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Nitric oxide donors induce apoptosis in glomerular mesangial cells, epithelial cells and endothelial cells

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Abstract

Renal mesangial cells exposed to inflammatory cytokines produce high concentrations of nitric oxide (NO) which may exert cytotoxic actions. We report here that glomerular mesangial cells, endothelial cells and epithelial cells in culture are themselves targets for NO and undergo apoptotic cell death upon exposure to high concentrations of NO. NO generated from different NO-releasing compounds as well as NO-saturated solution induce apoptosis in all three cell types as demonstrated by internucleosomal DNA fragmentation, an enrichment of cytosolic DNA/histone complexes, an increasing number of cellular 3′-OH-fragmented DNA ends and typical nuclear chromatin condensation. Induction of apoptosis was found to be dependent on protein synthesis and is preceded by expression of the tumour suppressor gene product p53 in mesangial cells. Induction of inducible NO synthase in mesangial cells by interleukin-1β leads to excessive formation of NO by the cells as measured by nitrite production. However, there was no evidence for apoptotic changes in mesangial cells triggered by endogenously produced NO. Co-cultures of glomerular endothelial or epithelial cells with interleukin-1β-activated mesangial cells expressing inducible NO synthase do not show apoptotic alterations in endothelial or epithelial cells. Moreover, preincubation of mesangial cells with interleukin-1β protects the cells from apoptosis induced by subsequent addition of exogenous NO thus suggesting that interleukin-1β not only triggers the expression of inducible NO synthase and massive NO formation but simultaneously stimulates a protecting principle in the cells. In summary, these results suggest that exogenous NO can induce apoptosis in all three types of intrinsic glomerular cells. However, whether endogenously produced NO can fulfil this function critically depends on a balance between a yet to be defined protective mechanism and inducible NO synthase expression in mesangial cells in response to interleukin-1β and eventually other inflammatory cytokines.

Keywords: Nitric oxide (NO); Nitric oxide (NO) donor; Mesangial cell; Apoptosis; Glomerular endothelial cell; Glomerular epithelial cell; Interleukin-1

1. Introduction

The one to one-and-a-half million glomeruli present in a human kidney are composed of at least four cell types: capillary endothelial cells, visceral epithelial cells (podocytes), parietal epithelial cells, and mesangial cells. The mesangium, a separate component of the glomerulus, is a highly specialized pericapillary tissue that is involved in the regulation of the glomerular circulation and filtration as well as in most pathological processes of the renal glomerulus. Mesangial cells orchestrate inflammatory processes in the renal glomerulus by increased mediator production, increased matrix production, and increased mesangial cell proliferation (Schlondorff, 1987; Pfeilschifter, 1989, 1994; Kashgarian and Sterzel, 1992). An important and highly versatile member of this orchestra of inflammatory mediators produced by mesangial cells is nitric oxide (NO) (Pfeilschifter, 1995). In recent years, NO has become established as a diffusible universal signalling molecule throughout the body. The constitutive isoforms of NO synthase produce small amounts of NO contributing to blood pressure regulation and certain aspects of neuronal plasticity. The production of large quantities of NO is coupled to cytokine- or endotoxin-stimulated expression of inducible NO synthase and plays an important role in immune regulation as a host defence against microorganisms and tumour cells. Pathological NO overproduction,

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however, may also damage healthy tissue (Nathan, 1992; Moncada and Higgs, 1993). The exact mechanisms by which NO exerts its cytotoxic and proinflammatory actions are not yet fully understood. Inhibition of iron-sulfur proteins like aconitase, complexes I and II of the mitochondria respiratory chain, and ribonucleotide reductase or NAD(H)-dependent covalent modification of glyceraldehye-3-phosphate dehydrogenase (Dimmeler et al., 1992; Mohr et al., 1996) and DNA damage as well as strand breaks (Wink et al., 1991; Nguyen et al., 1992; Fehsel et al., 1993) may contribute to the cytotoxic properties of NO. Recently, NO has been observed to trigger apoptotic cell death in murine macrophages (Albina et al., 1993; Sarih et al., 1993; Messmer et al., 1994, 1995), human chondrocytes (Blanco et al., 1995), rat cortical neurons (Bonfoco et al., 1995) and thymocytes (Fehsel et al., 1995; Sandau and Brüne, 1996). Apoptosis or programmed cell death is characterized by a specific sequence of cellular events which comprise cell shrinkage, nuclear condensation, and disintegration of the dying cell into apoptotic bodies which are destined to be rapidly phagocytosed by neighbouring cells. In contrast to necrosis no leakage of

