Molecular Human Reproduction Vol.12, No.11 pp. 677–685, 2006
Advance Access publication September 2, 2006 doi:10.1093/molehr/gal071

Nitration of p38 MAPK in the placenta: association of nitration with reduced catalytic activity of p38 MAPK in pre-eclampsia

R.P.Webster[1,3], D.Brockman[1] and L.Myatt[1,2]

[1]Department of Obstetrics and Gynecology and [2]Department of Molecular and Cellular Physiology, University of Cincinnati, College of Medicine, Cincinnati, OH, USA

[3]To whom correspondence should be addressed at: Department of Obstetrics and Gynecology, University of Cincinnati, College of Medicine, PO Box 670526, Cincinnati, OH 45267-0526, USA. E-mail: rose.webster@uc.edu

Peroxynitrite, a potent pro-oxidant formed from the interaction of superoxide and nitric oxide, has been widely reported to be nitrating tyrosine residues in proteins resulting in the formation of nitrotyrosine. Biological nitration of tyrosine, a footprint of oxidative injury, has been found to occur in various pathological states including pre-eclampsia, a leading cause of maternal mortality and increased perinatal mortality. Oxidative stress is a major contributor to endothelial dysfunction in pre-eclampsia. Previously, we have demonstrated increased nitrotyrosine immunostaining in placental villous vascular endothelium, surrounding vascular smooth muscle and villous stroma from pre-eclamptic or diabetic pregnancies. Immunoprecipitation (IP) with antinitrotyrosine antibodies followed by immunoblot analysis identified increased nitration of phospho-p38 mitogen-activated protein kinase (MAPK) in the pre-eclamptic placenta. The catalytic activity of p38 MAPK and concentration of phospho-p38 MAPK was also found to be reduced in placentae from pre-eclamptic pregnancies. Comparison of peptide masses of a 42-kDa protein obtained by mass spectrometry with masses of a theoretical tryptic digest of p38 MAPK that was modified by phosphorylation and nitration identified the protein to be p38 MAPK.

Key words: nitration/nitrotyrosine/p38 MAPK/peroxynitrite/pre-eclampsia

Introduction

Pre-eclampsia complicates about 5–7% of all pregnancies and is associated with fetal growth restriction, premature delivery and maternal death. It is diagnosed primarily by the onset of hypertension and proteinuria in the latter half of gestation. Vascular and endothelial dysfunction are well-documented characteristics of pre-eclampsia (Duley, 2003). Reduced endovascular trophoblast invasion and uteroplacental artery remodelling, the key pathologic features of pre-eclampsia, lead to relative hypoxia and increased oxidative stress in trophoblast tissue (Goldman-Wohl and Yagel, 2002). This results in the putative release of placental factors that may act upon the maternal systemic vasculature resulting in widespread oxidative stress and vascular dysfunction. Despite active research for many decades, the aetiology of this disorder, which is unique to human pregnancy, remains an enigma. It is obvious that a single mechanism responsible for the pre-eclampsia syndrome does not exist although several compelling studies demonstrate that hypertension may develop because of oxidative stress (Roberts *et al*., 2000; Vaziri *et al*., 2000, 2002; Barton *et al*., 2001; Makino *et al*., 2002; Zhou *et al*., 2002). Nitric oxide, as a vasodilator, appears to play a crucial role in the gradual decrease in the vascular resistance of the placenta throughout pregnancy enabling it to meet the growing needs of the fetus (Myatt *et al*., 1991; Bisseling *et al*., 2003). Nitric oxide produced by syncytiotrophoblasts also appears to prevent platelet adhesion to the trophoblast surface thereby maintaining blood flow in the intervillous space (Myatt *et al*., 1991). Increased expression of the type III endothelial nitric oxide synthase (eNOS) isoform has been reported in the placentae of pregnancies complicated by pre-eclampsia (Myatt *et al*., 1997). The combination of increased oxidative stress and nitric oxide in the placentae of pregnancies complicated by pre-eclampsia potentiates the formation of the potent pro-oxidant peroxynitrite (Ischiropoulos and al-Mehdi, 1995; Buttery *et al*., 1996). Peroxynitrite has the ability to nitrate tyrosine residues in proteins resulting in the formation of nitrotyrosine residues, which are used as fingerprints of peroxynitrite formation and action (Radi, 2004). We have found nitrotyrosine residues, which are markers of both oxidative stress and nitrative stress, in the villous vessels and stroma of placentae from pregnancies complicated by either pre-eclampsia or pregestational diabetes (Myatt *et al*., 1996, 2000; Lyall *et al*., 1998; Stanek *et al*., 2001). In addition, we have shown that peroxynitrite treatment of the normal placental vasculature *in vitro* leads to the formation of nitrotyrosine residues and alters vascular reactivity of the placenta to resemble that observed in placentae from pregnancies complicated by either pre-eclampsia or pregestational diabetes (Kossenjans *et al*., 2000).

p38 mitogen-activated protein kinase (MAPK), a member of the highly conserved MAPK superfamily, regulates diverse cellular processes in response to a plethora of extracellular stimuli (Ichijo, 1999). p38 MAPK is essential for placental organogenesis and for trophoblast growth and invasion (Adams *et al*., 2000; Li *et al*., 2003). p38 MAPK also has been reported to regulate placental lactogen gene in trophoblasts (Peters *et al*., 2000). Also, p38 MAPK has been implicated to regulate implantation and differentiation in mouse models (Scherle *et al*., 2000; Natale *et al*., 2004). It is well established that dual phosphorylation of p38 MAPK at threonine-180 and tyrosine-182 is a critical requirement

for its catalytic activity (Brancho *et al*., 2003) This particular tyrosine residue or any other tyrosine residues in the catalytic domain of the kinase may also be modified by nitrative stress resulting from the oxidative environment prevalent in pre-eclampsia, and this could potentially down-regulate the activity of p38 MAPK. Tyr-nitration of p38 MAPK has been both previously suggested (Kiroycheva *et al*., 2000) and tentatively identified (Bruckdorfer, 2001) in other systems.

In this study, we hypothesized that nitrated proteins would be increased in the placentae from pregnancies complicated by pre-eclampsia where oxidative stress and nitrative stress are observed. We have used a proteomic approach of immunoprecipitation (IP) combined with mass spectrometry to begin to selectively isolate and identify the nitrated proteins in the placenta.

Materials and methods

Products

Antinitrotyrosine antibody was obtained from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Protein A sepharose was purchased from Amersham Biosciences (Piscataway, NJ, USA). Non-radioactive p38 MAPK assay kit, phospho-p38 and p38 MAPK antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Polyclonal antibodies to phospho-p38 were raised by immunizing rabbits with a synthetic phospho-Thr180/Tyr183 peptide by the manufacturer. Antibodies used against p38 MAPK and phospho-p38 MAPK were against the alpha form of p38 MAPK. Western Blot Chemiluminescence reagent was purchased from Perkin Elmer (Boston, MA, USA). Precast 8–16% gradient gels were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Molecular weight markers used were broad range for silver staining from Amersham Biosciences Corp., kaleidoscopic marker from Invitrogen Life Technologies and molecular weight standards from Santa Cruz Biotechnology Inc. (SantaCruz, CA, USA) for westerns. The BCA kit for protein estimation was obtained from Pierce (Rockford, IL, USA).

Tissue preparation

Tissues were collected under a protocol approved by the Institutional Review Board at the University of Cincinnati Medical Center. Placentae were collected immediately following delivery from normal pregnancies (mean gestational age for this group was 39.4, Table I) and those complicated by severe pre-eclampsia (mean gestational age was 30.1, Table I). These two groups are not matched by gestational age. In this study, for the control group, only those placentae obtained from pregnancies without any other known complications were used. Each group comprised nine patients for most experiments except where indicated otherwise. Severe pre-eclampsia was defined as occurrence of hypertension (sustained blood pressure >160 mmHg systolic and 110 mmHg diastolic), edema and proteinuria (>3^+ protein on dip stick) after 20 weeks of gestation in a previously normotensive woman. Critical clinical characteristics of the patient groups are shown in Table I. Villous tissue was dissected out from beneath the chorionic plate, avoiding the basal plate, and stored at –80°C. Tissue was subsequently thawed and lysed at 4°C using a tissue tearor. Lysates (10% w/v) were prepared in lysis buffer 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS) 2%, 20 mM Tris, pH 7.5, 1 mM ethylene diammine tetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 2 μM leupeptin, 5.8 μM pepstatin, 20 μM 4-(2-aminoethyl)-bezenesulphonyl fluoride (AEBSF) and 5 μM *N*-tosyl-L-lysine chloromethyl ketone (TLCK). Lysates were centrifuged at 20 000 × *g* at 4°C for 5 min to remove debris, and supernatants were used for all experiments.

