Proapoptotic and redox state-related signaling of reactive oxygen species generated by transformed fibroblasts

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Oncogenic transformed fibroblasts are characterized by extracellular superoxide anion generation through a membrane-associated NADPH oxidase. After cellular glutathione depletion, extracellular reactive oxygen species (ROS) generated by transformed fibroblasts exhibit a strong apoptosis-inducing potential. As apoptosis induction under glutathione depletion is inhibited by catalase, the NADPH oxidase inhibitor apocynin, superoxide dismutase, the hydroxyl radical scavenger terephthalate and the iron chelator deferoxamine, the metal-catalysed Haber-Weiss reaction seems to be the responsible signaling mechanism. In contrast to extracellular ROS, intracellular ROS play no role for apoptosis induction in glutathione-depleted transformed fibroblasts initially, since a high level of intracellular catalase scavenges intracellular hydrogen peroxide. Intracellular catalase seems to be induced by extracellular hydrogen peroxide, as pretreatment of transformed fibroblasts with exogenous catalase downmodulates endogenous catalase and renders glutathione-depleted transformed cells susceptible for the effect of endogenous hydrogen peroxide. In contrast to transformed fibroblasts, nontransformed glutathione-depleted fibroblasts do not generate substantial extracellular ROS, but apoptosis is efficiently induced in these cells by intracellular ROS. Our data show that extracellular ROS of transformed fibroblasts exhibit redox-related signaling and at the same time represent a potential apoptosis-inducing hazard through the metal-catalysed Haber-Weiss reaction.

Keywords: transformed; reactive oxygen species; apoptosis; catalase

Introduction

The balance between cellular reactive oxygen species (ROS) generation and the cellular antioxidant tone prevents ROS-dependent damage and induction of apoptosis (for review see Bauer *et al*., 2000). Whereas transformed and nontransformed fibroblasts are both exposed to intracellular ROS derived from mitochondria (Fridovich, 1995; Boveris and Chance, 1973; Cadenas and Davies, 2000), only transformed cells are characterized by substantial extracellular ROS generation (Irani *et al*., 1997; Bauer, 2000).

Mitochondria are considered to be the major source for intracellular ROS (Fridovich, 1995; Boveris and Chance, 1973; Cadenas and Davies, 2000). About 4% of all electrons channelled into the respiratory chain seem to lead to superoxide anion generation (Fridovich, 1995). Superoxide anions can dismutate to hydrogen peroxide spontaneously or catalysed by mitochondrial superoxide dismutase. Whereas superoxide anions are confined to the site of their generation (i.e. the inner mitochondrial membrane), hydrogen peroxide can pass membranes readily and reach the cytosol (Antunes and Cadenas, 2000). Hydrogen peroxide causes lipid peroxidation through hydroxyl radical generation via the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow OH + OH^- + Fe^{3+}$) (Girotti, 1998; Mylonas and Kouretas, 1999). If the catalyst is recycled through superoxide anions ($O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+}$), the whole sequence is usually termed metal-catalysed Haber-Weiss reaction (for review see Kehrer, 2000). Though conventionally used, this term is conceptionally and historically unprecise, as outlined by Koppenol, 2001.

Hydrogen peroxide is scavenged by catalase, glutathione peroxidase/glutathione and by glutathione directly. The central role of glutathione for the antioxidant defense of the cell is overt as glutathione depletion causes rapid induction of apoptosis (Zucker *et al*., 1997). This reaction is blocked by antioxidants, pointing to the determining role of ROS in this process.

Extracellular ROS generation seems to represent a hallmark of transformed fibroblasts (Irani *et al*., 1997; for review see Bauer, 2000). Transformation by ras causes activation of rac, an essential regulatory constituent of a membrane-associated NADPH oxidase. As a consequence, ras-transformed cells generate extracellular superoxide anions. There is also evidence for extracellular superoxide anion production by src-transformed fibroblasts (Engelmann *et al*., 2000; Herdener *et al*., 2000) and by NRK 536 cells in which the transformed state has been reversibly induced by EGF and TGF-beta (Häufel and Bauer, 2001). More-

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over, membrane associated NAD(P)H oxidases seem to represent a rather general feature of transformed and tumor cells (Irani *et al*., 1997; Morre *et al*., 1995; Morre and Reust, 1997; Bittinger *et al*., 1998).

Extracellular superoxide anions generated by transformed fibroblasts are crucial for their proliferation and the maintenance of their transformed state (Irani *et al*., 1997; Suh *et al*., 1999). As the second side of the coin, transformed cell-derived extracellular ROS drive the efficiency and selectivity of natural control systems like TGF-beta-triggered fibroblasts, granulocytes, macrophages and certain B cell lines (Herdener *et al*., 2000; Paul and Bauer, 2001; Heigold and Bauer, 2002; Schimmel and Bauer, 2001; for review see Bauer, 2000). These effector cell systems induce apoptosis selectively in transformed cells, based on a fine interplay between effector cell derived peroxidase and NO on one and target cell-derived ROS on the other side.

Extracellular superoxide anions can spontaneously dismutate and form hydrogen peroxide (Bielski and Allen, 1977). Therefore, the extracellular space around transformed cells contains both superoxide anions and hydrogen peroxide. In the presence of iron ions, this combination of ROS should allow the metal-catalysed Haber-Weiss reaction, leading to the formation of hydroxyl radicals.

The aim of this study was to delineate whether extracellular ROS of transformed cells exhibit pro-apoptotic functions after glutathione depletion and whether they are involved in redox-related signaling.

Results

Glutathione was depleted in nontransformed 208 F rat fibroblasts and src- transformed 208 F src3 cells through buthionine sulfoximine (BSO) treatment. As reported earlier, BSO treatment caused depletion of reduced glutathione within 12 h below the level of detection (Zucker *et al*., 1997) and mediated induction of apoptosis in both cell lines (Figure 1). Apoptosis induction through BSO-mediated glutathione depletion was inhibited by 2 mM of the cell-permeable anti-oxidant N-acetylcysteine throughout the experiment (Figure 1A), indicating that apoptosis induction was mediated by ROS. Apoptosis induction in glutathione-depleted transformed fibroblasts was inhibited by the NADPH oxidase inhibitor apocynin during the first 22 h after BSO addition. At later time points, apocynin was no longer effective. These findings indicate the role of NADPH oxidase activity for the initial phase of apoptosis induction in transformed cells. Apocynin had no effect on nontransformed cells. Cell death induced by glutathione depletion was characterized as apoptosis through a positive TUNEL reaction (Zucker *et al*., 1997). In addition, cell death mediated by glutathione depletion was associated with a loss of mitochondrial membrane potential (data not shown). In line with this observation, bcl-2 overexpressing cells were partially protected from BSO-mediated apoptosis induction (Table 1).