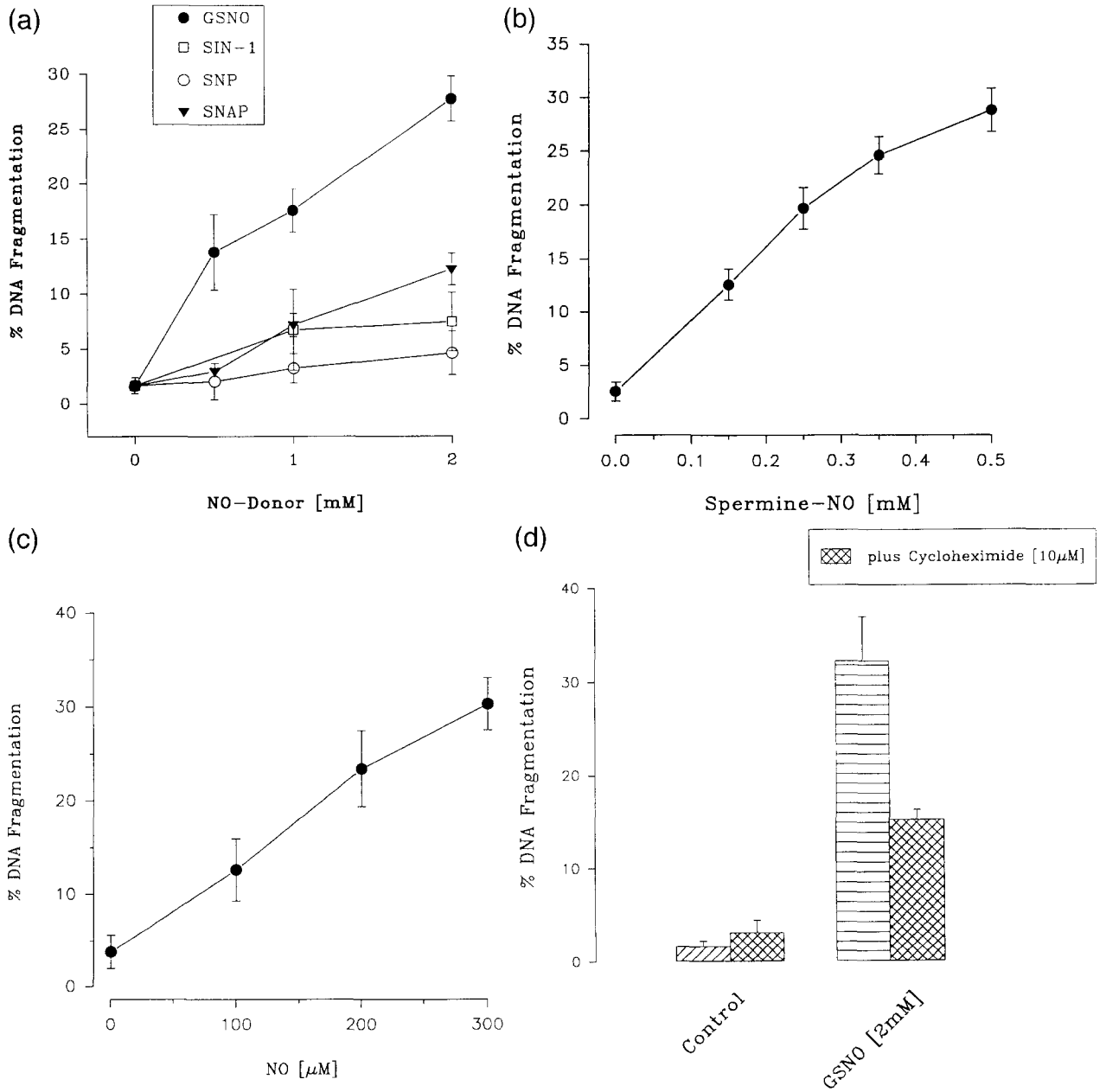

Fig. 1. Dose-dependent DNA fragmentation in mesangial cells, induced by various NO donors, and inhibition by cycloheximide. Cells were incubated for 24 h with the indicated concentrations of *S*-nitrosoglutathione (GSNO), SIN-1, sodium nitroprusside (SNP) and *S*-nitroso-*N*-acetylpenicillamine (SNAP) (a), spermine-NO (b) or authentic NO gas (c) in the absence or presence of cycloheximide (10 μM) (d) as indicated, followed by DNA extraction and quantitation of DNA fragmentation using the diphenylamine reaction. Data are means ± S.D. of four independent experiments.

the cell occurs. Apoptosis is a controlled biological strategy to remove unwanted cells in a given tissue and thus plays a crucial role in development, immunology and tumour biology (Cohen, 1993; Majno and Joris, 1995). In this report we present evidence that NO can induce apoptosis of glomerular mesangial, endothelial, and epithelial cells which may be of importance in the initiation as well as in the resolution phase of certain forms of glomerulonephritis.

2. Materials and methods

2.1. Cell culture

Renal glomeruli from male Sprague-Dawley rats or from female Wistar rats (80–100 g body weight) were isolated under sterile conditions by a sieving technique, and glomerular cells were cultured as described previously (Pfeilschifter et al., 1984). In a second step, single cells were cloned by limited dilution in 96-microwell plates to obtain pure cultures of epithelial cells and mesangial cells (Huwiler et al., 1993). Cells were identified as mesangial cells by (a) their typical stellate morphology, (b) positive staining for the intermediate filaments desmin and vimentin, which is considered to be specific for myogenic cells, (c) positive staining for Thy 1.1 antigen, (d) negative staining for Factor VIII-related antigen and cytokeratin, excluding endothelial and epithelial contaminations, respectively and (e) generation of inositol trisphosphate upon activation of angiotensin II AT_1 receptors was used as a functional criterion for characterizing the cloned cell line. Cells were identified as parietal epithelial cells by the following criteria (Holthöfer et al., 1991): (a) typical cobblestone morphology, (b) positive staining for cytokeratin and (c) negative staining with markers for mesangial cells (Thy 1.1 antigen, smooth muscle actin, desmin), endothelial cells (Factor VIII-related antigen), podocytes (podocyte antigen pp44, antibodies kindly provided by Professor W. Kriz and Dr. P. Mundel, University of Heidelberg), tubular epithelial cells (uvomorulin) and macrophages (ED1). For the experiments, passages 8–15 of mesangial cells and 12–18 of epithelial cells were used. Calf kidneys were obtained from a local slaughterhouse and were used to obtain glomerular endothelial cell cultures by a method previously described by Ballermann (1989) and modified by Briner and Kern (1994). Individual clones of endothelial cells were characterized by positive staining for Factor VIII-related antigen and uniform uptake of fluorescent acetylated low-density lipoproteins. Negative staining for smooth muscle actin and cytokeratin excluded mesangial cell and epithelial cell contaminations. Cells were utilized at passages 5–7. For co-culture studies of the cells, glomerular endothelial cells were plated as above in 35 mm diameter dishes and mesangial cells were plated into Millicell inserts (Millipore) and induced to produce NO by a 24 h preincubation with interleukin-1β (1–10 nM). The inserts containing the stimulated mesangial cells were subsequently placed into the dishes containing the endothelial cells and sit 1–2 mm above the endothelial cells. The bottom of the Millicell chamber is formed by a cellulose membrane containing 0.45 μm pores, which allow for diffusion of soluble effectors.

2.2. Quantitation of DNA fragmentation by the diphenylamine assay

DNA fragmentation was assayed as reported (McConkey et al., 1989). Briefly, following incubations, cells were centrifuged, resuspended in 250 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and lysed by adding 250 μl cold lysis buffer, containing 2 mM EDTA, 0.5% (v/v) Triton X-100, and 5 mM Tris-HCl, pH 8.0. Samples were allowed to lyse for 30 min at 4°C prior to centrifugation (15 min at 14000 $\times$ g) to separate intact chromatin (pellet) from DNA fragments (supernatant). Pellets were resuspended in 500 μl TE buffer and the DNA content of pellets and supernatants was measured using the diphenylamine reagent.

2.3. Analysis of DNA fragmentation by DNA agarose gel electrophoresis

Cells were lysed and centrifuged as described in order to separate DNA fragments from intact chromatin. DNA fragments in the supernatants were precipitated with 1 ml 100% ethanol and 50 μl 5 M NaCl at −20°C, overnight. After centrifugation (14000 $\times$ g for 15 min), pellets were incubated in 50 μl TE buffer containing 100 μg/ml RNase A at 37°C for 30 min, extracted with phenol/chlo-

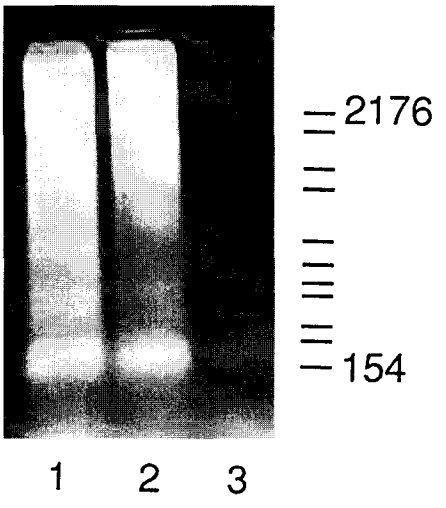

Fig. 2. DNA fragmentation in mesangial cells induced by spermine-NO. Cells were incubated for 20 h with spermine-NO 500 μM (lane 1) or 250 μM (lane 2) or with vehicle (control, lane 3) and intranucleosomal DNA fragmentation was analysed by agarose gel electrophoresis as described in Section 2. DNA markers are indicated.

roform/isoamylalcohol (25:24:1) and precipitated again overnight at −20°C. DNA fragments were electrophoretically separated on a 1% agarose gel and visualized by ultraviolet fluorescence after staining the gel with ethidium bromide (1 μg/ml).

