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ENDOTHELIN-1 RELEASE FROM ENDOTHELIAL CELLS IN CULTURE IS ELEVATED BOTH ACUTELY AND CHRONICALLY BY SHORT PERIODS OF MECHANICAL STRETCH

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Summary. The effects were examined of mechanical stretch on the release of endothelin-1 (ET-1) and prostacyclin (measured as 6-keto-prostaglandin (PG) $F_{1\alpha}$) from cultured endothelial cells. Stretching (0.2 Hz) for 20, 60 or 360 min caused immediate ($\leq$20 min) and secondary (up to 360 min) increases in ET-1 release. The secondary but not immediate release of ET-1 was prevented by actinomycin D (8×10^{-7} M) or cycloheximide (3.6×10^{-6} M). Neither compound affected the release of ET-1 from unstretched cells over 360 min. Stretching of the endothelial cells increased the accumulation of 6-keto-$PGF_{1\alpha}$ at 360 min but not at 20 min, suggesting that stretch does not produce a rapid, non-selective increase in autacoid production from endothelial cells. The intracellular amounts of ET-1 were approximately 20 times greater than those of big ET-1. Thus, endothelial cells contain stores of ET-1 that are released rapidly by stretch.

Mechanical forces alter the structure and function of many different cell types including smooth muscle cells, osteoblasts, bone cells and epithelial cells (1, 2, 3). Because vascular endothelial cells line the blood vessel wall they are continuously exposed to stretch, as a consequence of vessel pulsation, and shear stress from blood flow (see 4). These forces may well be the most important regulators of the release of vasodilator (5, 6, 7) and vasoconstrictor (8,9) autacoids both acutely and chronically.

Endothelin-1 (ET-1) is a 21 amino acid peptide produced by the endothelium and is the most potent mammalian vasoconstrictor peptide known (10). Many endothelial cell stimulants such as thrombin cause a delayed (i.e. hours), but not immediate (i.e. minutes), elevation in the production of ET-1 which appears to depend upon an increased synthesis of the peptide (11). Thus, it has not been clear why some stimuli, such as cold stress (12), can cause immediate increases in the circulating levels of endothelin, particularly as secretory granules cannot usually be detected in endothelial cells (11). Here we show that stretching of

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bovine aortic endothelial cells produces a rapid (≤20 min) and prolonged increase (≥360 min) in ET-1 release. This is due to release of ET-1 from stores within the endothelial cells for HPLC analysis revealed the amounts of big ET-1 stored intracellularly to be approximately 20-fold lower than those of mature ET-1. Thus, endothelial cells contain stores of ET-1 that can be released rapidly.

MATERIALS AND METHODS

Materials. The Locke's buffer had the following composition (mM): NaCl, 154; KCl, 5.6; $CaCl_2$, 2; $MgCl_2$, 1.0; $NaHCO_3$, 3.6; glucose, 5.6; HEPES, 10; pH 7.4. Actinomycin D, bovine serum albumin, cycloheximide, 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) and 6-keto-$PGF_{1\alpha}$ antiserum were purchased from Sigma Chemical Co., Poole, Dorset, U.K. ET-1 and porcine big ET-1 was obtained from Scientific Marketing Associates, Enfield, Middlesex, U.K. ^{125}I-ET-1, ^{125}I-Bolton and Hunter reagent (N-succinimidyl 3-(4-hydroxy-5-[^{125}I]-iodophenyl) propionate) and ^{3}H-6-keto-$PGF_{1\alpha}$ were bought from Amersham International, Aylesbury, Buckinghamshire, U.K. ^{125}I-porcine big ET-1 was prepared using ^{125}I-Bolton and Hunter reagent, and the product was purified by HPLC.

Culture of endothelial cells. Endothelial cells were isolated from bovine aortae as described previously (13). The cells were seeded onto individual round culture dishes (22 cm^2) with flexible bottoms (Petriperm, Bachoffer GMBH, Germany) and allowed to grow to confluence.