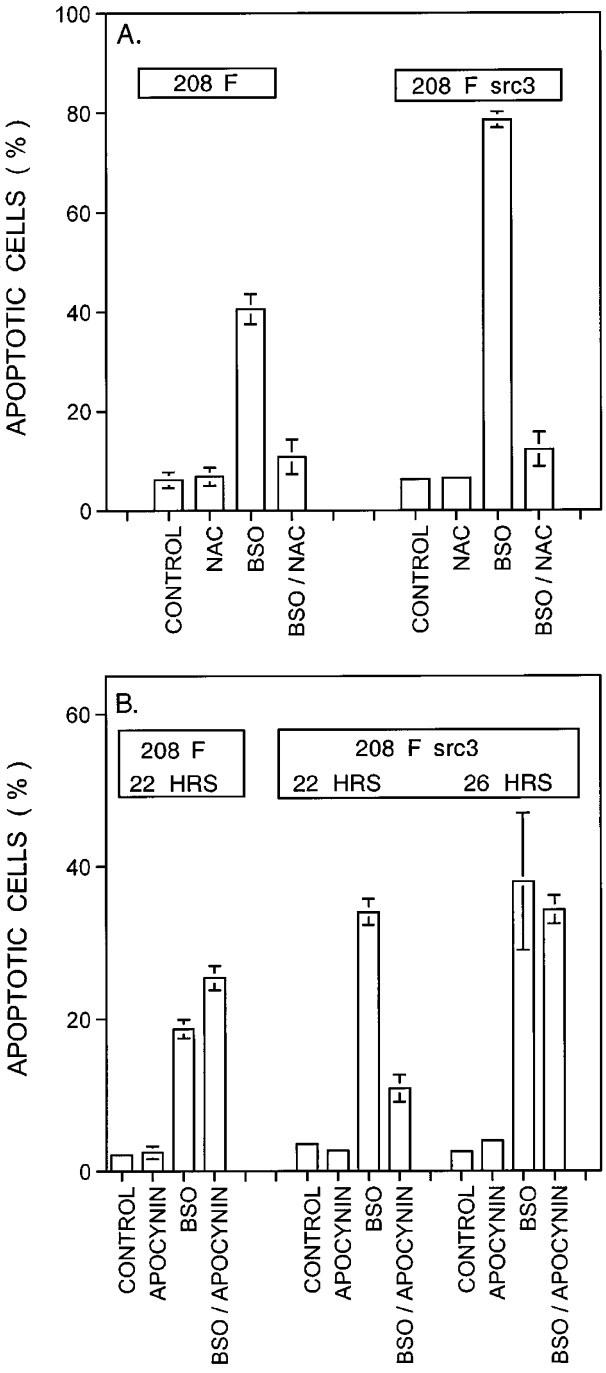

Figure 1 Glutathione depletion causes ROS-mediated apoptosis in nontransformed and transformed fibroblasts. Nontransformed 208 F and transformed 208 F src 3 rat fibroblasts were seeded in Costar 24 well tissue culture clusters (15 000 cells per well). The cells were treated without ('Control') or with 100 μM BSO ('BSO'). In addition, as indicated, assays received 2 mM of the cell-permeable antioxidant N-acetylcysteine (NAC) (**A**) or 50 μg/ml of the NADPH oxidase inhibitor apocynin (**B**). The percentage of apoptotic cells (characterized by membrane blebbing and/or nuclear condensation/fragmentation) was determined kinetically, using inverted phase contrast microscopy. The values determined at 40 h (**A**) or at 22 h and 26 h (**B**) after BSO addition are shown in the graph. All assays were performed in duplicate

The next experiments were performed to clarify whether extracellular ROS generated by transformed

cells play a role during apoptosis induction after glutathione depletion. The effect of extracellular catalase on apoptosis induction was initially tested. Since catalase cannot penetrate cellular membranes, its action is restricted to the detoxification of extracellular hydrogen peroxide. Nontransformed as well as transformed cells were treated with BSO in the presence or absence of extracellular catalase. As can be seen in Figure 2a, BSO-mediated apoptosis was not inhibited by catalase in nontransformed cells, indicating that exogenous hydrogen peroxide was not responsible for apoptosis induction in these cells. In glutathione-depleted transformed cells, however, apoptosis induction was completely blocked by catalase during the first 20 h after onset of apoptosis (Figure 2b). After this delay, apoptosis preceded with an uninhibited kinetics. This finding shows that extracellular hydrogen peroxide is the sole cause of apoptosis induction in transformed cells during the initial phase of apoptosis induction, but that it does not play a measurable role after 20 h. A second addition of catalase 19 h after the beginning of the experiment did not extend the time of inhibition. Similar to catalase, extracellular glutathione, which cannot penetrate membranes blocked apoptosis initially, whereas the cell-permeable antioxidant N-acetylcysteine blocked apoptosis throughout the experiment (Figure 2c).

To test for the generality of the phenomena described, 208F fibroblast harbouring an inducible ras oncogene (Schwieger *et al*., 2001) were either treated with the inducer IPTG for 2 days to induce ras expression and establishment of the transformed state, or they remained uninduced. Then glutathione was depleted by BSO application and the effects of N-acetylcysteine or catalase were determined. As can be seen in Figure 3, BSO induced apoptosis both in cells expressing or not expressing the ras oncogene. Apoptosis induction was ROS-dependent both in uninduced and in ras-expressing cells, as it was completely inhibited by N-acetylcysteine. Apoptosis-relevant ROS in uninduced cells were completely endogenous, as exogenous catalase had no inhibitory effect. In contrast, BSO-mediated apoptosis in ras-expressing cells was inhibited by catalase. These data point to the role of exogenous hydrogen peroxide for apoptosis induction in glutathione-depleted ras-expressing cells.

Table 1 Bcl-2 expression protects against apoptosis induction after glutathione depletion

Cell line	*BSO*	*Apoptotic cells (%)*
208 F src neo	−	5±2.7
208 F src neo	+	46.7±7.4
208 F src bcl-2	−	4.3±0.7
208 F src bcl-2	+	13.2±0.6

15 000 transformed 208 F src 3 cells, either carrying a control plasmid (208 F src 3 neo) or overexpressing bcl-2 (208 F src 3 bcl-2) where treated with 50 μM BSO or remained untreated. The percentage of apoptotic cells was determined after 48 h. Assays were performed in duplicate

