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Cell Injury, Repair, Aging and Apoptosis

Carbon Monoxide Inhibits Hypoxia/Reoxygenation-Induced Apoptosis and Secondary Necrosis in Syncytiotrophoblast

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Pre-eclampsia, a hypertensive disorder of pregnancy, affects 5 to 7% of pregnancies. Oxidative stress-induced placental injury and subsequent release of placental debris into the maternal circulation are key pathogenic events in the progression of pre-eclampsia. Women who smoke cigarettes throughout pregnancy are 33% less likely to develop this disorder than nonsmoking women. We postulated that elevated carbon monoxide concentrations in serum of smoking women inhibits apoptosis and debris shedding of trophoblast cells exposed to ischemia-reperfusion injury because carbon monoxide has cytoprotective effects on endothelial and smooth muscle cells in culture. This may be responsible for the reduced risk of pre-eclampsia in smoking women. To assess the cytoprotective properties of carbon monoxide within placental tissue, carbon monoxide treatments were administered to *in vitro* hypoxia/reoxygenation-insulted villous explants cultured from term human placenta. Induction of apoptosis was assessed using molecular and morphological approaches. Placental villous explants treated with carbon monoxide demonstrated 60% less hypoxia/reoxygenation-induced apoptosis in the differentiated syncytiotrophoblast layer compared with untreated explants undergoing a similar insult. In addition, retention of intact syncytial membranes was observed in carbon monoxide-treated explants. These observations indicate that carbon monoxide has potent anti-apoptotic properties within human placenta and may hold therapeutic potential in the treatment of pre-eclampsia. ***(Am J Pathol 2006, 169:774–783; DOI: 10.2353/ajpath.2006.060184)***

Pre-eclampsia (PE) affects 5 to 7% of all pregnancies and is a leading cause of maternal morbidity and mortality world wide.[1,2] PE is clinically characterized by maternal hypertension and proteinuria, typically presenting in the third trimester. The pathological processes responsible for this disorder are thought to originate early in gestation during placentation.[1–3] Trophoblast cells of the human placenta normally invade and remodel the maternal spiral arterioles at weeks 12 to 16 of gestation to expand vessel capacity and optimize nutrient and oxygen delivery to the developing fetus. In PE, the structural changes to the spiral arterioles are incomplete or absent.[1,4,5] The result is abnormally high vascular resistance with pulsatile blood flow to the placenta because the unaltered small diameter spiral arterioles remain responsive to vasoactive compounds in the maternal circulation.[6,7] This sets up an environment for ischemia-reperfusion injury. Ischemia-reperfusion injury is the result of generated free radicals on reintroduction of molecular oxygen into ischemic tissues. The outcome is widespread lipid and protein oxidative modifications, mitochondrial injury, and tissue apoptosis and necrosis.[8,9] In the placenta, areas of oxidative stress generate reactive oxygen species and the release of proinflammatory cytokines.[3,10]

The syncytiotrophoblast, a multinucleated trophoblast layer, lines the intervillous spaces and is in direct contact with the maternal circulation. This syncytium readily undergoes apoptosis and necrosis under conditions of oxidative stress.[6,11] As a result, placental debris is shed into the maternal circulation along with the generated free radicals and cytokines, each con-

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tributing to a heightened maternal systemic inflammatory response.[3,12–15] Elevated shedding of debris is postulated to be the pivotal step in the pathophysiology of PE because it transforms a placental disorder into a systemic maternal disease. The release of placental debris into the maternal circulation elicits, along with the heightened maternal inflammatory response, endothelial dysfunction resulting in the clinical disease.[3,16] At present, timely delivery is the only treatment for PE and is usually associated with prematurity of the infant and increased neonatal morbidity/mortality.

Several factors predispose women to develop PE including PE in a previous pregnancy, family history of PE, diabetes, and conception with a new sexual partner.[17–19] In contrast, a reduced risk for PE is found in women smoking cigarettes throughout pregnancy. The reduction in risk is 33% compared to nonsmoking women.[20] The mechanism through which smoking confers protection from PE is unknown. However women who use snuff, a form of smokeless tobacco, do not have a reduced risk for PE but rather an increased incidence compared with controls.[21] It is hypothesized that exposure to elevated concentrations of exogenous carbon monoxide (CO), one of the combustible byproducts of cigarette smoke, mediates the observed protective effects of smoking on PE.

Environmental pollution and cigarette smoking are major exogenous sources of CO exposure. Carbon monoxide is also endogenously produced throughout the body with important physiological functions including platelet aggregation, anti-inflammatory actions, and smooth muscle relaxation.[22–24] Heme oxygenase, the key enzyme involved in CO generation, is widely distributed throughout tissues and organs, including the placenta.[25,26] There is increasing evidence that CO is required for normal placental development and function; endogenous CO production may regulate trophoblast migration and organization[1,25,26] and play a hemodynamic role.[27] Further, women who have PE have significantly decreased CO concentrations in their exhaled breath compared with healthy pregnant women,[28,29] suggesting an endogenous regulatory role of this gaseous compound in the maintenance of healthy pregnancies.

