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Title: Rho A/ Rho kinase: Human Umbilical Artery mRNA Expression in Normal and Pre-Eclamptic Pregnancies and Functional Role in Isoprostane Induced Vasoconstriction.

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Running Title: Rho A/ Rho kinase Expression and Isoprostanes

Abstract

Pre-eclampsia represents a state of increased or prolonged vasoconstriction, partially linked to the potent vasocontractile effect of isoprostanes. The process of Rho A–mediated calcium sensitization is inherent to a state of prolonged contractility in many smooth muscle types. The aims of this study were 1), to investigate mRNA expression levels of Rho A and Rho kinase isoforms (I and II) in umbilical artery from normotensive and pre-eclamptic women, and 2), to determine whether the effects of two isoprostanes, 8-*iso* prostaglandin $F_{2\alpha}$ (8-*iso* $PGF_{2\alpha}$) and 8-*iso* prostaglandin E_2 (8-*iso* PGE_2), on umbilical artery tone, were mediated via the Rho kinase pathway. Real-time RT-PCR using primers for Rho A, ROCK I and ROCK II was performed on total RNA isolated from umbilical artery specimens obtained from normotensive and pre-eclamptic women. The effects of both isoprostanes (n=6) (in the absence and presence of the specific Rho kinase inhibitor Y-27632), on umbilical artery tone were measured, and compared with control recordings. Rho A mRNA expression levels were significantly lower in umbilical artery samples obtained from pre-eclamptic women (n=4) in comparison to those from normotensive women (n=6) ($P<0.05$). ROCK I and ROCK II mRNA levels were similar in both vessel types ($P>0.05$). Both isoprostanes exerted a significant concentration dependent vasocontractile effect (n=7)($P<0.001$) on umbilical artery. For 8-*iso* PGE_2 this effect was antagonised by Y-27632 (n=6) ($P<0.01$). The significant reduction of Rho A mRNA levels in umbilical arteries from pregnancies complicated by pre-eclampsia may serve to counteract the diminished perfusion associated with the pathophysiology of pre-eclampsia. The vasocontractile effect of 8-*iso* PGE_2 in pre eclampsia may in part be mediated via the Rho kinase pathway.

Introduction

Pre-eclampsia, is a hypertensive disorder affecting 3-5% of all pregnancies and is a leading cause of maternal and fetal morbidity and mortality (Walker 2000). It is associated with fetal growth restriction, premature birth and low birth weight babies (Walker 2000; Byrne & Morrison 2001). Pre-eclampsia is characterized by intense and prolonged vasospasm. This ultimately leads to elevated systemic vascular resistance and the clinical manifestation of maternal hypertension, which may result in decreased perfusion to organs including the kidney, uterus, placenta, liver and brain (Roberts & Cooper 2001). Central to this condition are mechanisms that regulate vascular smooth muscle contractility, namely signalling pathways that regulate vasoconstriction in the systemic circulation.

Research has indicated that the process of calcium sensitization (increase in smooth muscle tension and/or phosphorylation of myosin light chains at a constant $[Ca^{2+}]_i$ by inhibition of myosin light chain phosphatase (MLCP)), is of major importance in regulating the state of vasoconstriction of vascular smooth muscle (Somylo & Somylo 2000). It is now apparent that the small G protein, Rho A is associated with inhibition of MLCP (Uehata *et al*. 1997; Kunihiko *et al*. 1999). Although the precise mechanism of action is unknown, two target proteins of Rho A, ROCK I and its isoform ROCK II, which are collectively known as Rho kinases, have a major role in Rho A-mediated calcium sensitization. Upon activation they enhance Rho-mediated calcium sensitization and hence smooth muscle contractility. It is now clear that this Rho kinase pathway plays a central role in the pathogenesis of hypertension in animal models, in humans and in various situations of increased peripheral vascular resistance observed in hypertensive disorders (Chitaley *et al*. 2001) and the prolonged

84 enhanced arterial vasoconstriction in heart failure (Hisaoka *et al*., 2000). There is no
85 information pertaining to the role of the Rho pathway in feto-placental vasculature
86 during normal pregnancy or in pregnancies complicated by pre-eclampsia. The feto-
87 placental unit is apparently not innervated (Fox & Khong 1990) and hence the
88 regulation of blood flow to the placenta must depend on structural changes, the
89 influence of vasoactive factors and local signalling mechanisms.
90
91 It is known that isoprostanes, metabolites of arachidonic acid, are closely linked to the
92 severe vasoconstriction associated with pre-eclampsia (Walsh *et al*. 2000) and can
93 exert their action in part via the Rho kinase pathway (Janssen *et al*. 2001).
94 Isoprostanes are implicated in the pathogenesis of a wide variety of human disorders
95 and are used extensively as markers of oxidative stress (Roberts & Morrow 2000),
96 with markedly increased levels reported in disorders associated with increased
97 vascular constriction such as in angina (Cipollone *et al*. 2000), heart failure (Mallet *et*
98 *al*. 1998), pulmonary hypertension (Christman 1998) and pre-eclampsia (Barden *et al*.
99 1996; Staff *et al*. 1999; Walsh *et al*. 2000). To date there are minimal data outlining
100 the potential role of RhoA / Rho kinase in feto-placental vasculature, firstly in normal
101 pregnancies and pregnancies complicated by pre-eclampsia, and secondly in the
102 vasoconstrictor actions of isoprostanes. Therefore, the aims of this study were
103 twofold, firstly to investigate the mRNA expression levels of Rho A, ROCK I and
104 ROCK II in human umbilical artery in normal pregnancies and pregnancies
105 complicated by pre-eclampsia, and secondly to investigate the effects of two
106 isoprostanes, 8-*iso* $PGF_{2\alpha}$ and 8-*iso* PGE_2, on human umbilical artery tone and to
107 determine if their effects were mediated via the rho kinase pathway.
108

Materials and Methods

Tissue collection.

