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Excessive stimulation of poly(ADP-ribosyl)ation contributes to endothelial dysfunction in pre-eclampsia

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1 Pre-eclampsia is a serious pregnancy disorder associated with widespread activation of the maternal vascular endothelium. Recent evidence implicates a role for oxidative stress in the aetiology of this condition.

2 Reactive oxygen species, particularly superoxide anions, invokes endothelial cell activation through many pathways. Oxidant-induced cell injury triggers the activation of nuclear enzyme poly(ADP-ribose) polymerase (PARP) leading to endothelial dysfunction in various pathophysiological conditions (reperfusion, shock, diabetes).

3 We have studied whether the loss of endothelial function in pre-eclampsia is dependent on PARP activity. Endothelium-dependent responses of myometrial arteries were tested following exposure to either plasma from women with pre-eclampsia or normal pregnant women in the presence and absence of a novel potent inhibitor of PARP, PJ34. Additional effects of plasma and PJ34 inhibition were identified in microvascular endothelial cell cultures.

4 In myometrial arteries, PARP inhibition blocked the attenuation of endothelium-dependent responses following exposure to plasma from women with pre-eclampsia. In endothelial cell cultures, plasma from pre-eclamptics induced measurable oxidative stress and a concomitant increase in PARP activity and reduction in cellular ATP. Again, these biochemical changes were reversed by PJ34.

5 These results suggest that PARP activity plays a pathogenic role in the development of endothelial dysfunction in pre-eclampsia and promotes PARP inhibition as a potential therapy in this condition.
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Abbreviations: ADP, adenosine diphosphate; ANOVA, analysis of variance; ATP, adenosine triphosphate; BSA, bovine serum albumin; DCFH-DA, dichlorofluorescein diacetate; DMF, dimethylformamide; DNA, deoxy-ribo-nucleic acid; EDTA, ethylenediaminetetra-acetic acid; HNMR, proton nuclear magnetic resonance spectroscopy; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; IBR, individualised birth weight ratio; kDa, kilo-Daltons; LDH, lactate dehydrogenase; MS, mass spectrometry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; NAD+, nicotinamide adenine dinucleotide; NO, nitric oxide; PAGE, polyacrylamide gel electrophoresis; PARP, poly(ADP ribose) polymerase; PE, pre-eclampsia; PES, phenazine ethosulfate; PSS, physiological salt solution; ROS, reactive oxygen species; s.e.m., standard error of mean; TLC, thin-layer chromatography; α-MEM, alpha-modified minimum essential medium

Introduction

Poly(ADP-ribose) polymerase (PARP) is an abundant eukaryotic cell nuclear enzyme. In response to DNA breakage, PARP catalyses the covalent post-translation modification of nuclear proteins with poly(ADP-ribose) from nicotinamide adenine dinucleotide (NAD+) as a precursor (Virag & Szabo, 2002). Consequently, poly(ADP-ribosyl)ation plays a key role in a number of physiological cellular functions including participation in DNA base-excision repair, resistance to genotoxic stress, regulation of genomic stability and gene expression, regulation of transcription and proteasomal function and apoptosis. However, in contrast to these cytoprotective functions, overstimulation of PARP is associated with pathophysiological effects resulting from rapid depletion of the intracellular NAD+ and ATP pools; this slows the rate of glycolysis and mitochondrial respiration and leads to cellular dysfunction (Eliasson *et al*., 1997; Szabo *et al*., 1997; Zingarelli *et al*., 1998; Burkart *et al*., 1999; Oliver *et al*., 1999; Pieper *et al*., 1999a, b; Soriano *et al*., 2001). PARP activation is implicated in the pathogenesis of stroke (Eliasson *et al*., 1997), autoimmune β-cell destruction (Burkart *et al*., 1999; Pieper *et al*., 1999a), shock and inflammation (Szabo *et al*., 1997; Oliver *et al*., 1999) and diabetic vascular dysfunction (Soriano *et al*., 2001).

Pre-eclampsia is a multisystem disorder of pregnancy characterised by hypertension and proteinuria. It is a leading cause of maternal mortality and the World Health Organisation estimates that worldwide over 100,000 women die from pre-eclampsia each year. In the U.K., it is the most important cause of maternal death in recent decades (RCOG:

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Confidential Report, 1997–1999). Although the precise aetiology of pre-eclampsia is unknown, current thinking suggests it to be a two-step disease process. (Lain & Roberts, 2002; Roberts & Lain, 2002) In this model, inappropriate adaptation of the interface between the maternal vasculature and the developing placenta leads to inadequate perfusion of the fetoplacental unit (Lunell *et al*., 1984; Zhou *et al*., 1997). This stimulates the release of circulating factors that lead to maternal vascular dysfunction (Ashworth *et al*., 1998). Many studies have shown that the maternal vascular endothelium is the ultimate target of these factors and there is incontrovertible evidence that the normal protective role of this cell layer becomes compromised in pre-eclampsia (Roberts *et al*., 1989; McCarthy *et al*., 1993; Roberts & Redman, 1993; Friedman *et al*., 1995; Ashworth *et al*., 1997).

