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Attenuation of p53 expression and Bax down-regulation during phorbol ester mediated inhibition of apoptosis

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1 Nitric oxide (NO) caused apoptotic cell death in murine RAW 264.7 macrophages. Associated with apoptotic morphology we observed p53 up-regulation and increased Bax expression. 12-O-tetradecanoylphorbol-13-acetate (TPA), a protein kinase C (PKC) activator potently blocked NO-induced apoptosis. To gain insights into the mechanisms involved we investigated the effect of TPA on apoptotic conveying proteins such as p53 and Bax.

2 TPA (100 nM) attentuated p53 up-regulation elicited by the NO-releasing compounds, S-nitrosoglutathione (1 mM) and sodium nitroprusside (1 mM), and suppressed p53 protein accumulation in response to endogenously generated NO. Hence, TPA appeared to lower the steady state p53 level following its up-regulation by NO.

3 Mezerein, a stage 2 tumour promoter and PKC activating agent was equally active to TPA. Moreover, two potent PKC inhibitors, staurosporine (10 nM) and Gö 6976 (50 nM), reversed the inhibitory effect of TPA. However, bisinoylmaleimide I (up to 500 nM) was ineffective.

4 By extending the studies, we revealed a TPA-mediated p53 down-regulation in response to etoposide (50 μM), mitomycin C (5 μg ml^{-1}) and actinomycin D (2 μg ml^{-1}).

5 With the notion that TPA suppressed apoptotic DNA fragmentation in p53 antisense expressing cells as well, we searched for additional inhibitory actions of TPA. As well as affecting p53, TPA elicited a rapid decline of the steady state level of Bax within 30 min.

6 We concluded that down-regulation of two classical apoptotic promoting proteins contributes to the anti-apoptotic action of mezerein and TPA.

Keywords: Apoptosis; Bax down-regulation; nitric oxide; p53; phorbol ester

Introduction

Nitric oxide (NO) has gained attention as a pathophysiological signalling molecule (Moncada *et al*., 1991; Nathan, 1992). Physiological amounts of NO, generated by constitutive NO synthase isoenzymes (NOS, EC 1.14.13.39), account for smooth muscle relaxation or neurotransmission (Palmer *et al*., 1987). Pathophysiological quantities of NO, produced by a cytokine-inducible high output system can be directed against tumour cells, invading bacteria, or parasites as a first line of nonspecific immune defence. Lethal NO concentrations are associated with medical symptoms such as insulin-dependent diabetes mellitus type I, septic shock, rheumatoid arthritis and multiple sclerosis. NO has been demonstrated to cause apoptosis in pancreatic β-cells (Kaneto *et al*., 1995), chondrocytes (Blanco *et al*., 1995) and macrophages (Meßmer *et al*., 1995). Several cells like macrophages not only utilize NO in the defence against pathogens but are also vulnerable to the toxic effects of NO.

Activation of soluble guanylyl cyclase, followed by guanosine 3′:5′-cyclic monophosphate (cyclicGMP) generation, causes phosphorylation as a prime physiological NO action (Ignarro, 1990). In contrast, toxic or apoptotic NO-signalling is still an enigma. Possible mechanisms include interactions between NO and iron-sulphur enzymes or protein thiol groups (Nathan, 1992), the NAD(H)-dependent modification of glyceraldehyde-3-phosphate dehydrogenase (Mohr *et al*., 1996), or direct DNA damage (Wink *et al*., 1991). The latter often results in p53 protein accumulation, associated with a G_1-cell cycle arrest and/or induction of apoptosis (Liebermann *et al*., 1995). Previously, we demonstrated accumulation of p53 protein before apoptotic DNA fragmentation in response to a cytokine-inducible NO formation or the NO donors S-nitrosoglutathione (GSNO) and sodium nitroprusside (SNP) (Meßmer *et al*., 1994).

The nuclear phosphoprotein p53 has been characterized as a growth suppressor (Selter & Montenarh, 1994; for review). Thus, inactivation of p53 by mutation or gene deletion is commonly associated with human cancers. Wild-type p53 (wtp53) binds sequence-specifically to DNA and functions either as a transcriptional activator or repressor. Among the cellular proteins transcriptionally repressed by wtp53 are PCNA (proliferating cell nuclear antigen) (Mercer *et al*., 1991) and Bcl-2 (B-cell lymphoma protein 2) (Miyashita *et al*., 1994). p53 serves as a transcriptional activator of Gadd45 (growth arrest and DNA damage) (Kastan *et al*., 1992), Mdm-2 (mouse double minute) (Barak *et al*., 1993), Bax (Bcl-2 associated protein x) (Miyashita & Reed, 1995) and $p21^{WAF1/CIP1}$ (El-Deiry *et al*., 1993). Following its activation by p53, Gadd45 binds to PCNA, stimulates DNA excision repair and blocks cell cycle progression to the S phase (Smith *et al*., 1994). $p21^{WAF1/CIP1}$ inhibits the kinase activity of multiple cyclin-dependent kinase complexes, mediating p53-dependent cell growth inhibition (Harper *et al*., 1993). However, in the case of severe DNA damage, p53 causes apoptosis, in part by promoting Bax up-regulation (Miyashita *et al*., 1994).

Previously, we established that NO-induced apoptosis was inhibited by exposure to 12-O-tetradecanoylphorbol-13-acetate (TPA) and protein kinase C (PKC) activation (Meßmer *et al*., 1995). The role of PKC in apoptotic signalling has been studied, generally, in several different experimental systems (Lucas & Sànchez-Margalet, 1995; for review). For example, PKC activation blocks apoptotic cell death in rat thymocytes exposed to Ca^{2+} ionophores and glucocorticoids (McConkey *et al*., 1989), suppresses radiation-induced sphingomyelin hydrolysis and apoptosis in

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aortic endothelial cells (Haimovitz-Friedman *et al*., 1994b), and prevents ceramide induced programmed cell death in U937 monoblastic leukaemic cells (Obeid *et al*., 1993). Furthermore, basic fibroblast growth factor (bFGF) protected endothelial cells against radiation-induced apoptosis by translocation and activation of the α-isotype of PKC (Haimovitz-Friedman *et al*., 1994a). In rat freshly isolated hepatocytes, inhibition, as well as down-regulation of PKC caused apoptosis (Sánchez *et al*., 1992). Despite the probability that PKC mediates activation and phosphorylation of Bcl-2 (May *et al*., 1993) and prevents sphingomyelin hydrolysis (Jarvis *et al*., 1994b), questions regarding its inhibitory action on apoptotic cell death remain unanswered.

Here we investigated the effect of TPA on NO-induced apoptosis in the macrophage cell line RAW 264.7, concentrating on p53 and Bax expression. Our data demonstrated that TPA and mezerein, two PKC-activating compounds, attenuate p53 expression induced by NO or genotoxic stressors and stimulate down-regulation of the death molecule Bax.

Methods

Cell culture and cell treatment

The mouse monocyte/macrophage cell line RAW 264.7 was maintained in RPMI 1640 supplemented with 100 u ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 10% heat-inactivated foetal calf serum (complete RPMI). All experiments were performed with complete RPMI. For DNA fragmentation experiments, 4×10^6 cells were seeded into 6-well culture plates (10^6 cells ml^{-1}), allowed to adhere overnight and incubated with the selected substances after the medium was changed. To investigate p53 expression, 2×10^7 cells were cultured and treated in the same way (10 cm Petri-dishes, 20 ml/plate). If not otherwise indicated, TPA was added 30 min before the addition of the NO donors or DNA-damaging agents. Stimulation of endogenous NO production was achieved with a combination of LPS (10 μg ml^{-1}) and IFN-γ (50 u ml^{-1}) applied for various times (15, 18, 21 or 24 h, respectively) whereas TPA was added 12 h after lipopolysaccharide/interferon-γ (LPS/IFN-γ) addition to avoid interference with nitric oxide synthase induction.