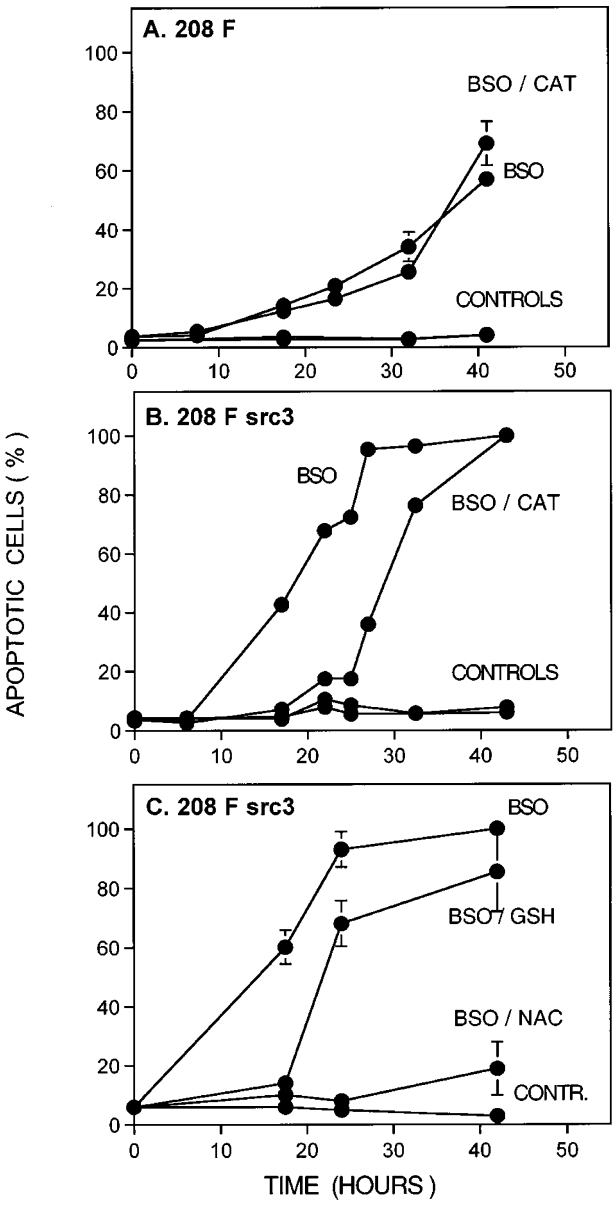

Figure 2 Apoptosis induction in glutathione depleted src-transformed cells is initially inhibited by extracellular catalase and extracellular glutathione. Nontransformed 208 F and transformed 208 F src3 rat fibroblasts were seeded in Costar 24 well tissue culture clusters (15 000 cells per well). The cells were treated with 100 μM BSO, in the absence or presence of 77 U/ml catalase (**A**,**B**), 2 mM glutathione (not cell-permeable) (**C**) or 2 mM N-acetylcysteine (cell-permeable) (**C**). Control assays received no BSO. The percentage of apoptotic cells (characterized by membrane blebbing and/or nuclear condensation/fragmentation) was determined kinetically using inverted phase contrast microscopy. All assays were performed in duplicate. Controls: untreated cells and cells treated with catalase

The known interdependencies of ROS (for review see Bauer, 2000; Bauer *et al*., 2000) allowed to speculate that alternatively to direct apoptosis induction by hydrogen peroxide, several other hydrogen peroxide-dependent signaling pathways were conceivable. These were (1) the iron-catalysed Haber-Weiss reaction; (2)

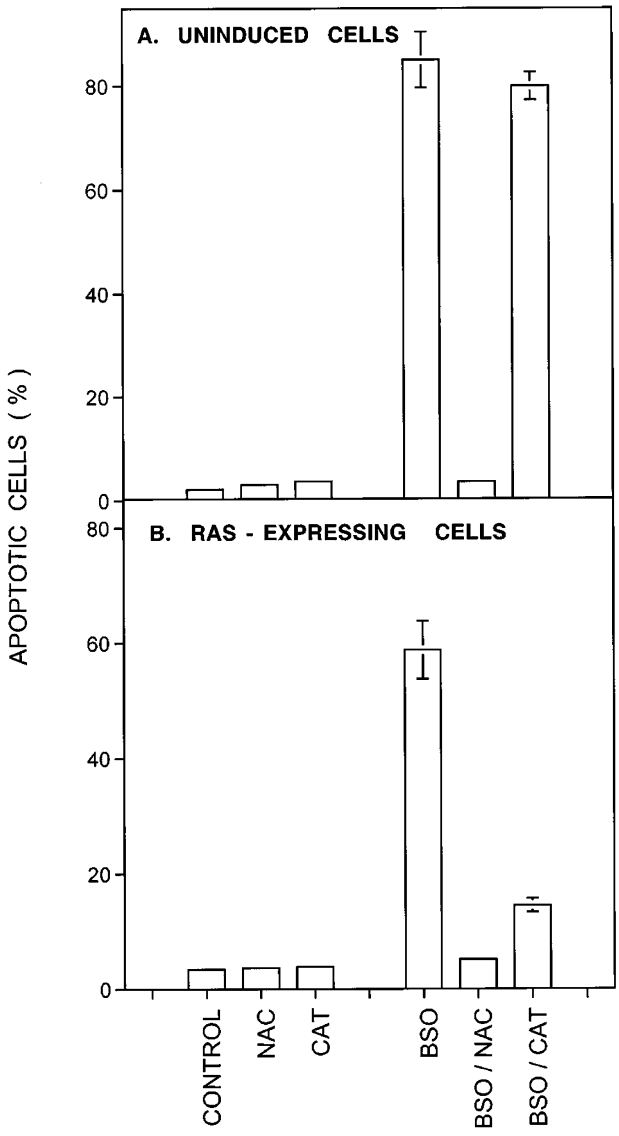

Figure 3 Apoptosis induction in glutathione-depleted ras-transformed cells depends on extracellular hydrogen peroxide. IR-1 fibroblasts (that carry an inducible ras oncogene) were kept in the absence or presence of 20 mM IPTG (inducer of ras oncogene expression) for 3 days. They were then seeded in Costar 24 well tissue culture clusters (15 000 cells per well). IPTG treatment was continued in the induced cell population. Assays received 100 μM BSO or not. In addition, assays received 115 U/ml catalase (CAT) or 2 mM N-acetylcysteine (NAC). The percentage of apoptotic cells (characterized by membrane blebbing and/or nuclear condensation/fragmentation) was determined kinetically, using inverted phase contrast microscopy. The values determined at 30 h are shown in the graph. All assays were performed in duplicate

conversion of hydrogen peroxide to hypochlorous acid (through peroxidase), followed by the reaction between hypochlorous acid with superoxide anions leading to the formation of hydroxyl radicals (Rosen and Klebanoff, 1979; Candeias *et al*., 1993; Herdener *et al*., 2000), and (3) interaction of hydrogen peroxide with nitric oxide, leading to the formation of hydroxyl radicals (Nappi and Vass, 1998). As can be seen in Figure 4, apoptosis induction in glutathione-depleted src-transformed was inhibited by the hydrogen peroxide scavenger catalase, the superoxide anion scavenger SOD, the hydroxyl radical scavenger terephthalate (Saran and Summer, 1999) and the iron chelator deferoxamine, whereas addition of $FeCl_2$ augmented apoptosis induction. The inhibitor of NO synthesis L-NAME, the HOCl scavenger taurine and 4-amino benzoyl hydrazide (ABH), a mechanism-based inhibitor of peroxidase had no effect on BSO-mediated apoptosis in transformed cells. These results indicate that apoptosis induction in BSO-treated transformed cells involved hydrogen peroxide, superoxide anions, ferrous ions and hydroxyl radicals and point to the metal-catalysed Haber-Weiss reaction as responsible mechanism.