Patient recruitment took place in the Department of Obstetrics and Gynaecology, University College Hospital Galway. Ethical Committee approval for tissue collection was obtained from the Research Ethics Committee at University College Hospital Galway and patient recruitment was by written informed consent. For mRNA expression studies, sections of umbilical cord were excised from the proximal segment of the cord (i.e., nearest placental attachment) immediately after vaginal delivery or elective caesarean section at term, from normotensive pregnancies and pregnancies complicated by pre-eclampsia. Umbilical artery was dissected free of Warton's jelly, immediately snap frozen in liquid nitrogen and stored at -80°C. The normotensive group were non-proteinuric patients with uncomplicated pregnancies. The criteria for pre-eclampsia were as follows: at least two separate blood pressure readings >140/90mmHg, and the presence of +1 protein, or more, by dipstick analysis on more than one occasion (Fleming *et al*. 2000). Women with known pre-existing cardiac or renal disease were excluded from the study. For organ tissue bath studies, sections of umbilical cord excised from the proximal segment of the cord immediately after elective caesarean section were placed in Krebs-Henseleit physiologic salt solution, pH 7.4, containing: 4.7mmol KCl l^{-1}, 118mmol NaCl l^{-1}, 1.2mmol Mg_2SO_4 l^{-1}, 1.2mmol $CaCl_2$ l^{-1}, 1.2mmol KPO_4 l^{-1}, 25mmol $NaHCO_3$ l^{-1}, and 11mmol glucose l^{-1}. Indomethacin (10μmol l^{-1}) was also added to the Krebs-Henseleit solution to prevent generation of cyclo-oxygenase metabolites of arachidonic acid. Cord was stored at 4°C and used within 12 hours of collection.

RNA Extraction and Reverse Transcription

Total RNA was isolated using TRIzol® reagent (Life Technologies, Grand Island, NY, USA) (Chomczynski 1993). All RNA samples were DNA-*free*™ treated (Ambion Inc., Austin, TX, USA) and checked by standard RT-PCR to ensure that RNA used for real-time fluorescence RT-PCR contained no contaminating genomic DNA. 1µg of RNA (DNA-*free*™ treated) (Ambion Inc.) was reverse transcribed into complementary DNA (cDNA) for use as a template for Polymerase Chain Reaction (PCR). The RNA samples were then denatured at 65°C for 10 minutes. Reverse transcription was performed at 42°C for 60 minutes in a reaction volume of 20µl containing the following: oligo dT primer (500ng), Moloney murine leukaemia virus (M-MLV) reverse transcription buffer (50mmol Tris-HCl l^{-1} pH 8.3, 75mmol KCl l^{-1}, 3mmol $MgCl_2$ l^{-1}, 10mmol dithiothreitol l^{-1} (DTT))(Promega, Madison, WI, USA), diethylpyrocarbonate (DEPC)-treated water (BDH, Dorset, England), deoxyribonucleotide triphosphates (dNTPs) (0.2mmol l^{-1}) (Promega) and 200U M-MLV reverse transcriptase (Promega). Reverse transcriptase activity was stopped by heating samples at 65°C for 10 minutes. Control RNA samples, in which no reverse transcription enzyme was added, were included to confirm that no genomic DNA contamination was present.

PCR

5µl of the RT reaction was then used in the subsequent PCR. PCR was performed in a final volume of 50µl containing 1.5mmol $MgCl_2$ l^{-1}, 20mmol Tris-HCl l^{-1}, 50mmol KCl l^{-1} pH 8.3 (Life Technologies, Grand Island, NY, USA), 1.25U Taq DNA polymerase (Life Technologies), 40µmol dNTPs l^{-1} (Promega) and 0.2pmol l^{-1} of each sense and antisense primer. cDNA amplification was carried out by an initial denaturation step of 5 minutes at 95°C followed by 45 cycles of denaturation at 94°C

for 20s, annealing at 55°C for 45s and elongation at 72°C for 45s. 5µl of each PCR product were then separated by gel electrophoresis on a 1.5% agarose gel. Products were separated alongside a 2-log DNA molecular weight ladder for sizing. Primers used were designed to published DNA and mRNA sequences from GenBank as previously reported (Moran *et al*. 2002; Friel *et al*. 2005)(Table 1).

One Step Real-Time Fluorescence RT-PCR

One step RT-PCR using specific primers for Rho A, ROCK I and ROCKII was performed on total RNA isolated from umbilical artery using the LightCycler™ (Roche Diagnostics, GmbH, Mannheim, Germany). Reagents from the RNA Amplification kit SYBR Green I (Roche Diagnostics GmbH, Mannheim, Germany) were used throughout the experiment. Standard curves containing a certain number of cDNA copies were generated for each of Rho A (1×10^9 cDNA copies, 1×10^7 cDNA copies, 1×10^6 cDNA copies), ROCK I (1×10^8 cDNA copies, 1×10^6 cDNA copies, 1×10^5 cDNA copies) and ROCK II (1×10^8 cDNA copies, 1×10^6 cDNA copies, 1×10^5 cDNA copies) genes. Copy number/µl of cDNA was calculated according to the following formula, available from the Roche Lightcycler™ website (Curley *et al*. 2004):

$$\frac{6 \times 10^{23}\ [\text{copies/mol}] \times \text{concentration [g/µl]}}{\text{molecular weight [g/mol]}} = \text{amount [copies//µl]}$$

