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

4

5

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25

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33 **Running Title:** Rho A/ Rho kinase Expression and Isoprostanes

34 **Abstract**

35 Pre-eclampsia represents a state of increased or prolonged vasoconstriction, partially  
36 linked to the potent vasocontractile effect of isoprostanes. The process of Rho A-  
37 mediated calcium sensitization is inherent to a state of prolonged contractility in many  
38 smooth muscle types. The aims of this study were 1), to investigate mRNA expression  
39 levels of Rho A and Rho kinase isoforms (I and II) in umbilical artery from  
40 normotensive and pre-eclamptic women, and 2), to determine whether the effects of  
41 two isoprostanes, 8-*iso* prostaglandin F<sub>2α</sub> (8-*iso* PGF<sub>2α</sub>) and 8-*iso* prostaglandin E<sub>2</sub> (8-  
42 *iso* PGE<sub>2</sub>), on umbilical artery tone, were mediated via the Rho kinase pathway. Real-  
43 time RT-PCR using primers for Rho A, ROCK I and ROCK II was performed on total  
44 RNA isolated from umbilical artery specimens obtained from normotensive and pre-  
45 eclamptic women. The effects of both isoprostanes (n=6) (in the absence and presence  
46 of the specific Rho kinase inhibitor Y-27632), on umbilical artery tone were  
47 measured, and compared with control recordings. Rho A mRNA expression levels  
48 were significantly lower in umbilical artery samples obtained from pre-eclamptic  
49 women (n=4) in comparison to those from normotensive women (n=6) (P<0.05).  
50 ROCK I and ROCK II mRNA levels were similar in both vessel types (P>0.05). Both  
51 isoprostanes exerted a significant concentration dependent vasocontractile effect  
52 (n=7)(P<0.001) on umbilical artery. For 8-*iso* PGE<sub>2</sub> this effect was antagonised by Y-  
53 27632 (n=6) (P<0.01). The significant reduction of Rho A mRNA levels in umbilical  
54 arteries from pregnancies complicated by pre-eclampsia may serve to counteract the  
55 diminished perfusion associated with the pathophysiology of pre-eclampsia. The  
56 vasocontractile effect of 8-*iso* PGE<sub>2</sub> in pre eclampsia may in part be mediated via the  
57 Rho kinase pathway.

58

## 59 **Introduction**

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

70

71 Research has indicated that the process of calcium sensitization (increase in smooth  
72 muscle tension and/or phosphorylation of myosin light chains at a constant  $[Ca^{2+}]_i$  by  
73 inhibition of myosin light chain phosphatase (MLCP)), is of major importance in  
74 regulating the state of vasoconstriction of vascular smooth muscle (Somylo & Somylo  
75 2000). It is now apparent that the small G protein, Rho A is associated with inhibition  
76 of MLCP (Uehata *et al.* 1997; Kunihiro *et al.* 1999). Although the precise mechanism  
77 of action is unknown, two target proteins of Rho A, ROCK I and its isoform ROCK  
78 II, which are collectively known as Rho kinases, have a major role in Rho A-mediated  
79 calcium sensitization. Upon activation they enhance Rho-mediated calcium  
80 sensitization and hence smooth muscle contractility. It is now clear that this Rho  
81 kinase pathway plays a central role in the pathogenesis of hypertension in animal  
82 models, in humans and in various situations of increased peripheral vascular  
83 resistance observed in hypertensive disorders (Chitale *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 fetoplacental vasculature  
86 during normal pregnancy or in pregnancies complicated by pre-eclampsia. The fetoplacental  
87 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 al.*  
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 fetoplacental 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<sub>2α</sub> and 8-*iso* PGE<sub>2</sub>, on human umbilical artery tone and to  
107 determine if their effects were mediated via the rho kinase pathway.