After glutathione depletion, endogenous ROS seemed to be the sole apoptosis inducers in nontransformed cells, whereas apoptosis induction in transformed cells was initially completely dependent on exogenous ROS and was then mediated by endogenous ROS. Why did endogenous ROS not contribute to the initial phase of apoptosis induction in transformed cells? One conceivable explanation for this effect was to assume a high intracellular concentration of catalase present in transformed cells. If this assumption was true, inhibition of intracellular catalase in glutathione-depleted transformed cells should render the cells sensitive to apoptosis induction, even if their extracellular hydrogen peroxide was scavenged by cell-impermeable glutathione. In the presence of an catalase inhibitor, intracellular hydrogen peroxide should then drive apoptosis induction. As can be seen in Figure 5, BSO-treated transformed cells showed marked apoptosis induction that was blocked by extracellular glutathione, indicating that until this time point only extracellular ROS had contributed to apoptosis induction and intracellular hydrogen peroxide must have been scavenged. When, however, 3-aminotriazole (a cell permeable catalase inhibitor) was added, BSO-treated transformed cells showed strong apoptosis induction even though their extracellular ROS was scavenged by extracellular glutathione. This finding indicates that during the initial phase after BSO treatment, intracellular hydrogen peroxide in transformed cells is completely scavenged by catalase.

As hydrogen peroxide has been shown to induce catalase expression in several other systems (Lai *et al*., 1996; Roehrdanz and Kahl, 1998; Barnes *et al*., 1999), it seemed likely that the high catalase level in transformed cells was regulated by extracellular hydrogen peroxide generated by these cells. If this working hypothesis was correct, pretreatment of transformed cells with catalase (in order to remove extracellular hydrogen peroxide) should possibly cause downmodulation of endogenous catalase and therefore render the transformed cells sensitive for apoptosis induction by endogenous hydrogen peroxide after glutathione depletion. To address this question, transformed cells were pretreated with catalase for one day or remained untreated. Then the intracellular

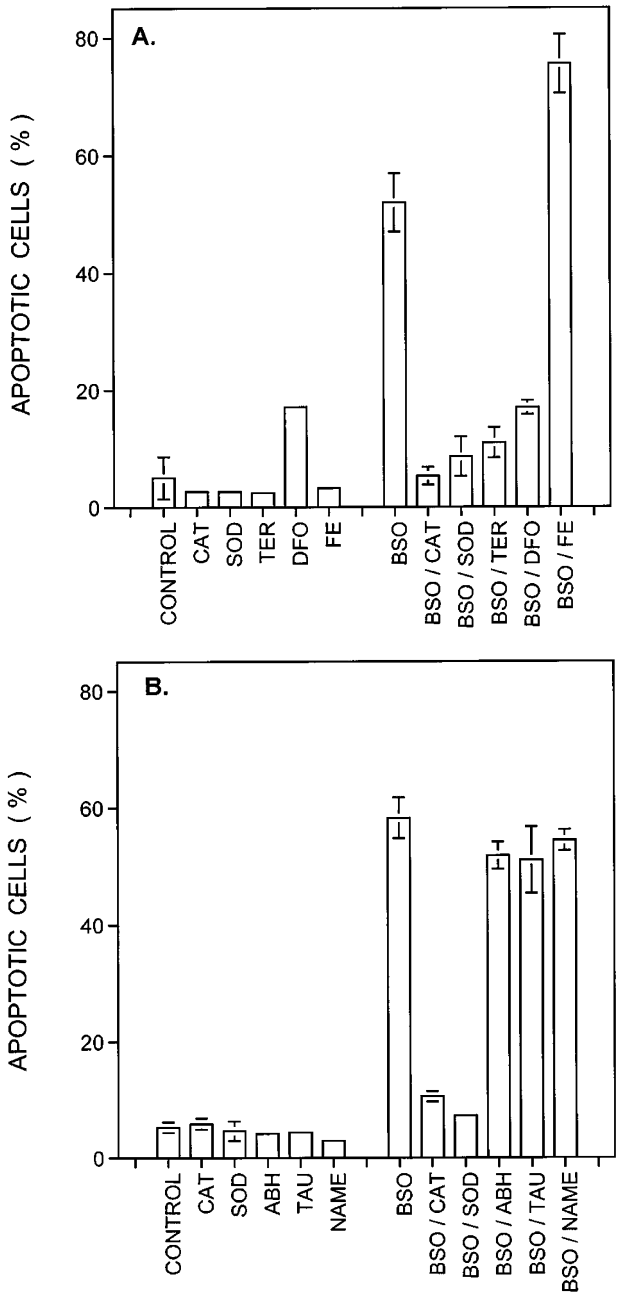

Figure 4 Apoptosis induction in glutathione-depleted transformed fibroblasts: evidence for the metal-catalysed Haber-Weiss reaction. 15 000 src-oncogene transformed 208 F src 3 cells were seeded per well in Costar 24 well tissue culture clusters. Assays received 100 μM BSO (or no addition). Where indicated, assays received the following additions: (**A**) 115 U/ml of the hydrogen peroxide scavenger catalase (CAT), 150 U/ml of the superoxide anion scavenger SOD, 400 μM of the hydroxyl radical scavenger terephthalate (TER), 50 μM of the iron chelator deferoxamine (DFO) or 20 μM $FeCl_2$. (**B**) 115 U/ml of the hydrogen peroxide scavenger catalase (CAT), 150 U/ml of the superoxide anion scavenger SOD, 100 μM of the mechanism-based peroxidase inhibitor ABH, 80 mM of the HOCl scavenger taurine (TAU), or 1.2 mM of the NO synthase inhibitor NAME. The percentage of apoptotic cells (characterized by membrane blebbing and/or nuclear condensation/fragmentation) was determined after 17 h (**A**) or 20 h (**B**), using inverted phase contrast microscopy. All assays were performed in duplicate

