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Effects of oxidative stress on expression of extracellular superoxide dismutase, CuZn-superoxide dismutase and Mn-superoxide dismutase in human dermal fibroblasts

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To determine the effect of oxidative stress on expression of extracellular superoxide dismutase (EC-SOD), CuZn-SOD and Mn-SOD, two fibroblast lines were exposed for periods of up to 4 days to a wide concentration range of oxidizing agents: xanthine oxidase plus hypoxanthine, paraquat, pyrogallol, α-naphthoflavone, hydroquinone, catechol, Fe^{2+} ions, Cu^{2+} ions, buthionine sulphoximine, diethylmaleate, *t*-butyl hydroperoxide, cumene hydroperoxide, selenite, citiolone and high oxygen partial pressure. The cell lines were cultured both under serum starvation and at a serum concentration that permitted growth. Under no condition was there any evidence of EC-SOD induction. Instead, the agents uniformly, dose-dependently and continuously reduced EC-SOD expression. We interpret the effect to be due to toxicity. Enhancement of the protection against oxidative stress by addition of CuZn-SOD, catalase and low concentrations of selenite did not influence the expression of any of the SOD isoenzymes. Removal of EC-SOD from cell surfaces by heparin also did not influence SOD expression. Mn-SOD was moderately induced by high doses of the first 11 oxidants. Apart from reduction at high toxic doses, there were no significant effects on the CuZn-SOD activity by any of the treatments. Thus EC-SOD, previously shown to be profoundly influenced by inflammatory cytokines, was not induced by its substrate or other oxidants. In a similar fashion, Mn-SOD, previously shown to be greatly induced and depressed by cytokines, was only moderately influenced by oxidants. We suggest that the regulation of these SOD isoenzymes in mammalian tissues primarily occurs in a manner co-ordinated by cytokines, rather than as a response of individual cells to oxidants.

INTRODUCTION

Extracellular superoxide dismutase (EC-SOD, EC 1.15.1.1) is a secretory tetrameric, Cu and Zn-containing glycoprotein [1,2]. It is the major SOD isoenzyme of extracellular fluids such as plasma and lymph [3–5], but exists primarily in the interstitial space of tissues [6–8], anchored to heparan sulphate proteoglycan in the glycocalyx of cell surfaces and in the connective-tissue matrix [8–10]. The concentration of EC-SOD in its tissue distribution volume is apparently high [8]. Whereas cytosolic CuZn-SOD and mitochondrial matrix Mn-SOD are expressed by virtually all cell types [11,12], EC-SOD is expressed and secreted by only a few [12]. The widely dispersed fibroblasts are apparently important sources of EC-SOD [12].

EC-SOD expression is markedly enhanced by interferon-γ (IFN-γ) and is depressed by interleukin-1α (IL-1α), tumour necrosis factor-α (TNF-α) and especially transforming growth factor-β (TGF-β) [13]. However, the effects of its substrate, the superoxide anion radical, and other types of oxidant are unknown. We here report the effects of a wide variety of agents that induce oxidative stress on fibroblast EC-SOD expression. To gain more complete information on the response of the fibroblasts, we also analysed the effects on CuZn-SOD and Mn-SOD expression.