500ng of the DNA-*free*™ treated RNA samples, in which no genomic contamination was present, were used in the subsequent one step real-time fluorescence RT-PCR. This reaction was performed in a final volume of 20µl containing 6mmol $MgCl_2$ l^{-1},

183 0.4µl enzyme mix, 4µl reaction mix, 2µl resolution solution, (Roche Diagnostics
184 GmbH, Germany), and 0.3µmol l^{-1} of each sense and antisense primer. The final
185 volume of 20µl was achieved using sterile water (Roche Diagnostics GmbH,
186 Germany). Reverse transcription was carried out at 55°C for 30 minutes. cDNA
187 amplification was carried out by an initial denaturation step at 95°C for 30s, followed
188 by 45 cycles of denaturation at 95°C with a 5s hold time, annealing at 55°C with a 10s
189 hold time and elongation at 72°C with a 15s hold time. The temperature transition rate
190 for the elongation step was 2°C/s. The temperature transition rate for each step was
191 20°C/s unless otherwise stated. Fluorescence data was acquired at the end of each
192 PCR cycle, as previously described (Friel *et al*., 2005). The LightCycler™ Software
193 version 3 (fit-points method), calculated cDNA copy numbers for each gene,
194 generated from their respective amplification curve crossing points (point at which
195 exponential amplification begins) and generated standard curve. This point is
196 equivalent to fluorescence data plotted on the logarithmic scale. Generated cDNA
197 copy numbers for Rho A, ROCK I and ROCK II were then normalized to the
198 housekeeping gene beta-actin. Melting curve analysis was performed by an initial
199 denaturation step of 95°C, cooling to 65°C for 10s and finally gradually increasing the
200 temperature to 95°C. Fluorescence was measured continually during the melting
201 curve cycle.
202
203 10µl of each PCR product were then separated by gel electrophoresis on a 1.5% (w/v)
204 agarose gel. Products were separated alongside a 2-log DNA molecular weight ladder
205 for sizing. cDNA copy numbers for Rho A , ROCK I and ROCK II generated

206 automatically via the LightCycler from their respective standard curves were
207 normalized to the housekeeping gene beta-actin.
208
209 Umbilical Artery Tissue Bath Experiments
210 Human umbilical artery was dissected free of Warton's jelly and cut into transverse
211 rings, approximately 3-5mm in length. Rings were suspended on stainless-steel hooks
212 and mounted in organ tissue baths under 2 grams tension as previously described
213 (Dennedy *et al*. 2002; Ravikumar *et al*. 2004). The tissue baths contained 10ml of
214 Krebs-Henseleit physiologic salt solution maintained at 37°C, pH 7.4 and gassed
215 continuously with 95%O_2/5%CO_2. Individual rings were allowed to equilibrate for at
216 least 90 minutes, during which time the Krebs-Henseleit physiologic salt solution was
217 changed every 15 minutes. After the equilibration period, rings were challenged with
218 60mM KCl. Once the maximum response to KCl was achieved, rings were washed
219 and allowed to equilibrate for 20 minutes, to allow base-line to be reached again. The
220 KCl challenge was repeated three times. Forty minutes after the final KCl washout
221 either 8-*iso* $PGF_{2\alpha}$ or 8-*iso* PGE_2 were added in a cumulative manner, at 20 minute
222 intervals, at concentrations of 1nmol l^{-1}, 10nmol l^{-1}, 100nmol l^{-1}, 1μmol l^{-1}, and
223 10μmol l^{-1}. The mechanical response of tissues was measured by calculation of the
224 mean amplitude of contraction for 20 minute periods using the PowerLab hardware
225 unit and Chart v3.6 software (AD Instruments, Hastings, UK). The mean amplitude
226 of contraction for the first 20 minutes (following the forty minute period after the final
227 KCl washout) was calculated and this value served as a control. Antagonism of the
228 effects of 8-*iso* $PGF_{2\alpha}$ and 8-*iso* PGE_2 were investigated by addition of the rho kinase
229 inhibitor, Y-27632 (10μmol l^{-1}) 30 minutes prior to the addition of 8-*iso* $PGF_{2\alpha}$ or 8-
230 *iso* PGE_2. Control strips were simultaneously run with bath exposure to vehicle, but

without addition of drug. The effects of 8-*iso* $PGF_{2\alpha}$, 8-*iso* PGE_2 alone and with Y-27632 were expressed in terms of g tension generated.

Drugs and Solutions

All chemicals were purchased from Sigma-Aldrich, Dublin, Ireland unless otherwise stated. 8-*iso* $PGF_{2\alpha}$ and 8-*iso* PGE_2 were obtained from Cayman Chemical, Ann Arbor, MI, USA. A stock solution (10mmol l^{-1}) of 8-*iso* $PGF_{2\alpha}$ or 8-*iso* PGE_2 was prepared in dimethylsulphoxide (DMSO). Series of dilutions were made with Krebs-Henseleit physiologic salt solution on the day of experimentation and maintained at room temperature for the duration of the experiment. Y-27632 was kindly donated by Welfide Corporation, Osaka, Japan. A stock solution (10mmol l^{-1}) of Y-27632 was made with deionised water. Series of dilutions were made with Krebs-Henseleit physiologic salt solution on the day of experimentation. A stock solution (100mmol l^{-1}) of indomethacin was made in DMSO. Fresh Krebs-Henseleit physiologic salt solution was made daily.