2.4. Cell death detection by analysis of cytosolic oligonucleosome-bound DNA

Confluent mesangial cells in 35 mm diameter dishes were washed twice with phosphate-buffered saline (PBS) and incubated in Dulbecco's modified Eagle's medium (DMEM). Cytosolic oligonucleosome-bound DNA was quantitated using a ELISA kit (Boehringer Mannheim) with a primary anti-histone antibody and a secondary anti-DNA antibody coupled to peroxidase according to the manufacturer's instruction. Absorbance values ($A_{450\,nm/486\,nm}$) are a relative measure for DNA fragmentation. Percentage of fragmentation is expressed in comparison to controls.

Table 1
Antioxidants inhibit NO-induced DNA fragmentation in mesangial cells

	Control	GSNO (2 mM)
–	4 ± 3	33 ± 3
NAC (1 mM)	6 ± 2	22 ± 6 *
Trolox (1 mM)	7 ± 3	14 ± 3 *

Cells were incubated for 24 h with vehicle (control) or *S*-nitrosoglutathione (GSNO, 2 mM) in the presence or absence of Trolox or *N*-acetylcysteine (NAC) as indicated, followed by DNA extraction and quantitation of DNA fragmentation using the diphenylamine reaction. Data indicate percentage of DNA fragmentation and are means ± S.D. of four independent experiments.

* Significant differences from control: $P < 0.05$, Student's *t*-test.

2.5. In situ detection of apoptosis by terminal desoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)

Following incubations, cells (3×10^3 cells/well) were washed with PBS and fixed on the chamber slide with 1%

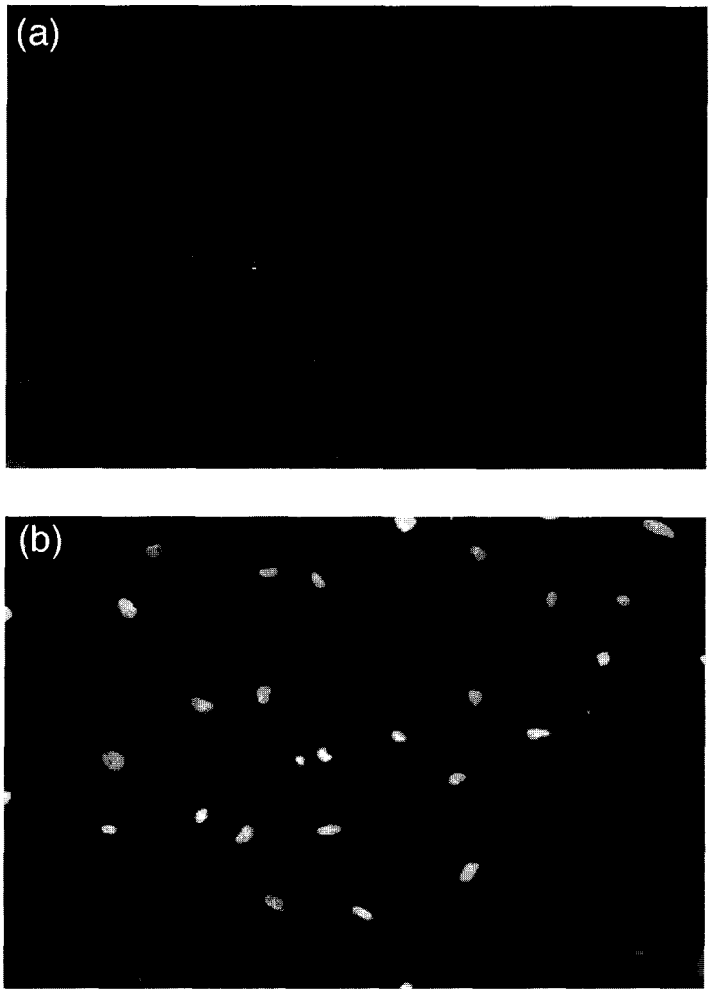

Fig. 3. In situ detection of spermine-NO-mediated apoptosis in mesangial cells by TUNEL staining ((a) and (b)) and of nuclear chromatin condensation ((c) and (d)). Mesangial cells were incubated for 16 h with vehicle (control, (a) and (c)) and with spermine-NO (500 μM, (b) and (d)). Thereafter in situ detection of apoptotic cells by TUNEL staining was performed as described in Section 2 ((a) and (b)) and nuclei were stained with Hoe-33258 in order to detect chromatin condensation ((c) and (d)).

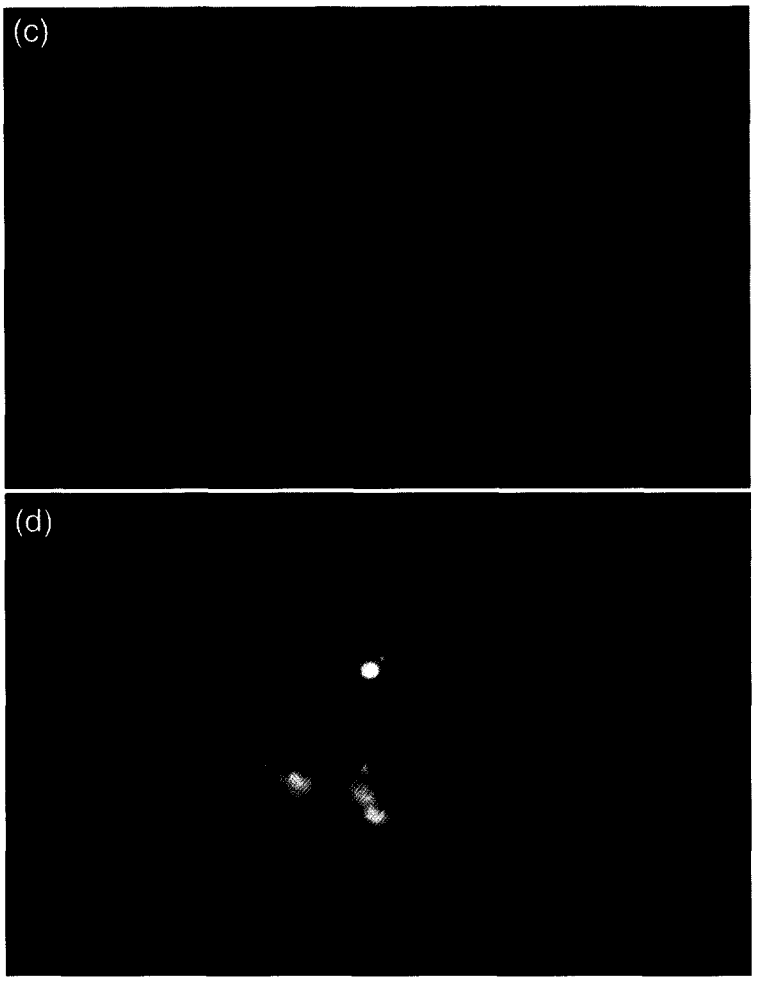

Fig. 3 (continued).

paraformaldehyde for 30 min at 4°C. Overnight, the cells were kept in 70% ethanol at −20°C, washed with PBS and incubated with terminal desoxynucleotidyl transferase (10 U/100 μl) and 20 μM biotin-dUTP for 1 h at 37°C. After washing, the staining solution (1 μg/100 μl streptavidin/rhodamine) was added and kept in a dark humid chamber for 1 h. The washed and dried chamber slide was embedded and the dye was analyzed with excitation 560 nm/emission 570 nm wavelength under a fluorescence microscope.

