Elevation in the ratio of Cu/Zn-superoxide dismutase to glutathione peroxidase activity induces features of cellular senescence and this effect is mediated by hydrogen peroxide

Judy B. de Haan, Francesca Cristiano, Rocco Iannello, Cecile Bladier\textsuperscript{1,+}, Michael J. Kelner\textsuperscript{2} and Ismail Kola* 

Molecular Genetics and Development Group, Institute of Reproduction and Development, Monash University, Clayton, Victoria 3168, Australia, \textsuperscript{1}Commissariat a l’Energie Atomique, Centre de Cadarache, Laboratoire de Phytotechnologie, 13108 St-Paul-lez-Durance, France and \textsuperscript{2}Department of Pathology, University of California, 200 West Arbor Drive, San Diego, California 92103–8320, USA

Received November 28, 1995; Accepted December 1, 1995

Although reactive oxygen species have been proposed to play a major role in the aging process, the exact molecular mechanisms remain elusive. In this study we investigate the effects of a perturbation in the ratio of Cu/Zn-superoxide dismutase activity (Sod1 dismutases .O\textsubscript{2}– to H\textsubscript{2}O\textsubscript{2}) to glutathione peroxidase activity (Gpx1 catalyses H\textsubscript{2}O\textsubscript{2} conversion to H\textsubscript{2}O) on cell growth and development. Our data demonstrate that Sod1 transfected cell lines that have an elevation in the ratio of Sod1 activity to Gpx1 activity produce higher levels of H\textsubscript{2}O\textsubscript{2} and exhibit well characterised markers of cellular senescence viz. slower proliferation and altered morphology. On the contrary, Sod1 transfected cell lines that have an unaltered ratio in the activity of these two enzymes, have unaltered levels of H\textsubscript{2}O\textsubscript{2} and fail to show characteristics of senescence. Furthermore, fibroblasts established from individuals with Down syndrome have an increase in the ratio of Sod1 to Gpx1 activity compared with corresponding controls and senescence earlier. Interestingly, cells treated with H\textsubscript{2}O\textsubscript{2} also show features of senescence and/or senescence earlier. We also show that Cip1 mRNA levels are elevated in Down syndrome cells, Sod1 transfectants with an altered Sod1 to Gpx1 activity ratio and those treated with H\textsubscript{2}O\textsubscript{2}, thus suggesting that the slow proliferation may be mediated by Cip1. Furthermore, our data demonstrate that Cip1 mRNA levels are induced by exposure of cells to H\textsubscript{2}O\textsubscript{2}. These data give valuable insight into possible molecular mechanisms that contribute to cellular senescence and may be useful in the evolution of therapeutic strategies for aging.

INTRODUCTION

Aerobic organisms generate toxic reactive oxygen species (ROS) during oxidative metabolism. Prokaryotic and eukaryotic organisms have evolved antioxidant defences to protect against ROS, predominant amongst which is the enzymatic antioxidant pathway. This pathway consists of essentially two steps: firstly, the dismutation of .O\textsubscript{2}– to H\textsubscript{2}O\textsubscript{2} by superoxide dismutases (Sod); and secondly, the conversion of H\textsubscript{2}O\textsubscript{2} to H\textsubscript{2}O which is catalysed by Gpx and/or catalase (1). Theoretically at least, the balance between the first and second step antioxidant enzymes may be critical: on the one hand too little Sod relative to Gpx and/or catalase could lead to an accumulation of .O\textsubscript{2}– which are toxic to macromolecules; and on the other hand, too much Sod relative to Gpx and/or catalase could lead to increased production of the H\textsubscript{2}O\textsubscript{2} intermediate, which in itself may be responsible for the genesis of ROS (viz. OH) that are even more noxious than .O\textsubscript{2}–. The conversion of H\textsubscript{2}O\textsubscript{2} to OH\textsuperscript{.} is catalysed by Fe\textsuperscript{2+} in the Fenton reaction (2).