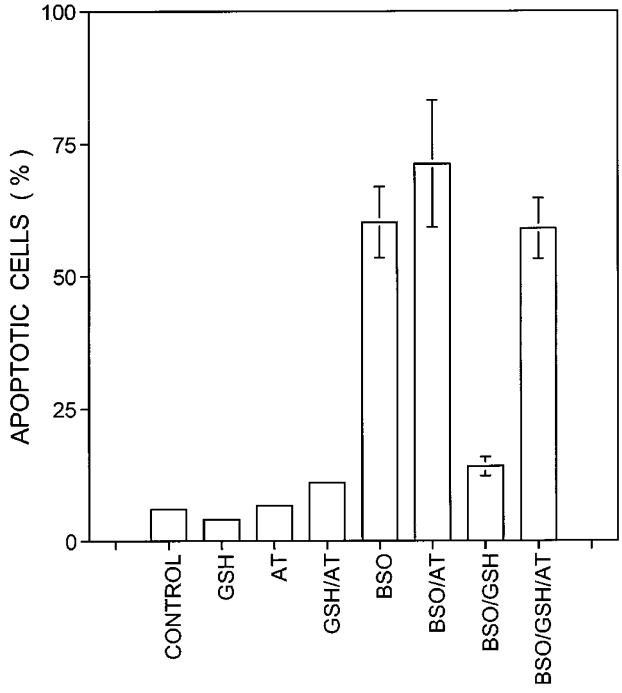

Figure 5 Intracellular catalase protects transformed cells from intracellular hydrogen peroxide. 15 000 transformed 208 F src3 cells were seeded in Costar 24 well tissue culture clusters (15 000 cells per well). Controls remained without BSO, which was added at a concentration of 50 μM. Extracellular ROS was scavenged by 2 mM glutathione (not cell permeable). Where indicated, assays received 25 mM of the catalase inhibitor 3-aminotriazole. The percentage of apoptotic cells (characterized by membrane blebbing and/or nuclear condensation/fragmentation) was determined after 17 h using inverted phase contrast microscopy. All assays were performed in duplicate

glutathione level was depleted through BSO treatment and the kinetics of apoptosis induction in the presence or absence of exogenous catalase was determined. As can be seen in Figure 6, transformed cells that had been pretreated with catalase showed BSO-mediated apoptosis induction that was no longer blocked by exogenous catalase, in contrast to cells that had not been pretreated with catalase. These findings indicate that scavenging of extracellular hydrogen peroxide during the pretreatment period had downmodulated endogenous catalase and therefore had sensitized the cells for the effects of their intracellular hydrogen peroxide, similar to the situation found in nontransformed cells.

A direct measurement of hydrogen peroxide scavenging activity in transformed cells, nontransformed cells and transformed cells pretreated with catalase (Figure 7) showed that transformed cells had indeed a fourfold higher catalase activity than nontransformed cells or catalase pretreated transformed cells.

Discussion

Our data confirm that glutathione depletion in fibroblasts causes induction of apoptosis since the cells

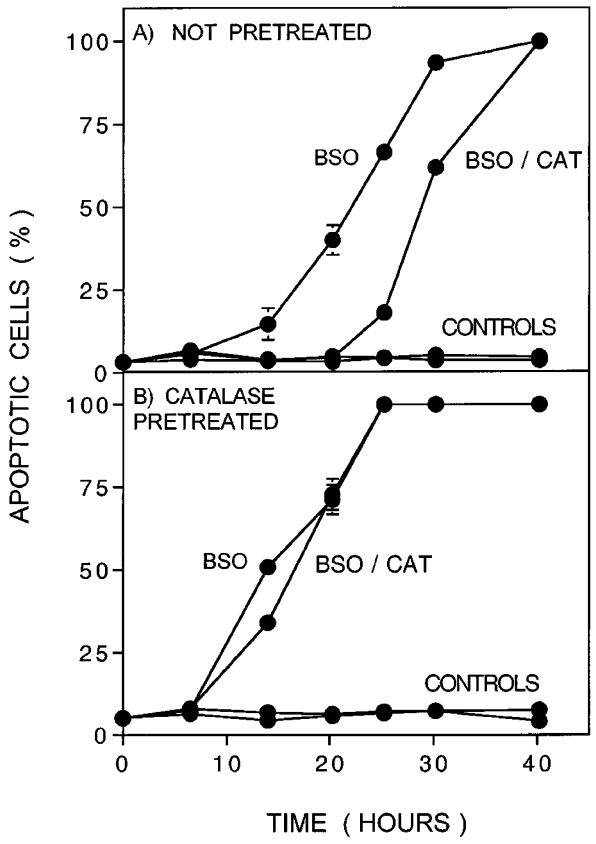

Figure 6 Modulation of the intracellular antioxidant tone by exogenous hydrogen peroxide. 15 000 transformed 208 F src3 cells were seeded in Costar 24 well tissue culture clusters. Cells were either not pretreated (**A**), or pretreated with 77 U/ml catalase (**B**) for 1 day. All assays were washed and then received BSO (100 μM) or not, in the absence or presence of 77 U/ml catalase. The percentage of apoptotic cells (characterized by membrane blebbing and/or nuclear condensation/fragmentation) was determined kinetically using inverted phase contrast microscopy. All assays were performed in duplicate. Time point 0 h represents the time of addition of BSO. Controls: untreated cells and cells treated with catalase in the absence of BSO

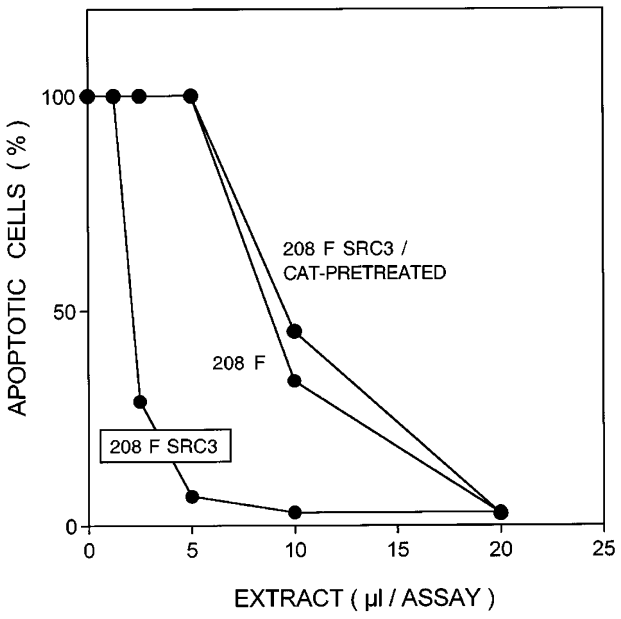

Figure 7 Direct measurement of catalase activity in transformed and nontransformed cells. 600 000 nontransformed 208 F cells, 208 F src 3 cells or 208 F src 3 cells in the presence of 77 U/ml catalase were cultured for 1 day. Cells were trypsinized, centrifuged, washed with PBS, centrifuged and resuspended in 200 μl PBS. Cells were sonicated and the extract was centrifuged to remove cell debris. Indicated volumes of the supernatant were added to assays containing 15 000 transformed 208 F src 3 cells in the presence of 4 mU/ml glucose oxidase. Glucose oxidase generates hydrogen peroxide and thus mediates apoptosis induction. The percentage of apoptotic cells was determined after 8 h. Addition of 25 mM of the catalase inhibitor 3-aminotriazole to parallel assays containing 20 μl of each of the three extracts and 4 mU/ml glucose oxidase caused a complete abrogation of apoptosis inhibition, indicating that the inhibitory principle in the extracts was indeed catalase