108

## 109 **Materials and Methods**

110 Tissue collection.

111 Patient recruitment took place in the Department of Obstetrics and Gynaecology,  
112 University College Hospital Galway. Ethical Committee approval for tissue  
113 collection was obtained from the Research Ethics Committee at University College  
114 Hospital Galway and patient recruitment was by written informed consent. For  
115 mRNA expression studies, sections of umbilical cord were excised from the proximal  
116 segment of the cord (i.e., nearest placental attachment) immediately after vaginal  
117 delivery or elective caesarean section at term, from normotensive pregnancies and  
118 pregnancies complicated by pre-eclampsia. Umbilical artery was dissected free of  
119 Warton's jelly, immediately snap frozen in liquid nitrogen and stored at -80°C. The  
120 normotensive group were non-proteinuric patients with uncomplicated pregnancies.  
121 The criteria for pre-eclampsia were as follows: at least two separate blood pressure  
122 readings >140/90mmHg, and the presence of +1 protein, or more, by dipstick analysis  
123 on more than one occasion (Fleming *et al.* 2000). Women with known pre-existing  
124 cardiac or renal disease were excluded from the study. For organ tissue bath studies,  
125 sections of umbilical cord excised from the proximal segment of the cord immediately  
126 after elective caesarean section were placed in Krebs-Henseleit physiologic salt  
127 solution, pH 7.4, containing: 4.7mmol KCl l<sup>-1</sup>, 118mmol NaCl l<sup>-1</sup>, 1.2mmol Mg<sub>2</sub>SO<sub>4</sub> l<sup>-1</sup>,  
128 1.2mmol CaCl<sub>2</sub> l<sup>-1</sup>, 1.2mmol KPO<sub>4</sub> l<sup>-1</sup>, 25mmol NaHCO<sub>3</sub> l<sup>-1</sup>, and 11mmol glucose l<sup>-1</sup>.  
129 Indomethacin (10µmol l<sup>-1</sup>) was also added to the Krebs-Henseleit solution to prevent  
130 generation of cyclo-oxygenase metabolites of arachidonic acid. Cord was stored at  
131 4°C and used within 12 hours of collection.

132

133 RNA Extraction and Reverse Transcription

134 Total RNA was isolated using TRIzol<sup>®</sup> reagent (Life Technologies, Grand Island, NY,  
135 USA) (Chomczynski 1993). All RNA samples were DNA-free<sup>™</sup> treated (Ambion  
136 Inc., Austin, TX, USA) and checked by standard RT-PCR to ensure that RNA used  
137 for real-time fluorescence RT-PCR contained no contaminating genomic DNA. 1µg  
138 of RNA (DNA-free<sup>™</sup> treated) (Ambion Inc.) was reverse transcribed into  
139 complementary DNA (cDNA) for use as a template for Polymerase Chain Reaction  
140 (PCR). The RNA samples were then denatured at 65°C for 10 minutes. Reverse  
141 transcription was performed at 42°C for 60 minutes in a reaction volume of 20µl  
142 containing the following: oligo dT primer (500ng), Moloney murine leukaemia virus  
143 (M-MLV) reverse transcription buffer (50mmol Tris-HCl l<sup>-1</sup> pH 8.3, 75mmol KCl l<sup>-1</sup>,  
144 3mmol MgCl<sub>2</sub> l<sup>-1</sup>, 10mmol dithiothreitol l<sup>-1</sup> (DTT))(Promega, Madison, WI, USA),  
145 diethylpyrocarbonate (DEPC)-treated water (BDH, Dorset, England),  
146 deoxyribonucleotide triphosphates (dNTPs) (0.2mmol l<sup>-1</sup>) (Promega) and 200U M-  
147 MLV reverse transcriptase (Promega). Reverse transcriptase activity was stopped by  
148 heating samples at 65°C for 10 minutes. Control RNA samples, in which no reverse  
149 transcription enzyme was added, were included to confirm that no genomic DNA  
150 contamination was present.

