Regulation of adrenomedullin release from human endothelial cells by sex steroids and angiotensin-II

Lachlan J Pearson, Christopher Rait, M Gary Nicholls[1], Timothy G Yandle[1] and **John J Evans**

Department of Obstetrics and Gynaecology and [1]Department of Medicine, Christchurch School of Medicine and Health Sciences, University of Otago, Christchurch, New Zealand

(Requests for offprints should be addressed to J J Evans; Email: john.evans@chmeds.ac.nz)

Abstract

It is well documented that there are gender differences in the incidence and patterns of cardiovascular diseases but the reasons are unclear. Sex steroids may modulate the behaviour of vascular endothelial cells, which in turn act by paracrine processes to alter adjacent vascular smooth muscle activity. We hypothesised that the sex steroids alter the percentage of vascular endothelial cells that secrete the vasodilator peptide, adrenomedullin and modify the adrenomedullin-stimulating action of angiotensin-II. The percentage of adrenomedullin-secreting human aortic endothelial cells was determined using the cell immunoblot method. Cells were incubated with selected concentrations of angiotensin-II, oestradiol and testosterone alone and sex steroids in combination with angiotensin-II. Adrenomedullin mRNA expression in endothelial cells was quantified by real-time PCR. It was observed that at 4 h, angiotensin-II increased the percentage of adrenomedullin-secreting cells in a concentration-dependent manner. Testosterone at physiological concentrations was observed to increase the number of adrenomedullin-secreting cells whilst oestradiol had no effect. Addition of testosterone to angiotensin-II resulted in less than additive increases in the number of cells secreting adrenomedullin. Oestradiol reduced the angiotensin-II-induced increase in adrenomedullin-secreting cells. Adrenomedullin mRNA expression was significantly increased by testosterone and there was also a trend for an increase in adrenomedullin mRNA expression, which occurred when cells were incubated with angiotensin-II. Our results point to a complex interplay between the sex steroids and angiotensin-II in regulating adrenomedullin production by human endothelial cells, which may contribute to gender-related differences in vascular disease in humans.

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Introduction

It is well established that there are gender-related differences in human vascular disease. Numerous studies have indicated that blood vessel contractility is sexually dimorphic and/or influenced by sex hormones (Orshal & Khalil 2004) and both testosterone and oestradiol have a number of effects on the vasculature. Oestradiol has many reported vascular benefits in women, such as improving endothelial-dependent vasodilation (Pinto *et al.* 1997, Virdis *et al.* 2000) and regulation of cell proliferation (Farhat *et al.* 1996). However, clinical trials of oestradiol in postmenopausal women have provided equivocal evidence of benefit (Khalil 2005). Investigations regarding the actions of testosterone have produced contradictory results (Kravariti *et al.* 2005, Rosano *et al.* 2005). For example, both vasodilatory (Webb *et al.* 1999) and anti-vasodilatory effects have been observed (Hutchison *et al.* 1997, Ceballos *et al.* 1999) and testosterone may be involved in the development of some forms of experimental hypertension (Song *et al.* 2004, Reckelhoff 2005). Endothelial cells have receptors for both oestradiol (Losordo *et al.* 1994) and testosterone (Sierra-Ramirez *et al.* 2004), by which these steroids can directly influence blood vessels and hence the development of vascular disease and hypertension.

It is known that a complex network of neuroendocrine factors regulate blood vessel function and many input signals modulate the production and/or action of each factor (Isumi *et al.* 1998). One peptide, adrenomedullin, has demonstrated vasodilator effects *in vitro* as well as hypotensive actions *in vivo* (Kitamura *et al.* 1993, Nakamura *et al.* 1997). Adrenomedullin plasma levels are elevated in patients with essential hypertension, congestive heart failure and renal failure (Ishimitsu *et al.* 1994, Kitamura *et al.* 1994, Kato *et al.* 1996). Further, low-dose i.v. infusion of the hormone elicits clear biological effects at pathophysiologically relevant plasma levels (Lainchbury *et al.* 2000). Adrenomedullin is produced by a wide range of cell types, but plasma adrenomedullin appears to be derived mainly from endothelial cells, which have been reported to secrete large amounts of the peptide (Hinson *et al.* 2000, Beltowski & Janroz 2004). Therefore, identifying factors which act on endothelial cells to modify adrenomedullin secretion is of vital interest for defining blood vessel physiology and pathophysiology. In addition, adrenomedullin receptors have been detected on endothelial cells (Kato *et al.* 1995), suggesting that a complete