Statistical Analysis

For the mRNA expression study, normalized cDNA copy numbers for each transcript, between both vessel types, were compared using the Student *t* test. For the organ tissue bath study, calculated mean g tension for control rings and rings exposed to either 8-*iso* $PGF_{2\alpha}$ (alone or with Y-27632) and 8-*iso* PGE_2 (alone or with Y-27632) were compared using Student *t* test. A P value of <0.05 for the Student *t* test was considered to be statistically significant. Comparisons of g tension, for each bath concentration of 8-*iso* $PGF_{2\alpha}$ (alone or with Y-27632) and 8-*iso* PGE_2 (alone or with Y-27632) were performed using ANOVA followed by Sheffe post hoc comparison

where appropriate. The statistical package SPSS for Windows version 11 (SPSS Inc., Chicago, Ill, USA) was used for these statistical calculations. The concentration of drug resulting in half the maximal effect (i.e. the EC_{50}) was measured and represented in pharmacological terms as its appropriate $-\log_{10}$ value (i.e. $-\log_{10}$ EC_{50}), which is also known as the pD_2 value. The mean maximum contractile (MMC) effect is the maximum contractile effect produced by the highest concentration of drug (i.e. 10μmol l^{-1}). Curve fitting was performed with the package Prism™ (Graphpad Software, San Diego, USA).

Results

Tissue Samples

For the mRNA expression study umbilical cords were obtained from 6 normotensive women and 4 pre-eclamptic women after delivery. All 6 normotensive women had elective caesarean sections. The reasons for elective caesarean section were previous caesarean section (n=5) and breech presentation (n=1). The mean patient age (year) ± SEM was 35.67 ± 2.06; median gestation 39 weeks (range 38-40); parity 0 (n=1), 1 (n=4), 3 (n=1). Of the 4 pre-eclamptic women, 1 had an elective caesarean section. The reason for the caesarean section was breech presentation. The mean patient age (year) ± SEM was 32.25 ± 3.90; median gestation 37.5 weeks (range 36-39); parity 0 (n=2), 1 (n=1), 2 (n=1).

For organ tissue bath studies umbilical arteries were obtained from a total of 12 women following delivery. Of these 12 women, 8 underwent elective caesarean section. The reasons for elective caesarean section included previous caesarean section (n=2), breech presentation (n=3), patient request (n=1), high head (n=1) and

281 macrosomia (n=1). The mean patient age (year) ± SEM was 33.50 ± 2.08; median
282 gestation 39.5 weeks (range 38-41); parity 0 (n=6), 1 (n=4), 2 (n=1), 3 (n=1).
283
284 Standard RT-PCR
285 Beta-actin, Rho A, ROCK I and ROCK II mRNA expression was detected in all
286 samples (Figure 1). Amplification of umbilical artery cDNA with the beta-actin
287 primer set yielded a 377bp PCR product. Amplification with the Rho A primer set
288 resulted in a 309bp PCR product and amplification with ROCK I and ROCK II
289 primers yielded 369bp and 390bp products. These products were sequenced (MWG-
290 Biotech Ltd., UK) and results verified that they were the appropriate parts of the beta-
291 actin, Rho A, ROCK I and ROCK II gene sequences. PCR of the reverse transcriptase
292 negative controls (RT-) showed no amplification confirming the absence of
293 significant genomic DNA contamination. Similarly, the PCR negative control (no
294 cDNA template) showed no amplification. Therefore, RNA in which no genomic
295 contamination was present was used for subsequent quantitative real-time
296 fluorescence RT-PCR.
297
298 One-Step Fluorescence RT-PCR
299 To compensate for any undue experimental error, analyses of each gene, for both
300 vessel types, were performed in triplicate. The mean values of these experiments were
301 used for statistical analysis. The four primer sets yielded RT-PCR products of the
302 expected sizes (data not shown). All patients showed expression of beta-actin, Rho A,
303 ROCK I and ROCK II mRNA. Standard curves generated for each of the genes under
304 investigation were used to determine their respective transcript number, per 0.5µg
305 total RNA, in both vessel types studied. Using the LightCycler™ Software version 3

(fit-points method), calculated cDNA copy numbers for each gene were generated from their respective amplification curve crossing points (point at which exponential amplification begins) as previously described (Friel *et al*. 2005). A representative recording of fluorescence plotted on the logarithmic scale corresponding to Rho A amplification in umbilical artery is shown in Figure 2. The melting peak analyses of Rho A, ROCK I and ROCK II showed specificity of product amplification (data not shown).

Umbilical Artery Expression

Beta-actin mRNA expression did not significantly differ between normotensive and pre-eclamptic umbilical arteries (Table 2), which indicated that beta-actin was suitable as a housekeeping gene for this vessel type. cDNA copy numbers for Rho A, ROCK I and ROCK II were therefore normalized to the beta-actin gene for determination of their absolute cDNA copy numbers per 0.5µg total RNA. Comparisons of cDNA copy numbers, between both groups, for Rho A, revealed that Rho A mRNA expression was significantly down-regulated in artery obtained from pre-eclamptic women in comparison to that measured in artery obtained from normotensive women ($P<0.05$). The cDNA copy numbers (per 0.5µg of total RNA) ± the standard error of the mean (SEM) for Rho A were: (normal) 7.0e+07 ± 7.6e+06 (n=6) and (pre-eclamptic) 4.8e+07 ± 4.5e+06 (n=4) (Figure 3). The mRNA expression levels of ROCK I and ROCK II were not significantly different between the two vessel types analysed ($P>0.05$). The cDNA copy numbers for ROCK I were: (normal) 1.3e+07 ± 8.7e+05 (n=6); (pre-eclamptic) 1.0e+7 ± 1.9e+06 (n=4) and for ROCK II were: (normal) 5.2e+07 ± 1.1e+07 (n=6); (pre-eclamptic) 3.0e+07 ± 6.7e+05 (n=4) (Figure 3).