151

152 PCR

153 5µl of the RT reaction was then used in the subsequent PCR. PCR was performed in a  
154 final volume of 50µl containing 1.5mmol MgCl<sub>2</sub> l<sup>-1</sup>, 20mmol Tris-HCl l<sup>-1</sup>, 50mmol  
155 KCl l<sup>-1</sup> pH 8.3 (Life Technologies, Grand Island, NY, USA), 1.25U Taq DNA  
156 polymerase (Life Technologies), 40µmol dNTPs l<sup>-1</sup> (Promega) and 0.2pmol l<sup>-1</sup> of each  
157 sense and antisense primer. cDNA amplification was carried out by an initial  
158 denaturation step of 5 minutes at 95°C followed by 45 cycles of denaturation at 94°C

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

164

#### 165 One Step Real-Time Fluorescence RT-PCR

166 One step RT-PCR using specific primers for Rho A, ROCK I and ROCKII was  
167 performed on total RNA isolated from umbilical artery using the LightCycler™  
168 (Roche Diagnostics, GmbH, Mannheim, Germany). Reagents from the RNA  
169 Amplification kit SYBR Green I (Roche Diagnostics GmbH, Mannheim, Germany)  
170 were used throughout the experiment. Standard curves containing a certain number of  
171 cDNA copies were generated for each of Rho A (1×10<sup>9</sup> cDNA copies, 1×10<sup>7</sup> cDNA  
172 copies, 1×10<sup>6</sup> cDNA copies), ROCK I (1×10<sup>8</sup> cDNA copies, 1×10<sup>6</sup> cDNA copies,  
173 1×10<sup>5</sup> cDNA copies) and ROCK II (1×10<sup>8</sup> cDNA copies, 1×10<sup>6</sup> cDNA copies, 1×10<sup>5</sup>  
174 cDNA copies) genes. Copy number/µl of cDNA was calculated according to the  
175 following formula, available from the Roche Lightcycler™ website (Curley *et al.*  
176 2004):

$$177 \quad \frac{6 \times 10^{23} \text{ [copies/mol]} \times \text{concentration [g/}\mu\text{l]}}{\text{molecular weight [g/mol]}} = \text{amount [copies//}\mu\text{l]}$$

179

180 500ng of the DNA-free™ treated RNA samples, in which no genomic contamination  
181 was present, were used in the subsequent one step real-time fluorescence RT-PCR.  
182 This reaction was performed in a final volume of 20µl containing 6mmol MgCl<sub>2</sub> l<sup>-1</sup>,



183 0.4µl enzyme mix, 4µl reaction mix, 2µl resolution solution, (Roche Diagnostics  
184 GmbH, Germany), and 0.3µmol l<sup>-1</sup> 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<sub>2</sub>/5%CO<sub>2</sub>. 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<sub>2α</sub> or 8-*iso* PGE<sub>2</sub> were added in a cumulative manner, at 20 minute  
222 intervals, at concentrations of 1nmol l<sup>-1</sup>, 10nmol l<sup>-1</sup>, 100nmol l<sup>-1</sup>, 1μmol l<sup>-1</sup>, and  
223 10μmol l<sup>-1</sup>. 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<sub>2α</sub> and 8-*iso* PGE<sub>2</sub> were investigated by addition of the rho kinase  
229 inhibitor, Y-27632 (10μmol l<sup>-1</sup>) 30 minutes prior to the addition of 8-*iso* PGF<sub>2α</sub> or 8-  
230 *iso* PGE<sub>2</sub>. Control strips were simultaneously run with bath exposure to vehicle, but

231 without addition of drug. The effects of 8-*iso* PGF<sub>2α</sub>, 8-*iso* PGE<sub>2</sub> alone and with Y-  
232 27632 were expressed in terms of g tension generated.

233

#### 234 Drugs and Solutions

235 All chemicals were purchased from Sigma-Aldrich, Dublin, Ireland unless otherwise  
236 stated. 8-*iso* PGF<sub>2α</sub> and 8-*iso* PGE<sub>2</sub> were obtained from Cayman Chemical, Ann  
237 Arbor, MI, USA. A stock solution (10mmol l<sup>-1</sup>) of 8-*iso* PGF<sub>2α</sub> or 8-*iso* PGE<sub>2</sub> was  
238 prepared in dimethylsulphoxide (DMSO). Series of dilutions were made with Krebs-  
239 Henseleit physiologic salt solution on the day of experimentation and maintained at  
240 room temperature for the duration of the experiment. Y-27632 was kindly donated by  
241 Welfide Corporation, Osaka, Japan. A stock solution (10mmol l<sup>-1</sup>) of Y-27632 was  
242 made with deionised water. Series of dilutions were made with Krebs-Henseleit  
243 physiologic salt solution on the day of experimentation. A stock solution (100mmol l<sup>-1</sup>)  
244 of indomethacin was made in DMSO. Fresh Krebs-Henseleit physiologic salt  
245 solution was made daily.

