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Nitric oxide protects human extravillous trophoblast cells from apoptosis by a cyclic GMP-dependent mechanism and independently of caspase 3 nitrosylation☆

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Abstract

Apoptosis is thought to play an important regulatory role in placental development and inappropriate trophoblast apoptosis has been implicated in complications of pregnancy such as pre-eclampsia. Here we show that apoptosis of a human extravillous trophoblast-derived cell line (SGHPL-4) can be regulated by nitric oxide (NO). Nitric oxide produced exogenously by the addition of NO donors was able to delay or inhibit apoptosis induced by a combination of tumour necrosis factor α and actinomycin D and to suppress the activity of caspase 3. Treatment with hepatocyte growth factor (HGF) stimulated expression of the inducible isoform of NO synthase and was also able to protect SGHPL-4 cells from caspase 3 activation and apoptosis. The inhibition of basal NO production with NO synthase inhibitors was shown to sensitise cells to apoptotic stimuli and to reduce the level of endogenous caspase 3 nitrosylation. The anti-apoptotic effects of NO in these extravillous trophoblast cells appear to be mediated through the production of cyclic GMP as inhibitors of soluble guanylate cyclase inhibited the protective effect of both HGF and NO donors.

Introduction

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 extravillous trophoblasts which invade the uterine wall and its blood vessels. At this stage the extravillous trophoblasts remodel the maternal spiral arteries, displacing smooth muscle and endothelial cells, 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. An inability of extravillous trophoblasts to fully invade into and modify the uterine environment has been implicated in disorders of pregnancy such as pre-eclampsia and intrauterine growth restriction (IUGR)[1] [1,2].

Nitric oxide (NO) is an important signaling molecule that acts in many tissues to regulate a diverse range of physiological processes including vasodilation, neuronal function, inflammation, and immune function [3,4]. Nitric oxide is derived from L-arginine in a reaction catalysed by the nitric oxide synthase (NOS) enzyme, of which three isoforms exist. Cells of the placenta express at least two of these

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[1] Abbreviations used: IUGR, intrauterine growth retardation; TNFα, tumor necrosis factor α; TRAIL, TNFα-related apoptosis-inducing ligand; HGF, hepatocyte growth factor; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; L-NAME, L-nitro-arginine-methyl ester; L-NMMA, N^G-monomethyl-L-arginine; PARP, poly(ADP-ribose) polymerase; ADMA, asymmetric dimethylarginine.

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isoforms, the calcium–calmodulin-dependent isoform eNOS and the calcium-independent isoform iNOS that is expressed following growth factor stimulation [5–8]. Nitric oxide is involved in pregnancy through its action as a vasodilator [9] and its role in regulating trophoblast function. Implantation of the blastocyst [10], trophoblast differentiation [11], and motility and invasion [12] may all be regulated by NO. Dysregulation of NO production has been implicated in pre-eclampsia and IUGR [13–16].

Apoptosis of trophoblast cells occurs during normal pregnancy [17,18], while excessive apoptosis of syncytiotrophoblasts and cytotrophoblasts, including the extravillous population, has been observed in pregnancies complicated with pre-eclampsia [19–22]. Excessive apoptosis of trophoblast cells is likely to compromise their ability to modify the uterine environment and may be a contributing factor to complications of pregnancy. Trophoblast apoptosis has been shown to be unusual in many respects; for example, cytotrophoblasts are resistant to CD95/Fas ligand [23]- and TRAIL [24]-induced apoptosis. Phosphatidylserine, typically expressed on the outer surface of apoptotic cells as a label for phagocytosis, is involved in trophoblast differentiation and is often expressed on the surface of healthy trophoblasts throughout the placenta [25,26].

Nitric oxide can act both to induce and to protect from apoptosis depending on the cell type and concentration [27–30]. Many of the inhibitory effects of NO on apoptosis are mediated through the elevation of cGMP levels and the activation of cGMP-dependent protein kinases, although cGMP-independent mechanisms have also been reported. One of the main cGMP-independent mechanisms of mediating the effects of NO is the nitrosylation and inactivation of apoptotic proteins such as caspase 3 [30,31].

There has been little research into the regulation of extravillous trophoblast apoptosis. Research into the role and regulation of these cells is hampered by the difficulty of isolating sufficient quantities of pure primary extravillous trophoblasts. In this article we have used the extravillous trophoblast-derived cell line SGHPL-4 as a model to investigate the hypothesis that NO is able to protect extravillous trophoblasts from apoptosis.

Materials and methods

Materials

Caspase 3 substrate (Z-DEVD-pNA) and anti-PARP polyclonal antibody were purchased from Biosource UK. TNFα was obtained from Serotec, UK, and HGF was purchased from R&D Systems (UK). The anti-iNOS antibody (clone N-20), Protein-G Plus-agarose and anti-caspase-3 monoclonal antibody, clone E-8 (and agarose conjugate), were from Santa Cruz (Autogen Bioclear, UK). Mouse IgG1 was from Sigma, UK. Nitric oxide donor, PAPA-NONOate, was purchased from Alexis Biochemicals (UK) as were the iNOS inhibitor 1400W and the soluble guanylate cyclase inhibitor NS2028. Camptothecin, the caspase inhibitor ZVAD-fmk, and the anti-nitrosocysteine polyclonal antibody were obtained from Calbiochem (UK).

Cell lines

SGHPL-4 cells are derived from first trimester human 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 [12,32,33]. SGHPL-4 cells were cultured using standard tissue culture techniques in Ham's F10 medium supplemented with glutamine (2 mM), penicillin (100 units/ml), streptomycin (0.1 mg/ml), and 10% (v/v) fetal calf serum.

Time-lapse digital image microscopy

Apoptosis was observed over time using an Olympus IX70 inverted microscope equipped with a Hamamatsu C4742-95 digital camera. The microscope and stage were enclosed within a heated chamber (37°C) and cells were cultured in 5% CO_2 in air. Images were captured every 15 min and analysed using Image Pro Plus software (Media Cybernetics, USA). In each treatment 40 cells per field of view were randomly chosen at the beginning of the time-lapse sequence and were scored according to whether they became apoptotic over the time course of the experiment. 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.

Antisense oligonucleotide transfection

Cells were transfected with antisense phosphorothioate derivatives of oligodeoxynucleotides according to the method of Noiri et al. [34]. Oligonucleotides were synthesized by MWG-Biotech AG (Milton Keyenes, UK) and were either antisense to iNOS mRNA or a scrambled oligonucleotide sequence as a control.

Caspase activity assays

Cells (5×10^6) were pelleted, resuspended in lysis buffer (10 mM Hepes, 2 mM EDTA, 0.1% (v/v) NP40, 5 mM DTT, 1 mM 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 was removed, and protein content was quantified using the Bradford assay. To determine caspase activity 178 μl of reaction buffer (100 mM Hepes, 20% (v/v) glycerol, 0.5 mM EDTA and 5 mM DTT) was mixed with up to

20 μl of cell lysate (containing 100–200 μg total protein) and 2 μl of a colorimetric caspase 3 substrate (Z-DEVD-pNA, 5 mM stock in DMSO). 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. Caspase activity was determined after 2 h and represented as the percentage change in absorbance of treated cells compared with untreated cells.

