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Mammalian Ste20-like protein kinase 3 plays a role in hypoxia-induced apoptosis of trophoblast cell line 3A-sub-E

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ABSTRACT

Mammalian Ste20-like protein kinase 3 (Mst3) is a key player in inducing apoptosis in a variety of cell types and has recently been shown to participate in the signaling pathway of hypoxia-induced apoptosis of human trophoblast cell line 3A-sub-E (3A). It is believed that oxidative stress may occur during hypoxia and induce the expression of Mst3 in 3A cells via the activation of c-Jun N-terminal protein kinase 1 (JNK1). This hypothesis was demonstrated by the suppressive effect of DL-α-lipoic acid, a reactive oxygen species scavenger, in hypoxia-induced responses of 3A cells such as Mst3 expression, nitrotyrosine formation, JNK1 activation and apoptosis. Similar results were also observed in trophoblasts of human placental explants in both immunohistochemical studies and immunoblot analyses. These suggested that the activation of Mst3 might trigger the apoptotic process in trophoblasts by activating caspase 3 and possibly other apoptotic pathways. The role of nitric oxide synthase (NOS) and NADPH oxidase (NOX) in hypoxia-induced Mst3 up-regulation was also demonstrated by the inhibitory effect of N^G-nitro-L-arginine and apocynin, which inhibits NOS and NOX, respectively. Oxidative stress was postulated to be induced by NOS and NOX in 3A cells during hypoxia. In conclusion, hypoxia induces oxidative stress in human trophoblasts by activating NOS and NOX. Subsequently, Mst3 is up-regulated and plays an important role in hypoxia-induced apoptosis of human trophoblasts.

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1. Introduction

Hypoxia is a condition of relatively low oxygen supply with respect to tissue metabolic demand (Neubauer, 2001). Cells under hypoxic stress may exhibit an adaptive response that increases the rates of metabolism and angiogenesis. Successful placentation and embryo development at the early stages of pregnancy occurs in a predominantly low oxygen environment with a partial pressure of oxygen (pO_2) of <20 mmHg (<2.6%) (Heazell et al., 2008; Khong et al., 1987; Rodesch et al., 1992). Organogenesis of the vascular system, placenta, lung and skeleton during embryonic and fetal development is also facilitated by hypoxic stress (Dunwoodie, 2008; Shinkai et al., 2005; Simon and Keith, 2008; Yue and Tomanek, 1999). However, hypoxia occurring during acute and chronic vascular disease, pulmonary disease, brain injury and cancer results in cell death (Harris, 2002; Shimizu et al., 1996). Various studies shown that the cell death induced by hypoxia can occur via apoptosis (Shimizu et al., 1996).

Apoptosis is an intrinsic mechanism that progressively eliminates excessively generated cells, the improperly developed cells, aged cells and cells with sustained genetic damages (Cohen and Duke, 1992; Golstein et al., 1991). A dysregulation of apoptotic cell death may be involved in the pathogenesis of a variety of human diseases, including cancer, autoimmune diseases, neurodegenerative disorders, and viral infection (Raff et al., 1993; Sarraf and Bowen, 1988; Vaux et al., 1994; Williams, 1991). Apoptosis also plays roles in the placental development, remodeling and aging (Huppertz and Kingdom, 2004; Rama and Rao, 2003; Smith et al., 1997). Hypoxia was shown to induce apoptosis in trophoblasts (Hung et al., 2001; Levy et al., 2000; Chen et al., 2010). Trophoblasts isolated from the placenta in pregnancies complicated by intrauterine fetal growth retardation (IUGR) and preeclampsia exhibit enhanced apoptosis in response to hypoxia compared with trophoblasts from uncomplicated pregnancies (Crocker et al., 2003; Hung et al., 2002). It is believed that hypoxia-induced trophoblast apoptosis further promotes the pathogenic progression of IUGR and preeclampsia. Several cellular factors, such as p53 (Crocker, 2007; Heazell et al., 2008; Levy et al., 2000), Bcl-2 family members (Hu

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and Zhou, 2006; Humphrey et al., 2008), NF-κB (Cindrova-Davies et al., 2007) and c-Jun N-terminal protein kinase (JNK)/p38 MAPK cascades (Cindrova-Davies et al., 2007; Humphrey et al., 2008; Jessmon et al., 2010), were shown to participate in the signaling of hypoxic responses in trophoblasts. However, the molecular mechanism leading to hypoxic injury of human placenta has not been fully defined.

Recently, mammalian Ste20-like protein kinase 3 (Mst3) was shown to mediate the apoptosis of human placenta from normal term delivery and the trophoblast cell line (Wu et al., 2008). Mst3 is a member of the GCK-III subfamily of human Ste20-like serine/threonine protein kinases with a molecular mass of around 50 kDa (Huang et al., 2002; Lee et al., 2004). It was shown to participate in the apoptosis of many cell lines upon the treatment with Fas-ligand or staurosporine (Lee et al., 2004). In human placenta, however, oxidative stress was shown to be responsible for the upregulation of Mst3 (Wu et al., 2008). Oxidative stress is induced by the overproduction of reactive oxygen species (ROS), is postulated to play a role in cell apoptosis (Hung et al., 2001; Heazell et al., 2007; Jauniaux et al., 2000; Redman and Sargent, 2000). ROS, including superoxide radicals (O_2^-) and its reduction products hydrogen peroxide (H_2O_2) and the hydroxyl radical, have been shown to induce cellular damage through membrane lipid peroxidization, DNA modification and protein denaturation. JNK1 was shown to be activated by oxidative stress and, subsequently, upregulated the Mst3 in trophoblasts (Wu et al., 2008). Although Mst3 was demonstrated to trigger the trophoblast apoptosis by oxidative stress, its role in the hypoxia-induced apoptosis of human trophoblast has not been fully explored.

In this study, Mst3 was demonstrated to play an important role in the signaling pathway of hypoxia-induced trophoblast apoptosis. Similar results were also observed in human explant cultures under hypoxic stress. Furthermore, this study shows that hypoxia may activate Mst3 in human trophoblasts by elevating oxidative stress. Both NOS and NOX were shown to play a role in generating ROS in human trophoblasts under hypoxic stress.

2. Materials and methods

2.1. Materials

Fetal bovine serum (FBS), α-MEM, penicillin G, streptomycin, trypsin and lipofectamine were obtained from GIBCO Life Technologies. N^G-nitro-L-arginine (L-NNA) and DL-α-lipoic acid (LA) were bought from Cayman. G418-sulfate was obtained from Invitrogen. SP600125 was obtained from Calbiochem. Hypoxyprobe™-1 Kit for the detection of tissue hypoxia was purchased from Chemicon. Other reagents were regent grade.