Carbon monoxide is of considerable interest in transplant research.[30] In animal models, transplanted organs that are either genetically modified to up-regulate endogenous CO production or harvested under high concentrations of CO before transplantation have significantly improved survival.[31–34] In addition, if the recipient of the allograft is exposed to relatively low doses of exogenous CO, via inhalation or treatment with the CO-releasing compound methylene chloride, both organ and recipient survival rates are improved.[34,35] Organs transplanted in the CO treatment group had fewer surrounding inflammatory cells, increased perfusion of the allograft, and fewer apoptotic cells.[34–37] The cytoprotective effects of CO have also been described in endothelial and smooth muscle cells in culture.[38,39]

It is hypothesized that the increased CO concentration in the serum of smoking women inhibits the apoptosis and debris shedding of the syncytiotrophoblast into the maternal circulation after an ischemia-reperfusion injury, thus reducing the development of PE. The objective of the current study was to determine whether CO had anti-apoptotic properties in syncytiotrophoblast of term human placenta after a hypoxia/reoxygenation (H/R) insult.

Materials and Methods

This research study and use of human placental tissues was approved by the Queen's University (Kingston, ON, Canada) research ethics board before initiation.

Reagents and Solutions

Villous explant culture medium contained 2.2 mg/ml $NaHCO_3$, 100 μg/ml streptomycin sulfate, 100 IU/ml penicillin G, 1 μg/ml insulin, and 2 μg/ml L-glutamine in CMRL-1066 (Invitrogen, Burlington, ON, Canada) supplemented with 5% heat-inactivated fetal calf serum.[40] All reagents, unless otherwise indicated, were purchased from Sigma-Aldrich Ltd. (Oakville, ON, Canada).

CO-saturated culture medium was prepared on the day of experiment by bubbling CO gas (99.5%; Praxair, Toronto, ON, Canada) through 30 ml of normal culture medium for 30 minutes. Three serial dilutions of the CO-saturated medium in normal medium were prepared and stored in air-tight sealed bottles. CO concentrations in all solutions were measured using gas-solid chromatography as described previously.[27]

Tissue Collection and Culture

Term human placentas (n = 13) were collected immediately after elective cesarean section from nonlaboring, nonsmoking, low-risk women at the Kingston General Hospital (Kingston, ON, Canada). Cubes of 2.5 cm of placenta, devoid of calcifications or tears, were dissected from 7 to 10 random sites across each placenta. The chorionic plate and basal plate were removed from each piece of tissue. The tissue was transferred to the laboratory in a sealed bottle containing ice-cold phosphate-buffered saline (PBS).[6]

Chorionic villous explants (5 to 10 mg each) were dissected on ice from the placental tissue cubes and rinsed 2× with ice-cold PBS and 3× with explant culture medium. Five explants were cultured in individual Costar Netwell supports (15-mm diameter, 74 μm mesh; Cole-Parmer, Anjou, QC, Canada) in 1.2 ml of culture medium[11] and exposed H/R as previously described[6] (Figure 1). Initially, explants were cultured under hypoxic conditions (pO_2 in medium = 12 to 16 mmHg[6]) for 3 hours then oxygenation for 3 hours using gas phase 21% O_2/5% CO_2/balance N_2 (pO_2 in medium = 133 to 152 mmHg). A subset of villous explants (n = 6 placentas, 30 explants) were subjected to a repeat of this gaseous cycle. The untreated group (n = 13 placentas, 65 explants) was bathed with normal culture medium and the CO-treated group (n = 13 placentas, 65 explants) was bathed in the CO-infused culture medium. Both treated and untreated explant

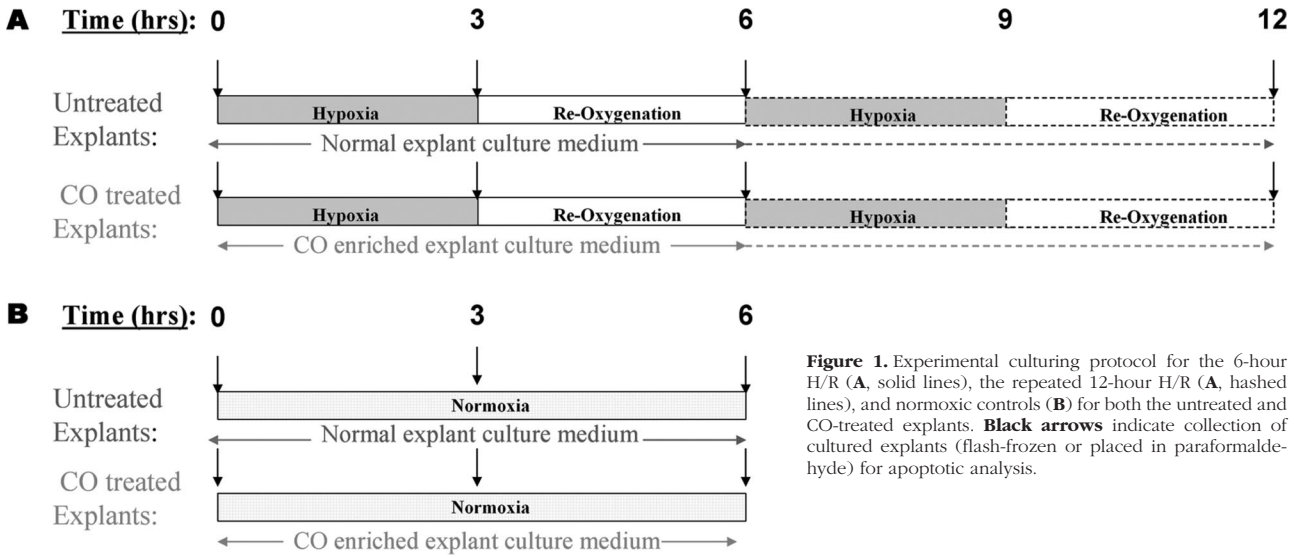

Figure 1. Experimental culturing protocol for the 6-hour H/R (**A**, solid lines), the repeated 12-hour H/R (**A**, hashed lines), and normoxic controls (**B**) for both the untreated and CO-treated explants. **Black arrows** indicate collection of cultured explants (flash-frozen or placed in paraformaldehyde) for apoptotic analysis.

