Abstract. Nitric oxide (NO) has recently been shown to mediate apoptosis induction selectively in transformed fibroblasts, in contrast to their nontransformed parental cells. Here we show that NO-mediated apoptosis induction in transformed fibroblasts can be divided into two major phases. During phase 1, peroxynitrite is generated by the interaction of extracellular superoxide anions with NO and the intracellular glutathione level is subsequently lowered. This defines the beginning of phase 2, in which NO-mediated signaling depends on intracellular superoxide anions exclusively. The resultant peroxynitrite seems to activate the mitochondrial permeability transition pore and thus triggers execution of apoptosis. Experimental depletion of intracellular glutathione causes a drastic decrease in the length of phase 1 in transformed cells and renders nontransformed cells sensitive to NO-mediated apoptosis induction. These findings allow the prediction that either induction of superoxide anion generation or glutathione depletion may render cells sensitive to NO-mediated apoptosis induction.

Nitric oxide (NO) is a free radical with diverse biological functions of central importance (1, for review see ref. 2). It arises from the guanodino group of L-arginine in a NADPH-dependent reaction catalyzed by constitutively expressed or inducible NO synthases (NOS). NO is able to pass cellular membranes (3), to decrease the intracellular glutathione pool (4), to regulate gene expression via interaction with the zinc finger transcription factor SP1 (5) and to up-regulate p53 gene expression (6). The neurotrophic function of NO switches to cell death induction in midbrain cultures after glutathione depletion (7).

Direct apoptosis induction by NO has been reported for thymocytes (8), human leukemia cells (9) and mesangial cells (6). In the study by Sandau and Brune (6), the apoptosis-inducing effect of NO was efficiently antagonized by the simultaneous presence of superoxide anions. Inhibition of mitochondrial respiration by NO, leading to ATP depletion, seems to represent one way of apoptosis induction in certain systems (10).

NO and superoxide anions form peroxynitrite in a diffusion controlled reaction (11-13). Peroxynitrite and its protonated form peroxynitrous acid are highly reactive species, with the potential to oxidize sulphhydryl groups (14), to nitrate tyrosine and tryptophan (15), to perform hydroxylation reactions (16) and to nitrosylate either directly (17) or after interaction with NO, leading to the formation of the nitrosylating molecule dinitrogentrioxide (18). In addition, peroxynitrite shows the potential for lipid peroxidation (19). Whereas NO does not directly interact with glutathione, peroxynitrite is readily scavenged by reduced glutathione (for review see ref. 20).

In many cellular systems, peroxynitrite rather than NO seems to be the responsible apoptosis inducer (21-23). This conclusion is based on several findings: 1) Up-regulation of mitochondrial SOD (i.e. removal of superoxide anions and thus prevention of peroxynitrite formation) caused resistance to NO-mediated apoptosis (24), whereas down-regulation of intracellular SOD augmented NO-mediated apoptosis (25). 2) Peroxynitrite has the potential for lipid peroxidation, a central step in apoptosis induction (19, 26), whereas NO inhibits lipid peroxidation (26,27). 3) Peroxynitrite-induced apoptosis depends on activation of caspase-3 (28), but NO inhibits caspases through nitrosylation (29). 4) Mitochondrial permeability transition represents a central step during execution of apoptosis. It can be efficiently triggered by peroxynitrite, but not by NO or superoxide anions alone (30).

NO represents a central tool used by several natural antitumor defense mechanisms. Effector cells like natural killer cells, monocytes, macrophages, Kupffer cells,
microglia, neutrophils, endothelial cells or TGF-beta-treated fibroblasts release NO that mediates apoptosis induction in transformed cells (for review see ref. 31).

This selective sensitivity of transformed cells seems to be linked to their extracellular superoxide anion production. It is based on membrane-associated NADPH oxidase, a characteristic feature of transformed cells, which is linked to oncogene expression (32, 33).

In a recent study, we directly demonstrated that NO induces apoptosis selectively in transformed fibroblasts (1). Apoptosis induction was blocked when the superoxide anion generating NADPH oxidase was inhibited by apocynin, when superoxide anions were scavenged by SOD or SOD mimetics, or when peroxynitrite was destroyed by the decomposition catalyst FeTPPS. These data allow the conclusion that the interaction of transformed cell-derived superoxide anions with NO leads to peroxynitrite formation. Peroxynitrite then seems to act as the ultimate apoptosis inducer. In line with this conclusion, addition of chemically synthesized peroxynitrite caused apoptosis in transformed as well as nontransformed cells with equal efficiency. Likewise, supplementation of nontransformed cells with a superoxide anion-generating enzyme system (xanthine oxidase) rendered them sensitive to apoptosis induction in the presence of NO. Therefore, extracellular superoxide anions seem to be the key for the selectivity of NO-mediated apoptosis induction, based on the generation of peroxynitrite.

In the present study, the process of NO-mediated apoptosis induction in transformed fibroblasts is separated into two distinct phases. During phase 1, extracellular superoxide anions interact with NO and form peroxynitrite. As a consequence, the intracellular glutathione level is lowered. Subsequently, during phase 2, intracellular superoxide anions and NO cause intracellular peroxynitrite formation and activation of the mitochondrial permeability transition pore. These data allow the prediction that cells can be rendered sensitive to NO-mediated apoptosis if they either generate extracellular superoxide anions or if they reduce their intracellular glutathione concentration.

