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Inhibitory effect of adrenomedullin on basal and tumour necrosis factor alpha-stimulated endothelin-1 synthesis in bovine aortic endothelial cells is independent of cyclic AMP

Stewart Barker*, Delphine M. Lees, Elizabeth G. Wood, Roger Corder

The William Harvey Research Institute, St. Bartholomew's and the Royal London School of Medicine and Dentistry, Queen Mary, University of London, London EC1M 6BQ, UK

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Abstract

Adrenomedullin (ADM) is a potent vasodilator and reverses the vasoconstrictor action of endothelin-1 (ET-1). These studies aimed to determine the effect of ADM on ET-1 synthesis in bovine aortic endothelial cells (BAEC) and to identify the possible mechanisms involved. In this cell model, ADM increased cyclic AMP production by BAEC with threshold concentrations of 100 pM and an EC_{50} of 1 nM. This effect was not blocked by co-treatment with the CGRP type 1 receptor antagonist $CGRP_{8-37}$. ADM caused a potent concentration-dependent inhibition of ET-1 release that was correlated with reduced preproET-1 mRNA levels. This reached a maximal reduction of 70% compared to basal levels after 2 and 6 hr exposure of BAEC to 1 nM ADM, with significant decreases at concentrations as low as 10 pM. However, a 100-fold discrepancy between the threshold ADM concentration for cyclic AMP production and inhibition of ET-1 release was observed. Treatment of BAEC with tumour necrosis factor alpha (TNFα; 10 ng/mL) caused a 2-fold increase over basal ET-1 release. ADM caused a more marked reduction in stimulated ET-1 synthesis with a threshold of 1 pM, and suppression of ET-1 release to basal levels at 100 nM. 8-Bromo cyclic AMP, showed no concentration-dependent inhibition of ET-1 release, yet caused a 50% reduction in TNFα-stimulated intercellular adhesion molecule-1 (ICAM-1) mRNA levels. Thus, physiological ADM concentrations inhibit ET-1 synthesis independently of cyclic AMP in BAEC at the level of preproET-1 mRNA expression. The high sensitivity of this inhibition implicates ADM as an important physiological regulator of endothelial ET-1 production.

Keywords: Adrenomedullin; Endothelin-1; Tumour necrosis factor alpha; Cyclic AMP; Calcitonin receptor-like receptor; Intercellular adhesion molecule-1

1. Introduction

ADM elicits potent direct vasodilatory effects in a number of vascular beds [1–4] and should therefore be an important factor to consider in patients suffering from chronic congestive heart failure, essential hypertension, pulmonary hypertension and atherosclerosis. Infusion of physiological amounts of ADM into the brachial artery evoked significant increases in forearm blood flow in human subjects [5] and human ADM gene delivery attenuated hypertension in rats [6]. These observations indicate that ADM does have an impact on vascular resistance at physiological concentrations.

ADM is synthesised and secreted by vascular smooth muscle cells (VSMC) and endothelial cells, with ADM release being modulated by a number of hormones and cytokines [7–9]. ADM release by VSMC is increased by TNFα [9], and in endothelial cells is increased by glucocorticoids, and decreased by staurosporine [10]. Specific binding sites for ADM are present on both VSMC and endothelial cells [1–4]. These binding sites share most pharmacological similarity with a putative cloned ADM-receptor [11], but so far, expression of this receptor species

* Corresponding author. Present address: School of Biological Sciences, Medical Sciences building, Room 4.43, Queen Mary, University of London, London E1 4NS, UK. Tel.: +44-20-7882-6316; fax: +44-20-8983-0531.

E-mail address: s.barker@mds.qmw.ac.uk (S. Barker).

Abbreviations: ADM, adrenomedullin; BAEC, bovine aortic endothelial cells; BPASMC, bovine pulmonary artery smooth muscle cells; CGRP, calcitonin gene-related peptide; CRLR, calcitonin receptor-like receptor; ET-1, endothelin-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IBMX, 3-isobutyl-1-methylxanthine; ICAM-1, intercellular adhesion molecule-1; RAMP, receptor-activity-modifying protein; RT-PCR, reverse transcriptase polymerase chain reaction; TNFα, tumour necrosis factor alpha; VSMC, vascular smooth muscle cells; AP-1, activator protein-1; HIF-1, hypoxia-inducible factor-1; STAT, signal transducer and activator of transcription; IRF-1, interferon regulatory factor-1.

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has not been reported in vascular cells. However, another ADM-responsive receptor, the calcitonin receptor-like receptor (CRLR) [12], is expressed in vascular endothelium [13]. This receptor can respond to either ADM or CGRP depending on the cell context, and on the expression of one of three accessory factors known as receptor-activity-modifying protein (RAMP) 1, 2, and 3 [14]. Rabbit aortic endothelial cells express both CRLR and RAMP2 and respond selectively to ADM [15]. This relationship has also been shown in human endothelial cells [16].

In recent years, there has been considerable effort focused on the regulation of ET-1 production and its contribution to cardiovascular pathologies [17,18]. ET-1 is synthesised mainly by endothelial cells, causing vasoconstriction by direct action on underlying VSMC. Its secretion is increased in response to numerous factors including inflammatory cytokines such as TNFα. Interestingly, ADM can reverse the vasoconstrictor effect of ET-1 in isolated porcine coronary artery strips [19] and ADM is capable of a similar effect in ischaemic cerebral arterioles *in vivo* [20]. These observations suggest that ADM may act as a locally-acting physiological antagonist of ET-1 in the vasculature. A balance between secretion of ET-1 and ADM could maintain a set point of vascular tone. Here we describe studies (in BAEC lines expressing abundant levels of CRLR mRNA) that demonstrate that physiological concentrations of ADM inhibit ET-1 synthesis (under basal and TNFα-stimulated conditions) by a mechanism that is independent of cyclic AMP.