showed membrane blebbing, chromatin condensation and fragmentation and DNA strand breaks detectable by the TUNEL reaction. As mitochondrial depolarization preceded morphological signs of apoptosis, and as bcl-2 (a regulator of the mitochondrial permeability transition pore (Kroemer *et al*., 1997; Hirsch *et al*., 1997)) inhibited apoptosis, the functional role of mitochondria during apoptosis mediated by glutathione depletion is shown. Apoptosis induction after glutathione depletion was not attenuated through expression of the src oncogene, whereas apoptosis induction by serum withdrawal has been reported to be inhibited by either bcl-2 overexpression or src oncogene expression (Johnson *et al*., 2000). This finding may be helpful to further dissect the underlying intracellular signaling pathways.

In line with our initial report (Zucker *et al*., 1997), apoptosis induction after glutathione depletion was shown to be mediated by reactive oxygen species, since it was completely inhibited by the cell-permeable antioxidant N-acetylcysteine. However, the differentiation between the effects of intracellular and extracellular ROS involved in apoptosis induction demonstrated a remarkable difference between transformed and nontransformed fibroblasts. In glutathione-depleted transformed cells, apoptosis induction was initially completely dependent on extracellular ROS, as it was inhibited by extracellular catalase, SOD and glutathione, and as it was augmented by ferrous ions. The effects of these cell-impermeable substances indicate the action of extracellular ROS. Membrane associated NADPH oxidase seems to be the responsible superoxide anion-generating enzyme in transformed cells, as apoptosis induction was blocked by apocynin, a specific inhibitor of NADPH oxidase (T'Hart, 1990; Stolk *et al*., 1994). In addition, extracellular ROS generation has been verified for the transformed cell lines used in this study, as they foster HOCl synthesis by extracellular myeloperoxidase (Engelmann *et al*., 2000) and allow extracellular peroxynitrite generation in the presence of NO donors (Heigold *et al*., 2002). An alternative mechanism for extracellular ROS generation through the interaction of gamma-glutamyltransferase with glutathione (Enoiu *et al*., 2000) does

not seem to be effective in our system, as apoptosis induction was inhibited by extracellular glutathione. Intracellular ROS action seemed to be ineffective during the initial phase of apoptosis induction in glutathione-depleted transformed cells. In contrast, apoptosis induction in glutathione-depleted nontransformed cells was not inhibited by extracellular catalase or SOD and therefore seemed to depend solely on intracellular ROS.

Since apoptosis induction in glutathione-depleted transformed cells was initially inhibited by extracellular catalase, extracellular SOD, terephthalate and deferoxamine, direct apoptosis-inducing effects of hydrogen peroxide or superoxide anions can be excluded. Apoptosis induction rather seems to dependent on an interplay of hydrogen peroxide, superoxide anions, ferrous and ferric ions and hydroxyl radicals. Based on the spectrum of molecular species defined through the action of the inhibitors, their redox potentials and known interdependencies (Bauer, 2000), the metal catalysed Haber-Weiss reaction (Kehrer, 2000) seems to be the cause for apoptosis induction (Figure 8). As superoxide anions show a free diffusion path length in the μm range (Saran and Bors, 1994), this sequence of reactions is confined to the direct vicinity of the cell membrane. Only hydroxyl radicals which are generated in a distance up to 40 nm (the free diffusion path length of hydroxyl radicals) from the cell membrane will eventually cause apoptosis induction. As hydrogen peroxide can pass cell membranes readily, it cannot be excluded that the metal-catalysed Haber-Weiss reaction is also occurring inside the cell. However, inhibition of apoptosis by extracellular catalase demonstrates that hydrogen peroxide is primarily formed outside transformed cells.

These findings are in agreement with several reports on apoptosis induction through hydroxyl radicals generated during the Fenton reaction (Li *et al*., 1997a,b; Rollet-Labelle *et al*., 1998; Oyama *et al*., 1999; Held *et al*., 1996; Aoshima *et al*., 1997; Xu *et al*., 1997; Rauen and De Groot, 1998), or by the metal catalysed Haber-Weiss reaction (Faure *et al*., 1996; Oettl *et al*., 1999; Shi and Dalal, 1992; Das *et al*., 1997). To our knowledge, our report is the first to show that transformed cell derived ROS can induce apoptosis in the cell that initially generated them, thereby utilizing the metal-catalysed Haber-Weiss reaction.

The apoptosis inducing effect of extracellular ROS can only be demonstrated in glutathione-depleted transformed cells. This indicates that the hydroxyl radical or the consequences of its apoptosis signaling are efficiently counteracted by intracellular glutathione-dependent mechanisms. Therefore, lowering the intracellular glutathione level through the apo/fas signaling pathway (Van den Dobbelsteen *et al*., 1996) might also lead to apoptosis induction by their extracellular ROS.

Intracellular hydrogen peroxide plays no apoptosis-inducing role initially in glutathione-depleted transformed cells. During the first 20 h, extracellular ROS is the major cause for apoptosis induction in these cells, since extracellular catalase and extracellular glutathione can completely block apoptosis during this time span. Endogenous hydrogen peroxide has no detectable effect in transformed cells during the initial phase and therefore seems to be effectively scavenged by intracellular catalase. Glutathione peroxidase should have no protective effect against hydrogen peroxide under the conditions of glutathione depletion, as its active cycle depends on reduced glutathione. The protective role of catalase was verified in an experimental approach in which glutathione was depleted in transformed cells and the apoptosis inducing effect of their extracellular ROS was specifically blocked by the addition of extracellular glutathione. In the presence of extracellular glutathione, apoptosis was inhibited despite intracellular glutathione depletion, thus demonstrating that intracellular hydrogen peroxide was efficiently neutralized in these cells. As addition of the catalase inhibitor 3-aminotriazole under these conditions caused marked apoptosis induction, the protective role of intracellular catalase during glutathione depletion was demonstrated. In contrast, nontransformed cells do not seem to have a similar degree of protection by intracellular catalase, as there is marked apoptosis induction in glutathione-depleted nontransformed cells mediated by intracellular ROS. Assuming that nontransformed and transformed fibroblasts contain comparable intracellular concentrations of hydrogen peroxide, our findings allow to predict that transformed fibroblasts should contain more intracellular catalase than nontransformed parental cells. This prediction was validated by direct measurement of catalase through determination of 3-aminotriazole-sensitive hydrogen peroxide scavenging activity in cell extracts. This finding is in line with the results obtained by Deichman *et al*. (1996, 1998) who have also shown an increased concentration of catalase in transformed cells compared to parental cells.