2.6. Staining of nuclei with Hoe-33258

Confluent mesangial cells in 35 mm diameter dishes were washed twice with PBS and incubated in DMEM with the indicated concentrations of compounds. Thereafter cells were fixed in 3% paraformaldehyde in phosphate buffer (100 mM, pH 7.4). From a stock solution Hoe-33258 (1 mg/ml) was added up to a final concentration of 30 μg/ml. The nuclei were viewed under a fluorescence microscope using standard excitation filters.

2.7. p53 quantification

p53 amount was quantified by immunoprecipitation followed by Western blot analysis as described previously (Messmer et al., 1994). Briefly, 7.5×10^6 cells were incubated for the times indicated, scraped off and lysed in 700 μl lysis buffer (50 mM Tris/HCl, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet-40, 1 mM phenylmethanesulfonyl fluoride, pH 8.0). Lysed cells were sonicated with a Branson sonifier (10 s, duty cycle 100%, output control 1). After centrifugation non-specific absorbents were removed 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) and 50 μl 50% protein A-sepharose. Immune complexes were washed 3 times with 500 μl SNNTE (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. Finally, samples were resuspended in 40 μl sample buffer (125 mM Tris/HCl, 2% sodium dodecylsulfate, 10% glycerin, 1 mM dithiothreitol, 0.002% bromephenol blue, pH 6.9),

proteins were resolved on 10% sodium dodecylsulfate-polyacrylamide gels, and blotted onto nitrocellulose sheets using 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 TBS (140 mM NaCl, 50 mM Tris/HCl, pH 7.2) containing 0.1% Tween-20 before blocking unspecific binding 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 unspecific binding was blocked as described. For detection, blots were incubated with [^{125}I]protein A (2 ng/ml protein A, 1 μCi in TBS/0.06% Tween-20/0.1% bovine serum albumin) for 2 h followed by quantitative determination of radioactivity using phosphor imaging.

2.8. Chemicals

S-Nitrosoglutathione was synthesized as described previously (Hart, 1985); *S*-nitroso-*N*-acetylpenicillamine (SNAP), (*Z*)-1-{*N*-[3-aminopropyl]-*N*-[4-(3-aminopropylammonio)butyl]-amino}-diazen-1-ium-1,2-diolate (spermine-NONOate) and [3-morpholinosydnonimine · HCl were from Alexis (Läufelfingen, Switzerland); Hoechst dye 33528, actinomycin D, cycloheximide, diphenylamine, protein A-sepharose, and sodium nitroprusside were from Sigma (Deisenhofen, Germany). Recombinant human interleukin-1β was generously supplied by Dr. C. Rordorf (Ciba, Basel, Switzerland). Cell death detection ELISA and in situ cell death detection kit were from Boehringer Mannheim (Rotkreuz, Switzerland). Cell culture media and nutrients were obtained from Gibco BRL (Basel, Switzerland) and all other chemicals were either from Merck (Darmstadt, Germany), or Fluka (Buchs, Switzerland).

3. Results

3.1. NO donors and authentic NO gas induce mesangial cell apoptosis

In a first step we used five different NO donors as well as NO gas and evaluated their capacity to induce apoptotic cell death in mesangial cells as measured by internucleosomal cleavage of nuclear DNA. Incubation of mesangial cells with increasing concentrations of *S*-nitrosoglutathione, sodium nitroprusside, *S*-nitroso-*N*-acetylpenicillamine, SIN-1 (Fig. 1a) spermine-NO (Fig. 1b) or media equilibrated with authentic NO gas (Fig. 1c) for 24 h caused a concentration-dependent DNA fragmentation. However, the extent of DNA fragmentation varied markedly with the NO donor used. Authentic NO gas and spermine-NO proved to be strongest inducers, whereas *S*-nitroso-*N*-acetylpenicillamine and SIN-1 were much less active while sodium nitroprusside displayed only a minor increase in DNA fragmentation (Fig. 1). NO gas at a concentration of 300 μM induced approximately 30% DNA fragmentation, whereas spermine-NO and *S*-nitrosoglutathione at 500 μM caused roughly 29% and 14% DNA fragmentation, respectively. DNA cleavage required protein synthesis since incubation of the cells with *S*-nitrosoglutathione in the presence of cycloheximide efficiently reduced DNA degradation, as shown in Fig. 1d. Antioxidants also reduced NO-induced DNA fragmentation (Table 1). When used at the same concentration (1 mM) Trolox, a vitamin E analogue, was much more potent than *N*-acetylcysteine (Table 1). Analysis of DNA by gel electrophoresis reveals the characteristic DNA ladder in mesangial cells exposed to spermine-NO (Fig. 2), thus confirming the results obtained with the diphenylamine reaction. To further extend these studies on the level of the single cell we performed in situ detection of apoptosis by labelling the cellular free 3′-OH ends in genomic DNA using the terminal desoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method. Exposure of mesangial cells to spermine-NO for 16 h results in a

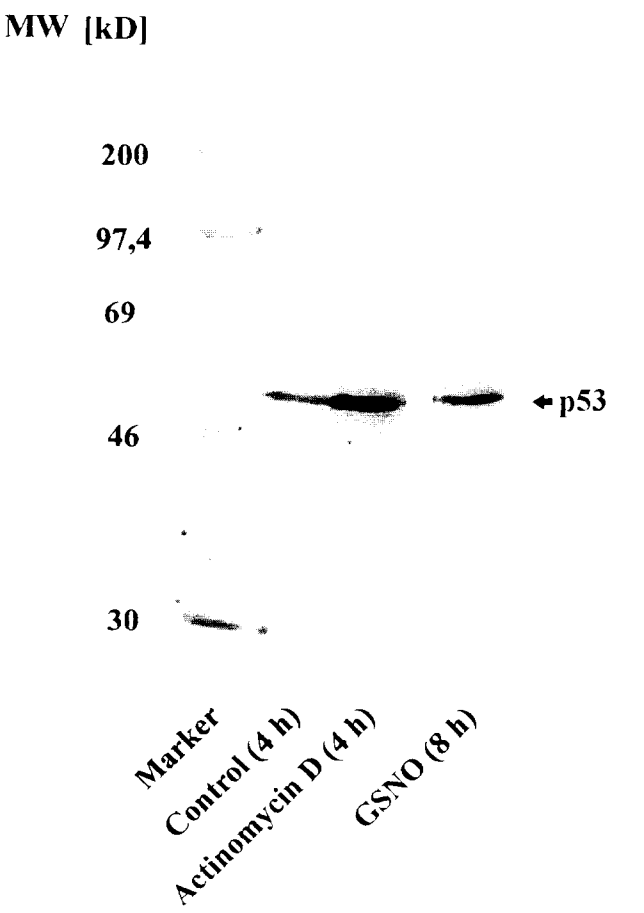

Fig. 4. Immunoprecipitation and Western blotting of p53 from mesangial in cells. Cells ($7,5 \times 10^6$) were treated for the times indicated with vehicle (control), actinomycin D (2 μg/ml) or *S*-nitrosoglutathione (GSNO) (2 mM) and p53 content was analysed as described in Section 2. The figure is representative of 3 similar experiments.