Table I. Clinical characteristics of pregnant women and their offspring

Characteristics	Normal	Pre-eclamptic
Mother's age (years mean ± SD)	28 ± 6.1	27±7.2
Gestational age (weeks, mean ± SD)	39.4 ± 0.8	30.1 ± 1.13
BP systolic	121 ± 10.5	169 ± 20.1
BP diastolic	69 ± 8.0	101 ± 9.3
Gravidity[a]	1–5	1–6
Fetal weight (mean ± SD)	3191 ± 211	1105 ± 341.6
Mode of delivery	2:7 (VD : CS)[b]	2:7 (VD : CS)

CS, Cesarean section and VD, vaginal delivery.
All the women used in this study did not differ significantly ($P > 0.05$, Student's *t*-test) in age. There was a significant ($P < 0.0001$, Student's *t*-test) difference in gestational age, blood pressure and fetal weight between the two groups.
[a]Two of the nine women in the normal group and four of nine in the pre-eclamptic group were pregnant for the first time.
[b]In both groups there were two vaginal deliveries and the rest were all Cesarean sections.

IP

IP was carried out essentially according to Rane *et al*. (2001) using supernatants containing 1 mg of protein diluted in IP buffer containing 20 mM Tris (pH 7.5), 1 mM EDTA, 1 mM EGTA, 200 μM AEBSF, 200 μM sodium orthovanadate, 0.5% Nonidet P-40, 0.5% Triton X-100, 20 mM sodium fluoride, 0.15 M sodium chloride, 2 μM leupeptin, 5.8 μM pepstatin and 5 μM TLCK. The lysates were precleared by incubation with 20 μl of protein A sepharose for 1 h at 4°C. Following centrifugation at 10 000 × *g* for 1 min, 10 μl of antinitrotyrosine antibody or p38 MAPK antibody or phospho-p38 MAPK antibody was incubated with the supernatants overnight at 4°C on a rotator. Protein A sepharose was then added followed by incubation for an additional 1 h at 4°C on the rotator. Beads were washed three times with lysis buffer, resuspended in 50 μl of 1× Laemmli buffer and boiled at 95°C for 5 min. Immunoprecipitates were separated by sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS–PAGE) and silver stained or Coomassie stained or western blotted as appropriate. To aid nitrotyrosine detection on western blots, a modification of the Laemmli buffer was used, replacing dithiotrietol (DTT) with 10 mM iodoacetamide (IAA). This prevents reduction of nitrotyrosine to aminotyrosine, which enables detection of nitrated proteins (Balabanli *et al*., 1999).

Western blotting

Following lysate preparation, proteins were separated on 8–16% gradient precast gels, transferred onto nitrocellulose membranes and blocked with 5% milk in 0.1% Tween, 20 mM Tris (pH7.5)-buffered saline (TTBS) (w/v) for 1 h. Blots were probed with antinitrotyrosine (1:2000), anti-p38 (1:500) or anti-phospho-p38 (1:500) antiserum in 5% bovine serum albumin/TTBS overnight and were detected using peroxidase-conjugated secondary antibody (1:10 000) in 5% milk/TTBS for 1 h. In the case of antinitrotyrosine, blots were blocked with 1% BSA, and both primary and secondary antibodies were made up in the same. Products were visualized by chemiluminescence (Perkin Elmer). Band intensity was measured using AlphaImager software from Alpha Innotech. Equal protein loading was confirmed by Ponceau S staining.

p38 MAPK activity assays

The assay was carried out essentially according to the manufacturer's protocol. Total activity of p38 MAPK was measured in placental lysates. Activity was also measured in phospho-p38 immunoprecipitates or nitrotyrosine immunoprecipitates as required. Reactions were carried out with kinase buffer containing 25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM sodium orthovanadate, 10 mM $MgCl_2$, 200 μM adenosine triphosphate (ATP) and 2 μg of activating transcription factor-2 (ATF-2) at 30°C for 30 min. Reactions were stopped with 25 μl of 3× Laemmli buffer, and reaction mixtures were boiled at 95°C for 5 min. Reaction mixtures were separated on 12% SDS–PAGE gels and western blotted for phospho-ATF-2. The blots were then quantified. Higher levels of phospho-p38 activity were reflected in higher amounts of phospho-ATF-2. This is an assay widely used in measurement of p38 MAPK activity (Samet *et al*., 1998; He *et al*., 2003). It has also been demonstrated that inhibitors of p38 MAPK can inhibit phosphorylation of ATF-2 (Lewis *et al*., 2005).

Statistical analysis

The significance of difference between the data from the two independent groups of samples (normotensive and pre-eclamptic) was determined by the non-directional two-tailed Student's *t*-test in preliminary experiments. Preliminary

experiments were carried out with three samples each. Power analysis was carried out on the preliminary data obtained, and this indicated that seven samples were necessary to obtain 90% power at a significance level of $P < 0.05$. Experiments carried out with nine samples had a power of 96.7% at the same significance level. In those experiments that were repeated with more samples (>3), the unidirectional one-tailed Student's *t*-test was used. Each experiment included at least triplicate experiments for each variable tested. All results are expressed as mean ± SEM of three independent experiments. Differences were considered to be significant when the *P* value < 0.05. Statistical analyses were carried out on densitometric data obtained from western blots.

Trypsin digestion

To obtain peptides for mass spectrometric analysis, antinitrotyrosine immunoprecipitated proteins from one representative pre-eclamptic placenta were separated by SDS–PAGE and Coomassie stained. The same sample was also separated by SDS–PAGE and western blotted for phospho-p38 MAPK. The phospho-p38-immunoreactive band on the film obtained after western blot was aligned to the Coomassie-stained gel, and the corresponding band was excised and digested with trypsin. Before digestion, the excised gel pieces were destained for 10 min, washing three times with 200 µl of 25 mM NH_4HCO_3/50% acetonitrile. For the protein reduction, a freshly prepared 10 mM DTT was added to the gels, enough to cover them, vortexed briefly and incubated at 56°C for 45 min. The samples were cooled to room temperature and DTT solution was removed followed by the addition of 55 mM solution of IAA in 25 mM NH_4HCO_3 enough to cover the gel pieces; this addition was done for alkylation. The IAA solution was removed and discarded; samples were cleaned with 25 mM NH_4HCO_3 for 10 min and then twice with 50% acetonitrile/25 mM NH_4HCO_3. Samples were then dried for 20 min in speed vac, and then trypsin solution was added. The gel pieces were rehydrated in 0.01 µg/µl of trypsin solution and then incubated at 4°C for 45 min. The trypsin solution was made in 25 mM NH_4HCO_3. All the trypsin solution was removed and a small amount of 25 mM NH_4HCO_3 solution added to keep the gels hydrated during digestion. The digestion was performed overnight at 37°C in a water bath. The peptides were extracted from the gels by using 5% formic acid and 50% acetonitrile/25 mM NH_4HCO_3 solution. Extraction of the peptides was done twice, solutions were combined, volumes were reduced to ~20 µl and 10 µl of the eluted peptide was loaded on a capillary column ready for (LC-MS) run. Two microlitres was applied by a thin film-spotting procedure for matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) analysis using α-cyanohydroxycinnamic acid as the matrix on stainless steel targets.

Mass spectrometry

Mass spectral data were obtained at the WM Keck Foundation Biotechnology Laboratory, Yale University, using a Micromass TofSpec SE and a 337-nm N_2 laser at an accelerating voltage of 25 kV in the positive linear ion mode. Peptide masses obtained by MALDI-MS analysis were searched against databases using the programs ProFound and Pepsea. These programs rely on the National Center for Biotechnology Information (NCBI) and European Molecular Biology Laboratory (EMBL) non-redundant databases, respectively, to identify the intact proteins. We then compared actual peptide masses obtained from our protein in this way with peptide masses obtained from a theoretical (Nicholson *et al*., 2005) tryptic digest of p38 MAPK (Database: Swiss Prot, accession number Q16539) modified by phosphorylation and nitration. Theoretical tryptic digest masses were generated using the MS-Digest program of Protein Prospector (http://prospector.ucsf.edu). Peptide mass matching was carried out with a mass tolerance of upto 1 Da. Peptide mass matching has been reported by others (Thongboonkerd *et al*., 2002; Singh *et al*., 2003; Emanuelsson *et al*., 2005) with over a dalton difference.

