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Nitric oxide inhibits polyamine-induced apoptosis in the human extravillous trophoblast cell line SGHPL-4

Philip R.Dash, Judith E.Cartwright and Guy St.J.Whitley[1]

Department of Biochemistry and Immunology, St.George's Hospital Medical School, University of London, London SW17 ORE, UK

[1]To whom correspondence should be addressed at: Department of Biochemistry and Immunology, St.George's Hospital Medical School, Cranmer Terrace, London SW17 ORE, UK. E-mail: g.whitley@sghms.ac.uk

BACKGROUND: Polyamines are regulators of proliferation and differentiation in mammalian cells. They are also known to regulate cell survival and apoptosis, although their precise function varies between cell types. We have investigated the effect of polyamines on the apoptosis of human extravillous trophoblasts. METHODS: Using the extravillous trophoblast-derived cell line SGHPL-4 we performed time-lapse microscopy studies to evaluate the induction of apoptosis following exposure to polyamines. RESULTS: The polyamines spermine, and to a lesser extent spermidine, were able to induce apoptosis in extravillous trophoblasts. The induction of apoptosis occurred rapidly and was accompanied by DNA fragmentation and morphological changes consistent with the onset of apoptosis. Apoptosis was inhibited by the broad-spectrum caspase inhibitor Z-VAD-fmk, although no activity was detected using assays for caspase-2, -3, -6, -8 or -9 activity. We demonstrated that an oxidation product of spermine accounted for the induction of apoptosis and implicated the formation of hydrogen peroxide as this oxidation product. We have also demonstrated that exposure to nitric oxide inhibited the onset of spermine-induced apoptosis. CONCLUSIONS: Spermine and spermidine induce apoptosis in extravillous trophoblasts following their oxidation and the production of hydrogen peroxide. Nitric oxide is able to inhibit this apoptosis.

Key words: apoptosis/extravillous trophoblast/hydrogen peroxide/nitric oxide/polyamines

Introduction

The regulation and balance between trophoblast invasion, proliferation and apoptosis are of key importance in the remodelling of the uterine environment and the rapid development of the placenta that occurs in early pregnancy. Cytotrophoblast cells from the fetus differentiate, migrate and invade into the uterine stroma in early pregnancy. The cytotrophoblast stem cells either fuse to form syncytiotrophoblasts or aggregate to form anchoring villous trophoblasts. The latter give rise to a sub-population known as extravillous trophoblasts, which invade the uterine wall and its blood vessels. Extravillous trophoblasts remodel the maternal spiral arteries, displacing smooth muscle and endothelial cells, in order to produce a blood vessel with a larger diameter, increased blood flow and reduced resistance. This is an essential step in establishing and maintaining a normal pregnancy and is necessary for the higher blood requirement of the fetus later in pregnancy (Pijnenborg *et al*., 1981).

The polyamines, spermine, spermidine and putrescine, are ubiquitous regulators of cell proliferation and differentiation (Sooranna and Das, 1995; Facchiano *et al*., 2001; Maccarrone *et al*., 2001). Putrescine is synthesized from arginine via ornithine and can be converted to spermidine and eventually to spermine through the sequential addition of propylamine groups. Their exact function within cells is still uncertain but they appear to exert a wide range of effects including ion channel control, protein synthesis and cell cycle regulation (Facchiano *et al*., 2001). In addition, high levels of polyamine synthesis have been demonstrated in cells stimulated to grow and divide, and also in neoplastic tissue (Pegg, 1988; Celano *et al*., 1989). It has also been suggested that polyamines may be important in placental growth and for a successful pregnancy (Sooranna and Das, 1995). Elevated levels of polyamines have been reported during pregnancy, with the highest levels detected during the first trimester (Sooranna and Das, 1995). The enzymes involved in the oxidation of polyamines have also been detected in maternal serum, the placenta and the decidua during pregnancy (Illei and Morgan, 1979; 1980).

In addition to their role in regulating many essential cellular processes, polyamines have also been implicated in the regulation of cell survival (Poulin *et al*., 1995; Stefanelli *et al*., 1998; 2000). In some cell types, such as smooth muscle cells (Facchiano *et al*., 2001), polyamines have been shown to induce apoptosis, while in others, such as thymocytes (Brune *et al*., 1991), they play a protective role.

The aim of this study was to determine the effect of polyamines on extravillous trophoblasts. We demonstrate that polyamines rapidly induce apoptosis in the extravillous trophoblast cell line SGHPL-4. We also show that the induction of apoptosis is mediated through the oxidation of polyamines by amine oxidases and the production of hydrogen peroxide. Nitric oxide (NO) is known to react with hydrogen peroxide and to share a common biosynthetic origin with the polyamines. It has also been shown to inhibit apoptosis in many cell types and we have demonstrated that it inhibits apoptosis in extravillous trophoblasts (Dash *et al.*, 2003). We therefore investigated the role of NO in the regulation of polyamine-induced apoptosis.

Materials and methods

Materials

The anti-poly (ADP) ribose polymerase (PARP) antibody was purchased from R&D Systems (Oxfordshire, UK). Nitric oxide donors, PAPA-NONOate and DPTA-NONOate, were purchased from Alexis Biochemicals (Nottingham, UK) as were the caspase substrates. The caspase inhibitor ZVAD-fmk was obtained from Calbiochem (Nottingham, UK), while TNFα was obtained from Serotec (UK). The amine oxidase inhibitor, pargyline, and catalase were purchased from Sigma (Poole, Dorset, UK) as was actinomycin D.

Cell lines

SGHPL-4 cells are derived from primary extravillous trophoblasts transfected with the early region of SV40. Characterization of these cells has confirmed an extravillous trophoblast-like phenotype including expression of cytokeratin-7, BC-1, HLA Class I, CD9, hPL and hCG (Choy and Manyonda, 1998; Cartwright *et al*., 1999; Shiverick *et al*., 2001). SGHPL-4 cells were cultured using standard tissue culture techniques in Hams F10 media supplemented with glutamine (2 mmol/l), penicillin (100 units/ml), streptomycin (0.1 mg/ml) and 10% (v/v) fetal calf serum. Jurkat cells were used as a positive control in some of the apoptosis assays as it is well established that apoptosis is induced in these cells following exposure to 10 μmol/l camptothecin for 4 h.