2. Materials and methods

2.1. Cell culture

BAEC lines (E11 and F2 clones) were derived by clonal selection from BAEC primary cultures [21]. Bovine pulmonary artery smooth muscle cells (BPASMC) were obtained by explant culture from fresh bovine pulmonary artery. Confluent BPASMC cultures exhibited characteristic smooth muscle cell morphology and stained positively for α-actin. All cells were routinely cultured in Dulbecco's modified Eagle medium containing 10% (v/v) fetal bovine serum and were washed twice in serum-free medium prior to use. All experiments were performed on confluent cultures using serum-free medium.

2.2. Assays

To measure ET-1 release, cells were incubated for 2 or 6 hr in the presence or absence of various treatments, and ET-1 in BAEC conditioned medium was quantified using a direct radioimmunoassay specific for the C-terminal peptide ET [16–21] (His-Leu-Asp-Ile-Ile-Trp). ^{125}I-ET-1 (Amersham Pharmacia Biotech) was used as tracer, and dilutions of ET-1 (Peptide Institute) were used as standard. Samples of conditioned media from BAEC were measured after appropriate dilution. Cross-reactivity of the following peptides in this assay are for ET-1 100%, ET-1 [19–21] (Ile-Ile-Trp) 0.0003%, and big ET-1 <0.015% [22]. Values thus obtained therefore represented accumulated ET-1 released into the medium over the duration of the treatment.

Cyclic AMP production was assayed by incubating cells for 30 min at room temperature with ADM or CGRP, in the presence or absence of $CGRP_{8–37}$, in medium containing the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; 1 mM). After boiling for 5 min and centrifugation at 14,000 *g* for 5 min to precipitate cell debris, cyclic AMP present in supernatants was assayed using a cyclic AMP-specific enzyme immunoassay (Amersham International plc.).

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR)

After removal of conditioned medium for ET-1 assay, BAEC or BPASMC monolayers were used for RT-PCR studies. Total cellular RNA was extracted and RT-PCR carried out using 100 ng of RNA per reaction [10]. RT-PCR reactions, using primers for preproET-1 RNA (5′-GAGTGTGTCTACTTCTGCCA-3′sense; 5′-TGATGGCCTCCAACCTTCTT-3′ antisense) and ICAM-1 (5′-CGACCACAGGAGCAACTTCT-3′ sense: 5′-TTCTGGATCTTCCGCTGGTA-3′antisense) based on bovine sequences ([23] and [24], respectively), were performed as described [21]. Primers for CRLR were designed based on sequence obtained from bovine cDNA (5′-AGACATCCAGCAAGCAACAG-3′ sense; 5′-CACCAGGATAAGCGTAGCTC-3′ antisense). For quantitation purposes, 23 cycles were used for preproET-1 and ICAM-1, 25 cycles (94° for 30 s; 65° for 30 s; 72° for 1 min) for CRLR, and 20 cycles for GAPDH [10]. The cycling conditions for each set of PCR primers was determined empirically to obtain a cycle number at which the intensity of the ethidium bromide stained DNA band was directly and linearly proportional to the amount of RNA template. Thus, the PCR cycling conditions were optimised for the linear phase of PCR amplification. All primers were obtained from Eurogentec UK Ltd. (Abingdon).

2.4. Peptides and data analysis

Rat ADM, rat CGRP and rat $CGRP_{8–37}$ used in these experiments were obtained from Peptide Institute (Osaka, Japan). Lyophilised peptides were dissolved in 5 mM acetic acid containing 10% (v/v) dimethylsulphoxide at stock concentrations of 20 μM for ADM and CGRP, and 200 μM for $CGRP_{8–37}$.

Statistical tests of significance were carried out using one way ANOVA followed by the Dunnett's multiple comparisons test as a post-*hoc* test to obtain *P* values

for individual comparisons. Data are expressed as mean ± SEM.

3. Results

The BAEC lines (E11 and F2) used in these studies expressed abundant levels of CRLR mRNA when compared with BPASMC (Fig. 1A). ADM increased cyclic AMP production with a threshold of 0.1 nM ADM (Fig. 1B). CGRP had no significant stimulatory effect on cyclic AMP production, except at 100 nM CGRP. Responses were unaffected by the presence of $CGRP_{8–37}$.

Treatment of these cells with ADM resulted in a potent concentration-dependent inhibition of ET-1 release. The threshold concentration causing a significant reduction in ET-1 release (accumulation in the culture medium over a 6 hr period) was 0.1 pM ADM and maximal inhibition (50% of basal release) occurred at 1 nM ADM (Fig. 2). CGRP at concentrations 10 nM had no significant effect on ET-1 release, although 100 nM CGRP reduced ET-1 production by $30.4 \pm 4.3\%$ ($n = 4$; $P < 0.01$) over a 6 hr incubation period (data not shown). Thus, both ADM stimulation of cyclic AMP production and inhibition of ET-1 release were ADM-specific effects.

The effect of ADM on steady state levels of preproET-1 mRNA (determined by using RT-PCR) was more pronounced at the earlier time point of 2 hr, reaching 30% of control values. This effect was maximal at ADM concentrations of 1 nM (Fig. 2), but showed a significant reduction at 10 pM ADM. After 6 hr, 1 nM ADM still exerted a potent inhibitory effect on preproET-1 mRNA levels, along with a significant reduction at 10 pM, while at 100 nM ADM the inhibition of preproET-1 mRNA expression was somewhat less than at the 2 hr time point. In comparison with the effect of ADM on preproET-1 mRNA, ET-1 release after 2 hr exposure matched the changes in mRNA levels, while the cumulative effect of 6 hr exposure showed a more pronounced inhibition of ET-1 release.