A disequilibrium between the activity of first and second step antioxidant enzymes occurs in various pathological processes and may contribute directly or indirectly to these processes. For instance, individuals with Down syndrome have three copies of the Sod1 gene and only two copies of the Gpx1 gene. Brooksbank and Balazs (3) have shown that cerebral cortex tissue derived from Down syndrome fetuses have increased Sod1 activity, unchanged Gpx1 activity, and a significant increase in lipid peroxidation after stimulation with FeSO\textsubscript{4} and ascorbic acid. It has been proposed (4) that this disequilibrium between Sod1 and Gpx1 may contribute to some of the pathological features that occur

\textsuperscript{*To whom correspondence should be addressed

\textsuperscript{+Present address: Molecular Genetics and Development Group, Institute of Reproduction and Development, Monash University, Clayton Vic 3168, Australia
The ratio of Sod1 to Gpx1 enzyme activity in control (C), adapted (AD) and non-adapted (NON-AD) Sod1 transfected cell lines. Non-adapted cell lines have significantly elevated ratios compared with adapted and parental cells. \( \ast p < 0.05 \) and \( \ast\ast p < 0.01 \) (t-test).

as part of the Down syndrome phenotype and indeed, transgenic mice over-expressing Sod1 develop morphological and biochemical changes at tongue neuromuscular junctions similar to those seen in tongues of individuals with Down syndrome (5,6). Interestingly these neuromuscular deteriorations are, morphologically at least, also similar to those seen in muscles of aging mice and rats (7). Consequently, it has been proposed that over-expression of Sod1 in Down syndrome plays a role in the premature aging of these individuals (8). Consistent with this hypothesis is our finding that Sod1 activity increases in the brains of mice during the aging process, and that this increase occurs against a background of unaltered Gpx1 activity. The increase in the ratio of Sod1 activity to Gpx1 activity is paralleled by an increase in lipid peroxidation (9). Thus, the balance between the activity of the first and second-step antioxidant enzymes may also be an important determinant in the aging process. Furthermore, this imbalance is also associated with the onset of epileptic seizures. Singh and Pathak (10) have demonstrated that an intracortical injection of FeCl3 into rats induced epileptic seizures and that this was associated with an increase in brain lipid peroxidation and a 60% increase in brain Sod activity, whereas the concomitant increase in Gpx1 and catalase activity was less than 20%. Thus, these data suggest that the ratio of Sod1 to Gpx1 and/or catalase activity may be important in the genesis of various perturbations in cellular functions.

In order to further investigate the role of an altered Sod1 to Gpx1 (and catalase) activity in the perturbation of cellular function, and more specifically in the induction of characteristics of cellular senescence, we focus on two cell systems that have elevated Sod1 activity relative to Gpx1 activity. In the first, Sod1 levels are elevated as a result of stable integration of additional copies of human Sod1 cDNA, while the second are Down syndrome cells where Sod1 levels are elevated as a consequence of an altered gene dosage for Sod1. We focus on three criteria as markers of senescence, namely cellular proliferation, morphology and the expression of senescent genes. With regard to senescent genes, we have focussed our attention on the Cip1 gene (11) [also known as Waf1 (12) and Sdi (13)]. The Cip1 gene is over-expressed (10- to 20-fold) in senescent cells compared with young cells, and the increase in Cip1 mRNA closely paralleled the onset of senescence and the loss of cellular proliferation. Furthermore, Cip1 mRNA levels rise significantly when young cells are made quiescent by deprivation of growth factors or contact inhibition (13). For these reasons, Cip1 has been proposed to play a role in the maintenance of the senescent phenotype.

Our data show that cells with altered Sod1 to Gpx1 activity ratios show characteristics of cellular senescence, i.e., they proliferate more slowly, have altered morphology and size, and they have elevated Cip1 levels. Furthermore, cells with an elevated Sod1 to Gpx1 activity ratio have, and secrete higher levels of H2O2 and, intriguingly these features of senescence can be induced by treatment of cells with H2O2. Thus, these data implicate an elevation in the ratio of Sod1 to Gpx1 activity in the induction of cellular senescence and suggest that this effect may be mediated via H2O2.
RESULTS

Cells with an altered Sod1 to Gpx1 activity ratio

Sod1 transfectants. Two populations of Sod1 transfectants were studied. The first, a sample of clones representing the vast majority of Sod1 transfectants in murine NIH3T3 cells (14), had up-regulated endogenous Gpx1 in response to elevated Sod1 levels (the adaptive response), such that the ratio of activity of Sod1 to Gpx1 in these cell-lines was relatively unchanged (range of Sod1 to Gpx1 activity ratio relative to controls was 0.87–1.38) (Fig. 1). This population of cells is referred to as adapted cell-lines henceforth. The second are a sample of clones representing a population that had elevated Sod1 levels but failed to demonstrate an increase in Gpx1 activity, such that the ratio of Sod1 to Gpx1 activity was significantly elevated (2.37–2.91) (Fig. 1). This population is referred to as non-adapted cell-lines. The levels of catalase (the other enzyme with peroxidase activity) was not significantly different in both these populations compared with that of the parental controls, whilst the levels of Sod2 were lower in the adapted clones and slightly higher in the non-adapted clones (15).