dramatic increase of TUNEL-positive cells (Fig. 3). In parallel with DNA fragmentation, cell morphology was studied. Chromatin condensation which may occur independent of DNA fragmentation (Sun et al., 1994) is another characteristic feature of ongoing apoptosis. Nuclear staining of mesangial cells treated with spermine-NO revealed typical apoptotic chromatin condensation which is not detectable in untreated cells (Fig. 3).

3.2. NO donor S-nitrosoglutathione stimulates p53 expression in mesangial cells

Expression of wild-type p53, a tumour suppressor gene, seems to be closely linked to apoptosis caused by most of the DNA-damaging agents (Lowe et al., 1993; Clarke et al., 1993). The wild-type nuclear phosphoprotein p53, originally characterized as a tumour suppressor protein (Hollstein et al., 1991), acts as a checkpoint control in the cell cycle, allowing the repair of damaged DNA. The block in G_1/S transition which results from p53 activation may cause programmed cell death in the case of severe DNA damage (Baker et al., 1990; Kastan et al., 1991). More recently, evidence has been presented suggesting that the p53 gene product directly participates in the apoptotic process (Caelles et al., 1994) and that nitric oxide can stimulate p53 accumulation in RAW 264.7 macrophages (Messmer et al., 1994). We therefore examined p53 expression in mesangial cells exposed to NO. Immunoprecipitation of p53 from mesangial cell lysates followed by Western blot analysis reveals that p53 is already expressed under basal conditions (Fig. 4). Addition of *S*-nitrosoglutathione for 8 h increased p53 protein level in mesangial cells by approximately 51%. As a positive control we also evaluated the effect of actinomycin D which causes a roughly 133% increase in p53 protein level (Fig. 4).

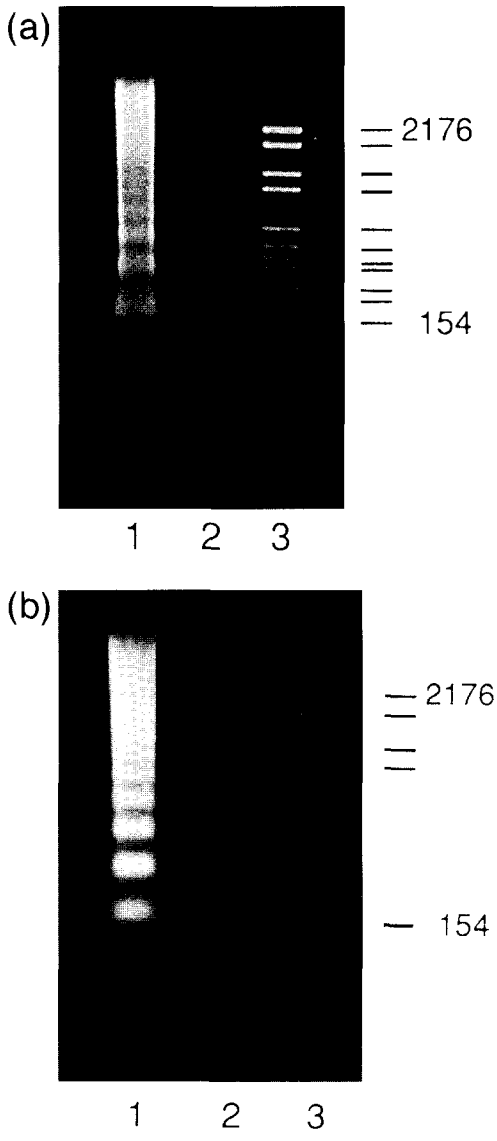

Fig. 5. DNA fragmentation in glomerular endothelial cells by NO donors. Endothelial cells were incubated for 20 h with (a) sodium nitroprusside or (b) *S*-nitrosoglutathione (1 mM, lane 1) or vehicle (lane 2) and DNA fragmentation was analysed by agarose gel electrophoresis as described in Section 2. DNA size marker are shown in lane 3.

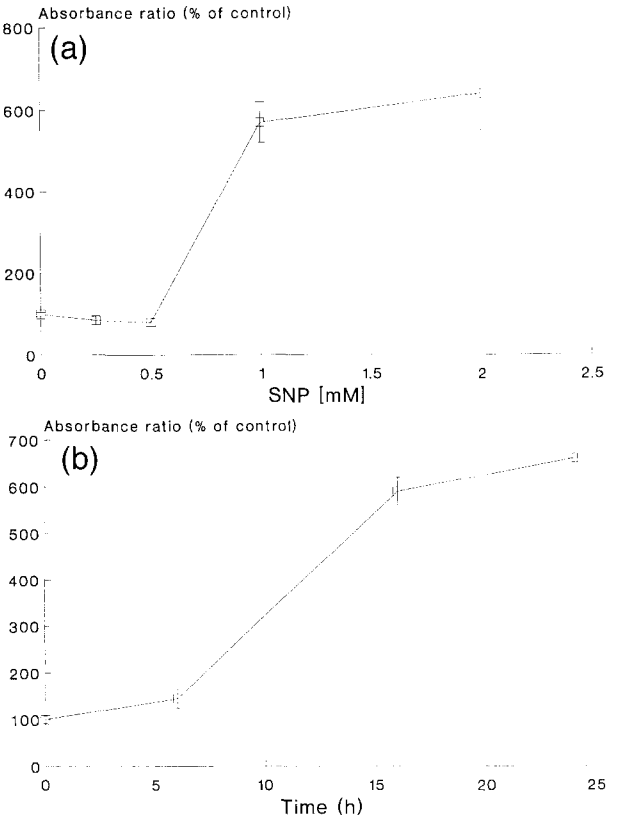

Fig. 6. Induction of DNA fragmentation in glomerular endothelial cells by sodium nitroprusside (SNP). Cells were stimulated for 20 h with the indicated concentrations of sodium nitroprusside (a) or with 1 mM sodium nitroprusside for the indicated time periods (b). Thereafter DNA fragmentation was quantified by an ELISA based on the detection of cytosolic histone/DNA complexes. The absorbance ratio $A_{405\,nm} / A_{486\,nm}$ was measured. Results are means ± S.D. of four independent experiments and are expressed as percentage of untreated control cells.