EXPERIMENTAL

Cell culture and study of the effect of oxidative stress

Human skin fibroblast lines were initiated from skin punch biopsy specimens obtained from healthy volunteers (ages 16, 3, 19, 9 and 33 years for the lines K1, K2, K4, K25 and C3 respectively), using Ham's F10 (Flow, Irvine, Scotland, U.K.) containing 10% (v/v) fetal calf serum (FCS; Gibco), 100 units/ml penicillin, 25 mM Hepes and 2 mM glutamine as medium. The lines were used between the 10th and 20th passages. The cells were mostly seeded into 12-well culture plates, bottom area 3.80 cm^2, and grown to near-confluence. The effects of oxidative stress were mainly studied with cells cultured in either 10% FCS or 0.5% FCS. Fibroblasts cultured in 0.5% FCS do not proliferate [13], whereas those cultured in 10% FCS increased their protein and DNA contents by 50–100% during the 4-day experiments. For the cells studied in 0.5% FCS, the medium was exchanged twice to medium with 0.5% FCS about 20 h before the start of the experiments. The experiments were started by exchange to 0.5 ml of medium with 10% or 0.5% FCS containing the indicated concentrations of oxidative-stress-inducing substances or without these substances (controls). Every 24 h, the media were collected and replaced with fresh media containing the oxidative-stress-inducing substances. At the end of the experiments, usually after 4 days (but in some cases also after 6 h or 24 h), the media were collected and the wells were washed three times with 0.15 M NaCl. To collect and homogenize the cells, 0.5 ml of ice-cold 50 mM sodium phosphate, pH 7.4, containing 0.3 M KBr, 10 mM diethylenetriaminepenta-acetic acid, 0.5 mM phenylmethanesulphonyl fluoride and 14 μg/ml aprotinin was added to each well. After sonication in the wells, with the plate bathed in ice/water, the homogenates were centrifuged (20000 *g* for 10 min) and the supernatants were

Abbreviations used: EC-SOD, extracellular superoxide dismutase; CuZn-SOD, Cu/Zn-containing superoxide dismutase; Mn-SOD, Mn-containing superoxide dismutase; FCS, fetal calf serum; IFN-γ, interferon-γ; IL, interleukin; TNF-α, tumour necrosis factor-α; TGF-β, transforming growth factor-β.

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collected for analysis. All samples were kept at −80 °C before assay.

SOD analysis

EC-SOD protein was determined in cell culture media and cell homogenates by an e.l.i.s.a. [14].

SOD enzymic activity in cell homogenates was determined by the direct spectrophotometric method employing KO_2 [15] as modified [16]. Cyanide (3 mM) was used to distinguish between the cyanide-sensitive isoenzymes CuZn-SOD and EC-SOD and the cyanide-resistant Mn-SOD. One unit corresponds to 8.3 ng of human CuZn-SOD, 8.6 ng of human EC-SOD and 65 ng of bovine Mn-SOD. The CuZn-SOD activity was obtained as total activity minus activity in the presence of 3 mM cyanide minus units of EC-SOD as calculated from the e.l.i.s.a. The KO_2 assay was performed at high pH and relatively high superoxide concentration. In comparison, the xanthine oxidase–cytochrome *c* assay [17] was carried out under more physiological conditions, i.e. neutral pH and low superoxide concentration. One unit in the 'KO_2 assay' corresponds to 0.024 unit of human CuZn-SOD and EC-SOD in the 'xanthine oxidase' assay and to 0.24 unit of Mn-SOD. The KO_2 assay is thus 10 times more sensitive for CuZn-SOD and EC-SOD activity than for Mn-SOD activity.

Protein and DNA analysis

For protein analysis, Coomassie Brilliant Blue G-250 was employed [18], standardized with human serum albumin. DNA was determined by fluorimetry as a complex with 2-[2-(4-hydroxyphenyl)-6-benzimidazolyl]-6-(1-methyl-4-piperazyl)-benzimidazol 3HCl (Hoechst 33258) [19] using calf thymus DNA as standard.

RNA extraction and Northern-blot analysis

Total cellular RNA was isolated from fibroblasts using the procedure of Chomczynski and Sacchi [20] with modifications described by Meltzer et al. [21]. Total RNA (8 μg per lane) was electrophoresed in formaldehyde-containing 1% (w/v) agarose gels, transferred to Nylon filters (Hybond N; Amersham plc, Amersham, Bucks., U.K.) and immobilized by u.v. linkage. The filters were then prehybridized for about 10 h and hybridized for 16 h with a hybridization solution containing 0.5 M Na_2HPO_4, 1% (w/v) BSA, 1 mM EDTA and 7% (w/v) SDS, pH 7.2, at 65 °C in a hybridization oven. For EC-SOD detection, a DNA probe was used, which corresponded to nucleotides 1018–1211 in the cDNA sequence [22]. For β-actin, a probe corresponding to cDNA nucleotides 363–428 [23] was used. ^{32}P-labelling was achieved by random priming (Megaprime-DNA labelling systems; Amersham). To allow rehybridization, boiling 0.1% SDS was twice poured on to the filters. Absorbance of photographic films exposed to the filters was determined with a two-dimensional densitometer (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