We examined both adapted and non-adapted cells for features of senescence. The reduced rate of cellular proliferation is a well characterised and widely accepted feature of senescence (16,17). Non-adapted cell-lines proliferated significantly slower than the adapted cell-lines and the parental controls, i.e., the non-adapted cell-lines had a significantly elevated cell doubling time (Fig. 2a). Interestingly, the cell doubling time of the adapted cell-lines was not significantly different from that of the parental cell-lines. Furthermore, our data also show that the uptake of 3H-thymidine was lower in the non-adapted cells compared with that of the parental controls—again, the uptake of 3H-thymidine was not significantly different between the parental controls and both the adapted cell-lines (Fig. 2b).

The second feature of senescence studied was cellular morphology and size (18,19). The morphology of non-adapted Sod1 transfectants was altered compared with controls. These cells were more translucent, had larger nuclei and a changed morphology [Fig. 3a(iii)]. The adapted clones, on the other-hand, were morphologically indistinguishable from the parental cell line [Figs 3a(i, ii)]. Stereological analysis was carried out to confirm the morphological changes in non-adapted Sod1 transfectants. The non-adapted clones had a significantly greater adherent surface area, cell volume and nucleus volume (Fig. 3b). The adapted clones were not significantly different from the parental cell line for any of the criteria evaluated stereologically (Fig. 3b).

Since Cip1 is a biochemical marker of senescence, we investigated the levels of Cip1 mRNA in NIH3T3 cells and compared these to both the adapted and non-adapted transfectants. Our data demonstrates that the constitutive mRNA levels for Cip1 are elevated in non-adapted Sod1 transfected clones (Fig. 4, lanes 7 and 9) compared with the adapted clones (Fig. 4, lanes 3 and 5) and the parental NIH3T3 cells (Fig. 4, lane 1). The levels of Cip1 mRNA are undetected or just detected in the parental and adapted untreated cells. However, in the non-adapted cells the levels of Cip1 are constitutively expressed at higher levels. Thus these data demonstrate that Cip1 levels are elevated in cells with higher ratios of Sod1 to Gpx1 activity.

Down syndrome cells. Next we studied Down syndrome cells where an imbalance in the Sod1 to Gpx1 activity ratio exists. Down syndrome arises due to an extra copy of chromosome 21.
Figure 4. Northern blot analysis of Cip1 mRNA levels in parental, adapted and non-adapted Sod1 transgenic cell lines. Non-adapted cells show higher levels of constitutive Cip1 expression (lanes 7 and 9) than control (lane 1) or adapted cells (lanes 3 and 5). Cip1 mRNA levels are induced in cell lines treated with 150 µM H2O2 for 5 h, compared with untreated counterparts (NIH3T3 cells, lane 2; FD5, lane 4; FC1, lane 6 and 2F6, lane 8), except in 3A7.

(or in some rare cases part of chromosome 21) (20). The Sod1 gene is located on human chromosome 21 (21) and Gpx1 on human chromosome 3 (22)—thus, in these individuals the gene dosage for Sod1/Gpx1 is elevated 1.5 fold, theoretically at least. We therefore studied primary cell lines derived from individuals with Down syndrome and compared these to age-matched controls for the Sod1 to Gpx1 activity ratio and the acquisition of features of senescence. It cannot necessarily be assumed that a gene dosage elevation in Sod1 or Gpx1 would result in an elevation in enzyme activity, especially since several studies have shown that in some cell types Gpx1 is upregulated in Down syndrome in the so-called adaptive response (23,24).

The Sod1 activity was significantly higher in the two Down syndrome primary cell-lines studied, while Gpx1 activity was not significantly different. Thus the ratio of Sod1 to Gpx1 activity was significantly higher in both Down syndrome cell-lines as compared with corresponding controls (Fig. 5). Interestingly, the levels of MnSod (Sod2) were not significantly different between the Down syndrome cell-lines and the corresponding age-matched controls (Fig. 6).