Undoubtedly, the ubiquitous nature and diverse functions of the vascular endothelium account for the complex multisystemic nature of this disease. In recent years, free radicals have emerged as likely promoters of maternal endothelial dysfunction. In pre-eclampsia, a rise in plasma markers of lipid peroxidation, including malondialdehyde (Hubel *et al*., 1996) and 8-epiprostaglandin-F2α (Barden *et al*., 1996), and a reduction in water- and lipid-soluble antioxidants in plasma (Mikhail *et al*., 1994) and placenta (Wang & Walsh, 1996) strongly suggest a state of oxidative stress. Reactive oxygen species (ROS), particularly superoxide anions, are a potential source of excessive oxidation. Superoxide anions can invoke endothelial cell activation and dysfunction through many pathways (Davidge, 1998). One possibility is that nitric oxide (NO), in combination with superoxide anions, yields peroxynitrite, a powerful oxidant, that disrupts the maternal endothelium (Beckman & Koppenol, 1996). In pre-eclampsia, imbalances in this system and overproduction of peroxynitrite have been established through immunolocalisation of nitrotyrosine to maternal subcutaneous microvessels (Roggensack *et al*., 1999).

In previous studies, we have demonstrated an attenuation in the endothelium-dependent relaxation of subcutaneous and myometrial vessels isolated from women with pre-eclampsia (Ashworth *et al*., 1997; Kenny *et al*., 2002). We have also shown that we can mimic this response through incubation of normal vessels with low levels of plasma from pre-eclamptic donors (Ashworth *et al*., 1998; Hayman *et al*., 2000). Among other changes, an increase in eNOS expression has been established in endothelial cell cultures following exposure to plasma from pre-eclamptic women (Davidge *et al*., 1995). From these observations, we hypothesise that a potential pathway to endothelial dysfunction is the overstimulation of PARP in response to peroxynitrite formation. Here, we investigated whether endothelium activation in pre-eclampsia is dependent on PARP activity.

Methods

This investigation conformed to the principles outlined in the World Medical Association Declaration of Helsinki, 2001. Ethical permission was obtained from the Hospital Ethics Committee and all participants gave their informed written consent.

Subjects

We obtained plasma samples from 12 women with pre-eclampsia. Pre-eclampsia was defined as a blood pressure of at least 140/90 mmHg on more than two occasions, in the presence of at least 0.5 mg of protein in a 24 h collection of urine arising after 20 weeks gestation. There was no pre-existing renal disease, and in all patients hypertension had resolved by the sixth week postpartum. Women with multiple pregnancies, or diabetes and those with chronic hypertension were excluded. Blood was taken from these patients at the time of diagnosis and before the commencement of any pharmacological treatment. Blood was also collected from 12 normal pregnant women. These women were normotensive and were individually matched for age, ethnicity, parity and gestation. In addition, we obtained myometrial arteries from biopsies taken from normal pregnant women, with uncomplicated pregnancies, undergoing elective Caesarean section at term for reasons such as breech presentation.

Plasma preparation

Blood was collected into precooled ethylenediaminetetra-acetic acid (EDTA) vacutainers. The vacutainers were immediately transported on ice to the laboratory and centrifuged at 500 g for 7 min at 4°C. The plasma was removed and stored in 1 ml aliquots at −80°C until experimental use. Equal volumes of plasma from the women with pregnancies complicated by pre-eclampsia were mixed to produce pooled plasma. A separate 'pool' was prepared in the same way from the matched normal pregnant controls.

Synthesis and pharmacological characterisation of PJ34

PJ34 was generated using an established method (Li *et al*., 2001). A suspension of 2-nitro-6(5H)-phenanthridinone in N,N-dimethylformamide (DMF) was reduced by iron and ammonium chloride to produce 2-amino-6(5H)-phenanthridinone. Chloroacylation of 2-amino-6(5H)-phenanthridinone with chloroacetyl chloride in pyridine/DMF yielded *N*-(6-oxo-5,6-dihyro-phenanthridin-2-yl)-chloroacetamide, which was then treated with dimethylamine in methanol or *N*,*N*-dimethylformamide to furnish *N*-(6-oxo-5,6-dihydro-phenanthridin-2-yl)-*N*,*N*-dimethylacetamide. The latter compound was then treated with HCl in ether and methanol to produce PJ34, *N*-(6-oxo-5,6-dihydro-phenanthridin-2-yl)-*N*,*N*-dimethylacetamide HCl. Product identity and purity were assessed using HNMR, MS, TLC and HPLC.