RESULTS

Design of experiments

The cells were usually cultured for 4 days, with media containing active substances exchanged each day, and the cells homogenized for analysis at the end of the experiment. The approach was based on previous experience with regulation by cytokines in which the response of Mn-SOD and especially EC-SOD developed over a period of several days [13]. The responses were studied both in 10% (v/v) FCS and under serum starvation, i.e. 0.5% FCS. The effects of the agents were studied in wide concentration intervals, with relatively small (3-fold) steps, beginning at concentrations exerting no or minimal observable effects and ending with concentrations producing toxicity, as demonstrated by loss of protein and DNA. As fibroblast lines are individuals and are known to differ in response, the effects of the agents tested were usually evaluated on two lines. Owing to the design of the experiments, which mostly involved analysis on two (sometimes four) wells for each data point, we only considered changes in concentration/activities differing by more than 30% from controls. This limitation should not result in any loss of information of biological relevance, as EC-SOD expression is known to respond with severalfold increases and decreases to the influence of various cytokines [13], and Mn-SOD is even more inducible [13,24–26].

Agents inducing oxidative stress

Oxidative stress was induced in a wide variety of ways as listed in Table 1 and shown in Figures 1(a)–1(d). Xanthine oxidase plus hypoxanthine primarily produces superoxide and hydrogen peroxide extracellularly [27]; pyrogallol autoxidizes to produce superoxide, probably both intracellularly and extracellularly [28]; paraquat is metabolically reduced by cells followed by autoxidation and superoxide radical formation [29]; α-naphthoflavone, hydroquinone and catechol are likely to produce oxidative stress by similar mechanisms; Fe^{2+} and Cu^{2+} induce oxidative stress in a variety of ways by catalysing one-step redox reactions [30]; buthionine sulphoximine [31] inhibits synthesis of the intracellular antioxidant glutathione; diethylmaleate reacts with and blocks both glutathione and other thiols in cells [32]; and finally, both *t*-butyl hydroperoxide and cumene hydroperoxide directly oxidize and may participate in free-radical-forming reactions with transition metal ions.

Treatment with all these oxidative agents uniformly, dose-dependently and continuously, depressed EC-SOD secretion into the culture medium (Table 1 and Figures 1a–1c). In no case at any dose was there evidence of enhancement of EC-SOD secretion. Except for the case with Fe^{2+} ions (Figure 1c), corresponding reductions in EC-SOD content were seen in the cell layers collected at the end of the experiments (Figures 1a, 1b and 1d; other results not shown).

In the case of Fe^{2+} ions, a peculiar pattern with regard to EC-SOD expression was seen (Figure 1c). There was a dose-dependent decrease in EC-SOD secretion and an increase in EC-SOD content in the cell homogenates. The effect was, as expected, seen at lower Fe^{2+} concentrations in 0.5% FCS than in 10% FCS. In separate experiments with the cell lines K1 and K3 terminated after 6 h and 24 h (results not shown), the same pattern was seen, although the increase in cell EC-SOD was proportionally smaller than after 4 days. To study the phenomenon further, we isolated RNA from fibroblasts exposed to 100 and 300 μM $FeCl_2$ for 6 h and 24 h and determined the relative EC-SOD mRNA contents by means of Northern-blot analysis. There was no significant difference between controls and the Fe^{2+}-treated cultures with regard to the EC-SOD mRNA content relative to the β-actin mRNA content (results not shown). We thus interpret the accumulation of EC-SOD in the cells caused by Fe^{2+} ions to be due to a secretion block, a notion further supported by the increases in protein/DNA ratios also seen (Figure 1c).