2.2. Histology and immunohistochemistry

Fresh placental tissues for explant culture were obtained from normal pregnant women undergoing elective Cesarean sections (ECS) at term without labor pain (36-week; $n = 6$) with informed consent. The placental specimens were cut into a size of 1 cm × 1 cm × 0.5 cm and incubated in α-MEM medium (containing 10% FBS and 100 units/mL penicillin and 100 (g/mL streptomycin) under the hypoxia (1% O_2, 5% CO_2 and 94% N_2) or the normal condition (20% O_2, 5% CO_2 and 75% N_2) at 37 °C for 24 h. The histological and immunohistochemistry analyses of the explants were performed as described previously (Wu et al., 2008). The placental specimens were examined by a pathologist to confirm the histological preservation of the microanatomical structure and the absence of any abnormality in placenta.

The fixed human placental specimens were incubated with anti-Mst3 (1:100 dilution, Santa Cruz), anti-nitrotyrosine (1:200 dilution, ABcam Cat#ab52309) or anti-active caspase 3 (1:100 dilution, Santa Cruz) antibody for 2 h. Placental specimens were washed once with PBST (0.13 M NaCl, 0.1 M NaH_2PO_4 and 0.05% Tween-20) and then incubated with peroxidase-conjugated antibody against rabbit or mouse IgG (1:300 dilution, Santa Cluz). The activity of antibody-conjugated peroxidase was determined with diaminobenzidine (DAB) or 3-amino-9-ethylcarbazole (AEC). Slides were then dried and sealed for microscopic analysis (Leica, DMLS). Images were obtained using a digital camera (Olympus, DP70) and processed by the free PLUS software supplied with the camera.

2.3. Cell culture and apoptosis assay

Human placental trophoblast, SV40 transformed cell line 3A-sub-E (3A) from American Type Culture Collection (ATCC # CRL-1584) was maintained in the α-MEM medium. Cells were incubated under hypoxia or 20% O_2 in a humidified 37 °C incubation chamber. The stable clones 3A(pNEO), 3A(siMst3)-1 and -2 (Wu et al., 2008) were maintained in the same cultural medium containing 0.3 mg/mL G418-sulfate (Invitrogen).The TUNEL (TdT-mediated, dUTP-incorporated nick-end labeling) staining of human placental specimens were performed by using Frag EL™ DNA Fragmentation Detection kit (Calbiochem). The specimens were covered with 60 μL TUNEL mix (TdT, HRP-12-dUTP, and cobalt chloride) at 37 °C for 1.5 h. A negative control was performed by omitting TdT. Human placental specimen treated with DNase I was used as a positive control. After incubation, specimens were rinsed three times with 1× PBS prior to incubating with 5 mg/mL DAB under room temperature for 10 min. The specimens were washed twice with 1× PBS and once with double-deionized H_2O before mounting for the microscopic analysis.The TUNEL assay of 3A cells was performed by using DeadEnd™ Fluorometric TUNEL System kit (Promega). Briefly, 3A cells (2×10^5 cells) were harvested and fixed with 0.1% formaldehyde for 10 min. After fixation, cells were washed three times with 1× PBS and then suspended in 5 mL 70% ice-cold ethanol. After incubating at −20 °C for at least 4 h, cells were then rinsed three times with 1× PBS and equilibrated with 100 μL of equilibration buffer (10 mM EDTA, 10 mM Tris–HCl, pH7.5, 10 mM DTT) at room temperature for 10 min. After equilibration, cells were incubated with 60 μL TUNEL mix (TdT, FITC-12-dUTP, and cobalt chloride) at 37 °C for 1 h. Reaction was terminated by adding 1 mL of 20 mM EDTA. Cells were then rinsed and suspended in 1× PBS for the analysis under a fluorescence-activated cell sorter Cytometer (CyFlow® SL, Partec). The negative control was prepared by omitting TdT in the TUNEL mix. Cell treated with DNase I was used as positive control.

For Annexin V binding assay, cells were collected from 100 mm dishes and washed twice with cold 1× PBS. Subsequently, cells were resuspended in 500 μL of 1× binding buffer (0.01 M Hepes/NaOH buffer, pH 7.4 containing 0.14 M NaCl, 2.5 mM $CaCl_2$) and stained with 5 μL of Annexin V-FITC and 5 μL of propidium iodide (50 μg/mL). After incubated at room temperature for 5 min in the dark, number of cells bound Annexin V-FITC was analyzed on flow cytometer (CyFlow® SL, Partec).

2.4. Immunoblot analysis

Cells lysate was prepared by incubating 3A cells (about 2×10^6) with 50 μL RIPA buffer (1% Triton X-100, 0.1% SDS, 50 mM Tris, pH 8.0,150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Na-deoxycholate, 5 μg/mL leupeptin, and 5 μg/mL aprotinin) at room temperature for 15 min. The particulates were removed from cell lysates by centrifugation at 4 °C and a speed of $16{,}100 \times g$ for 5 min. The explant cultures of fresh placental villous were transfected with