DNA fragmentation (Comet) assay

Cells were treated with 10 μmol/l spermine for 5 h, pelleted and resuspended in PBS. Frosted microscope slides were covered with normal melting point (NMP) agarose. Approximately 10^4 cells were mixed with 65 μl low melting point agarose and placed on top of the layer of NMP agarose. A further layer of NMP agarose (75 μl) was then placed on top of the cell suspension. The slides were then placed in cold lysis solution [2.5 mol/l NaCl, 100 mmol/l EDTA, 10 mmol/l Tris, adjusted to pH 10 with 1% (v/v) Triton X-100 added fresh] for 1 h at 4°C. Slides were then placed in a horizontal electrophoresis tank and covered with electrophoresis buffer (1 mmol/l EDTA, 300 mmol/l NaOH) for 30 min before being electrophoresed at 25 V for 20 min. The slides were neutralized using 0.4 mol/l Tris (pH 7.5) and DNA was visualized by fluorescent microscopy following the addition of ethidium bromide.

Time-lapse digital image microscopy

Apoptosis was observed over time using an Olympus I×70 inverted fluorescence microscope equipped with a Hamamatsu C4742–95 digital camera. The microscope and stage were enclosed within a heated and humidified chamber (37°C) in the presence of 5% CO_2 in air. Images and time-lapse sequences were analysed using Image Pro Plus software from Media Cybernetics. In each treatment 40 cells per field of view were randomly chosen, with three fields of view examined and repeated in triplicate. These cells were observed over time and were scored according to whether they became apoptotic. Apoptotic cells were also scored according to the time at which clear apoptotic morphology was first observed. Apoptotic morphology was considered as cytoplasmic and nuclear shrinkage and a change to a phase-bright appearance, often with the formation of membrane blebs/blisters.

Caspase activity assays

Cells (5×10^6) were pelleted, resuspended in 500 μl lysis buffer [10 mmol/l Hepes, 2 mmol/l EDTA, 0.1% (v/v) NP40, 5 mmol/l dithiothreitol (DTT), 1 mmol/l phenyl-methyl-sulphonyl-fluoride (PMSF), 10 μg/ml pepstatin A, 20 μg/ml leupeptin and 10 μg/ml aprotinin] and incubated on ice for 15 min. The lysates were centrifuged at 10 000 *g* for 2 min, the supernatant removed and protein content quantified using the Bradford assay. In a microtitre plate reader 178 μl of reaction buffer [100 mmol/l Hepes, 20% (v/v) glycerol, 0.5 mmol/l EDTA and 5 mmol/l DTT] was mixed with up to 20 μl of cell lysate (containing 100–200 μg total protein) and 2 μl of a colorimetric caspase substrate. Volumes were adjusted to 200 μl where necessary and plates incubated at 37°C. Absorbance was measured at 405 nm every 30 min to demonstrate enzyme activity. The value for caspase activity was determined after 2 h and is represented as a percentage of the caspase activity in untreated cells (modified from Kim *et al*., 1998).

Greiss reaction

NO was measured indirectly by the Greiss reaction to detect nitrite in the media. The reaction mixture was made by adding 1 ml of a sulphanilamide solution [2% (w/v) sulphanilamide, 5% (v/v) phosphoric acid] to 1 ml of a 0.2 % (w/v) solution of napthylethylenediamine. The reaction mixture (25 μl) was then added to the sample (75 μl) and the absorbance read at 540 nm. A standard curve was generated using serial dilutions of a stock solution of sodium nitrite (0–200 μmol/l).

Western blot analysis

Following SDS-PAGE, proteins were transferred onto Hybond P membrane (Amersham, UK). Membranes were blocked for 1 h in 5% (w/v) non-fat milk. The primary antibody incubation was performed for 1 h at a dilution of 1:1000 for both PARP and caspase-3. The secondary antibodies were used in both cases at a dilution of 1:1000. Detection was performed using Electrochemiluminescence (ECL) Plus (Amersham, UK) according to the manufacturer's instructions. Western blots shown are representative of at least three separate experiments.

Transient transfections

Transient transfections of SGHPL-4 cells were performed in 6-well plates in the presence of 10% FCS using poly-L-ornithine (15 000 Mw) mixed with DNA at a ratio of 0.9:1 (wt:wt). The fusion protein (iNOS-EGFP) plasmid used was produced by Purdie *et al*. (2002). Cells were incubated with 100 μmol/l chloroquine in tissue culture media for 30 min prior to the addition of the poly-L-ornithine/DNA mixture at a final concentration of 5 μg DNA per well. The cells were then incubated for 5 h, washed with PBS and fresh media added. Analysis of the cells was carried out 24 h post-transfection.