understanding of adrenomedullin control may clarify paracrine and autocrine processes, which contribute to vascular structure, function and dysfunction.

Another peptide, angiotensin-II, is well known for its vasoconstrictor action. It has been reported that angiotensin-II induces a rise in adrenomedullin levels *in vivo* (Onitsuka *et al.* 2005). Conversely, adrenomedullin inhibited the pressor activity of angiotensin-II (Charles *et al.* 2000, Luodonpaa *et al.* 2004) and also inhibited angiotensin–II–induced contraction in rat aortic rings, an action partially mediated by NO^- (Haulica *et al.* 2004). Hence, there appears to be indications of important physiological interactions between angiotensin-II and adrenomedullin (Charles *et al.* 2003). However, there are few studies that have investigated the interactions between adrenomedullin and angiotensin-II in endothelial cells.

In a previous study, it was reported that neither oestradiol nor testosterone influenced adrenomedullin mRNA levels in cultured rat aortic endothelial cells (Imai *et al.* 1995). On the other hand, both testosterone and oestradiol were shown to increase immunoreactive adrenomedullin secretion from rat aortic endothelial cells in culture (Isumi *et al.* 1998). We studied here the possible interaction between the two steroids, testosterone and oestradiol, with angiotensin-II and each separately in the modulation of adrenomedullin secretion. Although these compounds are concurrently present in blood vessels, their combined vascular effects are not established.

The cell immunoblot method, in which cells are incubated on a protein-binding membrane, has been extensively employed by our laboratory (Evans *et al.* 2002, 2003) and others (Arita 1993, Kusaka *et al.* 2000). The method uses singly dispersed cells, and in contrast to other methods in which cells are cultured in high density, is able to reduce effects of paracrine interactions, and can investigate functional diversity which may be present in endothelial cells (Frid *et al.* 2004). In this study, we used the cell immunoblot method to investigate whether testosterone, oestradiol and angiotensin-II, separately and together regulate adrenomedullin secretion at the level of the individual cell using human aortic endothelial cells.

Materials and Methods

Human endothelial cells

Human aortic endothelial cells were purchased (Clonetics, CA, USA) and cultured in endothelial basal medium (EBM) medium supplemented with endothelial growth medium (EGM) Bulletkit reagents (Cambrex, Walkersville, MD, USA). Cells were passaged on 10 cm collagen-coated dishes using trypsin/EDTA to release cells. Confluent cells at passages 7–9 were harvested using trypsin/EDTA and suspended in bicarbonate-free Dulbecco's modified Eagle medium (DMEM) medium containing N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES) buffer (pH 7·4) and 0·1% BSA. The cells were used within 1 h of harvest.

