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Cell Injury, Repair, Aging and Apoptosis

Nuclear Factor-κB, p38, and Stress-Activated Protein Kinase Mitogen-Activated Protein Kinase Signaling Pathways Regulate Proinflammatory Cytokines and Apoptosis in Human Placental Explants in Response to Oxidative Stress

Effects of Antioxidant Vitamins

Tereza Cindrova-Davies,*
Olivera Spasic-Boskovic,* Eric Jauniaux,†
D. Stephen Charnock-Jones,‡ and
Graham J. Burton*

From the Departments of Physiology, Development and Neuroscience and Obstetrics and Gynaecology,‡ University of Cambridge, Cambridge; and the Academic Department of Obstetrics and Gynaecology,† Royal Free and University College, London, United Kingdom*

Preeclampsia is a potentially fatal complication of human pregnancy characterized by hypertension, proteinuria, and edema. Placental oxidative stress is a key element in the pathogenesis of the syndrome and results in the release of a cocktail of factors, including proinflammatory cytokines and apoptotic debris, that in turn cause activation of the maternal endothelium. The intermediary molecular mechanisms underlying this release are unknown, but they represent a potential target for therapeutic interventions. We examined activation of signaling pathways during hypoxia-reoxygenation of villous explants *in vitro*. Hypoxia-reoxygenation activated the p38 and stress-activated protein kinase mitogen-activated protein kinase (MAPK) and the nuclear factor-κB pathways. Downstream consequences included increased tissue concentrations and secretion of tumor necrosis factor-α and interleukin-1β, increased expression of cyclooxygenase-2, and increased apoptosis. Administration of vitamins C and E to explants blocked activation of the p38 and stress-activated protein kinase MAPK and nuclear factor-κB pathways. Vitamin administration or p38 pathway inhibition also reduced cyclooxygenase-2 expression, tumor necrosis factor-α and interleukin-1β secretion, and the levels of apoptosis. We conclude that oxidative stress is a potent inducer of placental synthesis and release of proinflammatory factors. Most of these effects are mediated through the p38 MAPK and nuclear factor-κB pathways and can be effectively blocked by vitamins C and E *in vitro*. *(Am J Pathol 2007, 170:1511–1520; DOI: 10.2353/ajpath.2007.061035)*

Preeclampsia is the most important complication of human pregnancy worldwide and a major contributor to maternal and fetal morbidity and mortality. Despite much research, the pathophysiology remains elusive, although there is strong evidence that generation of placental oxidative stress is a key intermediary event.[1,2] The stress is thought to induce the placenta to release a cocktail of factors, including proinflammatory cytokines, antiangiogenic factors, and apoptotic debris, which culminates in an enhanced maternal inflammatory response.[3] However, the molecular mechanisms linking oxidative stress to release of these factors are not known. In part, this is due to the constraint that placental tissues are only available for study at the end of pregnancy, when the pathology is well established or secondary pathology may have been superimposed.

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D.S.C.-J. and G.J.B. contributed equally to this work.

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Address reprint requests to Professor Graham J. Burton, Department of Physiology, Development and Neuroscience, Downing Street, Cambridge CB2 3EG, UK. E-mail: gjb2@cam.ac.uk.

Early-onset preeclampsia, the most severe form of the syndrome, has its origins in the first trimester of pregnancy.[4] During this period, fetal extravillous trophoblast cells migrate into the endometrium, and their presence is associated with the physiological conversion of the maternal spiral arteries into dilated, flaccid conduits that ensure an inviolable blood supply to the conceptus. Trophoblast invasion is impaired in preeclampsia for a number of possible reasons, but the net result is absent, or severely reduced, conversion of the arteries.[5] We recently proposed that the retention of smooth muscle within the vessel walls leads to persistence of vasoreactivity and to intermittent perfusion of the intervillous space.[4,6] Consequently, the placental tissues are exposed to fluctuating oxygen concentrations and hence suffer repeated ischemia-reperfusion type injuries. In support of this theory, we and others have demonstrated that villi sampled from normal placentas delivered by caesarean section show increased oxidative stress when subjected to hypoxia-reoxygenation (H/R) compared with controls maintained under hypoxia alone.[7] These changes closely mimic those reported in preeclampsia and could be attenuated with desferrioxamine, the electron spin trap α-phenyl-*N*-tert-butylnitrone,[4,7] or carbon monoxide.[8] Hypoxia-reoxygenation also proved a potent stimulus for apoptotic changes within the syncytiotrophoblast[8,9] and induced secretion of tumor necrosis factor (TNF)-α.[10] Although these studies established that hypoxia-reoxygenation is a more physiological stimulus than hypoxia alone for generating the placental changes associated with preeclampsia, they did not explore the signaling pathways involved.

Evidence of the involvement of oxidative stress in the pathogenesis of preeclampsia stimulated several clinical trials to test the prophylactic benefits of antioxidant vitamins C and E in women at risk of preeclampsia. Although a beneficial effect of antioxidants was suggested in a small randomized trial of women at high risk of preeclampsia,[11] a recent multicenter randomized clinical trial (VIP trial) showed no benefit in prevention of the disease.[12] By contrast, we have previously demonstrated that vitamins can prevent hypoxia-reoxygenation-induced secretion of free fetal DNA from villous explants, suggesting that they can limit trophoblast damage *in vitro*.[13] In view of these conflicting *in vivo* and *in vitro* findings, we have identified the signaling pathways activated by acute hypoxia-reoxygenation *in vitro* and tested the effects of antioxidant vitamins C and E on these pathways and the downstream consequences induced.

