dismutase and GPx activities. Pioneering work of Orr and Sohal using transgenic *Drosophila* in which both catalase and superoxide dismutase had been over-expressed showed that the life span of these insects increased relative to controls [13]. In this respect, females behave as double transgenics that over-express both, superoxide dismutase and GPx.

Oestradiol prevents oxidative stress in vivo. The fact that females exhibit lower oxidative stress and higher expression of antioxidant enzymes prompted us to study whether these favourable conditions were due to the presence of oestrogens. Thus, we studied the peroxide production by mitochondria from control and ovariectomised female rats. Table I shows that ovariectomy caused a significant increase in the rate of peroxide production by mitochondria. Moreover, when ovariectomised rats were treated with oestradiol, the rate of mitochondrial peroxide production fell to values similar to ovariectomized controls. Thus, the lower peroxide production in females compared to males depends on oestrogens.

Oestradiol counteracts oxidative stress by a mechanism requiring interaction with its receptor. Oestrogens were reported to have antioxidant properties due to the presence of the phenolic group in the steroid structure [14]. However, regarding the dosage of oestrogen used in hormonal replacement therapies after menopause, it appears impossible that they act as chemical antioxidants *in vivo*. Indeed, 50 μg of oestradiol are routinely prescribed for women after menopause, whereas approximately 400 milligrams of vitamin E are recommended as a dietary supplement. Both compounds have similar molecular weights, thus, if oestradiol were to act as an antioxidant it should be 8000 times more potent than vitamin E to exert comparable antioxidant activity, which is not predicted based on structural considerations. With this guiding principle, we reasoned that oestradiol might rather act by the interaction with oestrogen receptors (ERs) than as an antioxidant. To test this, we used tamoxifen, a well-known modulator of the nuclear ER, which competes with oestrogen for binding to the receptor and, thus, interferes with the effects of oestradiol. In MCF7 cells, a cell line derived from mammary gland carcinoma, we tested the effect of oestradiol on peroxide levels and its possible interaction with tamoxifen. Tamoxifen completely inhibited the protective effect of oestradiol against oxidative stress in these cells, thus showing that the hormone acts by binding to ERs and not as a chemical antioxidant *per se* [15].

Cell signalling pathways are involved in the beneficial effects of oestrogens against oxidative stress. Having found that oestrogens exert their beneficial effects via ERs we asked whether other signalling pathways are involved too. As obvious from Figure 1a oestradiol, at concentrations normally found in the blood of pre-menopausal women, causes phosphorylation of ERK1-2, which is completely prevented when an inhibitor of the MAP kinase pathway, UO126, is co-incubated with oestradiol. This indicates the involvement of the MAP kinase pathway in the effects of oestrogen. This conclusion is further supported by the translocation of the p50 subunit of NFκB to the nucleus, an event which can be triggered by MAP kinases. Physiological concentrations of oestradiol increased NFκB activation and this activation was inhibited by incubation with the MAP kinase inhibitor UO126 (Figure 1b).

Oestrogens induce the expression of antioxidant enzymes. As mentioned above, the low concentrations of oestrogens present in plasma do not act as chemical antioxidants *in vivo*. However, they might be able to activate a signal transduction program which results in the up-regulation of antioxidant genes. This was found to be indeed the case. An increase in MnSOD and GPx-1 gene expression was demonstrated in MCF7 cells incubated with oestradiol (Figure 1c). Furthermore, when the NFκB pathway was blocked (by incubation with UO126) or the activation of NFκB was inhibited (by incubation with PDTC), the induction was prevented completely. Accordingly, oestradiol reduced peroxide levels to about 50% of that of controls. This effect again is attenuated by the inhibition of MAP kinase or NFκB activation (Figure 1d).