culture controls were kept under normoxic conditions throughout the entire 6-hour experimental period. A normoxic environment was established using a 5% O_2/90% N_2/5% CO_2 gas mixture resulting in pO_2 of 45 to 62 mmHg[6] in the culture medium, a concentration that is similar to the oxygen tension measured within the intervillous space of term placenta.[41,42] All explants were incubated at 37°C throughout the experiment. At time 0, 3, 6, and 12 hours (in the subset of repeated H/R insult experiments) explants were collected from all experimental groups and either flash-frozen for molecular analysis or fixed in paraformaldehyde for histological analysis.

Terminal dUTP Nick-End Labeling (TUNEL) Assay and Apoptotic Index

Explants collected at 0, 3, 6, and 12 hours were fixed using 4% paraformaldehyde, embedded in paraffin, and cut into serial sections. To assess apoptosis of the syncytiotrophoblast layer, an immunofluorescence TUNEL assay was performed according to the manufacturer's instructions (*In Situ* Cell Death Detection kit; Roche Molecular Biochemicals, Laval, QC, Canada). Negative controls were sections incubated without terminal deoxynucleotidyl transferase (TdT). All sections were mounted with an aqueous medium containing 4,6-diamidino-2-phenylindole (DAPI) (Vectashield Mounting Medium; Vector Laboratories, Burlington, ON, Canada).

All sections were observed by a blinded third party using a Leica inverted microscope (Leica Microsystems, Richmond Hill, ON, Canada) at a magnification of ×20. Digital images of the TUNEL (green) and DAPI (blue) staining were captured at three randomly selected fields, providing a minimum of 900 syncytiotrophoblast nuclei per slide. Digital images were captured and deconvolved using Slidebook 4.1 software (Intelligent Imaging Innovations, Inc., Denver, CO). TUNEL-positive nuclei (apoptotic nuclei) and DAPI-stained nuclei (total nuclei) were counted in the syncytiotrophoblast cell layer using Image-Pro Plus 5.1 software (Media Cybernetics Inc., Silver Spring, MD). The apoptotic index in each section was calculated as the percentage of syncytiotrophoblast nuclei stained TUNEL-positive divided by the total number of DAPI-stained nuclei found within the syncytiotrophoblast.

Immunohistochemical Staining for the p85 Fragment of Poly-ADP Ribose Polymerase (PARP)

Paraffin-embedded placental sections were randomly selected and deparaffinized by heating them at 40°C for 20 minutes followed by sequential incubations in Hemo-D and decreasing ethanol baths (each for 2 × 3 minutes). Endogenous peroxidase activity was quenched using 3% hydrogen peroxide and nonspecific binding was limited using 5% normal goat serum. The sections were reacted with a rabbit polyclonal anti-PARP p85 fragment antibody (1:500; Promega, Nepean, ON, Canada) for 4 hours at room temperature. A secondary anti-rabbit horseradish peroxidase conjugate (1:200; Vector Laboratories) was then added to the sections. Additional processing of the sections for the detection of the p85 fragment of PARP was performed according to the instructions provided with the Vectashield Elite ABC kit (Vector Laboratories). Colorimetric detection was achieved using diaminobenzidine as the peroxidase substrate. Quantification of the p85-fragmented PARP staining was performed using Image-Pro Plus 5.1 software. Western blot analysis (n = 6) of protein extracts from flash-frozen was used to confirm immunohistochemical staining for fragmented PARP. The membranes were probed with the anti-PARP p85 fragment antibody (1:250), stripped, and sequentially probed with anti-β-actin monoclonal antibody (1:10,000; Bio-Rad, Mississauga, ON, Canada). The p85 PARP fragment band densities were quantified using densitometric analysis (Image-Pro

Plus 5.1 software) and normalized to their corresponding β-actin band densities.

Morphological Assessment of Villous Tissue Using Semi-Thin and Ultra-Structural Sectioning

Initial morphological assessments were completed using toluidine blue-stained semithin sections (1 μm) at a magnification of $\times$40. Explants collected for electron microscope (EM) analysis were processed as previously described.[43] Briefly, these explants were fixed in 2% paraformaldehyde/0.5% glutaraldehyde and embedded in epoxy resin. Ultra-thin sections (70 nm) were cut and counterstained with uranyl acetate and lead citrate. Morphological visualization of the explants was performed using transmission EM (Hitachi 7000; Hitachi, Pleasanton, CA) at magnifications between $\times$3500 to $\times$15,000. Morphological analyses were focused on the syncytium as a whole. Specific attention was paid to the integrity of the syncytial membrane, chromatin condensation, and vacuole formation.

To assess the resemblance of ultrastructural syncytial damage caused through the *in vitro* H/R insult used in this study to that observed *in vivo* within PE placental tissue, villous explants were additionally dissected from term placentas of women diagnosed with PE (maternal hypertension, blood pressure $\geq$140/90; proteinuria, $\geq$0.3 g of urine protein/24 hours), as previously described. These explants were immediately fixed for semithin sectioning and EM analysis and subjected to similar morphological analysis detailed above.