Materials and Methods

Materials

Antibodies to the phosphorylated and total forms of p38, stress-activated protein kinase (SAPK), IκB, nuclear factor-κB (NF-κB), Hsp27, TNF-α, cleaved caspase-3, and cleaved caspase-9 were from Cell Signaling Technology (Beverly, MA). Anti-cyclooxygenase (COX)-2 was from Cayman Chemical (Ann Arbor, MI). Anti-4-hydroxy-2-nonenal (HNE) antibody was from Axxora (Nottingham, UK) and anti-Hsp90 from Stressgen Bioreagents Corp. (York, UK). The horseradish peroxidase-conjugated secondary antibodies were from Amersham Biosciences (Buckinghamshire, UK). Alexa 488 and Alexa 568 fluorescently labeled antibodies were from Molecular Probes Invitrogen Detection Technologies (Leiden, The Netherlands), and biotinylated secondary antibodies were from Vector Laboratories (Peterborough, UK). The PD169316 inhibitor was from Calbiochem (San Diego, CA); ascorbic acid, SB202190, and Trolox (water-soluble vitamin E) were from Sigma (Poole, UK). Secreted TNF-α was detected by colorimetric enzyme-linked immunosorbent assay using Quantikine kits from R&D Systems (Oxon, UK).

Explant Culture

Placentas ($n = 12$) were collected from normal-term singleton pregnancies delivered by elective caesarean section with informed written consent of the patients and permission of the Local Research Ethics Committee. Villous samples were taken midway between the chorionic and basal plates from the periphery of lobules free of visible infarction, calcification, hematoma, or tears. After a brief rinse in ice-cold phosphate-buffered saline, samples were placed into ice-cold transport medium (TCS large vessel endothelial cell basal medium; TCS CellWorks, Milton Keynes, UK) containing 2% fetal bovine serum, heparin, hydrocortisone, human epidermal growth factor, human basic fibroblast growth factor, 25 μg/ml gentamicin, and 50 ng/ml amphotericin B, 1 mmol/L vitamin C, and 1 mmol/L Trolox) that had been equilibrated with 5% O_2/90% N_2/5% CO_2.

Following transport to the laboratory on ice, placental samples were further dissected into small pieces (about 5 mm in diameter) in ice-cold culture medium in a glove box under 10% O_2/85% N_2/5% CO_2. Samples were cultured on individual Costar Netwell (24-mm diameter, 500-μm mesh; Corning Life Sciences, Acton, MA) supports in 4 ml of culture medium per well in six-well plates. Approximately 6 to 10 pieces were added to each well, depending on the experimental requirements. Placental explants were incubated in pregassed medium under: 1) normoxic conditions (controls) (10% O_2/85% N_2/5% CO_2) for 7 hours ($n = 6$) or 16 hours ($n = 6$), or 2) subjected to hypoxia (0.5% O_2/94.5% N_2/5% CO_2) for 1 hour and subsequent reoxygenation at normoxia (10% O_2/85% N_2/5% CO_2) for the following 6 hours ($n = 6$) or 15 hours ($n = 6$) (H/R treatment). All inserts were transferred into previously pregassed medium after 1 hour of incubation. The p38 inhibitor PD169316 was used at a concentration of 10 μmol/L and SB202190 at 30 μmol/L. Vitamin treatment included the addition of 2 mmol/L ascorbic acid and 1 mmol/L Trolox. In all experimental setups, medium was changed in a glove box under a low O_2 environment after the first hour of incubation. The p38 inhibitors and vitamins were added at the beginning of each experiment and also when medium was changed.

Western Blotting

Tissue homogenization to obtain protein lysate and subsequent sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting were performed as previously described.[13] Proteins were revealed and quantified using Image J software (National Institutes of Health, version 1.36b, *http://rsb.info.nih.gov/ij/*, accessed Sept 2006). Membranes were reprobed with antibody recognizing β-actin to control for protein loading. The values are expressed as a percentage of the control lysate (100%) for each experiment.

Statistical Analysis

All data are presented as means ± SEM. Statistical analysis was performed using StatView (SAS Institute Inc., Cary, NC). Normalized Western blot measurements were analyzed using repeated measures analysis of variance. Differences between two groups were evaluated using a paired Student's *t*-test. In all cases, results were considered significant at $P < 0.05$.

Colorimetric and Fluorescent Immunohistochemistry

Immunohistochemistry with diaminobenzidine detection was performed according to a protocol described recently.[13] For immunofluorescent staining, sections were incubated with primary antibodies overnight as in the colorimetric protocol, washed, and incubated for 1 hour at room temperature with species-specific Alexa 488 or Alexa 568 secondary fluorescent antibodies. Sections were washed in Tris-buffered saline and subsequently mounted in Vectashield mounting medium containing 4,6-diamidino-2-phenylindole (Vector Laboratories). Images were captured using a Leica confocal microscope (LeicaTCS-NT; Leica Instruments GmbH, Wetzlar, Germany).

Results

Effect of in Vitro *H/R on the Levels of Oxidative Stress*

Expression of the heat shock proteins Hsp27 and Hsp90, lipid peroxidation, and the formation of peroxynitrite were used as markers of oxidative stress. H/R strongly increased Hsp27, Hsp90, HNE, and nitrotyrosine concentrations in the explants after 7 and 16 hours of incubation (Figures 1, a–c, and 5, a–c), reflecting an increase of oxidative stress that was localized principally to the syncytiotrophoblast (Figure 5, a–c). The addition of the antioxidant vitamins C and E significantly reduced markers of oxidative stress in H/R-treated samples (Figures 1, a–c, and 5, a–c). All experiments were repeated at least six times.