Stretching of endothelial cells. The medium from the cells was removed and replaced with 2 ml of warmed (37 °C) Locke's buffer containing 5 % w/v bovine serum albumin, and in some experiments, cycloheximide (3.6×10^{-6} M) or actinomycin D (8×10^{-7} M). The culture dishes were placed on a stretching apparatus (14), inside an incubator (37 °C), and stretched at 0.2 Hz for 20, 60 or 360 min, or in control experiments left unstretched. Buffer from individual dishes was collected at 0, 20, 120 and 360 min and the amounts of ET-1 and 6-keto-$PGF_{1\alpha}$ (the stable hydrolysis product of PGI_2) determined by specific radioimmunoassay. In some experiments cells were lysed by addition of HCl (0.1 M) and the lysate from 8 separate plates pooled for separation by HPLC prior to detection of the contents of ET-1 and big ET-1 by selective radioimmunoassay. Stretching did not diminish cell viability, as assessed by the mitochondrial reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan (15).

HPLC analysis and radioimmunoassay. ET-1 and big ET-1 were separated by HPLC using a column of 5 μm TSK gel ODS-120T, (4.6 x 250 mm, TOSOH Corporation, Japan from Anachem, Luton, Bedfordshire). Samples were first acidified and extracted using TechElut SPE C_{18} columns (HPLC Technology, Macclesfield, Cheshire), eluted with 1 ml of 80% acetonitrile containing 0.1% trifluoroacetic acid, concentrated under a stream of N_2, and injected onto the HPLC system. The HPLC column was eluted at a flow rate of 1 ml min^{-1} with a gradient of acetonitrile in 0.1% trifluoroacetic acid using a Pharmacia LKB model 2249 pump. The gradient used was 0-20% over 3 min, 20-32% over 12 min, 32-40% over 15 min, and 40-48% over 8 min. The column effluent was monitored at 280 nm using a Pharmacia LKB model 2141 variable wavelength monitor. Fractions were collected every 30 s and numbers 50-65 were dried down for radioimmunoassay. Radioimmunoassay for ET-1 was performed using an antiserum raised to $ET\text{-}1_{(16\text{-}21)}$ (ref. 16) and for big ET-1 using an antiserum to porcine big ET-1 (Peptide Institute).

Statistics. Results are expressed as the mean ± s.e. mean of n dishes. Data was compared by unpaired *t*-test and $p<0.05$ taken as significant. Experiments were performed on at least 3 different experimental days.

RESULTS

Basal releases of ET-1 and 6-keto-PGF$_{1\alpha}$. The releases of ET-1 (figure 1) and 6-keto-PGF$_{1\alpha}$ (figure 2) from unstretched endothelial cells over 360 min were 215±33 fmol/ml (n=7) and 351±65 pmol/ml (n=7), respectively.

Effects of stretch on the release of ET-1. Stretching of the endothelial cells produced a rapid and significant increase in ET-1 accumulation at 20 min (unstretched, 68±30 fmol/ml, n=7; stretched, 171±17 fmol/ml, n=6, $p<0.05$), and at the subsequent time points (20, 120 and 360 min, figure 1). Stretching of the endothelial cells for 20 or 60 min produced the same increase in ET-1 accumulation at 360 min as did stretching for the whole experimental period (360 min) (figure 1).

Effects of stretch on the release of 6-keto-PGF$_{1\alpha}$. Stretch did not increase the release of 6-keto-PGF$_{1\alpha}$ at any time point except at 360 min (unstretched, 351±65 pmol/ml, n=7; stretched, 675±76 pmol/ml, n=7, $p<0.05$) (figure 2). Stretching for 20 or 60 min also similarly elevated the release of 6-keto-PGF$_{1\alpha}$ at 360 min ($p<0.05$).

Effects of actinomycin D or cycloheximide. Actinomycin D (8×10^{-7} M) or cycloheximide (3.6×10^{-6} M) greatly decreased the release of ET-1 caused by stretching, such