Transfections

The plasmid pBK/CMVΔp53asn was constructed by subcloning the coding sequences for amino acid 71–387 of the mouse p53 cDNA into the BamH I and EcoR I sites of pBK/CMV in antisense orientation (Meßmer & Brüne, 1996b). RAW 264.7 macrophages, 2×10^6, were grown in a 10 cm Petri dish. After overnight adhesion, cells were transfected either with 20 μg of the plasmid pBK/CMVΔp53asn or the control plasmid pBK/CMV lacking the p53 antisense gene (neomycin-vector control) by using the calcium phosphate precipitation method (Wigler *et al*., 1979). Stable transfected single clones were picked randomly after selection with 400 μg ml^{-1} geneticin and examined for p53 expression by immunoprecipitation and Western blot analysis.

S-nitrosoglutathione synthesis

GSNO (S-nitroso derivative of glutathione) was synthesized as described previously (Hart, 1985; Meßmer *et al*., 1994). Briefly, glutathione was dissolved in HC1 at 0°C before the addition of $NaNO_2$. The mixture was stirred at 0°C for 40 min followed by the addition of 2.5 volumes of acetone. The precipitate was filtered and washed once with 80% acetone, two times with 100% acetone, finally three times with diethylether and dried under vacuum. GSNO was characterized by high-performance liquid chromatrographic (h.p.l.c.)-analysis and u.v. spectroscopy.

Quantitation of DNA fragmentation

DNA fragmentation was essentially assayed as described previously (Meßmer *et al*., 1995). Briefly, after incubation cells were scraped off the culture plates, resuspended in 250 μl 10 mM Tris, 1 mM EDTA, pH 8.0 (TE-buffer) and incubated with an additional volume of lysis-buffer (5 mM Tris, 20 mM EDTA, pH 8.0, 0.5% Triton X-100) for 30 min at 4°C. After lysis, the intact chromatin (pellet) was separated from DNA fragments (supernatant) by centrifugation for 15 min at 13000 *g*. Pellets were resuspended in 500 μl TE-buffer and samples were precipitated by adding 500 μl 10% trichloroacetic acid at 4°C. Samples were pelleted at 4000 r.p.m. for 10 min and the supernatant was removed. After addition of 300 μl 5% trichloracetic acid, samples were boiled for 15 min. DNA contents were quantitated with the diphenylamine reagent (Burton, 1956). The percentage of DNA fragmented was calculated as the ratio of the DNA content in the supernatant to the amount in the pellet.

DNA agarose gel electrophoresis

For the preparation of DNA for agarose gel electrophoresis, cells were cultured, harvested, lysed and centrifuged as described above to separate DNA fragments from intact chromatin. Supernatants were precipitated overnight with 2 volumes ice-cold ethanol and 50 μl 5 M NaCl at -20°C, centrifuged at 13000 *g* for 15 min followed by incubation of the pellet in 500 μl TE-buffer supplemented with 100 μg ml^{-1} RNase A at 37°C for 30 min. Samples were extracted with phenol : chloroform : isoamylalcohol (25 : 24 : 1) and once again with chloroform : isoamylalcohol (24 : 1). DNA was precipitated and pellets were recovered by centrifugation (13000 *g*, 15 min), air dried, resuspended in 10 μl TE-buffer, supplemented with 2 μl sample buffer (0.25% bromophenol blue, 30% glyceric acid), and electrophoretically separated on a 1% agarose gel containing 1 μg ml^{-1} ethidium bromide for 2.5 h at 100 V. Pictures were taken by u.v. transillumination.

Quantitation of apoptotic cells

Macrophages (4×10^5) were grown in 12-well culture plates. After adhesion, cells were stimulated, followed by fixation with 3% paraformaldehyde for 5 min onto glass slides. Samples were washed with phosphate-buffered saline, stained with Hoechst dye H33258 (8 μg ml^{-1}) for 5 min, washed with distilled water, and mounted in KAISER'S glycerol gelatin. Nuclei were visualized with a Leitz fluorescence microscope. In each sample a minimum of 500 cells was counted and apoptotic nuclei were expressed as % of total nuclei.

Nitrite determination

Nitrite, a stable NO oxidation product, was determined by use of the Griess reaction (Ding *et al*., 1990). Cell-free culture supernatants were collected, adjusted to 4°C, and mixed with 1/10 volume sulphanilamide (dissolved in 1.2 M HCl) and 1/10 volume N-naphthylethylenediamine dihydrochloride. After 5 min at room temperature, the absorbance was measured at 560 nm with a reference wavelength of 690 nm. Nitrite concentrations were calculated by use of a $NaNO_2$ standard.

p53 quantification

p53 was quantified by immunoprecipitation followed by Western blot analysis as described previously (Meßmer *et al*., 1994). Briefly, cells were scraped off and lysed in 700 μl lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet-40, 1 mM PMSF, pH 8.0). Lysed cells were sonicated with a Branson sonifier (10 s, duty cycle 100%, output control 1). After centrifugation non-specific adsorbents were removed