Results

Nitrated proteins in the placenta

Our earlier observations of increased nitrotyrosine immunostaining in the pre-eclamptic placenta led us to our current hypothesis that there would be correspondingly higher levels of nitrated proteins in the pre-eclamptic placenta and initiated the search for nitrated proteins. To selectively isolate and identify the nitrated proteins, we first separated the antinitrotyrosine immunoprecipitated proteins by SDS–PAGE and silver stained the gels. Figure 1 (panel A) shows at least six or more proteins of sizes ranging from 20 to 90 kDa, identified by silver staining that are nitrated in both normal and pre-eclamptic placentae ($n = 3$, each group) examined. This was confirmed by western blot analysis of total lysates with antinitrotyrosine antibody, which also showed nitration in both normal and pre-eclamptic placentae (Figure 1, panel B, $n = 3$, each group) with similar molecular weight ranges of proteins being identified. Preincubation of the antinitrotyrosine antibody with 10 mM nitrotyrosine was used to check the specificity of the antibody, and this blocked the nitrotyrosine immunoreactivity in the lysates on western blot (Figure 1, panel B).

Identification of phospho-p38 as a nitrated protein and effect on catalytic activity of phospho-p38

Major differences in the levels of nitrated proteins between normal and pre-eclamptic placentae were found around 42 kDa range (Figure 1, panel A), suggesting that it might correspond to p38 MAPK, among other MAPKs. We explored the possibility that p38 MAPK was a target of increased nitration in the pre-eclamptic placenta. To achieve this aim, we immunoprecipitated nitrotyrosine-containing proteins with the antinitrotyrosine antibody and cross-blotted them with phospho-p38 MAPK antibody, which gave recognition of a band at 42 kDa (Figure 2A). The apparent intensity of this band was significantly greater ($n = 9$, $P < 0.0001$) in antinitrotyrosine immunoprecipitates from pre-eclamptic placentae than that from normal placenta (Figure 2B). Negative control of IP with protein A sepharose beads alone gave no bands on cross-blotting, confirming the specificity of the IP protocol (data not shown).

To directly examine whether Tyr-nitration affected functionality of p38 MAPK in the placenta, we tested antinitrotyrosine immunoprecipitates for their ability to phosphorylate ATF-2, a measure of the catalytic activity of p38 MAPK. There was no significant difference in overall p38 MAPK activity shown by antinitrotyrosine immunoprecipitates from pre-eclamptic placentae (Figure 2C and D) when compared with normotensive pregnancies. When activity was normalized to the amount of phospho-p38 MAPK present in the antinitrotyrosine immunoprecipitates, however, the specific catalytic activity obtained from pregnancies complicated by pre-eclampsia was 65% less ($P < 0.03$, Figure 2E) than that from normotensive pregnancies.

The catalytic activity was drastically reduced in phospho-p38 MAPK immunoprecipitates from pre-eclamptic pregnancies, and they exhibited 81% lower activity (Figure 3, panel A, $P < 0.05$) than in immunoprecipitates from normal placentae. It is significant that phospho-p38 immunoprecipitates from pre-eclamptic placentae also showed a higher extent of nitration (Figure 3, panel B) compared with normal. This demonstrates a correlation between higher level of nitration of phospho-p38 and lower catalytic activity. For additional evidence of nitration, we have also, in separate experiments, immunoprecipitated with antibody that recognizes total p38 MAPK (non-phosphorylated plus phosphorylated, Figure 3c) and cross-blotted with antinitrotyrosine antibody. This blot was also stripped and probed for total p38 (Figure 3d).

Western blotting of total placental lysates also revealed that the amount of phospho-p38 MAPK was significantly less in placentae of pregnancies complicated by pre-eclampsia compared with normotensive pregnancies ($P < 0.001$, Figure 4). Not surprisingly, the overall catalytic activity of phospho-p38 MAPK was 32% less ($P < 0.05$) in placental lysates from pregnancies complicated by pre-eclampsia compared with normotensive pregnancies (Figure 5).

Confirmation of nitration of p38 MAPK by MALDI-MS

To obtain unequivocal identification by MALDI-MS, we excised the protein band corresponding to phospho-p38 MAPK from an SDS–PAGE

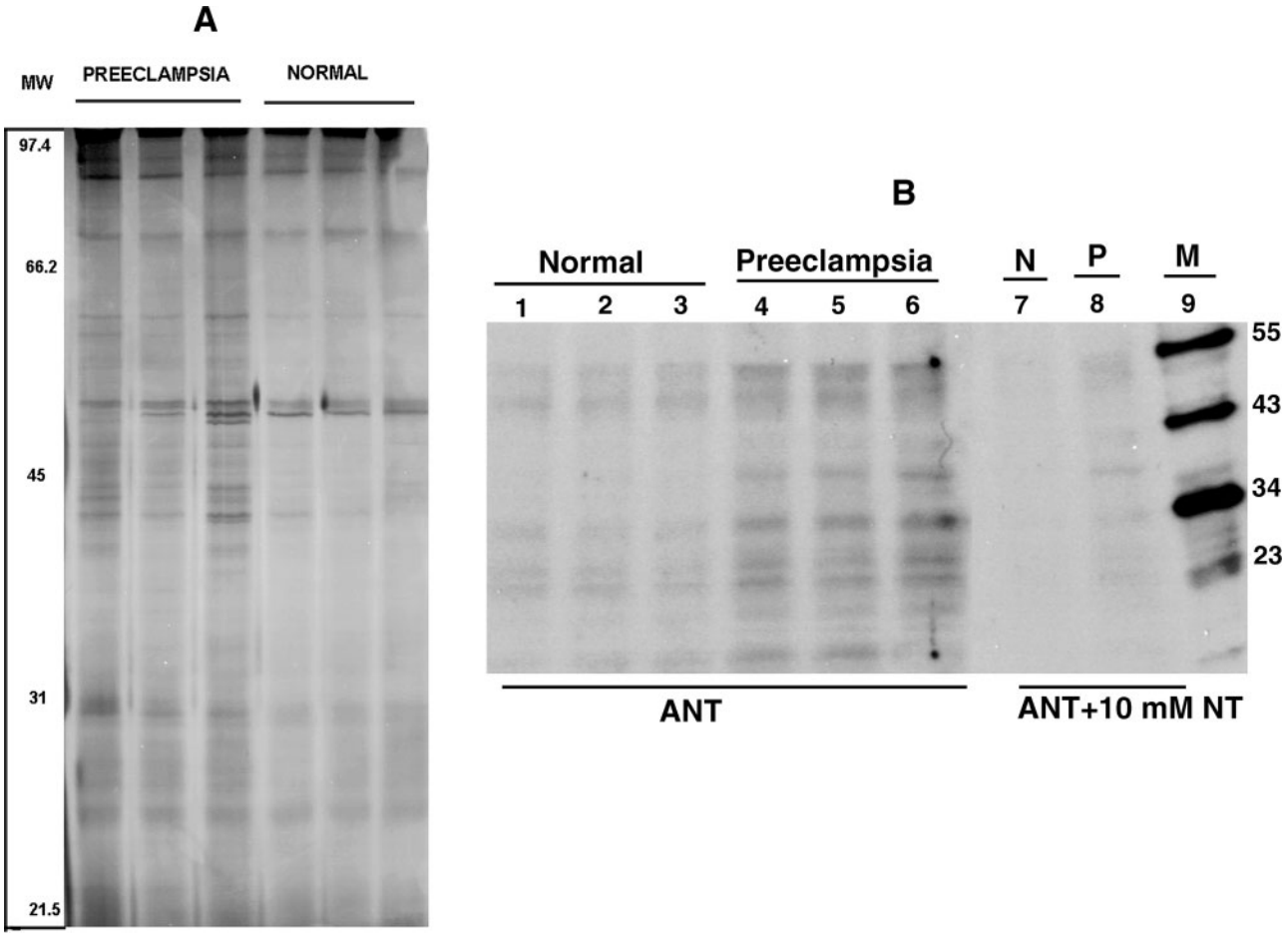

Figure 1. Detection of nitrated proteins from normal and pre-eclamptic placentae. (**A**) Placental lysates (1 mg) from either normal or pre-eclamptic placentae were subjected to immunoprecipitation with antinitrotyrosine antibody. The immunoprecipitates were then separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and silver stained. (**B**) Western blot of nitrated proteins in placenta placental lysates (50 ug of protein/lane, lanes 1–6) were subjected to immunoblot analysis using antinitrotyrosine antibodies. One representative sample from a normal (lane 7) and pre-eclamptic (lane 8) placenta were also subject to western analysis using antinitrotyrosine (ANT) antibody preadsorbed with 10 mM 3-nitrotyrosine (NT) to determine specificity of the antibody.