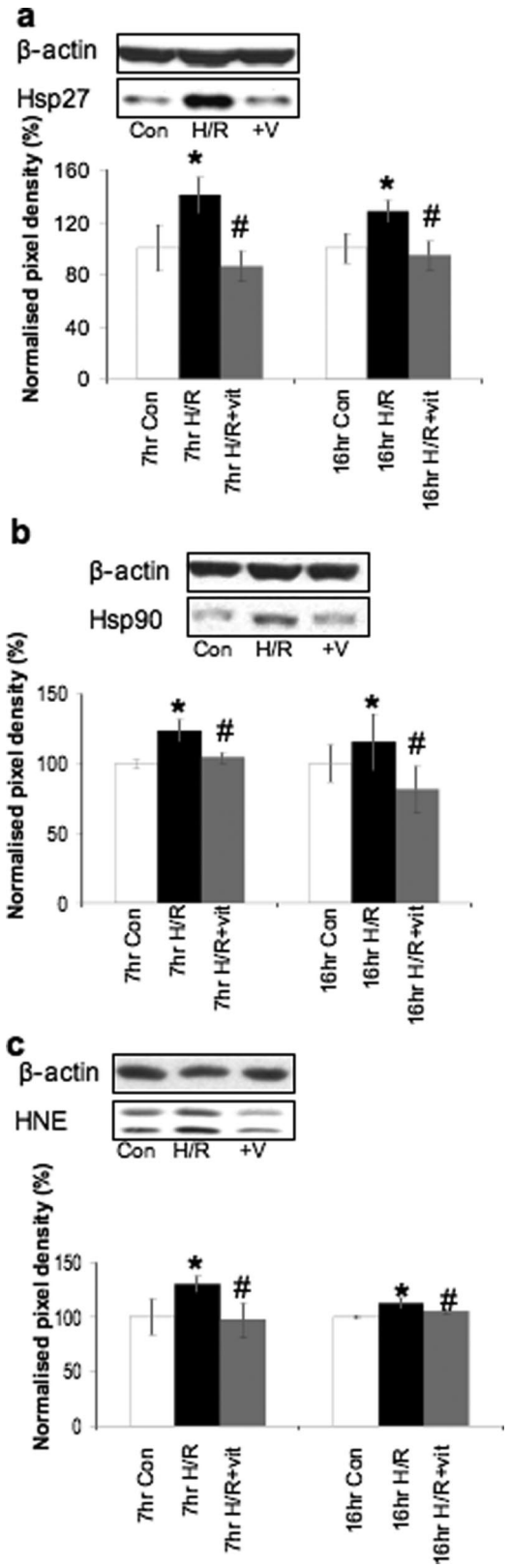

Figure 1. *In vitro* H/R stimulates the expression of oxidative stress markers; simultaneous addition of vitamins suppresses the effects. Protein lysates from placentas cultured under H/R in the presence or absence of vitamins C and E (V) for 7 hours ($n = 6$) or 16 hours ($n = 6$) were analyzed for Hsp27 (**a**), Hsp90 (**b**), and HNE (**c**). Representative blots show the 7-hour time point. $^{*}P < 0.05$ compared with normoxic control; $^{\#}P < 0.05$ compared with H/R treatment.