Myography

Myometrial biopsies were obtained at the time of delivery by Caesarean section from the upper lip of the lower segment incision from normal pregnant women ($n=6$). Biopsies were placed directly into ice-cold physiological salt solution (PSS) (see below for chemical composition). Myometrial small arteries (200–500 μm) were identified under a stereomicroscope, carefully dissected free from the surrounding connective tissue and cut into small lengths approximately 3 mm. Following dissection, myometrial vessels were incubated overnight for 18 h in PSS, 2% final concentration of plasma and 1 U μl^{-1} heparin at 4°C. Arteries were incubated with 2%

plasma from women with pre-eclampsia in the absence ($n=6$) and presence ($n=6$) of PJ34 (3 μM), the water-soluble phenanthridinone derivative of PARP, or from normal pregnant women, matched for gestation, age and parity, in the absence ($n=6$) and presence ($n=6$) of PJ34 (3 μM). Vessels were then mounted onto 40 μm steel wires in a wire myography (Danish Myotechnology, Aarhus, Denmark) containing 7 ml of PSS. Vessel lengths were measured using a calibrated eyepiece micrometer. The baths were warmed to 37°C and gassed with 95%O_2–5%CO_2. Small arteries were normalised to an internal circumference calculated at 0.9 of L_{100} (the calculated circumference of the vessel at a passive transmural pressure of 100 mmHg) for the remainder of the experiment. A sustained contraction was obtained by the addition of arginine vasopressin (10 nM) followed by the addition of incremental doses of the endothelium-dependent vasodilator, bradykinin (10 pM–1 μM). Vessels were then subjected to two washes with PSS until they returned to their basal tension and the protocol was then repeated.

Drugs and solutions

Myography All drugs were made fresh from stock solutions on the day of the experiment and dissolved in PSS. The composition of PSS was as follows: 1.6 mM $CaCl_2 \cdot H_2O$, 119 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.18 mM KH_2PO_4, 1.17 mM $MgSO_4 \cdot 7H_2O$, 0.023 mM Na_2EDTA and 5.5 mM glucose (pH 7.4 when gassed with 5% CO_2–95%O_2). Unless otherwise stated, all reagents were purchased from Sigma Chemical Co., Poole, U.K.

Endothelial cell culture

Bovine microvascular endothelial cells (B88s) were cultured in a humidified 5%CO_2 incubator at 37°C in alpha-modified minimum essential medium (α-MEM; GIBCO, Paisley, U.K.) containing 10% horse serum, 2 mM L-glutamine, nystatin (20 U ml^{-1}), kanamycin (20 $\mu g\,ml^{-1}$) and gentamicin (5 $\mu g\,ml^{-1}$). Experiments were typically performed in 96-well, white-walled, clear-bottomed tissue culture-treated plates (Corning Costar, High Wycombe, U.K.) using cells no greater than passage 6, seeded at a concentration of 50,000 cells $well^{-1}$ in 200 μl of media. Pooled plasma from women with pre-eclampsia and from control subjects, containing heparin at a final concentration of 1 U μl^{-1}, were added to the endothelial cells at a concentration of 2% in the absence and presence of PJ34 (3 μM). Unless otherwise stated, cells were cultured for a further 24 h before analysis.

Assay of cellular ATP

Direct measurements of ATP were made using the bioluminescence Vialight Kit (Biowhittaker Ltd, Wokingham, U.K.). For these experiments, cells were cultured in clear-bottomed culture plates (Corning Costar) and the bases covered before readings taken. Following two washes with PSS, nucleotides were released from the cultured B88s by the addition of 100 μl somatic cell nucleotide-releasing reagent. After 5 min at room temperature, 20 μl luciferin-luciferase nucleotide-monitoring reagent was added and the plate loaded into a Microlumat LB96P luminometer (EG & G Berthold, Vienna, Austria). Exactly 2 min later, ATP levels were measured using the Microwin Data Package (Microtek, Overath, Germany) and the results expressed as relative light units.

Assessment of PARP activity

PARP activation was assessed by inhibition, protein expression and the concomitant depletion of intracellular NAD+. In this case, NAD+ levels were measured by an enzymatic cycling assay (Carl & Marcella, 1973). Following plasma and inhibitor incubations, B88 cells were harvested by centrifugation and extracted with 0.4 M perchloric acid. After neutralisation with 2.5 M $KHCO_3$, cell debris was removed by centrifugation. An aliquot of the supernatant (10 ml) was added to 1 ml of reaction mixture (2 mM PES, 0.5 mM MTT, 600 mM ethanol, 200 mg of alcohol dehydrogenase in 120 mM Na/Bicine buffer, pH 7.8), and then incubated for 30 min at 37°C. The reaction was stopped by the addition of 1 ml of 12 mM iodoacetate. The absorbance was measured at 570 nm and the results corrected for protein and transformed using molar absorptivity calculations.

PARP expression was established by standard SDS–polyacrylamide gel electrophoresis (PAGE) procedures. Cell lysates were prepared using boiling lysis buffer (1% SDS, 1.0 mM sodium orthovanadate, 10 mM Tris (pH 7.4)). Separated proteins were transferred to nitrocellulose membrane and stained with Ponceau (0.1% (v v^{-1})) to verify equal loading and transfer. These were incubated with antibodies to PARP (1 : 1000 dilution, Oncogene Research Products, San Diego, U.S.A.). The primary antibody was detected by horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (Calbiochem-Novabiochem U.K. Ltd, Beeston, U.K.) and identified by enhanced chemiluminescence (Amersham Pharmacia Biotech, Little Chalfont, U.K.). Commercially obtained staurosporine-exposed Jurkat cells (BD Biosciences, Cowley, U.K.) were used to demonstrate PARP cleavage.