Detection of nitrosylated caspase 3

Caspase 3 was immunoprecipitated overnight with an anti-caspase 3 monoclonal antibody–agarose conjugate following preclearing of the lysate with mouse IgG1 and Protein-G Plus-agarose. Pellets were washed five times with PBS and finally resuspended in gel loading buffer in the absence of reducing agents. Following SDS–PAGE, proteins were transferred onto Hybond P membrane (Amersham, UK) and probed with an anti-nitrosocysteine polyclonal antibody at room temperature for 1 h at a 1:1000 dilution. Detection was preformed using ECL Plus (Amersham, UK) according to the manufacturer's instructions. To test the specificity of the antibody for nitrosocysteine some of the samples were treated with 1 mM $HgCl_2$, which selectively removes nitrosocysteine groups, prior to electrophoresis. Gel loading was controlled by probing with anti-caspase 3 monoclonal antibody. All treatments were prepared in triplicate. The bands were quantified using FluorChem software (Alpha Innotech Corporation, UK) and results normalised to control for differences in protein loading.

Western blot analysis

Apoptosis was induced as described above, with Jurkat cells used as a positive control. Following SDS–PAGE, proteins were transferred onto Hybond P membrane (Amersham, UK). When detecting poly(ADP-ribose) polymerase (PARP), membranes were blocked for 1 h in 5% (w/v) nonfat milk. Primary antibody incubation was performed for 1 h at room temperature at a dilution of 1:1000. Detection was performed using ECL Plus (Amersham, UK) according to the manufacturer's instructions. Western blots shown are representative of at least three separate experiments.

Statistics

The time taken to induce apoptosis in 50% of cells, the EC_{50} value, was determined by nonlinear regression using GraphPad Prism (GraphPad Software, USA). Triplicate EC_{50} values were then analysed for their statistical significance using a Student *t* test and error values were expressed as standard errors of the mean.

Results

Induction of apoptosis in trophoblasts

Incubation of cells with a combination of TNFα (30 ng/ml) and actinomycin D (800 ng/ml) induced apoptosis in 90% of SGHPL-4 cells within 16 h and was used throughout this study to induce apoptosis. Time-lapse digital image microscopy of a cell undergoing apoptosis is shown in Fig. 1A. Apoptosis was confirmed using the broad-spectrum caspase inhibitor ZVAD-fmk (50 μM), which protected cells from TNFα/actinomycin D-induced cell death (data not shown). Apoptosis was further demonstrated by the detection of cleaved poly(ADP-ribose) polymerase (PARP) by Western blot analysis (Fig. 1B).

NO donors delay or prevent the onset of apoptosis

The NO donor PAPA-NONOate spontaneously decomposes at physiological pH (with a half-life of 15 min) to produce NO. The effect of increasing doses of the NO donor (10–100 μM) on cell survival following exposure to TNFα/actinomycin D was studied. The time taken for TNFα/actinomycin D to induce apoptosis in 50% of SGHPL-4 cells was termed the EC_{50} and was typically 8.4 h (EC_{50} 8.39 ± 0.14 h) (Fig. 2A). When the cells were incubated in the presence of PAPA-NONOate there was a dose-dependent delay in the onset of apoptosis (Fig. 2B). The addition of 10 μM PAPA-NONOate significantly shifted the time taken to induce apoptosis in 50% of cells to 12.3 ± 1.43 h ($P = <0.001$). The use of higher concentrations of the NO donor resulted in an EC_{50} for the induction of apoptosis of 15.51 ± 0.88 h (50 μM PAPA-NONOate, $P \leq 0.001$) and 21.61 ± 2.06 h (100 μM PAPA-NONOate, $P < 0.001$). This last dose of the NO donor more than doubles the survival times of the cells compared with TNFα/actinomycin D alone (EC_{50} is increased 164%) (Fig. 2A).

Hepatocyte growth factor upregulates iNOS and delays the onset of apoptosis

Exposure of SGHPL-4 cells to HGF (10 ng/ml) has been shown to increase the expression of iNOS protein [12,35] and was confirmed in Fig. 3A. Treatment of SGHPL-4 cells overnight with HGF was found to significantly delay the onset of apoptosis induced with TNFα/actinomycin D (Fig. 3B). In the absence of HGF treatment TNFα/actinomycin D was able to induce apoptosis in 50% of cells in 9.29 ± 0.45 h; when the cells were treated with HGF this increased to 13.52 ± 0.24 h ($P < 0.0028$, $n = 3$). Treatment of cells with the iNOS-specific inhibitor 1400W for 24 h prior to the addition of HGF was able to inhibit this protective effect ($EC_{50} = 8.86 \pm 0.28$ h, $P < 0.046$, $n = 3$), suggesting that the HGF-induced protection from apoptosis involves the generation of NO by iNOS. The protection mediated by HGF exposure was comparable to that mediated by expo-

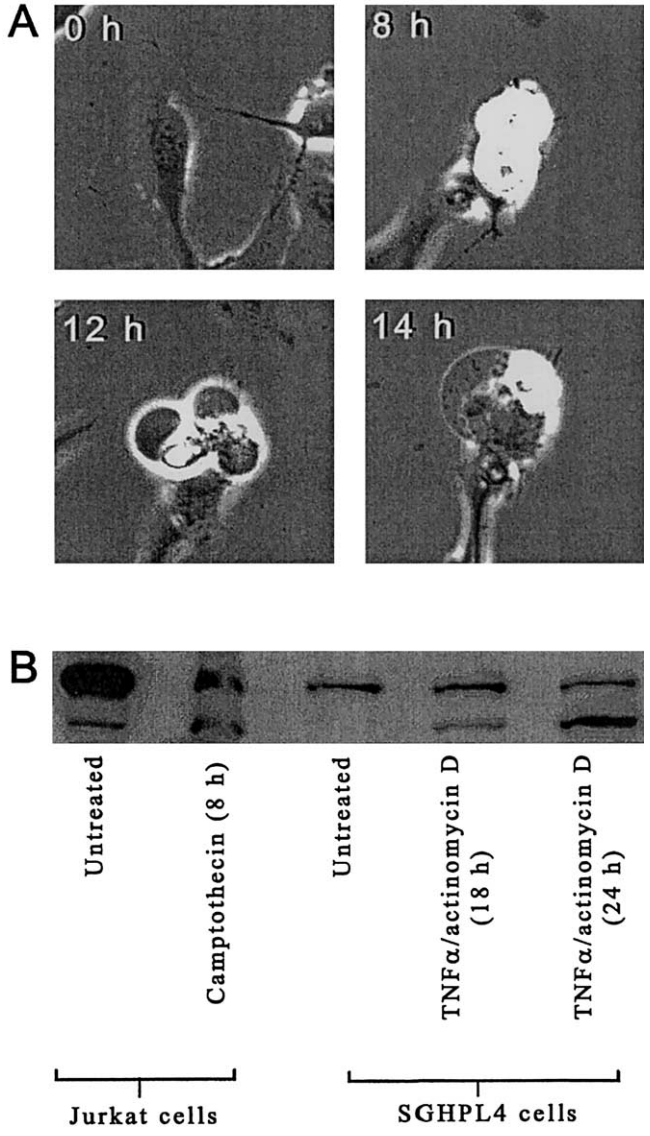


Fig. 1. (A) Time-lapse images of SGHPL-4 trophoblast cells undergoing apoptosis in response to treatment with a combination of TNFα (30 ng/ml) and actinomycin D (800 ng/ml). A typical cell at the start of the time course is shown at time zero. After 8 h exposure to TNFα/actinomycin D, cells begin to show cytoplasmic shrinkage and become phase bright. By 12 h membrane blebs and blisters are commonly observed. The process typically ends with the formation of large blisters, shown here after 14 h (B) Western blot analysis of PARP cleavage from the full-length 118-kDa fragment to the cleaved 85-kDa fragment demonstrates that the apoptotic morphology correlates with typical biochemical events associated with apoptosis. Jurkat cells are known to undergo apoptosis when treated with camptothecin and were therefore used as a positive control.