Cell immunoblot assay

Immobilon P membrane (Millipore Corporation, Bedford, MA, USA), a protein-binding membrane, was cut into strips and immersed in methanol for 20 s and allowed to dry. Glass incubation cylinders (internal diameter and height, 5 and 9 mm respectively) were sealed to the membrane using silicon grease and the unit was transferred to humidified six-well culture dishes (well diameter 35 cm). The membrane within each cylinder was incubated with 100 μl DMEM for 15 min and then the medium was aspirated off. An aliquot of cells (100 μl) was added and allowed to settle in the humidified chambers at 37 °C for 60 min. The supernatant was removed and discarded. Medium only or medium containing angiotensin-II (Bachem AG, Switzerland; 0·0001–10 μM), testosterone (Sigma-Aldrich; 3·5–3500 nM) or β-oestradiol (Sigma-Aldrich; 0·0037–3700 nM) was then added. Combinations of angiotensin-II (0·01–10 μM) with oestradiol (37 nM) or testosterone (350 nM) added together were also used. The cells were incubated for 4 h, after which the supernatant was removed. Glutaraldehyde (100 μl, 2·5% (v/v); EM grade, ProSciTech) was added for 1 h at room temperature. The cells were washed 3× (10 min each) in 100 μl Tris–HCl buffer (100 mM, pH 8·2). Blocking buffer (Tris–HCl buffer containing 3% BSA) was added for 1 h followed by 100 μl rabbit anti-adrenomedullin antiserum 1/3000 in Tris–HCl containing 0·3% BSA. The cells were incubated overnight at 4 °C and then washed 3× in phosphate buffer (pH 7·4) containing 0·3 M NaCl (PBS). Secondary antibody coupled to biotin (in PBS) was added at 1/1000 (goat anti-rabbit-IgG–biotin, Sigma-Aldrich) for 1 h and washed 3× in PBS. Adrenomedullin was visualised using alkaline phosphatase (Vectorstain kits, Vector Laboratories, Burlingame, CA, USA), which produced Vector Red product. Cells that secreted adrenomedullin were associated with a stained area on the Immobilon P membrane surrounding the cell. Control incubations were submitted to normal rabbit serum (Sigma-Aldrich) and to only secondary antibody to identify any non-specific binding.

The glass incubation cylinders were removed from the membrane strips. The membranes were passed through 70% ethanol, 100% ethanol and xylene and rehydrated and the cells counterstained with light green CI42095 (Gurr, product no. 34204; 1% in water). The cells were dehydrated in increasing concentrations of ethanol, cleared in xylene and permanently mounted. The number of cells that secreted adrenomedullin was manually counted by two independent observers blinded to the treatments. Secretion was observed as colour development around the outside of the cell. The number of secreting cells was converted to a percentage of the total number of cells in the counted sample; approximately 300 cells were counted in each incubation. Each treatment was repeated in three incubations during each separate experiment. Results are presented as mean ± S.E.M. For the dose–response studies of angiotensin-II and testosterone, the percentage of cells that secreted adrenomedullin in the groups at each concentration were compared to the results in incubations without added

peptide and statistically analysed by Student's *t*-test. The data were treated as a single set and submitted to the paired *t*-test in the experiment in which cells were exposed to testosterone that was added to angiotensin-II (incubations with angiotensin-II versus incubations with angiotensin-II + testosterone). Results of $P < 0.05$ were taken to indicate a statistically significant difference between groups.

Adrenomedullin RIA

The adrenomedullin RIA was based on the work of Lewis *et al.* (1998). Human aortic endothelial cells were grown in EGM-2 growth media to approximately 90% confluence in six-well plates. A 1 h preincubation of cells in serum-free DMEM 0·1% BSA was performed. The media were then replaced with fresh DMEM 0·01% BSA with or without either angiotensin-II (1 µM) or testosterone (100 ng/ml). After 4 h, the media were collected and immediately acidified with acetic acid (1 M). Triton-X 0·001% was added and the solution was boiled for 10 min. The samples were stored at −20 °C and were thawed and centrifuged, and supernatants mixed with an equal volume of 0·1% Triton-X. Samples were added under gravity to Sep-Pak C_{18} cartridges (Waters) and washed with 0·9% saline under low pressure (<17 kPa). Adrenomedullin was eluted with 2 ml of 80% isopropanol/0·013 M HCl into tubes containing 10 µl of 1% Triton. Tubes were air-dried and stored at −20 °C until assayed. Extracts were reconstituted with 300 µl phosphate-alkali treated casein (PATC) buffer and centrifuged. Antiserum and I-labelled adrenomedullin were added on ice, and the tubes were incubated at 4 °C for 24 h. Bound and free fractions were separated using secondary antibody. After centrifugation, the pellet was counted for radioactivity. Results were interpolated from a computer-fitted standard curve.