Table 1 Collection of data for oxidative treatments affecting SOD expression

The effect of oxidants on fibroblast SOD expression was determined as detailed in the Experimental section (see also Figures 1a to 1d). The Table presents the concentrations of oxidants tested (xanthine oxidase with 100 μM hypoxanthine), the human dermal fibroblast lines tested, data for 0.5% and 10% FCS in the medium, the lowest oxidant concentration to produce > 30% reduction in EC-SOD content in the 4th-day culture medium, the maximal enhancement in Mn-SOD activity per mg of protein in cell homogenates (-fold) and the oxidant concentration at which this occurred, the lowest oxidant concentration to produce > 30% reduction in CuZn-SOD activity per mg of protein in cell homogenates and the lowest oxidant concentration to produce > 30% reduction in cell protein and cell DNA. NS, no significant effect (less than 30% change). n.d., not determined.

Oxidant (concentrations tested)	Cell line	FCS concn. (%)	EC-SOD oxidant concn. at > 30% reduction	Mn-SOD		CuZn-SOD oxidant concn. at > 30% reduction	Protein oxidant concn. at > 30% reduction	DNA oxidant concn. at > 30% reduction
				Max. stimulation (-fold)	Oxidant concn. at max stimulation			
Xanthine oxidase	K3	0.5	50	NS	NS	NS	50	NS
(0.5, 1.5, 5.0, 15,	K25	0.5	50	1.6	15	NS	50	50
50 munits/ml)	K3	10	15	1.6	15	NS	50	NS
	K25	10	15	1.7	15	NS	50	50
Paraquat	K3	0.5	0.05	2.6	0.05	0.15	0.15	0.15
(0.016, 0.05,	K25	0.5	0.016	2.2	0.05	0.05	0.15	0.15
0.15, 0.45,	K3	10	0.016	2.5	0.15	0.15	0.15	0.15
1.5 mM)	K25	10	0.05	2.0	0.15	0.45	0.15	0.45
Pyrogallol	K3	0.5	30	7.7	300	300	100	300
(3, 10, 30, 100,	K25	0.5	30	10.6	300	NS	100	300
300 μM)	K3	10	30	1.4	100	NS	100	300
	K25	10	30	2.3	300	NS	100	NS
α-Naphthoflavone	K3	0.5	10	1.4	30	100	30	NS
(3, 10, 30, 100,	K25	0.5	10	2.4	30	100	10	NS
300 μM)	K3	10	30	2.4	100	300	30	NS
	K25	10	30	2.5	100	300	30	NS
Hydroquinone	K3	0.5	10	2.9	600	30	100	300
(10, 30, 100, 300,	K25	0.5	30	4.6	300	30	100	300
600 μM)	K3	10	30	3.3	600	300	100	100
	K25	10	30	4.1	600	300	300	300
Catechol	K3	0.5	300	2.4	300	600	300	600
(10, 30, 100, 300,	K25	0.5	10	3.6	600	600	30	600
600 μM)	K3	10	600	2.3	600	NS	600	600
	K25	10	30	2.3	600	NS	600	NS
$FeCl_2$	K3	0.5	30	2.3	1000	NS	NS	NS
(10, 30, 100,	K25	0.5	30	2.6	1000	NS	NS	NS
300, 1000 μM)	K3	10	300	NS	NS	NS	NS	NS
	K25	10	1000	1.5	300	NS	NS	NS
$CuSO_4$	K3	0.5	10	2.8	1000	300	300	300
(10, 30, 100,	K25	0.5	10	4.5	1000	300	300	300
300, 1000 μM)	K3	10	300	2.2	1000	1000	300	300
	K25	10	300	2.0	1000	300	300	300
Buthionine	K25	0.5	0.3	NS	NS	NS	NS	NS
sulphoximine	K25	10	0.1	3.0	1.0	NS	0.1	0.3
(0.03, 0.1, 0.3,								
1.0, 3.0 mM)								
Diethylmaleate	K3	0.5	0.03	NS	NS	1.0	1.0	1.0
(0.01, 0.03, 0.1,	K25	0.5	0.03	1.8	0.3	1.0	1.0	1.0
0.3, 1.0 mM)	K3	10	0.3	NS	NS	1.0	1.0	1.0
	K25	10	0.3	1.6	0.3	1.0	1.0	1.0
t-Butyl hydroperoxide	K3	0.5	0.3	NS	NS	NS	NS	NS
(0.01, 0.03, 0.1,	K3	10	0.3	NS	NS	NS	NS	NS
0.3, 1.0 mM)								
Cumene	C3	0.5	0.1	n.d.	n.d.	n.d.	0.3	0.3
hydroperoxide	K4	0.5	0.03	NS	NS	NS	0.3	0.3
(0.01, 0.03, 0.1,	C3	10	0.3	n.d.	n.d.	n.d.	0.3	0.3
0.3, 1.0 mM)	K4	10	0.3	NS	NS	NS	0.3	0.3
Selenite	K3	0.5	3	NS	NS	NS	10	NS
(0.1, 0.3, 1.0,	K25	0.5	3	NS	NS	NS	3	NS
3.0, 10 μM)	K3	10	3	NS	NS	NS	10	NS
	K25	10	3	NS	NS	NS	10	NS

