Eph receptors and zonation in the rat adrenal cortex

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Abstract

Although the zonation of the adrenal cortex has a clear functional role, the mechanisms that maintain it remain largely conjectural. The concept that an outer proliferative layer gives rise to cells that migrate inwards, adopting sequentially the zona glomerulosa, fasciculata and reticularis phenotypes, has yet to be explained mechanistically. In other tissues, Eph receptor (EphR)/ephrin signalling provides a mechanism for cellular orientation and migration patterns. Real-time PCR and other methods were used to determine the possible role of Eph/ephrin systems in the rat adrenal. mRNA coding for several members of the EphR family was detected, but out of these, EphA2 provided the closest parallel to zonal organisation. In situ hybridisation showed that EphA2 mRNA and EphA protein were predominantly located in the zona glomerulosa. Its transcription closely reflected expected changes in the glomerulosa phenotype, thus it was increased after a low-sodium diet, but decreased by pretreatment with the angiotensin-converting enzyme inhibitor, captopril. It was also decreased by ACTH treatment, but unaffected by betamethasone. mRNA coding for ephrin A1, the major ligand for the EphA receptors, was also detected in the rat adrenal, though changes evoked by the various pretreatments did not clearly reflect the expected changes in zonal function. Because the maintenance of cellular zonation requires clear positional signals within the adrenal cortex, these data support a role for Eph forward and reverse signalling in the maintenance of adrenocortical zonation.

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Introduction

Aspects of adrenocortical zonation remain enigmatic. Functionally, the accepted view is that the glomerulosa is the source of aldosterone secretion and the fasciculata and reticularis are the source of glucocorticoids (Deane 1962, Ogishima et al. 1992, Vinson et al. 1992, Ennen et al. 2005) and (depending on species) androgens as well (Deane 1962, Parker et al. 1983, Vinson et al. 1992, Rehman & Carr 2004). Within this system, aldosterone secretion is under the regulation of angiotensin (and other agents) and adrenocorticotropic hormone (ACTH) supports fasciculata and reticularis functions. Though this simple model probably needs adjustment and reappraisal (Vinson 2003, 2004), it nevertheless seems evident that the zonal arrangement is important in the maintenance of adrenocortical function, so it is important to determine how it arises.

The cell-migration theory, first proposed by Gottschau (Deane 1962, Gottschau 1883), holds that the outermost part of the cortex is the major site of adrenal cell proliferation, and the inner zones are the site of cell death. Though there are conflicting views (Wolkersdorfer & Bornstein 1998, Mitani et al. 2003), it does seem likely that adrenocortical cells are propagated centripetally, in cords, through the cortex (Wright & Vencina 1977, Bertholet 1980, Zajicek et al. 1986, McNicol & Duffy 1987, Stachowiak et al. 1990, Morley et al. 1996). Consequently, each adrenocortical cell sequentially adopts the phenotypes of glomerulosa, fasciculata and reticularis cells during its migration through the gland. We have argued that an implication of this mechanism is that individual adrenocortical cells have a polarity, each with apical and basal borders facing the capsule and medulla respectively (Vinson & Ho 1998, Vinson 2003).

This poses the question: what are the locally generated signals that determine phenotypic changes at specific sites in the gland? Is it attributable to a morphogenic landscape, in which non-cortical cells, such as neural or vascular cells, release local organisers that vary in nature at different latitudes (Ho & Vinson 1997, Vinson & Ho 1998)? Alternatively, does the signalling between adjacent cortical cells, linked to their polarity, provide sufficient information to promote specific cellular phenotypes at specific loci?