Materials and Methods

Materials. The NO donor sodium nitroprusside (SNP) was obtained from Sigma (Deisenhofen, Germany). It was kept as a 200 mM stock solution in medium at -20°C. SNP represents a classical NO donor, sometimes present in a two step reaction: SOD(Cu++) + O2- + O2 → SOD(Cu++) + O2. SOD(Cu++) + O2. SOD(Cu++) H+ + H+ + O2 → SOD(Cu++) + H2O2.

SOD (from bovine erythrocytes, Sigma) stock solutions (30,000 Units/ml in PBS) were kept at -20°C and only used once per aliquot. SOD represents an efficient scavenger of superoxide anions, in a two step reaction: SOD(Cu++) + O2. O2. SOD(Cu++) H+ + H+ + O2 → SOD(Cu++) + H2O2.

Peroxynitrite (synthesized from isoamylnitrite and hydrogen peroxide) was obtained as a 200 mM stock solution from Calbiochem (Deisenhofen, Germany). It was kept as a 200 mM stock solution in PBS at -20°C until use.


Determination of mitochondrial depolarisation.

Cells were stained with bisbenzimide in PBS for 30 minutes at room temperature, followed by washing steps with PBS. Cells were then stained with 1 Ìg/ml bisbenzimide in PBS before they were inspected by inverted fluorescence microscopy.

Apoptosis induction.

Cells were stained with bisbenzimide in PBS for 30 minutes at room temperature, followed by washing steps with PBS. Cells were then stained with 1 Ìg/ml bisbenzimide in PBS before they were inspected by inverted fluorescence microscopy.

A positive TUNEL reaction indicates the presence of free 3`hydroxyl groups of DNA, one of the hallmarks of apoptotic cells.

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.

All quantitative data in this paper were derived using this method. In parallel, control assays ensured that apoptotic cells characterized by morphological criteria as described above showed a positive TUNEL reaction, indicative of free 3`hydroxyl groups of the DNA, one of the hallmarks of apoptotic cells.

DNA strand breaks (free 3`hydroxyl groups) were detected by the TUNEL reaction (46, 49, 50), using a commercially available detection kit (Boehringer, Mannheim, Germany). Cells were fixed with 3% paraformaldehyde in PBS for 30 minutes at room temperature, washed with PBS for 30 minutes and then permeabized in 0.1% Triton X-100 / 0.1% sodium citrate in PBS for two minutes on ice. After two washing steps with PBS, the TUNEL assay kit (containing terminal deoxynucleotidyl transferase, fluoresceine-labeled dUTP and suitable buffer components) was added. Incubation at 37ÆC was for 60 minutes, followed by three washing steps with PBS. Cells were then stained with 1 Ìg/ml bisbenzimide in PBS for 30 minutes at room temperature, followed by two washing steps with PBS. The cells were visualized using an inverted fluorescence microscope with suitable filter sets.

Apoptosis induction.

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.

All quantitative data in this paper were derived using this method. In parallel, control assays ensured that apoptotic cells characterized by morphological criteria as described above showed a positive TUNEL reaction, indicative of free 3`hydroxyl groups of the DNA, one of the hallmarks of apoptotic cells.

DNA strand breaks (free 3`hydroxyl groups) were detected by the TUNEL reaction (46, 49, 50), using a commercially available detection kit (Boehringer, Mannheim, Germany). Cells were fixed with 3% paraformaldehyde in PBS for 30 minutes at room temperature, washed with PBS for 30 minutes and then permeabized in 0.1% Triton X-100 / 0.1% sodium citrate in PBS for two minutes on ice. After two washing steps with PBS, the TUNEL assay kit (containing terminal deoxynucleotidyl transferase, fluoresceine-labeled dUTP and suitable buffer components) was added. Incubation at 37ÆC was for 60 minutes, followed by three washing steps with PBS. Cells were then stained with 1 Ìg/ml bisbenzimide in PBS for 30 minutes at room temperature, followed by two washing steps with PBS. The cells were visualized using an inverted fluorescence microscope with suitable filter sets.

Determination of mitochondrial depolarisation.

Cells were stained with 5 µg/ml rhodamine 123 (Sigma) for 30 minutes in medium at 37° C. The medium was removed and the cells were washed two times with PBS before they were inspected by inverted fluorescence microscopy. In control cells, mitochondria were stained bright red, whereas cells with mitochondrial depolarisation showed no staining.

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 key experiments were repeated more than ten times, involving different investigators.

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

Results

The first experiment was performed in order to test for the differential response of transformed and nontransformed fibroblasts to increasing concentrations of the NO donor sodium nitroprusside and to define the role of extracellular superoxide anions in this process. Therefore, the indicated concentrations of SNP were added to either transformed or nontransformed fibroblasts. Parallel assays received SOD or not. Under the conditions of the experiment, SOD scavenges extracellular superoxide anions efficiently, but cannot reach intracellular superoxide anions. Apoptosis induction was measured at the indicated times (Figure 1).