Phytoestrogens mimic some of the beneficial effects of estrogens. The beneficial effects oestradiol as an up-regulator of longevity-related genes indicates that its administration might be beneficial particularly for

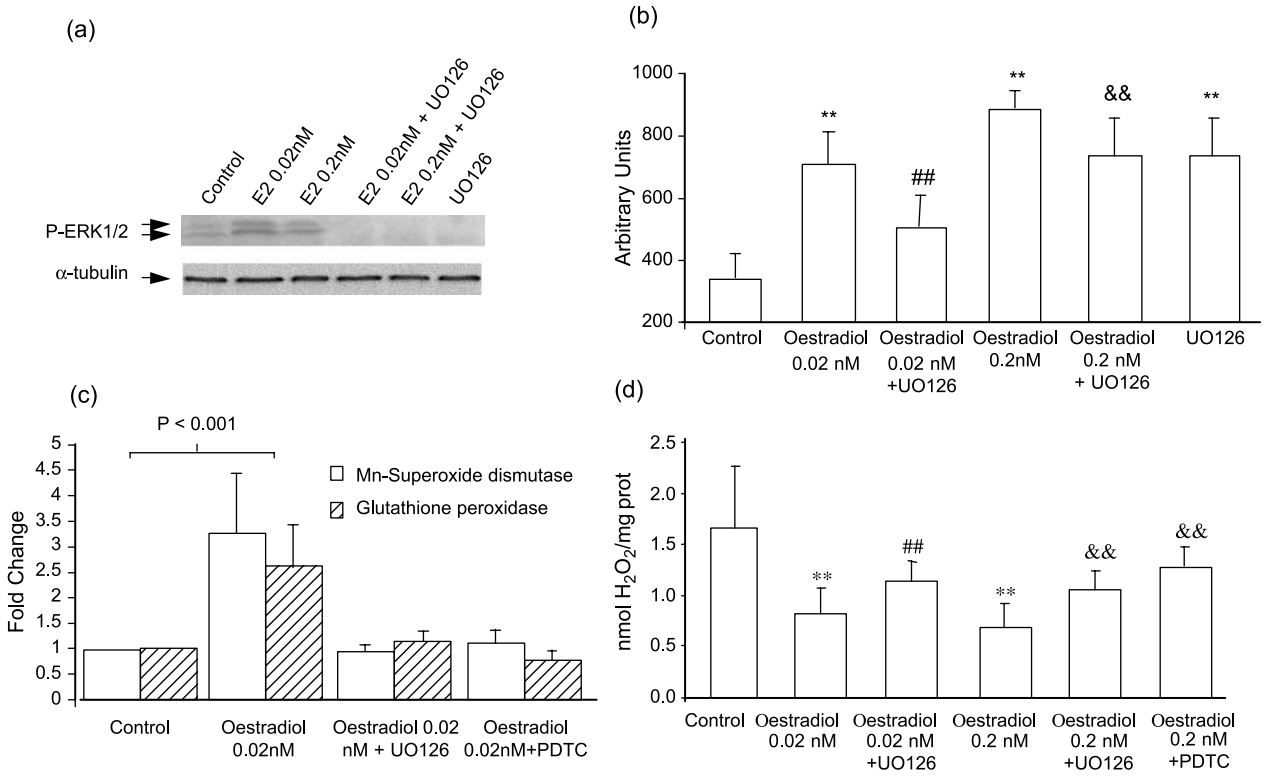

Figure 1. Effects of oestradiol on cellular signalling pathways involved in antioxidant defence. Panel a: oestradiol activates phophorylation of ERK 1 and 2. The effect is prevented by MAP Kinase inhibitor UO126. Panel b: oestradiol induces NFκB translocation: levels of the active p50 subunit of NFκB were measured in nuclear extracts from cells treated with physiological concentrations of oestradiol alone or with 1 μM UO126 as indicated. Panel c: oestradiol induces transcription of MnSOD and GPx-1, mediated by MAP kinases (the effect is inhibited by UO126) and by NFκB (effect inhibited by PDTC). Panel d: oestradiol lowers peroxide levels in cells via by MAP kinases (the effect is partially prevented by UO126) and by NFκB (effect partially prevented by PDTC). In all cases, data are expressed as mean ± SD for 4–10 different experiments. The statistical significance is expressed as ** ($p < 0.01$ vs. control), ## ($p < 0.01$ vs. oestradiol 0.02 nM) and && ($p < 0.01$ vs. oestradiol 0.2 nM). Neither UO126 nor PDTC alone affected MAP kinases or NKκB or H_2O_2 levels.