246

#### 247 Statistical Analysis

248 For the mRNA expression study, normalized cDNA copy numbers for each transcript,  
249 between both vessel types, were compared using the Student *t* test. For the organ  
250 tissue bath study, calculated mean g tension for control rings and rings exposed to  
251 either 8-*iso* PGF<sub>2α</sub> (alone or with Y-27632) and 8-*iso* PGE<sub>2</sub> (alone or with Y-27632)  
252 were compared using Student *t* test. A P value of <0.05 for the Student *t* test was  
253 considered to be statistically significant. Comparisons of g tension, for each bath  
254 concentration of 8-*iso* PGF<sub>2α</sub> (alone or with Y-27632) and 8-*iso* PGE<sub>2</sub> (alone or with  
255 Y-27632) were performed using ANOVA followed by Sheffe post hoc comparison

256 where appropriate. The statistical package SPSS for Windows version 11 (SPSS Inc.,  
257 Chicago, Ill, USA) was used for these statistical calculations. The concentration of  
258 drug resulting in half the maximal effect (i.e. the  $EC_{50}$ ) was measured and represented  
259 in pharmacological terms as its appropriate  $-\log_{10}$  value (i.e.  $-\log_{10} EC_{50}$ ), which is  
260 also known as the  $pD_2$  value. The mean maximum contractile (MMC) effect is the  
261 maximum contractile effect produced by the highest concentration of drug (i.e.  
262  $10\mu\text{mol l}^{-1}$ ). Curve fitting was performed with the package Prism™ (Graphpad  
263 Software, San Diego, USA).

264

## 265 **Results**

### 266 Tissue Samples

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

276

277 For organ tissue bath studies umbilical arteries were obtained from a total of 12  
278 women following delivery. Of these 12 women, 8 underwent elective caesarean  
279 section. The reasons for elective caesarean section included previous caesarean  
280 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)  $\pm$  SEM was  $33.50 \pm 2.08$ ; 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  $0.5\mu\text{g}$   
305 total RNA, in both vessel types studied. Using the LightCycler™ Software version 3

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

313

#### 314 Umbilical Artery Expression

315 Beta-actin mRNA expression did not significantly differ between normotensive and  
316 pre-eclamptic umbilical arteries (Table 2), which indicated that beta-actin was suitable  
317 as a housekeeping gene for this vessel type. cDNA copy numbers for Rho A, ROCK I  
318 and ROCK II were therefore normalized to the beta-actin gene for determination of  
319 their absolute cDNA copy numbers per 0.5 $\mu$ g total RNA. Comparisons of cDNA  
320 copy numbers, between both groups, for Rho A, revealed that Rho A mRNA  
321 expression was significantly down-regulated in artery obtained from pre-eclamptic  
322 women in comparison to that measured in artery obtained from normotensive women  
323 ( $P < 0.05$ ). The cDNA copy numbers (per 0.5 $\mu$ g of total RNA)  $\pm$  the standard error of  
324 the mean (SEM) for Rho A were: (normal)  $7.0e+07 \pm 7.6e+06$  (n=6) and (pre-  
325 eclamptic)  $4.8e+07 \pm 4.5e+06$  (n=4) (Figure 3). The mRNA expression levels of  
326 ROCK I and ROCK II were not significantly different between the two vessel types  
327 analysed ( $P > 0.05$ ). The cDNA copy numbers for ROCK I were: (normal)  $1.3e+07 \pm$   
328  $8.7e+05$  (n=6); (pre-eclamptic)  $1.0e+7 \pm 1.9e+06$  (n=4) and for ROCK II were:  
329 (normal)  $5.2e+07 \pm 1.1e+07$  (n=6); (pre-eclamptic)  $3.0e+07 \pm 6.7e+05$  (n=4) (Figure  
330 3).