Villous Tissue Integrity Assessment

Villous tissue integrity was analyzed by measuring the release of lactate dehydrogenase (LDH) into the culture medium after 6 hours of explant incubation. LDH concentration was assessed at the Kingston General Hospital core laboratory using a commercially available kit (L-lactate enzymatic colorimetric kit; Roche Diagnostics, Indianapolis, IN) that measures the reduction of NAD to NADH, a reaction that couples the conversion of L-lactate to pyruvate in the presence of LDH. The rate of NADH formation is directly proportional to the catalytic LDH activity and is determined by measuring the increase in absorbance at 340 nm.

Statistical Analysis

All data sets were analyzed for Gaussian distribution using the D'Agostinon and Pearson omnibus normality test. All data are presented as means $\pm$ SEM. Statistical analysis was performed using two-way analysis of variance with a Bonferroni posttest; treatment (untreated versus CO treated) and culture time (3 hours versus 6 hours versus 12 hours) were identified as possible interacting variables. In the instance of only one experimental time point (ie, LDH analysis), differences between the two treatment groups were examined using a paired Student's *t*-test. Statistical significance was set at $P < 0.05$ for all tests performed.

Results

CO Concentrations

Baseline CO concentration in normal culture medium measured 3.9 μmol/L ($\pm$1.6), whereas that in CO-saturated culture medium was 244 $\pm$ 5.8 μmol/L. CO in the three serial dilutions of the saturated medium were 181 μmol/L ($\pm$16.9), 145 μmol/L ($\pm$20.57), and 116 μmol/L ($\pm$25.3). After 3 hours of explant culture, CO remaining in the culture medium was 15.9 μmol/L ($\pm$1.9), 12 μmol/L ($\pm$2.2), 10.2 μmol/L ($\pm$1.8), and 3.7 μmol/L ($\pm$1.4), respectively. The first dilution of the saturated medium (181 μmol/L) had a CO concentration similar to that measured in the umbilical cord blood of smoking women.[44] Therefore this CO dilution was used for all subsequent testing.

CO Treatment Decreased H/R-Induced Apoptosis of Syncytiotrophoblast Cells

Explants fixed immediately after dissection, without culture, exhibited minimal levels of syncytiotrophoblast apoptosis (1.1 $\pm$ 0.4%). After the 3-hour hypoxic portion of the 6-hour H/R insult, both the untreated and CO-treated explants exhibited similar increases in their apoptotic index (11.8 $\pm$ 1.8% versus 8.4 $\pm$ 1.5%). After the subsequent 3 hours of reoxygenation with 21% O_2, the untreated explants demonstrated a twofold increase in syncytial apoptosis. The CO-treated explants, however, demonstrated no further increases in TUNEL-positive staining, indicating no further increases in apoptosis (Figures 2 and 3A). In the subset of placental explants subjected to a second 6-hour H/R insult, the protective effects of CO were maintained with 18.9 $\pm$ 3.5% and 27.4 $\pm$ 2.7% apoptosis seen in the CO-treated and untreated syncytiotrophoblast, respectively ($P < 0.05$). The untreated and treated controls kept under normoxic (5% O_2) conditions throughout the experimental incubation exhibited minimal levels of apoptosis at all time points tested (5.1 $\pm$ 1.4% versus 3.3 $\pm$ 1.2% at 6 hours; Figure 3B).

CO Treatment Decreased Immunohistochemical Staining of Fragmented PARP after H/R Insult

Explants fixed immediately after delivery exhibited low levels of fragmented PARP. Reactivity was predominantly located in the syncytiotrophoblast. After 6 hours of H/R insult CO-treated explants had less staining for fragmented PARP in both the syncytiotrophoblast and stroma compared with untreated explants ($P < 0.05$, Figure 4). Explants maintained under normoxic conditions throughout (both untreated and CO-treated), demonstrated minimal staining for fragmented PARP. Substitution of the primary antibody with a nonimmune rabbit IgG resulted in