Up to more than 20 hours, there was no detectable apoptosis induction under any of the conditions of the experiment. Thirty-three and 45 hours after addition of SNP, transformed fibroblasts showed apoptosis induction, which was both dependent on the concentration of SNP and on the time. Apoptosis was seen for all concentrations of SNP applied, with a plateau at 0.5 mM. Apoptosis induction in transformed cells was nearly completely blocked by SOD at both time points, demonstrating the role of extracellular superoxide anions for this process. In contrast to transformed cells, nontransformed cells did not show any apoptotic response at 33 or 45 hours after SNP addition. At 55 hours after addition of SNP, 100 percent apoptosis induction was seen in transformed cells for any concentration of SNP applied, whereas nontransformed cells did not yet respond to the NO donor. Transformed cells receiving SNP and SOD showed apoptosis induction up to 30 percent now, indicating that the inhibitory effect of SOD became gradually ineffective at this time point. Sixty-seven hours after the beginning of the experiment, nontransformed cells, which had not shown apoptosis induction at all time points before, were now showing a significant response at SNP concentrations higher than 0.25 mM. This reaction was completely blocked by SOD, pointing to a role of extracellular superoxide anions in this process. In summary, this experiment demonstrates the fast and sensitive response of transformed cells to NO, in contrast to the late response of nontransformed cells which is only seen at high concentrations of NO. NO-mediated apoptosis induction in transformed as well as the weaker and later effect in nontransformed cells seem to depend on extracellular superoxide anion generation.
Figure 1. Differential response of transformed and nontransformed fibroblasts to NO. 15,000 transformed 208 F src3 or nontransformed 208 F cells were seeded into 24-well tissue culture clusters with 1 ml of medium. Six hours later, SNP was added at the indicated concentrations. Addition of SNP was defined as time point zero of the experiment. Parallel assays received SOD (150 U / ml). All assays were performed in duplicate. The percentage of apoptotic cells (characterized by membrane blebbing and / or nuclear condensation / fragmentation) was determined at the indicated times, using inverted phase contrast microscopy.
As shown in Figure 1, the kinetics of NO-mediated apoptosis induction in transformed cells was characterized by a lag-phase of more than 20 hours (termed "phase 1" in this paper), followed by a steep increase in the number of apoptotic cells (termed "phase 2"). To test for the

As indicated, cells were treated without or with 0.25 or 0.025 mM SNP throughout the experiment (marked as 0.25 or 0.025). In other assays, cells were treated with either 0.25 mM or 0.025 mM SNP during phase 1 (i.e. the lag-phase until the onset of apoptosis induction), medium was removed, cells were washed with fresh medium and incubation continued in the presence of the indicated concentration. For example, 0.25/0.025 indicates an assay where 0.25 mM SNP was present in phase 1, followed by 0.025 mM SNP in phase 2. All assays were performed in duplicate. The percentage of apoptotic cells (characterized by membrane blebbing and/or nuclear condensation/fragmentation) was determined kinetically, using inverted phase contrast microscopy.
Figure 4. Role of extracellular and intracellular superoxide anions and of peroxynitrite during NO-mediated apoptosis induction.

6,000 transformed 208 F src3 cells were seeded in 48-well tissue culture clusters with 0.4 ml of medium. After the cells had attached, SNP was added at a concentration of 0.25 mM. Control assays did not receive SNP. As indicated, some of the SNP-containing assays received 100 U/ml of the cell impermeable superoxide anion scavenger SOD, or 40 μM of the cell-permeable SOD mimetics MnTMPyP, MnTBAP, FeTMPyP or 40 μM of the peroxynitrite decomposition catalyst FeTPPS from the beginning of the experiment (upper graph) or 26 hours after addition of SNP (lower graph). Control assays ensured that the inhibitors added to cells alone did not induce apoptosis (data not shown). All assays were performed in duplicate. The percentage of apoptotic cells (characterized by membrane blebbing and/or nuclear condensation/fragmentation) was determined kinetically, using inverted phase contrast microscopy.
dependence on NO during phases 1 and 2, transformed cells were treated with SNP or not. At the beginning of phase 2 and in the middle, the NO donor was removed in some of the assays, the cells were washed with fresh medium and incubation continued in the absence of the NO donor. SNP-treated cells showed a lag-phase (phase 1) of about 40 hours in this experiment. It was followed by a steep increase in apoptotic cells (Figure 2). Removal of the NO donor at either 42 or 48 hours caused a complete stop in the process of apoptosis induction, compared to the control with continuous SNP presence. These data show that NO has to be supplied continuously during phases 1 and 2 in order to achieve maximal apoptosis induction.

Though NO has to be present during both phases in order to achieve maximal apoptosis induction, the requirement for the concentration of NO might be different for phases 1 and 2. To address this question, transformed cells were treated with 0.25 mM SNP or the suboptimal concentration of 0.025 mM SNP throughout the experiment or during phases 1 or 2, in adequate combinations. As shown in Figure 2 B, a strong apoptosis-inducing effect was achieved by the continuous presence of 0.25 mM SNP, whereas 0.025 mM SNP only showed a late and marginal effect. If the cells were treated with 0.25 mM SNP initially and then the NO donor was removed at the onset of phase 2, apoptosis induction was completely prevented. If, however, the cells were treated with 0.25 mM SNP during phase 1, followed by a wash step at the onset of phase 2 and 0.025 mM SNP was then added, apoptosis induction continued with high efficiency. This effect was not obtained when the suboptimal concentration was applied during phase 1, followed by the higher concentration during phase 2. These data demonstrate that phase 2 requires lower concentrations of NO than phase 1 for optimal efficiency and that the higher concentration of NO during phase 1 is absolutely necessary.