sure to 10 μM PAPA-NONOate, but is not as effective as a 100 μM dose of the NO donor. To confirm the role of iNOS in HGF-mediated protection cells were transfected with antisense oligonucleotides designed to inhibit the translation of iNOS mRNA (Fig. 3C). It was found that HGF was unable to inhibit apoptosis in cells transfected with iNOS antisense oligonucleotides, confirming the hypothesis that iNOS transcription is necessary for the anti-apoptotic action of HGF. No effect of scrambled oligonucleotides was observed.

Caspase 3 activity is reduced by an NO donor and HGF

Both exogenous NO in the form of the NO donor PAPA-NONOate and endogenous NO in the form of HGF-stimulated iNOS expression were able to reduce apoptosis as measured by time-lapse microscopy. To investigate whether

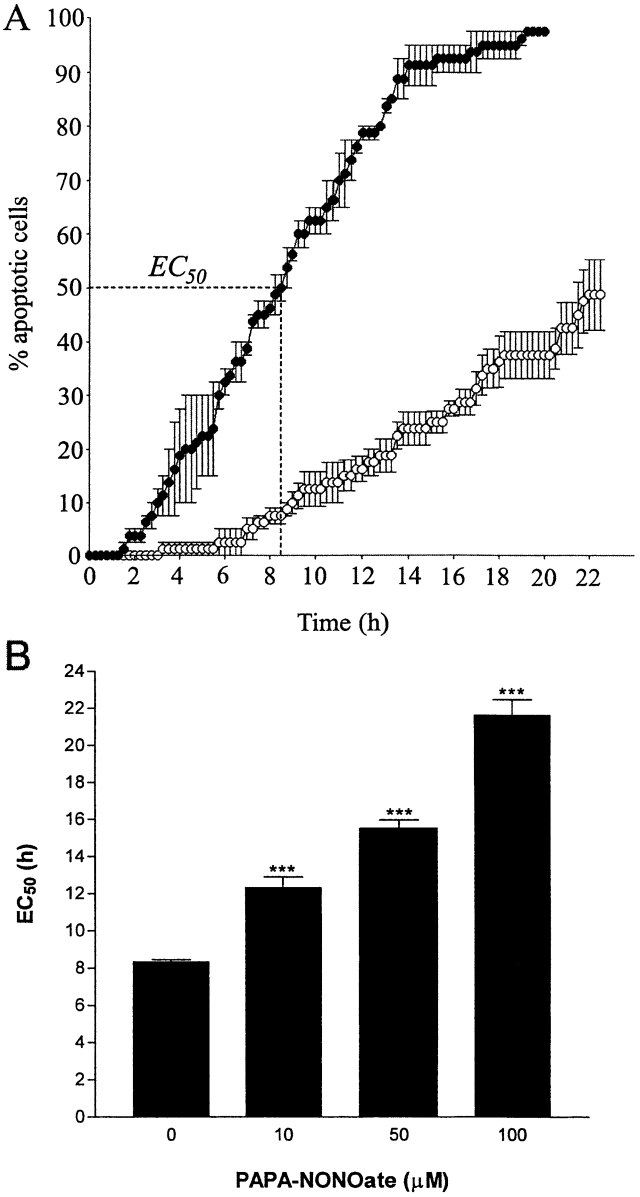
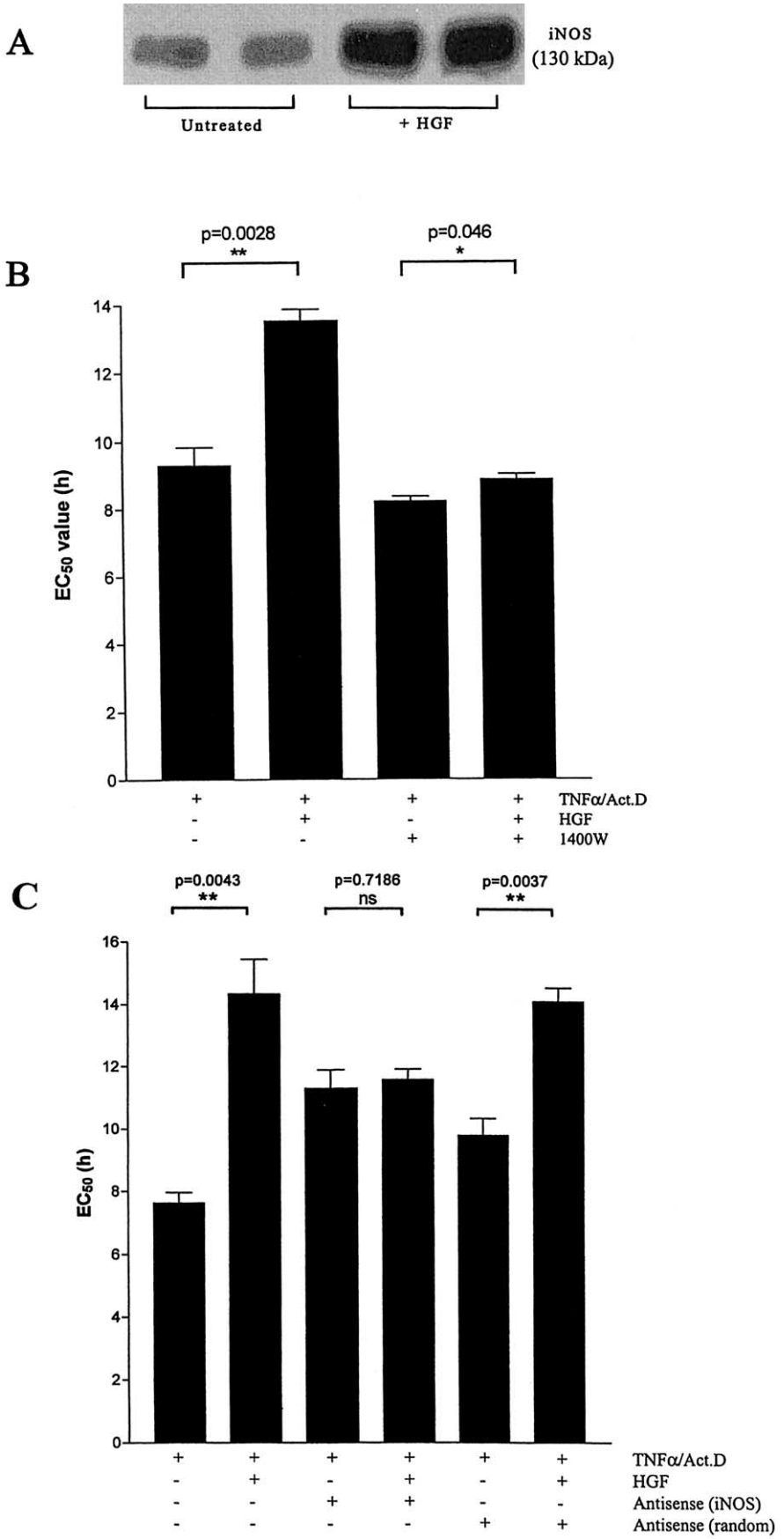