gel on which the antinitrotyrosine immunoprecipitate from a pre-eclamptic placenta had been fractionated and digested with trypsin (Table II). Because of the extensive nitration combined with phosphorylation that presumably prevented a definitive identification of the protein, masses obtained by MALDI-MS did not match any peptide sequences in any available database. Accordingly, the sequences were subsequently compared with those obtained by theoretical tryptic digest of p38 MAPKα (Database: Swiss Prot., accession number Q16539). This theoretical digest was carried out after modification (enabled by the software) of its serine, threonine and tyrosine residues by phosphorylation and nitration of tyrosine. Interestingly, eight of the peptide masses from the excised phospho-p38 MAPK band matched those of the theoretical digest masses of p38 MAPK modified by phosphorylation and nitration in all the possible residues of serine, threonine and tyrosine (Table I). In a database search using MOWSE, three masses are considered enough for a positive identification. The peptide 166–186 (Table I) contains the sites of dual phosphorylation, T(180) and Y(182), that is critical for p38 MAPK catalytic activity. The mass spectrometry data thus indicate that phospho-p38 MAPK is nitrated in the pre-eclamptic placentae.

Discussion

The data presented here demonstrate that p38 MAPK is a nitrated protein in the placentae, with the amount of nitrated protein being significantly higher ($P < 0.0001$) in the placentae of pregnancies complicated by pre-eclampsia than in normal pregnancies. It needs to be borne in mind that these data were generated using two distinct groups of completely normal and severe pre-eclamptic women. The two patient groups used were significantly ($P < 0.0001$) different in their gestational ages. The primary reason for not using age-matched normals was due to the fact that no pregnancies which deliver at 28–32 weeks can be considered as true normals. Most, if not all, of them have some underlying pathology (Barrett, 2002; Badr *et al*., 2005; Crider *et al*., 2005). Although risk factors for preterm birth include among others preterm premature rupture of membranes (pPROM), cervical insufficiency, pathologic uterine distension, uterine anomalies, intrauterine infection/inflammation and social factors, the final common pathway appears to be activation of the inflammatory cascade (Engel *et al*., 2005), which was yet another reason for not using age-matched normals. Moreover, because oxidative stress and, therefore by association, nitrative stress have been reported (Wang and Walsh, 1998; Casanueva and Viteri, 2003; Jauniaux *et al*., 2003) to increase with increasing gestational age, a true normal preterm group would have potentially exhibited even lower levels of nitrated protein and thus increased the intensity of the differences that we have obtained. This study specifically looked at early onset severe pre-eclampsia so as to ensure maximal phenotypic differences.

The combined tools of IP and mass spectrometry confirm the nitration of phospho-p38 MAPK from human placental lysates. We speculated

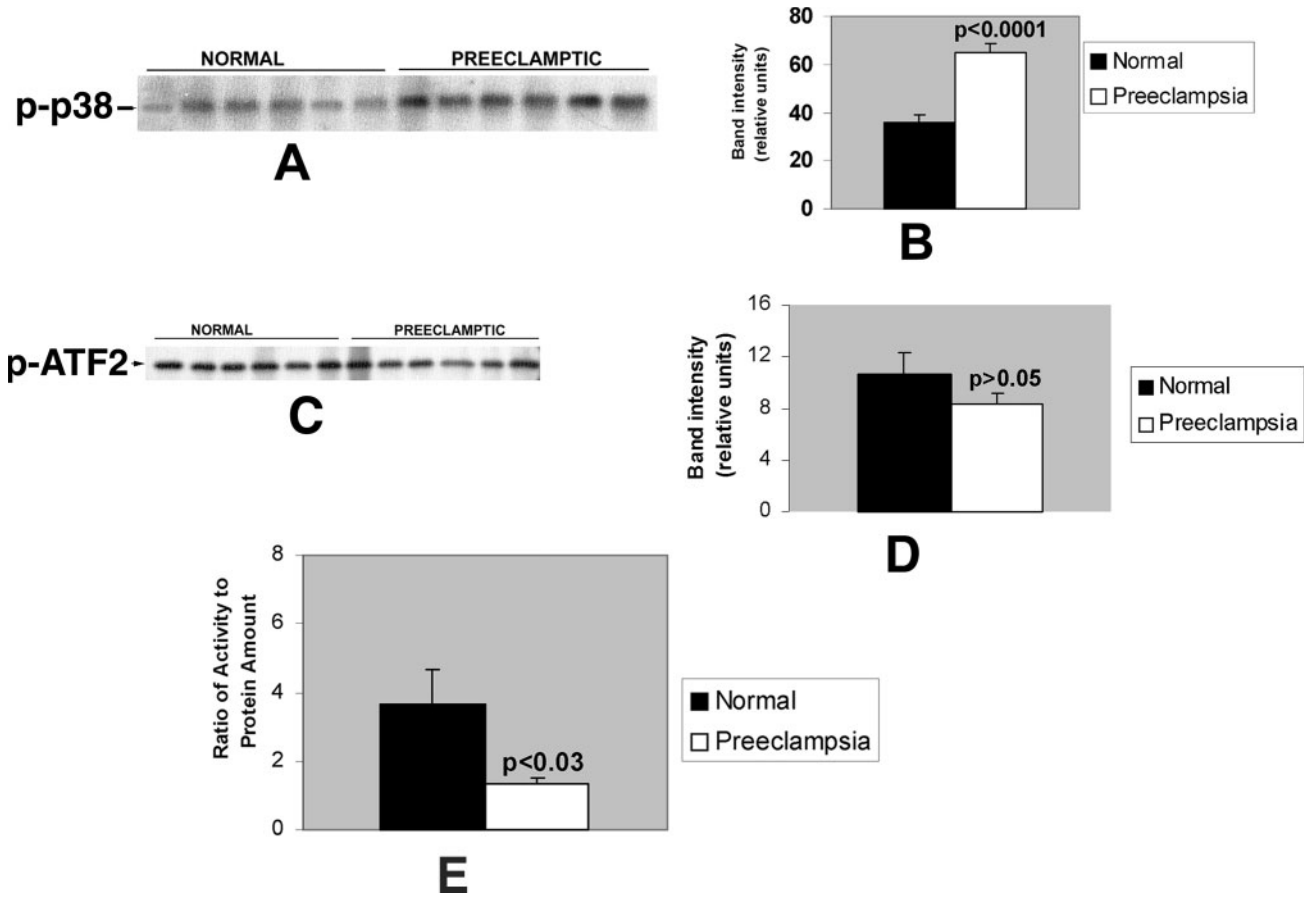

Figure 2. Expression of phospho-p38 mitogen-activated protein kinase (MAPK) in antinitrotyrosine immunoprecipitates from pre-eclamptic placenta. (**A**) Placental protein (1 mg) was immunoprecipitated with antinitrotyrosine antibody, and the immunoprecipitates were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Nitrocellulose membrane was then probed with phospho-p38 MAPK antibody. Control immunoprecipitations (Ips) without antinitrotyrosine antibody showed no evidence of non-specific binding. Blot shown here shows six of the nine samples tested. (**B**) The intensity of phospho-p38 immunoreactive band was quantified, and data are expressed as mean ± SEM. Intensity of phospho-p38 was significantly higher ($P < 0.0001$, $n = 9$) in pre-eclampsia when compared with normotensive. (**C**) Nitrotyrosine immunoprecipitates were resuspended in kinase buffer to determine their ability to phosphorylate ATF-2. Blot shows data obtained for normal and pre-eclamptic placentae ($n = 9$each). This figure shows data obtained with six samples. (**D**) Band intensity of phospho ATF-2 formed by phospho-p38 in antinitrotyrosine immunoprecipitates was quantitated using the AlphaImager software. Data shown (mean ± SEM) are from three separate experiments, each experiment consisting of triplicates. The difference between the two groups was not significant ($P > 0.05$, Student's *t*-test). (**E**) Ratio of band intensity of phospho ATF-2 formed (activity) by nitrotyrosine immunoprecipitates to that of phospho-p38 MAPK (protein expression) in nitrotyrosine immunoprecipitates was calculated for the two groups. Band intensity of phospho ATF-2 in antinitrotyrosine immunoprecipitates measures activity of phospho-p38 MAPK. Band intensity of phospho-p38 in antinitrotyrosine immunoprecipitates measures expression of phospho-p38. Activity to expression ratio of p38 MAPK was significantly less (35%, $P < 0.03$, $n = 9$) in pre-eclampsia (Student's *t*-test).

that the extensive nitration combined with the phosphorylation prevents a definitive identification of the protein from conventional database searches. However, eight peptide masses of the unidentified protein match the theoretical tryptic digest of p38 MAPK, when it was modified by nitration and phosphorylation in its serine, threonine and tyrosine residues. Although such an extensive modification may not take place in an *in vivo* situation, this assumption was necessary so as not to miss any peptides that could be potentially modified. Peptide matches obtained cover 33% of the entire p38 MAPK sequence and essentially confirm the identification of nitrated p38 MAPK. It is highly likely that nitration of p38 MAPK interferes with its interaction with other proteins. Several reports (Choi, 2000; Hagemann and Blank, 2001; Taylor and Starnes, 2003) do exist concerning interaction of active p38 MAPK with other proteins and also the ability of p38 MAPK to regulate various gene functions (Ono and Han, 2000). Nitration of p38 MAPK demonstrated here would therefore adversely impair several critical functions at both cellular and genetic levels.