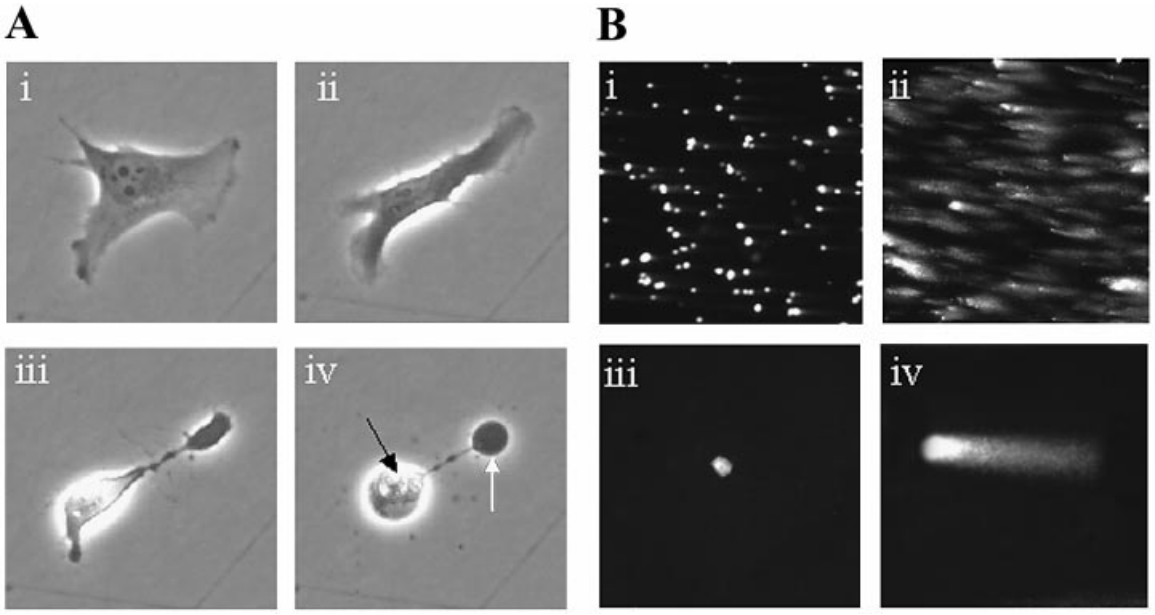


Figure 1. (**A**) Morphological changes over time in an SGHPL-4 extravillous trophoblast cell in response to 10 μmol/l spermine; original magnification ×100. (i) Untreated cell showing normal morphology; (ii) the same cell 3 h after the addition of spermine, now showing signs of cytoplasmic shrinkage; (iii) 4 h exposure to spermine results in further cytoplasmic shrinkage, nuclear condensation and a phase bright appearance; (iv) after 5 h exposure to spermine the cell has shrunk further and the appearance of membrane blebs and blisters (black arrow) and apoptotic bodies (white arrow) can be observed. (**B**) Detection of DNA damage in individual cells using the comet assay. Untreated cells show few signs of DNA damage (i and iii), while cells treated with 10 μmol/l spermine (ii and iv) show evidence of DNA damage in the majority of cells (i and ii objective = ×4; magnification ×40; iii and iv objective = ×10; magnification ×100).

Statistics

The time taken to induce apoptosis in 50% of cells, the EC_{50} value, was determined by non-linear regression using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Triplicate EC_{50} values were then analysed for their statistical significance using Student's *t*-test and error values expressed as standard errors.

Results

Spermine rapidly induces apoptosis in extravillous trophoblasts

Extravillous trophoblast cells, SGHPL-4, were exposed to spermine at doses ranging from 5–100 μmol/l. Time-lapse microscopy was used to monitor the effects of spermine on the SGHPL-4 cells over time. It was found that at doses of ⩾10 μmol/l, spermine rapidly induced typical apoptotic morphology. No effect was observed at a dose of 5 μmol/l (data not shown). A typical example of the morphological changes induced by spermine is shown in Figure 1A. Cells begin to undergo apoptosis after 3–4 h of exposure to spermine and display signs of cytoplasmic shrinkage, nuclear condensation and the appearance of membrane blebs or blisters. In addition the cells become phase-bright. These morphological changes are accompanied by DNA fragmentation, a hallmark of apoptosis. The fragmentation of the DNA was detected using the comet assay (Figure 1B). Fragmented DNA uncoils under assay conditions and migrates out of the nucleus during electrophoresis, as seen in panels (ii) and (iv). By contrast intact DNA from untreated cells remains in the nucleus [panels (i) and (iii].

Spermine and spermidine, but not putrescine, are able to induce apoptosis in extravillous trophoblasts

Spermine is synthesized from spermidine, which in turn is synthesized from putrescine. To determine whether these other polyamines are similarly able to induce apoptosis in extravillous trophoblasts, SGHPL-4 cells were exposed to a range of doses of spermine, spermidine and putrescine. The onset of apoptosis was followed by time-lapse microscopy and the results are shown in Figure 2. Doses of spermine of ⩾10 μmol/l induced apoptosis in 100% of SGHPL-4 cells after ~8 h exposure (Figure 2A), with the time taken to induce apoptosis in half of the cells (the EC_{50} value) of 5.7 h (±0.26). Spermidine at concentrations of ⩾100 μmol/l also induced apoptosis over a similar time-scale (Figure 2B). However, at the 10 μmol/l dose, spermidine appeared to be much less effective than spermine at inducing apoptosis, with an EC_{50} of >20 h. Putrescine was ineffective at inducing apoptosis at any of the concentrations used (Figure 2C).

Spermine-induced apoptosis is independent of PARP and caspase-3 cleavage

To investigate the mechanism of apoptosis induction, the processing of caspase-3 and the cleavage of PARP was investigated by Western blot analysis. The cleavage of the DNA repair enzyme PARP is a common characteristic of apoptosis, as can be seen in camptothecin-treated Jurkat cells (Figure 3A). It can be seen that in Jurkat cells some of the full length PARP protein (116 kDa) is cleaved to form a protein of 85 kDa when the cells undergo apoptosis. However, when

SGHPL-4 cells were treated with spermine, no PARP cleavage was observed at any time-point. To demonstrate that PARP cleavage can occur in SGHPL-4 cells, these cells were treated with a combination of TNFα and actinomycin D (Figure 3B). This treatment induces apoptosis through the activation of death receptor signalling and leads to PARP cleavage. These results suggest that spermine-induced cell death is independent of PARP cleavage.