The protection of transformed glutathione-depleted cells by catalase was abrogated after 15–20 h. Abrogation of protection was not due to inactivation of catalase, as renewal of the enzyme had no effect. It rather seemed to be due to the fact that intracellular ROS became effective after this period.

The intracellular level of catalase in transformed cells seems to be regulated by exogenous hydrogen peroxide generated by the cells. Measurement of catalase in the extract of catalase-pretreated transformed cells showed that they had a fourfold lower content of catalase, similar to nontransformed cells. Pretreatment of transformed cells with catalase or N-acetylcysteine prior to BSO treatment rendered the apoptosis induction characteristics completely: Apoptosis induction in catalase-pretreated glutathione-depleted transformed cells was no longer inhibited by exogenous catalase, indicating that under these conditions, endogenous ROS was the major driving force for apoptosis induction. Therefore, pretreatment with catalase must have caused a downmodulation of endogenous catalase, resulting in effective apoptosis induction by intracellular hydrogen peroxide after glutathione depletion. Regulation of intracellular

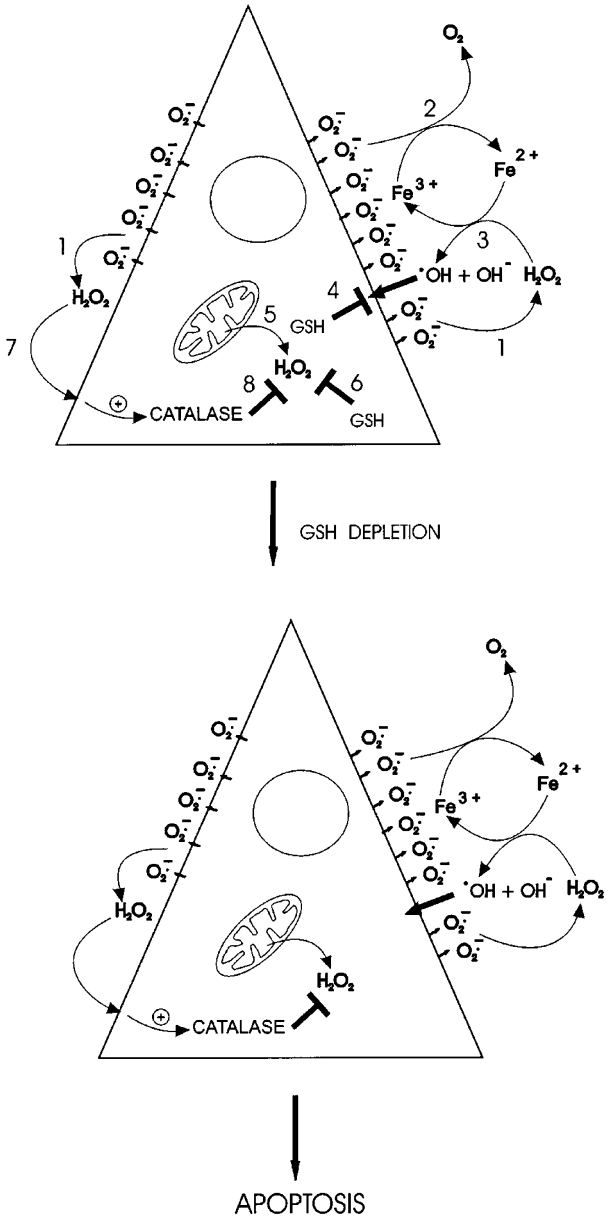

Figure 8 Proapoptotic and redox state-related signaling of reactive oxygen species generated by transformed fibroblasts. Transformed fibroblasts (triangle) generate extracellular superoxide anions through membrane associated NADPH oxidase (Irani *et al*., 1997; Engelmann *et al*., 2000; Herdener *et al*., 2000). Superoxide anions spontaneously dismutate and form hydrogen peroxide (reaction 1). In addition, superoxide anions reduce ferric ions to ferrous ions (reaction 2). Ferrous ions interact with hydrogen peroxide in the classical Fenton reaction, thereby generating hydroxyl radicals (reaction 3). The apoptosis-inducing effect of hydroxyl radicals is counterbalanced by intracellular glutathione (reaction 4). Therefore, apoptosis induction is only seen after intracellular glutathione depletion. Mitochondria generate substantial concentrations of hydrogen peroxide (reaction 5). Intracellular hydrogen peroxide is scavenged by intracellular glutathione (reaction 6). In addition, in transformed cells, extracellular hydrogen peroxide induces a high intracellular concentration of catalase (reaction 7), which also scavenges intracellular hydrogen peroxide (reaction 8). In glutathione-depleted transformed cells, apoptosis induction seems to depend initially only on the extracellular metal-catalysed Haber-Weiss reaction, but not on intracellular hydrogen peroxide, which is most likely been scavenged by intracellular catalase. Nontransformed fibroblasts lack substantial extracellular ROS generation and exhibit lower intracellular catalase concentrations than transformed fibroblasts. Therefore, glutathione depletion in nontransformed cells leads to apoptosis induction mediated solely by intracellular hydrogen peroxide. The evidence for the effect of the extracellular metal-catalysed Haber-Weiss reaction is based on inhibition of apoptosis in glutathione-depleted transformed fibroblasts by scavenging superoxide anions (through SOD), hydrogen peroxide (through catalase), hydroxyl radicals (through terephthalate) or iron ions (through deferoxamine) (Figure 4). The evidence for the effect of intracellular catalase has been derived from experiments in which scavenging of extracellular ROS of glutathione depleted transformed cells by extracellular glutathione blocked apoptosis (Figure 5). If intracellular catalase was blocked by 3-aminotriazole under these conditions, apoptosis induction was restored, pointing to the role of intracellular hydrogen peroxide. Induction of intracellular catalase by extracellular hydrogen peroxide has been demonstrated through pretreatment of transformed cells with catalase (leading to a downmodulation of intracellular catalase) which was determined functionally (Figure 6) and through direct measurement of catalase in cell extracts (Figure 7)

catalase by exogenous hydrogen peroxide is in line with similar findings in other systems (Lai *et al*., 1996; Roehrdanz and Kahl, 1998; Barnes *et al*., 1999).