males, to reach a life span similar to that of females. However, there is considerable evidence that oestrogen replacement therapy after menopause may have setbacks [16]. Phytoestrogens might mimic the favourable effects of oestrogens without their major side effects. Beneficial effects of phytoestrogens have been reported repeatedly [17] and, to our knowledge, very few, if any, detrimental effects have been reported. Genistein is one of the major phytoestrogens present in soya [18]. It is able to decrease oxidative stress at concentrations that can be considered as nutritionally relevant, i.e. those normally found in the blood of people in the Far East who eat relatively large quantities of soya in their normal diet. This concentration is, however, significantly higher than the one found in people living in the Western world. As shown for oestradiol, effects of phytoestrogens are mediated by ERs. Moreover, the cell signalling pathways involved (MAP kinases and NFκB) are the same as required for oestradiol (unpublished results from this laboratory).

Taken together, oestradiol and certain phytoestrogens up-regulate antioxidant enzymes by a mechanism requiring the activation of MAP kinase and NFκB, pathways usually mediated by oxidative stress. This is in line with the often reported oxidative effects of oestrogens. How this is achieved remains to be investigated. Classical actions of oestrogens and phytoestrogens are exerted by their binding to the nuclear ER which then activates gene expression via the oestrogen responsive element (ERE) in the promoters of target genes. As shown here, induction of MnSOD and GPx-1 may also be obtained by other transcription factors such as NFκB. Recently, cell surface ERs have been described [19] that may activate gene expression via classical cytosolic signalling pathways. Since none of the genes whose expression was up-regulated by oestradiol contains an ERE in its promoter but putative NFκB sites, we propose the following pathway for the induction of GPx-1 and MnSOD: interaction of oestrogen with a membrane ER leads to activation of MAP kinases followed by activation of NFκB and in turn gene expression.

Adaptation to physical exercise

Pioneering work by Packer and his colleagues revealed that exercise generates ROS [20]. It soon became apparent, however, that exercise also caused the up-regulation of antioxidant enzymes [21] and that there

was no overall increase in oxidative stress. Work from our group [22] revealed that exercise generated oxidative stress only when it was exhaustive. Non-exhaustive exercise caused an increased production of ROS which could be counterbalanced by the concurrent increase in antioxidant defences, and, therefore, unbalanced oxidation known as oxidative stress [23] was not observed. Thus, it is important to differentiate between non-exhaustive exercise, which is not accompanied by oxidative stress, and exercise to exhaustion, which does confer a state of oxidative stress.

In setting out to determine the mechanism by which exercise causes an increased production of ROS, we came across the generally accepted idea that, since exercise causes an increase in oxygen consumption by mitochondria, it also causes an increase in free radical formation by these organelles. This, however, is based on the misconception that the proportion of ROS formed by mitochondria is in the range of 2% of the total oxygen consumed. Very early work by the group of Britton Chance [24,25] revealed that the approximately 2% of oxygen are converted to free radicals only when mitochondria are at the resting state 4 [26]. However, when mitochondria are in state 3, i.e. actively producing ATP from ADP with a high electron flow to oxygen, then the proportion of oxygen converted to free radicals falls to a tenth of that found in the resting state. With these calculations in mind, the role of mitochondria in the formation of free radicals in exercise should be reconsidered and perhaps alternative sources for ROS should be identified. Work by Michael Reid [27], Ilva Ellsten [28], and Malcolm Jackson [29] indicated that there might be extracellular sources of superoxide associated with exercise. We examined the role of xanthine oxidase and the possible effect of allopurinol, a well known, widely used inhibitor of this enzyme. Allopurinol applicated to rats at a concentration of 32 mg/kg by intraperitoneal injection, prevented the oxidation of glutathione and lipid peroxidation associated with exhaustive exercise [30]. This indicated that xanthine oxidase might be a source of ROS. To test this hypothesis *in vivo*, we performed experiments with cyclists of the professional cycling team US Postal during the Tour de France. Administration of allopurinol prevented muscle damage associated with the strenuous exercise performed by these cyclists [31]. The group of Malcolm Jackson observed that superoxide radicals generated during periods of contractile activity by skeletal muscle is extracellular [32]. They reported that the increase in superoxide radical concentration in the extracellular fluid may not be derived from mitochondrial but from membrane-bound oxidoreductase(s).