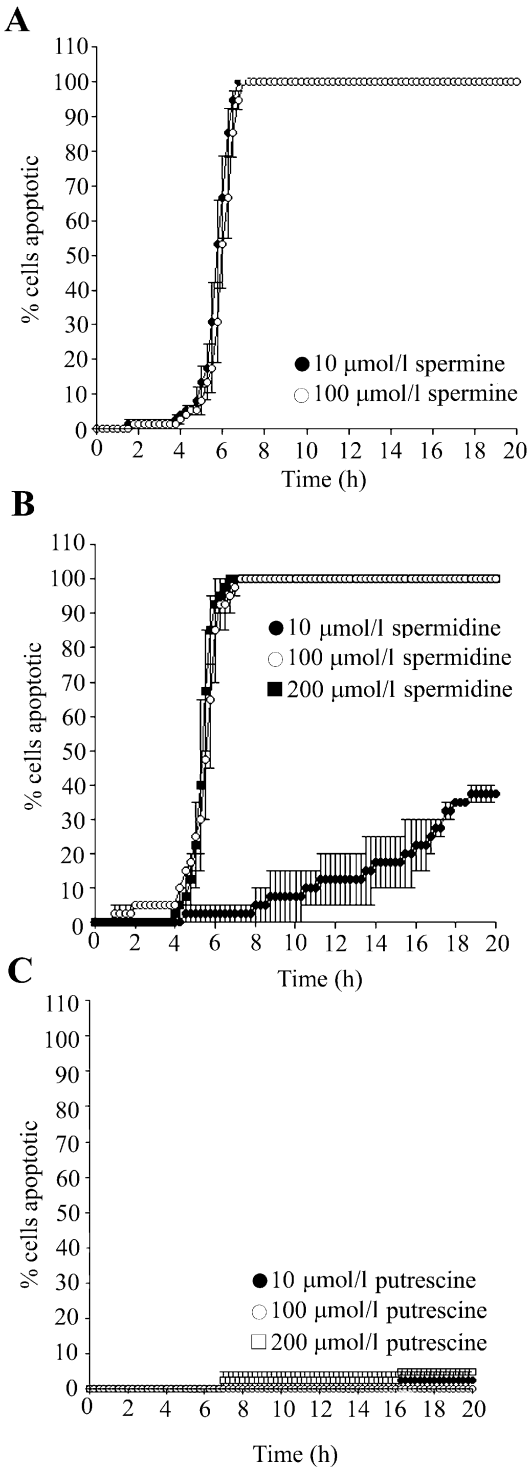


Figure 2. Survival of cells following treatment with different doses of polyamines as measured by time-lapse microscopy. (**A**) Induction of apoptosis with spermine (10 and 100 μmol/l). Values shown are the mean ± SEM of triplicate experiments. (**B**) Induction of apoptosis with spermidine (10, 100 and 200 μmol/l). Values shown are the mean ± SEM of triplicate experiments. (**C**) Survival of cells treated with putrescine (10, 100 and 200 μmol/l). Values shown are the mean ± SEM of triplicate experiments.

The processing of caspase-3 to an active form was investigated by Western blot analysis to detect the presence of cleavage products following caspase processing (Figure 3C). Jurkat cells treated with camptothecin showed a reduction in the amount of full-length caspase-3 (32 kDa) and the appearance of cleavage products at 17 and 11 kDa. However, spermine treatment produced no detectable cleavage of caspase-3 at any time-point, suggesting that spermine-induced cell death was independent of caspase-3 processing.

Spermine-induced apoptosis is independent of caspase-2, -3, -6, -8 and -9 activity but is inhibited by the broad spectrum caspase inhibitor Z-VAD-fmk

The involvement of caspases in spermine-mediated cell death was investigated by measuring the level of caspase activity following exposure to 10 μmol/l spermine for 5 h. Cell lysates were tested for caspase-2, -3, -6, -8 and -9 activity. Caspase activity was expressed as a percentage of untreated SGHPL-4 cells, while a combination treatment of TNFα and actinomycin D was used as a positive control (Figure 4A). Spermine-treated cells showed no increased activity in any of the caspases tested compared with untreated cells. By contrast, cells treated with TNFα and actinomycin D showed increased activity in all the caspases, particularly caspases -3 and -6. Caspase activity was also assayed at different time-points (1, 2, 4, 6, 7 and 8 h exposure to 10 μmol/l spermine), but no activity was detected.

To test for the involvement of any caspase activity, cells were treated with the broad spectrum caspase inhibitor Z-VAD-fmk prior to the addition of 10 μmol/l spermine. Apoptosis was then monitored using time-lapse microscopy and the comet assay. It was found that treatment with spermine induced DNA fragmentation in 100% of cells, as measured by the comet assay, while the addition of Z-VAD-fmk reduced the number of cells showing evidence of DNA damage to ~35% (Figure 4B). Analysis of cell death using time-lapse microscopy also demonstrated that pre-treatment of cells with Z-VAD-fmk significantly reduced the amount of cell death (Figure 4C). These results suggest that there is some caspase activity in SGHPL-4 cells during spermine-induced apoptosis, although not caspases -2, -3, -6, -8 or -9.

Oxidation of spermine contributes to the induction of apoptosis

It has been reported that oxidation products of polyamines may account for their ability to induce apoptosis in other cell types. To determine whether this is the case for spermine-induced extravillous trophoblast apoptosis, SGHPL-4 cells were pre-incubated with 3 mmol/l pargyline, an inhibitor of amine oxidases (Figure 5). Treatment with pargyline was found to significantly ($P < 0.001$) inhibit the onset of apoptosis induced by spermine (the EC_{50} value increased from 5.5 to