The next experiments were performed to elucidate the role of extracellular and intracellular superoxide anions during phases 1 and 2. Therefore, transformed cells were treated with SNP or not. Some of the assays received SOD or the cell permeable SOD mimetic MnTMPyP, either at the beginning of phase 1 or at the beginning of phase 2. The presence of either SOD or MnTMPyP from the beginning of the experiment prevented NO-mediated apoptosis completely, indicating the initial role of extracellular superoxide anions for apoptosis induction (Figure 3). Addition of SOD at the beginning of phase 2 caused no inhibitory effect on NO-mediated apoptosis induction, whereas the addition of the cell permeable SOD mimetic MnTMPyP at the beginning of phase 2 caused complete inhibition. These data show that superoxide anions are required during both phases, but that intracellular superoxide anions are exclusively required during phase 2 of NO-mediated apoptosis induction.

For a further substantiation of this central finding for NO-mediated apoptosis, transformed cells were treated with SNP and several specific scavengers were added either from the beginning of the experiment or at the beginning of phase 2 only. The following scavengers were applied: cell impermeable SOD, the cell permeable SOD mimetics MnTBAP, MnTMPyP and FeTMPyP and the peroxynitrite decomposition catalyst FeTPPS. As shown in Figure 4, all compounds blocked SNP-mediated apoptosis when added from the beginning of the experiment, confirming the role of superoxide anions and peroxynitrite formation for NO-mediated apoptosis induction. When added during phase 2 exclusively, SOD had no inhibitory effect on SNP-mediated apoptosis induction, whereas all the other scavengers had. This finding confirms the role of intracellular superoxide anions and of intracellular peroxynitrite formation during phase 2.

As peroxynitrite can be scavenged by glutathione directly or by the combined action of glutathione peroxidase and glutathione (51), the role of glutathione peroxidase during...
Figure 6. Glutathione depletion enhances NO-mediated apoptosis induction in transformed cells and sensitizes nontransformed cells for NO-mediated apoptosis. Lack of role for extracellular superoxide anions.

15,000 transformed 208 F src 3 or nontransformed 208 F cells were seeded in Costar 24-well tissue culture clusters with 1 ml of medium. Six hours after attachment of the cells, the medium was renewed and BSO was added at a concentration of 12.5 μM. This concentration of BSO causes a gradual, but not complete, depletion of glutathione, as determined by monochlorobimane staining. After 12 additional hours, SNP was added at the indicated concentrations. Some of the assays received 150U/mlSOD in addition. All assays were performed in duplicate. The percentage of apoptotic cells (characterized by membrane blebbing and / or nuclear condensation / fragmentation) was determined at the indicated times, using inverted phase contrast microscopy.
the two phases of NO-mediated apoptosis induction was studied. Transformed cells were treated with SNP or not, in the absence or presence of mercaptosuccinate, an inhibitor of glutathione peroxidase. As shown in Figure 5, inhibition of glutathione peroxidase did not affect the length of the lag-phase of apoptosis induction and therefore does not seem to influence phase 1. It rather caused a steeper increase in the apoptotic response during phase 2. This finding points to a modulatory role of glutathione peroxidase exclusively during step 2 of NO-mediated apoptosis induction. It also allows the speculation that reduced glutathione might be present in abundance at the beginning of phase 1 and that additional peroxynitrite decomposition by glutathione peroxidase is not required for optimal protection initially. The effect of inhibition of glutathione peroxidase on apoptosis induction at the end of phase 1 might indicate that the level of reduced glutathione had been lowered during phase 1. This is indeed the case. SNP-treatment caused a discrete decrease in the intracellular glutathione level in transformed cells, as seen directly after staining with monochlorobimane (data not shown). When cells were treated with 6 µM BSO, the intracellular glutathione level was decreased to approximately half the concentration of untreated control cells, without causing apoptosis induction (data not shown). When SNP was added in addition to 6 µM BSO, the intracellular glutathione level decreased beyond the level of detection during phase 1 and then apoptosis occurred.

If phase 1 of NO-mediated apoptosis induction in transformed cells was characterized as consumption of intracellular glutathione, experimental glutathione depletion should allow immediate onset of phase 2, i.e. the shift from extracellular to intracellular superoxide anion use and a marked sensitization with respect to the NO concentration.

Figure 7. NO-mediated apoptosis induction in glutathione-depleted cells depends on intracellular superoxide anions and peroxynitrite formation. 15,000 transformed 208 F src 3 cells were seeded in Costar 24-well tissue culture clusters with 1 ml of medium. Six hours after attachment of the cells, the medium was renewed and BSO was added at a concentration of 12.5 µM. After 12 additional hours, SNP was added at a concentration of 0.25 mM. As indicated, some of the assays received 100 U/ml SOD (not cell-permeable), or 40 µM of the cell-permeable superoxide dismutase mimetic MnTMPyP or the peroxynitrite decomposition catalyst FeTPPS. All assays were performed in duplicate. The percentage of apoptotic cells (characterized by membrane blebbing and / or nuclear condensation / fragmentation) was determined after five hours, using inverted phase contrast microscopy. Parallel assays without SNP treatment showed SNP-dependent apoptosis after 52 hours, which was completely blocked by SOD or MnTMPyP, demonstrating that the SOD used in this experiment was active (data not shown).