In Fig. 2, the threshold concentration of ADM which inhibited ET-1 release was considerably lower than that required for cyclic AMP stimulation (Fig. 1B). To determine whether the effects of ADM on ET-1 were independent of cyclic AMP synthesis the cell-permeable analogue

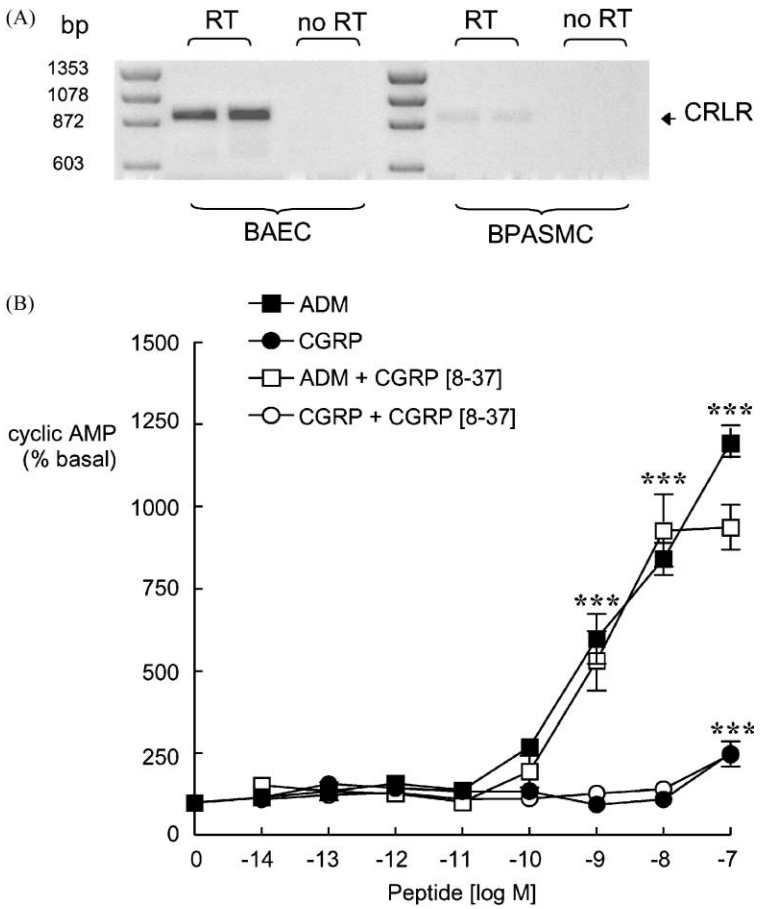

Fig. 1. (A) Comparison of relative abundance of CRLR mRNA using RT-PCR with 100 ng of total RNA from BAEC and BPASMC. M: molecular size markers (200 ng) from *Hae*III-digested ϕX174 RF DNA (Gibco); bp: base pairs. Duplicate reactions with (RT) and without (no RT) reverse transcriptase. (B) Cyclic AMP production by BAEC following incubation with increasing doses of ADM (filled squares), or CGRP (filled circles), alone, or in the presence of 100 nM $CGRP_{8–37}$ (open squares and open circles, respectively). Data expressed as % basal value (2.1 ± 0.2 pmol/10^6 cells/30 min; $n = 4$); $^{***}P < 0.001$.

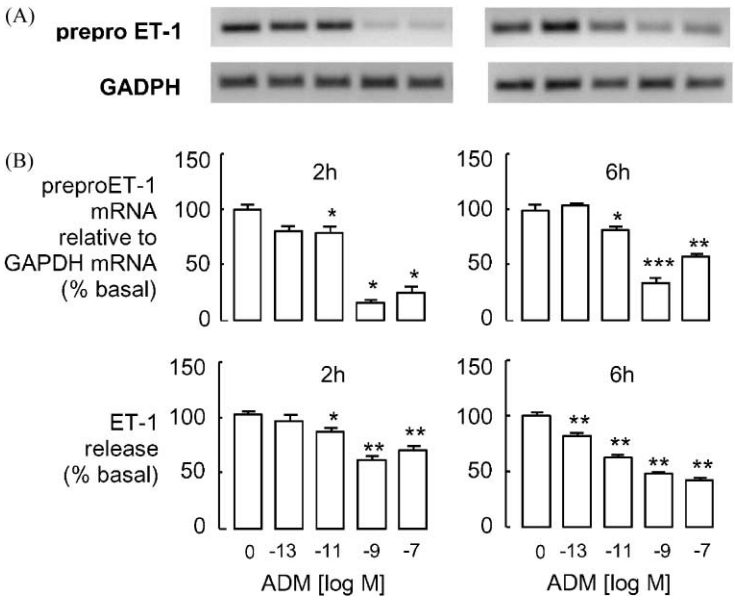

Fig. 2. (A) Representative RT-PCR results for preproET-1 mRNA (upper panels) and GAPDH mRNA (lower panels) in BAEC incubated for 2 hr (left hand panels) or 6 hr (right hand panels) with or without increasing concentrations of ADM. (B) Pooled data showing effect of increasing concentrations of ADM on preproET-1 mRNA levels (relative to GAPDH levels) and expressed as % of basal values (upper panels), and ET-1 release (lower panels). Data expressed as mean ± SEM; $^{**}P < 0.01$; $^{*}P < 0.05$ ($n = 4$–8). Mean basal release over 2 hr = 7.2 ± 0.2 fmol/cm^2; over 6 hr = 14.2 ± 2.0 fmol/cm^2.

of cyclic AMP, 8-bromo cyclic AMP, was used to examine its effect on ET-1 release and preproET-1 mRNA levels. ADM caused a concentration-dependent inhibition of ET-1 release under both basal and TNFα-stimulated conditions (Fig. 3A), with a significant threshold inhibition of TNFα-stimulated ET-1 release at 1 pM ADM. No concentration-dependent effect was observed with 8-bromo cyclic AMP under either basal or TNFα-stimulated conditions (Fig. 3B).