The overall reduced concentration of phospho-p38 MAPK that we observed in the pre-eclamptic placentae may be because of several reasons. It may be hypothesized that gestational age could influence activity and expression of p38 MAPK in the pre-eclamptic group. However, to our knowledge there have been no reports on the change in p38 MAPK activity or expression in the placenta with gestational age. Yet, because our data presented here (Figure 2A) demonstrate increased nitration of phospho-p38 in the pre-eclamptic placentae, we propose that the reduction in the amount of phospho-p38 MAPK is due to nitration. It is possible that the nitration of phospho-p38 MAPK reduces its affinity for the specific antibody as previously suggested by Kiroycheva *et al*. (2000). We have also estimated the catalytic activity of p38 MAPK in total placental lysates where the assay was not compromised by the use of immunoprecipitating antibodies. Here, again the catalytic activity of p38 MAPK was significantly ($P < 0.05$, Figure 5) less in pre-eclampsia. We have established the decreased activity of p38 MAPK in pre-eclampsia in three different ways, namely by measuring activity in antinitrotyrosine, phospho-p38 immunoprecipitates and also in total placental lysates. On the basis of data presented here and our *in vitro* data (Webster *et al*., 2006) that demonstrated total inhibition of catalytic activity on nitration of p38

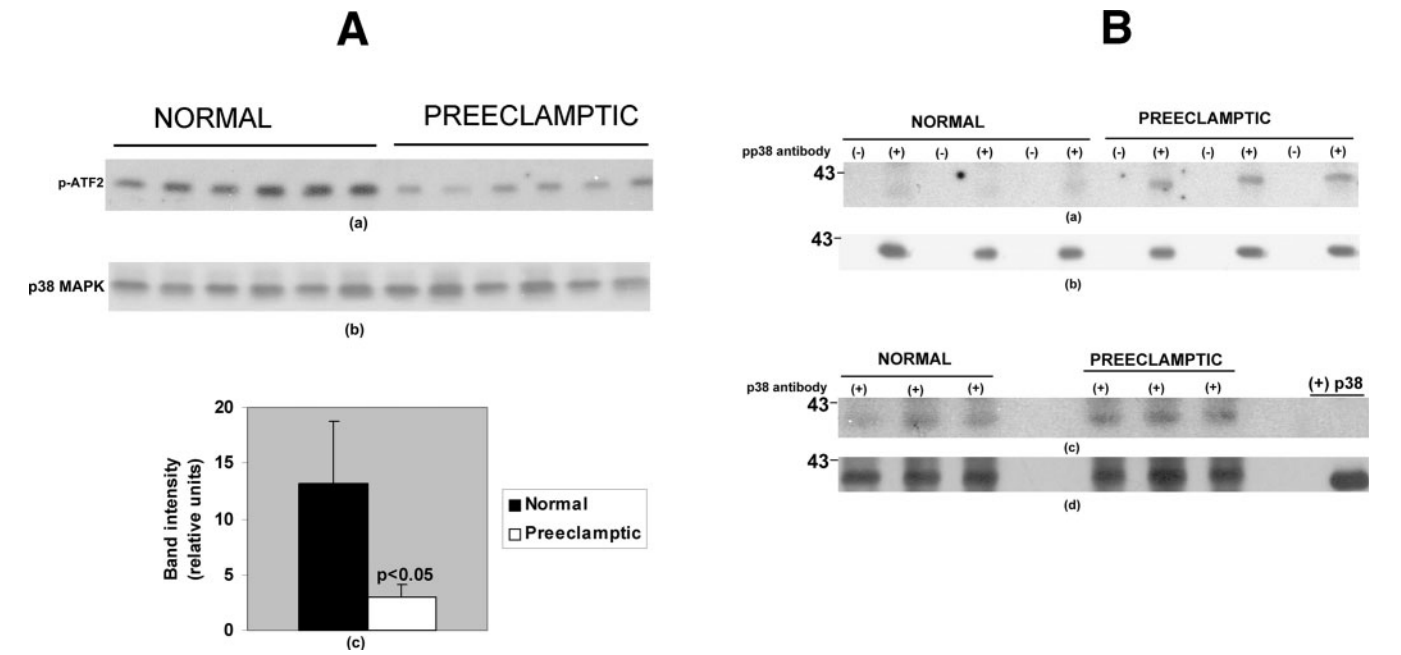

Figure 3. Panel A shows formation of phospho ATF-2 by phospho-p38 mitogen-activated protein kinase (MAPK) immunoprecipitates from placenta. (**a**) Phospho-p38 immunoprecipitates were resuspended in kinase buffer to estimate their ability to phosphorylate ATF-2. The figure shows six of the nine samples tested. (**b**) The above blot was stripped and probed for total p38 MAPK to verify protein loading. (**c**) Bands corresponding to phospho ATF-2 formed by phospho-p38 immunoprecipitates from normal and pre-eclamptic were quantified by Alphaimager software. Data shown (mean ± SEM) are representative of three separate experiments, each done in triplicates. Activity of p38 MAPK was observed to be significantly ($P < 0.05$, $n = 9$) higher in pre-eclampsia than in normal. Panel B shows nitration in p38 MAPK immunoprecipitates from placenta. Protein (1 mg) was immunoprecipitated with either (a) phospho-p38 MAPK or (c) total p38 MAPK antibody, the immunoprecipitates were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and probed with antinitrotyrosine antibody. Each of the blots were then stripped and probed for (b) phospho-p38 and (d) total p38.

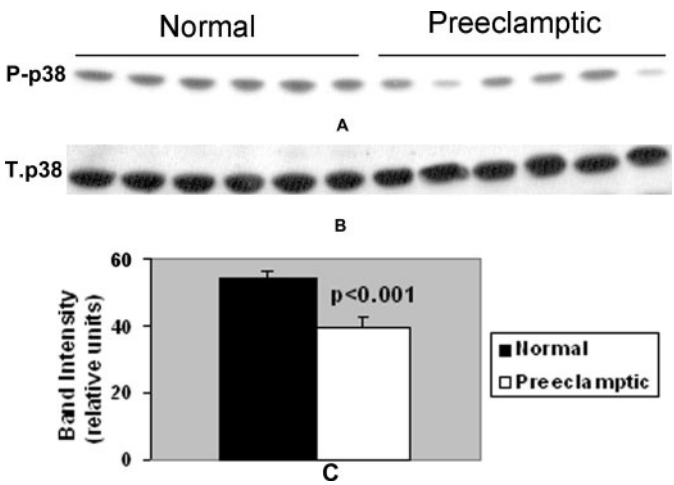

Figure 4. Level of phospho-p38 mitogen-activated protein kinase (MAPK) in placenta. (**A**) Lysate (20 ug) from normal and pre-eclamptic pregnancies was separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blotted for phospho-p38 MAPK. This figure shows six of the nine samples tested. (**B**) The blot was stripped and probed for total p38 to verify protein loading. (**C**) Scanning densitometry revealed that phospho-p38 levels in pre-eclamptic placentae were less ($P < 0.001$, $n = 9$, Student's *t*-test) than in normal. Values are expressed as mean ± SEM.