Figure 8. NO-mediated apoptosis induction in transformed fibroblasts utilizes the mitochondrial pathway of apoptosis. 6,000 transformed 208 F src3 cells were seeded in 48-well tissue culture clusters in 0.4 ml of medium. Where indicated, SNP was added at a concentration of 0.25 mM. SNP-containing assays or control assays without SNP received 50 µM bongkrekic acid at 20 hours (onset of phase 2). All assays were performed in duplicate. The percentage of apoptotic cells (characterized by membrane blebbing and / or nuclear condensation / fragmentation) was determined kinetically, using inverted phase contrast microscopy. CON = control without addition.
Figure 9. Modulation of NO-mediated apoptosis induction in transformed fibroblasts through manipulation of their glutathione level or inhibition of NADPH oxidase.

6,000 transformed 208 F src3 cells were seeded in 48-well tissue culture clusters in 0.4 ml of medium. SNP was added at a concentration of 0.25 mM. Assays received the indicated concentrations of BSO (inhibitor of glutathione synthesis) or apocynin (inhibitor of NADPH oxidase) (APO). As indicated, SOD (100 U/ml) was added in parallel assays. The percentage of apoptotic cells (characterized by membrane blebbing and/or nuclear condensation/fragmentation) was determined kinetically, using inverted phase contrast microscopy. BSO treatment with 6 μM caused a 50% reduction of the intracellular glutathione concentration after 48 hours. In the presence of 25 μM BSO, glutathione was below the level of detection after 48 hours. In the presence of 25 μM BSO, glutathione was below the level of detection after 48 hours. Control assays (data not shown) ensured that assays without SNP did not show apoptosis within the time frame of the experiment, except for the assays containing 12.5 μM or 25 μM BSO, which showed apoptosis 25 hours later than the assays containing the respective BSO concentration plus SNP. The late apoptosis induction by the high BSO concentrations in this experiment is explained by the metal-catalyzed Haber Weiss reaction of extracellular hydrogen peroxide and superoxide anions (52). Please note that apoptosis induction by SNP in the presence of low concentrations of BSO or apocynin is blocked by SOD, whereas inhibition by SOD becomes less efficient with increasing concentrations of BSO.
In addition, transformed and nontransformed cells should react in a similar way under these conditions, because they differ in extracellular superoxide anion generation but not in the availability of intracellular superoxide anions. To address this question, transformed and nontransformed cells were treated with buthionine sulfoximine (BSO), an inhibitor of glutathione synthetase, before the NO donor was added. In order to avoid complete glutathione depletion, which allows apoptosis induction by extracellular metal catalyzed Haber Weiss reaction (52), the BSO concentration had been adjusted to achieve only partial inhibition of glutathione synthetase. As determined by monochlorobimane staining in parallel, this resulted in a discrete, but not complete, decrease of the intracellular glutathione concentration. Under these conditions, the kinetics and concentration dependency of NO-mediated apoptosis induction changed substantially and dramatically compared to control cells (Figure 6). Even at 2 hours after addition of SNP, BSO-pretreated transformed cells showed a concentration-dependent increase in apoptotic cells in the range of 0.06 to 0.25 mM SNP, followed by a decrease at higher concentrations of SNP. The response of nontransformed cells was weaker at this time point, but followed a similar dose response. In contrast to the experiment described in Figure 1, SOD had no inhibitory effect on NO-mediated apoptosis induction in cells with partial glutathione depletion. Up to 8 hours after addition of SNP, apoptosis induction increased further, with the optimum at 0.25 mM and without interference by SOD. Apoptosis induction was observed both for transformed and nontransformed cells. Twenty hours after addition of SNP, apoptosis induction was more or less complete for transformed and nontransformed cells at any concentration of SNP applied. In summary, this experiment demonstrates that partial glutathione depletion: i) abrogates the relative selectivity of NO-mediated apoptosis induction for transformed cells; ii) allows an early onset of apoptosis directly after addition of the NO donor; iii) causes an increased sensitivity of apoptosis induction with respect to the concentration of the NO donor; and iv) allows apoptosis induction by NO that does not seem to depend on extracellular superoxide anions.

In order to test for the effects of intracellular superoxide anions after experimental glutathione depletion (in analogy to the situation during phase 2), transformed fibroblasts where pretreated with BSO and then SNP was added in the absence or presence of SOD, the cell-permeable SOD mimetic MnTMPyP or the cell permeable peroxynitrite decomposition catalyst FeTPPS (Figure 7). As expected, BSO-pretreated cells showed a strong response to SNP even five hours after its addition (Figure 7), whereas control cells without BSO treatment showed no response at this time point. SNP-mediated apoptosis induction in BSO-pretreated cells was not significantly affected by SOD, but was completed blocked by MnTMPyP and substantially blocked by FeTPPS. These data show that NO-mediated apoptosis induction in glutathione-depleted cells: i) depends exclusively on intracellular superoxide anions and ii) is mediated by peroxynitrite. Taken together, these results demonstrate that glutathione depletion reflects the major effect during phase 1 of SNP-mediated apoptosis induction.
We have recently shown that NO-mediated apoptosis induction is preceded by mitochondrial depolarisation (1). To test for a functional relevance of mitochondrial depolarisation for NO-mediated apoptosis induction, bongkrekic acid, an inhibitor of mitochondrial permeability transition, was added to SNP-containing assays at the onset of phase 2. As shown in Figure 8, addition of bongkrekic acid at the onset of phase 2 caused a complete stop to apoptosis induction. This finding demonstrates that NO-mediated apoptosis induction in transformed cells is executed by the mitochondrial pathway of apoptosis induction.