That the polarity of the adrenocortical cell cords, signalling centripetally via cell–cell contact, might in combination give sufficient information to establish zonation has not been considered, though related processes have been described in other tissues, in which the Eph receptor (EphR)/ephrin signalling system has been shown to have a special significance. In particular, in the small intestine of the mouse, progeny of the stem cells that border the intervillus pockets migrate in precise patterns. Wnt signalling results in β-catenin stabilisation and interaction with T-cell factor (TCF) transcription factors, leading in turn to inverse regulation of EphB2/B3 receptors and their ligand ephrin B1 (Batlle et al. 2002). The receptors
restrict cell intermingling and distribute cell populations to
discrete locations within the intestinal epithelium. Similar
expression gradients of EphRs and ligands may determine tissue
patterning in other systems, for example in the subventricular
divisions of the brain in which EphA/ephrin signalling regulates cell
migration and the balance between proliferation and differen-
tiation, or in the topographic maps of neuronal connections

As Wnt, β-catenin and TCF-like transcription factors
interact with steroidogenic factor (SF-1; Gummow et al.
2003, Kennell et al. 2003) and have crucial roles in adrenal
and gonadal) development (Luo et al. 2003, Else & Hammer 2005), it is appropriate to examine the
possibility of EphR/ephrin signalling in the rat adrenal cortex.
To demonstrate the possibility of specific EphR/ephrin
involvement, the tissues were taken from control animals, and
from animals subjected to treatments known to have specific
actions on adrenal cell differentiation and phenotype, and thus
zonal expression. These were low-sodium diet or ACTH
treatment, which enhance glomerulosa and fasciculata
eexpression respectively, and treatment with the angiotensin
converting enzyme (ACE) inhibitor captopril or betametha-
sone, which reduce the circulating levels of the major stimulants
of the glomerulosa and fasciculata, namely angiotensin II and
ACTH (e.g. Vinson et al. 1992, Raza et al. 2005).

Materials and Methods

Animals
Adult male Wistar rats 12–14 weeks old weighing 180–220 g
were obtained from commercial suppliers, and maintained
briefly at Queen Mary, University of London under standard
conditions of light and temperature, in accordance with
appropriate guidelines for animal care. As required, animals
received treatments as follows: ACTH-treated animals were
injected 5 days subcutaneously with 100 μg Depot
Synacthen (Ciba-Geigy, UK) and betamethasone (Betnesol,
Glaxo-Wellcome, UK) was provided in drinking water,
0.2 g/100 ml, for 7 days. Captopril was also supplied in
drinking water (0.5 mg/ml) for 2 weeks. In all of these cases,
controls were untreated. All of these animals were
maintained on standard laboratory diets. For dietary sodium
studies, control rats were maintained on a diet of wholemeal
flour (Sainsburys, Ltd, London, UK) supplemented with 1%
NaCO3 and 1% NaCl, with access to distilled water for
3 weeks. The low-sodium diet omitted the 1% NaCl.
Animals were killed by stunning and cervical dislocation,
and adrenals were snap frozen and stored in liquid nitrogen,
or fixed in 4% (w/v) paraformaldehyde in PBS and stored at
4°C prior to in situ hybridisation.

Preparation of RNA and real-time PCR
Tissues were homogenised in Trizol (Invitrogen) using an
Ultra-Turrax T25 polytron homogeniser and RNA extracted
according to the manufacturers’ instructions. RNAs were
treated with 5 U/μl RNase-free DNase for 20 min at room
temperature to destroy genomic DNA. Complete cDNA was
synthesised from 1 μg of each RNA using random hexamers
(500 nM, Promega), RNAsin (40U, Promega) and Super-
script III Reverse Transcriptase (Invitrogen) for 60 min at
37°C to destroy genomic DNA. Complete cDNA was