Fig. 4A represents a separate experimental dataset and shows the effect of either ADM (1 nM) or 8-bromo cyclic AMP (10 μM) on ET-1 release with or without the addition of TNFα (10 ng/mL) during either a 2 or 6 hr incubation. The effects were similar but more pronounced during a 6 hr incubation. This sustained inhibition of ET-1 production by ADM follows some time lag and reflects more closely the effects on preproET-1 mRNA at 2 hr (Fig. 4B left panel) than does the 2 hr secretion data (Fig. 4A; 2 hr). TNFα alone caused an increase in ET-1 release. ADM reduced ET-1 release under both basal and TNFα-stimulated conditions. 8-Bromo cyclic AMP was without significant effect. Total RNA from these cells, at the end of the 2 hr incubation, was analysed by RT-PCR. 8-Bromo cyclic AMP (10 μM) had no effect on preproET-1 mRNA levels under basal or TNFα-stimulated conditions (Fig. 4B) while ADM (1 nM) markedly reduced both basal and TNFα-stimulated preproET-1 mRNA levels. To confirm that 8-bromo cyclic AMP was having an effect on these cells we investigated ICAM-1 gene expression. ICAM-1 is known to be up-regulated by TNFα and down-regulated by cyclic AMP. Fig. 4C shows ICAM-1 mRNA levels after 2 hr treatment with ADM (1 nM) or 8-bromo cyclic AMP (10 μM) with and without TNFα. As expected, TNFα increased ICAM-1 mRNA levels in parallel with preproET-1 mRNA.

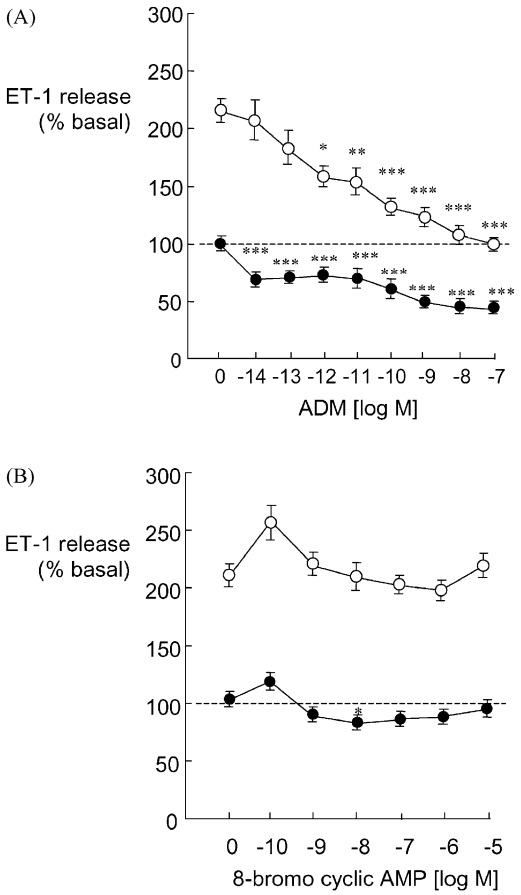

Fig. 3. (A) Concentration-dependent inhibition of ET-1 release by adrenomedullin in the presence (open circles) and absence (closed circles) of TNFα (10 ng/mL) during a 6 hr incubation. (B) Lack of effect of 8-bromo cyclic AMP under similar conditions ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, $n = 12$). Dashed lines denote 100% basal ET-1 release. Mean basal release 18.2 ± 0.9 fmol/cm^2/6 hr.