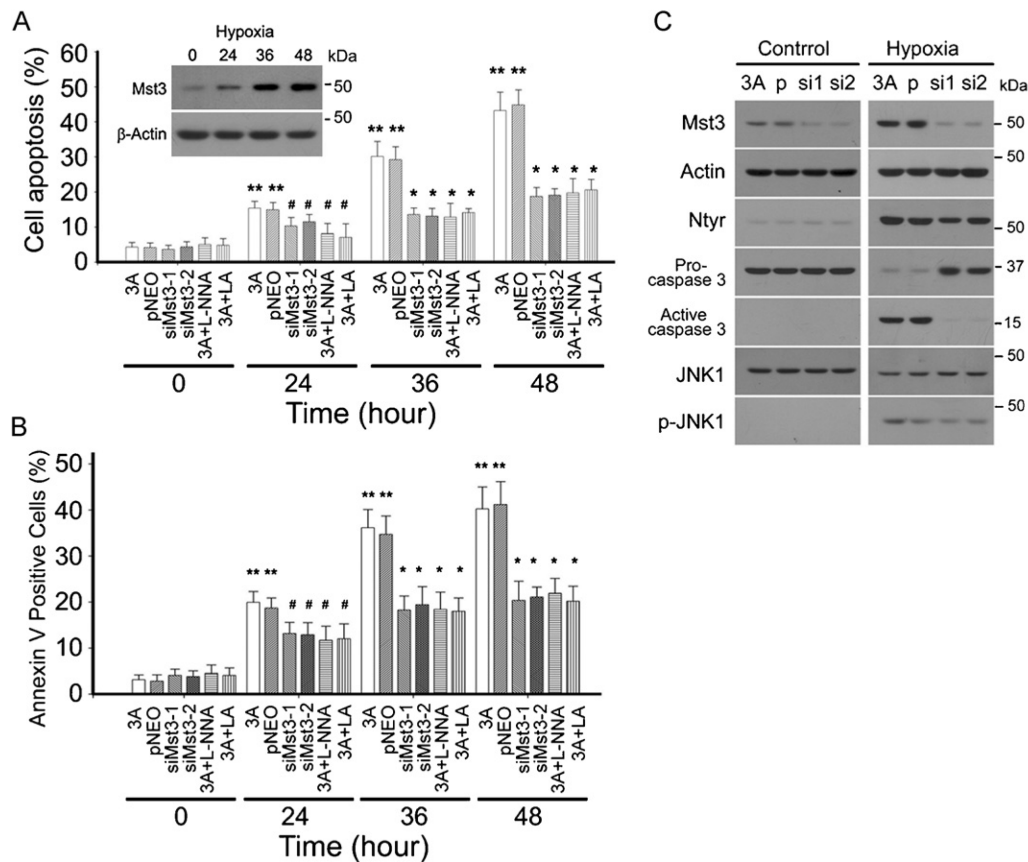

Fig. 1. Role of Mst3 in hypoxia-induced human trophoblast apoptosis. (A) The apoptosis of human trophoblasts was studied by treating with hypoxia in the presence (3A, pNEO, siMst3-1 and siMst3-2) or absence of 100 μM L-NNA (3A + L-NNA) or 10 μM LA (3A + LA) at 37 °C for 0, 24, 36 and 48 h. Following incubation, cells were subjected to TUNEL assay. Data are presented as means ± S.D. from three independent experiments. 3A and 3A(pNEO) cells are used as control. $^{**}P < 0.001$ versus controls at time 0, $^{*}P < 0.01$ versus controls at each time point and $^{\#}P < 0.05$ versus controls at 24 h. (B) Hypoxia-induced Annexin V binding was inhibited in 3A(siMst3)-1 and -2 clones. Cells were treated as described in (A). After treatment, cells were subjected to Annexin V binding analysis. Data are presented as mean ± S.D. from at least three independent experiments. $^{**}P < 0.001$ versus controls at time 0, $^{*}P < 0.01$ versus controls at each time point and $^{\#}P < 0.05$ versus controls at 24 h. (C) The level of Mst3, NTyr, active caspase 3 and p-JNK1 in cells treated with hypoxia for 36 h was determined by immunoblotting. Pro-caspase 3, JNK1 and β-actin were used as internal controls. The siMst3-1, siMst3-2 and pNEO are 3A(siMst3)-1 and 3A(siMst3)-2 and 3A(pNEO) stable clones, respectively.

pNEO or pNEO/siMst3 plasmid by Lipofectamine transfection kit (METAFECTENE®EASY, Biontex). The placental villi were then cultured under normal or hypoxic condition at 37 °C for 36 h prior to crude extract preparation.

Cell lysates or tissue crude extracts (40 μg each) were loaded on a 10% SDS-polyacrylamide electrophoresis gel (SDS–PAGE). Following electrophoresis, the protein bands were transferred to a polyvinylidine difluoride (PVDF) membrane (Millipore) by electroblotting. Immunoblot analysis was performed using antibodies specifically against Mst3 (1:2000 dilution; Santa Cruz), nitrotyrosine (1:2000 dilution, ABcam), pro-caspase 3 (1:2000 dilution, Santa Cruz), active caspase 3 (1:2000 dilution, Santa Cruz), JNK1 (1:2000 dilution, Santa Cruz), p-JNK1 (phospho-JNK1) (1:2000 dilution, Biovision) and β-actin (1:2000 dilution; Santa Cruz). Protein bands were visualized by using ECL Chemiluminescence kit.

2.5. Measurement of NO production

3A cells, 3A(pNEO) and 3A(siMst3) cells were seeded in a 96-well plates with a density of 0.5×10^4 cells/well. After culturing at 37 °C for 16 h, cells were placed in a fresh medium and incubated under hypoxia without and with 100 μM L-NNA or 10 μM LA at 37 °C for 0, 24, 36, and 48 h. At indicated times, the conditioned medium (50 μL) was collected for NO measurement. The quantitative analysis of NO production was performed by measuring the accumulation of nitrite in the conditioned medium by a microplate assay following manufacturer's protocol (Nitric Oxide Detection Kit, Stressgen, USA). Sodium nitrite was used as a standard. The absorbance of the reaction mixtures was determined at 540 nm on a microplate reader (BMG Fluostar Galaxy).

3. Results and discussion

3.1. Mst3 is essential in hypoxia-induced apoptosis of human trophoblasts

Mst3, a human counterpart of the yeast Ste20-like serine/threonine protein kinase, has been shown previously to trigger apoptosis of human trophoblasts in response to oxidative stress (Wu et al., 2008). However, its role in hypoxia-induced trophoblast apoptosis is unknown. Therefore, the role of Mst3 in hypoxia-induced apoptosis was investigated using 3A cells as a model. As shown in Fig. 1A, 3A cells under hypoxic stress exhibited a significant increase in the apoptotic cell level from 4.3 ± 1.4% at 0 h to 15.4 ± 2.0%, 30.1 ± 4.3% and 43.2 ± 5.3% at 24, 36, and 48 h, respectively (Fig. 1A). Similar results were observed in 3A(pNEO) cells, a stable clone containing the pNEO vector only. Interestingly, the level of endogenous Mst3 in 3A cells under hypoxic stress was induced in a time-dependent manner (Fig. 1A, inset and Fig. 2A, hypoxia panel). This result indicates that Mst3 in trophoblasts can be up-regulated by hypoxic stress and may correlate with hypoxia-induced trophoblast apoptosis. In comparison, Mst3 levels in 3A