Except for *t*-butylhydroperoxide and cumene hydroperoxide, the oxidative agents induced moderate inductions in Mn-SOD activity (Table 1 and Figures 1a–1c). The largest induction was seen with pyrogallol. The Mn-SOD induction was mainly seen at oxidative stress intensities that also induced loss of protein, DNA and CuZn-SOD activity from the cell cultures. However, the relationship between the concentration of oxidant at which there was onset of induction of Mn-SOD activity and the concentration at which toxic effects occurred differed between the oxidants. There were agents, such as cumene hydroperoxide,

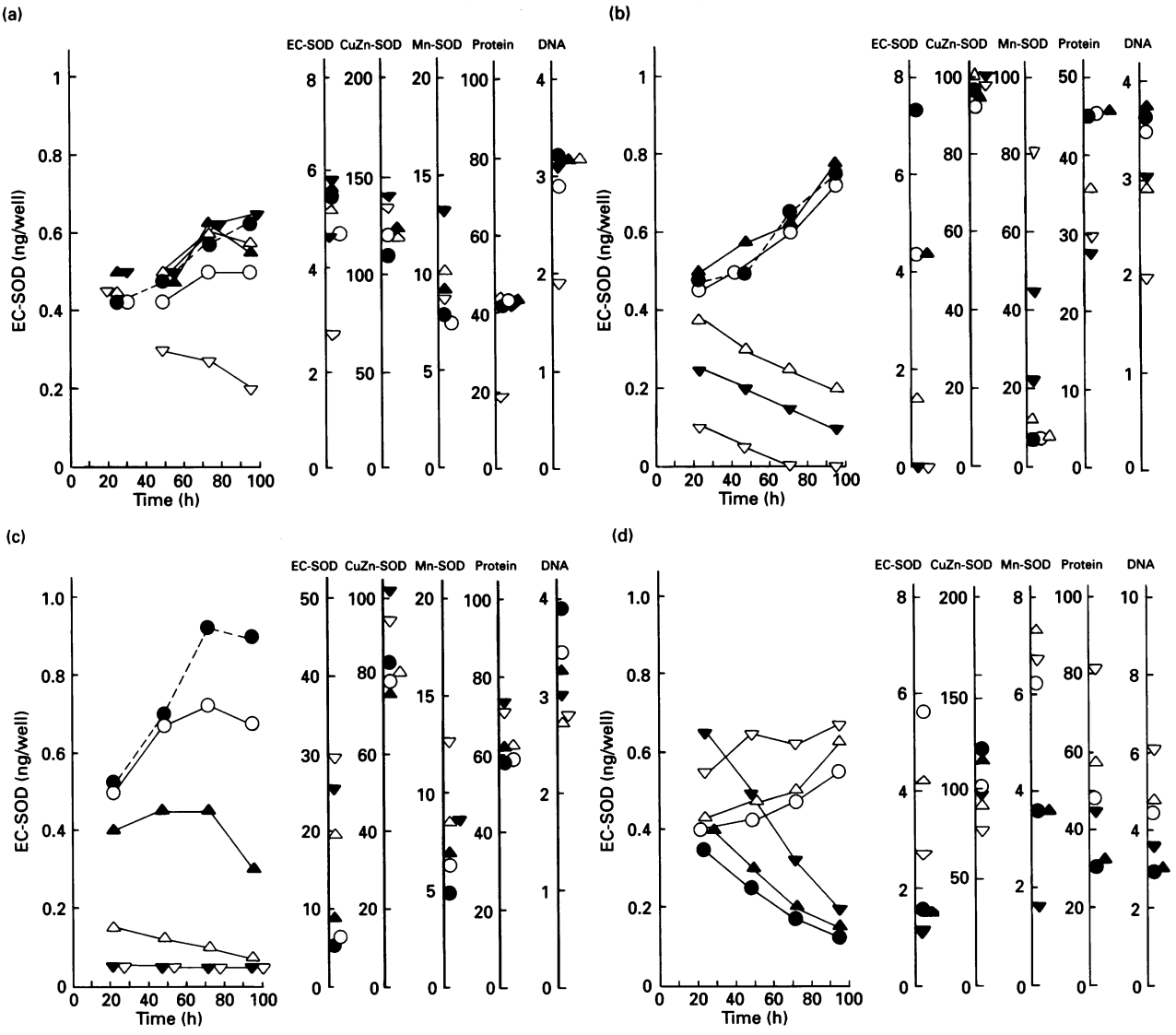

Figure 1 Effect of oxidative stress on fibroblast SOD synthesis

(**a**) Xanthine oxidase (○, 0.5 munits/ml; ▲, 1.5 munits/ml; △, 5 munits/ml; ▼, 15 munits/ml; ▽, 50 munits/ml; ●, control) plus hypoxanthine (100 μM); (**b**) pyrogallol (○, 3 μM; ▲, 10 μM; △, 30 μM; ▼, 100 μM; ▽, 300 μM; ●, control); (**c**) $FeCl_2$ (○, 10 μM; ▲, 30 μM; △, 100 μM; ▼, 300 μM; ▽, 1000 μM; ●, control); (**d**) high oxygen partial pressure (○, 20% O_2, 0.5% FCS; △, 20% O_2, 2% FCS; ▽, 20% O_2, 10% FCS; ●, 88% O_2, 0.5% FCS, ▲, 88% O_2, 2% FCS; ▼, 88% O_2, 10% FCS). Fibroblast lines were cultured in 3.8 cm^2 wells in culture media with different FCS concentrations: (**a**), (**b**) and (**c**), K25 in 0.5% FCS; (**d**) K25 in 0.5, 2 or 10% FCS. Culture media containing the indicated concentrations of oxidants were changed daily for 4 days. In (**d**), the cells were cultured in either ambient O_2 or 