331

## 332 Effects of Isoprostanes on Umbilical Artery

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

346

## 347 Effects of Rho Kinase Antagonism on Umbilical Artery

348 8-*iso* PGE<sub>2</sub> 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<sub>2α</sub> induced contractions were not significantly antagonised (P>0.05)(Figure 4).  
351 The MMC and pD<sub>2</sub> values (± SEM) for antagonised 8-*iso* PGF<sub>2α</sub> and 8-*iso* PGE<sub>2</sub> are  
352 detailed in Table 3. There was no significant difference in pD<sub>2</sub> values for antagonised  
353 8-*iso* PGF<sub>2α</sub> and 8-*iso* PGE<sub>2</sub> in comparison to 8-*iso* PGF<sub>2α</sub> (P>0.05) and 8-*iso* PGE<sub>2</sub>  
354 (P>0.05) alone.

355

356 **Comment**

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

366

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



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

388

389 It is evident that isoprostanes contribute significantly to the prolonged  
390 vasoconstriction that occurs in pre-eclampsia. Using umbilical artery ring  
391 preparations, with standard in vitro techniques, we have demonstrated that the two  
392 isoprostanes 8-*iso* PGE<sub>2</sub> and 8-*iso* PGF<sub>2α</sub>, both exert a potent vasoconstrictor effect as  
393 has been demonstrated previously (Oliveira *et al.* 2000). By preincubation with a  
394 specific Rho kinase inhibitor it is clear from our experiments that 8-*iso* PGE<sub>2</sub> is unable  
395 to elicit the same response after Rho kinase inhibition, indicative of the fact that the  
396 Rho kinase pathway is involved in the vasoconstrictor effect of 8-*iso* PGE<sub>2</sub>. These  
397 results were not found for the vasoconstrictor of 8-*iso* PGF<sub>2α</sub>. There is no obvious  
398 reason why the effects of 8-*iso* PGF<sub>2α</sub> were apparently different to those of 8-*iso*  
399 PGE<sub>2</sub>, but it is evident that a different mechanism for 8-*iso* PGE<sub>2</sub> exists, which  
400 operates at least in part via the Rho kinase pathway. On speculation, this difference  
401 observed in relation to antagonism with Y-27632 can only be due to a relative  
402 difference in potencies observed in tissues, whereby 8-*iso*-PGE<sub>2</sub> is more potent  
403 (Oliveira *et al.*, 2000; Tazzeo *et al.*, 2003). There are no signalling pathways to our  
404 knowledge, known to operate via 8-*iso*-PGE<sub>2</sub> and 8-*iso*-PGF<sub>2α</sub> 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<sub>2</sub>, 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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430 **Acknowledgements**

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432 Galway for their assistance in patient recruitment and obtaining biopsy specimens.

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**528 Figure 1**

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

534

**535 Figure 2**

536 Quantitative real-time fluorescence RT-PCR amplification curve for Rho A mRNA  
537 expression in human umbilical artery (both normal and pre-eclamptic). Fluorescence  
538 is plotted on the y-axis and PCR cycle number on the x-axis. Continuous lines  
539 represent the Rho A cDNA standards ( $1 \times 10^9$  and  $1 \times 10^7$  cDNA copy numbers). Closed  
540 circles represent normal samples (n=6), open circles represent pre-eclamptic samples  
541 (n=4) and closed squares represent the water control.

542

**543 Figure 3**

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

552

**553 Figure 4**

554 The effects of 8-*iso* PGF<sub>2α</sub> (alone and following Y-27632 addition) on human  
555 umbilical artery tone. The graph depicts the effects of cumulative increases in bath  
556 concentration of 8-*iso* PGF<sub>2α</sub> (1nM-10μM) at 20 minute intervals. Open squares  
557 represent 8-*iso* PGF<sub>2α</sub> (following Y-27632 addition) and closed squares represent 8-  
558 *iso* PGF<sub>2α</sub> (alone). Contractility (g Tension) is shown on the y-axis, and the  
559 concentration of 8-*iso* PGF<sub>2α</sub> is shown on the x-axis. Values plotted are means.  
560 Vertical error bars represent the standard error of the mean (SEM).