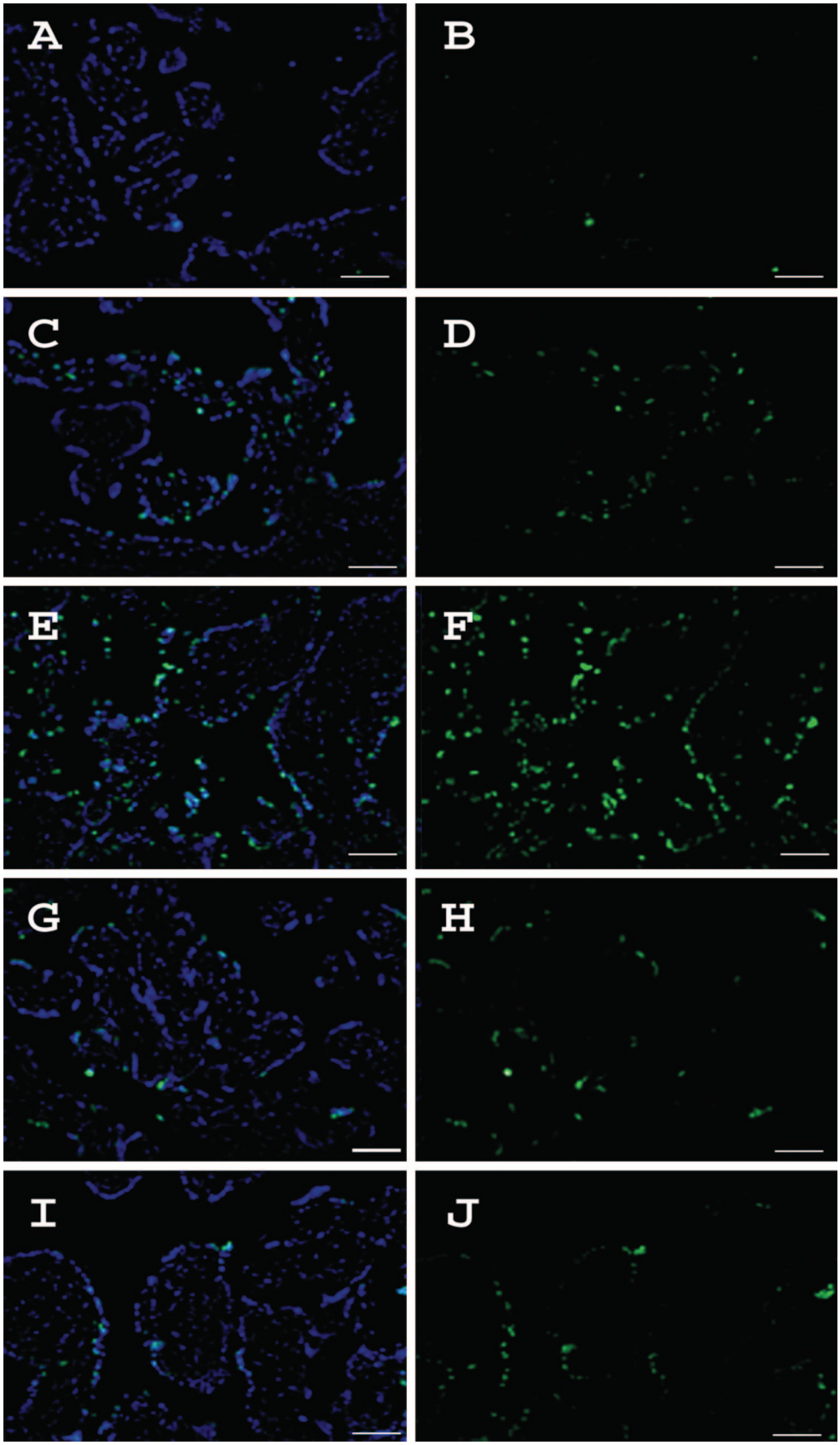

Figure 2. Immunofluorescent labeling for DAPI-positive (blue) and TUNEL-positive (green) nuclei of villous explants sampled immediately after delivery (**A**, **B**), untreated explants after 3 hours of hypoxia (**C**, **D**), untreated explants after 6 hours of H/R (**E**, **F**), CO-treated explants after 3 hours of hypoxia (**G**, **H**), and CO-treated explants after 6 hours of H/R (**I**, **J**). CO-treated explants demonstrated fewer TUNEL-positive syncytiotrophoblast nuclei compared with untreated explants after a 6-hour H/R insult. Scale bar = 50 μm.

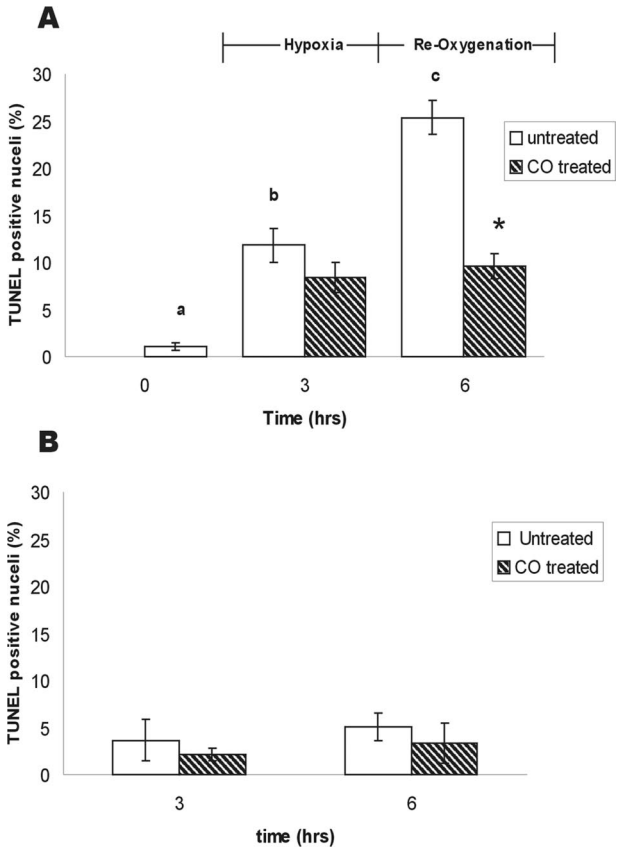

Figure 3. Percentage of TUNEL-positive nuclei in untreated versus CO-treated villous explants throughout time during a 6-hour H/R insult (**A**) and in untreated and treated controls incubated under normoxic conditions (5% O_2) for 6 hours (**B**). CO treatment significantly inhibited development of H/R-induced apoptosis in the syncytiotrophoblast. Minimal levels of apoptosis were apparent in the syncytiotrophoblast of both the untreated and CO-treated control groups. Data are presented as means ± SEM for 13 placentas (**A**) and 5 placentas (**B**). Group mean values with different letters are statistically different from each other using analysis of variance ($P < 0.05$). *$P < 0.05$ between two groups using paired Student's *t*-test.