88% O_2 with daily media changes. At the end of the experiment, the cells were homogenized and analyses were made on culture media for EC-SOD and on cell homogenates for SOD isoenzymes (results in ng/mg of protein for EC-SOD and units/mg of protein for CuZn-SOD and Mn-SOD), protein (μg/well) and DNA (μg/well) as described in the Experimental section. The data presented for (**a**)–(**c**) are the means of results from two wells, and for (**d**) the means of four wells.

t-butylhydroperoxide (Table 1), selenite and high oxygen partial pressure (see below and Figure 1d) that apparently induced toxicity but did not induce Mn-SOD activity. Induction of Mn-SOD activity was thus not simply due to high stress intensity. Cell cultures were also collected 6 h and 24 h after treatment with several oxidants at the concentrations indicated in Table 1: xanthine oxidase/hypoxanthine, paraquat, pyrogallol, $FeCl_2$, buthionine sulphoximine and *t*-butyl hydroperoxide. There was no enhancement of Mn-SOD activity with any of the treatments after 6 h, and only minor enhancement with some after 24 h: paraquat (1.8-fold), pyrogallol (1.5-fold) and $FeCl_2$ (1.8-fold).

There was no evidence for induction of CuZn-SOD activity by any of the oxidative treatments in cultures collected after 4 days (Table 1) or after 6 h and 24 h as described in the preceding paragraph. Instead, with most of the treatments, dose-dependent reductions in activity were seen, beginning near the concentrations that also induced losses in protein and DNA from the cultures.