561

562

**563 Figure 5**

564 The effects of 8-*iso* PGE<sub>2</sub> (alone and following Y-27632 addition) on human placental  
565 artery tone. The graph depicts the effects of cumulative increases in bath  
566 concentration of 8-*iso* PGE<sub>2</sub> (1nM-10μM) at 20 minute intervals. Open squares  
567 represent 8-*iso* PGE<sub>2</sub> (following Y-27632 addition) and closed squares represent 8-*iso*  
568 PGE<sub>2</sub> (alone). Contractility (g Tension) is shown on the y-axis, and the concentration  
569 of 8-*iso* PGE<sub>2</sub> is shown on the x-axis. Values plotted are means. Vertical error bars  
570 represent the standard error of the mean (SEM). \* 8-*iso* PGE<sub>2</sub> versus Y-27632 & 8-  
571 *iso* PGE<sub>2</sub>, P<0.05; \*\* 8-*iso* PGE<sub>2</sub> versus Y-27632 & 8-*iso* PGE<sub>2</sub>, P<0.001.

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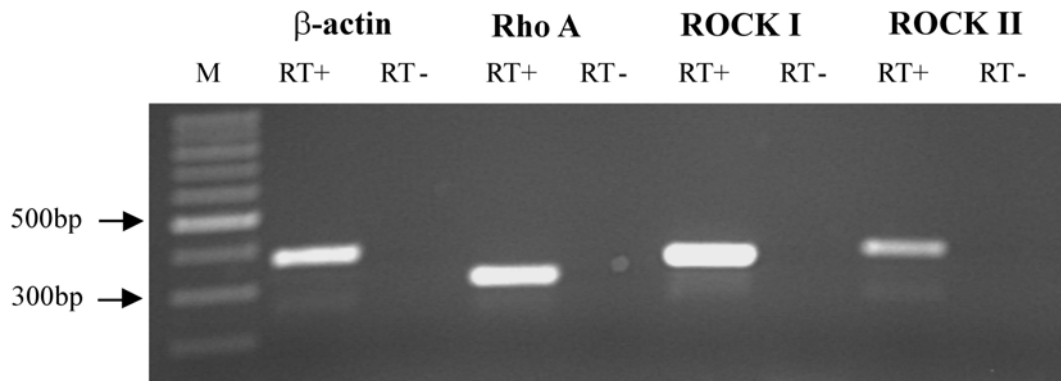
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580 **Figure 1**

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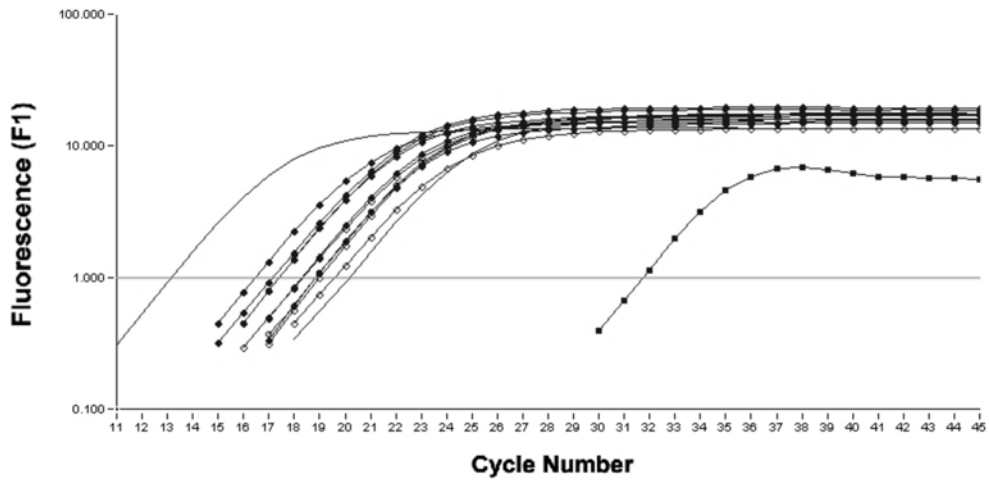
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600 **Figure 2**