Table 1 Real-time PCR: primers

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>EphrinA1</td>
<td>CCA ACA TTA CGA GGA CGA CTCT</td>
<td>GGG CTC GCA TGT CAC ATA CTC</td>
</tr>
<tr>
<td>EphrinA2</td>
<td>AGT CTA CTG GAA CCG CAG CAA</td>
<td>TAG CCG CCG CCA TCA C</td>
</tr>
<tr>
<td>EphrinA3</td>
<td>CCA TGC CCGCCA GA AGA A</td>
<td>CTT TCA TCC TCA GAC ACT TCC AA</td>
</tr>
<tr>
<td>EphrinA4</td>
<td>CAG CGG GAA GGG TGT GA</td>
<td>GCT GGA ATT GCA CCG TAC CT</td>
</tr>
<tr>
<td>EphrinA5</td>
<td>CGT TAT GTC CTG TAC GTG AA</td>
<td>TTC CCA CTC TCT GAA CCC TTT G</td>
</tr>
<tr>
<td>EphrinB1</td>
<td>CGT AAC GCC TGA GCA GTT GA</td>
<td>AGC C TG GT GTC GGT TGT GAC</td>
</tr>
<tr>
<td>EphrinB2</td>
<td>CCT ACA GAG CAC ATG GAA AGC A</td>
<td>GCC AGA GAG ATC CCA TCA ATT C</td>
</tr>
<tr>
<td>EphrinB3</td>
<td>TTC TGC GAG TGG GAC AAA GTC</td>
<td>GGT CTC TCT CCA TGG GCA TTT</td>
</tr>
<tr>
<td>EphrinB4</td>
<td>CCG GAG TGT CCA TTC GGC TAC</td>
<td>TCA CTT GGT CTT TGA GTC CCA G</td>
</tr>
<tr>
<td>EphrinB5</td>
<td>AGT CTG AAG ATC ATC ACA AGC</td>
<td>CAC ATC CTT CCA GTA TCT TAC AC</td>
</tr>
<tr>
<td>EphrinB6</td>
<td>ATG TCC AGG ACC ACG ACC GCC TTG</td>
<td>GCC ATG CAT CTG CTG CAT CTG</td>
</tr>
<tr>
<td>EphrinB7</td>
<td>CGC GTC AAG CAG GGT ATC TAC T</td>
<td>ACC AGT ACT TGG CCA AGG GTT G</td>
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<td>EphrinB8</td>
<td>GAC ATC CTC GTA ATG CCA GAA T</td>
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<tr>
<td>EphrinB9</td>
<td>CCA TAA GCC CTC TTC TGG ACC A</td>
<td>ACA CTT GGA TGC CGG TCG TGT</td>
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<tr>
<td>EphrinB10</td>
<td>ACC ATC ACC GCT GTG TCT TCC</td>
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<tr>
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<tr>
<td>EphrinB12</td>
<td>CAG TGG CCC ATC TGG CAT GT</td>
<td>CTT AGC AGA TCT TCT GCA GT</td>
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<tr>
<td>EphrinB13</td>
<td>GGA CAG GCC TTC CCA GCC GCTC</td>
<td>TGG CAG GTC TCC CAG GGA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CCC TCA AGA TTG TCA GCA ATG C</td>
<td>GTC CTC ATG GTA GCC CAG GAT</td>
</tr>
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</table>
were used to establish empirically the appropriate PCR conditions for each of the genes reported before comparative experiments were carried out. Total RNA concentrations were quantified using an Eppendorf UV Biophotometer (Merck) and 1 µg was used per cDNA synthesis. For GAPDH, cDNA was quantified similarly and 100 ng cDNA was used per reaction. Initial 40 cycle 20 µl PCRs were performed for each primer pair using cDNA from rat adrenal glands or brain with 0.5 µM of each primer prior to performing quantitative real-time PCR. For those genes found to be expressed in the adrenal gland, parallel 25 µl real-time PCRs were set up each containing 1 µl (25 ng) cDNA and 300 ng of each primer. A 40 cycle PCR was performed at 60 °C on a Stratagene MX3000P QPCR system (Stratagene, Cedar Creek, TX, USA) followed by a thermal dissociation step to allow analysis of the product for purity. DNA synthesis was monitored using SYBR green (Stratagene). Preliminary experiments confirmed that GAPDH mRNA itself was unaffected by the treatments and normalisation of candidate gene expression against GAPDH was used to permit comparison between cDNAs. Each measurement was performed in triplicate on the tissue from four different animals on each of at least 3 separate days with reverse transcriptase-free samples for each RNA acting as negative controls. Primer sequences are shown in Table 1.