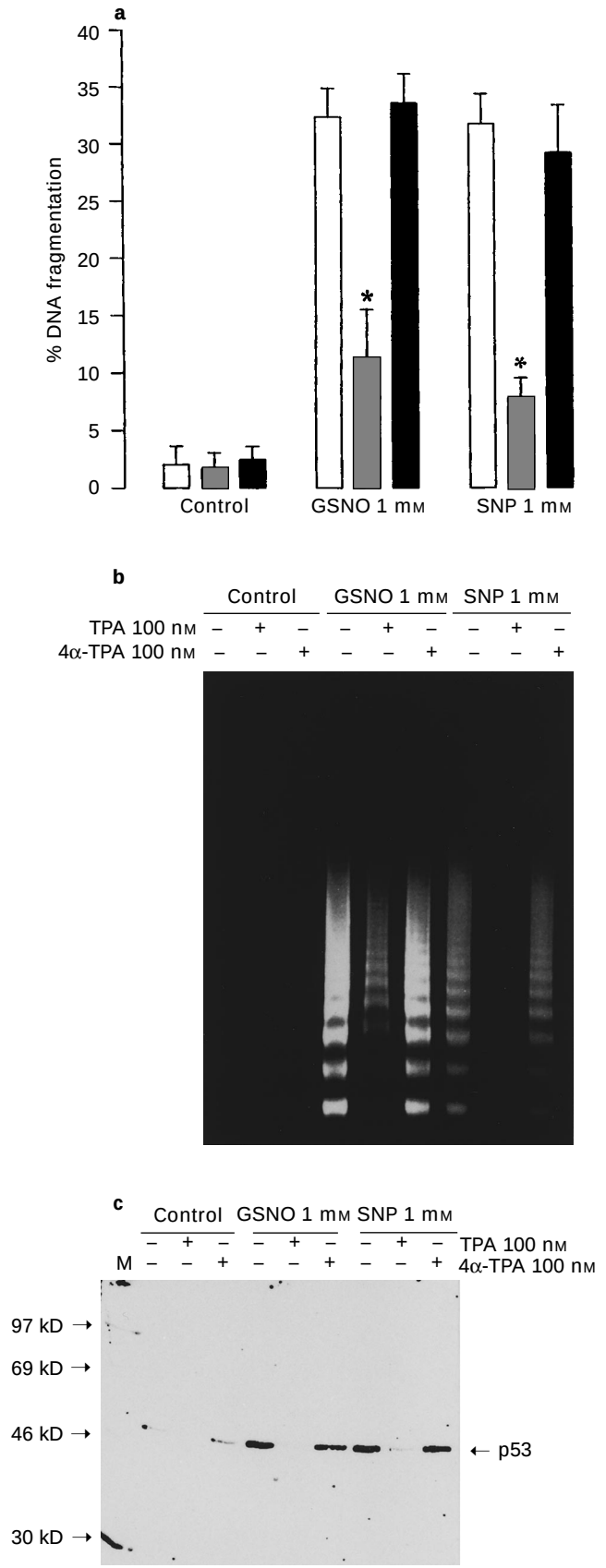

Figure 1 TPA prevents NO-induced p53 accumulation and attenuates apoptotic DNA fragmentation. RAW 264.7 macrophages (4×10^6 (a), 8×10^6 (b), 2×10^7 (c) cells) were incubated either for 8 h (a and b) or 4 h (c) with GSNO (1 mM), SNP (1 mM), TPA (100 nM), and 4α-TPA (100 nM), as indicated. TPA and 4α-TPA were added 30 min before NO donors. (a) DNA fragmentation was quantitated with the diphenylamine reaction. In (a): open columns, control; hatched columns, + 100 nM TPA; solid columns, + 100 nM 4α-TPA. DNA fragments were visualized on 1% agarose gels stained with ethidium bromide (b) and p53 protein was immunoprecipitated and determined by Western blotting as described in Methods (c). M refers to ^{14}C-labelled molecular weight markers. (a) Data are means ± s.d. of at least four individual experiments, whereas in (b) agarose gels and (c) Western blots are representative of three similar assays. TPA significantly attenuated apoptotic DNA fragmentation compared with incubations without TPA (*$P\leqslant0.01$).

from the resulting supernatant with 40 μl 50% (v/v) protein A-sepharose. p53 was immunoprecipitated by adding 200 μl hybridoma supernatant (clone PAb 122, kindly provided by Hans Stahl, Universität des Saarlandes, Homburg, Germany) and 50 μl 50% protein A-sepharose. Immunecomplexes were washed 3 times with 500 μl SNNTE buffer (5% sucrose, 1% Nonidet-40, 0.5 M NaCl, 50 mM Tris, 5 mM EDTA, pH 7.4) and another time with 1 ml SNNTE buffer. Finally, samples were resuspended in 40 μl sample buffer (125 mM Tris, 2% sodium dodecylsulphate (SDS), 10% glycerin, 1 mM dithiothreitol (DTT), 0.002% Bromophenol blue, pH 6.9), proteins were resolved on 10% SDS-polyacrylamide gels and blotted onto nitrocellulose sheets by use of the semi-dry blot system from Pharmacia (0.8 mA/cm^2, 1.25 h, 25 mM Tris/192 mM glycine as buffer system). The sheets were washed twice with Tris buffered saline (TBS; 140 mM NaCl, 50 mM Tris, pH 7.2) containing 0.1% Tween-20 before nonspecific binding was blocked with TBS/2% bovine serum albumin. The p53 antibody was added (hybridoma supernatant against p53; clone PAb122; 1 : 6 in TBS/0.2% bovine serum albumin) and incubated overnight at 4°C. Nitrocellulose sheets were washed 5 times and nonspecific binding was blocked as described. For detection, blots were incubated with [^{125}I]-protein A (2 ng ml^{-1} protein A, 1 μCi in TBS/0.06% Tween-20/0.1% bovine serum albumin) for 2 h followed by quantitative determination of radioactivity with the phosphor image system (Molecular Dynamics).

Alternatively, cells were metabolically labelled with [^{35}S]-methionine (100 μCi ml^{-1}) for 4 h in methionine-free medium containing 10% foetal calf serum with or without 1 mM GSNO +/− 100 nM TPA. Cell lysates were prepared and immunoprecipitation was performed with p53-specific monoclonal antibodies, PAb122 and PAb248 (PAb248 was kindly provided by Peter Wagner, Universität des Saarlandes, Bad Homburg, Germany). Immunoprecipitates were analysed by SDS-polyacrylamide gel electrophoresis, quantitative video densitometry, or radioactivity was quantitatively analysed by phosphor image analysis.

Bax Western blot analysis

Cell lysis was achieved with lysing buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet-40, 1 mM PMSF, pH 8.0) and sonication (Branson sonifier; 10 s, duty cycle 100%, output control 40%), followed by centrifugation ($4000\times g$, 5 min), and protein determination with the Bradford method (Bradford, 1976). Proteins were normalized to 100 μg/lane, resolved on 15% polyacrylamide gels and blotted onto nitrocellulose sheets basically following the method described for p53. Filters were incubated with the rabbit anti-mouse Bax antibody (clone P-19, 0.5 μg ml^{-1}) overnight at 4°C. Detection was by horse-radish peroxidase-conjugated goat anti-rabbit monoclonal antibodies (1 : 5000) for 1 h at room temperature by enhanced chemiluminence (ECL) (Amersham). Video densitometry quantification was performed with a Vilber Lourmat BIO1D V 6.02c.

Materials

Hoechst dye 33258, protein A sepharose, diphenylamine, bovine serum albumin, 12-O-tetradecanoylphorbol-13-acetate (TPA), 4α-TPA, etoposide, mitomycin C, actinomycin D, cycloheximide, saccharin, diethylstilbestrol (DES), mezerein,

H_2O_2, lipopolysaccharide LPS, (*E. coli* serotype 0127 : B8), staurosporine and SNP were purchased from Sigma (Deisenhofen, Germany). Gö 6976 and bisindoylmaleimide I hydrochloride were ordered from Calbiochem (Bad Soden, Germany). [^{125}I]-protein A was from DuPont New England Nuclear (Dreieich, Germany). The ECL detection reagents and [^{35}S]-methionine were from Amersham (Braunschweig, Germany). RNase A from bovine pancreas and recombinant murine interferon-γ (IFN-γ) were from Boehringer Mannheim (Mannheim, Germany). The rabbit polyclonal anti-mouse Bax antibody (clone P-19) was from Santa Cruz (Ismaning, Germany), RPMI 1640 from Biochrom (Berlin, Germany) and cell culture supplements, foetal calf serum and agarose were from Gibco (Berlin, Germany). All other chemicals were of the highest grade of purity commercially available.