Fig. 2. (A) Effect of the NO donor PAPA-NONOate (100 μM) on the survival times of SGHPL-4 cells exposed to TNFα (30 ng/ml)/actinomycin D (800 ng/ml), as determined by time-lapse digital image microscopy. Closed circles, TNFα/actinomycin D; open circles, TNFα/actinomycin D + 100 μM PAPA-NONOate. The time taken to induce apoptosis in 50% of the cells (the EC_{50} value) was determined by nonlinear regression analysis. Values shown are means ± SEM of triplicate experiments. (B) Effect of increasing dose of the NO donor PAPA-NONOate on cell survival as determined by time-lapse microscopy analysis. The EC_{50} values were determined from the survival curves using nonlinear regression analysis. Values shown are means ± SEM of nine experiments. ***P = 0.0001.


A
iNOS
(130 kDa)
Untreated
+ HGF
B
p=0.0028
**
p=0.046
*
EC50 value (h)
0
2
4
6
8
10
12
14
+ + + + TNFα/Act.D
- + - + HGF
- - + + 1400W
C
p=0.0043
**
p=0.7186
ns
p=0.0037
**
EC50 (h)
0
2
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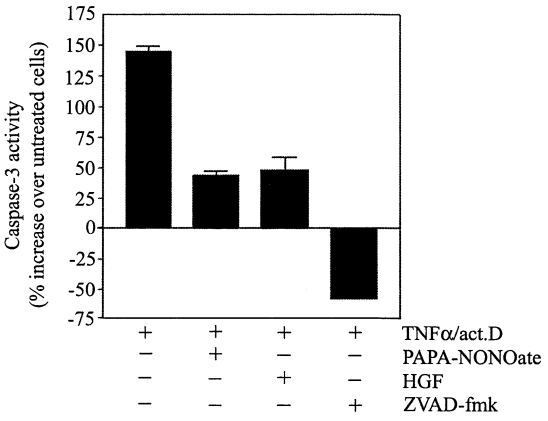

Fig. 4. Effect of the NO donor PAPA-NONOate (100 μM) and HGF (10 ng/ml) on the activity of caspase 3 in lysates from cells treated with TNFα (30 ng/ml)/actinomycin D (800 ng/ml) for 18 h. The broad-spectrum caspase inhibitor ZVAD-fmk (50 μM) was also used. Values shown are means ± SEM of triplicate experiments.

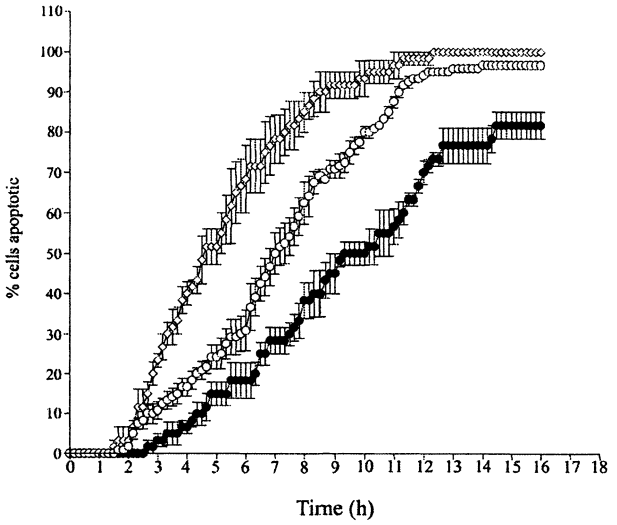

Fig. 5. Time course of apoptosis, as determined by time-lapse microscopy, induced by TNFα/actinomycin D in cells pretreated with the iNOS inhibitor 1400W (2 mM, open circles) or the general NOS inhibitor L-NAME (3 mM, open diamonds). Cells treated with TNFα/actinomycin D only are also shown (closed circles). Values shown are means ± SEM of triplicate experiments.

increased survival times correlated with decreased caspase activity, the activity of caspase 3 was measured. Both PAPA-NONOate and HGF were able to significantly reduce the activity of caspase 3 following exposure of cells to TNFα and actinomycin D (in both cases $P < 0.001$, $n = 3$), although neither was as effective as the broad-spectrum caspase inhibitor ZVAD-fmk (Fig. 4).

Inhibition of NO production sensitizes cells to apoptotic stimuli

The ability of endogenous levels of NO production to protect SGHPL-4 cells from apoptosis was investigated using inhibitors of NOS. L-NAME (3 mM) competitively inhibits all isoforms of NOS and was found to sensitise cells to apoptosis induced by TNFα/actinomycin D (Fig. 5), significantly reducing the time taken to induce apoptosis in 50% of cells from 8.75 ± 0.8 to 4.31 ± 0.29 h ($P = 0.006$, $n = 3$). A similar effect was observed when the NOS inhibitor L-NMMA was used (data not shown). The role played by iNOS was investigated using the iNOS-specific inhibitor 1400W (2 mM). It was found that inhibition of iNOS also sensitised the cells to apoptosis but was less effective than the use of general NOS inhibitors, reducing the time taken to induce apoptosis in 50% of cells from 8.75 to 6.93 ± 0.38 h ($P < 0.001$, $n = 3$).

Role of cGMP signaling in the NO-mediated anti-apoptotic effect

It has been proposed that NO may mediate its anti-apoptotic effects through a number of mechanisms including the activation of cGMP signaling [29,36,37] and the nitrosylation of the caspases, in particular through the nitrosylation of caspase 3 [31,37,38].

To investigate the role of cGMP signaling in protecting SGHPL-4 cells from apoptosis, cells were incubated in the presence of the soluble guanylate cyclase inhibitor NS2028 (5 μM) prior to addition of the NO donor PAPA-NONOate (Fig. 6A) or HGF (Fig. 6B). Treatment with 10 μM NO donor increased the EC_{50} from 8.4 ± 0.14 to 12.3 ± 1.43 h ($P < 0.001$, $n = 9$), while pretreatment with the guanylate cyclase inhibitor reversed this protection, leading to an EC_{50} of 9.36 ± 1.30 h ($P < 0.0039$, $n = 9$), suggesting that at least part of the effect of NO donors is mediated through the production of cGMP from guanylate cyclase. Addition of 100 μM PAPA-NONOate increased the EC_{50} of apoptosis induction from 8.4 ± 0.14 to 21.61 ± 2.06 h ($P < 0.0001$, $n = 9$). Treatment of cells with NS2028 in addition to TNFα/actinomycin D and the 100 μM dose of the NO

Fig. 3. (A) Western blot analysis showing iNOS expression (130 kDa) in SGHPL-4 cells either without HGF or following overnight (16 h) exposure to 10 ng/ml HGF. (B) Survival times (EC_{50} values), as determined by time-lapse microscopy, of SGHPL-4 cells treated with TNFα (30 ng/ml)/actinomycin D (800 ng/ml) in the presence or absence of overnight exposure to HGF (10 ng/ml). The iNOS inhibitor 1400W (2 mM) was added 24 h prior to the addition of HGF to investigate the role of iNOS in the effect of HGF on SGHPL-4 cells. Values shown are means ± SEM of triplicate experiments. (C) Survival times (EC_{50} values), as determined by time-lapse microscopy, of SGHPL-4 cells treated with TNFα (30 ng/ml)/actinomycin D (800 ng/ml). Cells were treated with overnight exposure to HGF (10 ng/ml) following transfection with either iNOS antisense oligonucleotides or randomly scrambled antisense oligonucleotides.