3.3. NO donors induce apoptosis in glomerular endothelial and epithelial cells

In a next step we evaluated the effects of exogenous NO on pure cultures of glomerular endothelial cells and parietal epithelial cells, two cell types that are in close vicinity to mesangial cells. Incubation of glomerular endothelial cells with sodium nitroprusside (Fig. 5a) or *S*-nitrosoglutathione (Fig. 5b) for 20 h induces massive DNA fragmentation as visualized by gel electrophoresis. This

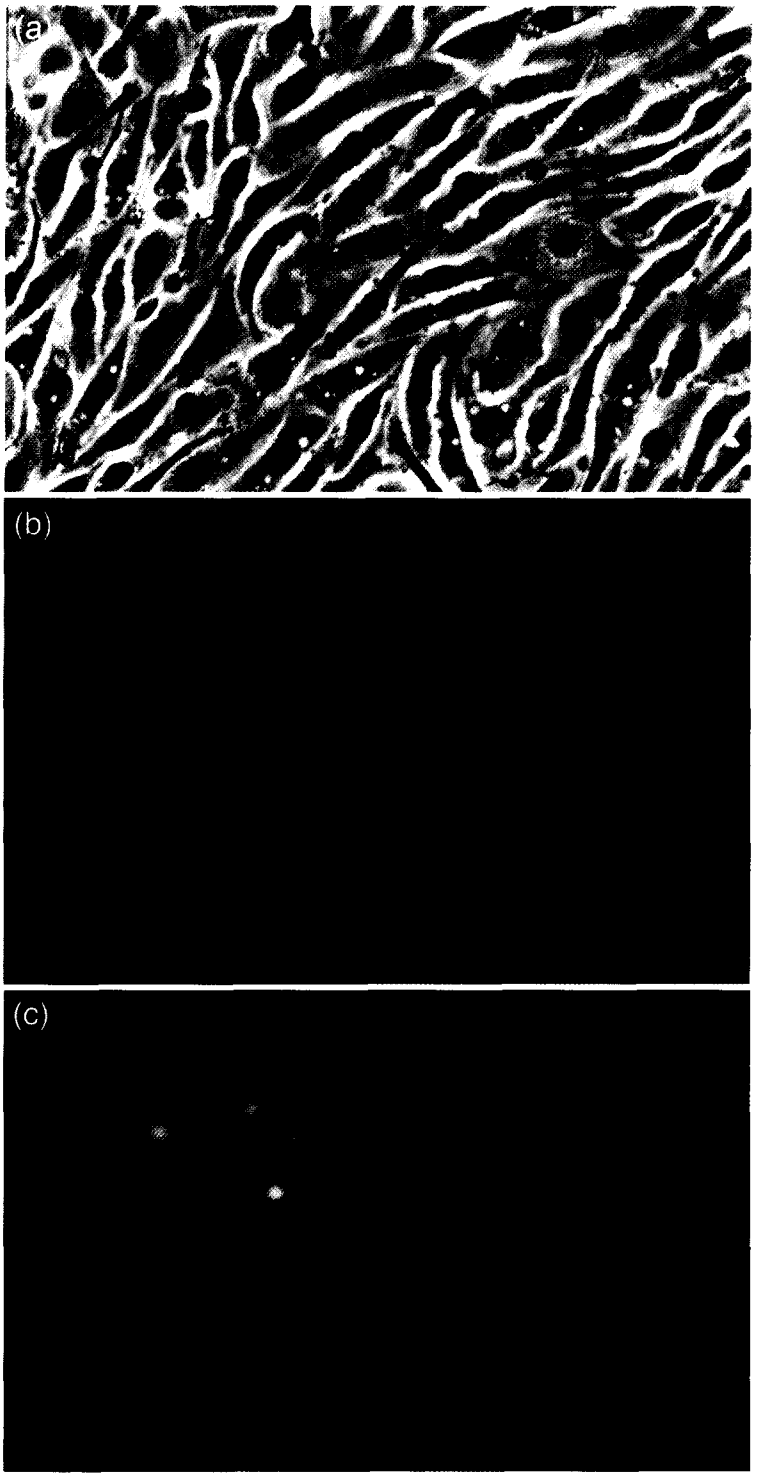

Fig. 7. Detection of NO-induced nuclear chromatin condensation in glomerular endothelial cells ((a)–(c)) and epithelial cells ((d)–(f)). (a) and (d), phase-contrast photograph of untreated (control) cells; (b) and (e), staining of nuclei with Hoe-33258 in untreated control cells; (c) and (f), staining of nuclei with Hoe-33258 in cells treated with sodium nitroprusside (2 mM, (c)) or *S*-nitrosoglutathione (3 mM, (f)) for 20 h.