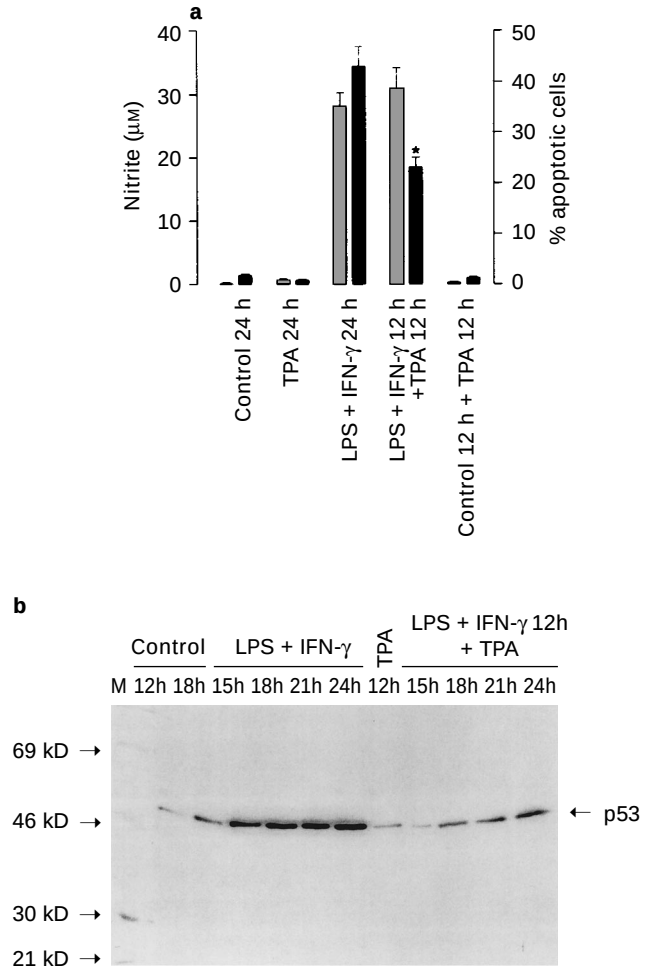

Figure 2 Effect of TPA on nitrite production, apoptotic cell death and p53 protein levels in response to endogenously generated NO. RAW 264.7 macrophages were seeded into 12-well culture plates (2×10^5 cells ml^{-1}; 2 ml/well) (a), or into 10 cm Petri-dishes (2×10^7 cells) (b), allowed to adhere overnight and incubated without further addition (control), with 100 nM TPA or 10 μg ml^{-1} LPS, and 50 u ml^{-1} IFN-γ for the times given. TPA was supplemented for indicated times following preincubation with LPS/IFN-γ for 12 h to avoid interference with NOS induction. (a) After 24 h nitrite was measured with the Griess reaction (stippled columns) and apoptotic cells (solid columns) were determined after nuclear staining with the Hoechst dye 33258 as described in Methods. Values are means $\pm$ s.e. of four to five independent determinations. *$P<0.01$ compared to incubations without TPA. (b) After the times indicated (total incubation time), p53 protein was immunoprecipitated with the monoclonal antibody PAb122 and determined by Western blotting as described. M indicates ^{14}C-molecular weight markers. The blot is representative of three similar experiments.

Statistical analysis

Each experiment was performed at least three times and statistical analysis were performed by use of two tailed Student's *t* test.

Results

TPA prevents NO-induced p53 up-regulation

NO-releasing agents, such as GSNO and sodium nitroprusside (SNP), induced apoptosis in RAW 264.7 macrophages. Internucleosomal DNA degradation determined quantitatively by the diphenylamine reaction or qualitatively by agarose gel electrophoresis (Figure 1a, b) was selected as a reliable apoptotic parameter. GSNO or SNP, exposure for 8 h, elicited 30–35% DNA degradation (Figure 1a) and lead to the appearance of a distinctive apoptotic DNA-ladder (Figure 1b). A 30 min pre-exposure to the phorbol ester TPA suppressed NO-induced DNA degradation to values around 10–15% and prevented DNA-laddering. 4α-TPA, a stereoisomer of TPA that is ineffective in PKC activation, induced no protection.

Knowing that NO donors cause p53 accumulation before DNA fragmentation, we tested for the effect of the phorbol esters. Western blotting occasionally revealed low p53 expression under resting conditions (Figure 1c) while the NO donors GSNO (1 mM) and SNP (1 mM) caused a marked, 10 to 20 fold up-regulation of the tumour suppressor within 4 h. Pretreatment with 100 nM TPA suppressed basal p53 protein expression and prevented NO donor-induced p53 accumulation, while 4α-TPA again was ineffective.

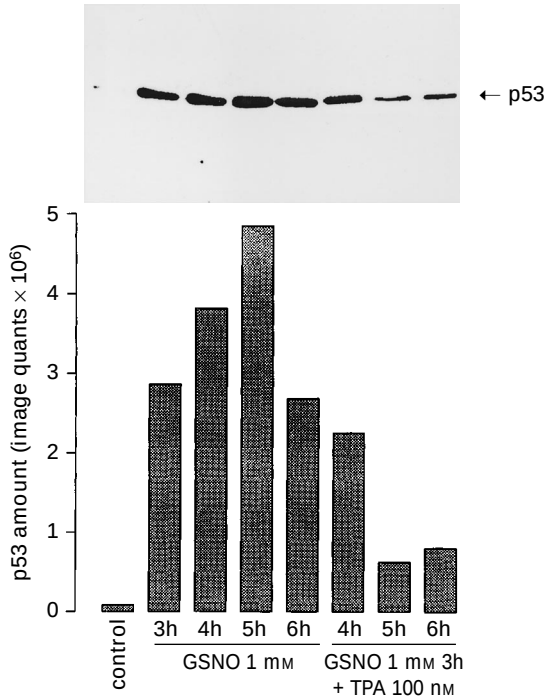

Figure 3 TPA promotes a decrease in the steady-state p53 protein level. Macrophages (2×10^7 cells) were incubated without any addition (control, 3 h) or with 1 mM GSNO for 3 to 6 h, respectively. TPA (100 nM) was supplemented 3 h after the addition of GSNO and incubations went on to reach a total exposure of 4, 5 and 6 h, respectively. p53 was determined by immunoprecipitation, Western blotting and bound [^{125}I]-protein A. Radioactivity was quantitated with the phosphor imager system (Molecular Dynamics). The blot is typical of five similar determinations.

We then concentrated on inducible type NOS expression and treated RAW macrophages with lipopolysaccharide (LPS) and interferon-γ (IFN-γ). The stable NO oxidation product nitrite was below the detection limit under control conditions with only 2% of the cells becoming apoptotic (Figure 2a). Stimulating macrophages for 24 h with a combination of LPS and IFN-γ resulted in nitrite accumulation in the culture supernatant with roughly 40% of all cells becoming apoptotic. Apoptotic death was blocked with an iNOS inhibitor (i.e. N^G-monomethyl-L-arginine), thereby showing a link between NO production and cell destruction (data not shown). In further experiments, we avoided any interference of TPA with NOS induction by adding the phorbol ester 12 h after LPS/IFN-γ addition, which gave enough time for the cytokines to up-regulate NOS. Following the addition of LPS/IFN-γ and TPA all experiments were continued for an additional 12 h period. These experimental conditions left nitrite release unaltered but lowered apoptotic cell death from about 42% to a TPA-inhibited response of around 22%. TPA alone was not toxic. LPS/IFN-γ addition caused p53 up-regulation after 15 h and the protein remained elevated up to 24 h (Figure 2b). When TPA was added 12 h after the addition of LPS/IFN-γ, p53 accumulation was less pronounced.

For optimal suppression, TPA required a preincubation period of 30 min to inhibit DNA fragmentation measured by the diphenylamine assay. When TPA was added from 30 min up to 4 h after the NO donors its inhibitory potency gradually declined (data not shown).

Activation of PKC by TPA is known to phosphorylate the carboxyl-terminal domain of human p53 protein, resulting in reduced immunoreactivity of a p53 antibody (PAb421) (Takenada *et al*., 1995). Hence, it was of interest to see whether TPA caused decreased p53 protein expression or mediated reduced immunoreactivity of the p53 antibody used (PAb122). Therefore, we probed with a mouse p53 specific antibody (PAb248) which recognizes a conserved conformational domain of the antigen, not affected by phosphorylation. Because PAb248 recognized non-denatured p53 only, we exposed RAW 264.7 macrophages to 100 nM TPA and/or 1 mM GSNO in the presence of [^{35}S]-methionine followed by p53 immunoprecipitation with the monoclonal antibodies PAb122 and PAb248. Experiments with PAb122 (relative density of the corresponding p53 protein band; OD = 4.0 with 1 mM GSNO versus a relative density of OD = 2.7 with 1 mM GSNO/100 nM TPA following a 3 h incubation time) and PAb248 (relative density of the protein band; OD = 6.1 with 1 mM GSNO versus a relative density of OD = 4.3 after 1 mM GSNO/100 nM addition) revealed attentuated p53 protein expression in response to the combination of GSNO and TPA. These results strengthen our hypothesis that TPA attenuates p53 protein expression.