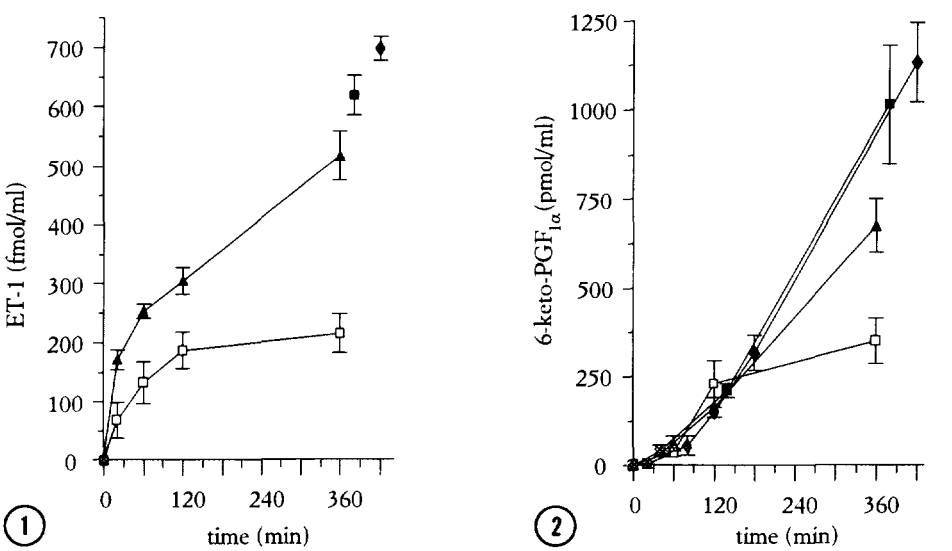

Figure 1. Stretch increases the release of ET-1 from bovine aortic endothelial cells. Stretching of endothelial cells for 20 (■), 60 (◆), or 360 min (▲) increased the accumulation of ET-1 above that from unstretched cells (□) at all time points. Each point represent the mean ± s.e.m. from 3-7 experiments. Data for 20 and 60 min of stretch have been omitted at the lower time points for clarity.

Figure 2. Stretch increases the release of 6-keto-PGF$_{1\alpha}$ from bovine aortic endothelial cells. Stretching of endothelial cells for 20 (■), 60 (◆), or 360 min (▲) increased the accumulation of 6-keto-PGF$_{1\alpha}$ above that from unstretched cells (□) at 360 min. Each point represent the mean ± s.e.m. from 3-7 experiments.

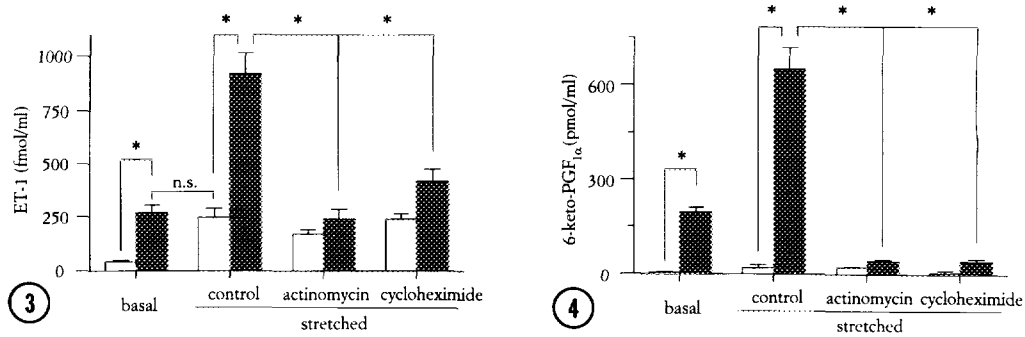

Figure 3. Actinomycin D (8×10^{-7} M) or cycloheximide (3.6×10^{-6} M) prevent the elevation in ET-1 production caused by stretching at 360 min but not at 20 min. Each bar with a vertical line represents the mean ± s.e.m. from 3-5 experiments. Open bars, release at 20 min; closed bars, release at 360 min.

Figure 4. Actinomycin D (8×10^{-7} M) or cycloheximide (3.6×10^{-6} M) prevent the elevation in 6-keto-$PGF_{1\alpha}$ production caused by stretching at 360 min. Each bar with a vertical line represents the mean ± s.e.m. from 3-5 experiments. Open bars, release at 20 min; closed bars, release at 360 min.

that the accumulation of ET-1 at 360 min was not significantly different from that at 20 min (figure 3). The release of ET-1 from unstretched cells over 360 min was unaffected by either agent (control, 267±36 fmol/ml, n=5; +actinomycin D, 218±48 fmol/ml, n=5; +cycloheximide, 385±31 fmol/ml, n=5).