ROS mediate the adaptive response to exercise in muscles. It remained to be analyzed whether free radicals produced during exercise would act as signals for the adaptation of the muscle cell to stress. Experiments reported in Figure 2 show that this is indeed the case. Activation of NFκB in rat skeletal muscles during exercise, as measured, by EMSA peaked at approximately four hours after exercise and was maintained for 24 h after its completion. By 48 h after exercise, DNA binding to NFκB had disappeared. Phosphorylation of the IκB subunit of the NFκB complex increased immediately after the completion of exhaustive exercise and then fell to pre-exercise levels within approximately four hours, whereas unphosphorylated IκB behaved in the opposite way. Translocation of the p65 subunit of NFκB to the nucleus occurred within one to four hours after exercise and was no longer observed after 24 h (Figure 2, upper panel) [33]. Exercise also caused a significant increase in the expression of the genes for MnSOD, iNOS, and eNOS. The induction was completely blocked when animals had been treated with allopurinol to decrease the formation of radicals associated with exercise (Figure 2, lower panel) [30].

These findings clearly indicate that ROS generated during exercise act as signals to increase the production of enzymes relevant to the adaptation of muscle cells to exercise. Moreover, these findings lead us to reconsider the 'wisdom' of taking antioxidant supplements during training. In all likelihood, antioxidant supplements should not be recommended before training as they interfere with muscle cell adaptation [34]. Indeed, when rats were trained, the expression of antioxidant enzymes and of other enzymes relevant to cell function was increased. When antioxidants were given, these adaptations were, however, hampered [30]. On the other hand, antioxidants may be administered before competition, when exercise is likely to be exhaustive and results in the generation of ROS that overwhelm the defensive mechanisms. A clear example of this protective effect was found in the case of cyclists taking part in the Tour de France: when given allopurinol, they had lower increases in the activity of creatine kinase and aspartate amino transferase [31].

Genetic models: Oxidative stress and aging

A key tenet of the oxidative stress hypothesis of ageing is that the intra-cellular flux of ROS fosters oxidative damage, which in turn contributes to the tissue and organ deficits observed in older individuals. One way to test this hypothesis is to bolster the antioxidative defenses by means of transgenic methodology, with the prediction that such manipulation could reduce ROS levels, attenuate oxidative stress and pertinent damage, and thereby slow down the aging process.

Transgenic drosophila

Multiple efforts have been made in the realm of antioxidant overexpression of antioxidant proteins in *Drosophila melanogaster*, with particular focus on the