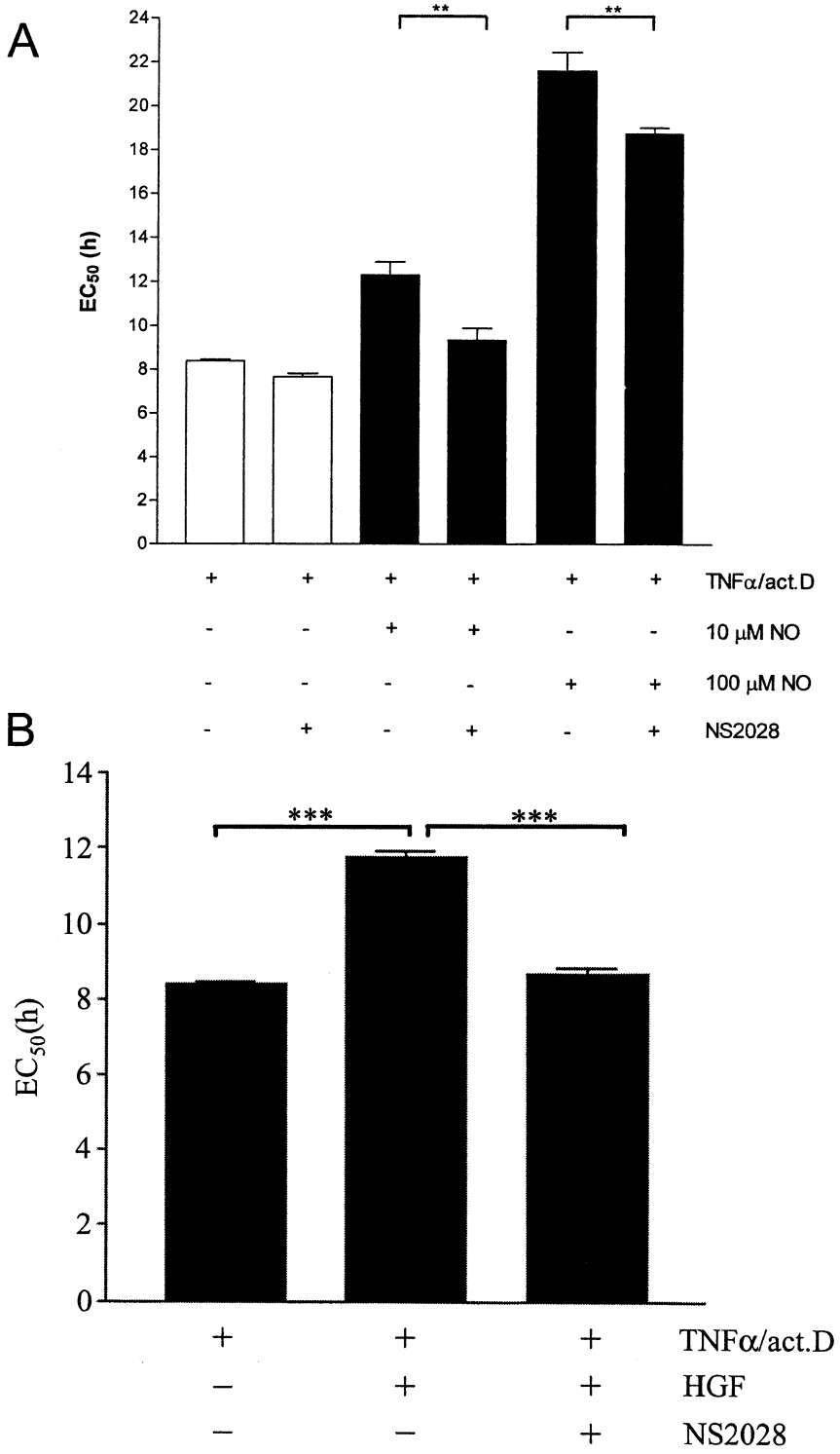

Fig. 6. (A) Effect of the guanylate cyclase inhibitor NS2028 on the survival times (EC_{50} value) of SGHPL-4 cells, as determined by time-lapse microscopy. Cells were exposed to TNFα (30 ng/ml)/actinomycin D (800 ng/ml) in the presence or absence of either 100 or 10 μM PAPA-NONOate and the guanylate cyclase inhibitor NS2028 (5 μM). Values shown are means ± SEM of nine experiments. (B) Cells were exposed to TNFα/actinomycin D in the presence or absence of HGF (10 ng/ml) and the guanylate cyclase inhibitor NS2028 (5 μM). Values shown are means ± SEM of triplicate experiments. $^{**}P < 0.001$, $^{***}P < 0.0001$.

donor significantly, but not completely, reversed the protective effect of NO (EC_{50} = 18.74 ± 0.61 h, P = 0.0086, n = 9), suggesting that some of the effects of the higher concentration of NO donor are independent of cGMP signaling.

When the guanylate cyclase inhibitor was added to cells

treated with HGF the result was a reversal of the protective effect, similar to that observed with the 10 μM dose of the NO donor. Treatment with HGF increased the EC_{50} for apoptosis induction from 8.4 to 11.8 h (Fig. 6B), while treatment with HGF and NS2028 reduced the EC_{50} to 8.6 h. This suggests that the anti-apoptotic effects of HGF are mediated through the production of cGMP from guanylate cyclase. Inhibition of guanylate cyclase, in the absence of PAPA-NONOate or HGF had no significant effect on the induction of apoptosis (data not shown).

Role of caspase 3 nitrosylation in the anti-apoptotic effect of high doses of NO donors

Inhibition of caspase 3 activity by nitrosylation is one of the major cGMP-independent mechanisms through which NO has been shown to regulate apoptosis. Treatment of cells with the NO donor PAPA-NONOate prior to the induction of apoptosis with TNFα/actinomycin D was able to significantly reduce the activity of caspase 3 (Fig. 4). The mechanism through which NO inhibits caspase 3 activity was investigated using an antibody against nitrosocysteine groups. The level of caspase 3 nitrosylation was compared between untreated cells and cells treated with either an inhibitor of NOS (L-NAME) or with PAPA-NONOate. Caspase 3 was immunoprecipitated and electrophoresed under nonreducing conditions by SDS–PAGE. Western blot analysis with anti-nitrosocysteine antibody revealed that caspase 3 is heavily nitrosylated in untreated cells and that the degree of nitrosylation is not significantly increased by the addition of an NO donor (Fig. 7) ($P = 0.64$). The addition of an NO donor directly to caspase 3 immunoprecipitated from untreated cells was also unable to increase the degree of nitrosylation (data not shown). Inhibition of basal NO synthesis by L-NAME produced a significant (42 $\pm$ 13%, $P = 0.007$) decrease in the degree of nitrosylation, suggesting that basal NO production may be responsible for nitrosylating; and therefore inactivating, caspase 3.