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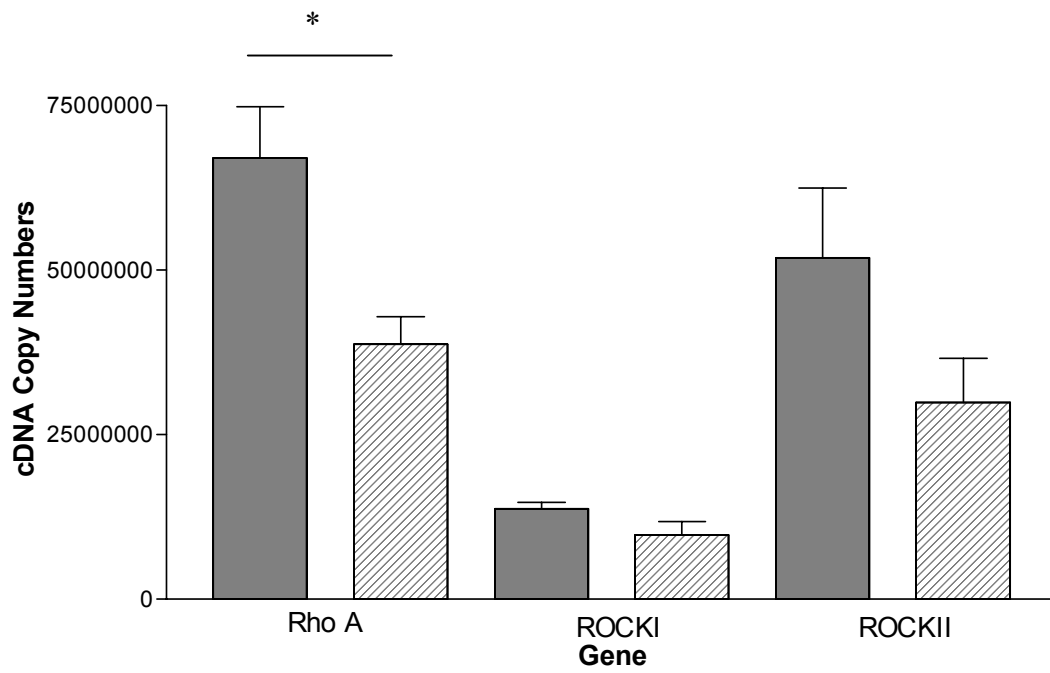
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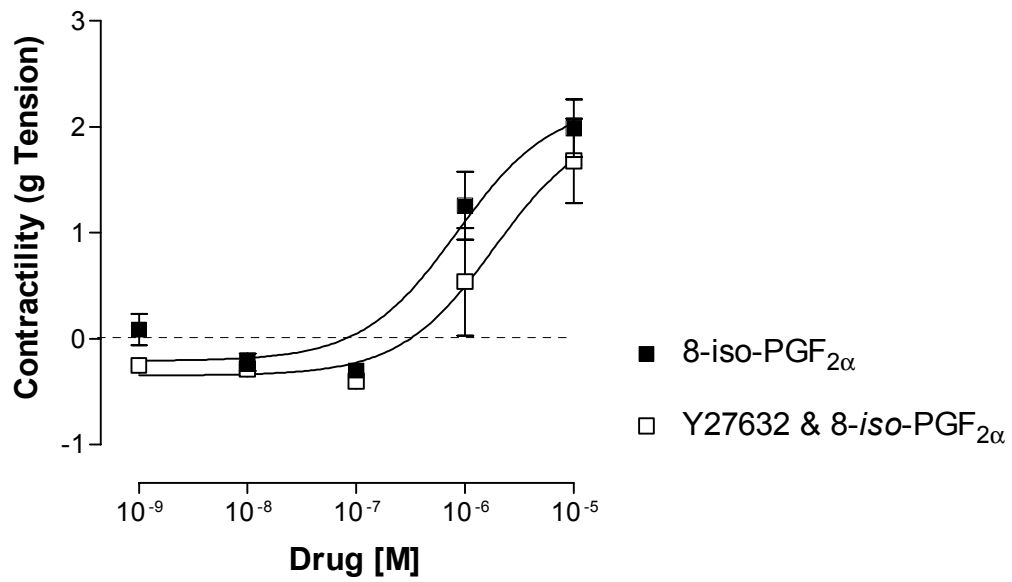
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639 **Figure 4**