Oxidative stress detection

2′,7′-Dichlorofluorescein diacetate (DCFH-DA) was used as an indicator of intracellular ROS. B88 cells were loaded with the fluorescent substrate DCFH-DA for 15 min at 37°C, and then incubated for 4 h with either 2% pre-eclampsia plasma or control plasma in the presence or absence of PJ34 (3 μM). DCFH-DA is cleaved intracellularly to DCFH, which in turn is oxidised by reactive oxygen metabolites to fluorescent 2′,7′-dichlorofluorescein (DCF). The B88 cells were dispersed by trypsinisation and their fluorescence intensity determined by an EPICS Elite Flow Cytometer (Beckman-Coulter, High Wycombe, U.K.). Intracellular reactive oxygen was expressed by the percentage of positive cells and mean fluorescence units. Xanthine-derived superoxide (1 mM xanthine/100 mM xanthine oxidase) and angiotensin II (10 μM) were used as positive controls.

Nitrotyrosine recognition

The immune recognition of nitrotyrosine residues in whole-cell lysates was achieved by PAGE and Western blotting. Experimental cell numbers were increased to obtain enough protein for gel loading. Typically, 1×10^6 cells were used per well. Following exposure to plasma, adherent cells were

lysed using 2× concentrated boiling electrophoresis sample buffer (125 mM Tris (pH 6.8), 4% SDS, 10% glycerol, 0.006% bromophenol blue, 1.8% β-mercaptoethanol). Cells were scraped from the culture flask and centrifuged to remove insoluble debris. Before loading, whole-cell lysates were boiled for a further 3 min. For positive controls, bovine serum albumin (BSA) (1 mg ml^{-1}) was treated with peroxynitrite (24 mM, 5 min) in accordance with the manufacture's instructions (Upstate Ltd, Milton Keynes, U.K.). PAGE and Western blotting were conducted as described for PARP as above. Nitrosylated proteins were recognised using a mouse monoclonal anti-nitrotyrosine antibody (Abcam Ltd, Cambridge, U.K.). Equal loading and transfer were verified with amino blue–black stain (0.1% (w v^{-1})). Under these conditions, anti-α-actin recognition proved inadequate.

Assessment of cell viability

Trypan blue exclusion of adherent cells and lactate dehydrogenase (LDH) release into the culture media were used to establish B88 culture viability. LDH was measured using a colorimetric assay for the catalysed reduction of 1.6 mM pyruvate in Tris-buffer (pH 7.5). LDH activity was determined from the conversion rate of 0.2 mM NADH to NAD+ at 37°C and was measured by the change in absorbance at 340 nm over a 3-min period. For trypan blue exclusion, nonviable cells in five random microscopic fields of view were expressed as a percentage of total cell number. LDH results are given as enzymatic units per litre (U l^{-1}).

Statistical analysis

All data were analysed using the SPSS for Windows statistical package (version 10.1, SPSS Inc., SPSS UK Ltd, Woking, U.K.). Demographic and clinical data from the pre-eclampsia and control groups were compared using the Mann-Whitney *U*-test. The effect of PJ34 on the plasma-induced alteration of responses to bradykinin in myometrial vessels was compared using repeated measures ANOVA. Data generated from the cell culture experiments were first tested for normal distribution using the Shapiro–Wilks' test. Differences were detected using a paired *t*-test and were corrected where necessary for multiple comparisons. Unless otherwise stated, all data are expressed as means ± s.e.m. In all cases, significance was determined at $P<0.05$.

Results

Patient demographics

Patient details for the two groups are detailed in Table 1. Body mass index was calculated from weight taken at the first trimester booking visit and was not significantly different in the normal pregnant group compared with pre-eclamptics. There was no significant difference in age between groups. The pre-eclampsia patients had significantly raised mean arterial pressures, which were taken as the maximum recorded value in the 24 h immediately prior to blood sampling, and a significantly shorter gestation period. Individualised birth weight ratios (IBRs) were calculated for each pregnancy. The IBR centile is a corrected birth weight for gestational age centile, which is dependent on maternal ethnicity, height, weight, parity and fetal sex (Gardosi, 1998). Compared to normal pregnancy, the pre-eclampsia group had babies of significantly lower birth weight but not IBR.

Wire myography

Responses to bradykinin are represented as a mean of the two relaxation curves. There were no significant differences in

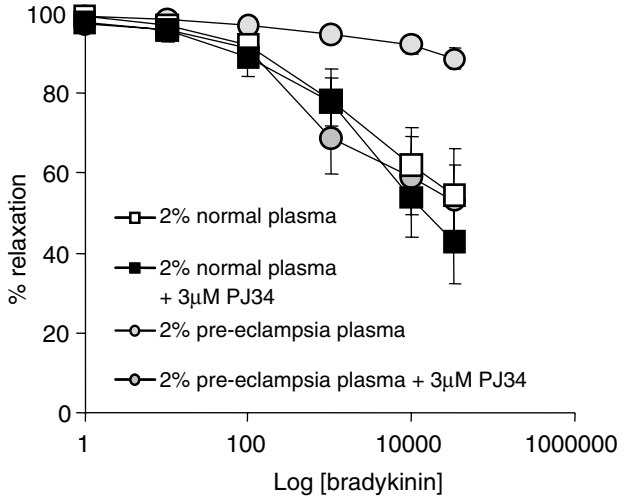

Figure 1 Effect of the PARP inhibitor, PJ34, on endothelium-dependent responses to bradykinin, following the prior incubation of myometrial vessels with plasma from women with pre-eclampsia and from normal pregnancy. The experiment was repeated on six separate occasions. Data represented as means ± s.e.m. (*$P<0.001$, repeated measures ANOVA).