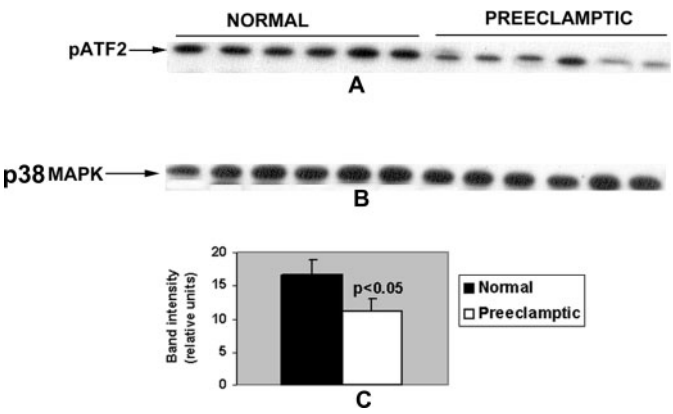

Figure 5. Activity of phospho-p38 mitogen-activated protein kinase (MAPK) in placental lysates. (**A**) Placental lysate (20 μg protein) was reacted with ATF-2 in kinase buffer. Phospho ATF-2 levels detected by western blot are taken as a measure of p38 MAPK activity. (**B**) The above blot was stripped and probed for total p38 to verify protein loading. (**C**) Scanning densitometry showed increased ($P < 0.05$) amounts of phospho-ATF-2 in normal placental lysates when compared with pre-eclamptic. Data shown (mean ± SEM) are from six of the total nine tested. Differences were significant at $P < 0.05$ (Student's *t*-test).

MAPK, we suggest that the reduced catalytic activity of p38 MAPK observed in the pre-eclamptic placenta is due to nitration.

Many proteins including manganese superoxide dismutase (MnSOD) (Yamakura *et al*., 1998), p53 (Yamakura *et al*., 1998; Chazotte-Aubert *et al*., 2000; Cobbs *et al*., 2001), cytochrome P450 (Roberts *et al*., 1998), Ca-ATPase of the sarcoplasmic reticulum (Viner *et al*., 1996), mitochondrial creatine kinase (Stachowiak *et al*., 1998), tyrosine hydroxylase (Ara *et al*., 1998) and prostacyclin synthase (Zou *et al*., 1997, 1999a,b) have been reported to be nitrated. Protein nitration has been most commonly shown to inhibit protein function, e.g. MnSOD (MacMillan-Crow *et al*., 1996), p53 (Chazotte-Aubert *et al*., 2000), NFκB (Park *et al*., 2005) and tyrosine hydoxylase (Ara *et al*., 1998). However, in some cases, nitration either has no effect, e.g. protein kinase epsilon (Balafanova *et al*., 2002), or activates protein function as is the case with cyclooxygenase-2 (Salvemini, 1997), poly ADP ribose polymerase (Szabados *et al*., 1999) and fibrinogen (Gole *et al*.,

Table II. Identification of p38 mitogen-activated protein kinase (MAPK) as a nitrated protein

Masses obtained	Masses matched	Start	End	Peptide
1359.7	1360.6	1	10	MSQERPTFYR
993.4	993.6	67	73	KRTYRELR
1954.1	1954	122	136	KLTDDHVQFLIYQILR
2746.3	2747.1	166	186	KILDFGLARHTDDEMTGYVATR
1483.7	1484.7	221	233	RTLFPGTDHIDQLK
2516.3	2517	249	267	KKISSESARNYIQMPK
1323.7	1322.7	257	267	RNYIQSLTQMK
1171.7	1172.5	288	296	KMLVLDSKR

1MSQERPTFYRQELNKTIWEVPERYQNLSPVGSGAYGSVCAAFDTKTGLRV
51AVKKLSRPFQSIIHA**KRTYRELR**LLKHMKHENVIGLLDVFTPARSLEEFN
101DVYLVTHLMGADLNNIVKCQ**KLTDDHVQFLIYQILRGLKY**IHSADIIHRD
151LKPSNLAVNEDCEL**KILDFGLARHTDDEMTGYVATRWYRA**PEIMLNWMHY
201NQTVDIWSVGCIMAELLTG**RTLFPGTDHIDQLKL**ILRLVGTPGAELL**KKI**
251SSESARNYIQSLTQMPKMNFANVFIGANPLAVDLLE**KMLVLDSDKR**ITAA
301QALAHAYFAQYHDPDDEPVADPYDQSFESRDLLIDEWKSLTYDEVISFVP
351PPLDQEEMES

Comparison of peptide masses obtained from a 42 KDa protein with theoretical tryptic digest of p38 MAPK (Database: Swiss Prot., accession number Q16539) with its S, T and Y residues modified by phosphorylation and nitration. The total coverage is 33%. Peptides covered in p38 MAPK (sequence given above) are shown in bold letters.

2000). In addition, cytoskeletal proteins, such as actin and tubulin, have also been shown to be nitrated (Ischiropoulos, 1998), which has been postulated to have a physiological role *in vivo*.

Proteins that are tyrosine nitrated have been reported to be poor substrates for tyrosine kinases (Gow *et al*., 1996; Kong *et al*., 1996). This would result in decreased phosphorylation of p38 MAPK when it is nitrated and may explain the decreased activity and amounts of phospho-p38 MAPK seen in our experiments as well as in those of Kiroycheva *et al*. (2000). Proteolytic degradation that has been seen to occur for other nitrated proteins (Grune *et al*., 1998) could also reduce the intact amount of a protein. Another possibility is that nitration on tyrosine leads to displacement of phosphotyrosine which has been reported to occur in lyn kinase (Mallozzi *et al*., 2001; Minetti *et al*., 2002). In the case of lyn kinase, phosphotyrosine displacement leads to activation of the kinase. However, the reduced catalytic activity of p38 MAPK that we observed seems to rule out such a possibility. Nitration has also been reported to be mimicking phosphorylation in the case of src kinase (Minetti *et al*., 2002).

The detection of nitration in both normal and pre-eclamptic placenta suggests that nitration may be a normal negative regulator for phosphorylation. It has been shown that both peroxynitrite (Jope *et al*., 2000) and nitric oxide (Li *et al*., 2001) have the ability to induce phosphorylation of p38 MAPK protein. Yet, there are reports (Kiroycheva *et al*., 2000) of peroxynitrite causing reduced expression of phospho-p38 MAPK protein. Our results show an association between the reduced amounts of phospho-p38 MAPK and increasing levels of nitration in the placenta. This increased nitration is also associated with lowered p38 MAPK catalytic activity.

Potentially, the nitration of p38 MAPK observed in term placenta could be because of a biphasic effect of peroxynitrite. Initially, peroxynitrite induces phosphorylation and then the increasing concentrations of peroxynitrite bring about nitration of the kinase. Such a biphasic effect of peroxynitrite has been reported in other situations (Jope *et al*., 2000). Higher levels of peroxynitrite caused by increased oxidative stress in pre-eclampsia may lead to increased nitration of p38 MAPK. Increased nitration of p38 MAPK correlates with the higher levels of nitrotyrosine staining, detected in the pre-eclamptic placenta (Myatt *et al*., 1996).

Pre-eclampsia is associated with abnormal trophoblast invasion and development of the placenta (Roberts and Cooper, 2001; Roberts and Lain, 2002; McMaster *et al*., 2004). Targeted inactivation of the p38 MAPKα is accompanied by early embryonic lethality in mice and is associated with defects in placental development because of loss of labyrinth and reduced spongiotrophoblast layers in the placenta, reduced vascularization of labyrinth and increased apoptosis (Adams *et al*., 2000). Nitric oxide and progesterone may synergistically activate p38 MAPK to induce apoptosis in endometrial epithelial cells (EECs), a process that may facilitate implantation (Li *et al*., 2001). If this is so, then inactivation of p38 MAPK following nitration may be one more potential cause for poor trophoblast invasion and consequently, implantation observed in pre-eclampsia (Waite *et al*., 2002). If this is the case though, substantial nitration of p38 MAPK may have to occur before implantation. Our data presented here provide another possible cause for inadequate implantation observed in pre-eclampsia. These data along with earlier reports by Miller *et al*. (1996) of elevated nitrative response in animal models of fetal growth restriction clearly suggest a link between nitrative stress and fetal growth restriction which accompanies pre-eclampsia.

Our data clearly demonstrate a coexistence of loss of function and nitration of p38 MAPK. We have identified a critical signalling molecule p38 MAPK to be nitrated. Our data demonstrate that the increased nitration of p38 MAPK is associated with a decrease in p38 MAPK catalytic activity which could be one of the causes for both poor implantation and growth restriction observed in pre-eclampsia. It is necessary to use both *in vitro* studies and *in vivo* cell culture to demonstrate the direct effect of nitration on functionality of p38 MAPK. Such studies are currently in progress in our laboratory.