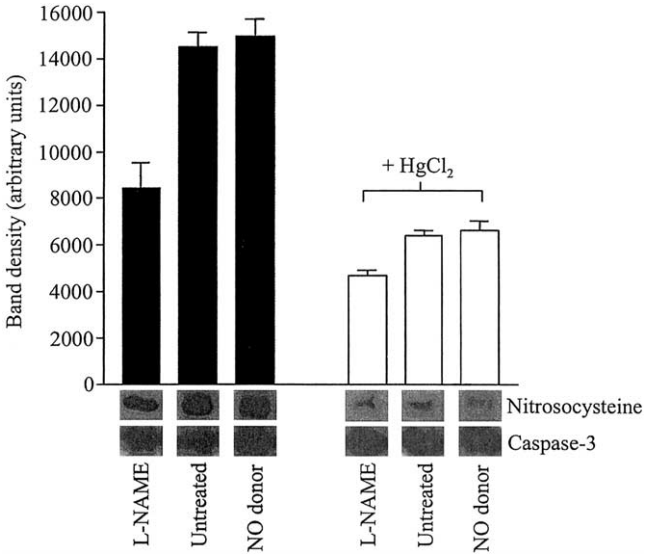

Fig. 7. Western blot analysis to detect nitrosylation of immunoprecipitated caspase 3. Cells were either not treated or treated with the NOS inhibitor L-NAME (3 mM) or the NO donor PAPA-NONOate (100 μM) for 24 h. Each treatment was performed in triplicate and bands were quantified using FluorChem software (Alpha Innotech Corporation, UK). Loading was normalised by probing the blot with an anti-caspase 3 antibody and quantifying the resulting autoradiograph. Some of the samples were also treated with $HgCl_2$ to selectively remove nitrosocysteine groups [31]. This treatment reduced the degree of nitrosylation in all samples by an average of 48.6%.

Discussion

Inappropriate extravillous trophoblast apoptosis has been implicated in the failure of trophoblasts to fully invade and modify the uterine environment, potentially leading to complications of pregnancy such as pre-eclampsia [19,21,39]. Nitric oxide inhibits apoptosis in a number of cell types including leukocytes [40], hepatocytes [37], and endothelial cells [41] and induces apoptosis in other cell types such as thymocytes [42], macrophages [43], and smooth muscle cells [44]. Using the extravillous trophoblast cell line SGHPL-4, we have investigated the role of NO in regulating extravillous trophoblast apoptosis.

Nitric oxide donors are commonly used to increase the concentration of NO experimentally and may mimic the effect of exogenous NO [37,41]. The NO donor PAPA-NONOate was able to significantly delay the onset of apoptosis induced by TNFα/actinomycin D and to significantly reduce caspase 3 activity. In the placenta, the extravillous trophoblast may be exposed to NO produced by surrounding cells such as endothelial cells, macrophages, smooth muscle cells, and neighboring trophoblasts. Nitric oxide exogenously produced by neighboring cells may be an important mechanism regulating apoptosis in trophoblasts.

Two isoforms of NO synthase have been identified in the human placenta, the constitutively expressed eNOS and the inducible isoform (iNOS), and immunocytochemical studies have shown expression of both isoforms by trophoblasts including the extravillous population [5–8]. Treatment of the extravillous line SGHPL-4 with HGF stimulated the expression of iNOS, significantly delayed the onset of apoptosis, and inhibited the activity of caspase 3 induced by TNFα/actinomycin D. Addition of the iNOS specific inhibitor 1400W to cells treated with HGF reversed its protective effect, suggesting that the increased survival times of these cells may be, in part, due to the effect of HGF on iNOS expression/activity. This conclusion was strengthened through the use of antisense oligonucleotides specific to iNOS. These oligonucleotides block the translation of iNOS mRNA and prevent HGF from stimulating the production of additional iNOS protein. In these circumstances HGF was found to be unable to produce an anti-apoptotic effect, suggesting that its protective effects are mediated mainly through the transcription of iNOS and the production of NO.

The effects of stimulating iNOS expression with HGF are particularly interesting since HGF is known to be essential for normal placental development [45] and has effects on extravillous trophoblast invasion and motility [12]. It is possible that an additional role for HGF during pregnancy is the protection of trophoblasts from apoptosis.

Cells treated with either a general NOS inhibitor (L-NAME) or an iNOS-specific inhibitor (1400W) were significantly more sensitive to apoptosis induced with TNFα/actinomycin D than untreated cells, suggesting that basal NO production affords the cells significant protection from apoptotic stimuli and that at least some of this basal production may be attributed to iNOS. We have previously demonstrated that SGHPL-4 cells express eNOS [12] and have shown that they do not express nNOS (unpublished data). It seems likely that while the HGF-stimulated production of NO from iNOS leads to enhanced protection from apoptosis, the basal response of extravillous trophoblast cells to apoptotic stimuli is regulated by NO produced from both iNOS and eNOS. Endogenous inhibitors of NO synthesis such as asymmetric dimethylarginine (ADMA) have been reported to increase in pregnancies complicated with pre-eclampsia [13]. It is possible that decreased NO production in extravillous trophoblasts, either through inhibition of eNOS and iNOS by ADMA or through a reduction in growth factors that stimulate iNOS expression, might result in increased sensitivity to apoptosis.

A number of mechanisms have been proposed to explain the role that NO plays in regulating apoptosis. Generally these mechanisms have been classified as cGMP dependent or independent. Nitric oxide is able to activate cGMP signaling through interaction with the heme group of guanylate cyclase [46]. The production of cGMP leads to the activation of cGMP-dependent protein kinases and suppression of caspase activity [37]. Studies using the inhibitor of soluble guanylate cyclase, NS2028, revealed a role for cGMP signaling in the protective effect of both low doses (10 μM) and higher doses (100 μM) of the NO donor PAPA-NONOate. However, at the higher doses a significant proportion of the protective effect appears to be independent of cGMP production. We have previously shown that HGF stimulates cGMP production in SGHPL-4 cells [12]. The anti-apoptotic effects of HGF also appeared to be mediated through cGMP signaling as the use of guanylate cyclase inhibitors significantly compromised the ability of HGF to protect the cells. This result provides further evidence that the anti-apoptotic effects of HGF are mediated, at least in part, through the production of NO. It also suggests that high doses of NO may inhibit apoptosis through both cGMP-dependent and -independent mechanisms.

The anti-apoptotic effects of NO can also be mediated through a number of mechanisms independent of cGMP signaling, in particular the nitrosylation of some of the caspases, such as caspase 3 [30,31]. Nitrosylation occurs in the cysteine group located in the active site and leads to inactivation of the enzyme. Caspase activity assays demonstrated that exposure of extravillous trophoblasts to the NO donor PAPA-NONOate was able to significantly reduce the activity of caspase 3. To determine whether this effect was through direct nitrosylation of caspase 3 or through an indirect mechanism, caspase 3 was immunoprecipitated and analysed for NO modified cysteine residues. Caspase 3 in untreated trophoblasts was found to be heavily nitrosylated, suggesting that basal NO production plays an important role in the regulation of caspase 3 activity. Surprisingly, the treatment of cells or immunoprecipitated caspase 3 with the NO donor was unable to increase the degree of nitrosylation, suggesting that in untreated SGHPL-4 cells caspase 3 is fully nitrosylated. This suggests that the suppression of caspase 3 activity observed following treatment with NO donors and HGF is mediated through an indirect mechanism, possibly involving cGMP signaling, rather than through direct nitrosylation.