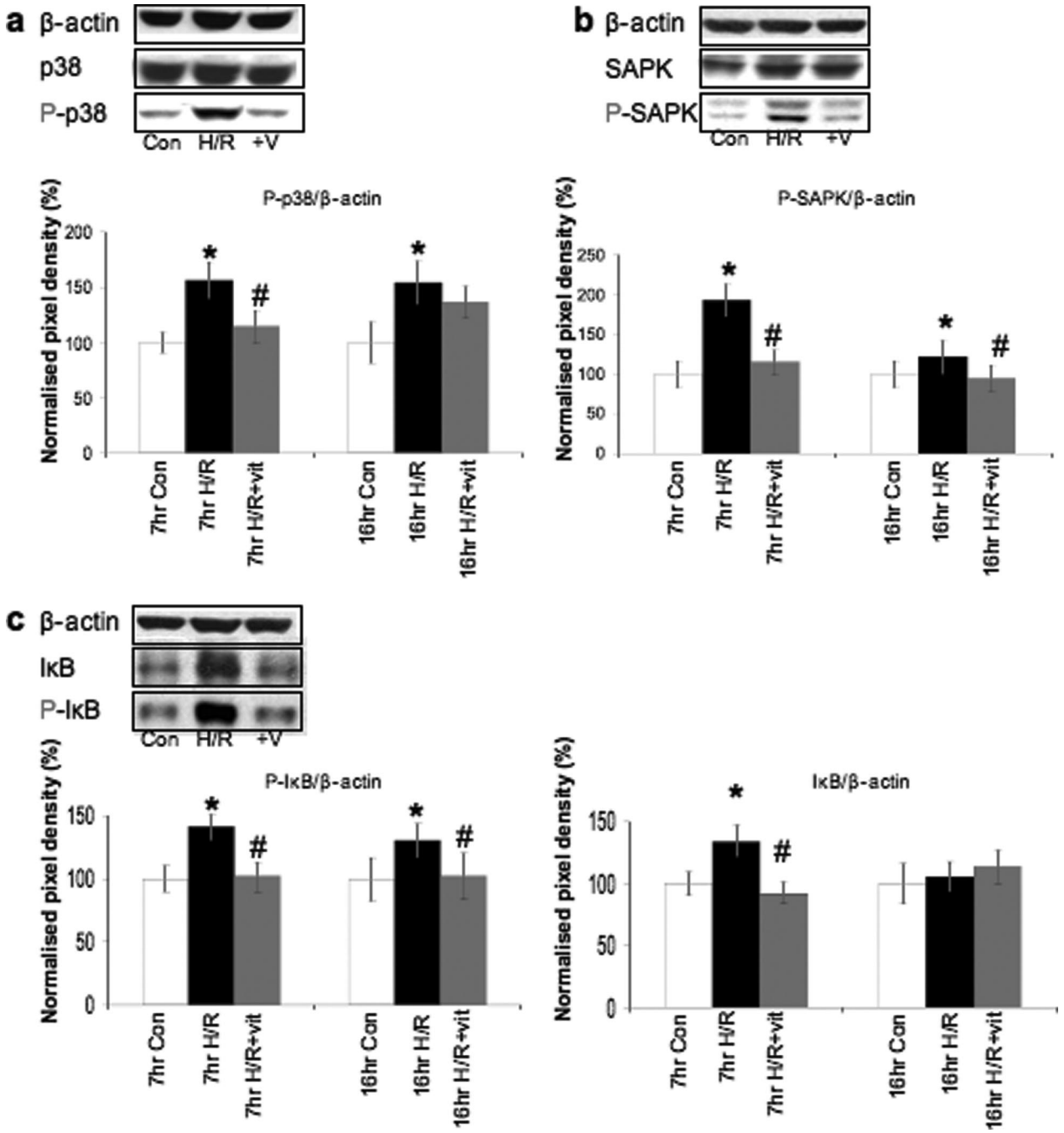

Figure 2. *In vitro* H/R activates p38 MAPK, SAPK, and NF-κB pathways; simultaneous addition of vitamins or a p38 inhibitor suppresses the effects of H/R. Protein lysates from placentas cultured under H/R in the presence or absence of vitamins C and E (V) or PD169316 (P-I) for 7 hours ($n = 6$) or 16 hours ($n = 6$) were analyzed for P-p38, p38 (**a**); P-SAPK, SAPK (**b**); and P-IκB, IκB (**c**). Representative blots show the 7-hour time point. $^{*}P < 0.05$ compared with normoxic control; $^{\#}P < 0.05$ compared with H/R treatment.

H/R Activates the p38 and SAPK MAPKs and the NF-κB Pathway in Vitro*: Effects of Antioxidant Vitamins*

H/R stimulated phosphorylation of the p38 and SAPK MAPK pathways after 7 and 16 hours of incubation, whereas total p38 and SAPK levels remained unchanged (Figure 2, a and b). The addition of vitamins suppressed phosphorylation of p38 at 7 hours and that of SAPK stress kinase at both time points (Figure 2, a and b). The p38 pathway has been implicated in the production of proinflammatory cytokines, suggesting a possible role in the regulation of NF-κB activity. Phosphorylation and subsequent degradation of the IκB protein allows NF-κB translocation to the nucleus, where it regulates gene expression. Hence, the phosphorylation status of IκB serves to determine activation of this pathway. H/R greatly elevated phosphorylation of IκB at 7 and 16 hours of incubation (Figure 2c). Total IκB expression was increased in H/R-treated samples