Other treatments

Selenite exerts two opposing effects. The selenium content in medium with 0.5% FCS is too low to allow maximal glutathione peroxidase expression [33], which, however, occurs even with the lowest selenite dose tested. The cellular protection against hydroperoxides is thereby enhanced. At high doses, selenite exerts toxic effects [33], possibly as a result of formation of oxyradicals [34]. At 3 μM and above, selenite dose-dependently

continuously depressed EC-SOD expression under all experimental conditions (Table 1). There was no significant effect on CuZn-SOD and Mn-SOD. At 10 μM toxicity occurred, as demonstrated by some loss of protein. In cultures harvested after shorter times, 6 h and 24 h, no significant effects on any parameter were seen.

Two fibroblast lines, K3 and K25 with 0.5%, 2% or 10% FCS in the medium, were cultured in 20% O_2 or 88% O_2. Culture in 88% O_2 resulted, in both cell lines under all conditions, in a continuous reduction in EC-SOD expression (Figure 1d). There was no significant change in the CuZn-SOD activity, whereas the Mn-SOD activity was roughly halved in both fibroblast lines. Corresponding experiments were also analysed after 6 h and 24 h. In those experiments no significant changes were observed (results not shown).

Citiolone has been reported to enhance CuZn-SOD activity [35,36]. Citiolone (0.03, 0.1, 0.3, 1, 3 mM) tested on one cell line (K3 grown in 0.5% FCS or 10% FCS) dose-dependently continuously reduced EC-SOD expression to about 30% of the control level at 1 mM at day 4. There was no significant effect on Mn-SOD or CuZn-SOD at any dose and only minor reductions in protein and DNA content of cells grown in 10% FCS at the highest dose (results not shown).

The effect of catalase (0.5, 1.5, 15, 50 μg/ml), bovine CuZn-SOD (10, 50 μg/ml) and combinations of the two were tested on two fibroblast lines, K3 and K25, grown in 0.5% FCS for 4 days. There was no effect of these antioxidant enzymes on any of the cell parameters analysed (results not shown). Addition of heparin (30 μg/ml), to remove any EC-SOD bound to the fibroblast cell surfaces [10], had no effect on expression of EC-SOD or the other SOD isoenzymes.

DISCUSSION

Two fibroblast lines were exposed to a wide variety of oxidative stress treatments in a wide dose range under serum starvation or at a serum concentration that supported proliferation. Analyses were made on four daily collected culture media and on cell layers harvested after 4 days, and in most cases also after 6 h and 24 h. In none of the cases was there any evidence of induction of EC-SOD expression. Instead, in all cases there was a dose-dependent continuous depression of EC-SOD expression seen both in culture media and in the cell homogenates. The one exception to this rule was the increase in cell EC-SOD content induced by Fe^{2+} treatment (Figure 1c), which, however, was apparently due to a secretion block. Enhancement of the protection against oxidative stress by addition of SOD, catalase and low concentrations of selenite had no effect on EC-SOD expression. We conclude that EC-SOD expression is not induced by its substrate or any other oxidative agent. Loss of EC-SOD expression occurred regularly at lower stress intensities than those that resulted in overt toxicity, as demonstrated by loss of protein and DNA from the cultures. We interpret this uniform effect to be due to toxicity rather than to specific depression of EC-SOD expression induced by oxidative stress.