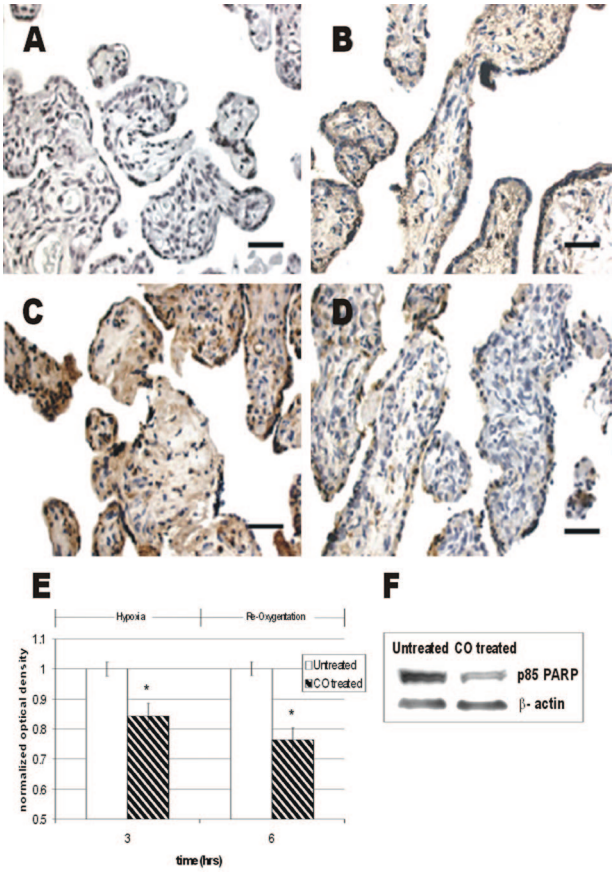

Figure 4. Immunohistochemical staining for the p85 fragment of PARP in villous explants immediately after delivery (**B**), in untreated (**C**) and CO-treated (**D**) explants after a 6-hour H/R insult, and in the negative control (**A**). Densitometric analysis of the immunohistochemical staining (**E**) demonstrates heightened presence of fragmented PARP within the syncytium of untreated explants after H/R insult compared to matched explants treated with CO for six placentas. *$P < 0.05$, paired Student's *t*-test. Confirmation of immunohistochemical staining results was obtained using Western blot analysis, representative image shown (**F**). Scale bar = 50 μm.

the absence of staining. These results were confirmed using Western blot analysis (Figure 4F).

Morphological Assessment of Untreated and CO-Treated Explants

Morphological assessment of tissue integrity was performed using semithin sectioning and transmission EM. Light microscopy of semithin sections revealed a typical nuclear distribution within the syncytiotrophoblast, normal distribution of chromatin within the nuclei, minimal cytoplasmic vacuolization, and intact syncytial membranes in explants collected immediately after delivery (Figure 5A). After the H/R insult the CO-treated explants exhibited similar morphological traits (Figure 5B). The untreated explants however, demonstrated several morphological hallmarks of apoptosis (Figure 5C). A number of nuclei demonstrated peripheral chromatin condensation, and the majority of nuclei were clumped together into syncytial knots. Large vacuoles were present within the syncytiotrophoblast and, in localized regions, the syncytium appeared to be separating from the underlying stroma. These alterations were also seen by ultrastructural analysis (Figure 5, E–G). In addition, the EM study demonstrated fresh explants had an intact membrane with clearly visible microvilli (Figure 5M). In explants treated with CO throughout the H/R insult, this membrane was still intact but had reduced numbers of shortened microvilli (Figure 5N). The syncytiotrophoblast membrane and its microvilli were generally absent in the untreated explants after H/R (Figure 5O).

Ultrastructural similarities were observed between fresh placental explants collected from PE placentas and untreated explants exposed to a 6-hour H/R insult (Figure 5, D, H, L, and P). The PE placental explants also demonstrated several hallmark features of syncytial apoptosis including pronounced peripheral chromatin condensation, microvilli clubbing with partial loss of syncytial membrane integrity, along with several syncytial knots.

Villous Tissue Integrity

Released cytoplasmic LDH was measured in culture medium bathing the explants. Medium bathing the explants