TPA mediates p53 down-regulation

We tested whether TPA affected the steady state p53 protein level. RAW 264.7 macrophages were exposed to GSNO which

Table 1 Effect of mezerein on NO-induced DNA fragmentation

		% DNA fragmentation			
		Control	*GSNO (500 μM)*	*GSNO (1 mM)*	*SNP (1 mM)*
Control		2.0±1.0	27.3±3.8	33.2±2.9	31.3±1.7
Merezein	1 nM	1.6±1.1	28.2±4.3	32.9±2.6	33.8±2.9
	10 nM	1.5±0.5	24.0±4.0	31.1±2.9	27.7±2.8
	50 nM	2.0±1.1	10.4±2.8*	21.1±4.2*	17.7±3.2*
	100 nM	2.1±0.7	7.9±3.8*	15.4±4.6*	14.3±4.1*

Cells (4×10^6) were pretreated for 1 h with different concentrations of the PKC activator mezerein, exposed for 8 h to the NO donors GSNO and SNP in the presence of mezerein, followed by quantitative DNA fragmentation analysis. The data presented are mean values±s.e. of at least three independent determinations. *$P<0.05$ compared to incubations without mezerein.

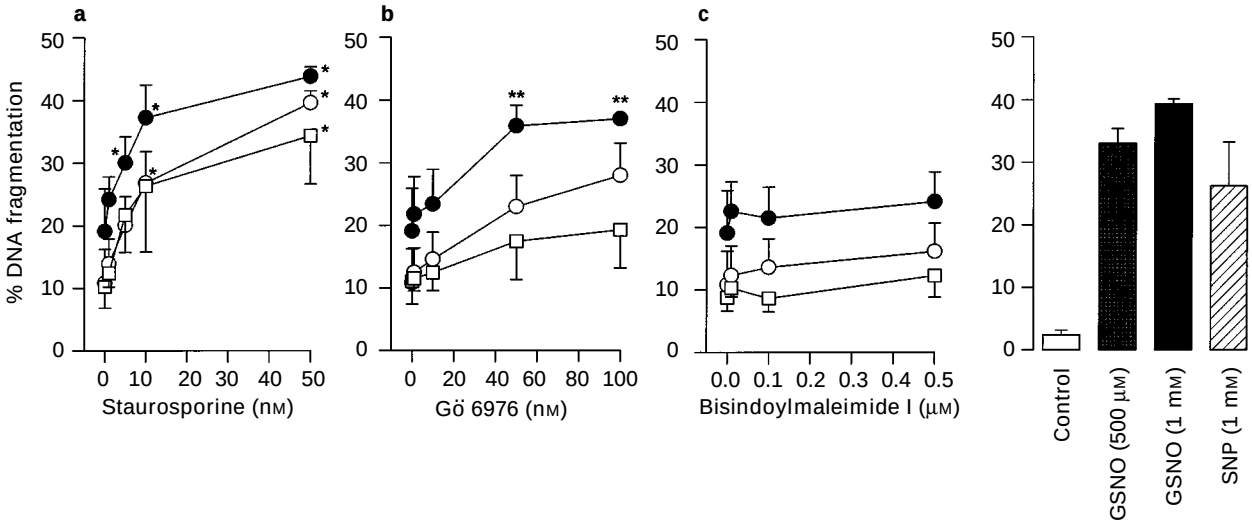

Figure 4 Effect of PKC inhibitors on TPA-inhibited apoptotic DNA fragmentation. RAW 264.7 macrophages were either incubated with GSNO (500 μM or 1 mM) and SNP (1 mM) or exposed to 100 nM TPA and the NO donors in the presence of PKC inhibitors (a) staurosporine, (b) Gö 6976 and (c) bisindoylmaleimide I. PKC inhibitors were preincubated for 1 h, whereas TPA was added 30 min before NO donors. DNA fragmentation was quantitated with the diphenylamine reaction as described in Methods. (○) TPA 100 nM + GSNO 500 μM, (●) TPA 100 nM + GSNO 1 mM and (□) TPA 100 nM + SNP 1 mM. Data are means and vertical lines show s.d. of at least 3 separate experiments. Significantly different values (*$P\leqslant0.001$; **$P\leqslant0.05$) compared to incubations in the absence of inhibitors.

induces a roughly 15 fold up-regulation of p53 within 3 h; the protein showed increased expression up to 6 h. Addition of TPA 3 h after the addition of GSNO led to a rapid decline of p53. This was most obvious 2 h after phorbol ester treatment (Figure 3). In contrast, p53 mRNA remained unchanged (data not shown).

Effect of the tumour promoter mezerein and PKC inhibitors on NO-induced apoptosis

A common property of some tumour promoters is the ability to block apoptosis (Wright *et al*., 1994). To generalize our studies we used mezerein, another PKC activating tumour promoter. Acute exposure to mezerein at a concentration of 50 or 100 nM significantly lowered GSNO- as well as SNP-induced apoptotic DNA fragmentation (Table 1).

As a consequence of previous experiments, we probed for NO-induced p53 accumulation and demonstrated that mezerein, comparable to TPA, markedly attenuated p53 up-regulation (data not shown).

In order to test whether the death-suppressing effect of TPA is PKC-mediated, we performed PKC down-regulation experiments and probed with some PKC inhibitors. Prolonged incubation of cells with high TPA concentrations is known to down-regulate some PKC isoenzymes. Preincubation of RAW 264.7 macrophages for 24 h with 500 nM TPA followed by another 8 hr incubation with 100 nM TPA in the presence of NO donors resulted in only a marginal inhibitory effect of TPA (Meßmer *et al*., 1995). To block the phorbol ester-induced inhibition, we preincubated cultured cells with three potent PKC inhibitors before the addition of TPA and GSNO/SNP. Staurosporine which is one of the most potent but relatively nonselective PKC inhibitors ($IC_{50}=6$ nM; Martiny-Baron *et al*., 1993) completely antagonized the inhibitory effect of TPA ($IC_{50}=5$ nM; Figure 4). However, the results with Gö 6976 and bisindoylmaleimide I are somewhat contradictory. Gö 6976, which selectively inhibits the Ca^{2+}-dependent PKC isoenzymes α and $\beta 1$ ($IC_{50}=3$ nM; Martiny-Baron *et al*., 1993) also antagonized the protective effect of TPA ($IC_{50}=30-100$ nM). Controls revealed that staurosporine and Gö 6976 alone were not inhibitory (data not shown). In contrast, bisindoylmaleimide I, which is known to inhibit all PKC isoenzymes ($IC_{50}=10$ nM; Toullec *et al*., 1991) did not reverse TPA inhibition (Figure 4). Similar effects were observed when mezerein was used (data not shown).

TPA attenuates cell death signals elicited by genotoxic stressors

To confirm the protection elicited by TPA, we exposed RAW 264.7 macrophages to DNA damaging agents, such as etoposide, mitomycin C and actinomycin D. For comparison we also studied the action of cycloheximide, H_2O_2, GSNO and SNP on p53 expression and apoptotic DNA laddering. The DNA-damaging agents, with the exception of cycloheximide, evoked p53 accumulation within 4 h (Figure 5b). Actinomycin D was most active, whereas mitomycin C was less potent. Etoposide, GSNO and SNP showed intermediate responses. The relatively low efficacy of mitomycin C was comparable, for both a 4 h or 8 h incubation period. Cycloheximide elicited apoptotic DNA cleavage in the absence of p53 protein expression which reflects a p53-independent signalling pathway. Also, all DNA-damaging agents induced characteristic apoptotic DNA-laddering (data not shown).