With several of the oxidative treatments, induction of Mn-SOD activity was seen. This enhancement in activity, however, was moderate and was generally seen at stress intensities that began to reduce the content of protein and DNA in the fibroblast cultures. The most distinct induction was caused by pyrogallol in cultures grown under serum starvation. The induction was relatively slow and seen after 4 days, but not with certainty after 6 h and only in some cases and to a minor extent after 24 h. This relatively slow enhancement in Mn-SOD activity was similar to that induced by IL-1 and TNF; the enhancements after 24 h were about a third as large as those seen after 4 days [13]. Absent or only moderate inductions of Mn-SOD activity by oxidative stress in various cultured mammalian cell lines have been observed in several other studies (e.g. [37–39]). The moderate effect of oxidative stress contrasts sharply with the major induction of Mn-SOD activity that can be produced by cytokines such as TNF [13,24] and IL-1 [13,25]. TNF-α and IL-1α induce the Mn-SOD activity of the present fibroblast lines 30- to 40-fold [13]. The pattern of response to the oxidants in the present study did not allow designation of a single inducing oxidant species. It can be noted that pyrogallol, α-naphthoflavone, hydroquinone, catechol and hydrogen peroxide (and probably other oxidants) may all activate via the antioxidant-responsive element [40], which consequently may be involved in inducing Mn-SOD synthesis. Another candidate for involvement is NF-κB, which is activated by oxidants (as well as by IL-1 and TNF) [41]. Indeed, there are three sites (nucleotides -497 to -486 and 2696 to 2705 on the coding strand and nucleotides 7125 to 7134 on the non-coding strand) that fulfil the suggested requirements of the core sequence of the antioxidant-responsive element in the part of the rat Mn-SOD gene sequenced so far [42]. Similarly, multiple elements potentially responding to NF-κB were detected [41]. Mn-SOD induction was only seen at high-oxidative-stress intensity, and may also have been due to a more or less specific interference with, for example, factors involved in the vast response to the cytokines. Finally, it has been shown that oxidants can induce several cell types [43,44] to secrete, for example IL-1 and IL-6, which, via an autocrine loop, may subsequently enhance the Mn-SOD activity.

We found no evidence for induction of CuZn-SOD by oxidative stress. We were also unable to reproduce in fibroblasts the previously reported induction by citiolone [35]. Instead, losses of CuZn-SOD activity were regularly seen, although generally at oxidative-stress intensities that also resulted in loss of cell protein and DNA. We interpret these effects to be due to toxicity. Similar findings of lack of response of CuZn-SOD to oxidative stress are reported in a variety of cell types by others [e.g. 26,37–39,45]. However, there are also reports demonstrating induction by oxidative stress in endothelial cells [46,47] and Chinese hamster fibroblasts [48]. *In vivo* exposure to hyperoxia has been reported to both enhance [49] and not influence [26,50,51] the CuZn-SOD activity of rat [49,50] and mouse [26] lungs and rat brain [51]. Exposure of guinea pigs to high oxygen partial pressure enhances both the CuZn-SOD and Mn-SOD activities of subsequently isolated neutrophils and alveolar macrophages [52]. *In vivo* endotoxin treatment in combination with *in vivo* hyperoxia or followed by exposure of lung slices to 95% O_2 [53] as well as hyperthermia [54] also enhances CuZn-SOD synthesis or activity. Thus, although in most situations studied there seems to be no response of CuZn-SOD to oxidative stress, some cell types in some species do respond with enhanced CuZn-SOD activity. However, in all cases reported the response is small. Similarly, most studies indicate that CuZn-SOD in a variety of cell types is not influenced by any cytokines (e.g. [13,24–26]), although one study with pulmonary artery endothelial cells reports a modest induction by TGF-β, IL-1 and TNF-α [46]. In contrast, changes in CuZn-SOD activity are widely reported to occur during development, especially in the perinatal period (e.g. [55–57]), and as a result of cell differentiation [58–60]. Finally, there are very large (more than 10-fold) differences in CuZn-SOD activity between human tissues [6,11] and between different human cell types [6,11,61].

In conclusion, EC-SOD expression is not influenced by its substrate or any type of oxidative stress but is profoundly

influenced by cytokines involved in the inflammatory response [13]. Likewise Mn-SOD expression in mammalian cells is greatly influenced by such cytokines [13,24,25] and reacts only unspecifically or to a limited extent to oxidative stress. Finally, CuZn-SOD appears to be essentially constitutive with a cell-specific variation. The wide cell and tissue differences in CuZn-SOD activity observed may reflect differences in 'anticipated' stress. Unicellular organisms, such as bacteria [62] and yeast [63], are known to respond directly to oxidative stress with enhanced expression of SODs and other antioxidant systems. The response of mammalian SOD isoenzymes to oxidative stress appears to be fundamentally different. The expression of EC-SOD and Mn-SOD by mammalian cells is apparently primarily regulated as a part of a co-ordinated tissue response orchestrated by cytokines, rather than by direct effects of oxidants on individual cells.

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