Actinomycin D or cycloheximide significantly ($p<0.05$) depressed the release of 6-keto-$PGF_{1\alpha}$ from unstretched cells over 360 min (control, 194±16 pmol/ml, n=5; +actinomycin D, 23±3 pmol/ml, n=5; +cycloheximide, 46±7 pmol/ml, n=5) and completely prevented the increased accumulation caused by stretching (figure 4). Neither agent affected the amounts of 6-keto-$PGF_{1\alpha}$ accumulated at 20 min.

Intracellular content of ET-1 and big ET-1. The intracellular content of big ET-1 was appoximately 20-fold less than that of ET-1 (table 1). Interestingly, stretching for 360 min increased the intra- and extracellular amounts of both ET-1 and big ET-1.

Table 1. The amounts of ET-1 and big ET-1 at 0 and 360 min intracellularly and extracellularly in unstretched or stretched endothelial cells. Each data point represents samples pooled from 8 culture plates which were then fractionated by HPLC and analysed by radioimmunoassay.

Peptide	Time	Unstretched cells		Stretched cells	
(fmol/plate)	(min)	Intracellular	Extracellular	Intracellular	Extracellular
ET-1	0	191	0	191	0
	360	199	60	236	167
big ET-1	0	5.9	0	5.9	0
	360	8.8	7.2	7.9	29.5

DISCUSSION

Here we show that stretching of endothelial cells causes both an immediate (≤20 min) release of ET-1 and an increase in the production and release of ET-1 over the subsequent 360 min. This prolonged elevation in ET-1 production is not dependent upon continued stretching of the cells as stretching for 20, 60 or 360 min produced the same increase in ET-1 release at 360 min.

Actinomycin D (which inhibits the transcription of mRNA) or cycloheximide (which inhibits protein synthesis) did not affect the increase in ET-1 release in the first 20 min of stretch, but did prevent the further increase seen at 360 min. Interestingly, the release of ET-1 from cells stretched for 20 min was not different from that released by unstretched cells in 360 min, and the release of ET-1 from unstretched cells was not affected by either actinomycin D or cycloheximide. This suggests that for periods of ≤360 min the release of ET-1 from static endothelial cells is supportable by preformed intracellular stores. Stretching may cause a rapid release of these stores which are then replenished by *de novo* peptide synthesis. This conclusion is supported by studies which show within endothelial cells staining for ET-1 (17) and the presence of ET-1-rich structures following subcellular fractionation (18). It appears unlikely that the endothelial cells store ET-1 in the form of its precursor big ET-1 for the intracellular amounts of big ET-1 in our cells were 20-fold less than those of ET-1. This data is in accordance with that of others who have also found the intracellular amount of ET-1 in endothelial cells to exceed that of big ET-1 (19). Interestingly, the release of ET-1 from unstretched cells plateaued after 120 min, suggesting that the mechanical disturbance caused by changing the medium at t=0 caused a transient increase in ET-1 production, similar to, but not as marked as that produced by stretching.

Stretching of endothelial cells also increased the accumulation of 6-keto-$PGF_{1\alpha}$ at 360 min but not at the earlier time points. This suggests that the degree of stretch we used was a relatively gentle stimulus for mechanical forces are potent, immediate releasers of prostaglandins (20, 21). The elevated release of 6-keto-$PGF_{1\alpha}$ was not seen when cells were treated with actinomycin D or cycloheximide indicating that it was secondary to increased mRNA transcription and protein synthesis. Thus, stretch may regulate the synthesis of the enzymes responsible for prostacyclin formation, such as phospholipase A_2, cyclo-oxygenase and prostacyclin synthase. As for ET-1 this increase in protein synthesis appears to be an early response to stretching because stretching of the endothelial cells for 20, 60 or 360 min caused similar elevations in 6-keto-$PGF_{1\alpha}$ at 360 min. Actinomycin D or cycloheximide also depressed the release of 6-keto-$PGF_{1\alpha}$ from unstretched cells over 360 min, a finding that reflects the short half-life of endothelial cell cyclo-oxygenase (approximately 20-30 min; 22).

Thus, stretching of endothelial cells causes the immediate release of ET-1 and influences the release of both ET-1 and prostacyclin at longer time periods. The release of ET-1 is thus dependent both upon *de novo* synthesis and upon release from pre-formed intracellular stores.

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