Real-time PCR

Human aortic endothelial cells were grown to approximately 90% confluence on six-well plates in EGM. One hour prior to the experiment, the medium was replaced with serum-free DMEM 0·1% BSA. At time zero, cells were incubated in fresh DMEM 0·1% BSA without or with testosterone (350 nM) or angiotensin-II (1 µM). At time points −1, 0, 1 and 3 h, RNA was extracted using the RNeasy Mini Kit (Qiagen) as specified in the RNeasy mini handbook. Concurrently, other cells were incubated in unchanged EGM in the presence or in the absence of testosterone before RNA extraction at 3 h. Cells were washed with physiological PBS, lysed in lysis buffer provided in the kit, removed from the plate with a cell scraper and homogenised using a syringe and needle. Homogenate was mixed with 70% ethanol and added to RNeasy mini columns. RNA was eluted with 40 µl water. RNA quantity and purity were analysed with a spectrophotometer (ND-1000, NanoDrop). Agarose gel electrophoresis was performed to test for RNA degradation. All RNA was diluted to 27 ng/µl and reverse transcribed with

a blend of oligo–dT and random primers (Qiagen) in a Thermal Cycler (Palm Cycler, Corbett Research). cDNA levels were measured and diluted to under 50 ng/µl. Semiquantitative PCR using Syber Green PCR kit (Qiagen) was performed with a real-time DNA amplification system (Rotagene 3000, Corbett Research). Bioinformatically validated primers included a housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (Qiagen Cat no. QT 00079247) and adrenomedullin (Qiagen Cat no. QT00223846). Product purity was checked with a melt curve and Ct values were derived using Rotagene 3000 software (Corbett Research). A standard curve was generated from RNA extracted from human aortic endothelial cells. Both genes had amplification efficiencies greater than 0·9 and had linear logarithmic concentration curves with regression coefficients greater than 0·98. The Ct method was selected for analysis of samples as stated in the Quantitect Primer Assay Handbook (Qiagen). All PCRs were performed in at least duplicate. Controls included a no template control and negative control.

Results

Approximately, one-tenth (9·7 ± 0·7%) of the endothelial cells in basal medium were observed to secrete the vasodilatory peptide, adrenomedullin, during the 4 h incubation period. Exposure of the cells to angiotensin-II for 4 h induced an increase in the number of adrenomedullin-secreting cells in a concentration-dependent manner (Fig. 1). A significant increase was observed at 1 nM angiotensin-II.

Testosterone added to the incubation medium likewise caused an increase in the numbers of cells secreting adrenomedullin in a concentration-dependent manner (Fig. 2). In contrast, oestradiol had no significant effect on
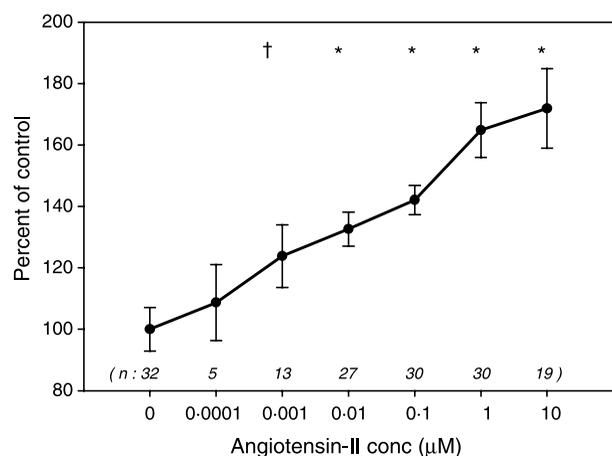