331

332 Effects of Isoprostanes on Umbilical Artery

333 Both 8-*iso* $PGF_{2\alpha}$ and 8-*iso* PGE_2 exerted a significant concentration dependent
334 vasocontractile effect on human umbilical artery. This is graphically represented as a
335 histograph in Figure 4 for 8-*iso* $PGF_{2\alpha}$, and in Figure 5 for 8-*iso* PGE_2. The MMC
336 effect (in g tension) and the pD_2 values (± SEM) are detailed in Table 3. Calculated
337 increases in g tension for control rings and rings exposed to 8-*iso* $PGF_{2\alpha}$ were
338 compared by Student *t* test. Analysis revealed a significant contractile effect at
339 increasing 8-*iso* $PGF_{2\alpha}$ concentrations of 1μmol l^{-1} ($P<0.01$) and 10μmol l^{-1}
340 ($P<0.001$). Similarly, calculated increases in g tension for control rings and rings
341 exposed to 8-*iso* PGE_2 were compared by Student *t* test. Again, analysis revealed a
342 significant contractile effect at increasing 8-*iso* PGE_2 concentrations of 1μmol l^{-1}
343 ($P<0.001$) and 10μmol l^{-1} ($P<0.001$). 8-*iso* PGE_2 induced vasocontractions were
344 significantly greater than those induced by 8-*iso* $PGF_{2\alpha}$ ($P<0.05$). There was no
345 significant difference between pD_2 ($P>0.05$) values for both compounds.

346

347 Effects of Rho Kinase Antagonism on Umbilical Artery

348 8-*iso* PGE_2 induced contractions were significantly antagonised by the specific rho
349 kinase inhibitor Y-27632 ($P<0.01$). This is demonstrated graphically in Figure 5. 8-*iso*
350 $PGF_{2\alpha}$ induced contractions were not significantly antagonised ($P>0.05$)(Figure 4).
351 The MMC and pD_2 values (± SEM) for antagonised 8-*iso* $PGF_{2\alpha}$ and 8-*iso* PGE_2 are
352 detailed in Table 3. There was no significant difference in pD_2 values for antagonised
353 8-*iso* $PGF_{2\alpha}$ and 8-*iso* PGE_2 in comparison to 8-*iso* $PGF_{2\alpha}$ ($P>0.05$) and 8-*iso* PGE_2
354 ($P>0.05$) alone.

355

Comment

Pre-eclampsia is one of the major disorders of obstetrics practice which contributes to maternal and perinatal morbidity and mortality. An understanding of the biological processes that result in the adverse maternal and fetal consequences is lacking. The factors regulating the feto-placental vasculature during normal pregnancy, and in pre-eclampsia, are poorly understood. The Rho A / Rho kinase system is closely linked to prolonged states of smooth muscle contraction, or vasoconstriction, and is closely linked to hypertensive disorders in animal and human models. For these reasons, we hypothesised that the Rho A / Rho kinase system may be linked to normal feto-placental circulatory regulation and the changes that occur in pre-eclampsia.

We have demonstrated that the mRNA expression of Rho A appears to be down regulated in umbilical arteries in association with pre-eclampsia. An obvious interpretation of this finding is that there is reduced expression, with presumably reduced activity of the Rho A / Rho kinase pathway in these vessels, in association with pre-eclampsia, which may facilitate greater vasodilatation or enhanced fetal blood flow. These findings therefore imply that Rho A/ ROCK does not influence the increased vasoconstriction seen in association with PET. These data are preliminary, and there are limitations in concluding from these findings. The total RNA for these results was extracted from total human umbilical artery preparations, and hence includes the endothelium and the vascular smooth muscle layer. This was the deliberate design of the experiments, as it would have been technically difficult to denude these vessels, and these samples were all snap frozen in the operating theatre from women with pre-eclampsia or normal pregnancy. Further attempts to explore this issue, i.e., to evaluate and quantify Rho A / Rho kinase pathway expression or

activity in the vascular smooth muscle, would require methods that are not as accurate in terms of quantitation, such as immunohistochemical techniques. The other issue, which needs to be addressed, is that of the protein expression and that would require Western Blotting experiments. As a preliminary finding however, it is apparent from our experiments that Rho A is down regulated at the mRNA level in total umbilical artery vessels from women with pre-eclampsia in comparison to control women with normal pregnancies.