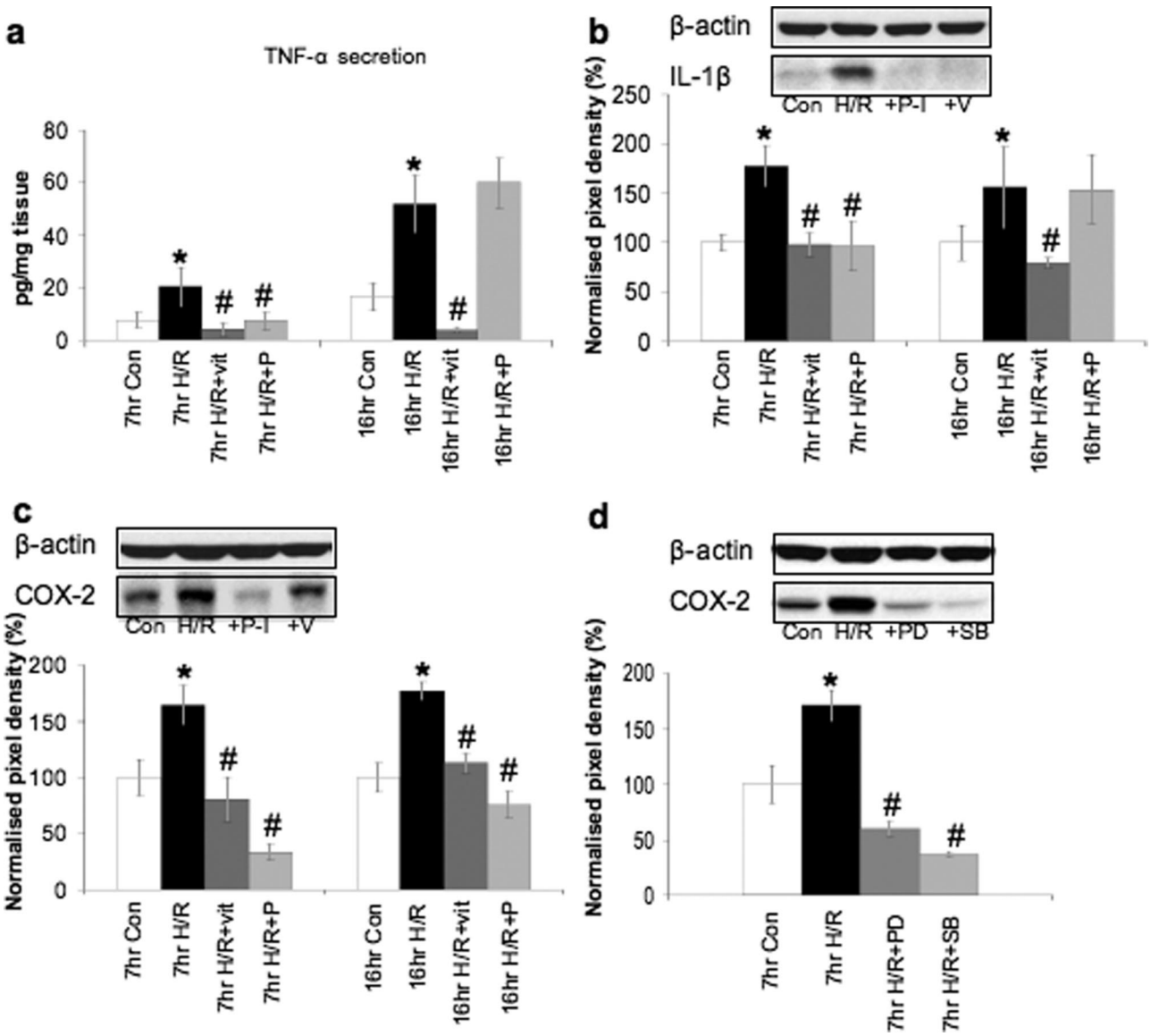

Figure 3. *In vitro* H/R activates the tissue production and secretion of inflammatory cytokines and COX-2; the addition of vitamins or the p38 inhibitor suppresses the effects of H/R. **a:** Supernatants from placentas cultured under H/R in the presence or absence of vitamins C and E (V) or PD169316 (P-I) for 7 hours (n = 6) or 16 hours (n = 6) were analyzed by enzyme-linked immunosorbent assay for the secretion of TNF-α. Data were normalized against wet tissue weights. Corresponding protein lysates were analyzed with IL-1β (**b**) and COX-2 (**c**, **d**). **d:** Effect of the two p38 inhibitors, PD169316 (PD) and SB202190 (SB), was comparable. Representative blots show the 7-hour time point. $^{*}P < 0.05$ compared with normoxic controls; $^{\#}P < 0.05$ compared with H/R.

after 7 hours of incubation but returned to basal level at 16 hours, reflecting IκB degradation. The addition of vitamins significantly suppressed phosphorylation of IκB by H/R (Figure 2c). Immunohistochemistry demonstrated nuclear translocation of phospho-NF-κB to syncytial nuclei, indicative of activation and involvement in the regulation of gene expression (Figure 5d).

In Vitro *H/R-Induced Stimulation of Proinflammatory Cytokines, COX-2, and Apoptosis Can Be Suppressed by Antioxidant Vitamins or p38 Inhibition*

NF-κB exerts its effects by regulating genes encoding cytokines, chemokines, adhesion molecules, growth factors, and inducible proinflammatory enzymes such as COX-2. Therefore, the tissue expression of COX-2 and the levels of TNF-α and interleukin (IL)-1β were examined. Vitamins blocked the NF-κB pathway, and their effect on these downstream pathways was thus of interest. The p38 MAPK pathway has also been shown to regulate inflammatory cytokines and COX-2.[14,15] To test involvement of this pathway, we used the p38 inhibitors PD169316 and SB202190 in conjunction with H/R. Application of both inhibitors yielded very similar results (Figure 3d), and for simplicity, only data from experiments using PD169316 are shown here.

Secretion of TNF-α from the explants into the supernatant was measured at 7 and 16 hours by enzyme-linked immunosorbent assay. H/R caused a significant increase in TNF-α secretion, which increased with time