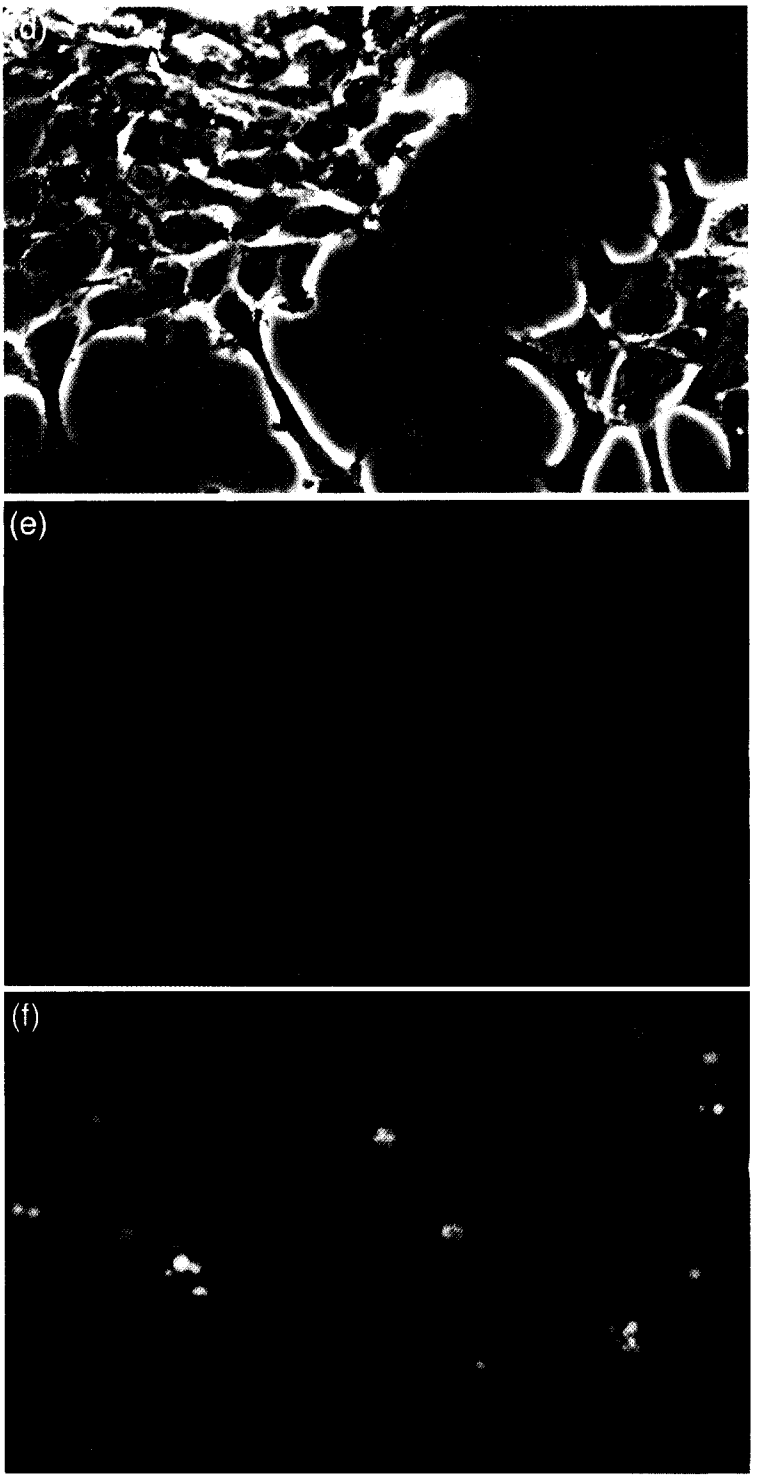

Fig. 7 (continued).

process is paralleled by the appearance of cytosolic DNA/histone complexes as shown in Fig. 6a which occurs after a lag period of approximately 7 h (Fig. 6b). A phase-contrast photograph of control glomerular endothelial cells is shown in Fig. 7a. Staining of the cells with Hoe-33258 results in a homogenous and diffuse nuclear staining (Fig. 7b). After an incubation with sodium nitroprusside for 20 h the vast majority of the cells are detached from the culture plate and float in the supernatant. After carefully spinning down the cells and staining with Hoe-33258 the nuclei of nearly all of the cells display a condensed and fragmented chromatin, a characteristic fea-

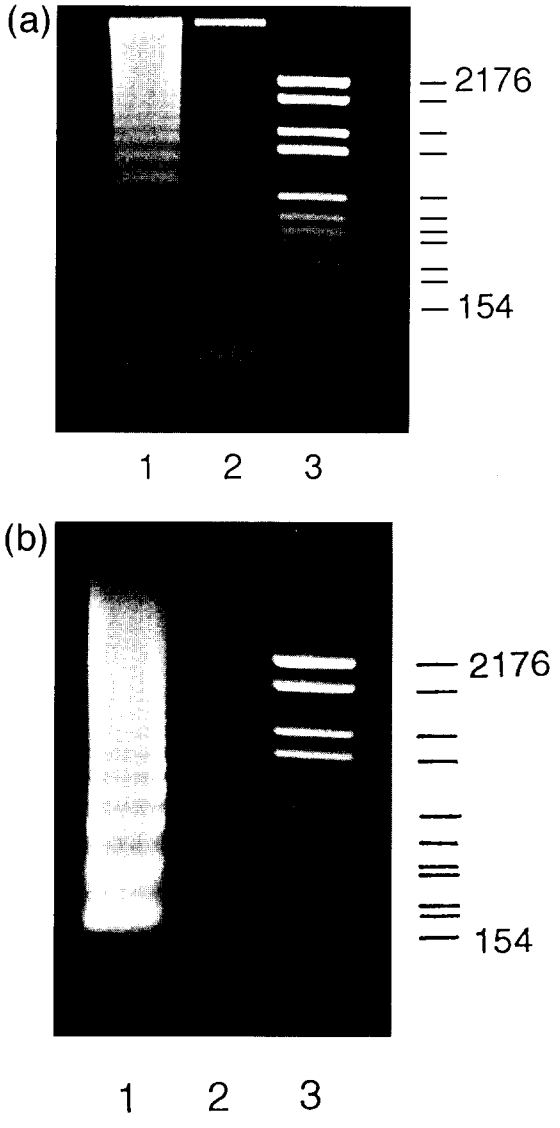

Fig. 8. DNA fragmentation in glomerular epithelial cells by NO donors. Epithelial cells were incubated for 24 h with (a) sodium nitroprusside or (b) *S*-nitrosoglutathione (1 mM, lane 1) or vehicle (lane 2) and DNA fragmentation was analysed by agarose gel electrophoresis as described in Section 2. DNA size markers are shown in lane 3.

ture of apoptosis (Fig. 7c). Comparably, glomerular epithelial cells exposed to the NO donors sodium nitroprusside or *S*-nitrosoglutathione show an excessive DNA fragmentation (Fig. 8) and characteristic chromatin condensation (Fig. 7f).

3.4. Induction of endogenous NO synthesis by interleukin-1β does not trigger apoptosis of mesangial cells or cocultured endothelial and epithelial cells

In previous studies we have shown that interleukin-1β induces the expression of an inducible NO synthase in mesangial cells with a dramatic increase in endogenous NO production (Pfeilschifter and Schwarzenbach, 1990; Pfeilschifter et al., 1992; Mühl and Pfeilschifter, 1995). Our experiments imply that various NO donors induce mesangial cell apoptosis. Therefore we examined whether endogenously formed NO triggers similar effects. Mesangial cells were exposed to interleukin-1β (1–10 nM) for different time periods (up to 48 h) which causes an extensive production of NO as measured by nitrite formation in the cell culture supernatant and by detection of NO synthase expression in parallel cell cultures by Western blot analysis. Under these conditions we consistently failed to detect specific biochemical (diphenylamine assay, DNA agarose elctrophoresis, cytosolic DNA/histone ELISA) or morphological (Hoe-33258 staining) apoptotic changes (data not shown). Moreover, co-cultures of interleukin-1β-induced mesangial cells with glomerular endothelial or glomerular epithelial cells did not cause any detectable apoptotic alterations in these cells (data not shown).

3.5. Interleukin-1β protects mesangial cells from apoptosis caused by exogenous NO

The resistence of mesangial cells and co-cultured glomerular endothelial and epithelial cells to endogenously produced NO was unexpected as we had obtained clear evidence for exogenous NO to be apoptogenic in all three types of glomerular cells examined. Assumingly the amount of NO produced in interleukin-1β-stimulated mesangial cells is not sufficient to initiate the apoptotic process or, alternatively, interleukin-1β not only induces the expression of inducible NO synthase but also stimulates a protecting mediator in mesangial cells. To examine this hypothesis we compared the apoptosis-inducing capability of spermine-NO in control and interleukin-1β-pretreated mesangial cells. As shown in Fig. 9, pretreatment of mesangial cells with interleukin-1β attenuates DNA frag-

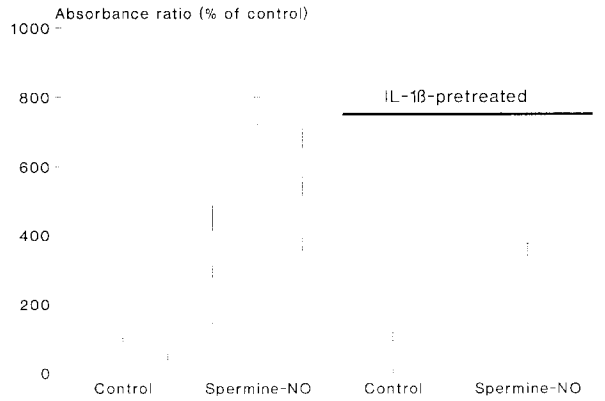

Fig. 9. Interleukin-1β protects mesangial cells from spermine-NO-induced DNA fragmentation. Cells were stimulated for 20 h with vehicle (control) and spermine-NO (500 μM) or pretreated with interleukin-1β (1 nM) for 20 h and subsequently exposed to spermine-NO (500 μM) for another 20 h. Therafter DNA fragmentation was quantified by an ELISA based on the detection of cytosolic histone/DNA complexes. The absorbance ratio A_{405nm} / A_{486nm} was measured. Results are means + S.D. of four independent experiments and are expressed as percentage of untreated control cells.

mentation after exposure to spermine-NO by approximately 50%. These results suggest that interleukin-1β stimulates a protecting factor in mesangial cells that may prevent endogenously produced NO from triggering apoptosis of the cells.