A portion of the protective effect of high doses of NO has been shown to be independent of cGMP signaling. While caspase 3 nitrosylation can be ruled out as a mechanism for the cGMP-independent effects of high-dose NO, it is possible that other caspases are not nitrosylated in their native state and are susceptible to higher doses of NO. It is also possible that other, as yet unidentified, proteins involved in cell survival could be nitrosylated by NO. We have preliminary evidence that a number of proteins in trophoblasts are nitrosylated by high doses of NO (unpublished data) and these are currently being studied.

Inhibition of nitric oxide synthases with L-NAME significantly reduced the degree of caspase 3 nitrosylation and increased the sensitivity to apoptotic stimuli. These results suggest that endogenously produced NO is able to directly regulate apoptosis in trophoblasts through the nitrosylation of caspase 3. Previous studies in T lymphocytes have also demonstrated that basal nitrosylation of caspase 3 is an important regulatory mechanism [31] and have suggested that following the induction of apoptosis two steps are required for caspase activation: the processing of caspase 3 zymogen to its active form and the denitrosylation of the active site cysteine.

Physiological production of NO in trophoblasts results in significant anti-apoptotic effects. In most cases the increased NO production does not completely inhibit apoptosis, but instead delays its onset. In a rapidly dividing tissue such as the placenta the balance between cell division and cell death through apoptosis is crucial to regulating normal tissue development. Small decreases in the responsiveness of cells to apoptotic stimuli may lead to a shift in this balance in favor of cell division, with implications for the growth rate of the tissue. Conversely, inhibition of NO synthesis leads to increased sensitivity of cells and may lead to a shift toward apoptosis. During pregnancy this may occur due to increases in the levels of endogenous inhibitors of NO synthases such as ADMA [13] or if iNOS expression in trophoblasts is not sufficiently stimulated, for example, by decreases in HGF levels or through downregulation of

the HGF receptor. Even small changes in the sensitivity of trophoblasts to apoptotic stimuli may compromise their ability to invade the uterine decidua and to fully modify the spiral arteries, potentially leading to complications of pregnancy such as pre-eclampsia.

References

[1] J.W. Meekins, R. Pijnenborg, M. Hanssens, I.R. McFadyen, A. van Asshe, A study of placental bed spiral arteries and trophoblast invasion in normal and severe pre-eclamptic pregnancies, Br. J. Obstet. Gynaecol. 101 (1994) 669–674.

[2] J.C.P. Kingdom, In vivo assessment of failed trophoblastic invasion of the spiral arteries in pre-eclampsia, Br. J. Obstet. Gynaecol. 106 (1999) 1328–1329.

[3] C.J. Lowenstein, S.H. Snyder, Nitric-oxide, a novel biologic messenger, Cell 70 (1992) 705–707.

[4] D.D. Browning, M.P. McShane, C. Marty, R.D. Ye, Nitric oxide activation of p38 mitogen-activated protein kinase in 293T fibroblasts requires cGMP-dependent protein kinase, J. Biol. Chem. 275 (2000) 2811–2816.

[5] T.J. Zarlingo, A.L. Eis, D.E. Brockman, W. Kossenjans, L. Myatt, Comparative localization of endothelial and inducible nitric oxide synthase isoforms in haemochorial and epitheliochorial placentae, Placenta 18 (1997) 511–520.

[6] D. Martin, K.P. Conrad, Expression of endothelial nitric oxide synthase by extravillous trophoblast cells in the human placenta, Placenta 21 (2000) 23–31.

[7] N. Yoshiki, T. Kubota, T. Aso, Expression and localization of inducible nitric oxide synthase in human non-pregnant and early pregnant endometrium, Mol. Hum. Reprod. 6 (2000) 283–287.

[8] E. Hambarisoumian, R.K. Srivastava, M.M. Seibel, Differential expression and regulation of inducible nitric oxide synthase (iNOS) mRNA in human trophoblasts in vitro, Am. J. Reprod. Immunol. 45 (2001) 78–85.

[9] D.J. Williams, P.J.T. Vallance, G.H. Neild, J.A.D. Spencer, F.J. Imms, Nitric oxide-mediated vasodilation in human pregnancy, Am. J. Physiol. Heart Circ. Physiol. 41 (1997) H748–H752.

[10] S. Gagloti, C. Scavone, E. Bevilacqua, Participation of the mouse implanting trophoblast in nitric oxide production during pregnancy, Biol. Reprod. 62 (2000) 260–268.

[11] F. Lyall, A. Jablonka-Shariff, R.D. Johnson, L.M. Olson, D.M. Nelson, Gene expression of nitric oxide synthase in cultured human term placental trophoblast during in vitro differentiation, Placenta 19 (1998) 253–260.

[12] J.E. Cartwright, D.P. Holden, G.S. Whitley, Hepatocyte growth factor regulates human trophoblast motility and invasion: a role for nitric oxide, Br. J. Pharmacol. 128 (1999) 181–189.

[13] D.P. Holden, S.A. Fickling, G.S. Whitley, S.S. Nussey, Plasma concentrations of asymmetric dimethylarginine, a natural inhibitor of nitric oxide synthase, in normal pregnancy and preeclampsia, Am. J. Obstet. Gynecol. 178 (1998) 551–556.

[14] C.P. Weiner, R.G. Knowles, S. Moncada, Induction of nitric oxide synthases early in pregnancy, Am. J. Obstet. Gynecol. 171 (1994) 838–843.

[15] R.A. Rutherford, A. McCarthy, M.H. Sullivan, M.G. Elder, J.M. Polak, J. Wharton, Nitric oxide synthase in human placenta and umbilical cord from normal, intrauterine growth-retarded and pre-eclamptic pregnancies, Br. J. Pharmacol. 116 (1995) 3099–3109.

[16] A. Pettersson, T. Hedner, I. Milsom, Increased circulating concentrations of asymmetric dimethyl arginine (ADMA), an endogenous inhibitor of nitric oxide synthesis, in preeclampsia, Acta Obstet. Gynecol. Scand. 77 (1998) 808–813.

[17] R. Halperin, S. Peller, M. Rotschild, I. Bukovsky, D. Schneider, Placental apoptosis in normal and abnormal pregnancies, Gynecol. Obstet. Invest. 50 (2000) 84–87.

[18] M.P. Thiet, V. Suwanvanichkij, K. Hasselblatt, J. Yeh, Apoptosis in human term placenta: a morphological and gene expression study, Gynecol. Obstet. Invest. 50 (2000) 88–91.

[19] E. DiFederico, O. Genbacev, S.J. Fisher, Preeclampsia is associated with widespread apoptosis of placental cytotrophoblasts within the uterine wall, Am. J. Pathol. 155 (1999) 293–301.