Acknowledgements

This work was supported by NIH RO1 grant HL07529. Mass spectrometric services were obtained from W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University.

References

Adams RH, Porras A, Alonso G, Jones M, Vintersten K, Panelli S, Valladares A, Perez L, Klein R and Nebreda AR (2000) Essential role of p38alpha MAP kinase in placental but not embryonic cardiovascular development. Mol Cell 6,109–116.

Ara J, Przedborski S, Naini AB, Jackson-Lewis V, Trifiletti RR, Horwitz J and Ischiropoulos H (1998) Inactivation of tyrosine hydroxylase by nitration following exposure to peroxynitrite and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Proc Natl Acad Sci USA 95,7659–7663.

Badr LK, Abdallah B and Mahmoud A (2005) Precursors of preterm birth: comparison of three ethnic groups in the middle East and the United States. J Obstet Gynecol Neonatal Nurs 34,444–452.

Balabanli B, Kamisaki Y, Martin E and Murad F (1999) Requirements for heme and thiols for the nonenzymatic modification of nitrotyrosine. Proc Natl Acad Sci USA 96,13136–13141.

Balafanova Z, Bolli R, Zhang J, Zheng Y, Pass JM, Bhatnagar A, Tang XL, Wang O, Cardwell E and Ping P (2002) Nitric oxide (NO) induces nitration of protein kinase Cepsilon (PKCepsilon), facilitating PKCepsilon translocation via enhanced PKCepsilon–RACK2 interactions: a novel mechanism of no-triggered activation of PKCepsilon. J Biol Chem 277,15021–15027.

Barrett JR (2002) Environmental links to early deliveries. Environ Health Perspect 110,A78–A79.

Barton CH, Ni Z and Vaziri ND (2001) Enhanced nitric oxide inactivation in aortic coarctation-induced hypertension. Kidney Int 60,1083–1087.

Bisseling TM, Wouterse AC, Steegers EA, Elving L, Russel FG and Smits P (2003) Nitric oxide-mediated vascular tone in the fetal placental circulation of patients with type 1 diabetes mellitus. Placenta 24,974–978.

Brancho D, Tanaka N, Jaeschke A, Ventura J-J, Kelkar N, Tanaka Y, Kyuuma M, Takeshita T, Flavell RA and Davis RJ (2003) Mechanism of p38 MAP kinase activation in vivo. Genes Dev 17,1969–1978.

Bruckdorfer KR (2001) The nitration of proteins in platelets. C R Acad Sci III 324,611–615.

Buttery LD, Springall DR, Chester AH, Evans TJ, Standfield EN, Parums DV, Yacoub MH and Polak JM (1996) Inducible nitric oxide synthase is present within human atherosclerotic lesions and promotes the formation and activity of peroxynitrite. Lab Invest 75,77–85.

Casanueva E and Viteri FE (2003) Iron and oxidative stress in pregnancy. J Nutr 133,1700S–1708S.

Chazotte-Aubert L, Hainaut P and Ohshima H (2000) Nitric oxide nitrates tyrosine residues of tumor-suppressor p53 protein in MCF-7 cells. Biochem Biophys Res Commun 267,609–613.

Choi ME (2000) Mechanism of transforming growth factor-beta1 signaling. Kidney Int Suppl 77,S53–S58.

Cobbs CS, Samanta M, Harkins LE, Gillespie GY, Merrick BA and MacMillan-Crow LA (2001) Evidence for peroxynitrite-mediated modifications to p53 in human gliomas: possible functional consequences. Arch Biochem Biophys 394,167–172.

Crider KS, Whitehead N and Buus RM (2005) Genetic variation associated with preterm birth: a HuGE review. Genet Med 7,593–604.

Duley L (2003) Pre-eclampsia and the hypertensive disorders of pregnancy. Br Med Bull 67,161–176.

Emanuelsson CS, Boros S, Hjernoe K, Boelens WC and Hojrup P (2005) Screening for transglutaminase-catalyzed modifications by peptide mass finger printing using multipoint recalibration on recognized peaks for high mass accuracy. J Biomol Tech 16,197–208.

Engel SA, Erichsen HC, Savitz DA, Thorp J, Chanock SJ and Olshan AF (2005) Risk of spontaneous preterm birth is associated with common proinflammatory cytokine polymorphisms. Epidemiology 16,469–477.

Goldman-Wohl D and Yagel S (2002) Regulation of trophoblast invasion: from normal implantation to pre-eclampsia. Mol Cell Endocrinol 187,233–238.

Gole MD, Souza JM, Choi I, Hertkorn C, Malcolm S, Foust RF 3rd, Finkel B, Lanken PN and Ischiropoulos H (2000) Plasma proteins modified by tyrosine nitration in acute respiratory distress syndrome. Am J Physiol Lung Cell Mol Physiol 278,L961–L967.

Gow AJ, Duran D, Malcolm S and Ischiropoulos H (1996) Effects of peroxynitrite-induced protein modifications on tyrosine phosphorylation and degradation. FEBS Lett 385,63–66.

Grune T, Blasig IE, Sitte N, Roloff B, Haseloff R and Davies KJ (1998) Peroxynitrite increases the degradation of aconitase and other cellular proteins by proteasome. J Biol Chem 273,10857–10862.

Hagemann C and Blank JL (2001) The ups and downs of MEK kinase interactions. Cell Signal 13,863–875.

He Z, Cho YY, Liu G, Ma WY, Bode AM and Dong Z (2003) p38 Mitogen-activated protein kinase regulation of JB6 Cl41 cell transformation promoted by epidermal growth factor. J Biol Chem 278,26435–26442.

Ichijo H (1999) From receptors to stress-activated MAP kinases. Oncogene 18,6087–6093.

Ischiropoulos H (1998) Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species. Arch Biochem Biophys 356,1–11.

Ischiropoulos H and al-Mehdi AB (1995) Peroxynitrite-mediated oxidative protein modifications. FEBS Lett 364,279–282.

Jauniaux E, Hempstock J, Greenwold N and Burton GJ (2003) Trophoblastic oxidative stress in relation to temporal and regional differences in maternal placental blood flow in normal and abnormal early pregnancies. Am J Pathol 162,115–125.

Jope RS, Zhang L and Song L (2000) Peroxynitrite modulates the activation of p38 and extracellular regulated kinases in PC12 cells. Arch Biochem Biophys 376,365–370.

Kiroycheva M, Ahmed F, Anthony GM, Szabo C, Southan GJ and Bank N (2000) Mitogen-activated protein kinase phosphorylation in kidneys of beta (s) sickle cell mice. J Am Soc Nephrol 11,1026–1032.

Kong S-K, Yim MB, Stadtman ER and Chock PB (1996) Peroxynitrite disables the tyrosine phosphorylation regulatory mechanism: lymphocyte-specific tyrosine kinase fails to phosphorylate nitrated cdc2(6–20) NH2 peptide. Proc Natl Acad Sci USA 93,3377–3382.

Kossenjans W, Eis A, Sahay R, Brockman D and Myatt L (2000) Role of peroxynitrite in altered fetal-placental vascular reactivity in diabetes or preeclampsia. Am J Physiol Heart Circ Physiol 278,H1311–H1319.

Lewis JS, Vijayanathan V, Thomas TJ, Pestell RG, Albanese C, Gallo MA and Thomas T (2005) Activation of cyclin D1 by estradiol and spermine in MCF-7 breast cancer cells: a mechanism involving the p38 MAP kinase and phosphorylation of ATF-2. Oncol Res 15,113–128.

Li HY, Chang SP, Yuan CC, Chao HT, Ng HT and Sung YJ (2001) Nitric oxide induces extensive apoptosis in endometrial epithelial cells in the presence of progesterone: involvement of mitogen-activated protein kinase pathways. Mol Hum Reprod 7,755–763.

Li HY, Chang SP, Yuan CC, Chao HT, Ng HT and Sung YJ (2003) Induction of p38 mitogen-activated protein kinase-mediated apoptosis is involved in outgrowth of trophoblast cells on endometrial epithelial cells in a model of human trophoblast–endometrial interactions. Biol Reprod 69,1515–1524.

Lyall F, Gibson JL, Greer IA, Brockman DE, Eis AL and Myatt L (1998) Increased nitrotyrosine in the diabetic placenta: evidence for oxidative stress. Diabetes Care 21,1753–1758.

MacMillan-Crow LA, Crow JP, Kerby JD, Beckman JS and Thompson JA (1996) Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts. Proc Natl Acad Sci USA 93,11853–11858.