Figure 1 The effect of increasing concentrations of angiotensin-II on the percentage of endothelial cells that secreted adrenomedullin after 4 h incubation. A concentration-dependent effect was observed (*$P < 0.001$; [†]$P < 0.05$ compared with control incubations performed in the absence of angiotensin-II).
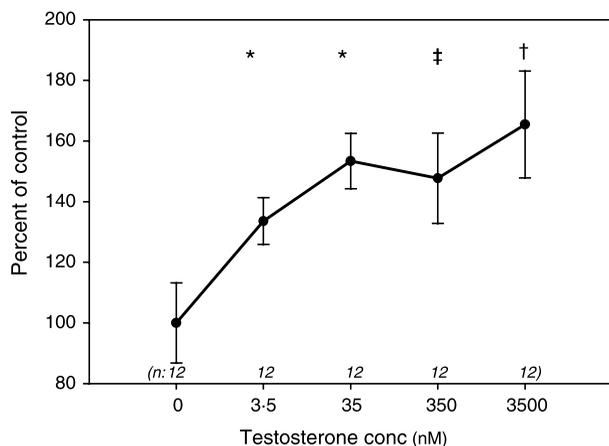
Figure 2 The effect of increasing concentrations of testosterone on the percentage of endothelial cells that secreted adrenomedullin after 4 h incubation. A concentration-dependent effect was observed (*$P<0.001$; †$P<0.01$; ‡$P<0.05$ compared with control incubations performed in the absence of testosterone).

the percentage of cells in basal medium that secreted adrenomedullin (Fig. 3). Treatment of endothelial cells with testosterone and angiotensin-II together significantly increased the percentage of cells that secreted adrenomedullin compared to that with angiotensin-II alone ($P<0.05$; Fig. 4). The increases induced by angiotensin-II and testosterone in combination were less than additive compared to their independent actions. On the other hand, oestradiol exhibited a significant inhibition on the stimulatory effect of angiotensin-II ($n=14$, paired t-test, $P<0.05$).

RIAs of the supernatants of incubated endothelial cells confirmed the release of adrenomedullin under basal conditions (22 fmole/10^6 cells) at 4 h, but no significant changes in adrenomedullin concentrations were observed following exposure to testosterone, oestradiol or angiotensin-II.
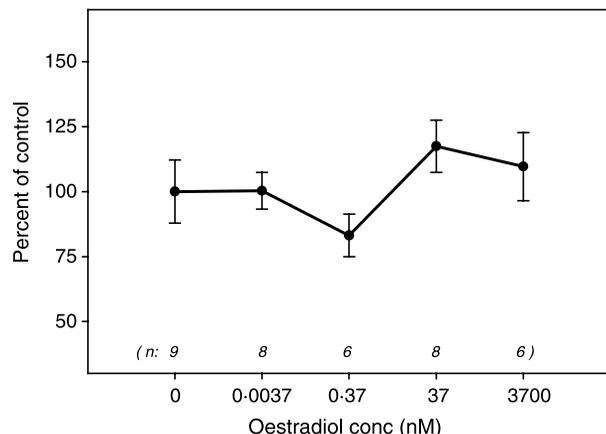

Figure 3 No significant effect of a series of concentrations of oestradiol was observed on the percentage of endothelial cells that secreted adrenomedullin after 4 h incubation.
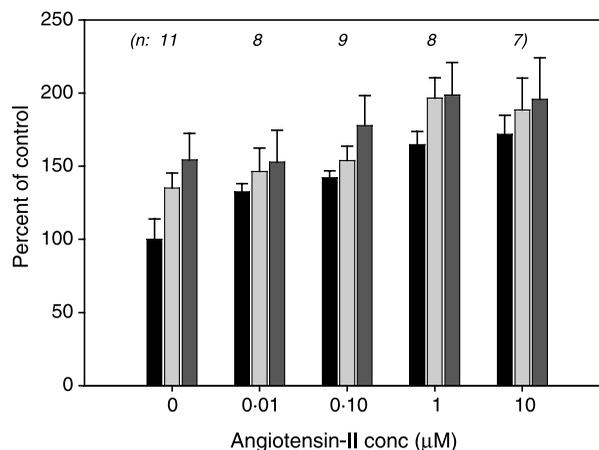
Figure 4 The effect of angiotensin alone and in combination with testosterone on the percentage of endothelial cells that secreted adrenomedullin. Dark bar, angiotensin-II only (at 0 μM the incubation contained medium only); light grey bar, angiotensin-II+testosterone 3·5 nM; medium grey bar, angiotensin-II+testosterone 350 nM. The addition of testosterone to angiotensin-II increased the percentage of cells that secreted adrenomedullin over that induced by angiotensin-II alone ($P<0.05$, paired t-test) in a manner that was less than additive.