It is evident that isoprostanes contribute significantly to the prolonged vasoconstriction that occurs in pre-eclampsia. Using umbilical artery ring preparations, with standard in vitro techniques, we have demonstrated that the two isoprostanes 8-*iso* PGE_2 and 8-*iso* $PGF_{2\alpha}$, both exert a potent vasoconstrictor effect as has been demonstrated previously (Oliveira *et al*. 2000). By preincubation with a specific Rho kinase inhibitor it is clear from our experiments that 8-*iso* PGE_2 is unable to elicit the same response after Rho kinase inhibition, indicative of the fact that the Rho kinase pathway is involved in the vasoconstrictor effect of 8-*iso* PGE_2. These results were not found for the vasoconstrictor of 8-*iso* $PGF_{2\alpha}$. There is no obvious reason why the effects of 8-*iso* $PGF_{2\alpha}$ were apparently different to those of 8-*iso* PGE_2, but it is evident that a different mechanism for 8-*iso* PGE_2 exists, which operates at least in part via the Rho kinase pathway. On speculation, this difference observed in relation to antagonism with Y-27632 can only be due to a relative difference in potencies observed in tissues, whereby 8-*iso*-PGE_2 is more potent (Oliveira *et al*, 2000; Tazzeo *et al*, 2003). There are no signalling pathways to our knowledge, known to operate via 8-iso-PGE_2 and 8-iso-$PGF_{2\alpha}$ directly. Finally, a

405 further limitation in interpreting these data relate to the fact that while the
406 cyclooxygenase pathway was blocked, the lipoxygenase pathway was not.
407
408 In summary, these findings highlight the potential importance of the Rho A / Rho
409 kinase pathway in the umbilical artery circulation in normal pregnancy, and raise the
410 question of reduced expression at the mRNA level for Rho A in pre-eclampsia. The
411 factors regulating these potential changes require further investigation. Future studies
412 include the assessment of the protein expression of the various components of the Rho
413 A / Rho kinase pathway in normal pregnancy and in pregnancies complicated by pre-
414 eclampsia. Finally, from a functional point of view, the vasocontractile effect of 8-*iso*
415 PGE_2, a potent isoprostane linked to pre-eclampsia appears to be mediated at least in
416 part via the Rho kinase pathway.
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Figure 1

Representative agarose gel stained with ethidium bromide demonstrating expression of β-actin, Rho A, ROCK I and ROCK II in human umbilical artery (normotensive). Reverse transcriptase-negative controls (RT-) for both genes are shown alongside reverse transcriptase-positive (RT+) PCR products. M represents the 2-log DNA molecular weight ladder.

Figure 2

Quantitative real-time fluorescence RT-PCR amplification curve for Rho A mRNA expression in human umbilical artery (both normal and pre-eclamptic). Fluorescence is plotted on the y-axis and PCR cycle number on the x-axis. Continuous lines represent the Rho A cDNA standards ($1x10^9$ and $1x10^7$ cDNA copy numbers). Closed circles represent normal samples (n=6), open circles represent pre-eclamptic samples (n=4) and closed squares represent the water control.

Figure 3

Rho A, ROCK I, ROCK II and beta-actin mRNA expression in human umbilical artery from normal pregnancies (N;n=6) and pre-eclamptic pregnancies (PET;n=4) by real-time Fluorescence RT-PCR. cDNA copy numbers are shown on the y-axis and the genes investigated on the x-axis. The histogram depicts Rho A, ROCK I and ROCK II cDNA copy numbers normalized to the housekeeping gene beta-actin. Grey columns represent normal samples. Columns with diagonal grey stripes represent pre-eclamptic samples. Vertical error bars represent standard error of the mean (SEM). ∗ N versus PET $P<0.05$.

Figure 4

The effects of 8-*iso* $PGF_{2\alpha}$ (alone and following Y-27632 addition) on human umbilical artery tone. The graph depicts the effects of cumulative increases in bath concentration of 8-*iso* $PGF_{2\alpha}$ (1nM-10μM) at 20 minute intervals. Open squares represent 8-*iso* $PGF_{2\alpha}$ (following Y-27632 addition) and closed squares represent 8-*iso* $PGF_{2\alpha}$ (alone). Contractility (g Tension) is shown on the y-axis, and the concentration of 8-*iso* $PGF_{2\alpha}$ is shown on the x-axis. Values plotted are means. Vertical error bars represent the standard error of the mean (SEM).

Figure 5

The effects of 8-*iso* PGE_2 (alone and following Y-27632 addition) on human placental artery tone. The graph depicts the effects of cumulative increases in bath concentration of 8-*iso* PGE_2 (1nM-10μM) at 20 minute intervals. Open squares represent 8-*iso* PGE_2 (following Y-27632 addition) and closed squares represent 8-*iso* PGE_2 (alone). Contractility (g Tension) is shown on the y-axis, and the concentration of 8-*iso* PGE_2 is shown on the x-axis. Values plotted are means. Vertical error bars represent the standard error of the mean (SEM). * 8-*iso* PGE_2 versus Y-27632 & 8-*iso* PGE_2, $P<0.05$; ** 8-*iso* PGE_2 versus Y-27632 & 8-*iso* PGE_2, $P<0.001$.

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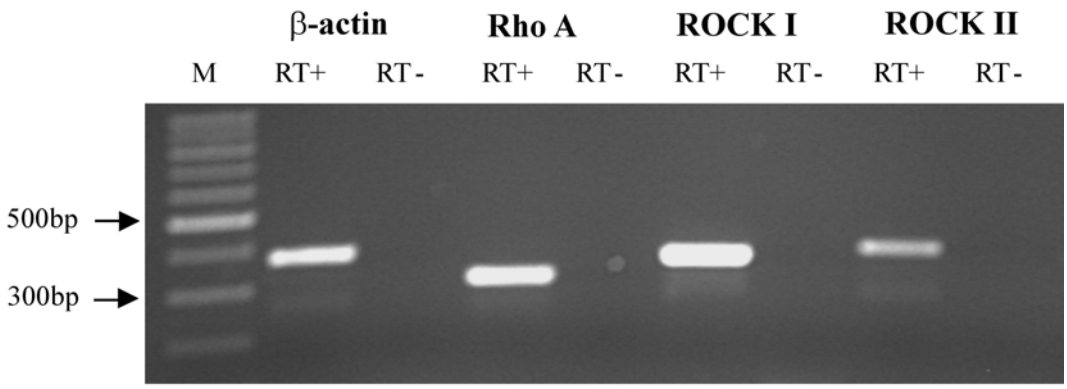
580 **Figure 1**