Table 1 Patient demographic details from which plasma pools were created

	Normotensive (n = 12)	*Pre-eclampsia* (n = 12)
Maternal age (years)	28 ± 2 (17–35)	27 ± 2 (18–45)
Parity	1 ± 0 (0–3)	1 ± 0 (0–3)
Maximum systolic blood pressure (mmHg)**	118 ± 3 (100–132)	153 ± 4 (140–178)
Maximum diastolic blood pressure (mmHg)**	73 ± 3 (60–88)	101 ± 2 (90–114)
Body mass index (kg m^{-2})	25 ± 2 (20–38)	30 ± 3 (21–64)
Gestation at delivery (days)*	281 ± 2 (266–291)	250 ± 8 (196–289)
Fetal birth weight**	3527 ± 175 (2380–4380)	2444 ± 234 (730–3510)
Individualised birth ratio (centile)	54 ± 9 (11–100)	38 ± 9 (0–100)

Data represented as means ± s.e.m., ranges are within parentheses (*$P<0.005$, **$P<0.0001$).

responses to bradykinin in vessels incubated with heparin alone (data not shown) and in vessels incubated with plasma from normal pregnant women (Figure 1), either with or without the addition of PJ34 (R_{max} control plasma ($n=6$), $45.4\pm11.4\%$ vs R_{max} control plasma with PJ34 ($n=6$), $57.2\pm10.8\%$). The incubation of vessels with plasma from women with pre-eclampsia significantly attenuated the response to bradykinin, as compared to plasma from normal pregnant equivalents (Figure 1: R_{max} control plasma ($n=6$), $45.4\pm11.4\%$ vs R_{max} pre-eclampsia plasma ($n=6$), $11.4\pm3.9\%$). The responses to bradykinin of vessels incubated in the combined presence of plasma from women with pre-eclampsia and PJ34 produced responses similar to those incubated with plasma from normal women (R_{max} pre-eclampsia plasma ($n=6$), $11.4\pm3.9\%$ vs R_{max} pre-eclampsia plasma with PJ34 ($n=6$), $53.3\pm8.9\%$) (Figure 1).

Endothelial cell ATP

B88 cells were incubated with 2% pooled plasma from patients with pre-eclampsia or normal pregnant control plasma in the presence or absence of PJ34 (3 μM). There was a significant decrease in the level of luminescence in the presence of pooled plasma from women with pre-eclampsia as compared to the observed levels in the presence of plasma from normal pregnant women (Figure 2). The additional presence of PJ34 was associated with a significant elevation in ATP levels in cells incubated with pooled plasma from the pre-eclamptic donors (Figure 2).

Endothelial cell viability

The marked reduction in cellular ATP, following incubation in pooled plasma from pre-eclamptic women, could not be explained by a direct increase in cell death, as LDH released to the media was unchanged (Figure 3). Similarly, trypan blue exclusion was unaffected ($99.5\pm0.04\%$, PE plasma vs $99.8\pm0.03\%$, normal pregnancy plasma). PJ34 in turn had no direct impact on cell viability ($99.8\pm0.10\%$, trypan blue exclusion $n=3$) and 2% serum from both groups had no discernable increase in LDH activity (data not shown).

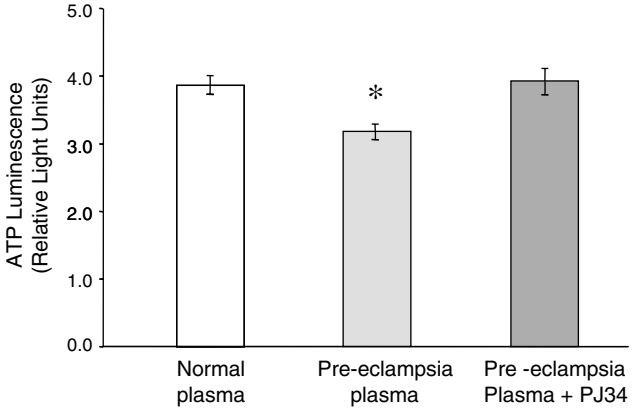

Figure 2 Effect of 2% plasma from women with pre-eclampsia and from normal pregnant women on the ATP levels of cultured B88 cells. Pre-eclampsia plasma induced a significant reduction in ATP as measured by lucigenin-enhanced chemiluminescence (*$P<0.05$). The PARP inhibitor PJ34 (3 μM) reversed this response. Results are depicted as means$\pm$s.e.m. for five separate experiments.