The findings shown in this paper allow several predictions for the modulation of NO-mediated apoptosis induction in transformed and nontransformed fibroblasts: 1) lowering the glutathione level in transformed cells should cause an earlier onset of NO-mediated apoptosis induction, whereas partial inhibition of superoxide anion generation of transformed cells should delay NO-mediated apoptosis induction; 2) lowering the glutathione level in transformed cells should gradually shift the usage of extracellular to intracellular superoxide anions; and 3) lowering the glutathione level in nontransformed cells should render these sensitive to NO-mediated apoptosis induction.

Each of these predictions was confirmed experimentally. The combination of increasing concentrations of BSO (an inhibitor of glutathione synthesis) with SNP caused a concentration-dependent decrease of phase 1 (Figure 9). The decrease of the length of phase 1 was paralleled by a shift in effectiveness of extracellular versus intracellular superoxide anions. Whereas SNP-mediated apoptosis induction in the absence of BSO or in combination with low concentrations of BSO was completely blocked by SOD, apoptosis induction in the presence of the highest concentration of BSO was not affected by SOD at all. The effect of SOD increased with the decrease of BSO, indicating a switch between the usage of intracellular and extracellular superoxide anions, dependent on the glutathione level of the cells. Whereas glutathione depletion caused a decrease in the duration of phase 1 of NO-mediated apoptosis induction, inhibition of extracellular superoxide anion synthesis caused its delay (Figure 9). As expected, the residual apoptosis induction in the presence of apocynin, an inhibitor of NADPH oxidase, was completely blocked by SOD.

Finally, nontransformed NRK rat fibroblasts, which show no apoptotic response to NO under standard conditions, were pretreated with increasing concentrations of BSO and then SNP was added. As seen in Figure 10, BSO or SNP treatment alone caused no or only marginal apoptosis induction in these cells. Pretreatment with BSO, followed by SNP addition, caused a strong apoptotic response. The time point of the onset of apoptosis was dependent on the concentration of BSO applied, with the earliest reaction seen for the highest BSO concentration in combination with SNP.

Discussion

Our data define the role of extracellular and intracellular superoxide anions, intracellular glutathione and the mitochondrial pathway for apoptosis induction mediated by NO in transformed fibroblasts. In this system, peroxynitrite formation through NO and superoxide anion interaction seems to be a necessary prerequisite for apoptosis induction. There is no evidence for a direct apoptosis-inducing effect of NO alone in this cell system. These data confirm the recently described differential response of transformed fibroblasts to NO, compared to their nontransformed parental cells. They define two phases during NO-mediated apoptosis induction and allow predictions for mechanisms that might sensitize cells for NO-mediated apoptosis induction and thus contribute either to NO-based control of transformed cells or to pathophysiological conditions.

As the apoptotic response of transformed fibroblasts to NO was completely blocked by SOD (when SOD was added simultaneously with the NO donor), extracellular superoxide anions seem to be the primary key that defines the sensitivity and reactivity of cells towards NO-mediated apoptosis induction. This finding is in good agreement with the activity of membrane-associated NADPH oxidase in oncogenically transformed cells (31, 32). The requirement of NO-mediated apoptosis induction for extracellular superoxide anions is further confirmed by the inhibitory effect of apocynin, an inhibitor of NADPH oxidase. The requirement for superoxide anions is best explained by the rapid interaction of NO with superoxide anions, leading to the formation of the apoptosis inducer peroxynitrite (11-13). In addition, the role of peroxynitrite is directly demonstrated through inhibition of NO-mediated apoptosis by the specific peroxynitrite decomposition catalyst FeTPPS. The apoptosis-inducing potential of peroxynitrite has been recently shown for transformed and nontransformed cells (1). Therefore, peroxynitrite seems to represent a nonselective apoptosis inducer, which cannot differentiate between transformed and nontransformed cells. However, peroxynitrite is selectively generated when NO meets superoxide anion-generating transformed cells and warrants their selective apoptosis induction.

However, as seen in Figure 1, the difference between transformed and nontransformed cells does not represent an all or nothing effect. Rather, transformed cells seem to generate higher concentrations of extracellular superoxide anions than their nontransformed parental cells. Therefore, the response of transformed cells to NO is much earlier and requires less NO than the late response of nontransformed cells, which requires higher concentrations of NO. The
Figure 11. Differential role of extra- and intracellular superoxide anions for nitric oxide-mediated apoptosis induction.

Phase 1: Transformed fibroblasts (triangle) are characterized by extracellular superoxide anion generation through membrane-associated NADPH oxidase. The interaction of NO with extracellular superoxide anions (reaction # 1) causes the formation of peroxynitrite (ONOO). NO that did not interact with extracellular superoxide anions but passed the cellular membrane has a chance to form intracellular peroxynitrite after interaction with intracellular, mitochondria-derived superoxide anions (reaction # 2). However, this peroxynitrite is efficiently scavenged by glutathione (GSH). During phase 1, the formation of extracellular peroxynitrite causes a gradual consumption and depletion of intracellular glutathione.