We then compared Down syndrome and control fibroblasts for characteristics of cellular senescence. The cells were analysed for (i) proliferation and (ii) morphology. Down syndrome fibroblasts proliferated significantly slower than control fibroblasts; Down syndrome fibroblasts had a cell doubling time approximately 1.8 fold greater than that of control fibroblasts (Fig. 7). The Down syndrome cells are morphologically much larger than the control cells; this difference is clearly evident in the photomicrographs shown in Figure 8a. Stereological analysis of the adherent surface area and mean cell volume of these cell lines demonstrate a significant increase in both of these parameters in Down syndrome cell-lines (Fig. 8b). In addition to the above two criteria, we also investigated the expression of the Cip1 gene as a biochemical marker of senescence. Our data demonstrate that the constitutive mRNA levels for Cip1 are elevated in Down syndrome cells compared with control fibroblasts (Fig. 9).

To evaluate whether these primary cells actually senesce earlier, we measured the replicative life-span of Down syndrome fibroblasts and compared these to that of control cells. The Down syndrome cells replicated for between 20–25 passages and then ceased to replicate further even though they were viable. The control cell-lines replicated for approximately 50 passages before they ceased to proliferate further. Thus, these data demonstrate that primary cell lines established from individuals with Down syndrome that had an altered Sod1 to Gpx1 activity ratio underwent true senescence.

Figure 5. Sod1 and Gpx1 enzyme activity, and the Sod1 to Gpx1 activity ratios of control and Down syndrome (DS) cells. CCD45 and D532 are age-matched control and DS cells respectively; likewise HF5 and D539 are age-matched control and DS cells respectively. In both cases, Sod1 activity is significantly increased in DS cells compared with controls, while Gpx1 activity is unchanged for both DS cell lines compared with controls. Thus the Sod1/Gpx1 activity ratio is significantly increased in both DS cell lines compared with controls. *p <0.05 and **p <0.01 (t-test).
The role of $H_2O_2$ in the induction of features of cellular senescence

Since (i) $H_2O_2$ is the intermediate between the two steps of the antioxidant pathway (25); (ii) theoretically at least, an elevation in Sod1 activity relative to Gpx1 activity could result in an accumulation of this intermediate; and (iii) $H_2O_2$ gives rise to OH through the Fenton reaction (2), we compared non-adapted, adapted and parental NIH3T3 cells for levels of $H_2O_2$.

The intracellular levels of $H_2O_2$ in the non-adapted clones were significantly higher than those of the parental controls, whilst those of the adapted clones were not significantly altered (Fig. 10i). Furthermore, the levels of $H_2O_2$ released into the medium by the non-adapted clones were significantly greater compared with levels from the adapted cells and parental controls; the latter two were not statistically different from one another (Fig. 10ii). It should be noted that the absolute levels of $H_2O_2$ secreted into the medium may be an underestimate since $H_2O_2$ is a very labile molecule; nevertheless, the comparison of relative levels secreted by the different cell lines is valid and the data are consistent with those measuring the intracellular levels. Thus non-adapted cells show characteristics of senescence and produce higher levels of $H_2O_2$, while adapted cells that do not show features of senescence (and resemble the parental cells), have similar levels of $H_2O_2$ to the parental cells.

To investigate the role of $H_2O_2$ in the induction of features of cellular senescence we treated the parental NIH3T3 cells and primary diploid fibroblast cells (CCD45) with increasing concentrations of $H_2O_2$. The CCD45 cells were included since they are a primary cell line and undergo senescence after 50 passages in culture. Exposure of both cell types to $H_2O_2$ inhibited cell proliferation in a dose-dependent fashion as evidenced by cell counts over a 24 h time period [Fig. 11(i) and (ii)] and $^3$H-thymidine incorporation over a 3 h time period after exposure to $H_2O_2$ for 3 and 12 h (NIH3T3 cells) and 24 h (CCD45) [Fig. 11b(i) and (ii)]. In addition, treatment of both NIH3T3 cells and CCD45 cells with $H_2O_2$ also induced changes in cell morphology [Fig. 12a(i) and (ii)] and stereology [Fig. 12b(i) and (ii)] in a manner analogous to that for Down syndrome cells and the non-adapted Sod1 transfectants. Furthermore, our data demonstrate an elevation in mRNA levels of Cip1 in NIH3T3 (Fig. 4, lane 2) and in the adapted cells treated with 150 μM $H_2O_2$ for 5 h (Fig. 4, lanes 4 and 6) as well as in NIH3T3 cells treated with 10 and 30 μM $H_2O_2$ for 24 h (Fig. 13). Cip1 mRNA levels were also induced in some, but not all non-adapted clones (Fig. 4, lane 8). These data strongly suggest that the characteristics of cellular senescence induced in cells with an altered Sod1 to Gpx1 activity ratio is mediated via $H_2O_2$.