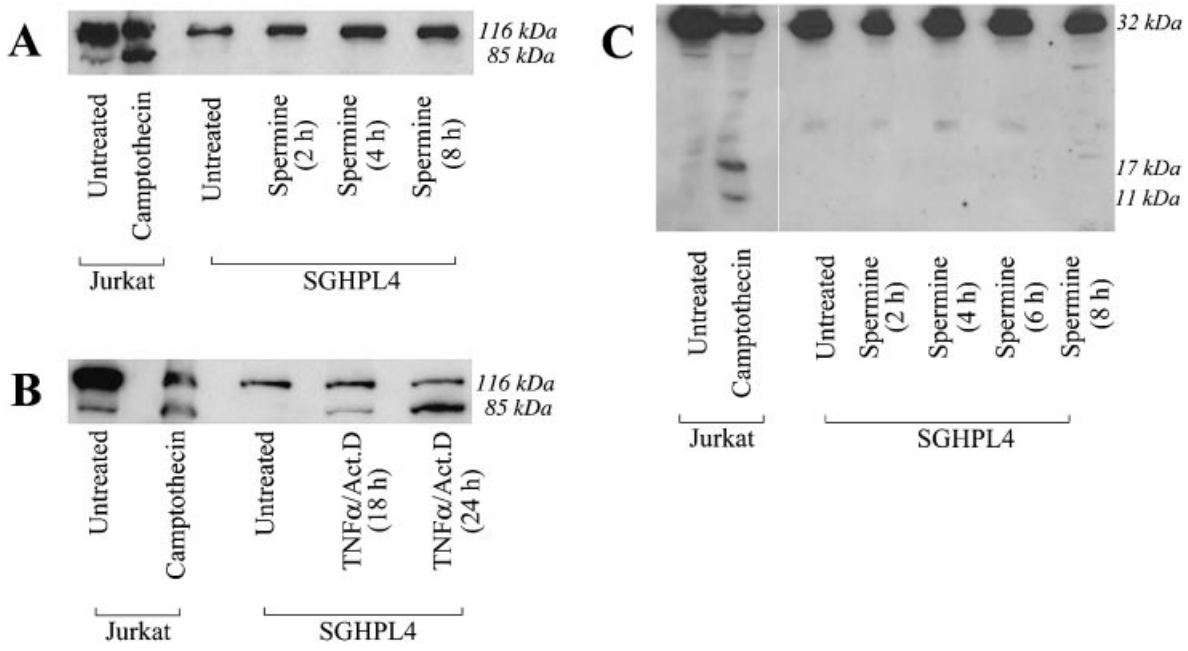


Figure 3. (**A**) Western blot analysis of PARP in its full-length form (116 kDa) and its cleaved product (85 kDa). SGHPL-4 cells were treated with spermine (10 μmol/l) for different periods of time (the positive control for PARP cleavage was Jurkat cells treated with 10 μmol/l camptothecin for 4 h). (**B**) Western blot analysis of PARP cleavage in SGHPL-4 cells following treatment with TNFα (32 ng/ml) and actinomycin D (800 ng/ml) for different periods of time. (**C**) Western blot analysis of caspase-3 processing during apoptosis induced with spermine (10 μmol/l) for different periods of time (the positive control for caspase-3 processing was Jurkat cells treated with 10 μmol/l camptothecin for 4 h).

14.7 ± 0.8 h), and also to reduce the number of cells that are apoptotic over the time-course of the experiment. This result indicates that the induction of apoptosis by spermine was, at least in part, a consequence of its oxidation.

Catalase inhibits spermine-induced apoptosis

Oxidation of polyamines produces hydrogen peroxide, ammonia and the corresponding aldehyde. There is some evidence that any of these products may be toxic, but the strongest evidence exists for the role of hydrogen peroxide as an inducer of apoptosis. To determine whether hydrogen peroxide is responsible for spermine-induced apoptosis, cells were pre-incubated with catalase (Figure 6). Catalase is located in peroxisomes and functions to convert hydrogen peroxide into water and oxygen. It was found that catalase (1000 IU/ml) significantly ($P < 0.001$) inhibited the onset of apoptosis induced with spermine (10 μmol/l). This result suggests that spermine-induced cell death is a catalase-sensitive process and implicates hydrogen peroxide as being, at least in part, responsible for the effect of spermine on extravillous trophoblasts.

Nitric oxide inhibits spermine-induced apoptosis

The effect of NO on spermine-induced apoptosis was investigated using NO donors and by transfecting SGHPL-4 cells to over-express the inducible isoform of nitric oxide synthase (iNOS). Incubation of SGHPL-4 cells with two NO donors, DPTA-NONOate and PAPA-NONOate, demonstrated a dose-dependent inhibition of spermine-induced apoptosis as measured by time-lapse microscopy (Figure 7A,B). Although both NO donors release the same concentration of NO, PAPA-NONOate releases NO more rapidly than DPTA-NONOate (a half-life of NO release of 15 min compared with 3 h). At doses of 100 μmol/l, PAPA-NONOate was able to almost completely inhibit apoptosis in cells treated with 10 μmol/l spermine (Figure 7B). A fusion protein of GFP and iNOS was transfected into SGHPL-4 cells to determine if the endogenous production of NO was also able to inhibit spermine-induced apoptosis. NO produced from the transfected cells significantly delayed the onset of apoptosis (Figure 7C), to a degree that was comparable with the NO donor DPTA-NONOate. As a measure of the NO produced by transfected cells, the amount of nitrite in the media was determined by the Greiss reaction. It was found that iNOS-over-expressing cells produced 3.1 fold (± 0.32) more NO than untransfected cells.

Basal NO production was inhibited by treatment with the general NOS inhibitor L-NAME (5 mmol/l). No significant difference in sensitivity to spermine (10 μmol/l) induced apoptosis was observed (data not shown).

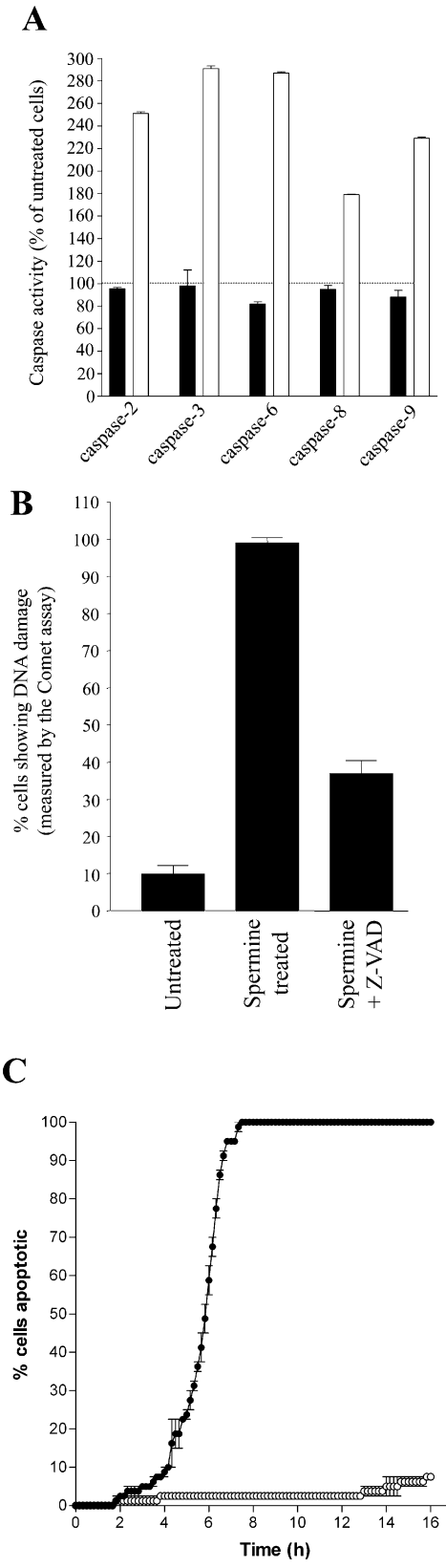
Discussion

Polyamines such as spermine, spermidine and putrescine play a critical, though little understood, role in the regulation of cellular proliferation and differentiation (Facchiano *et al*., 2001). They are also thought to play a role in placental growth and the establishment of a successful pregnancy (Sooranna and Das, 1995), although little is known of their precise role in this area. Placental extravillous trophoblasts invade and remodel the maternal spiral arteries in early pregnancy in order to increase the supply of blood to the fetus later in the pregnancy.