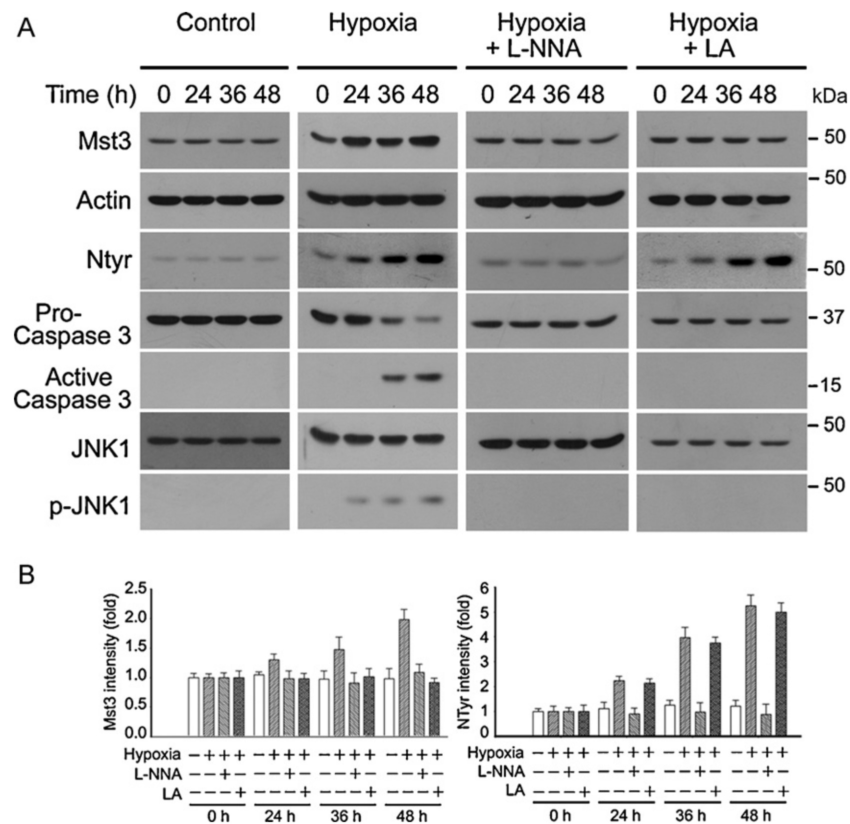

Fig. 2. Immunoblot analysis of Mst3 and related proteins in human trophobalsts. (A) 3A cells were treated with hypoxia only (hypoxia) or with either 100 μM L-NNA (hypoxia + L-NNA) or 10 μM LA (hypoxia + LA) at 37 °C for 0, 24, 36 and 48 h. 3A cells without any treatment were used as the input control. The level of Mst3, activated caspase 3, NTyr and p-JNK1 was determined by immunoblotting. Pro-caspase 3, JNK1 and β-actin were used as internal controls. The results represented here are from one of three independent experiments. (B) Quantification of the Mst3 and NTyr level in the cell lysates. Mst3 and NTyr bands were normalized with the β-actin signal in whole cell extract.

cells were unchanged under normal conditions, even after 48 h of culturing (Fig. 2A, control panel and B, left panel).

The role of Mst3 in hypoxia-induced apoptosis was then studied by using 3A(siMst3)-1 and -2, stable clones with selective knockdown of Mst3, as models (Wu et al., 2008). Western blot analysis revealed that Mst3 levels were effectively reduced in both 3A(siMst3)-1 and -2 cells, even under hypoxic stress (Fig. 1C). Compared with control cells, hypoxia-induced apoptosis was markedly reduced by 33–56% in 3A(siMst3)-1 and -2 cells (Fig. 1A). The level of apoptosis of hypoxia-treated 3A(siMst3)-1 cells was 3.6 ± 1.2%, 10.3 ± 2.4%, 13.6 ± 1.9%, 18.7 ± 2.5% and 19.1 ± 1.8% at 0, 24, 36 and 48 h, respectively (Fig. 1A). A similar result was also observed in 3A(siMst3)-2 cells. Investigation of phosphatidylserine (PS) exposure on the outer leaflet of the plasma membrane of 3A cells, which is a characteristic of early apoptosis (Savill and Haslett, 1995), further confirmed the role of Mst3. Consistent with the TUNEL assay result (Fig. 1A), the percentage of Annexin V positive cells increased upon hypoxia treatment from 3.1 ± 1.0% at 0 h to 19.9 ± 2.3%, 36.1 ± 4.0% and 40.2 ± 4.8% at 24, 36 and 48 h, respectively (Fig. 1B). Similar results were also observed for the 3A(pNEO) clone. The hypoxia-induced PS exposure was suppressed by 28–56% in 3A(siMst3)-1 and -2 stable clones (Fig. 1B, si-1 and si-2). These results indicate that Mst3 plays an important role in hypoxia-induced apoptosis of human trophoblasts. In addition, the correlation between hypoxia-induced trophoblast apoptosis and the increase of Mst3 level in cells was also suggested.

The p53 was shown previously to play a role in the hypoxia-induced trophoblast apoptosis (Crocker, 2007; Heazell et al., 2008; Levy et al., 2000). Recently, p53 was indicated to be down-regulated by hypoxia in human syncytiotrophoblasts. However, down-regulation of p53 in response to hypoxia was postulated to be a survival strategy for cells to eliminate the apoptosis tranduced by the p53 pathway (Chen et al., 2010). A p53-independent pathway may be activated during the apoptosis of syncytiotrophoblasts exposed to extreme hypoxia. Interestingly, the activity of p53 was shown to be down-regulated in SV40-transformed human cell lines (Ludlow, 1993; Digweed et al., 2002). Although the activity of p53 in 3A cell, a SV40 transformed human placental trophoblast cell line, has not been studied, the nullification of p53 properties in 3A cells was hypothesized. Based on these observations Mst3 is postulated to work in a manner independent of p53 and play a role in the hypoxia-induced trophoblast apoptosis.

3.2. Effect of oxidative stress in hypoxia-induced apoptosis

Oxidative stress was postulated to be elevated in the placenta under the phathophysiological conditions (Hung et al., 2001; Heazell et al., 2007; Jauniaux et al., 2000; Redman and Sargent, 2000). Thus, DL-α-lipoic acid (LA), a potent ROS scavenger, was used to explore the role of oxidative stress in the hypoxic responses of 3A cells. As shown in Fig. 1A, hypoxia-induced apoptosis of 3A cells was inhibited by 40–50% with 10 μM LA, suggesting the involvement of oxidative stress in the hypoxic responses of trophoblasts. Although LA was shown recently to inhibit NF-κB activation through antioxidant-independent mechanism, high concentrations of LA (>300 μM) is required for this process (Ying et al., 2010). At concentrations of 100 μM or lower (e.g., 10 μM) LA should merely exhibit antioxidant activity. Immunoblot analysis showed that hypoxia-induced expression of Mst3 in 3A cells was markedly suppressed by LA (Fig. 2A, hypoxia + LA). Although the exact mech-

Table 1
NO production of parental 3A and 3A stable clones under hypoxia.

Time (h)	3A		3A (peon)		3A (siMst3)-1		3A (siMst3)-2	
	Normal (μM)	Hypoxia (μM)	Normal (μM)	Hypoxia (μM)	Normal (μM)	Hypoxia (μM)	Normal (μM)	Hypoxia (μM)
0	7.8 ± 2.4	7.3 ± 1.7	8.1 ± 1.9	7.9 ± 2.2	7.2 ± 2.1	8.3 ± 1.7	7.6 ± 2.8	7.5 ± 1.9
12	7.0 ± ±1.5	13.2 ± 2.4	7.3 ± 2.1	13.6 ± 1.9	8.1 ± 2.4	12.3 ± 2.0	8.2 ± 1.9	13.0 ± 2.3
24	8.9 ± 1.8	23.6 ± 2.2	9.0 ± 2.5	22.5 ± 1.8	9.3 ± 1.9	20.2 ± 3.0	9.0 ± 2.4	20.8 ± 2.7
36	9.2 ± 2.1	35.5 ± 3.4	10.0 ± 1.9	36.9 ± 3.2	10.0 ± 2.6	33.4 ± 2.5	9.8 ± 2.7	34.2 ± 2.7

anism of hypoxia to induce oxidative stress is unclear so far, the oxidative stress is shown to play a role in the hypoxia-induced apoptosis of human trophoblast.