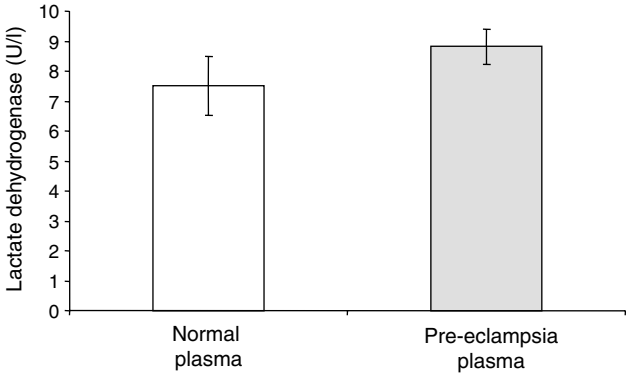

Figure 3 Effect of 2% plasma from pre-eclamptic and normal pregnant women on LDH liberation from B88 cells cultured over 24 h. Pre-eclampsia-derived plasma had no significant effect on cell viability. Results are depicted as means$\pm$s.e.m. for six separate experiments.

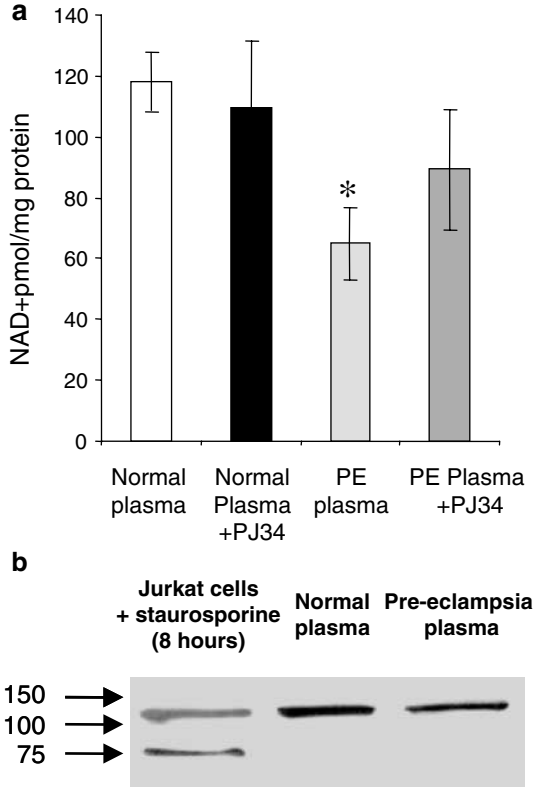

Figure 4 (a) PARP activity was assessed by NAD+ levels through the enzymatic conversion of ethanol to acetaldehyde. Cellular NAD+ was significantly reduced in B88 cells incubated in 2% plasma from women with pre-eclampsia (PE) compared to normal pregnant controls (*$P<0.01$). PJ34 partially restoring NAD+ levels in the pre-eclamptic group and the significant effect of pre-eclamptic plasma was lost. Results are depicted as means$\pm$s.e.m. for six separate experiments. (b) PARP protein expression of B88 cells incubated in 2% plasma from both study groups. Jurkat cells treated with staurosporine were used to demonstrate PARP cleavage.

PARP activity

NAD+, measured in B88 cells, was significantly reduced following incubation in 2% pooled plasma from the pre-eclampsia group compared with normal pregnancy controls (Figure 4a). This reduction was partially reversed by coin-

cubation with PJ34, which returned levels in the pre-eclamptic group to those comparable to normal plasma. The protein expression of PARP was not significantly altered by plasma incubations (Figure 4b) or by the presence of the PARP inhibitor (data not shown). Within these studies, the lack of PARP cleavage suggests that neither 2% normal pregnancy nor 2% pre-eclampsia plasma actively induced endothelial cell apoptosis.

Oxidative stress induction

Oxygen free radical metabolites were detected in B88 cells stimulated with xanthine/xanthine oxidase and angiotensin II (Figure 5a). The incubation of B88s in 2% pre-eclampsia plasma showed a significant increase in DCF-derived fluorescence over normal pregnancy equivalents, suggesting that oxidative stress was initiated by plasma from pre-eclamptic donors (Figure 5b). Notably, these elevations never reached the levels of the controlled stimulations. Currently, the nature of free radicals recognised in the DCFH-DA assay is unclear. Following exposure of cells to plasma from both pre-eclamptic and normal pregnant donors, the activation of peroxynitrite