Using an extravillous trophoblast cell line, SGHPL-4, we have investigated the effects of polyamines on these cells.

Polyamines induce apoptosis in many cell types, including endothelial cells and smooth muscle cells (Facchiano *et al*., 2001) but are also known to protect from apoptosis in other cell types such as thymocytes (Brune *et al*., 1991). In extravillous trophoblasts we found that polyamines rapidly induced apoptosis, in the case of spermine at doses of 10 µmol/l and upwards. The onset of apoptosis occurred only a few hours after exposure to spermine and typically led to 100% apoptosis within 4–5 h. Such rapid induction of apoptosis in the entire cell population is unusual and may indicate that these cells are particularly sensitive to the induction of apoptosis by spermine. Spermidine was also an effective inducer of apoptosis, but to a lesser extent than spermine, while putrescine had little effect.

It is unclear how polyamines such as spermine are able to induce apoptosis, although there is much speculation in the literature. It has been proposed that polyamines can directly activate caspases (Stefanelli *et al*., 1998; 1999), promote cytochrome C release from the mitochondria (Stefanelli *et al*., 2000), activate transglutaminase activity (Facchiano *et al*., 2001) or produce toxic metabolites such as aldehydes (Szabo *et al*., 1994) or hydrogen peroxide (Maccarrone *et al*., 2001). We examined characteristic biochemical markers of apoptosis such as PARP cleavage and caspase-3 processing in order to



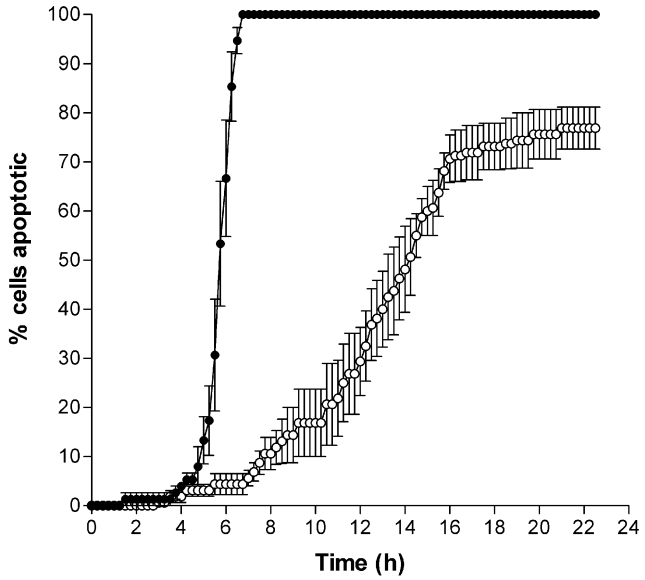


Figure 5. Effect of the amine oxidase inhibitor pargyline on the induction of apoptosis by 10 µmol/l spermine as measured by time-lapse microscopy. Closed circles = spermine (10 µmol/l); open circles = spermine (10 µmol/l) + pargyline (3 mmol/l). Values shown are the mean ± SEM of triplicate experiments.

Figure 4. (**A**) Caspase activity assays to determine the activation of caspases-2, -3, -6, -8 and -9 during apoptosis induced in SGHPL-4 cells by 5 h treatment with spermine (10 µmol/l, black bars) or a combination of TNFα (32 ng/ml) and actinomycin D (800 ng/ml, white bars). Values shown are the mean ± SEM of triplicate experiments. (**B**) The effect of the broad spectrum caspase inhibitor, Z-VAD-fmk (50 µmol/l) on the induction of apoptosis with 10 µmol/l spermine as measured by the comet assay. (**C**) The effect of the broad spectrum caspase inhibitor, Z-VAD-fmk (50 µmol/l) on the induction of apoptosis by spermine as measured by time lapse microscopy. Closed circles = spermine (10 µmol/l); open circles = spermine (10 µmol/l) + Z-VAD-fmk (50 µmol/l). Values shown are the mean ± SEM of triplicate experiments.

determine how spermine was able to induce apoptosis in extravillous trophoblasts. It was found that spermine treatment resulted in morphological changes and DNA fragmentation consistent with apoptosis, but showed no signs of either PARP cleavage or processing of caspase-3. A more detailed examination of caspase activity demonstrated no increased activity of caspases-2, -3, -6, -8 or -9 at any time-point following treatment with spermine. Despite this, treatment of cells with the broad-spectrum caspase inhibitor Z-VAD-fmk was able to significantly inhibit cell death. This suggests that some caspase activity was involved in the cell death, but that it proceeds independently of the major caspase activation pathways. This was an unexpected finding as, to our knowledge, polyamines have not previously been shown to induce apoptosis in this way in other cell types.

This unusual mechanism of apoptosis induction was further investigated by examining the potential role of the metabolic products of polyamines. There is evidence in some cell types that the induction of cell death by polyamines is due to their oxidation products rather than through direct effects (Szabo *et al*., 1994; Maccarrone *et al*., 2001). Polyamines are typically oxidized by a class of enzymes known as amine oxidases which include specific polyamine oxidases such as spermine oxidase. It has been shown that amine oxidases are present in the serum, amniotic fluid and retroplacental serum during pregnancy, and can be produced by the decidua at high local concentrations (Illei and Morgan, 1979; 1980).