The mitochondrial respiratory chain is thought to be one of the major sources of intracellular ROS production, presumably from complexes I and III of the electron transport chain (Manoli et al., 2007; Poyton et al., 2009). However, several lines of evidence have shown that endothelial nitric oxide synthase (eNOS) may be a source of ROS, which promotes oxidative stress in placentas of pregnancies complicated with preeclampsia (Many et al., 2000; Noris et al., 2004; Orange et al., 2003). NOS has also been shown to generate both NO and O_2^- in tissues under hypoxic stress (Wang et al., 2000; Xia et al., 1996). It is most possibly that various ROS may be formed by the excess production of O_2^- by NOS (Fujita et al., 2008).

3.3. Role of NOS in hypoxia-induced trophoblast apoptosis

Human placenta was shown to contain all three forms of NOS, i.e., endothelial NOS, neuronal NOS and inducible NOS (Iida et al., 1997). It is not clear which type of NOS will participate in the hypoxia-induced apoptotic pathway. Therefore, N^G-nitro-L-arginine (L-NNA), a non-selective NOS inhibitor, was used to explore the role of NOS in human trophoblast during hypoxic stress. When cells were co-treated with hypoxia and 100 μM L-NNA for 24, 36, and 48 h, the level of apoptosis of 3A cells decreased from 15.4 ± 2.0% to 8.1 ± 2.9%, from 30.1 ± 4.3% to 12.8 ± 4.0% and from 43.2 ± 5.3% to 19.7 ± 4.1%, respectively (Fig. 1A). These results demonstrate that NOS plays a role in hypoxia-induced apoptosis of human trophoblasts. Immunoblot analysis showed that hypoxia-induced expression of Mst3 in 3A cells was effectively suppressed by 100 μM L-NNA (Fig. 2A, hypoxia + L-NNA and Fig. 2B, left panel). As expected, the H_2O_2-induced responses, such as the up-regulation of Mst3 and activation of JNK1 and caspase 3 (Wu et al., 2008), could be effectively inhibited by LA (Supplemental results, Fig. S1). In contrast, L-NNA was ineffective on the H_2O_2-induced responses in 3A cells. This observation suggests that ROS may be generated downstream to NOS activation. However, the presence of other sources for ROS that are also induced by hypoxia cannot be ruled out.

Peroxynitrite ($ONOO^-$), a highly reactive pro-oxidant, can be formed from the interaction of NO and O_2^- (Wang et al., 2000; Xia et al., 1996) and quickly modify proteins at tyrosine residues to form nitrotyrosine (NTyr) (Noris et al., 2004; Webster et al., 2008). Therefore, the formation of NTyr may indicate the elevation of NO and oxidative stress in response to hypoxia (Fujita et al., 2008; Stanek et al., 2001) and can be readily determined by the appearance of a major nitrated protein at around 55 kDa by immunoblotting (Satoh et al., 2008). The formation of NTyr in 3A cells increased in response to hypoxia in a time-dependent manner (Fig. 2A, hypoxia panel). Compared to untreated cells, the levels of NTyr in 3A cells increased under hypoxic stress by two to five folds (Fig. 2B, right panel). Interestingly, the hypoxia-induced formation of 55 kDa nitrated protein could be effectively suppressed by L-NNA (Fig. 2A, hypoxia + LNNA panel), but not by LA (hypoxia + LA panel). Although LA was shown to be a potent scavenger of various ROS, it bound O_2^- poorly (Biewenga et al., 1997; Fujita et al., 2008). This may partially explain why LA did not suppress the hypoxia-induced formation of NTyr in 3A cells. This result also indicates that the activity of NOS may not be regulated by ROS.

The relationship between NOS and Mst3 in the signaling of hypoxia-induced trophoblast apoptosis was further investigated by measuring NO release in stable clones with selective knockdown of Mst3. The level of NO in 3A, 3A(pNEO), 3A(siMst3)-1 and 3A(siMst3)-2 cells was almost undetectable under normal cultural conditions (Table 1). Once treated with hypoxia, however, a similar level of NO was found to be released from all cell types tested in a time-dependent manner (Table 1). This result is consistent with the finding in Fig. 1C and suggests that hypoxia-induced NO production is unaffected by the selective knockdown of Mst3. These results confirm, at least partially, the hypothesis that NOS can be induced by hypoxia and generates both NO and O_2^-.

3.4. NOX is involved in hypoxia-induced Mst3 up-regulation

It has been demonstrated that NADPH oxidase (NOX) plays a role in generating ROS in human placentas in normal and preeclampic women (Cui et al., 2006; Raijmakers et al., 2004). Superoxide production in human placenta can be inhibited by DPI, a specific inhibitor of NOX (Raijmakers et al., 2004). Furthermore, NOX1 expression is significantly increased in syncytiotrophoblast and endothelial cells in the placentas of patients with preeclampsia (Cui et al., 2006). Hence, the role of NOX in hypoxia-induced trophoblast apoptosis was investigated by apocynin, a potent and specific inhibitor of NOX. Hypoxia-induced apoptosis of 3A cells was inhibited by 23–60% with apocynin (Fig. 3A). When cells were co-treated with hypoxia and 100 μM apocynin for 24, 36, and 48 h, the level of apoptosis of 3A cells decreased from 17.3 ± 2.7% to 13.4 ± 1.7%, from 34.3 ± 3.9% to 14.8 ± 2.9% and from 52.1 ± 4.7% to 20.5 ± 3.7%, respectively (Fig. 3A). The suppressive effect of apocynin in the hypoxic responses of 3A cells, including Mst3 expression and the activation of JNK1 and caspase 3, was also demonstrated on immunoblot analysis (Fig. 3B). This result suggests that NOX may be involved in hypoxia-mediated signaling in human trophoblasts. Recently, apocynin was reported to suppress collagen-induced platelet aggregation by an unidentified target (Dharmarajah et al., 2010). This observation suggests that NOX may not be the only target affected by apocynin in trophoblasts. However, whether this unknown target participates in the hypoxia-induced apoptotic pathway remains to be elucidated. The presence of apocynin did not further enhance the suppressive effect of selective knockdown of Mst3 in hypoxia-induced apoptosis (Fig. 3A). Compared to L-NNA (Fig. 1A), apocynin exhibits a similar inhibitory effect in the hypoxia-induced trophoblast apoptosis, suggesting the existence of a linkage between NOS and NOX in the hypoxia-mediated signaling pathway. Furthermore, the above results also suggest that Mst3 may be the major downstream effector of NOS and NOX signaling.