Furthermore, we established a linear correlation between p53 protein levels and the degree of DNA fragmentation subsequently induced GSNO, SNP, actinomycin D, etoposide and mitomycin C (data not shown). Collectively, we assumed apoptotic signalling elicited by genotoxic stress in RAW 264.7 cells to be, at least in part, p53-dependent.

In further experiments we studied TPA protection in relation to genotoxic stressors and cycloheximide (Figure 5a). TPA pretreatment significantly lowered etoposide-induced DNA fragmentation and suppressed mitomycin C- and actinomycin D-induced DNA cleavage, whereas DNA degradation caused by cycloheximide was not affected. Our results imply p53 repression by TPA, related to all agents tested. Exceptionally, cycloheximide alone suppressed p53 (data not shown).

TPA blocks GSNO-induced apoptosis in the absence of p53

Further experiments, with p53 antisense RNA transfectants, addressed the question of whether TPA-attenuated p53 accumulation suffices to prevent NO-induced cell death. RAW 264.7 macrophages were stably transfected with the plasmid pBK/CMVΔp53asm (Meßmer & Brüne, 1996b). Two different

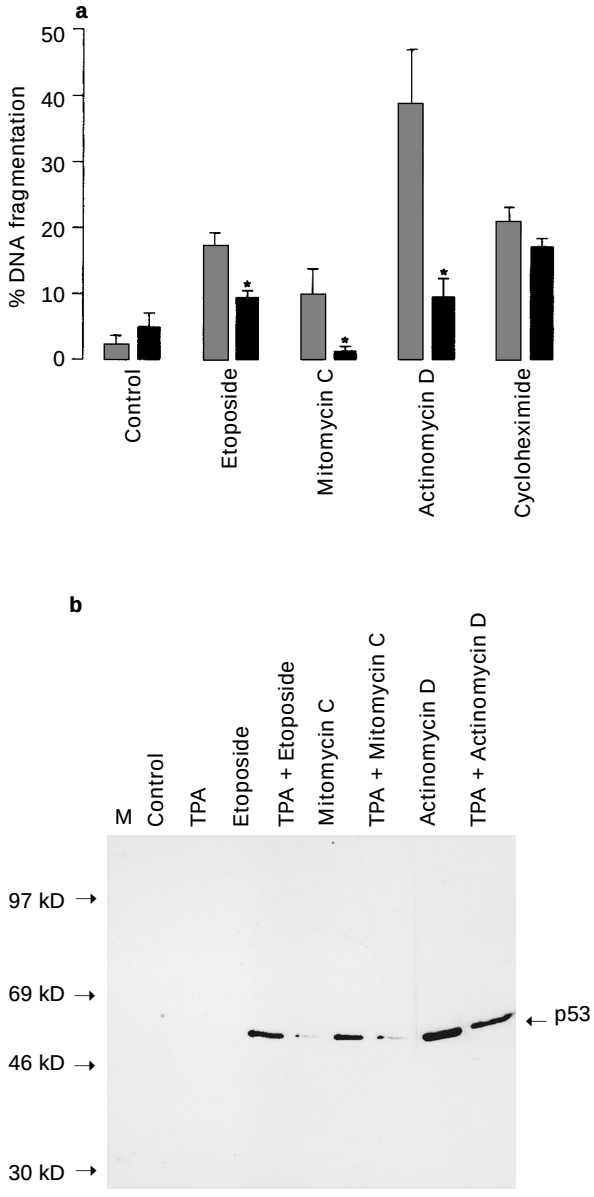

Figure 5 TPA antagonizes p53 up-regulation and DNA fragmentation in response to DNA-damaging agents. Cells were exposed for 8 h (a) or for 4 h (b) to 50 μM etoposide, 5 μg ml^{-1} mitomycin C, 2 μg ml^{-1} actinomycin D or 10 μM cycloheximide. TPA (100 nM) was added 30 min before the agonists. Hatched columns, control; solid columns, + TPA 100 nM. DNA fragmentation was measured with the diphenylamine reaction (a) and p53 protein determination was done by immunoprecipitation and Western blotting (b). (a) Values are means $\pm$ s.d. of four individual determinations. Significant difference ($^*P \leqslant 0.01$) compared to incubations without TPA.

clones, RΔp53asn-1 and RΔp53asn-11, exhibited substantially reduced p53 accumulation following GSNO stimulation (Table 2a). GSNO dose-dependently cleaved DNA (30–35% DNA cleavage; Table 2) in neomycin-vector control transfectants (Rneo-2). In p53 antisense RNA expressing clones (RΔp53asn-1 and RΔp53asn-11) DNA cleavage was substantially reduced (12–18% DNA fragmentation). TPA significantly suppressed NO-induced DNA fragmentation in neomycin-vector control transfectants. However, in clones with antisense antagonized p53 levels, TPA further suppressed GSNO-induced DNA degradation to below 6%. This may result from incomplete p53 elimination by p53 antisense RNA, or more likely may suggest additional TPA inhibitory mechanisms.

TPA mediates Bax down-regulation

In the search for additional TPA-inhibitory mechanism we considered Bax, a classical apoptosis promoting protein. We tested whether TPA affects Bax expression. Western blot analysis revealed constitutively expressed Bax (Figure 6). Intriguingly, TPA promoted Bax down-regulation within 30 min to 1 h (Figure 6a). Following TPA addition Bax levels disappeared after 4 h, and partially reappeared after 6–8 h. Mezerein (100 nM) gave identical results (Figure 7a). Addition of GSNO stimulated a small but significant increase in the Bax steady state protein level (Figure 6b, c). TPA pretreatment not only attenuated GSNO-induced apoptosis and p53 accumulation, but also stimulated Bax down-regulation when GSNO was present. Obviously, these conditions allowed Bax to recover more rapidly (Figure 6b). Extending these observations, we allowed GSNO to induce Bax for 3 h followed by the addition of TPA. Under these conditions TPA again was most effective in down-regulating Bax (Figure 7c).

Furthermore, we questioned whether Bax down-regulation is selective for PKC activation (TPA, mezerein) or whether protein kinase A stimulation would be effective as well. The lipophilic cyclic AMP analogue 8-(4-chlorophenylthio)-cyclic

Table 2 Effect of TPA on GSNO-induced apoptosis in the absence of p53

A

	p53 (relative units)	
	Control	*GSNO (1 mM)*
RAW 264.7	1	9.0±4.4
Rneo-2	0.8±0.1	8.1±0.6
RΔp53asn-1	0.3±0.0	0.6±0.3
RΔp53asn-11	0.4±0.4	2.1±1.3

B

	% DNA fragmentation		
	Control	*GSNO (500 μM)*	*GSNO (1 mM)*
Rneo-2			
−TPA	0.4±0.7	31.8±2.3	33.0±0.8
+TPA	1.6±0.4	7.7±4.8	17.3±4.5
RΔp53asn-1			
−TPA	0.7±0.7	12.2±4.8*	17.5±2.5*
+TPA	0.9±0.5	2.1±0.9**	5.4±2.3**
RΔp53asn-11			
−TPA	1.7±1.9	15.7±1.3*	16.7±2.6*
+TPA	0.7±0.9	1.7±1.1**	5.6±2.0**

(A) Raw 264.7 cells, a neomycin-vector control transfected clone (Rneo-2) and two p53 antisense RNA transfectants (RΔp53asn-1, RΔp53asn-11) were incubated for 4 h with 1 mM GSNO or remained untreated (control) and analysed for p53 protein expression by Western blot analysis. Quantitative p53 protein determination was done by phosphor image analysis. p53 image quant data of unstimulated RAW 264.7 macrophages served as a control (relative level of 1) and all other values were calculated proportionally. Values are means±s.d. of 3 independent experiments. (B) The neomycin-vector control clone (Rneo-2) and two p53 antisense RNA transfectants (RΔp53asn-1 and RΔp53asn-11) were exposed for 8 h to the NO donor GSNO (500 μM or 1 mM) and 100 nM TPA as indicated. TPA was added 30 min before GSNO. DNA fragmentation was quantitated by the diphenylamine reaction. Mean values±s.d. ($n=4$) are shown. *$P\leqslant 0.01$ compared to Rneo-2 incubated without TPA and **$P>0.01$ compared to Rneo-2 preincubated with TPA.