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600 **Figure 2**

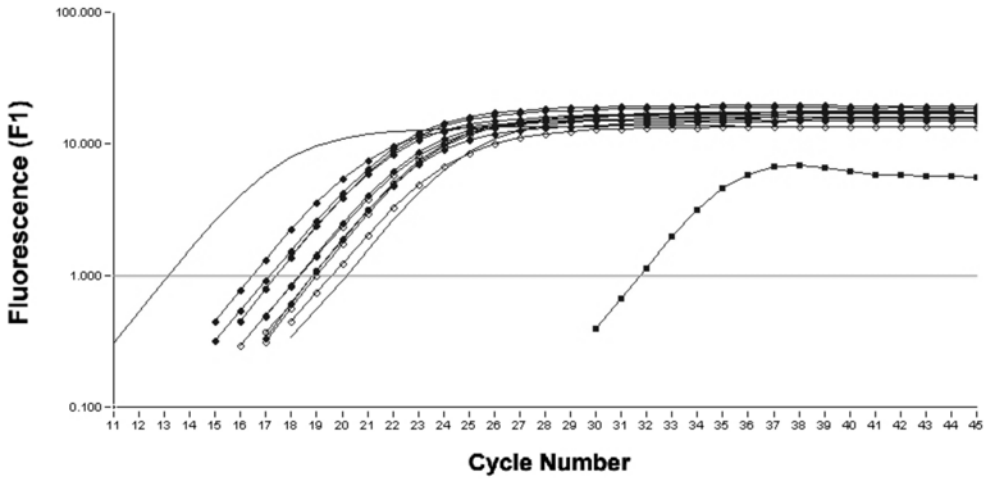

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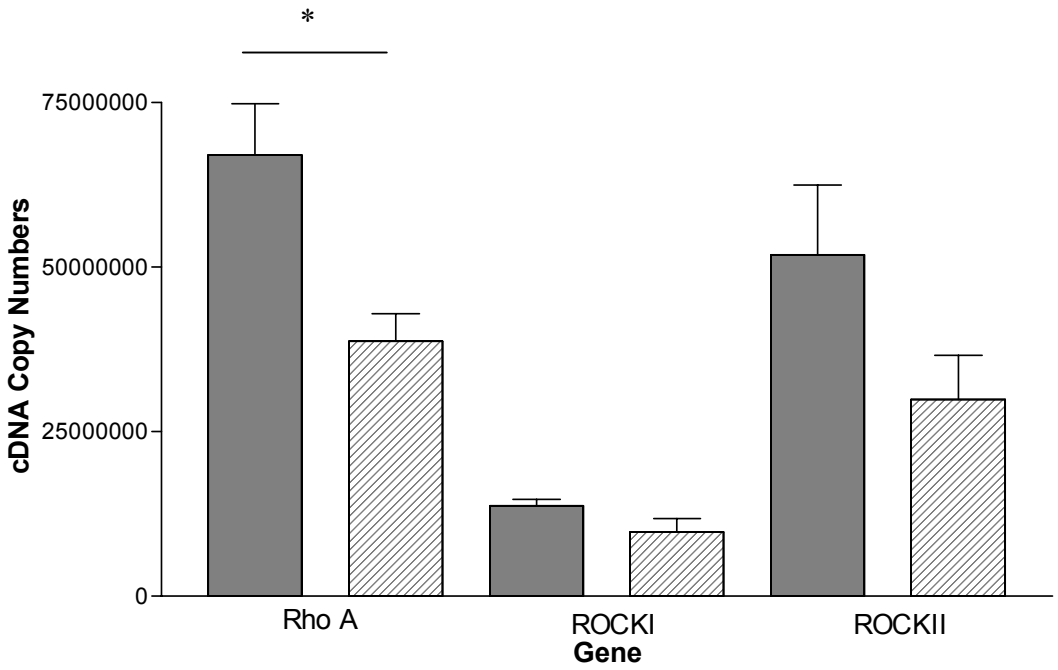
Figure 3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cDNA Copy Numbers
Rho A
ROCKI
ROCKII
Gene