Makino A, Skelton MM, Zou A-P, Roman RJ and Cowley AW Jr (2002) Increased renal medullary oxidative stress produces hypertension. Hypertension 39,667–672.

Mallozzi C, Di Stasi AM and Minetti M (2001) Nitrotyrosine mimics phosphotyrosine binding to the SH2 domain of the src family tyrosine kinase lyn. FEBS Lett 503,189–195.

McMaster MT, Zhou Y and Fisher SJ (2004) Abnormal placentation and the syndrome of preeclampsia. Semin Nephrol 24,540–547.

Miller MJ, Voelker CA, Olister S, Thompson JH, Zhang XJ, Rivera D, Eloby-Childress S, Liu X, Clark DA and Pierce MR (1996) Fetal growth retardation in rats may result from apoptosis: role of peroxynitrite. Free Radic Biol Med 21,619–629.

Minetti M, Mallozzi C and Di Stasi AM (2002) Peroxynitrite activates kinases of the src family and upregulates tyrosine phosphorylation signaling. Free Radic Biol Med 33,744–754.

Myatt L, Brewer A and Brockman DE (1991) The action of nitric oxide in the perfused human fetal-placental circulation. Am J Obstet Gynecol 164,687–692.

Myatt L, Rosenfield RB, Eis AL, Brockman DE, Greer I and Lyall F (1996) Nitrotyrosine residues in placenta. Evidence of peroxynitrite formation and action. Hypertension 28,488–493.

Myatt L, Eis AL, Brockman DE, Greer IA and Lyall F (1997) Endothelial nitric oxide synthase in placental villous tissue from normal, pre-eclamptic and intrauterine growth restricted pregnancies. Hum Reprod 12,167–172.

Myatt L, Kossenjans W, Sahay R, Eis A and Brockman D (2000) Oxidative stress causes vascular dysfunction in the placenta. J Matern Fetal Med 9,79–82.

Natale DR, Paliga AJ, Beier F, D'Souza SJ and Watson AJ (2004) p38 MAPK signaling during murine preimplantation development. Dev Biol 268,76–88.

Nicholson IC, Ayhan M, Hoogenraad NJ and Zola H (2005) In silico evaluation of two mass spectrometry-based approaches for the identification of novel human leukocyte cell-surface proteins. J Leukoc Biol 77,190–198.

Ono K and Han J (2000) The p38 signal transduction pathway: activation and function. Cell Signal 12,1–13.

Park SW, Huq MD, Hu X and Wei LN (2005) Tyrosine nitration on p65: a novel mechanism to rapidly inactivate nuclear factor-{kappa}B. Mol Cell Proteomics 4,300–309.

Peters TJ, Chapman BM, Wolfe MW and Soares MJ (2000) Placental lactogen-I gene activation in differentiating trophoblast cells: extrinsic and intrinsic regulation involving mitogen-activated protein kinase signaling pathways. J Endocrinol 165,443–456.

Radi R (2004) Nitric oxide, oxidants, and protein tyrosine nitration. Proc Natl Acad Sci USA 101,4003–4008.

Rane MJ, Coxon PY, Powell DW, Webster R, Klein JB, Pierce W, Ping P and McLeish KR (2001) p38 Kinase-dependent MAPKAPK-2 activation functions as 3-phosphoinositide-dependent kinase-2 for Akt in human neutrophils. J Biol Chem 276,3517–3523.

Roberts JM and Cooper DW (2001) Pathogenesis and genetics of pre-eclampsia. Lancet 357,53–56.

Roberts JM and Lain KY (2002) Recent Insights into the pathogenesis of pre-eclampsia. Placenta 23,359–372.

Roberts ES, Lin H, Crowley JR, Vuletich JL, Osawa Y and Hollenberg PF (1998) Peroxynitrite-mediated nitration of tyrosine and inactivation of the catalytic activity of cytochrome P450 2B1. Chem Res Toxicol 11,1067–1074.

Roberts CK, Vaziri ND, Wang XQ and Barnard RJ (2000) Enhanced NO inactivation and hypertension induced by a high-fat, refined-carbohydrate diet. Hypertension 36,423–429.

Salvemini D (1997) Regulation of cyclooxygenase enzymes by nitric oxide. Cell Mol Life Sci 53,576–582.

Samet JM, Graves LM, Quay J, Dailey LA, Devlin RB, Ghio AJ, Wu W, Bromberg PA and Reed W (1998) Activation of MAPKs in human bronchial epithelial cells exposed to metals. Am J Physiol 275,L551–L558.

Scherle PA, Ma W, Lim H, Dey SK and Trzaskos JM (2000) Regulation of cyclooxygenase-2 induction in the mouse uterus during decidualization. An event of early pregnancy. J Biol Chem 275,37086–37092.

Singh S, Powell DW, Rane MJ, Millard TH, Trent JO, Pierce WM, Klein JB, Machesky LM and McLeish KR (2003) Identification of the p16-Arc subunit of the Arp 2/3 complex as a substrate of MAPK-activated protein kinase 2 by proteomic analysis. J Biol Chem 278,36410–36417.

Stachowiak O, Dolder M, Wallimann T and Richter C (1998) Mitochondrial creatine kinase is a prime target of peroxynitrite-induced modification and inactivation. J Biol Chem 273,16694–16699.

Stanek J, Eis AL and Myatt L (2001) Nitrotyrosine immunostaining correlates with increased extracellular matrix: evidence of postplacental hypoxia. Placenta 22,S56–S62.

Szabados E, Fischer GM, Toth K, Csete B, Nemeti B, Trombitas K, Habon T, Endrei D and Sumegi B (1999) Role of reactive oxygen species and poly-ADP-ribose polymerase in the development of AZT-induced cardiomyopathy in rat. Free Radic Biol Med 26,309–317.

Taylor RP and Starnes JW (2003) Age, cell signalling and cardioprotection. Acta Physiol Scand 178,107–116.

Thongboonkerd V, Gozal E, Sachleben LR Jr, Arthur JM, Pierce WM, Cai J, Chao J, Bader M, Pesquero JB, Gozal D *et al.* (2002) Proteomic analysis reveals alterations in the renal kallikrein pathway during hypoxia-induced hypertension. J Biol Chem 277,34708–34716.

Vaziri ND, Wang XQ, Oveisi F and Rad B (2000) Induction of oxidative stress by glutathione depletion causes severe hypertension in normal rats. Hypertension 36,142–146.

Vaziri ND, Ni Z, Oveisi F, Liang K and Pandian R (2002) Enhanced nitric oxide inactivation and protein nitration by reactive oxygen species in renal insufficiency. Hypertension 39,135–141.

Viner RI, Huhmer AF, Bigelow DJ and Schoneich C (1996) The oxidative inactivation of sarcoplasmic reticulum Ca(2+)-ATPase by peroxynitrite. Free Radic Res 24,243–259.

Waite LL, Atwood AK and Taylor RN (2002) Preeclampsia, an implantation disorder. Rev Endocr Metab Disord 3,151–158.

Wang Y and Walsh SW (1998) Placental mitochondria as a source of oxidative stress in pre-eclampsia. Placenta 19,581–586.

Webster RP, Macha S, Brockman D and Myatt L (2006) Peroxynitrite treatment in vitro disables catalytic activity of recombinant p38 MAPK. Proteomics July 28 [epub ahead of print].

Yamakura F, Taka H, Fujimura T and Murayama K (1998) Inactivation of human manganese-superoxide dismutase by peroxynitrite is caused by exclusive nitration of tyrosine 34–3-nitrotyrosine. J Biol Chem 273,14085–14089.

Zhou XJ, Vaziri ND, Wang XQ, Silva FG and Laszik Z (2002) Nitric oxide synthase expression in hypertension induced by inhibition of glutathione synthase. J Pharmacol Exp Ther 300,762–767.

Zou M, Martin C and Ullrich V (1997) Tyrosine nitration as a mechanism of selective inactivation of prostacyclin synthase by peroxynitrite. Biol Chem 378,707–713.

Zou M, Yesilkaya A and Ullrich V (1999a) Peroxynitrite inactivates prostacyclin synthase by heme-thiolate-catalyzed tyrosine nitration. Drug Metab Rev 31,343–349.

Zou MH, Leist M and Ullrich V (1999b) Selective nitration of prostacyclin synthase and defective vasorelaxation in atherosclerotic bovine coronary arteries. Am J Pathol 154,1359–1365.

Submitted on May 5, 2006; resubmitted on July 12, 2006; accepted on July 16, 2006