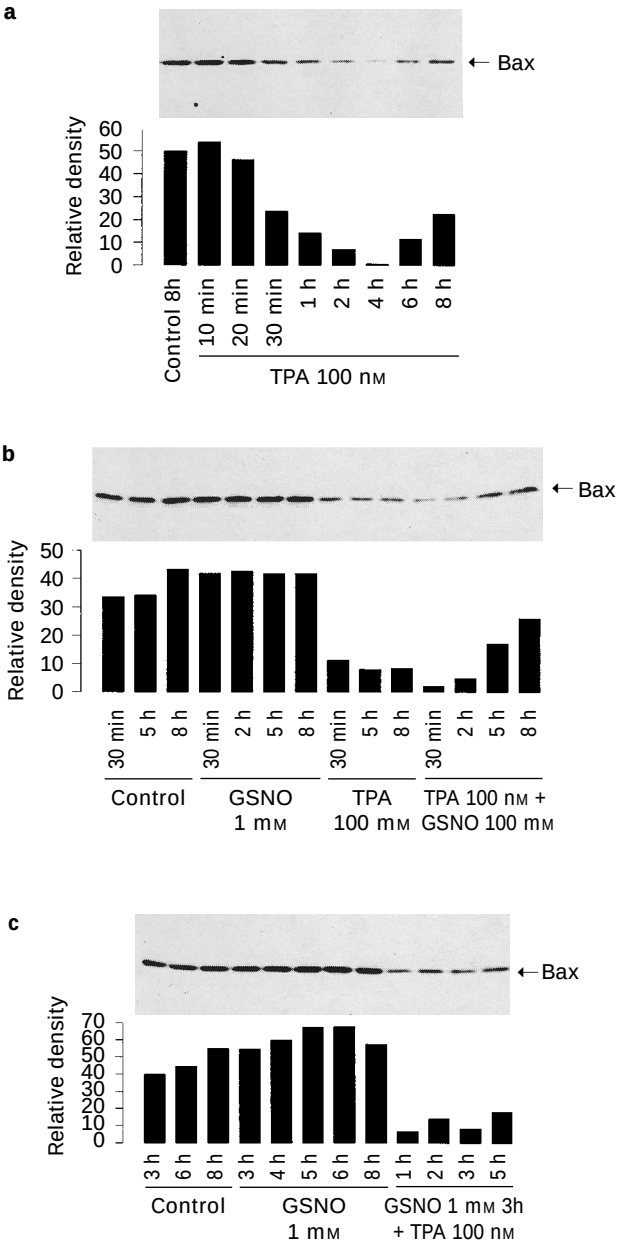

Figure 6 TPA promotes Bax down-regulation. RAW 264.7 macrophages, cultured as outlined in Methods, were treated with GSNO (1 mM) and TPA (100 nM) as indicated. Untreated cells (controls) were harvested at different times as shown. (a) Time-dependent Bax down-regulation by TPA. (b) GSNO caused Bax up-regulation, while TPA down-regulated the basal level of Bax. When TPA was added 30 min before GSNO, a decline in Bax still occurred. (c) TPA mediated the disappearance of Bax in GSNO prestimulated RAW 264.7 macrophages. TPA was added 3 h after GSNO for an additional 1 to 5 h period. Proteins were normalized to 100 μg/lane and the amount of Bax was determined by Western blot analysis and video densitometry. Each blot is representative of three similar experiments.

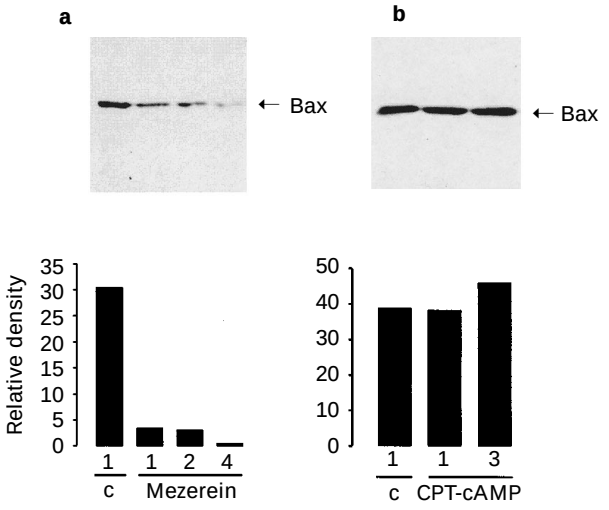

Figure 7 Effect of mezerein and a lipophilic cyclic AMP analogue on Bax steady state protein level. RAW 264.7 macrophages were treated with 100 nM mezerein (a) or 1 mM 8-(4-chlorophenylthio)-cylic AMP (CPT-cAMP) (b) for the times indicated. Untreated cells c were harvested after 1 h. Other details are as in Figure 7. Each blot is representative of three similar experiments.

AMP 7 which activates protein kinase A, did not influence Bax steady state protein levels (Figure 7b). Also, lipophilic cyclic GMP analogues were ineffective (data not shown).

Discussion

In the present study, tumour promoters such as TPA (12-O-tetradecanoylphorbol-13-acetate) or mezerein blocked apoptotic cell death in RAW 264.7 macrophages induced by NO and genotoxic stressors, such as etoposide, mitomycin C and actinomycin D. Theoretically, we propose that attenuation of p53 accumulation and down-regulation of the steady state level of Bax is associated with the anti-apoptotic action of PKC activating tumour promoters.

Previously, we established NO-mediated RAW 264.7 macrophage apoptosis as a model system (Meßmer *et al*., 1995) similar to the scenario demonstrated for peritoneal macrophages (Albina *et al*., 1993; Sarih *et al*., 1993). In macrophages endogenous NO production stimulated by LPS and IFN-γ suffices to induce programmed cell death. Apoptosis was morphologically characterized by chromatin condensation and biochemically, by DNA-laddering (Figure 2) (Meßmer *et al*., 1995). To study NO signalling and to gain insights into mechanistic signal interactions apart from cytokine transduction and NO synthase induction we used compounds that induce release of NO spontaneously as experimental tools.