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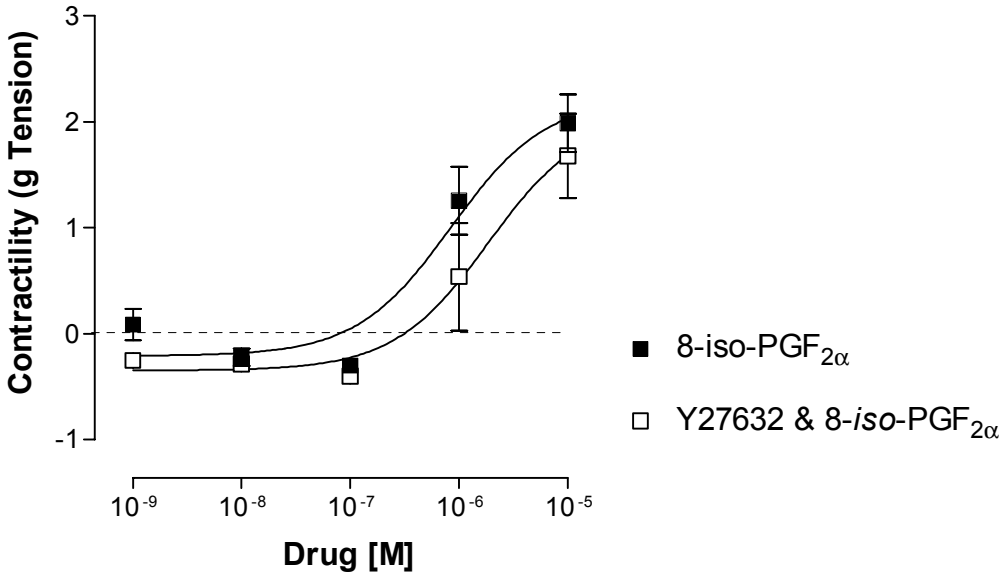
639 **Figure 4**



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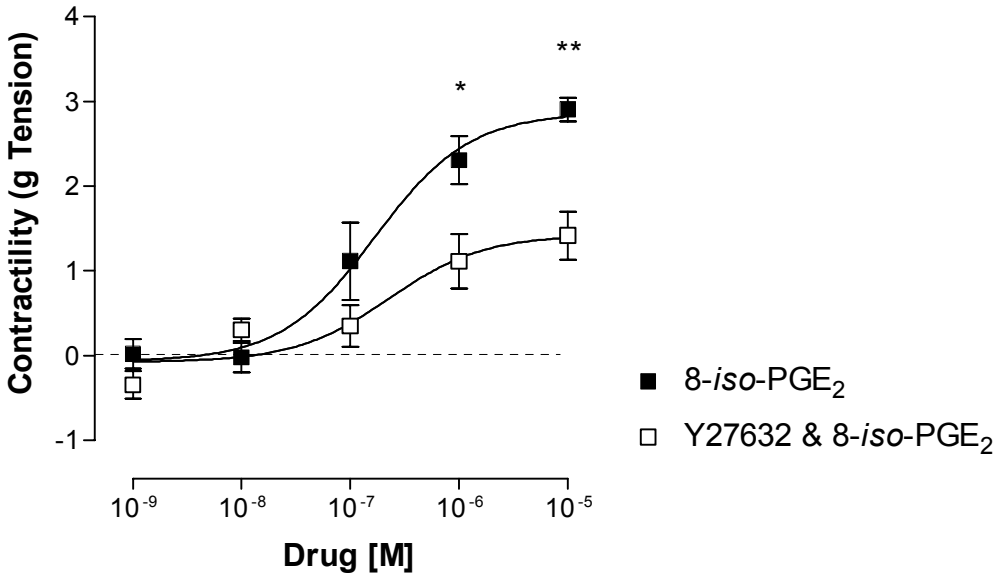
654 **Figure 5**



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671 **Table 1.** Primers used for standard RT-PCR and real-time fluorescence RT-PCR

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RT-PCR Primers		
Human Rho A	sense	5’-CTCATAGTCTTCAGCAAGGACCAGTT-3’
(Accession Code: L25080)	antisense	5’-ATCATTCCGAAGATCCTTCTTATT-3’
Human ROCK I	sense	5’-GAAGAAAGAGAAGCTCGAGAAGAAGG-3’
(Accession Code: XM_008814)	antisense	5’-ATCTTGTAGCTCCCGCATCTGT-3’
Human ROCK II	sense	5’-AATTCACTGTGTTTCCCTGAAGATA-3’
(Accession Code: XM_002676)	antisense	5’-TTCATTTTTCCTTGATTGTATGGAA-3’
Human Beta-actin	sense	5’-CAACTCCATCATGAAGTGTGAC-3’
(Accession Code: M10277)	antisense	5’-GCCATGCCAATCTCTCATCTTG-3’

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687 **Table 2.** cDNA copy numbers ± the standard error of the mean (SEM) for Rho A,
688 ROCK I, ROCK II and β-actin in human umbilical artery (both normal and pre-
689 eclamptic)
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Gene	Normal	Pre-eclamptic
Rho A	7.0e+07 ± 7.6e+06	4.8e+07* ± 4.5e+06
ROCK I	1.3e+07 ± 8.7e+05	1.0e+07 ± 1.9e+06
ROCK II	5.2e+07 ± 1.1e+07	3.0e+07 ± 6.7e+05
β-actin	2.8e+08 ± 4.5e+07	4.1e+08 ± 9.6e+07

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692 Values presented are means ± the standard error of the mean.
693 *P<0.05 v Normal
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Table 3. Effects of 8-*iso* PGE_2 and 8-*iso* $PGF_{2\alpha}$ alone and antagonised by Y-27632 on Human Umbilical Arterial Tone

	Umbilical Artery	
Drug	**Contractility (g tension)**	**pD_2**
8-*iso* PGE_2	2.91 ± 0.14 (n=7)	6.77 ± 0.13
8-*iso* PGE_2 + Y-27632	1.42 ± 0.28* (n=6)	6.65 ± 0.49
8-*iso* $PGF_{2\alpha}$	1.99 ± 0.27 (n=7)	6.07 ± 0.35
8-*iso* $PGF_{2\alpha}$ + Y-27632	1.68 ± 0.40 (n=6)	5.73 ± 0.18

Values presented are MMC means ± the standard error of the mean.

*$P<0.01$ versus 8-*iso* PGE_2 alone