To investigate whether there were effects on other processes in the cell, reverse transcriptase-PCR was performed after endothelial cells had been exposed to angiotensin-II (1 μM) or testosterone (350 nM). In concert with the observation that both the peptide and the sex steroid increased the number of cells that secreted adrenomedullin, both hormones induced an increase in the mean level of expression of adrenomedullin mRNA (Fig. 5), the effect being statistically significant for testosterone.

Discussion

This investigation examined the effects on adrenomedullin secretion of angiotensin-II and sex steroids in human aortic endothelial cells. Adrenomedullin has been recognised to have potent vasodilatory activity (Ishimitsu *et al*. 1994, Kitamura *et al*. 1994, Kato *et al*. 1996, Lainchbury *et al*. 2000). However, its interactions at the level of the endothelial cells with other vasoactive hormones remain to be defined. In contrast to adrenomedullin, angiotensin-II has potent vasoconstricting activity. In that, there is a clear epidemiological data, indicating gender differences in vascular disease, we hypothesised that peptides which influence vascular tone, namely angiotensin-II and adrenomedullin, may interact with sex steroids.

We showed that incubation with testosterone at concentrations as low as 3·5 nM over 4 h increased the proportion of cells that secreted adrenomedullin. This concentration is within the physiological range for testosterone in the plasma of healthy males (10–30 nM). Our results, showing that
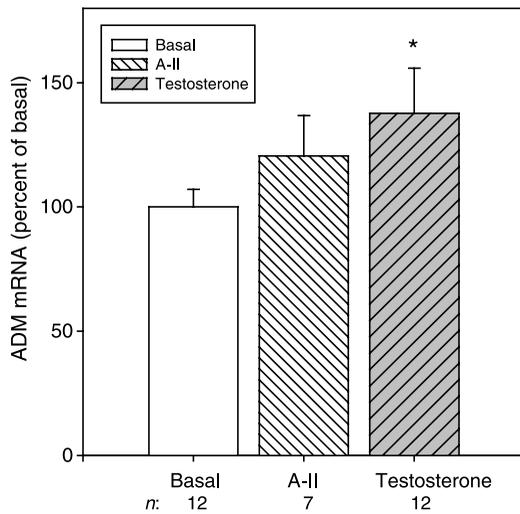
Figure 5 The effects of angiotensin-II or testosterone on adrenomedullin mRNA expression. The mRNA expression was determined relative to a housekeeping gene (GADPH) and the levels in cells exposed to hormone were calculated as a percentage of the expression in control incubations without hormone. An increase in mean adrenomedullin mRNA was observed in incubations with testosterone (*$P = 0.05$, paired *t*-test) and angiotensin-II.

testosterone increases the number of cells secreting adrenomedullin, reveal that testosterone has a direct effect on endothelial cells. Furthermore, it appears that the effect of testosterone on the vascular system may have an indirect component through the secretion of this vasodilator peptide. These results may partly explain observations that there is an inverse relationship between testosterone concentrations and blood pressure in men and that there can be beneficial effects of testosterone on the vasculature (Khaw & Barrett-Connor 1988, Jones *et al.* 2004).