The effect of apocynin in the formation of NTyr, an indicator of NOS activation during hypoxic stress, was further studied to explore the relationship between NOS and NOX in the signaling of hypoxia-induced trophoblast apoptosis. Interestingly, the

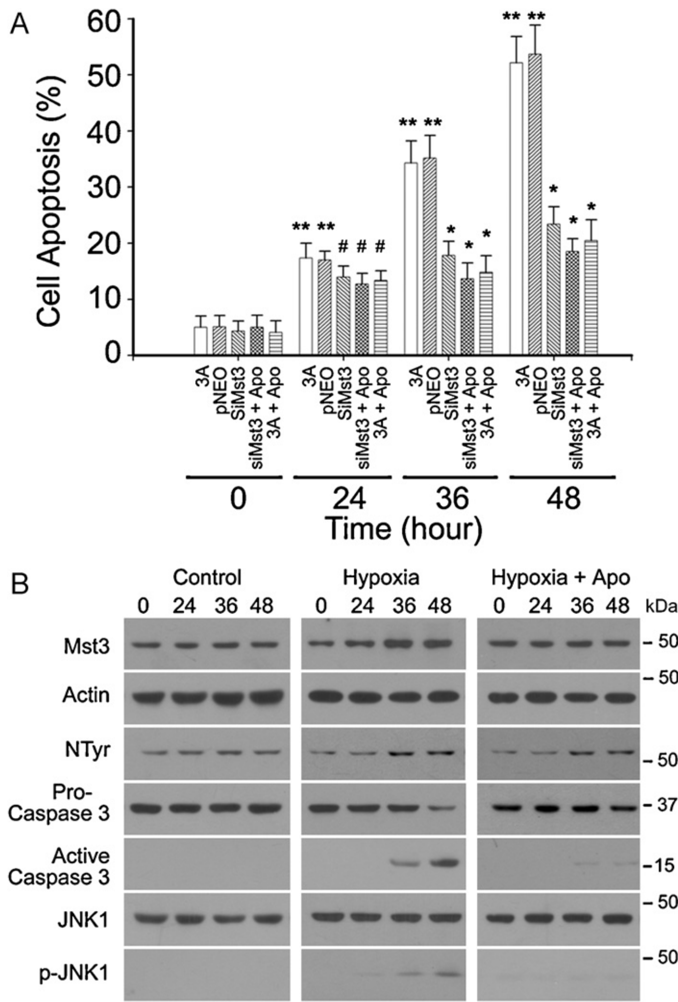

Fig. 3. Role of NOX in hypoxia-induced trophoblast apoptosis. (A) 3A cells were cultured under hypoxic conditions in the absence or presence of 100 μM apocynin at 37 °C for 0, 24, 36 and 48 h. Following incubation, cells were subjected to TUNEL assay. 3A cells grown under normal condition were used as controls. Data is presented as means ± S.D. from three independent experiments. $^{**}P < 0.001$ versus controls at time 0, $^{*}P < 0.01$ versus controls at each time point and $^{\#}P < 0.05$ versus controls at 24 h. (B) Cells treated with hypoxia in the absence (hypoxia) or the presence of 100 μM apocynin (hypoxia + Apo) were subjected to immunoblotting to determine the level of Mst3, NTyr, active caspase 3 and p-JNK1. 3A cells (3A) grown under normal conditions were used as a control. Pro-caspase 3, JNK1 and β-actin were used as internal controls. The represented results are from one of three independent experiments.

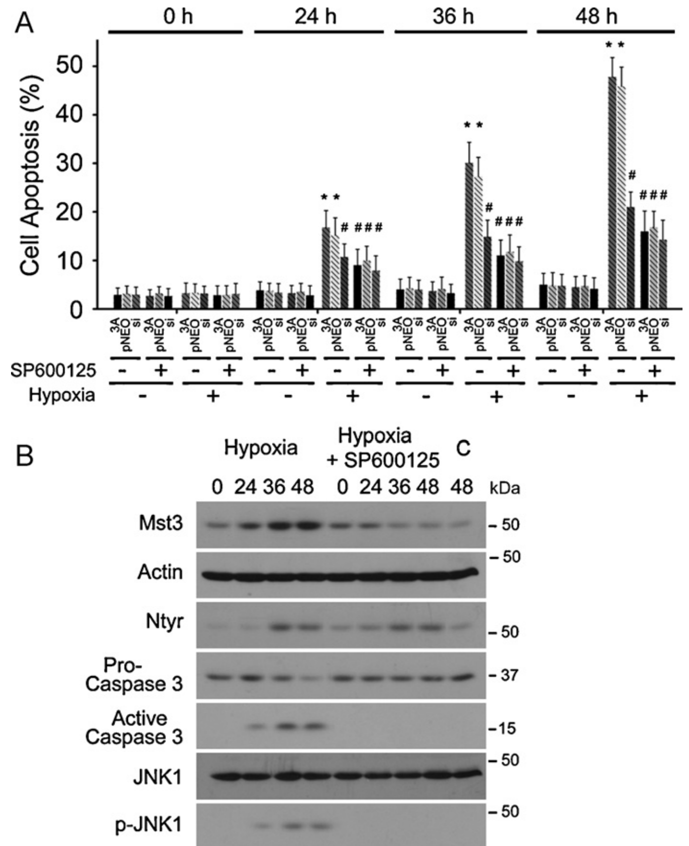

Fig. 4. Effect of SP600125 in the hypoxia-induced cell apoptosis. (A) 3A cells were pretreated with 1 nM SP600125 at 37 °C for 3 h before treatment with hypoxia at 37 °C for 0, 24, 36 and 48 h. Following incubation, cells were subjected to TUNEL assay. 3A cells grown under normal condition were used as controls. Data are presented as means ± S.D. from three independent experiments. $^{*}P < 0.001$ versus controls at time 0 and $^{\#}P < 0.01$ versus controls at each time point. (B) Immunoblot analysis of 3A cells with (hypoxia + SP600125) or without (hypoxia) pretreatment of 1 nM SP600125 at 37 °C for 3 h prior to hypoxia treatment at 37 °C for 0, 24, 36 and 48 h. 3A cell grown under normal condition at 37 °C for 48 h was used as a control (3A). Cell lysates (40 μg each) were separated on a 10% SDS–PAGE and analyzed for Mst3, activated caspase 3, NTyr and p-JNK1 by immunoblotting. Actin, pro-caspase 3 and JNK1 were used as internal controls. Represented results were from one of two independent experiments.

hypoxia-induced NTyr formation in 3A cells was affected little by apocynin (Fig. 3B), but was effectively suppressed by L-NNA (Fig. 2A, hypoxia + L-NNA), suggesting that the production of NO and O_2^- by NOS was unaffected by the activity of NOX. These results were in consistent with the previous observation that both inhibitors exerted similar inhibitory effect to the hypoxia-induced apoptosis of 3A cells (Figs. 1A and 3A). Although the relationship between and NOS and NOX is unclear so far, it is possible that NOX acts downstream to NOS in the hypoxia-mediated signaling pathway.