The products of polyamine oxidation are hydrogen peroxide, ammonia and the relevant aldehyde, all of which have been reported to induce cellular stress and apoptosis. Pargyline, an inhibitor of amine oxidases, and catalase, an enzyme involved in the metabolism of hydrogen peroxide, were able to significantly increase the survival times of extravillous trophoblast cells exposed to spermine, suggesting that at least some of its apoptosis-inducing effect is due to its oxidation and the formation of hydrogen peroxide. The inability of putrescine to induce apoptosis may reflect the fact that putrescine is oxidized by separate enzymes to spermine and spermidine (Morgan, 1987; Sessa and Perin, 1994). It is likely that oxidation occurs outside the cells in the culture medium, as it has been shown that amine oxidases are present in serum and we have observed that apoptosis does not occur in the absence of serum (unpublished data). This hypothesis is supported by the effect of catalase, which is too large a molecule to readily penetrate the plasma membrane.

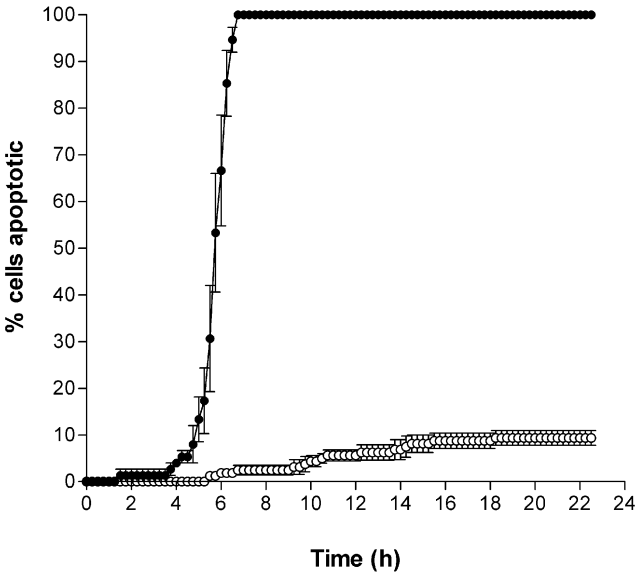


Figure 6. Effect of catalase on the induction of apoptosis induced by 10 μmol/l spermine as measured by time-lapse microscopy. Closed circles = spermine (10 μmol/l); open circles = spermine (10 μmol/l) + catalase (1000 IU/ml). Values shown are the mean ± SEM of triplicate experiments.

Hydrogen peroxide is a well-known reactive oxygen species capable of initiating lipid peroxidation, cellular stress and apoptosis. There is some evidence that, in certain circumstances, hydrogen peroxide is able to inhibit caspase activity (Hampton *et al*., 1998). It is possible that, in extravillous trophoblasts, hydrogen peroxide produced from polyamines suppresses caspase activity but promotes cellular stress, ultimately leading to the activation of specific apoptotic pathways that are independent of the major caspases. The addition of hydrogen peroxide directly to the tissue culture media was found to induce cell death in SGHPL-4 cells at doses comparable to that which can be theoretically produced from the oxidation of 10 μmol/l spermine (data not shown). However, this cell death was morphologically distinct from that induced by spermine and occurred over a much longer period, suggesting that the production of hydrogen peroxide is necessary, but not sufficient, for the induction of apoptosis by spermine.

We and others have previously shown that the signalling molecule NO is involved in pregnancy through its action as a vasodilator (Williams *et al*., 1997) and as an important regulator of trophoblast functions such as implantation, differentiation, motility, invasion and apoptosis (Lyall *et al*., 1998; Cartwright *et al*., 1999; 2002; Gagioti *et al*., 2000). NO is derived from L-arginine in a reaction catalysed by NOS, of which three isoforms exist. Cells of the placenta express at least two of these isoforms, the calcium-calmodulin dependent isoform, eNOS, and the calcium independent isoform, iNOS, that is expressed following growth factor stimulation (Zarlingo *et al*., 1997; Martin and Conrad, 2000; Yoshiki *et al*., 2000; Hambartsoumian *et al*., 2001).

The effect of NO on polyamine-induced extravillous trophoblast apoptosis was investigated using NO donors and iNOS over-expression. Exogenous NO from the NO donors DPTA-NONOate and PAPA-NONOate both inhibited spermine-induced apoptosis. DPTA-NONOate (half-life of decomposition to release NO of 3 h) delayed the onset of apoptosis, while the highest dose of PAPA-NONOate (100 μmol/l, half-life of 15 min) completely prevented apoptosis in 90% of trophoblast cells treated with spermine. NO produced endogenously by cells transfected to over-express iNOS also significantly inhibited apoptosis, with effects comparable with those of DPTA-NONOate. We have previously demonstrated that SGHPL-4 cells express eNOS and iNOS (Cartwright *et al*., 1999; 2002). Inhibition of this basal NO production with the