Our findings diversify the comprehensive view on the role of extracellular ROS generated by transformed cells. Extracellular ROS seem to be beneficial for the transformed cell, but also bear the chance of detrimental effects under certain conditions. The beneficial effects of extracellular ROS for transformed cells comprises induction of proliferation (Irani *et al*., 1997), maintenance of the transformed state (Jürgensmeier *et al*., 1997; Suh *et al*., 1999) and upregulation of intracellular catalase. Extracellular ROS of transformed cells exhibit potential detrimental effects for the cells as they are key elements for intercellular induction of apoptosis (Herdener *et al*., 2000; Paul and Bauer, 2001; Heigold and Bauer, 2002; for review see Bauer, 2000) and for self-destruction mediated by the metal ion catalysed Haber-Weiss reaction after glutathione depletion.

The multiple, partially adverse biological effects of transformed cell derived ROS are intriguing. Their exact knowledge and the possibility to modulate them may bear a chance for experimental and therapeutic manipulation of potential tumor cells.

Materials and methods

Materials

SOD (from bovine erythrocytes) was obtained from Sigma and kept as a stock solutions 30 000 Units/ml PBS at −20°C. Taurine (Sigma) was kept as a stock solution of 500 mM in medium at −20°C. Taurine represents a specific scavenger of HOCl (Aruoma *et al*., 1988).

Catalase from Aspergillus niger (77 U/μl) was obtained from Sigma.

Terephthalate was obtained from Sigma. It was kept as a stock solution of 40 mM in water at −20°C. Terephthalate represents a specific scavenger of hydroxyl radicals (Saran and Summer, 1999). ABH (4-aminobenzoyl hydrazide) (Acros Organics, Geel, Belgium) was dissolved in DMSO at a

concentration of 1 M. It was then diluted with medium to a concentration of 1 mM (stock solution). The stock solution was kept at −20°C. ABH represents a mechanism-based inhibitor of peroxidase (Kettle *et al*., 1997).

N-omega-nitro-L-arginine methylester hydrochloride (L-NAME) was obtained from Sigma. Stock solutions (60 mM) in medium were kept at −20°C. L-NAME inhibits nitric oxide synthetase. Apocynin was obtained from Calbiochem. Stock solutions (2.5 mg/ml) in medium were kept at −20°C. Apocynin (4-Hydroxy-3-methoxyacetophenone) represents a specific inhibitor of NADPH oxidases (T'Hart, 1990; Stolk *et al*., 1994).

Buthionine sulfoximide (BSO) was obtained from Sigma. It was dissolved in medium at a concentration of 10 mM. It was applied at a final concentration of 100 μM. BSO inhibits glutathione synthesis and therefore leads to glutathione depletion within 12 h.

Generation of cell extracts is described in the legend to Figure 7.

Cell lines and cell culture

Cell lines Nontransformed rat fibroblasts 208 F, their src oncogene-transformed derivatives 208 F src3 and 208 F fibroblasts with an inducible H-RAS oncogene (named IR-1) were a generous gift of Drs C Sers and R Schäfer, Charité, Berlin. RAS expression in IR-1 cells was mediated by 20 mM IPTG and caused morphological transformation after two days. 208 F src3 and induced IR-1 cells show criss cross morphology, form colonies in soft agar and are sensitive for intercellular induction of apoptosis by TGF-beta pretreated fibroblasts (Beck *et al*., 1997; Schwieger *et al*., 2001) whereas parental 208 F cells do not exhibit these features of transformed cells.

Bcl-2 overexpressing 208 F src3 cells and 208 F src3 cells, containing a control plasmid without Bcl-2 were obtained through transfection of 208 F src3 cells with either the pRc neo plasmid containing the bcl-2 gene at the polylinker cloning site or with the pRc neo plasmid, using the standard calcium phosphate technique. Plasmid containing cells were selected through cultivation in the presence of 1 mg/ml G 418.

Fibroblasts were kept in Eagle's Minimal Essential Medium, containing 5% fetal calf serum that had been heated for 30 min at 56°C prior to use. Medium was supplemented with penicillin (40 U/ml), streptomycin (50 μg/ml), neomycin (10 μg/ml), moronal (10 U/ml) and glutamine (280 μg/ml). Cell culture was performed in plastic tissue culture flasks. Cells were passaged once or twice weekly.

Determination of apoptosis

Assays were performed in Costar 24 well tissue culture clusters. Typically, 15 000 cells were seeded per assay.

After the indicated time of culture and specific treatment, the assays were checked for apoptotic cells based on the classical morphological criteria for apoptosis using phase contrast microscopy as described before (Jürgensmeier *et al*., 1994; Beck *et al*., 1997; Hipp and Bauer, 1997). Apoptotic cells were either attached or rounded and showed (a) membrane blebbing, or (b) membrane blebbing and nuclear condensation/fragmentation, or (c) nuclear fragmentation/condensation without blebbing (these cells seem to represent later stages of apoptosis where the blebs have been already lost). Care was taken to differentiate apoptotic cells from nonapoptotic rounded cells with intact nuclei, reflecting mitotic stages. To determine the percentage of apoptotic cells at least 200 cells were inspected per assay and the ratio between the number of apoptotic cells and the total number of cells inspected (apoptotic + intact) was calculated. Assays were performed in duplicate.

Control experiments ensured that chromatin condensation/fragmentation was paralled by DNA strand breaks, detectable by the TUNEL reaction, following the method described by Gorczyca *et al*., 1993. Photographs of TUNEL-positive apoptotic fibroblasts after glutathione depletion have been presented in our initial study (Zucker *et al*., 1997).

The involvement of mitochondria during apoptosis was determined through staining with rhodamine 123 (5 μg/ml). Intact cells show specific mitochondrial staining under the fluorescence microscope, whereas cells with depolarized mitochondria (typical for the early stage of ROS-mediated apoptosis) do not exhibit mitochondrial staining.

Determination of the glutathione level

Cells were stained with monochlorobimane as described in Zucker *et al*., 1997. BSO treatment (100 μM) for 12 h caused a loss of intracellular glutathione below the level of detection. Representative photographs are shown in Zucker, 1997.

Extracellular ROS generation

The reactions described in this paper can be attributed to extracellular superoxide anions and their dismutation product hydrogen peroxide, due to the inhibition experiments described in Figure 4. Our data are based on biological activities of ROS and not on their direct biochemical demonstration. Therefore we have no data on the fluxes, steady state or final concentrations of ROS reached.

Statistical analysis

In all experiments, assays were performed in duplicate. The mean values (from duplicate assays within the same experiment) and the empirical standard deviations were calculated and are shown in the figures. Absence of standard deviation bars for certain points indicates that the standard deviation was too small to be reported by the graphic program, i.e. that results obtained in parallel were nearly identical. Empirical standard deviations were calculated merely to demonstrate how close the results were obtained in parallel assays within the same experiment and not with the intention of statistical analysis of variance, which would require larger numbers of parallel assays.

The Yates continuity corrected chi-square test was used for the statistical determination of significances.

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