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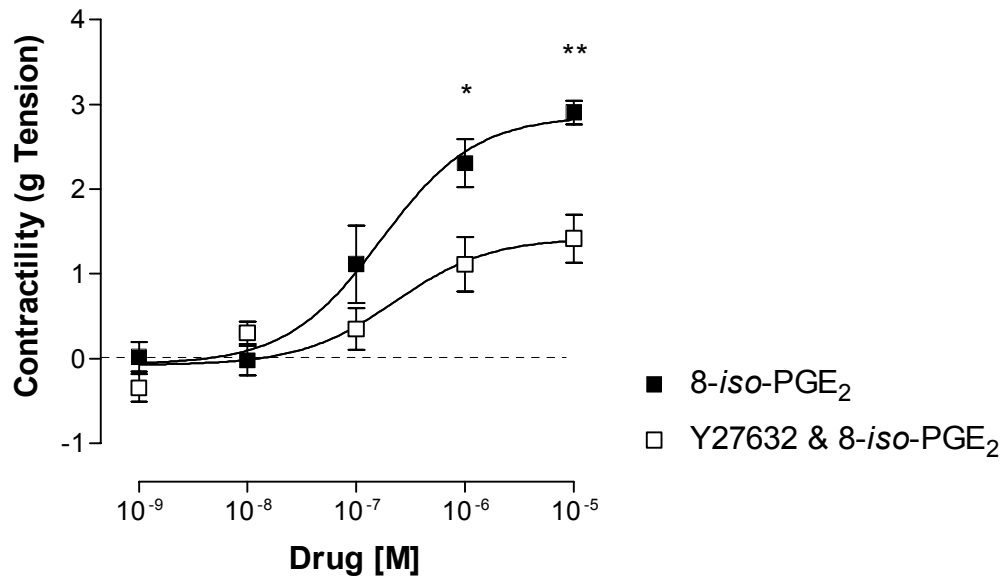
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654 **Figure 5**

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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**

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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'-ATCTTG TAGCTCCCGCATCTGT-3'
Human ROCK II	sense	5'-AATTC ACTGTGTTTCCCTGAAGATA-3'
(Accession Code: XM_002676)	antisense	5'-TTCATTTTTCTTGATTGTATGGAA-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  $\pm$  the standard error of the mean (SEM) for Rho A,  
 688 ROCK I, ROCK II and  $\beta$ -actin in human umbilical artery (both normal and pre-  
 689 eclamptic)

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<b>Gene</b>	<b>Normal</b>	<b>Pre-eclamptic</b>
<b>Rho A</b>	7.0e+07 $\pm$ 7.6e+06	4.8e+07* $\pm$ 4.5e+06
<b>ROCK I</b>	1.3e+07 $\pm$ 8.7e+05	1.0e+07 $\pm$ 1.9e+06
<b>ROCK II</b>	5.2e+07 $\pm$ 1.1e+07	3.0e+07 $\pm$ 6.7e+05
<b><math>\beta</math>-actin</b>	2.8e+08 $\pm$ 4.5e+07	4.1e+08 $\pm$ 9.6e+07

691

692 Values presented are means  $\pm$  the standard error of the mean.

693 \*P<0.05 v Normal

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707 **Table 3.** Effects of 8-*iso* PGE<sub>2</sub> and 8-*iso* PGF<sub>2α</sub> alone and antagonised by Y-27632 on  
 708 Human Umbilical Arterial Tone

Umbilical Artery		
Drug	Contractility (g tension)	pD <sub>2</sub>
8- <i>iso</i> PGE <sub>2</sub>	2.91 ± 0.14 (n=7)	6.77 ± 0.13
8- <i>iso</i> PGE <sub>2</sub> + Y-27632	1.42 ± 0.28* (n=6)	6.65 ± 0.49
8- <i>iso</i> PGF <sub>2α</sub>	1.99 ± 0.27 (n=7)	6.07 ± 0.35
8- <i>iso</i> PGF <sub>2α</sub> + Y-27632	1.68 ± 0.40 (n=6)	5.73 ± 0.18

717

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

719 \*P<0.01 versus 8-*iso* PGE<sub>2</sub> alone