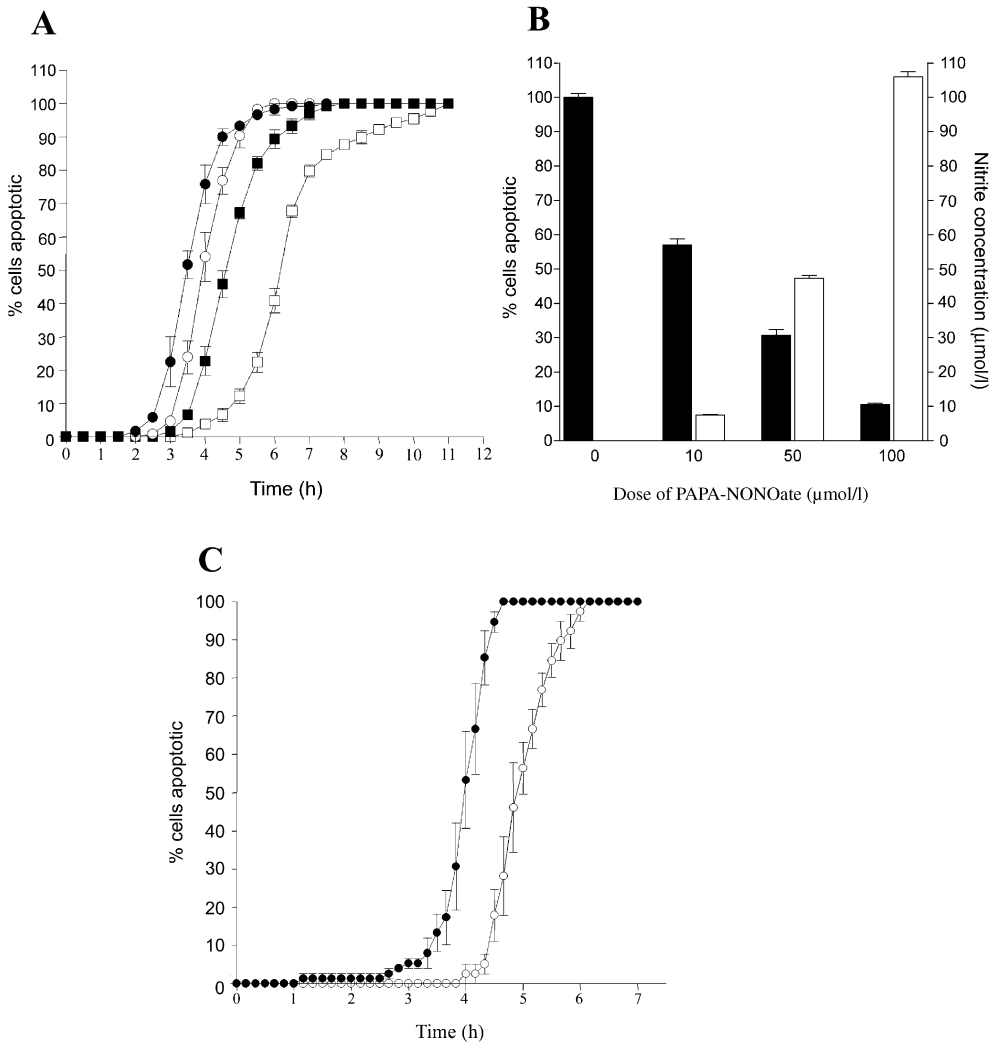


Figure 7. The effect of nitric oxide on apoptosis induced with 10 µmol/l spermine. (**A**) Effect of increasing doses of the NO donor DPTA-NONOate (half-life = 3 h) on spermine-induced apoptosis as measured by time-lapse microscopy. Closed circles = spermine (10 µmol/l) only; open circles = spermine + 10 µmol/l DPTA-NONOate; closed squares = spermine + 50 µmol/l DPTA-NONOate; open squares = spermine + 100 µmol/l DPTA-NONOate. Values shown are the mean ± SEM of triplicate experiments. (**B**) Effect of increasing doses (0–100 µmol/l) of the NO donor PAPA-NONOate (half-life = 15 min) on spermine-induced apoptosis. The white bars represent the concentration of nitrite detected in the media using the Greiss reaction and are an indication of the amount of NO the cells are exposed to. The black bars represent the percentage of cells that became apoptotic during a 24 h exposure to spermine, as measured by time-lapse microscopy. Values shown are the mean ± SEM of triplicate experiments. (**C**) Effect of transiently transfecting SGHPL-4 cells to over-express the inducible isoform of nitric oxide synthase (iNOS). Cells were transiently transfected with a gene for a fusion protein of GFP and iNOS. Successfully transfected cells were identified by their expression of GFP and were followed over time by time-lapse microscopy to determine their response to spermine (10 µmol/l) induced apoptosis. Closed circles = spermine only; open circles = GFP-iNOS transfected cells + spermine. Values shown are the mean ± SEM of triplicate experiments.

general NOS inhibitor L-NAME had no effect on the sensitivity of cells to spermine-induced apoptosis. This was unexpected, as we have previously demonstrated that basal NO production in extravillous trophoblasts plays an important role in protecting these cells from apoptotic stimuli such as TNFα and actinomycin D. This result suggests that the relatively low concentration of NO produced basally by these cells is insufficient to offer any significant protection from the apoptotic effects of hydrogen peroxide. It is possible that NO levels must be stimulated to rise above a threshold to have any protective effect from spermine, and that this occurs when iNOS is over-expressed in cells or when NO donors are used. This may also occur physiologically when iNOS expression is stimulated by growth factors or cytokines, when eNOS activity is enhanced following phosphorylation or due to increased NO production by cells in the local environment.

NO can react with hydrogen peroxide to form a variety of reaction products and can protect from reactive oxygen species toxicity (Wink *et al.*, 1993). We have implicated hydrogen peroxide production as a constituent of spermine-induced apoptosis and it is therefore possible that rapid production of NO is needed to counter the effects of hydrogen peroxide. There are a number of ways in which NO may act to prevent the effects of hydrogen peroxide. It may react with it in the

media and convert it to other, shorter lived species such as peroxynitrite or the hydroxyl radical (Filep *et al*., 1997). These are more reactive species than hydrogen peroxide which, if produced in the media, may react harmlessly with serum proteins before they are able to diffuse into cells and cause damage. Another possibility is that hydrogen peroxide toxicity is mediated through lipid peroxidation and subsequent cellular stress, as it has been suggested that NO may be able to inhibit lipid peroxidation by interfering with the propagation stage of the peroxidation chain reaction (Hogg *et al*., 1993; Kelley *et al*., 1999). Since both amine oxidases and polyamines are present in the placenta the synthesis of hydrogen peroxide is possible. Under these circumstances NO may play an important role in regulating the exposure of the placenta to potentially damaging reactive oxygen species.

As well as its potential role in limiting the damage caused by hydrogen peroxide and other reactive oxygen species, it is known that NO can exert its anti-apoptotic effects through the direct suppression of caspase activity following nitrosylation of the active site cysteine residue (Mannick *et al*., 1999). We have previously demonstrated that NO can suppress caspase activity in SGHPL-4 cells following induction of apoptosis with TNFα and actinomycin D, and that even basal NO production is sufficient for the complete nitrosylation of caspase-3 (Dash *et al.*, 2003). Although no activity of caspases-2, -3, -6, -8 or -9 was detectable in cells treated with spermine it remains possible that as yet unidentified caspases may be inhibited by NO through nitrosylation.

Further work will seek to determine the mechanisms through which NO protects extravillous trophoblasts from polyamine- and hydrogen peroxide-induced apoptosis. Understanding the balance between the factors which can regulate oxidative stress within the placental environment may have profound implications for trophoblast function in early pregnancy.

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