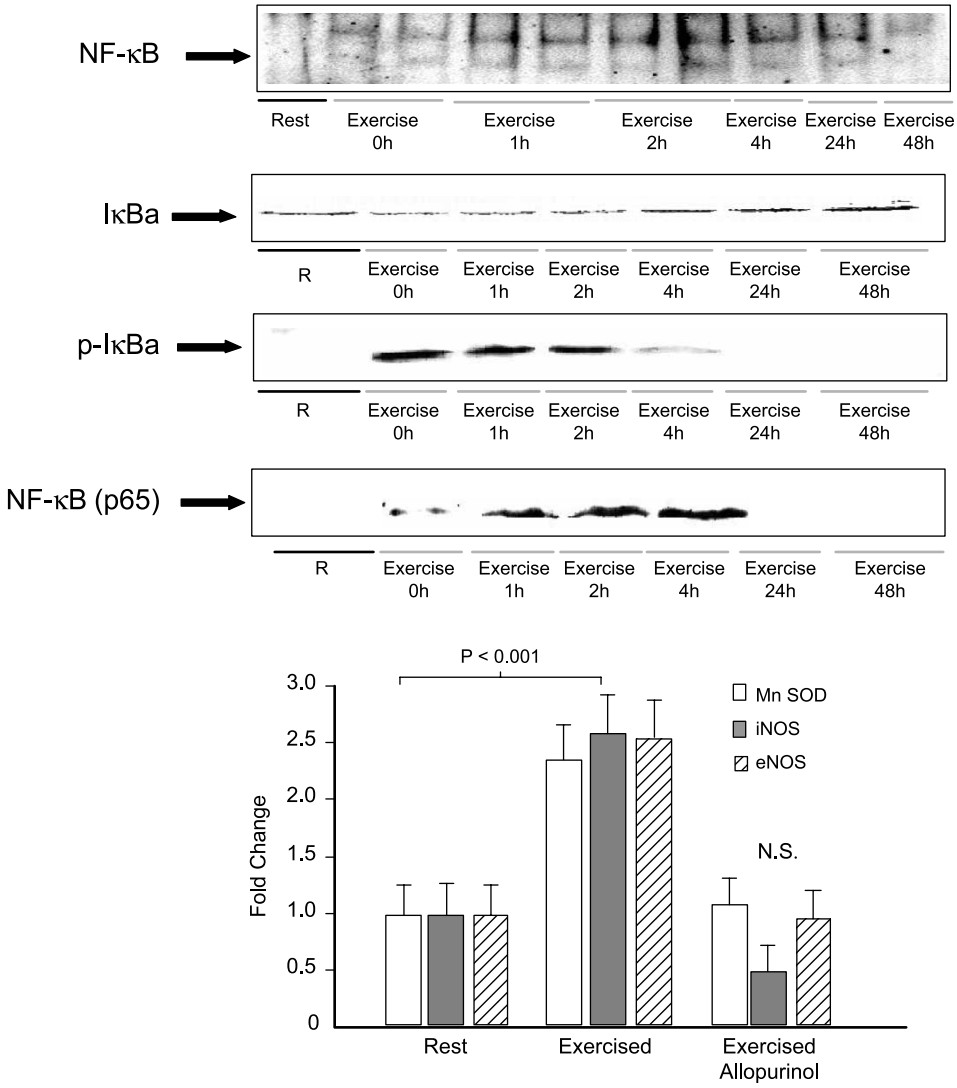

Figure 2. Moderate exercise activates NFKB and up regulates the expression of SOD, eNOS and iNOS in skeletal muscles of rats. Top panel — Time course of the activation of NFκB during exercise as measured by EMSA, phosphorylation of IκB and translocation of the p65 subunit into the nucleus. Lower panel — Moderate exercise up-regulates the expression of MnSOD, eNOS and iNOS. Treatment with allopurinol prevents this effect. Results are mean ± SD for 6–8 different experiments.

so-called primary axis of antioxidant defenses (SOD + catalase). Early studies in this realm were quite encouraging. Overexpression of SOD and catalase together using native promoters [35], overexpression of SOD1 using a regulatable constitutive overexpression system [36] and overexpression of SOD1 directed specifically to motor neurons resulted in robust life span increases up to 50% [37]. In most of these studies relatively little attention was paid to measuring the impact on ROS flux and reduction in ROS levels inferred from the known biochemical effects of antioxidant enzymes. Nevertheless, certain endpoint measures, e.g. steady state levels of oxidative damage, provide some validation of the presumed impact on ROS levels caused by increasing antioxidant defenses. As these studies progressed it became, however, clear that the salutary effects of antioxidant overexpression on life span were to some degree dependent on genetic background. The strongest positive lifespan effects tended to be associated with controls having shorter life spans, suggesting that in robust populations SOD1 overexpression has only small beneficial effects on longevity [38]. An interesting wrinkle to this conclusion is provided by a study conducted by the Promislow group, where the effects of human SOD1 overexpression in motor neurons were tested in ten different genotypes derived from recently caught, long-lived wild-type flies [39]. For males, a significant beneficial effect on longevity was observed in only one case out of ten, while in females the longevity effect was observed six out of ten cases. Thus, the impact of SOD1 overexpression on longevity is not only dependent on genotype, but in flies at least, the females appear to be more predisposed to exhibiting beneficial effects from this approach.

Transgenic mammals

How well do the relatively small benefits observed in *Drosophila* translate into the mammalian model? Transgenic mouse SOD1 over-expressors have been developed in several laboratories, and it has been noted that, in cases of acute stress, such as hyperoxia and induced trauma, some protection was conferred [40,41]. In only one case have effects on longevity been reported [42]. In this study, a ubiquitous 2–5 fold expression in homozygous transgenics resulted in a slight reduction in life span, while the more modest over-expression of 1.5- to 3-fold observed in the heterozygote, had no impact on life-span. Without a more thorough investigation of genotype effects, it is premature to draw any conclusions as to the potential effectiveness of this approach in mammals. The results, reported so far have not been encouraging.