4. Discussion

These experiments indicate that glomerular mesangial cells, endothelial cells, and epithelial cells exposed to NO can undergo cell death with biochemical and morphological changes characteristic of apoptosis. Previously, Baker et al. (1994) have reported that human and rat mesangial cells deprived of growth factors exhibit typical morphological features of apoptosis and we have demonstrated that activation of the cyclic AMP signalling pathway induces apoptosis in mesangial cells which depends on translation but is independent of NO production (Mühl et al., 1996). In vivo the presence of apoptotic bodies in glomeruli of patients with proliferative glomerulonephritis has been described by Harris (1988) and mesangial cells as well as infiltrating neutrophils have been reported to undergo apoptosis in experimental models of glomerulonephritis (Bagchus et al., 1986; Savill et al., 1992; Baker et al., 1994; Shimizu et al., 1995). One of these models is the Thy 1.1 model of self-limited mesangial proliferative glomerulonephritis in the rat (Bagchus et al., 1986). It is induced by injection of complement-fixing antibodies to the Thy 1.1 antigen present on the plasma membrane of mesangial cells which causes an acute complement-dependent mesangial cell damage (mesangiolysis) within 24 h. This is followed by mesangial cell proliferation with excess deposition of matrix which peaks by 5–10 days and results in mesangial hypercellularity. Subsequently the number of mesangial cells and the amount of matrix return to normal over a further 40 days or so (Savill and Johnson, 1995).

There is good evidence that apoptosis of mesangial cells is involved in the early lytic phase in Thy 1.1 nephritis after injection of monoclonal antibodies in vivo (Bagchus et al., 1986) and in vitro (Sato et al., 1996) and thus may account for the rapid mesangiolysis observed during the first 24 h of the disease. Furthermore, apoptosis is observed in resolving mesangial proliferative nephritis. Baker et al. (1994) and Shimizu et al. (1995) have reported that increased apoptosis initially correlates with mesangial cell mitosis, but persists longer and that the majority of cells undergoing programmed cell death are mesangial cells. Apoptosis thus crucially contributes to restoration of normal glomerular cell numbers in the recovery phase of the disease. The mechanisms underlying mesangial cell apoptosis in early and late phases in Thy 1.1 nephritis are unknown. It has been speculated whether mesangial cell survival factors deficiency or active, cell death promoting factors trigger mesangial cell apoptosis (Baker et al., 1994). In this context it is important to note that in the Thy 1.1 glomerulonephritis model a significant NO production has been associated with the phase of mesangiolysis (Cattell et al., 1993). Furthermore, Narita et al. (1995) have shown that blocking NO production in vivo by administration of the NO synthase inhibitor N^G-monomethyl-L-arginine prevents mesangial cell lysis by 90%, thus suggesting a fundamental role of NO in immune-mediated mesangial cell lysis. In turn this may underscore NO's role as a major candidate for initiating mesangial cell apoptosis, at least in the early phase of Thy 1.1 nephritis. Recent studies implicate infiltrating macrophages as a major source of NO formation in glomerulonephritis (Cattell and Cook, 1995), although intrinsic glomerular cells, especially mesangial cells, may substantially contribute to NO synthesis after expression of inducible NO synthase (Pfeilschifter, 1995). Irrespective of the source of NO production our present study provides evidence that NO can trigger apoptosis of mesangial cells as well as glomerular endothelial and epithelial cells. NO has been shown to induce apoptosis in macrophages (Albina et al., 1993; Sarih et al., 1993; Messmer et al., 1995), thymocytes (Fehsel et al., 1995) and chondrocytes (Blanco et al., 1995). Several proposals have been forwarded to account for cytotoxicity. Mechanisms may comprise inhibition of cellular energy supply by interference of NO with oxidative metabolism and respiration as well as with glycolysis, or direct DNA damage (Wink et al., 1991). We have demonstrated in this report that NO causes increased accumulation of p53 which may indicate a relevant signalling step promoting apoptosis of mesangial cells. However, in contrast to RAW 264.7 macrophages where endogenously generated NO after endotoxin and interferon-γ stimulation of the cells induces apoptosis, expression of inducible NO synthase and NO production in interleukin-1β-stimulated mesangial cells does not initiate programmed cell death. These obervations suggest distinct and cell-specific mechanisms of NO-stimulated apoptosis. Interleukin-1β stimulation of mesangial cells not only causes the expression of inducible NO synthase but also induces other important enzymes and mediators. This includes secretory and cytosolic phospholipases A_2 (Pfeilschifter et al., 1989; Schalkwijk et al., 1993), cyclooxygenase-2 and prostaglandins (Nüsing et al., 1996; Pfeilschifter et al., 1989), NADPH oxidase components and oxygen radicals (Radeke et al., 1990; Radeke et al., 1991), and a variety of cytokines and matrix components (for review see Pfeilschifter, 1994). It may be that treatment of the cells with interleukin-1β alters the quantity of protecting molecules against oxygen toxicity, i.e. the levels of intracellular reduced glutathione or the protein levels of bcl-2 or some other members of this family. The importance of radical interactions, especially considering NO and superoxide, has recently been highlighted in chondrocyte apoptosis (Blanco et al., 1995). The balance between NO and oxygen radicals was shown to determine

chondrocyte survival or the type of cell death (apoptosis versus necrosis). Alternatively, phospholipase A_2 or eicosanoids generated by cyclooxygenase 2 may modulate the apoptosis-inducing capability of NO in mesangial cells. Furthermore, we have recently shown that interleukin-1β triggers ceramide production by sphingomyelin hydrolysis and stimulates the mitogen-activated protein kinase cascade in mesangial cells (Huwiler et al., 1996). Ceramide is considered an endogenous regulator of apoptosis (Pushkareva et al., 1995), whereas activation of the classical p42 and p44 isoforms of mitogen-activated protein kinase exerts a potent inhibitory effect on induction of apoptosis via the stress-activated protein kinases (Xia et al., 1995). Future studies of glomerular cell apoptosis have to examine the nature of the proposed protecting principle induced in interleukin-1β-stimulated mesangial cells in order to further characterize the role of NO in initiation and resolution of immunologic injury to kidney mesangium in glomerulonephritis.

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