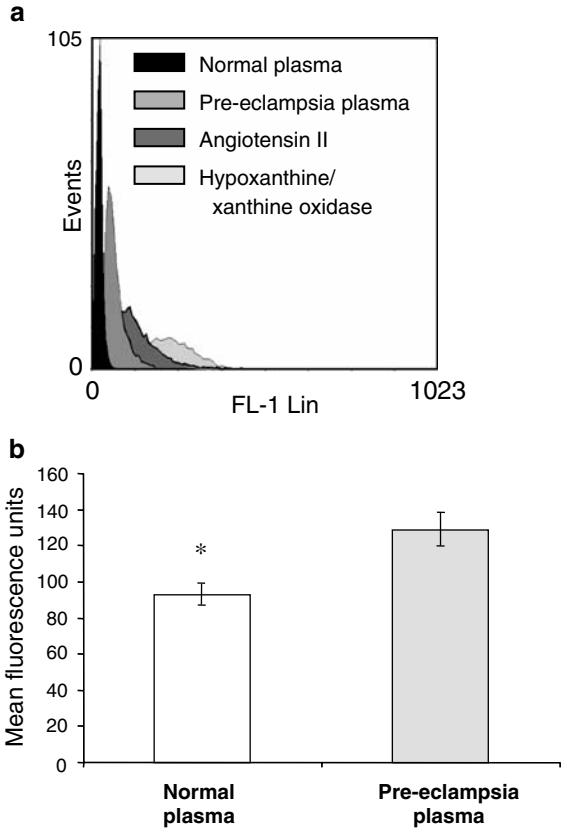

Figure 5 Effect of 2% plasma from pre-eclampsia patients and normal pregnant women on the induction of reactive oxygen metabolites in B88 cells, as measured by DCFH-DA. (a) is an overlaid histogram showing a typical increase in positive cells and mean fluorescence units following either treatment of B88 cells with angiotensin II, hypoxathine/xanthine oxidase or 2% plasma from pre-eclamptic donors. (b) shows the significant increase in fluorescence intensity of B88 cells incubated in 2% plasma from pre-eclamptic pregnancies ($*P<0.05$). Data represented as means$\pm$s.e.m. for 12 separate experiments.

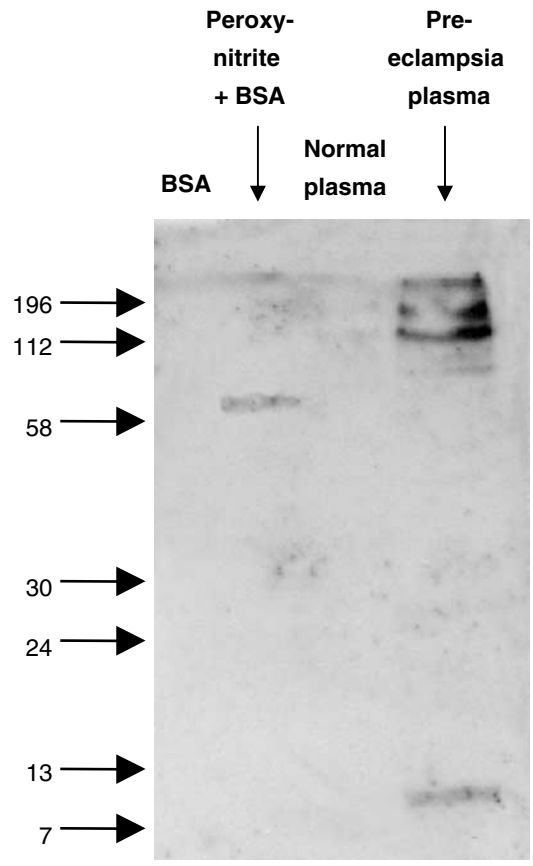

Figure 6 A representative immunoblot for nitrosylated proteins in B88 cell lysates following 24 h exposure to either 2% plasma from pre-eclamptic donors or 2% normal pregnancy controls. The positive nitrosylation of protein tyrosine residues was generated by peroxynitrite treatment of BSA. Whole-cell lysates from 1×10^6 cells were loaded per well. The experiment was repeated on four separate occasions and positive nitrosylation was observed solely in cells treated with pre-eclamptic plasma.

was confirmed in whole-cell lysates using a monoclonal anti-nitrotyrosine antibody (Figure 6). Only few protein bands were evident, but a restricted number of high molecular weight proteins (above 80 kDa) and a single low molecular weight band at 10 kDa showed consistent nitrosylation in the B88 cells treated with 2% plasma from pre-eclamptic women ($n=4$ repeats). The proteins of cells exposed to 2% normal pregnancy plasma never possessed the same degree of nitrosylation (Figure 6).

Discussion

There is overwhelming evidence linking pre-eclampsia with the development of endothelial dysfunction (Roberts *et al*., 1989; McCarthy *et al*., 1993; Roberts & Redman, 1993; Friedman *et al*., 1995; Ashworth *et al*., 1997; Kenny *et al*., 2002). However, the cellular mechanisms involved have yet to be established. Many groups have shown that plasma from women with pre-eclampsia has the capacity to alter endothelial cells *in vitro* (Baker *et al*., 1995; Scalera *et al*., 2001; Zhang *et al*., 2003). In this study, we have confirmed this finding, but unlike previous investigations, we have also defined a mechanism to explain these plasma-induced anomalies.

Previous investigations have demonstrated attenuated endothelium-dependent relaxation in subcutaneous and myometrial vessels isolated from women with pre-eclampsia (McCarthy *et al*., 1993; Knock & Poston, 1996; Ashworth *et al*., 1997; Vedernikov *et al*., 2001; Kenny *et al*., 2002). This attenuation has been mimicked in normal vessels by incubation with low levels of plasma from pre-eclamptic subjects (Ashworth *et al*., 1998; Hayman *et al*., 2000). Again we have reaffirmed this concept, but in these experiments we have reversed these detrimental effects by blocking the activity of PARP, an enzyme strongly linked with endothelial dysfunction in diabetes mellitus and other vascular conditions (Eliasson *et al*., 1997; Zingarelli *et al*., 1998; Soriano *et al*., 2001).