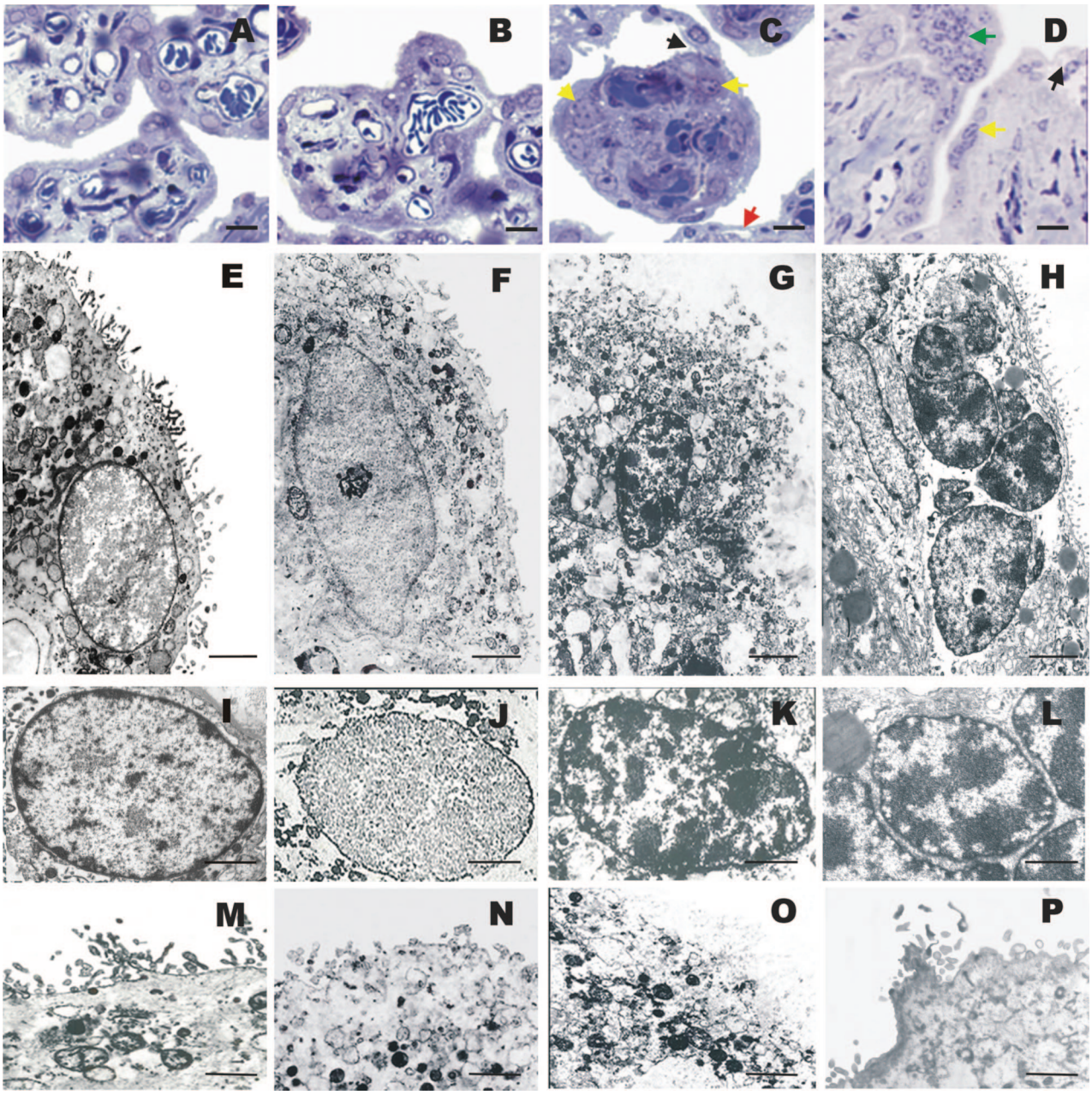

Figure 5. Representative semithin sections of villous explants immediately after delivery (**A**), CO-treated explants after a 6-hour H/R insult (**B**), and untreated explants after a 6-hour H/R insult (**C**). Formation of cytoplasmic vacuolization (**red arrow**), nuclear apoptotic changes (**yellow arrow**), and localized separation of the syncytiotrophoblast layer from the underlying stroma (**black arrow**) were observed in the untreated explants. These morphological changes associated with apoptosis were also seen in fresh explants collected from PE placentas (**D**), with several areas of syncytial knots (**green arrow**). By contrast, explants treated with CO maintained morphology similar to that seen in fresh placental tissue. Additional morphological analysis using representative electron micrographs of healthy villous explant syncytium immediately after delivery (**E**, **I**, **M**), CO-treated villous explant syncytium after a 6-hour H/R insult (**F**, **J**, **N**), untreated villous explant syncytium after a 6-hour H/R insult (**G**, **K**, **O**), and explants collected from PE placenta (**H**, **L**, **P**). Abundant euchromatin is distributed in the syncytial nuclei of fresh villous explants (**I**) and is preserved in the syncytial nuclei of the CO-treated villous explants (**J**). An intact syncytial membrane with abundant microvilli was apparent on the apical surface of the fresh villous explants (**M**). The syncytial membrane of CO-treated explants also remained intact; however, slight clubbing of the microvilli became apparent (**N**). In the untreated explants the syncytial nuclei (**K**) were often small and round with peripheral chromatin condensation. Untreated explants also demonstrated an increase in cytoplasmic vacuolization and a complete loss of syncytial membrane integrity (**O**). These morphological changes closely mimicked those observed within the syncytium of PE placentas. Scale bars = 25 μm (**A–D**); 2 μm (**E–H**); 1.5 μm (**I**, **J**, **L**); 1 μm (**K**); 0.5 μm (**M–P**).

treated with CO throughout the H/R insult had significantly lower levels of LDH compared with medium from untreated explants (59.11 ± 7.6 U LDH/mg total protein versus 76.91 ± 9.6 U LDH/mg total protein; $P < 0.05$).

Discussion

Smoking throughout pregnancy is associated with a 33% reduced risk for PE.[20] Mechanisms through which smok-

ing confers protection from this disorder are unclear. Oxidative stress induced placental injury and subsequent shedding of placental debris into the maternal circulation have been identified as key pathogenic events in the progression of PE.[1,3] A previous *in vitro* model of ischemia-reperfusion-induced injury to human placental tissues was established by exposing cultured placental explants to H/R, resulting in increased markers of oxidative stress, syncytial apoptosis, and loss of syncytial membrane integrity.[6,11] The current study applied this model to understand potential mechanisms through which cigarette smoke reduces the risk of PE. We have demonstrated that *in vitro* exposure to fluctuating oxygen tensions results in syncytiotrophoblast cell death and shedding of the apical microvillous border, tissue injury that was shown to closely resemble that observed in fresh PE placental explants. In addition, these processes were shown to be effectively attenuated in the presence of CO.