Mitochondria-targeted overexpression of catalase and MnSOD

As transgenic methodology has advanced, more focused approaches have become feasible, permitting tissue-specific as well as organelle-specific expression. Indeed, the capacity to direct expression to targets that are particularly under stress may serve as a more effective means to test the Oxidative Stress Hypothesis of Ageing. In two recent studies, one in *Drosophila* and one in mouse, over-expression of catalase in mitochondria was achieved via transgenic methodology. In the case of *Drosophila*, ectopic expression of catalase resulted in severe reduction of H_2O_2 generated by mitochondria as well as a greater resistance to oxidative stress [43]. In spite of this, there was a slight negative effect on longevity under normal conditions, a result, which presents somewhat of a dilemma. Surprisingly, when a similar strategy was adopted in the mouse model, there was a significant increase in median life span (19%) [44]. This was accompanied by an apparent reduction in oxidative damage as inferred by attenuation of aconitase inactivation and reduced generation of mitochondrial deletions as well as a delay in the appearance of cardiac pathology and cataracts. What might explain the discrepancy between these two distinct outcomes? One possible explanation could lie in the design of the transgenic constructs. In the case of the fly transgenic, the regulatory sequences used were derived from the native regulatory domain, thus resulting in the targeting of catalase to mitochondria in tissues where catalase is already present in the cytosol. In the mouse, expression of the catalase transgene is under the control of a constitutive actin promoter, thus shoring up the mitochondrial antioxidative capacity in tissues that are particularly at risk. Another interesting possibility, discussed by the authors, is based on the observation that in the mouse, mitochondrial catalase expression is mosaic in nature. In flies, severe reduction of H_2O_2 flux in certain tissues may disrupt redox regulation and counteract the positive effects conferred by the antioxidant function of catalase. In the mosaic mouse transgenic, the presence of a significant fraction of cells not expressing the catalase transgene may permit normal redox signalling and thus allow the positive effects of catalase as an antioxidant enzyme to prevail. If this is correct, then dissecting the interplay between antioxidant and signalling functions will be critical in gaining full advantage of antioxidant interventions.

Concluding remarks

The role of free radicals in cellular biology has been well established over the last three decades. However, the majority of the studies published up to the last five years, have concentrated on the damaging role of these molecules in cell physiology. Classical papers such as that of Rebecca Gerschman [45], who pointed out that radiation damage could be similar to that caused by ageing or Harman's classical papers establishing the free radical theory of ageing [46], contributed greatly to establishing the role of free radicals as damaging agents to cells. Pioneering work in the 70s by the group of Britton Chance [1] together with the discovery that free radicals, by activation of xanthine oxidase, were involved in the ischemia reperfusion syndrome, helped to establish the causal role of these radicals in cellular damage and, eventually in disease processes and ageing.

However, in recent years, a new role for oxidant species as mediators in cell physiology has been proposed and experimental evidence has provided ample support for this concept. Needless to say, that nitric oxide, itself being a free radical, was critical in changing the paradigm of these radicals from being damaging agents to molecules relevant to cellular signalling.

In this review, referring to physiological and genetic models, we have pointed out that radicals can be considered as signals and moreover as stimulators of cellular defences. They may, therefore, be considered as molecules that increase cellular defence systems and eventually cellular and probably, organismic longevity. The hormetic properties of free radicals, i.e. the fact that controlled rates of production of these radicals may increase the expression and activity of superoxide dismutase or GPx, indicate that radicals may be "good" for the cells and thus have a clear physiological benefit for the maintenance of cellular homeostasis, particularly when the cells are challenged by normal insults which occur in cellular physiology.

Acknowledgements

This work was supported by Grants from CICYT (BFI-2001-2849 to J.V.), from CICYT (PM 99/0148

to J.V.) and from Instituto de Salud Carlos III, RCMN (C03/08), Madrid, Spain.

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