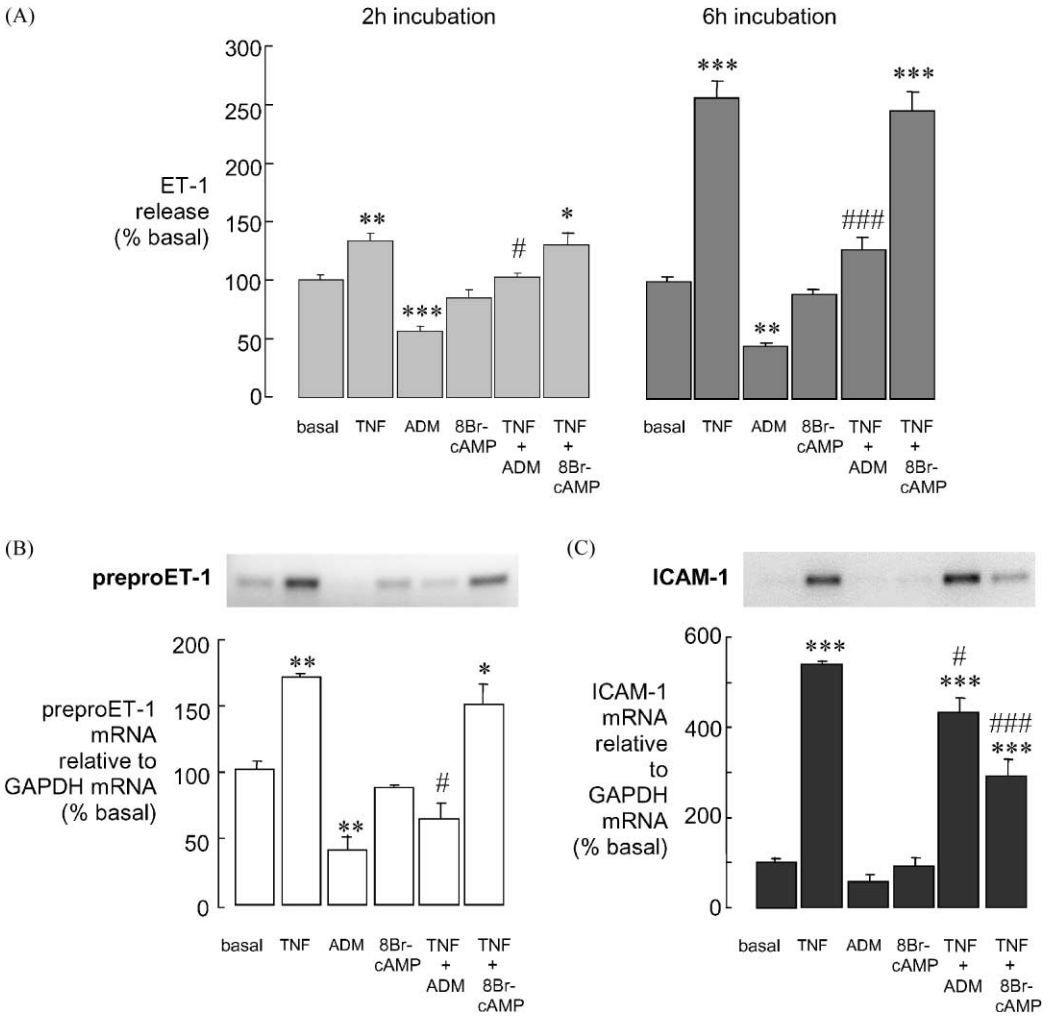

Fig. 4. (A) Reduction in basal and TNFα (10 ng/mL)-stimulated ET-1 release by ADM (1 nM) during 2 hr (left panel) and 6 hr (right panel) incubation. No significant effect of 8-bromo cyclic AMP (10 μM) was observed. Significantly different from basal ($^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$) or TNFα-stimulated ($^{###}P < 0.001$; $^{#}P < 0.05$) ($n = 8$). Mean basal release over 2 hr = 6.8 ± 0.6 fmol/cm^2; over 6 hr = 18.5 ± 1.6 fmol/cm^2. (B) Reduction in basal and TNFα (10 ng/mL)-stimulated preproET-1 mRNA levels by ADM (1 nM) but not by 8-bromo cyclic AMP (10 μM) (2 hr incubation). (C) Reduction of TNFα-stimulated increase in ICAM-1 mRNA levels by ADM (1 nM) and more potently by 8-bromo cyclic AMP (10 μM) (2 hr incubation). Data shown as representative agarose gel (above) and band density normalised to GAPDH mRNA levels (below). Significantly different from basal ($^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$) or TNF-stimulated ($^{#}P < 0.05$; $^{###}P < 0.001$) ($n = 3$–4).

In contrast, however, the TNFα-stimulated increase in ICAM-1 mRNA was reduced by 50% by 8-bromo cyclic AMP. This shows that while the expected effect of the cyclic AMP analogue with respect to ICAM-1 expression did occur, no effect of this compound was observed in relation to inhibition of ET-1 production.

4. Discussion

The studies described here show that ADM causes a potent, and sustained, concentration-dependent inhibition of ET-1 release in BAEC. The abundant expression of CRLR mRNA in the BAEC used for these studies suggests that CRLR mediates this effect. The low level of CRLR mRNA in BPASMC is in agreement with studies on other VSMC [25]. The effects of ADM were clearly mediated via a specific ADM-receptor since CGRP was unable to inhibit ET-1 release except at 100 nM. At this concentration it seems likely that CGRP is a partial agonist on high affinity ADM-receptor as the CGRP-receptor antagonist $CGRP_{8–37}$ did not abolish the effects of CGRP on cyclic AMP production (Fig. 1B). A correlation between high levels of CRLR transcript and ADM binding was recently reported in comparing rat lung (known to be a rich source of endothelial cells) with other rat tissues [26]. Furthermore, in transient expression studies of CRLR and RAMP1 and 2 [27], it appears that ADM binding is the ‘default’ phenotype for CRLR alone, and that this is enhanced by RAMP2. There is clearly a cell- and tissue-specific binding profile for ADM and CGRP which can be explained on the basis of different levels of expression of RAMP1 and 2 [14]. Where a particular cell or tissue expresses both of these accessory proteins a mixed profile of ADM and

CGRP binding should be expected. Thus, in artery segments of different diameters, the responses to ADM and CGRP reflected the ratio of RAMP2 to RAMP1, with levels of CRLR [28]. In rat testicular peritubular myoid cells (TPMC), ADM is the more potent of the two agonists although these cells respond to both ADM and CGRP to produce cyclic AMP [29]. Based on other studies one might therefore expect TPMC to express both RAMP1 and 2, but to express more of the latter. The action of exogenous ADM on these cells would presumably be able to act through a CRLR–RAMP1 (CGRP) and CRLR–RAMP2 (ADM) receptor which could explain why a CGRP-receptor selective antagonist, $CGRP_{8–37}$, and an ADM-receptor antagonist, $ADM_{22–52}$, are both able to inhibit the actions of ADM in these cells. On this basis we would predict that in endothelial cells, which have been shown to express RAMP2 and CRLR alone [15,27], ADM would be the sole agonist and that $CGRP_{8–37}$ would have no antagonist effect on this CRLR–RAMP2 receptor system.