DISCUSSION

The data in this study have shown that cells with an alteration in the ratio of Sod1 to Gpx1 activity demonstrate features of cellular senescence. Studies focussed on (i) NIH3T3 cells with an alteration in the Sod1 to Gpx1 activity ratio and (ii) cells derived from individuals with Down syndrome. NIH3T3 cells that failed to upregulate Gpx1 in response to increased Sod1 activity (i.e. had an altered Sod1 to Gpx1 activity ratio) displayed slower proliferation and had altered morphology and size. In addition these cells expressed increased amounts of the Cip1 gene, a gene known to be overexpressed in senescent cells (26). Furthermore, these cells express higher levels of fibroactin and are unresponsive to certain growth factors (15); these are also features characteristic of senescent cells. Importantly this study also shows that cells transfected with Sod1, but that upregulate Gpx1 such that the Sod1 to Gpx1 activity ratio is unchanged from that of parental controls (adapted cells), show none of these features of cellular senescence and are indistinguishable from their parental controls—these adapted cells can be thought of as ‘naturally rescuing’ the phenotype. Furthermore, primary cells derived from individuals with Down syndrome that also have an altered Sod1 to Gpx1 activity ratio, display features characteristic of senescent cells namely, slower proliferation, altered morphology and size, and increased expression of the senescent gene Cip1. Indeed, these primary cells undergo true senescence in that they have a much shorter replicative life-span than corresponding controls.

The findings of this study show that the ratio of the activity of the first and second step antioxidant enzymes, rather than the absolute levels of these enzymes per se, is the important determinant in alteration of cellular function. The primary evidence that supports this proposal is the data from the Sod1 transfectant experiments. Cells that have adapted to elevated Sod1 (by increasing Gpx1 levels) are indistinguishable from non-transfectants, at least for the parameters of senescence investigated in this study; whereas, cells that have not adapted to elevated Sod1 display a perturbation in cellular function. It is unclear, whether the elevated Sod1 to Gpx1 activity ratio is
Figure 8. (a) Photomicrograph showing the morphology of control (i) and Down syndrome (ii) cells. (magnification ×250); (b) stereological comparison of Down syndrome cell lines with controls. Both the adherent surface area and cell volume of Down syndrome cell lines were significantly increased compared with their corresponding controls. *p < 0.05 and **p < 0.01 (t-test).

Figure 9. Northern blot analysis of Cip1 mRNA levels in fibroblasts of a Down syndrome cell line compared with a control line. The amount of Cip1 (relative to 18S rRNA) in Down syndrome and control cells is shown at the bottom of the figure (data has been normalized relative to the controls). Down syndrome fibroblasts show increased levels of Cip1 mRNA compared with control cells.

directly responsible for the induction of the features of cellular senescence as shown in this study or whether it is mediated indirectly via other changes in these cells, especially since Sod2 levels are higher in the non-adapted clones (15). However, the fact that the Down syndrome fibroblasts do not show alterations in Sod2 but still show the same features of senescence as the
Production of H$_2$O$_2$, the intermediate between the first and second step of the enzymatic antioxidant pathway. Firstly, we have demonstrated that non-adapted Sod1 transfectants (cells with an increase in Sod1 to Gpx1 enzyme activity) have and secrete higher levels of H$_2$O$_2$ compared with adapted transfectants and untransfected controls; and that the non-adapted cells, but not the adapted cells or parental controls, display features of cellular senescence. Secondly, and more strikingly, is the finding that treatment of NIH3T3 cells as well as primary fibroblast cells with H$_2$O$_2$ led to slower proliferation, altered morphology and altered expression of genes such as Cip1. It should be noted that this upregulation of Cip1 in cells with an elevated ratio of Sod1 to Gpx1 and cells treated with H$_2$O$_2$ is independent of p53 since (i) the levels of p53 mRNA is unaltered, and (ii) the level of binding to a p53 consensus oligonucleotide in nuclear lysates of these cells is unaltered (data not shown). Thus, it is unlikely that the features of cellular senescence such as slower proliferation and upregulation of Cip1 mRNA are due to non-specific events such as DNA damage. Furthermore, independently of ourselves, others (31) have also recently shown that the treatment of cells with H$_2$O$_2$ leads to the induction of a 'state of senescence' in cells.