Carbon monoxide, a major combustible product of cigarette smoke, is elevated in maternal and feto-placental serum of smoking women. Umbilical cord arterial blood has a threefold higher CO concentration (149 $\pm$ 6 μmol/L versus 46 $\pm$ 5 μmol/L) in smokers compared with nonsmokers as measured by solid-gas chromatography (G.N. Smith and S.A. Bainbridge, unpublished data). A similar concentration of exogenous CO is capable of significant decreases in placental perfusion pressure[27] (ie, placental vascular resistance) indicating CO found in the serum of smokers is capable of hemodynamic control within the placenta. Therefore, elevated CO in smokers may be capable of maintaining spiral arterioles and placental blood vessels in a dilated state, thus preventing or limiting placental oxidative stress associated with pulsatile blood flow. In addition, the results of the current study demonstrate that exogenous CO treatment attenuates H/R-induced apoptosis in human placental trophoblast cells. Because markers of apoptosis are increased in the placentas of women with PE (Figure 5),[45,46] the elevated CO in the serum of smoking women may also reduce their risk of developing PE by attenuating ischemia-reperfusion-induced apoptotic cascades in the placenta.

Carbon monoxide may inhibit both the apoptotic and apo-necrotic pathways in trophoblast cells. Apo-necrotic injury is described as depletion of cellular energy stores after an overwhelming insult, with subsequent conversion of tightly organized deconstruction of a cell into unregulated cell death.[47] Leakage of cytoplasmic LDH into the culture medium, along with morphological analysis demonstrating loss of syncytial membrane integrity in untreated explants, suggests the apoptotic-inducing insult used may have been so great as to induce an apo-necrotic cascade. This insult is postulated to be involved in the pathophysiology of PE.[3,11] Ultrastructural analysis of fresh placental explants collected from PE placentas in this study supports the presence of apo-necrotic cascades in PE placenta because syncytial membrane integrity was compromised in localized regions. The syncytiotrophoblast acts as the endothelium of the intervillous space, as such is in direct contact with the maternal circulation. Loss of apical membrane integrity in this cellular layer would contribute to shedding of syncytiotrophoblast microfragments along with other fetal products such as DNA and asymmetric dimethylarginines into the maternal compartment; these products are measured at increased concentrations in the serum of PE women.[14,48] Treatment of explants with exogenous CO, at concentrations similar to those measured in serum of smoking women, limited leakage of LDH into the surrounding culture medium and resulted in retention of intact syncytial membranes. Microvilli of these explants demonstrated slight clubbing in localized regions; however, the morphological differences observed between the untreated and CO-treated explants clearly demonstrate a cytoprotective effect of CO in the syncytium after an overwhelming oxidative stress insult. CO may limit shedding of placental debris into the maternal circulation, suggesting a potential mechanism through which smoking women have a reduced risk of developing PE. Nonetheless, it must be noted that this study, and hence the conclusions drawn from this study, pertain specifically to an *in vitro* model of ischemia/reperfusion injury in term human placenta. Ongoing observational studies comparing syncytiotrophoblast microfragment shedding in nonsmoking versus smoking women throughout pregnancy will help solidify whether the results of the current study do in fact hold true *in vivo*.

An overall goal of the study was to understand mechanisms that limit the progression of placental damage leading to PE. Carbon monoxide has been identified as a cytoprotective compound in placental tissues and may contribute to the decreased risk of PE in smoking women. It may be possible to use CO, or its mechanisms of action, in the prevention or treatment of nonsmoking women identified at high risk of developing PE.

Several groups have examined the therapeutic potential of CO in organ transplant research through up-regulation of endogenous CO production and/or treatment with exogenous CO sources (eg, inhalation, pharmacological agents). Carbon monoxide increases viability and survival of transplanted organs by inhibiting inflammatory and apoptotic cascades.[34–37] Similarities have previously been drawn between PE placentas and allograft-rejected organs.[1] Inadequate perfusion, oxidative stress, and the presence of activated macrophages secreting proinflammatory and proapoptotic compounds are well-documented insults in both instances. This study is the first to demonstrate a similar therapeutic potential of CO in the prevention and treatment of PE.

An approach to elevate local CO as a treatment of PE may be through increased expression of the enzyme heme oxygenase, particularly in placental tissue where it is widely distributed.[26] In addition to increasing endogenous CO, this approach would increase production of bilirubin and biliverdin, both potent anti-oxidants.[49] Therapeutic heme oxygenase/CO up-regulation has been found to be effective at increasing the success of organ transplantation.[30,33,37] An alternative approach would be the use CO mimetics or compounds acting through second messenger pathways directly involved in CO's cytoprotective effects. Soluble guanylyl cyclase (sGC)[39,50] and p38 MAP kinase[38] have been identified as potential

mediators of CO's anti-apoptotic action in smooth muscle and endothelial cell preparations. In-depth mechanistic studies are currently underway to pinpoint specific messenger systems underlying the cytoprotection offered by CO within placental tissue.

The negative outcomes associated with cigarette smoking throughout pregnancy outweigh the potential benefits gained through a reduced risk for PE. By examining mechanisms through which cigarette smoke confers protection from PE we can identify compounds, such as CO, that are capable of attenuating pathological processes linked to the development of PE. Gained from this knowledge will be the ability to design novel therapeutics for women at high risk of developing this disorder.

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