The actions of ADM in a number of different tissues have been generally assumed to be the consequence of an increase in cyclic AMP production, with a consistent threshold of cyclic AMP stimulation of around 0.1 nM ADM [1–4]. Since both of the putative cloned ADM receptors have been characterised as mediators of ADM-stimulated cyclic AMP production [11,12], this suggested a cyclic AMP-dependent mechanism. We have also previously shown that ADM stimulates cyclic AMP formation in endothelial cells [30]. Indeed, ADM did increase cyclic AMP production by these cells (at concentrations above 0.1 nM). However, this increase cannot account for the effect of ADM on ET-1 release which occurred at much lower ADM concentrations, but rather this suggests that the ADM-dependent inhibition of ET-1 release is independent of cyclic AMP. In support of this conclusion, we found that 8-bromo cyclic AMP, a stable analogue of cyclic AMP, was unable to mimic the effects of ADM on ET-1 synthesis, whilst still having an appropriate inhibitory effect on ICAM-1 expression. The effect of ADM on ET-1 release was maintained over a 6 hr incubation period and this sustained inhibition of ET-1 release was preceded by rapid reductions in preproET-1 mRNA levels suggesting an effect on preproET-1 gene transcription (Fig. 4). In contrast, Kohno *et al.* [31] have shown that ADM can inhibit thrombin and PDGF-stimulated ET-1 production in rat VSMC. Furthermore, they were able to show that this effect could be mimicked by 8-bromo cyclic AMP. In rat VSMC cultures they were unable, however, to show any inhibitory effect on basal ET-1 production. These differences emphasise the point that each cell type may exhibit strikingly different mechanisms of gene regulation in response to ADM. Given their proximity in the vasculature the fact that VSMC and endothelial cell responses to ADM are different must be important in maintaining a balance of ADM and ET-1 secretion by these two cell types.

According to other reports, the accumulation of ADM secreted by endothelial cells in culture can range between 2 and 20 pM over a 2 hr period (extrapolating from values for a 24 hr incubation period) [8,9]. In the latter report TNFα caused a doubling of the amount of ADM secreted. In our experiments exogenously added ADM was having a significant inhibitory effect on ET-1 secretion at concentrations between 0.1 and 1 pM for both TNFα-stimulated and basal ET-1 production (Fig. 3A). This would suggest that endogenous ADM secretion was having a minimal or no effect in our studies. These differences probably reflect the effects of different culture conditions on a given cell type from different species. However, irrespective of any contribution from endogenous ADM secretion, we were able to show a clear and extremely potent inhibition of ET-1 production by bovine endothelial cells, which was not dependent on cyclic AMP production.

A number of factors have been shown to reduce ET-1 gene transcription in endothelial cells [17], including shear stress, calcium ionophore, and forskolin [22], but the precise mechanisms of these inhibitory effects are not established. No functional response element for cyclic AMP-responsive element binding protein (CREB) has been previously described in the ET-1 gene promoter, although use of MatInspector V2.2 (based on TRANSFAC 4.0 [32]) reveals three potential CREB binding sites in the 5′-flanking region of the ET-1 gene (EMBL accession number J05008). The cyclic AMP analogue dibutyryl cyclic AMP has been shown to reduce both serum- and thrombin-stimulated, but not basal, ET-1 synthesis in human endothelial cells and rat VSMC [31,33]. However, our data indicates that inhibition of both basal and TNFα-stimulated ET-1 synthesis by ADM occurs at concentrations below those at which ADM causes increases in cyclic AMP, suggesting an alternative mechanism. TNFα is a well-established regulator of gene transcription in endothelial cells exerting its actions through several different transcription factors including GATA-2, NF-κB, and modulators of phorbol ester-activated pathways such as protein kinase C (PKC). These transcription factors have been implicated in transcriptional regulation of the ET-1 gene by TNFα [34]. TNF-stimulation of endothelial arginine transport occurs through PKC activation [35] and TNFα had no effect on cellular cyclic AMP levels in human aortic endothelial cells [36]. Thus, there is no evidence that TNFα should itself increase cyclic AMP in our experiments. In another system, both TNFα and dibutyryl cyclic AMP were shown to increase ET-2 gene expression but through independent pathways [37].

Other possible mechanisms which require further investigation follow observations that prostaglandin-dependent decreases in preproET-1 mRNA levels are cyclic GMP-dependent [38]. ADM has also been shown to evoke vasodilation not only through cyclic AMP but also through the nitric oxide/cyclic GMP pathway [39,40]. One report suggests that ADM can cause increases in intracellular

calcium in endothelial cells [41] but we could not confirm this in our previous studies [30]. Other mechanisms through which ADM has been proposed to act include effects on potassium and calcium channels [3,4,42]. In addition, little is currently known with respect to coupling of CRLR to other signal transduction pathways in endothelial cells since this receptor has so far only been characterised with respect to cyclic AMP generation [14,42].

Regulation of ET-1 gene expression is an extremely complex process. It has been thoroughly established that basal expression involves the binding of GATA-2 and c-Jun and c-Fos (activator protein-1 (AP-1)) to their specific response elements in the ET-1 gene promoter [43]. However, in certain situations of stress it can be seen that GATA-2 and AP-1 function in conjunction with additional transcription factors, for example, under hypoxic conditions with hypoxia-inducible factor-1 (HIF-1), to activate ET-1 gene expression in endothelial cells [44]. Similarly, cytokines such as TNFα and interferon-gamma can activate ET-1 expression in VSMC [45], via the actions of a number of transcription factors, and this may involve use of NF-κB, signal transducer and activator of transcription (STAT), and interferon regulatory factor-1 (IRF-1) promoter elements. It is therefore conceivable that the inhibitory action of ADM on both basal and TNFα-stimulated ET-1 gene expression may result from effects of ADM–CRLR signalling on one or several of these transcription factors.