Phase 2: After (gradual) glutathione depletion, NO interaction with intracellular superoxide anions causes the formation of intracellular peroxynitrite, which is no longer scavenged by glutathione. Peroxynitrite will now activate the mitochondrial permeability transition pore and thus initiate the final steps of apoptosis induction, mediated by caspases.

The concept of phase 1 has been derived: i) from the kinetics of NO-mediated apoptosis induction, ii) by the effect of extracellular SOD, iii) from the data obtained by manipulation of the glutathione level and iv) from direct measurements of the glutathione level. The concept of phase 2 has been established from the findings: i) that only cell-permeable SOD mimetics and a peroxynitrite decomposition catalyst prevented NO-mediated apoptosis at this stage, ii) that bongkrekic acid (an inhibitor of mitochondrial permeability pore activity) prevents NO-mediated apoptosis and iii) that mitochondrial depolarisation as well as caspase 3 activity were directly measured (1).

Nontransformed fibroblasts generate much less extracellular superoxide anions than transformed cells and therefore either show an extremely long phase 1 or no reaction at all. However experimental glutathione depletion renders transformed as well as nontransformed cells sensitive to immediate onset of phase 2.
response of nontransformed cells is due to superoxide anion generation, as it is completely blocked by SOD. The marginal superoxide anion generation by nontransformed cells may be related to superoxide anion generation during the control of proliferation (53).

NO-mediated apoptosis induction in transformed cells was separated into two distinct phases in this study. Phase 1 extends from the addition of the NO donor to the onset of the apoptotic response. This lag-phase shows a high degree of variability between different experiments. The reason for this variability is not completely understood yet. Phase 1 is followed by phase 2, which is characterized as a rather steep increase in the percentage of apoptotic cells. Efficient apoptosis induction seems to require the presence of NO during both phases, as removal of the NO donor during phase 2 leads to a sudden halt in apoptosis induction.

The effect of NO seems to be mediated through peroxynitrite formation throughout both phases, as apoptosis induction depends on superoxide anions all the time and as the peroxynitrite decomposition catalyst FeTPPS inhibits NO-mediated apoptosis induction, even if it is added during phase 2 only. Phases 1 and 2 show different requirements with respect to the effective concentration of NO. Suboptimal concentrations of the NO donor only show a marginal effect when they are supplied throughout the experiment, but they allow a maximal effect when they are added to cells that were in contact with optimal concentrations of the NO donor during phase 1 exclusively. This shift of requirement for the NO concentration will be discussed in more detail in the context of glutathione depletion during NO action, below.

The transmission from phase 1 to phase 2 is paralleled by a shift in the requirement for extracellular superoxide anions to intracellular superoxide anions. SOD, which cannot penetrate cells (36, 37), shows strong inhibition of NO-mediated apoptosis induction when it is added at the beginning of phase 1, but has no inhibitory effect when added at the beginning of phase 2. This finding is in contrast to the strong inhibitory effect of several cell-permeable SOD mimetics during phase 2. Therefore, the effects following extracellular peroxynitrite formation seem to characterize phase 1, whereas intracellularly formed peroxynitrite seems to be the exclusive driving force during phase 2. Intracellular peroxynitrite formation has also been shown to be relevant for apoptosis induction in neuronal cells in the study by Keller et al. (24). As discussed below, the mitochondrial permeability transition pore seems to be the target for peroxynitrite formed during phase 2.

Phase 1 can be shortened or omitted by experimental depletion of the intracellular glutathione level of transformed cells through the addition of BSO, an inhibitor of glutathione synthesis. Increasing concentrations of BSO cause a parallel decrease of the intracellular glutathione level and lead to a parallel decrease of the length of the lag-phase (phase 1). An increase in the BSO concentration correlates with a gradual shift in the effectiveness of extracellular towards intracellular superoxide anions, as seen in the decreasing degree of inhibition by SOD (see Figure 9).

Glutathione therefore seems to represent a central modulator of NO-mediated apoptosis induction. In glutathione-competent fibroblasts, NO that passes membranes readily seems to have no chance to induce apoptosis. This finding indicates that NO has no direct apoptosis effect in these cells and that peroxynitrite, which might be formed through the interaction of NO with mitochondria-derived superoxide anions, must be readily scavenged by glutathione. Extracellular peroxynitrite, formed through the interaction of extracellular superoxide anions with NO, seems to cause a gradual decrease of the intracellular glutathione level during phase 1. The following results are in line with this conclusion: 1) experimental manipulation of the intracellular glutathione level of transformed cells modulates the length of phase 1; 2) the decrease of intracellular glutathione can be directly measured during phase 1 through the use of monochlorobimane; 3) experimental depletion of the intracellular glutathione level of nontransformed cells renders the cells sensitive to NO-mediated apoptosis induction; 4) after experimental depletion of intracellular glutathione, transformed as well as nontransformed cells respond to NO in a reaction that is not dependent on extracellular superoxide anions (as it is no longer blocked by SOD), but that is dependent on intracellular superoxide anions and peroxynitrite formation, as it is inhibited by cell-permeable SOD mimetics and by peroxynitrite decomposition catalysts; 5) after experimental glutathione depletion, the dependency on the concentration of the NO donor is drastically shifted to smaller concentrations. Similarly, the requirement for NO at the onset of phase 2 is shifted to smaller concentrations. This finding seems to reflect the fact that peroxynitrite formed intracellularly in glutathione-depleted phase 2 cells is no longer efficiently counterbalanced by the remaining glutathione.