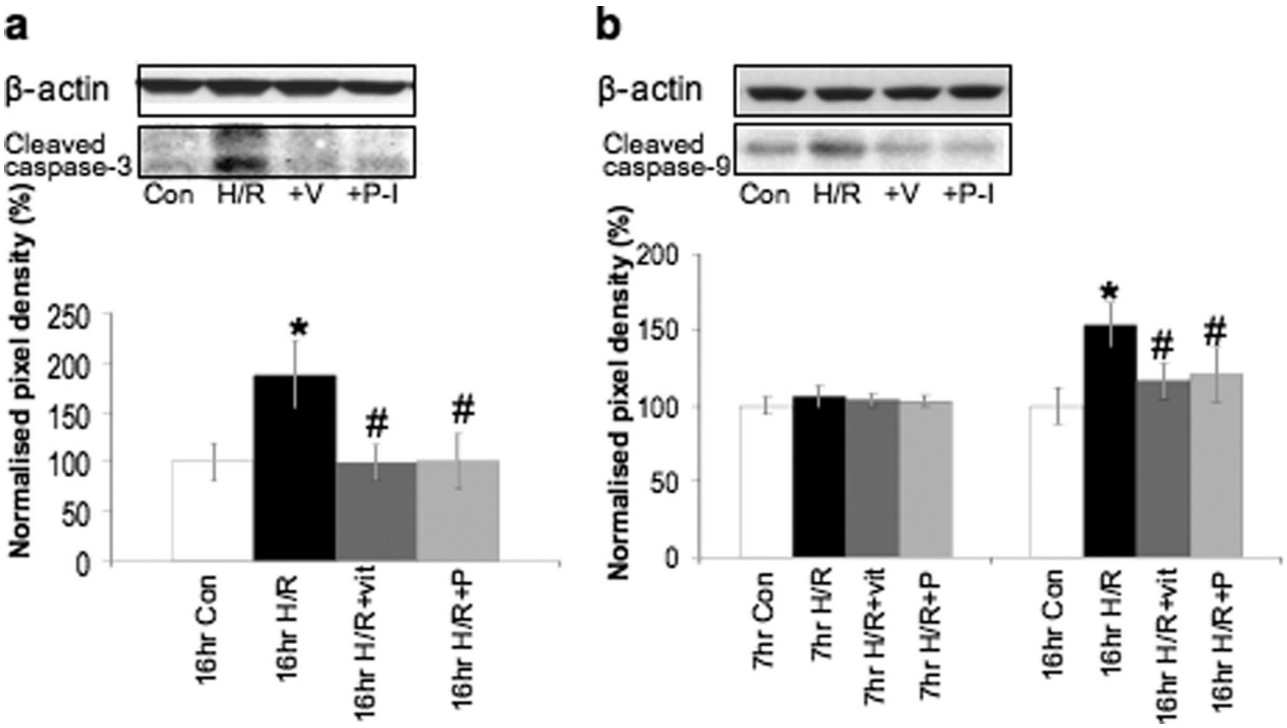

Figure 4. *In vitro* H/R stimulates apoptosis; the addition of vitamins or the p38 inhibitor suppress the effects of H/R. Protein lysates from placentas cultured under H/R in the presence or absence of vitamins C and E (V) or PD169316 (P-I) for 7 hours ($n = 6$) or 16 hours ($n = 6$) were analyzed for cleaved caspase-3 (undetectable at 7 hours) (**a**) and cleaved caspase-9 (**b**). Representative blots show the 16-hour time point. $^{*}P < 0.05$ compared with normoxic control; $^{\#}P < 0.05$ compared with H/R treatment.

(Figure 3a). The addition of vitamins powerfully suppressed this effect at both time points. The p38 inhibitor suppressed TNF-α secretion at 7 hours but had no effect at 16 hours (Figure 3a). Tissue production of IL-1β (Figure 3b) and TNF-α (data not shown) was also stimulated by H/R. The addition of vitamins prevented the H/R stimulation of IL-1β after 7- and 16-hour incubation. PD169316 only reduced tissue concentrations of IL-1β at 7 hours and had no effect at 16 hours (Figure 3b). Secretion of IL-1β was not measured. H/R-induced tissue production of TNF-α increased in a similar manner as that of IL-1β (not shown). H/R also stimulated a significant increase in COX-2 protein concentrations at both time points compared with normoxic controls, and this was localized mainly to the trophoblast (Figures 3c and 5e). Again, this increase was suppressed by vitamins and p38 kinase inhibitors at 7 and 16 hours (Figures 3, c and d, and 5e).

H/R stimulated the activation of molecules required for apoptosis in the placental explants as seen by an increase in the concentrations of cleaved caspase-3 (Figure 4a) and cleaved caspase-9 at 16 hours (cleavage of caspase-3 was undetectable at 7 hours) (Figure 4b). During apoptosis cytokeratins within trophoblast cells are cleaved by active caspase-3 to yield a specific product detected by the M30 antibody.[16] M30 staining confirmed an increase in trophoblast apoptosis following H/R (Figure 5f). Crucially, both PD169316 and vitamins C and E significantly reduced cleavage of both caspases to control levels in placental explants subjected to H/R and reduced the incidence of M30 staining (Figures 4, a–b, and 5f).

Discussion

The aim was to investigate the acute effects of oxidative stress on the human placenta to understand the pathophysiology of the placental changes that underlie preeclampsia. We have demonstrated that placental explants challenged with hypoxia-reoxygenation *in vitro* show a marked increase in the levels of oxidative stress and activation of the p38 and SAPK MAPK and the NF-κB pathways. The addition of the antioxidant vitamins C and E effectively suppressed concentrations of markers of oxidative stress, levels of apoptosis, and secretion of TNF-α and IL-1β induced by H/R at 7 and 16 hours. They also suppressed the levels of COX-2 and inhibited the phosphorylation of p38, SAPK, and IκB. A p38 inhibitor was used to evaluate the effect of the p38 pathway on the downstream effects of H/R. Inhibition of p38 effectively suppressed H/R-induced COX-2 expression at 7 and 16 hours and the level of apoptosis-associated markers at 16 hours. However, it only suppressed TNF-α and IL-1β expression and secretion at 7 hours and had no effect after 16 hours of culture. We used the pharmacological inhibitor of p38, PD169316, to address these questions. Similar results were obtained using a second pharmacological inhibitor of p38 signaling, SB202190. Both pharmacological agents are frequently used to block the p38 pathway. However, it should be noted that the specificity of the two p38 kinase inhibitors, PD169316 and SB202190, is questionable as they can also inhibit JNK/SAPK and may also inhibit other upstream kinases.[17] We are