The potent inhibitory effects of ADM on ET-1 release described here indicate that physiological concentrations of ADM are likely to be a physiological regulator of ET-1 gene expression. Indeed, evidence that exposure of rat VSMC to ET-1 results in increased secretion of ADM [8], via the endothelin type B receptor [46], suggests an important reciprocal interplay between these two peptides as part of local vascular homeostatic mechanisms. One could envisage a balance between ADM and ET-1 under basal conditions while under certain conditions of stress this balance could be shifted. Hence, studies to investigate the regulation of CRLR and RAMP2 in endothelial cells, and the downstream control of ET-1 gene expression, are likely to provide novel insights into mechanisms that protect against diseases such as hypertension or atherosclerosis.

References

[1] Kangawa K, Kitamura K, Minamino N, Eto T, Matsuo H. Adrenomedullin: a new hypotensive peptide. J Hypertension 1996;14(Suppl 5): S105–10.

[2] Richards AM, Nicholls MG, Lewis L, Lainchbury JG. Adrenomedullin (Review). Clin Sci 1996;91:3–16.

[3] Samson WK. Adrenomedullin and the control of fluid and electrolyte homeostasis. Ann Rev Physiol 1999;61:363–89.

[4] Jougasaki M, Burnett Jr. JC. Adrenomedullin: potential in physiology and pathophysiology. Life Sci 2000;66:855–72.

[5] Cockcroft JR, Noon JP, Gardnermedwin J, Bennett T. Haemodynamic effects of adrenomedullin in human resistance and capacitance vessels. Br J Clin Pharmacol 1997;44:57–60.

[6] Dobrzynski E, Wang C, Chao J, Chao L. Adrenomedullin gene delivery attenuates hypertension, cardiac remodelling, and renal injury in deoxycorticosterone acetate-salt hypertensive rats. Hypertension 2000;36:995–1001.

[7] Sugo S, Minamino N, Kangawa K, Miyamoto K, Kitamura K, Sakata J, Eto T, Matsuo H. Endothelial cells actively synthesize and secrete adrenomedullin. Biochem Biophys Res Commun 1994;201:1160–6.

[8] Sugo S, Minamino N, Shoji H, Kangawa K, Matsuo H. Effects of vasoactive substances and cAMP-related compounds on adrenomedullin production in cultured vascular smooth muscle cells. FEBS Lett 1995;369:311–4.

[9] Isumi Y, Shoji H, Sugo S, Tochimoto T, Yoshioka M, Kangawa K, Matsuo H, Minamino N. Regulation of adrenomedullin production in rat endothelial cells. Endocrinology 1998;139:838–46.

[10] Barker S, Wood EG, Clark AJL, Corder R. Cloning of bovine preproadrenomedullin and inhibition of its basal expression in vascular endothelial cells by staurosporine. Life Sci 1998;62:1407–15.

[11] Kapas S, Catt KJ, Clark AJL. Cloning and expression of cDNA encoding a rat adrenomedullin receptor. J Biol Chem 1995;43: 25344–7.

[12] Aiyar N, Rand K, Elshourbagy NA, Zeng Z, Adamou JE, Bergsma DJ, Li Y. A cDNA encoding the calcitonin gene-related peptide type 1 receptor. J Biol Chem 1996;271:11325–9.

[13] Han Z-Q, Coppock HA, Smith DM, Van Noorden S, Makgoba MW, Nicholl CG, Legon S. The interaction of CGRP and adrenomedullin with a receptor expressed in the rat pulmonary vascular endothelium. J Mol Endocrinol 1997;18:267–72.

[14] Mclatchie LM, Fraser NJ, Main MJ, Wise A, Brown J, Thompson N, Solari R, Lee MG, Foord SM. RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. Nature 1998;393:333–9.

[15] Muff R, Leuthäuser K, Bühlmann N, Foord SM, Fischer JA, Born W. Receptor activity modifying proteins regulate the activity of a calcitonin gene-related peptide receptor in rabbit aortic endothelial cells. FEBS Lett 1998;441:366–8.

[16] Kamitani S, Asakawa M, Shimekake Y, Kuwasako K, Nakhara K, Sakata T. The RAMP2/CRLR complex is a functional adrenomedullin receptor in human endothelial and vascular smooth muscle cells. FEBS Lett 1999;448:111–4.

[17] Rubanyi GM, Polokoff MA. Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. Pharmacol Rev 1994;46:325–415.

[18] Nussdorfer GG, Rossi GP, Maledowicz LK, Mazzochi G. Autocrine–paracrine endothelin system in the physiology and pathology of steroid-secreting tissues. Pharmacol Rev 1999;51:403–37.

[19] Nishimura J, Seguchi H, Sakihara C, Kureishi Y, Yoshimura H, Kobayashi S, Kanaide H. The relaxant effect of adrenomedullin on particular smooth muscles despite a general expression of its mRNA in smooth muscle, endothelial and epithelial cells. Br J Pharmacol 1997;120:193–200.

[20] Wang X, Yue T-L, Barone FC, White RF, Clark RK, Willette RN, Sulpizio AC, Aiyar NV, Ruffolo Jr. RR, Feuerstein GZ. Discovery of adrenomedullin in rat ischemic cortex and evidence for its role in exacerbating focal brain ischemic damage. Proc Natl Acad Sci USA 1995;92:11480–4.

[21] Corder R, Barker S. The expression of endothelin-1 and endothelin-converting enzyme-1 (ECE-1) are independently regulated in bovine aortic endothelial cells. J Cardiovasc Pharmacol 1999;33:671–7.

[22] Corder R, Khan N, Änggård EE, Vane JR. Calcium ionophores inhibit the release of endothelin-1 from endothelial cells. J Cardiovasc Pharmacol 1993;22:S42–5.

[23] Imai T, Hirata Y, Emori T, Yanagisawa M, Masaki T, Marumo F. Induction of endothelin-1 gene by angiotensin and vasopressin in endothelial cells. Hypertension 1992;19:753–7.