This study suggests that the slower proliferation of cells with an elevation in the ratio of Sod1 to Gpx1 activity and cells treated with H$_2$O$_2$ may be mediated by the elevated expression of Cip1 seen in these cells. Cip1 is a cyclin-dependent kinase (Cdk)-inhibitor that forms inhibitory complexes with many different cyclins and their associated Cdk5s and may be a universal Cdk inhibitor (11,32) thereby inhibiting the passage of cells through specific phases of the cell-cycle. Cip1 mRNA levels are increased 10–20 fold in senescent cells compared with cycling cells and this increase may contribute to maintaining the senescent phenotype (13). The exact mechanism(s) by which elevated levels of H$_2$O$_2$ induce Cip1 mRNA is unknown and remains to be elucidated. However, H$_2$O$_2$ is known to be able to affect gene expression and increase the binding of various transcription factors such as NF-kB (33) to their respective cis-binding elements in the promoters/enhancers of cellular genes. It may be that H$_2$O$_2$ is acting in a similar manner to upregulate the expression of Cip1 and/or other genes whose expression is modulated during cellular senescence. Indeed, the oxidative stress hypothesis of aging (34), extends the free radical theory of aging by proposing that the level of oxidative stress [dependent upon the rate of oxidant generation and antioxidant defences (35)] exerts an effect on gene expression. If H$_2$O$_2$ is an important mediator of cellular senescence, as suggested by this study, then therapeutic strategies that prevent the production of H$_2$O$_2$ and/or scavenge H$_2$O$_2$ may prove useful.

**MATERIALS AND METHODS**

**Cell culture**

All cells were cultured in DMEM plus 10% fetal calf serum at 37°C in 5% humidified CO$_2$. HF5 and CCD45 are human control fibroblast cell lines and D532 and D539 are primary fibroblast cell lines established from individuals with Down syndrome. CD45, D532 and D539 were obtained from ATCC, CCD45 and D532 were age-matched (2–3 months old) and HF5 and D539...
Figure 12. Morphological changes in cells after treatment with H₂O₂. (a) Photomicrographs showing the effects of (i) 25 µM H₂O₂ on the morphology of NIH3T3 cells after 15 h (magnification ×250). (A), untreated NIH3T3 cells; and (B), H₂O₂-treated cells; (ii) 200 µM H₂O₂ on CCD45 cells, (A), untreated cells; and (B), H₂O₂-treated cells. In both cases, H₂O₂ treated cells are larger, have bigger nuclei and a different shape from control cells; (b) stereological analysis of (i) NIH3T3 and (ii) CCD45 cells treated with H₂O₂. Histograms indicate the mean ± S.E.M. In both cases treated cells had significantly increased adherent surface area, and cell and nucleus volumes compared with untreated cells. *p <0.05 and **p <0.01 (t-test).

were derived from skin of children. All human cell lines were used at similar passage number. FD5, FC1, 2F6 and 3A7 are murine fibroblasts transfected with the human Sod1 cDNA and overexpress human Sod1 (14).

Growth curves and stereological analysis
Cells were plated at 1 × 10⁴ cells/ml and allowed to attach to the surface of the dish for 24 h prior to commencement of the
and removed from the dish by trypsinization. Cell counts were
3H-thymidine assayed after precipitation with 20% TCA and
3H-thymidine uptake
McCoy (16). Stereological analysis was performed according to
number against time according to the method of Segal and
assessed by trypan blue exclusion. Cell doubling time in h was
determined for stereological analysis. Viability of attached cells were
analysis. At various time points, cells were collected by
trypsinization and counted using a haemocytometer or photo-
graphed for stereological analysis. Viability of attached cells were
assessed by trypan blue exclusion. Cell doubling time in h was
calculated from the slope of a semi-logarithmic plot of cell
number against time according to the method of Segal and
McCoy (16). Stereological analysis was performed according to
Weibel (36) using the point counting method. Briefly, cells were
photographed using phase optics while attached to the dish to
determine adherent surface area. Trypsinized cells were photo-
photographed using phase optics while attached to the dish to
determine the number of points overlying individual cells. In
two and three respectively 100 points per photograph were counted. Three
or four photographs were analysed per cell line and the results are
shown as mean ± S.E.M.