3.5. Role of caspase 3 and JNK

JNK1 have been demonstrated to be activated in human placenta and trophoblast cell lines and acts as an upstream regulator of Mst3 in the oxidative stress-induced apoptosis signaling pathway (Wu et al., 2008). The involvement of JNK1 in the hypoxia-induced apoptosis signaling pathway was investigated by SP600125. SP600125 was demonstrated to specifically inhibit the activity of JNK at low concentrations (around nanomolar range) and exhibit inhibitory effect to many other protein kinases at high concentrations (micromolar range) (Bennett et al., 2001). Interestingly, the hypoxia-induced apoptosis of 3A and 3A(pNEO) cells could be effectively suppressed by 1 nM SP600125 by 45–67% and 36–64%, respectively (Fig. 4A). Immunoblot analysis also demonstrated that the active JNK1 (p-JNK1) was elevated in human trophoblast in response to hypoxia (Figs. 2A, 3B and Supplemental results Fig. S2B, hypoxia panel). The total JNK1 level, however, was unchanged. Inhibition of JNK activity by SP600125 leads to the inhibition of hypoxia-induced Mst3 expression and caspase 3 activation without affecting the formation of NTyr in 3A cells (Fig. 4B). Moreover, the activation of JNK1 by hypoxia was significantly inhibited by L-NNA, LA (Fig. 2A) and apocynin (Fig. 3B). These results suggest that JNK1 acts as a mediator that bridges the upstream NOS and NOX and the downstream Mst3 in the hypoxia-induced signaling pathway. Interestingly, hypoxia-induced JNK1 activation was moderately affected by the selective knockdown of Mst3 (Fig. 1C). It is possible that the activity of JNK1 can be regulated by Mst3, presumably through a feedback control.

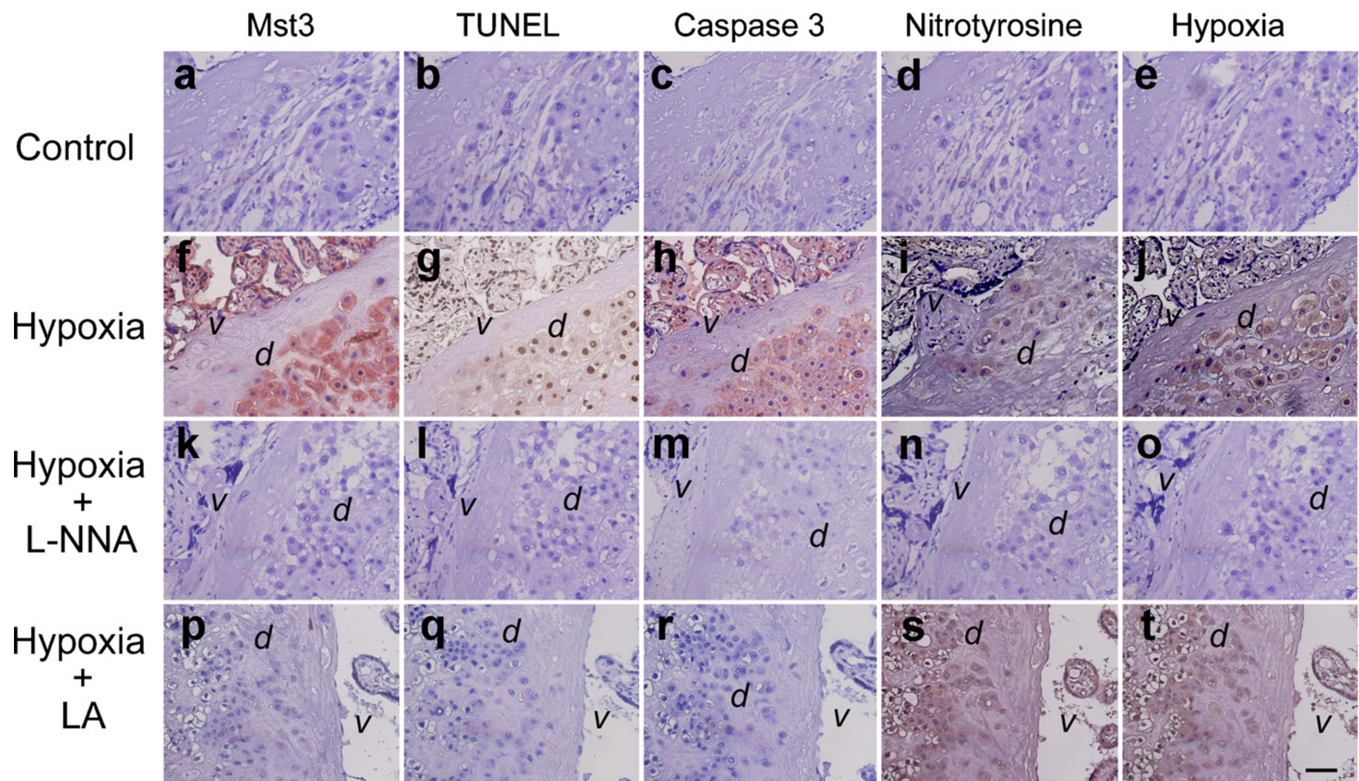

Fig. 5. Immunohistochemical study of fresh human placental explants. Fresh human placental specimens were incubated in cultivation medium under hypoxia in the absence (f–j) and presence of 100 μM L-NNA (k–o) or 10 μM LA (p–t) at 37 °C for 24 h prior to immunohistochemical study. Human placental specimens cultured under normal conditions were used as negative controls (a–e). The human placental specimens were analyzed for Mst3 expression (AEC stain) (a, f, k and p), DNA fragmentation (TUNEL assay; DAB stain) (b, g, l and q), caspase 3 activation (AEC stain) (c, h, m and r), pimonidazole–protein adduct formation (hypoxia marker; DAB stain) (e, j, o and t), and NTyr formation (DAB stain) (d, i, n and s), respectively. A representative bar is 100 μm and applies to all panels. (v) placental villous tissue; (d) decidural tissue.