[20] A.D. Allaire, K.A. Ballenger, S.R. Wells, M.J. McMahon, B.A. Lessey, Placental apoptosis in preeclampsia, Obstet. Gynecol. 96 (2000) 271–276.

[21] O. Genbacev, E. DiFederico, M. McMaster, S.J. Fisher, Invasive cytotrophoblast apoptosis in pre-eclampsia, Hum. Reprod. 14 (1999) 59–66.

[22] N. Ishihara, H. Matsuo, H. Murakoshi, J.B. Laoag-Fernandez, T. Samoto, T. Maruo, Increased apoptosis in the syncytiotrophoblast in human term placentas complicated by either preeclampsia or intrauterine growth retardation, Am. J. Obstet. Gynecol. 186 (2002) 158–166.

[23] S.G. Payne, S.C. Smith, S.T. Davidge, P.N. Baker, L.J. Guilbert, Death receptor Fas/Apo-1/CD95 expressed by human placental cytotrophoblasts does not mediate apoptosis, Biol. Reprod. 60 (1999) 1144–1150.

[24] T.A. Philips, J. Ni, G.H. Pan, S.M. Ruben, Y.F. Wei, J.L. Pace, J.S. Hunt, TRAIL (Apo-2L) and TRAIL receptors in human placentas: implications for immune privilege, J. Immunol. 162 (1999) 6053–6059.

[25] F. Shu, M. Sugimura, N. Kanayama, H. Kobayashi, T. Kobayashi, T. Terao, Immunohistochemical study of annexin V expression in placentae of preeclampsia, Gynecol. Obstet. Invest. 49 (2000) 17–23.

[26] B. Huppertz, H.G. Frank, J.C.P. Kingdom, F. Reister, P. Kaufmann, Villous cytotrophoblast regulation of the syncytial apoptotic cascade in the human placenta, Histochem. Cell Biol. 110 (1998) 495–508.

[27] X. Zhou, S.A. Gordon, Y.M. Kim, R.A. Hoffman, Y. Chen, X.R. Zhang, R.L. Simmons, R. Ford, Nitric oxide induces thymocyte apoptosis via a caspase-1-dependent mechanism, J. Immunol. 165 (2000) 1252–1258.

[28] E. Battinelli, J. Loscalzo, Nitric oxide induces apoptosis in megakaryocytic cell lines, Blood 95 (2000) 3451–3459.

[29] C. De Nadai, P. Sestili, O. Cantoni, J.P. Lievremont, C. Sciorati, R. Barsacchi, S. Moncada, J. Meldolesi, E. Clementi, Nitric oxide inhibits tumor necrosis factor-alpha-induced apoptosis by reducing the generation of ceramide, Proc. Nat. Acad. Sci. USA 97 (2000) 5480–5485.

[30] J.R. Li, C.A. Bombeck, S.F. Yang, Y.M. Kim, T.R. Billiar, Nitric oxide suppresses apoptosis via interrupting caspase activation and mitochondrial dysfunction in cultured hepatocytes, J. Biol. Chem. 274 (1999) 17325–17333.

[31] J.B. Mannick, A. Hausladen, L. Liu, D.T. Hess, M. Zeng, Q.X. Miao, L.S. Kane, A.J. Gow, J.S. Stamler, Fas-induced caspase denitrosylation, Science 284 (1999) 651–654.

[32] M.Y. Choy, I.T. Manyonda, The phagocytic activity of human first trimester extravillous trophoblast, Hum. Reprod. 13 (1998) 2941–2949.

[33] K.T. Shiverick, A. King, H.G. Frank, G.S. Whitley, J.E. Cartwright, H. Schneider, Cell culture models of human trophoblast II: trophoblast cell lines—a workshop report, Placenta 22 (2001) S104–S106.

[34] E. Noiri, T. Peresleni, N. Srivastava, P. Weber, W.F. Bahou, N. Peunova, M.S. Goligorsky, Nitric oxide is necessary for a switch from stationary to locomoting phenotype in epithelial cells, Am. J. Physiol. 270 (1996) C794–C802.

[35] Cartwright, J., Tse, W.K., Whitley, G.S. Hepatocyte growth factor induced human trophoblast motility involves phosphatidylinositol

3-kinase, mitogen activated protein kinase and inducible nitric oxide synthase. Exp. Cell Res. 279 (2002) 219–226.
[36] Y.M. Kim, H.T. Chung, S.S. Kim, J.A. Han, Y.M. Yoo, K.M. Kim, G.H. Lee, H.Y. Yun, A. Green, J.R. Li, R.L. Simmons, T.R. Billiar, Nitric oxide protects PC12 cells from serum deprivation-induced apoptosis by cGMP-dependent inhibition of caspase signaling, J. Neurosci. 19 (1999) 6740–6747.
[37] Y.M. Kim, R.V. Talanian, T.R. Billiar, Nitric oxide inhibits apoptosis by preventing increases in caspase-3-like activity via two distinct mechanisms, J. Biol. Chem. 272 (1997) 31138–31148.
[38] D.T. Hess, A. Matsumoto, R. Nudelman, J.S. Stamler, S-nitrosylation: spectrum and specificity, Nat. Cell Biol. 3 (2001) E46–E49.
[39] F. Reister, H.G. Frank, J.C. Kingdom, W. Heyl, P. Kaufmann, W. Rath, B. Huppertz, Macrophage-induced apoptosis limits endovascular trophoblast invasion in the uterine wall of preeclamptic women, Lab. Invest. 81 (2001) 1143–1152.
[40] J.B. Mannick, X.Q. Miao, J.S. Stamler, Nitric oxide inhibits Fas-induced apoptosis, J. Biol. Chem. 272 (1997) 24125–24128.
[41] S. Dimmeler, J. Haendeler, M. Nehls, A.M. Zeiher, Suppression of apoptosis by nitric oxide via inhibition of interleukin-1 beta-converting enzyme (ICE)-like and cysteine protease protein (CPP)-32-like proteases, J. Exp. Med. 185 (1997) 601–607.
[42] K. Fehsel, K.D. Kroncke, K.L. Meyer, H. Huber, V. Wahn, V. Kolbbachofen, Nitric-oxide induces apoptosis in mouse thymocytes, J. Immunol. 155 (1995) 2858–2865.
[43] U.K. Messmer, D.M. Reimer, J.C. Reed, B. Brune, Nitric oxide induced poly(ADP-ribose) polymerase cleavage in RAW 264.7 macrophage apoptosis is blocked by Bcl-2, FEBS Lett. 384 (1996) 162–166.
[44] E. Nishio, K. Fukushima, M. Shiozaki, Y. Watanabe, Nitric oxide donor SNAP induces apoptosis in smooth muscle cells through cGMP-independent mechanism, Biochem. Biophys. Res. Commun. 221 (1996) 163–168.
[45] D.E. Clark, S.K. Smith, A.M. Sharkey, H.M. Sowter, D.S. Charnock-Jones, Hepatocyte growth factor scatter factor and its receptor c-met: localisation and expression in the human placenta throughout pregnancy, J. Endocrinol. 151 (1996) 459–467.
[46] J.W. Denninger, M.A. Marletta, Guanylate cyclase and the (NO)-N-/cGMP signaling pathway, Biochim. Biophys. Acta Bioenergetics 1411 (1999) 334–350.