3H-thymidine uptake
Cells were plated at 1 × 10^5 cells/ml, allowed to attach to the
surface of the dish and grow for 24 h. Thereafter cells were pulsed
with 1 mCi/ml 3H-thymidine for 3 h, rinsed twice with medium
and removed from the dish by trypsinization. Cell counts were
performed on an aliquot of cells. An aliquot was sonicated and
3H-thymidine assayed after precipitation with 20% TCA and
filtration onto Whatman filter paper. Radioactivity was detected
after addition of scintillant and analysis on a Beckman β-counter.

Sod1 and Gpx1 assays
Sod1 and Gpx1 enzyme assays were carried out according to de
Haan et al. (37). Briefly, cells were lysed in 65 mM phosphate buffer
containing 1% Triton-X 100 and centrifuged at 105 000 g for 1 h at
4°C. The supernatant was used to measure both Sod1 and Gpx1
activity. Total Sod activity was measured after the addition of 250 µl
supernatant to 25 µl of xanthine (1.142 mg/ml), 25 µl of
hydroxylammonium chloride, 125 µl of H_2O_2, and 75 µl of xanthine
oxidase (0.1 IU/ml). The mixture was incubated at 25°C for 20 min.
Thereafter, 0.5 ml of sulphonic acid (3.3 mg/ml) and 0.5 ml of
α-naphthylamine (1 ng/ml) were added and further incubated at
room temperature for 20 min. The absorbance was measured at 530
nm. The addition of 125 µl of KCN (4 mM) in place of water
specifically inhibits Sod1 activity. Thus subtraction of the Sod
activity remaining after KCN treatment (ie. Sod2) from the total Sod
activity gives the Sod1 activity of the sample.

Gpx1 activity was assayed after addition of 100 µl of supernatant
to 800 µl of assay solution (62.5 mM potassium phosphate buffer,
ph 7.0; 1 mM EDTA; 1 mM NaN_3; 0.2 mM NADPH; 1 IU
glutathione reductase, and 0.31 mM reduced glutathione; the assay
solution is pre-incubated at room temperature for 5 min) and 100 µl
of 25 mM H_2O_2. The change in absorbance was measured at 340
nm for 5 min (29).

Determination of intracellular and secreted H_2O_2 levels
Intracellular H_2O_2 levels were determined according to Nasr-
Esfahani et al. (38). Briefly, 1 × 10^-5 M (final concentration)
2’,7’-dichlorodihydrofluorescein diacetate (DCHFDA) was
added to the culture medium of adherent cells for 15 min at 37°C.
Thereafter cells were rinsed twice with medium and the
fluorescence of individual cells measured using a quantitative
fluorescence microscope (Leitz Wetzlar fluovert F5; Mag. ×400)
over a 10 min period. The measuring diaphragm was set to the
size of an average cell and a 6.25% transmission neutral density
filter (Leitz N16) was placed in the path of the excitation light to
minimize photo-oxidation of DCHFDA and cell damage. H_2O_2
secreted into serum and dye-free medium was assayed using a
scopoletin/horseradish peroxidase method (39).

Preparation of the Cip1 probe
The Cip1 cDNA probe was obtained by reverse transcrip-
tase polymerase chain reaction (RT-PCR) from 1-day neonatal murine
lung using the following primers: 5’-AGC CTG ACA GGT AAG
-3’ and 5’-TCA GCC ACA GGC ACC
-3’. Primers were designed accord-
in Northern blot analysis
Total RNA was extracted from cells according to the method of
Chomczynski and Sacchi (40). Twenty µg total RNA was
denatured in formamide/formaldehyde/ MOPS buffer at 55°C for
15 min. Northern blots were prepared by electrophoresis through
17.5% formaldehyde/1.5% agarose gels for 15 h. Thereafter,
RNA was transferred to Hybond-N nylon membranes (Amer-
sham) and bound to filters by UV crosslinking. Labeling of
probes, hybridization conditions and washing stringencies were
as described by de Haan et al. (9)
REFERENCES