Endogenously or exogenously generated NO accounts for accumulation of the tumour suppressor protein p53 (Meßmer *et al*., 1994). The p53 response is fast, compared to other markers like DNA-laddering. Generally, p53 accumulates in response to DNA damage by prolonging the protein half-life. NO-induced oxidative damage and DNA deamination in RAW 264.7 macrophages probably explain p53 up-regulation following its generation (de-Rojas-Walker *et al*., 1995). Supportive evidence has come from *in vitro* studies where NO caused DNA strand breaks and deamination (Wink *et al*., 1991; Nguyen *et al*., 1992). Further, we have suggested a close correlation betwen p53 accumulation and apoptosis in RAW 264.7 macrophages (Meßmer & Brüne, 1996a). In addition, the apoptotic action of DNA damaging agents such as etoposide (topoisomerase II inhibitor), mitomycin C (alkylating agent that cross links DNA and causes single strand DNA breaks) and actinomycin D (intercalates between adjacent GC base pairs of DNA and inhibits RNA biosynthesis) can also be correlated with p53 up-regulation. We conclude that RAW 264.7 macrophage cell death induced by NO and genotoxic stress is, at least in part, p53-dependent. This hypothesis is supported by the finding that RAW cells stably transfected with plasmids encoding p53 antisense RNA, displayed partial resistance towards NO-induced apoptosis (Table 2; Meßmer & Brüne, 1996b).

Macrphage apoptosis elicited by NO or other genotoxic stressors was inhibited by the tumour promoting agent TPA (Figures 1, 2 and 5). Although the role of PKC activating compounds like TPA in blocking apoptosis has not been characterized, other studies have corroborated the anti-apoptotic effects due to PKC activation or potentiation of cell death following PKC inhibition. An important feature of tumour promotion in chemical carcinogenesis is the inhibition of cell death by apoptosis. Mezerein, a stage 2 tumour promoter and PKC activating agent (Jarvis *et al*., 1994a) produced protection comparable to TPA. This suggests that inhibition of NO-induced apoptosis by TPA and mezerein can be attributed to PKC activation. In agreement with this are experiments in which down-regulated PKC was shown to produce only marginal inhibition (Meßmer *et al*., 1995). Also, PKC inhibitors like staurosporine and Gö 6976 antagonized the protective effects of TPA. Although staurosporine is a potent PKC inhibitor, it also inhibits S6 kinase, phosphorylase kinase, cyclic AMP-dependent protein kinase, and *src* kinase with similar potency (Martiny-Baron *et al*., 1993). In contrast, Gö 6976 is a potent and selective inhibitor of PKC α and β1 (Martiny-Baron *et al*., 1993) and effectively antagonizes TPA in our system. Therefore, it was assumed that PKC α and β1 are likely targets for the action of TPA in RAW 264.7 macrophages. However, bisindoylmaleimide I, a potent inhibitor of all PKC isoenzymes (Toullec *et al*., 1991) did not reverse the inhibitory action of TPA or mezerein. Possibly, different specificities of PKC isoenzymes, down-regulation of PKC isoenzymes or drug metabolism may explain this discrepancy. Although, the hypothesis that the inhibitory effect of TPA is soley PKC-dependent has not been fully proven. Other potential targets of phorbol esters, like activation of vav, a GDP exchange protein (Coppola *et al*., 1991) and chimerin binding (Hall *et al*., 1993), should be considered. Besides the antiapoptotic action of TPA, cytokine-elicited pathways, most likely mediated by activation of nuclear factor-κB (NF-κB), are considered important in controlling programmed death pathways (Barinaga, 1996). This implies cytokine involvement not only in iNOS up-regulation associated with cell death but also in activation of cellular protection.

For the pathology of cancer, it seems relevant that the anti-apoptotic potency of tumour promoters is due to attenuated p53 and Bax expression. The nuclear phosphoprotein p53 is a prominent tumour suppressor, post-translationally modified by phosphorylation (Meek, 1994). Major phorphorylation-sites within p53 are located in the N- and C-terminal domain of the protein. Although PKC phosphorylation is within the C-terminal regulatory domain (Baudier *et al*., 1992), the consequences of phosphorylation are still controversial. Our results showed that TPA attenuated p53 expression and/or accelerated protein down-regulation as the steady state level of the protein decreased (Figure 3) whereas the mRNA level remained unchanged (data not shown). These are supported by the finding that TPA promotes p53 down-regulation when overexpressed in K562 cells, thereby preventing apoptosis (Magnelli *et al*., 1995). Furthermore, TPA attenuated a DNA damage-induced p53-DNA binding activity by decreasing the half-life of the protein (Price & Calderwood, 1993). A p53 decrease as a result of phosphorylation has also been shown for $p34^{cdc2}$ kinase (Lin & Desiderio, 1993). TPA-mediated p53 down-regulation rather than reduced PAb122 immunoreactivity was verified by using PAb248, an antibody that recognizes a conserved conformational domain, not affected by PKC phosphorylation. Taking into account that wild-type p53 can both

promote and suppress cell proliferation, these opposing functions may correlate with an altered p53 conformation (Milner, 1994). Phosphorylation, protein stability and the tertiary protein structure are likely candidates for modulating p53-dependent signalling pathways. In conclusion, we hypothesize that tumour promoters which are able to activate PKC either directly or indirectly promote p53 down-regulation causing inhibition of apoptotic signalling cascades.

Similar to p53, we noticed a decline in the steady-state level of Bax in response to TPA (Figures 6 and 7b). Bax down-regulation was fast and transient. We noticed some minor variations in the time course of down-regulation ranging from 30 min to 2 h. Although GSNO induced a small but signficant Bax increase, TPA still reduced the steady state level of the protein. The reappearance of Bax after 6–8 h following its down-regulation may indicate PKC desensitization, resulting in a reduced inhibitory potency of TPA.

Since the level of Bax in p53 antisense RNA expressing and RAW 264.7 parent cells were comparable, we concluded that Bax is not directly regulated by p53 protein expression (Meßmer & Brüne, unpublished observations). TPA-mediated Bax down-regulation may refer to a TPA inhibitory action apart from a p53 involvement in p53 antisense RNA expressing cells. This seems relevant for systems which lack functional p53. For example, $p53^{-/-}$ cells like HL-60 and U937 myeloid leukaemia cells which are $p53^{-/-}$ were protected from ceramide-induced apoptosis by phorbol esters, mezerein and bryostatin 1 (Jarvis *et al*., 1994a). As a likely explanation, it is now assumed that TPA mediates Bax down-regulation in those experimental systems. Bax is a Bcl-2-related protein that promotes cell death, whereas Bcl-2 suppresses apoptosis in part by heterodimerization with its counterpart Bax (Oltavi *et al*., 1993). Besides protein-protein interactions, little is known about the regulation of Bax. For Bcl-2 a few studies have indicated that it is regulated by serine phosphorylation. In lymphoid cells, ocadaic acid has been shown to induce Bcl-2 dephosphorylation thereby decreasing apoptosis (Haldar *et al*., 1995), while in prostate cancer cell lines phosphorylation of Bcl-2 was correlated to reduce heterodimerization with Bax (Haldar *et al*., 1996). In contrast, haematopoietic cells respond to the PKC-agonist bryostatin 1 with Bcl-2 hyperphosphorylation and inhibition of apoptosis (May *et al*., 1993). Our results point to down-regulation of the steady-state Bax level within 30 min to 1 h as a result of TPA action. This may correlate very well with TPA-mediated inhibition of apoptosis which requires a preincubation period of 30 min for optimal protection. The fundamental role of Bax in addition to Bcl-2 is further supported by our finding that RAW 264.7 macrophage programmed cell death is fully blocked by Bcl-2 overexpression (Meßmer *et al*., 1996). Intriguingly, whereas Bcl-2 gene transfer still allowed p53 accumulation in response to NO, TPA addition abrogated NO-initiated signalling pathways in this respect. These differences with respect to intracellular signalling in the regulation of apoptosis and the question of whether the effects of TPA on p53 and Bax are broadly applicable will be addressed in further studies.

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