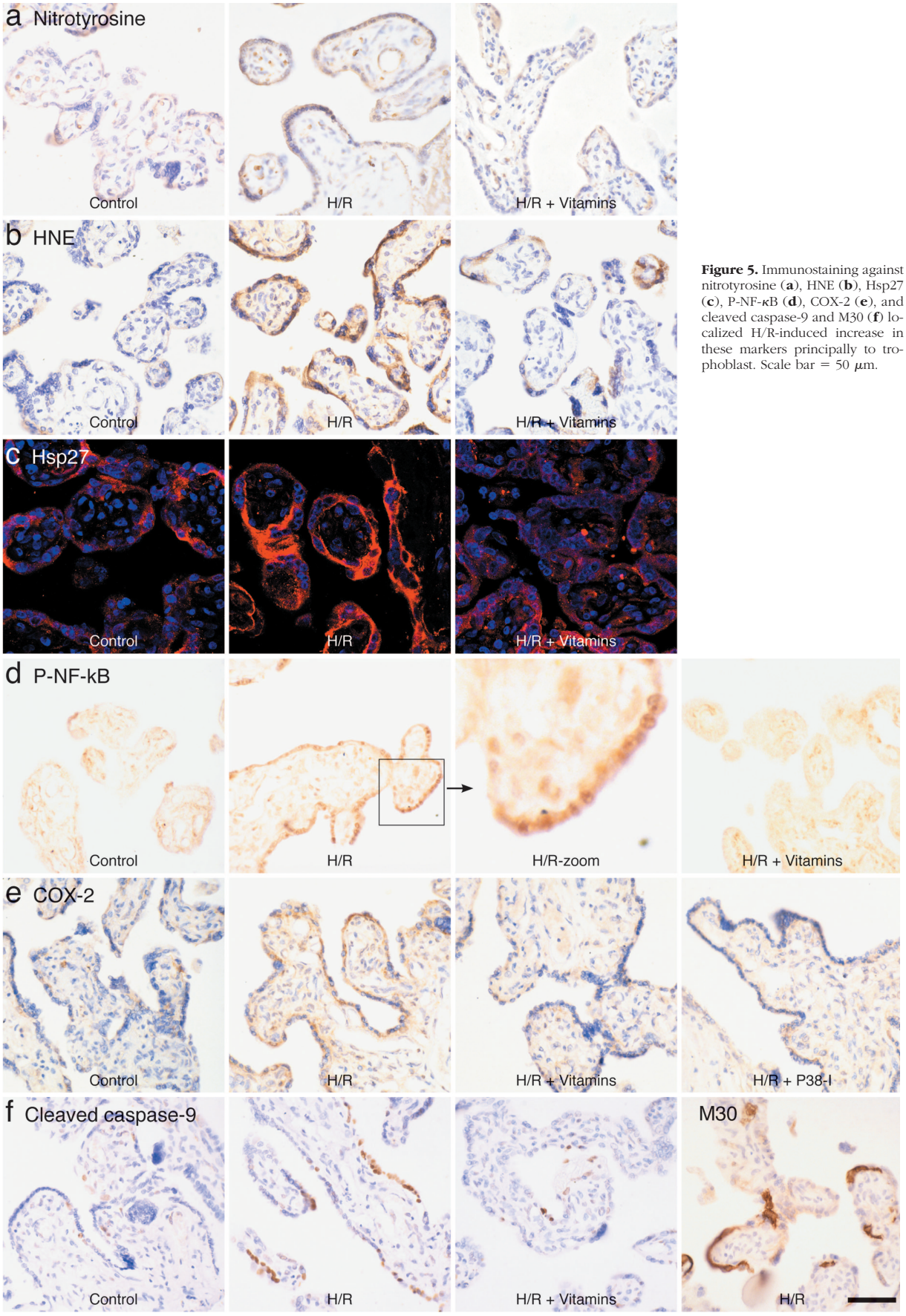

Figure 5. Immunostaining against nitrotyrosine (**a**), HNE (**b**), Hsp27 (**c**), P-NF-κB (**d**), COX-2 (**e**), and cleaved caspase-9 and M30 (**f**) localized H/R-induced increase in these markers principally to trophoblast. Scale bar = 50 μm.

aware of these difficulties, and we used both inhibitors at the lowest possible doses. However, we cannot exclude nonspecific effects of these inhibitors. We performed parallel experiments, in which explants were challenged with 1 mmol/L hydrogen peroxide (H_2O_2) under normoxic conditions for 7 or 16 hours. Remarkably, H/R and H_2O_2 treatments resulted in the same changes listed above, and the addition of vitamins was just as effective in suppressing H_2O_2-induced effects. For simplicity, only H/R effects are presented in this article.

The addition of vitamins to H/R-treated samples suppressed most effects of H/R *in vitro*. Vitamins C and E act in concert to scavenge reactive oxygen species, with vitamin C being required to recycle vitamin E. In agreement with our results, vitamin C has been reported to suppress TNF-α-induced nuclear translocation of NF-κB, NF-κB-dependent reporter transcription, and IκBα phosphorylation in human cell lines and primary endothelial cells.[18] In another study, the addition of vitamins C and E inhibited intracellular reactive oxygen species production and activation of the NF-κB, PKR, eIF-2α, protein kinase C, and p38 MAPK pathways.[19] Intracellular vitamin C has been shown to protect human umbilical vein endothelial cells from H/R-induced apoptosis, preventing loss of mitochondrial membrane potential, the release of cytochrome *c*, and activation of caspase-9 and caspase-3 during H/R.[20] It would thus seem that the beneficial effects of vitamins in our system are mediated through inhibition of NF-κB by vitamin C.

Vitamin E is known to inhibit the p38 MAPK pathway in smooth muscle cells and to prevent activation of NADPH oxidase in monocyte mitochondria, resulting in lower levels of intracellular reactive oxygen species.[21] α-Tocopherol also inhibits protein kinase C activation necessary for smooth muscle cell activation[22,23] and prevents human platelet aggregation via a protein kinase C-dependent mechanism.[24] In addition, vitamin E down-regulates the expression of intercellular cell adhesion molecule-1 and vascular cell adhesion molecule-1 in endothelial cells,[25] and inhibits cyclooxygenase activity in macrophages from aged mice by reducing peroxynitrite production.[26,27] These effects of vitamin E, namely p38 and COX-2 inhibition and reduced peroxynitrite formation, are in agreement with the findings of our study.