Caspase 3 was shown previously to be regulated by Mst3 in human trophoblasts (Wu et al., 2008). A similar result was also observed in human trophoblasts under hypoxic stress (Fig. 1C). Caspase 3 was inactive in 3A cells without treatment and significantly activated by hypoxia. The hypoxia-induced caspase 3 activation could be suppressed by selective knockdown of Mst3, presumably through suppressing the activity of Bax (Lin et al., 2010). Expectedly, the hypoxia-induced activation of caspase 3 can also be inhibited by L-NNA, LA (Fig. 2A), apocynin (Fig. 3B) and SP600125 (Fig. 4B). However, a caspase-independent pathway was also hypothesized to be triggered by Mst3 in response to stress (Lin et al., 2010).

3.6. Hypoxia induces apoptosis of trophoblasts in fresh human placental explants

The role of Mst3 in hypoxia-induced trophoblast apoptosis was further studied by using fresh human placental explants ($n = 6$) as a model (Genbacev et al., 1993; Wu et al., 2008). Fresh human placental tissue specimens (1 cm × 1 cm × 0.3 cm) were incubated under hypoxic conditions for 24 h prior to imunohistochemcial analysis. The immunohistograms of explants clearly showed characteristics of apoptosis, including the expression of Mst3 (reddish staining, Fig. 5f), DNA fragmentation (TUNEL assay; Fig. 5g) and caspase 3 activation (Fig. 5h), all of which were induced in villous and extravillous trophoblasts by hypoxia. The existence of pimonidazole–protein adduct (brownish DAB staining; Fig. 5j) in the human placental explant confirms the occurrence of hypoxia (Westbury et al., 2007). Explants cultured under normal conditions were used as a control and exhibited little or no signs of Mst3 expression, DNA fragmentation, caspase 3 activation, pimonidazole–protein adduct formation or NTyr formation (Fig. 5a–e). These results suggest that hypoxia plays a role in inducing apoptosis in trophoblasts, presumably through the activation of Mst3.

Consistent with previous observations, both L-NNA and LA effectively inhibited hypoxia-induced Mst3 expression (Fig. 5k and p), DNA fragmentation (Fig. 5l and q) and caspase 3 activation (Fig. 5m and r) in human explants. NTyr formed in villous and extravillous trophoblasts of human explants under hypoxic stress (Fig. 5i; dark brown DAB staining) and was only blocked by L-NNA (Fig. 5n). LA is unable to suppress the formation of pimonidazole–protein adduct (Fig. 5s) and NTyr formation (Fig. 5t). These results confirm the hypothesis that NOS participates in the signaling pathway of hypoxia-induced trophoblast apoptosis. Furthermore, oxidative stress may occur downstream to NOS reactivity. The formation of pimonidazole–protein adducts in the hypoxia-treated explant was also unexpectedly suppressed by L-NNA (Fig. 5o). It is possible that L-NNA may influence the reductive activation of pimonidazole in hypoxia and hence reduce the formation of pimonidazole–protein adducts.

The role of Mst3 in the hypoxic response of human placental explants was partially confirmed by selectively knocking down of endogenous Mst3 from human placental specimens of normal pregnant women ($n = 3$) (Wu et al., 2008). The tissue lysates were then prepared and subjected to immunoblot analysis. As shown in Fig. S2 (Supplemental results), endogenous Mst3 in placental specimens was reduced by approximately 50% by siRNA. Compared to normal explants, hypoxia-induced caspase 3 activation was markedly suppressed by selective knockdown of Mst3

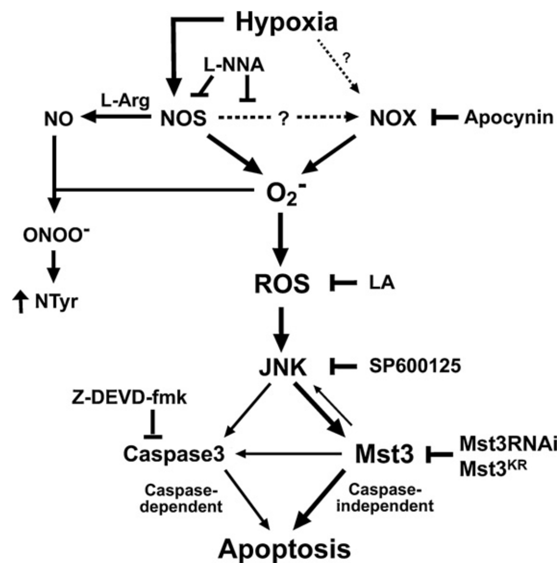

Fig. 6. Schematic illustration of the possible signaling pathway of hypoxia-induced apoptosis of human trophoblasts. Hypoxia induces apoptosis of human trophoblasts via activation of nitric oxide synthase (NOS), which generates NO and O_2^-. ROS is then elevated due to the excessive production of O_2^-. NADPH oxidase (NOX) may also contribute to the release of O_2^- in response to the treatment of hypoxia. Subsequently, ROS induces the upregulation of Mst3 via the activation of JNK. The apoptosis can then be induced by Mst3 through both caspase-dependent and -independent pathways. The proposed major pathways are indicated by the large arrows.

(Supplemental results, Fig. S2). The hypoxia-induced JNK1 activation and NTyr formation in explant culture, however, were only slightly affected by the selective knockdown of Mst3.

4. Conclusion

Mst3 is the human counterpart of the yeast Ste20-like serine/threonine protein kinase with a molecular mass of 47.5 kDa. It has been shown to trigger apoptosis of many cells lines in response to environmental cues (Huang et al., 2002; Lin et al., 2010; Wu et al., 2008). In human trophoblasts, Mst3 has previously been shown to trigger apoptosis in response to oxidative stress (Wu et al., 2008). In this study Mst3 was further demonstrated to participate in the signaling pathway of hypoxia-induced apoptosis of human trophoblasts. The schematic diagram detailing the hypoxia-induced apoptotic signaling pathway in human placenta is proposed in Fig. 6. In this proposed pathway, hypoxia may first activate NOS, which generates both NO and O_2^- under stress (Orange et al., 2003; Xia et al., 1996). NO interacts with O_2^- and forms $ONOO^-$, which may then exert a nitrative stress such as formation of NTyr to cells (Myatt, 2010). In this proposed apoptotic signaling pathway NOX may also be activated and genrate O_2^- (Many et al., 2000; Noris et al., 2004; Orange et al., 2003). Although it is not clear how NOX was activated, it could be activated directly by hypoxia or indirectly through the activation of NOS, presumably through nitrative stress (Fig. 6). The generated O_2^- then further breaks down and produces ROS, which induces oxidative stress in trophoblasts. Subsequently, JNK is activated by oxidative stress and up-regulates Mst3. Mst3 further triggers both caspase-dependent and caspase-independent apoptotic pathways in human trophoblasts (Fig. 6). However, the caspase-dependent mechanism may play a minor role in the proposed hypoxia-induced apoptosis of trophoblasts (Lin et al., 2010; Wu et al., 2008).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biocel.2011.01.015.

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