Glutathione peroxidase does not seem to be involved in phase 1-specific effects, as its inhibition does not influence the length of phase 1. It instead plays a modulatory role during phase 2, after the intracellular glutathione level has been decreased.

Mitochondria seem to be the central target for NO-mediated apoptosis induction, as mitochondrial depolarisation precedes apoptosis induction and as bongkrekic acid, an inhibitor of the mitochondrial permeability transition pore, causes a strong inhibitory effect on apoptosis induction. Activation of the mitochondrial permeability transition pore through peroxynitrite during NO-mediated apoptosis induction is in line with the ability of peroxynitrite to interact...
with the pore (30) and by the dependency of NO-mediated apoptosis induction on peroxynitrite formation. As a consequence, the known effects of mitochondrial depolarisation and release of cytochrome c (54) may lead to subsequent activation of caspases 9 and 3 and the final state of apoptosis execution. Interaction of peroxynitrite with the permeability transition pore seems to be the central event during phase 2.

Taken together, our findings allow establishment of the following model for NO-mediated apoptosis induction in transformed fibroblasts (Figure 11): NO generated experimentally by NO donors or by NO-producing cells in vivo first seems to interact with extracellular superoxide anions that are generated by membrane-associated NADPH oxidase. As a consequence extracellular peroxynitrite is formed and the intracellular glutathione level is then gradually diminished through peroxynitrite action. This process has been termed phase 1. Phase 1 can be shortened if the intracellular glutathione level is experimentally lowered through inhibition of glutathione synthesis. Phase 1 seems to switch to phase 2 as soon as the intracellular glutathione level is so low that intracellular peroxynitrite, formed through the interaction of NO (that has passed the cell membrane) with intracellular superoxide anions (derived from mitochondria or intracellular superoxide anion generating enzymes), is no longer scavenged by glutathione or glutathione peroxidase. Therefore, phase 2 is exclusively blocked by intracellular SOD mimetics or by peroxynitrite decomposition catalysts. As a result of intracellular peroxynitrite action, the mitochondrial permeability transition pore may be activated and the mitochondrial pathway of apoptosis induction seems to become effective. Therefore, phase 2 is paralleled by mitochondrial depolarisation and apoptosis induction can be blocked by bongkrekic acid, an inhibitor of the mitochondrial permeability transition pore.

As transformed cells show strong extracellular superoxide anion generation, they are able to go through phase 1 efficiently and even at moderate concentrations of the NO donor. Nontransformed cells, that show much less extracellular superoxide anions, show a much longer phase 1 than transformed cells, but only at relatively high concentrations of the NO donor. This difference seems to define the remarkable sensitivity of transformed fibroblasts to natural antitumor systems, such as granulocytes, macrophages or intercellular induction of apoptosis by neighboring cells. When, however, phase 1 is exerted through experimental depletion of the intracellular glutathione level, transformed as well as nontransformed cells show phase 2 reactions immediately and with a similar efficiency.

Though this mode of apoptosis induction by NO has been found initially for transformed fibroblasts, it allows several predictions for NO-mediated apoptosis induction in other biological systems. It can be predicted that any stimulus that induces extracellular superoxide anion generation may sensitize the affected cells to NO-mediated apoptosis induction. This interplay between NO production and enhanced superoxide anion generation has been recently shown to be responsible for apoptosis induction in beta cells (55). Infection by microorganisms and the action of cytokines are other good candidates for the induction of superoxide anion generation (56,57), thus possibly causing a sensitization towards NO-mediated apoptosis. This interplay may be especially relevant in the nervous system, where NO is present as a specific signaling molecule or may be released by microglial cells (58). As a consequence, the resultant peroxynitrite formation may cause apoptotic death and loss of cells in this compartment. Alternatively, the modulation of the intracellular glutathione level may immediately allow the onset of phase 2 reactions, leading to intracellular peroxynitrite formation and apoptotic death. Active glutathione extrusion after infection or the activation of receptors may contribute to apoptosis induction in this context. The modulatory role of glutathione for NO-mediated apoptosis has been shown in many other systems (7, 59, 60).

NO-mediated apoptosis induction in fibroblasts has been shown here to depend strictly on peroxynitrite formation, with extracellular and intracellular superoxide anions as the key elements and glutathione as modulator. In other cell systems, direct effects of NO on mitochondrial respiration (10), direct glutathione-depletion by NO (4) and the effect of NO on the regulation of gene activity (5) may possibly cause alternative ways for NO-mediated apoptosis induction, which may or may not interact with the signaling pathway described here.

Acknowledgements

We thank S. Heinzelmann for critical comments on the manuscript. This work was supported by the ‘Dr. Mildred Scheel Stiftung für Krebsforschung’ (grant 10-1177-Ba3), the ‘Müller-Fahnenbergstiftung Freiburg’ and the ‘Clotten Stiftung Freiburg’, Germany.

References