Inhibitors of the p38 pathway (PD169316 and SB202190) were equally effective in suppressing apoptosis and COX-2 expression and to some degree that of the proinflammatory cytokines. The p38 pathway has been implicated in the posttranscriptional regulation of TNF-α, IL-1, and COX-2 mRNAs.[14,15] In our study, placental explants subjected to oxidative stress showed increased secretion or tissue levels of TNF-α and IL-1β. The function of these cytokines in normal pregnancy and delivery is not fully established. TNF-α, IL-1, and IL-6 have been detected in human amniotic fluid during pregnancy and labor[28,29] and in preterm labor in the presence of chorioamnionitis.[30] These cytokines activate the prostaglandin biosynthetic pathway primarily via induction of COX-2. Investigations in a number of cell types have demonstrated that IL-1β-induced COX-2 expression involves activation of members of the NF-κB family of transcription factors and the SAPK, ERK1/2, and p38 MAPK cascades.[31–33] There is therefore the possibility of developing an autocrine feed-forward system. In human amnion cells harvested after labor, there is an increased constitutive activity of NF-κB, which functions to increase the COX-2 expression and seems to contribute to the "functional" progesterone withdrawal through an interaction with the progesterone receptor.[34] Inhibition of the p38 MAPK pathway significantly suppressed H/R-induced COX-2 expression at 7 and 16 hours, but it only suppressed TNF-α and IL-1β secretion at 7 hours and had no effect at 16 hours, suggesting that inflammatory cytokine regulation is not dependent on p38 signaling alone or that the inhibitors were overwhelmed in the longer cultures. Vitamin treatment significantly reduced NF-κB activation, and that was associated with significantly reduced levels of COX-2 and inflammatory cytokines at 7 and 16 hours. These results confirm that both p38 and NF-κB pathways are involved in stimulating COX-2 expression and TNF-α and IL-1β secretion. They also raise the possibility that placental oxidative stress may play a role in initiating or augmenting uterine contractions during labor. Our findings may be applicable to other cell types, particularly decidual and placental macrophages that also contribute to the production of inflammatory cytokines and may play a role in mediating labor contractions.[35–37]

Although a beneficial effect of antioxidants was suggested in a small randomized trial where women at risk of preeclampsia were supplemented with vitamins C and E,[11] a recent multicenter randomized clinical trial (VIP trial) showed no benefit in prevention of the disease.[12] The reasons for the disparity between our results and the *in vivo* data are not clear but may reflect differences in cellular concentrations or accessibility of the vitamins. Questions also arise as to whether supplementing with vitamins from 14 to 20 weeks is early enough in pregnancy to prevent the onset of the disease. It has been shown that oral dosing with vitamin C (200 mg/day) maintains a plasma concentration between 10 and 160 μmol/L.[38] However, intracellular concentrations of ascorbic acid can range between 1.4 and 3.4 mmol/L,[39] and so we used vitamin C concentrations of 1 to 2 mmol/L in our study. Vitamin C (2 mmol/L) was found to be the most effective in reducing the effects of H/R. Plasma concentrations of vitamins C in the VIP trial were around 100 to 130 μmol/L in the supplemented group, which are considerably lower, but the intracellular concentrations achieved in placental tissues are not known. The daily doses of vitamins C and E used in the trial were below the maximum daily tolerable intake recommended by the Institute of Medicine of the United States,[40] although it should be noted that the UK population studied already consumed considerable amounts of supplements in their diet. Consequently, additional intake may not have had much effect. This possibility could be addressed soon by two ongoing trials involving supplementation with antioxidant vitamins in developing nations where dietary deficiency is more likely (a multicenter World Health Organization trial and a trial in Brazil).

In the VIP trial, α-tocopherol was used as a vitamin E supplement. Vitamin E consists of four tocopherol homologues, and among them only α-tocopherol has been used in clinical studies.[41] Recent evidence demonstrated that γ-tocopherol has several specific pharmacological properties such as anti-inflammatory and antiproliferative effects in addition to its antioxidative effects.[42,43] γ-Tocopherol, but not α-tocopherol, reduces the neointima proliferation in insulin resistance independently of its effects on superoxide production.[44] The metabolic differences between different tocopherols may therefore need to be considered when studying the *in vivo* effects of vitamin E.

A recent Australian trial also found no benefits of vitamin C and E supplementation in the prevention of preeclampsia.[45] This trial was conducted on healthy nulliparous women who had a low risk of preeclampsia. The VIP trial focused on high-risk patients who are more likely to develop preeclampsia. As a consequence, the study population was very heterogeneous, including women with chronic hypertension, diabetes, renal disease, previous preeclampsia, obesity, antiphospholipid syndrome, and multiple pregnancies. Preventative intervention could thus have a varying effect on the different subgroups involved. The study group in the previous successful small trial[11] was notably more homogeneous, with women being recruited on the basis of abnormal two-stage uterine artery Doppler screening, which has a high predictive value for preeclampsia.[46] More trials are currently in progress in the United States, Canada, Mexico, and Brazil.[47,48]

An analogous situation has been reported in the field of cardiovascular pathology. Strong evidence exists that oxidative stress is pivotally involved in the pathogenesis of atherosclerosis. Many *in vitro*, cellular, and animal studies show convincing evidence of the protective effects of vitamin E against a variety of types of oxidative stress.[49] α-Tocopherol has been shown to reduce atherosclerotic lesions,[50] smooth muscle cell proliferation,[51] and platelet adherence and aggregation[52] in animal models. It can also improve endothelial function in humans.[53] Several cohort studies suggested reduced cardiovascular risk in persons taking vitamin E supplements.[54–56] However, these initial hopeful reports were followed by the negative results of almost all large randomized trials that failed to confirm a role for vitamin E supplementation in cardiovascular prevention.[41] The most recent trial additionally showed that not only does long-term vitamin E supplementation fail to prevent cancer or major cardiovascular complications, it may increase the risk for heart failure.[57]

Several important conclusions can be drawn on the basis of our findings. First, the acute stress of hypoxia-reoxygenation *in vitro* leads to the same placental changes that characterize what has previously been considered the more chronic stress of preeclampsia. These findings provide support for our hypothesis that intermittent perfusion of the placenta, secondary to reduced trophoblast invasion, initiates the placental changes that stimulate preeclampsia. Second, we were able to prevent the changes *in vitro* using the antioxidant vitamins C and E and to some extent by blocking the p38 MAPK pathway. Our findings suggest that devising strategies to minimize placental oxidative stress is still a worthwhile target in the prevention of preeclampsia.

Acknowledgment

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