[24] Staunton DE, Marlin SD, Stratowa C, Dustin ML, Stringer TA. Primary structure of ICAM-1 demonstrates interaction between

members of the immunoglobulin and integrin supergene families. Cell 1988;52:925–33.
[25] Autelitano DJ, Tang F. Co-expression of prepro-adrenomedullin with a putative adrenomedullin receptor gene in vascular smooth muscle. Clin Sci 1999;96:493–8.
[26] Chakravarty P, Suthar TP, Coppock HA, Nicholl CG, Bloom SR, Legon S, Smith DM. CGRP and adrenomedullin binding correlates with transcript levels for calcitonin receptor-like receptor and receptor activity modifying proteins (RAMPs) in rat tissues. Br J Pharmacol 2000;130:189–95.
[27] Bühlmann N, Leuthäuser K, Muff R, Fischer JA, Born W. A receptor activity modifying protein (RAMP) 2-dependent adrenomedullin receptor is a calcitonin gene-related peptide receptor when co-expressed with human RAMP1. Endocrinology 1999;140:2883–90.
[28] Sams A, Knyihár-Csillik E, Engberg J, Szok D, Tajti J, Bodi I, Edvinsson L, Vécsei L, Jansen-Olesen I. CGRP and adrenomedullin receptor populations in human cerebral arteries: *in vitro* pharmacological and molecular investigations in different artery sizes. Eur J Pharmacol 2000;408:183–93.
[29] Santiemma V, Rossi F, Guerrini L, Markouizou A, Pasimeni G, Palleschi S, Fabbrini A. Adrenomedullin inhibits the contraction of cultured rat testicular peritubular myoid cells induced by endothelin-1. Biol Reprod 2001;64:619–24.
[30] Barker S, Kapas S, Corder R, Clark AJL. Adrenomedullin acts via stimulation of cyclic AMP and not via calcium signalling in vascular cells in culture. J Human Hypertension 1996;10:421–3.
[31] Kohno M, Kano H, Horio T, Yokokawa K, Yasunari K, Takeda T. Inhibition of endothelin production by adrenomedullin in vascular smooth muscle cells. Hypertension 1995;25:1185–90.
[32] Quandt K, Frech H, Karas H, Wingender E, Werner T. MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. Nucl Acids Res 1995;23:4878–84.
[33] Stewart DJ, Cernacek P, Mohamed F, Blais D, Cianflone K, Monge J-C. Role of cyclic nucleotides in the regulation of endothelin-1 production by human endothelial cells. Am J Physiol 1994;266: H944–51.
[34] Marsden PA, Brenner BM. Transcriptional regulation of the endothelin-1 gene by TNFα. Am J Physiol 1992;262:C854–61.
[35] Pan M, Wasa M, Lind DS, Gertler J, Abbott W, Souba WW. TNF-stimulated arginine transport by human vascular endothelium requires activation of protein kinase C. Ann Surg 1995;221:590–600.
[36] Langeler EG, Fiers W, van Hinsbergh VW. Effects of tumour necrosis factor on prostacyclin and barrier function of human endothelial cell monolayers. Arterioscler Thromb 1991;11:872–81.
[37] Lambert GL, Barker S, Lees DM, Corder R. Endothelin-2 synthesis is stimulated by the type-1 tumour necrosis factor receptor and camp: comparison with endothelin-converting enzyme-1 expression. J Mol Endocrinol 2000;24:273–83.
[38] Prins BA, Hu R-M, Nazario B, Pedram A, Frank HJL, Weber MA, Levin ER. Prostaglandin E2 and prostacyclin inhibit the production and secretion of endothelin from cultured endothelial cells. J Biol Chem 1994;269:11938–44.
[39] Matsunaga K, Iwasaki T, Yonetani Y, Kitamura K, Eto T, Kangawa K, Matsuo H. Nitric oxide-associated relaxing effects of adrenomedullin in rat aorta. Drug Dev Res 1996;38:62–6.
[40] Hayakawa H, Hirata Y, Kakoki M, Suzuki Y, Nishimatsu H, Nagata D, Suzuki E, Kikuchi K, Kangawa K, Matsuo H, Sugimoto T, Omata M. Role of nitric oxide-cGMP pathway in adrenomedullin-induced vasodilation in the rat. Hypertension 1999;33:689–93.
[41] Shimekake Y, Nagata K, Ohta S, Kambayashi Y, Teroaka H, Kitamura K, Eto T, Kangawa K, Matsuo H. Adrenomedullin stimulates two signal transduction pathways, cAMP accumulation and Ca^{2+} mobilisation, in bovine aortic endothelial cells. J Biol Chem 1995;270:4412–7.
[42] Hinson JP, Kapas S, Smith DM. Adrenomedullin, a multifunctional regulatory peptide. Endocrinol Rev 2000;21:138–67.
[43] Kawana M, Lee M-E, Quertermous EE, Quertermous T. Co-operative interaction of GATA-2 and AP-1 regulates transcription of the endothelin-1 gene. Mol Cell Biol 1995;15:4225–31.
[44] Yamashita K, Discher DJ, Hu J, Bishopric NH, Webster KA. Molecular regulation of the endothelin-1 gene by hypoxia. Contributions of hypoxia-inducible factor-1, GATA-2, and p300/CBP. J Biol Chem 2001;276:12645–53.
[45] Woods M, Mitchell JA, Wood EG, Barker S, Walcot NR, Rees GM, Warner TD. Endothelin-1 is induced by cytokines in human vascular smooth muscle cells: evidence for intracellular endothelin converting enzyme. Mol Pharmacol 1999;55:902–9.
[46] Jougasaki M, Schirger JA, Simari RD, Burnett Jr. JC. Autocrine role for the endothelin-B receptor in the secretion of adrenomedullin. Hypertension 1998;32:917–22.