In addition to regulating genomic stability and transcription and proteasomal function, the most widely recognised role of PARP is in DNA-base excision repair and DNA-damage signalling. In endothelial cells treated with high glucose, as well as in diabetes and hyperglycaemia, a sequence of events has been proposed whereby reactive nitrogen and oxygen species trigger DNA single-strand breaks, which in turn induce the rapid and excessive activation of PARP, an event which quickly depletes the intracellular stores of NAD+ (Soriano *et al*., 2001; Virag & Szabo, 2002). This depletion slows glycolysis, electron transport and ATP formation, and finally initiates acute endothelial dysfunction and vascular instability (Eliasson *et al*., 1997; Szabo *et al*., 1997; Burkart *et al*., 1999; Pieper *et al*., 1999a; 2000).

In these experiments, we have demonstrated that plasma from women with pre-eclampsia induces oxidative stress and peroxynitrite damage in cultured endothelial cells and have provided evidence to suggest that PARP activation, in response to this insult, may suppress NAD+ and ATP levels to an extent where deregulation of the maternal vasculature is inevitable. We therefore propose strong parallels in pre-eclampsia with the endothelial injury and vascular dysfunction of diabetes.

Currently, the evidence of oxidative stress in the placenta and plasma in pre-eclampsia is substantial, with lipid peroxidation markers increased and water-soluble and lipid-soluble antioxidants reduced (Hubel, 1999). Although the origins of ROS in this condition are unknown, potential sources are activated neutrophils and endothelial mitochondria (Lee *et al*., 2003; Yorek, 2003). Superoxide anions and its metabolites, hydrogen peroxide and peroxynitrite, are all endogenous inducers of DNA single-strand breakages, the obligatory trigger of PARP activity (Pieper *et al*., 2000). To observe oxidant generation *in vitro*, we have used DCFH-DA, a general indicator of intracellular peroxide, and a nonspecific marker of oxidative stress. Using this technique, we were able to demonstrate reactive oxygen generation in endothelial cells exposed to plasma from pre-eclamptic donors. In long-term cultures, we also showed more specific evidence of peroxynitrite formation, a highly reactive oxidant. These *in vitro* observations concur with the previous pattern of nitrotyrosine found in the maternal vascular endothelium of pre-eclamptic women *in vivo* (Roggensack *et al*., 1999). They also support a more recent study by Scalera *et al*. (2001), who showed that serum from healthy pregnant women actively suppresses oxidative stress in human umbilical vein endothelial cells, and that this mechanism was altered in pre-eclampsia.

Endothelial dysfunction in the maternal vasculature in pre-eclampsia has been repeatedly reaffirmed by an marked elevation in soluble adhesion molecules such as VCAM, ICAM and E-selectin, and an increase in circulating von Willebrand factor, endothelin-1 and cellular fibronectin (Roberts *et al*., 1991; Coata *et al*., 2002). Although an early study highlighted a cytotoxic effect of serum from pre-eclamptic donors, more recent evidence supports an outcome of endothelial activation as opposed to premature decline (Roberts *et al*., 1992). Our own data suggest that the active plasma factor in pre-eclampsia does not induce apoptosis or necrosis within endothelial cells, at least under the culture period specified, but instead attenuates endothelial-dependent vasoreactivity, through a PARP-dependent pathway.

Further evidence points to an increase in microvascular permeability in pre-eclamptic complications. We know that the permeability of endothelial monolayers can be perturbed by serum from women with pre-eclampsia, and that this loss of barrier efficiency is associated with lipid peroxides and interleukin-8 (Zhang *et al*., 2003). These factors, in addition to plasma tumour necrosis factor alpha (Conrad & Benyo, 1997; Anim-Nyame *et al*., 2003), are elevated in pre-eclampsia and, as such, could be an alternative stimulus for the PARP activation observed. A direct involvement of PARP in the regulation and expression of adhesion molecules in endothelial cells has also been defined (Zingarelli *et al*., 1998). Consequently, PARP could have an additional role in neutrophil–endothelial interactions, promoting systemic inflammation, neutrophil respiratory burst and progression towards a hypercoagulatory state.

Irrespective of the causative factor of oxidative stress in pre-eclampsia, we propose that PARP activation plays an important role in maternal endothelial disruption. In this limited study, PJ34 was shown to partially reverse endothelial parameters following induction. However, in diabetic models, PJ34 has proved considerably more successful. Currently, a series of novel PARP inhibitors are in various stages of preclinical development (Soriano *et al*., 2001; Southan & Szabo, 2003).

Although the circulating factor in pre-eclampsia remains ill-defined, knowledge of its influence on the maternal endothelium may lead to important therapeutic breakthroughs. Here, we provide evidence linking PARP activity to reactive oxygen damage in the endothelium. From this study, we would tentatively promote PARP inhibition as a suitable strategy for reinstating normal vascular behaviour in pre-eclampsia.

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