By contrast, oestradiol had no detectable effect on the numbers of cells that secreted adrenomedullin. This may reflect a true absence of any effect or alternatively oestradiol may have effects outside the time parameter used in this study. The latter possibility is supported by investigations of other vascular systems, including prostacyclin production in endothelial cells (Mikkola *et al.* 1995) and AT1 receptor gene expression in smooth muscle cells (Nickenig *et al.* 1998), which have revealed that oestradiol may have a maximum effect at 12 h or greater. Endothelial cells can convert testosterone to oestradiol (Mukherjee *et al.* 2002) and it has been suggested that effects attributed to testosterone may be in part a result of its conversion to oestradiol (Sierra-Ramirez *et al.* 2004). However, our observation that testosterone increased the number of adrenomedullin-secreting endothelial cells, and that oestradiol had no significant effect, indicates that the actions of testosterone exhibited here were unlikely to be dependent on its conversion to oestradiol.

Angiotensin-II caused an increase in the number of adrenomedullin-secreting cells in a concentration-dependent

manner. In this study, a significant increase in adrenomedullin-secreting cells was detected at $0.001~\mu M$ angiotensin and maximum stimulation was observed at about $1~\mu M$. Previous experiments in our laboratory have indicated that endothelin-1 increases the number of cells secreting adrenomedullin (Evans *et al.* 2002). It is evident therefore that adrenomedullin is released from endothelial cells in response to two potent vasoconstrictors, angiotensin-II and endothelin-1. In that some actions of angiotensin-II are via stimulation of endothelin-1 (Riggleman *et al.* 2001, Pollock 2005), it is possible that the adrenomedullin response to angiotensin-II observed in the present study resulted from augmented endothelin-1 production in endothelial cells.

Although we observed the number of endothelial cells secreting adrenomedullin increased following exposure to testosterone or angiotensin-II, over the same time period, RIA did not detect a global change in the amounts of adrenomedullin released. This observation suggests that paracrine effects *in situ* between neighbouring endothelial cells and endothelial and smooth muscle cells may occur and have a role in the physiological regulation of vascular behaviour yet remain undetected when conventional methods, such as RIA, are used to determine the magnitude of hormone responses.

Our investigations regarding the effect of hormones on adrenomedullin mRNA levels were consistent with angiotensin-II and testosterone enhancing the production of ADM via increased mRNA expression as well as its secretion from endothelial cells. The results in relation to mRNA in this study were somewhat more robust for testosterone than angiotensin-II. Our data are at variance with others (Imai *et al.* 1995) who utilised Northern blotting and reported that testosterone did not have a detectable effect on adrenomedullin mRNA expression in endothelial cells. The reasons behind this discrepancy are not immediately apparent but could relate to different experimental circumstances or differences regarding the origin of the endothelial cells.

To our knowledge, this is the first study to investigate *in vitro* interactions between sex hormones and angiotensin-II on adrenomedullin release from endothelial cells, in the absence of other blood-borne factors that could complicate interpretation. The cell immunoblot method enables direct cellular responses to stimulation to be investigated. The method is very sensitive, detecting small changes in secretion from single cells within a population over relatively short time periods. Changes have been reported using the cell immunoblot within 1 h for secretion of angiotensin-II and endothelin-1 by endothelial cells (Kusaka *et al.* 2000). Vascular peptide measurements *in vitro* following exposure to compounds of interest have often used longer incubation periods prior to RIA (Isumi *et al.* 1998, Tomoda *et al.* 2001), and interpretation of the amount of peptide in the supernatant is potentially confounded by protein degradation or feedback processes acting on the cell. Clearly, the cell immunoblot methodology provides information that may be difficult or impossible to obtain by other techniques.

We conclude that testosterone increases the percentage of human endothelial cells that secrete adrenomedullin. Our results support the notion that testosterone may contribute to vasodilatory responses, partially through the release of adrenomedullin by endothelial cells. However, in the conditions of this study oestradiol had no significant effect on the number of adrenomedullin-secreting cells. Angiotensin-II recruited cells to the adrenomedullin-secreting population, and since angiotensin-II is produced by endothelial cells (Kifor & Dzau 1987, Kusaka *et al.* 2000), it may act in a paracrine/autocrine manner in regulating adrenomedullin release in the complex network that modulates vascular tone and growth.

Acknowledgements

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