In situ hybridisation (ISH)

Sixteen micrometre sections of paraformaldehyde-fixed paraffin wax-embedded glands were cut using a Leica RM2145 (Leica Microsystems (UK), Milton Keynes, UK) and in situ hybridisation performed as described elsewhere (Knoll et al. 2001, Polvani et al. 2003). Digoxigenin-labelled RNA in situ probes were generated from 1 kb PCR fragments of rat EphA2 and EphA3 cloned in pGEM-T easy (Promega) vector. The primers used to generate the cloned fragments were EphA2: forward 5'-TGG GAC CTG-ATG CAA AAC AT-3' and reverse 5'-CCA CCT TCT CGT-AGC CTT CTT-3' and EphA3: forward 5'-TTG TCA-CCT CTC CAT CCT CAT-3', reverse 5'-GAT AAC ATT-TCT TGG TGC GG-3'. Signals were detected using alkaline phosphatase conjugated anti-digoxigenin (Roche) and BM purple substrate (Roche). Sense probes were used as a negative control.

**Figure 1** PCR analysis of EphA and EphB receptor transcription in rat adrenal gland (Ad) and brain (Br). Reverse transcription-free samples (~RT) acted as negative controls. Size markers are 100 bp ladder (Smart ladder, Eurogentech). When using brain cDNA as the template, 200–300 bp products were detected for each receptor. EphA2, 3, 4 and 7, and EphB2, 3 and 6 are expressed in the adrenal gland.
Localisation of EphA receptor protein

Ligand binding domain alkaline fusion proteins (LAP) were generated as described previously (Flanagan & Leder 1990, Brennan et al. 1997) and used according to published procedure (Brennan et al. 1997, Brennan & Fabes 2003). LAP binding was performed on 16 μm cryosections of fixed tissue.

Results

Eph receptors

Evidence for transcription of mRNA coding for several EphR subtypes in the rat adrenals is shown in Fig. 1. EphA2, A3, A4 and A7, and EphB3 and B6 are all expressed in the rat adrenal gland. In a preliminary set of experiments, changes in EphR expression levels in response to prior dietary sodium restriction or captopril treatment were determined. Of those EphR expressed within the adrenal, only EphA2 gene transcription showed responses that are clearly associated with zona glomerulosa phenotype, in that transcription was enhanced 50% by a low-sodium diet, but reduced by captopril to 75% of control values. The transcription of other subtypes was variably affected or unaffected (not shown). Based on these preliminary data, subsequent experiments focussed on EphA2 expression. Figure 2 shows that EphA2 receptor transcription is localised in the glomerulosa, as is also the primary site for ephrin A ligand binding, which is an indirect indication of the presence of EphA receptor. The transcription of mRNA coding for the A3 subtype also mostly occurs in the glomerulosa (Fig. 3), although in this case unaffected by a low-sodium diet or captopril (data not shown).

Finally, further analysis of the regulation of EphA2R transcription is consistent with its glomerulosa localisation, since ACTH pretreatment, which may be expected to enhance the fasciculata at the expense of glomerulosa function, diminishes EphA2R mRNA production, although betamethasone treatment has no effect (Fig. 4).

Ephrins

Ephrin ligands are expressed in adrenal tissue with ephrinA1, the predicted cognate ligand for EphA2, showing the highest level of expression (Fig. 5A). Real-time PCR also identified ephrin A1 in adrenal tissue, and Fig. 5B shows the changes evoked by the different treatments.

Discussion

Ten EphA and six EphB receptors have been identified in vertebrates, classified according to their binding preferences for either the six glycosylphosphatidylinositol-anchor-linked ephrin A ligands or the three transmembrane ephrin B ligands. The significance of these interactions for cellular organisation and differentiation is that signalling between adjacent cells can occur in two directions, ‘forward’ via the EphR and ‘reverse’ via the ephrin ligand, but EphR/ephrin signalling can evoke repulsion as well as adhesion and attraction, thus governing cell migration, and leading to the possibility of cell segregation and the establishment of discrete populations of similar cells (for reviews, see Kullander & Klein 2002, Pasquale 2005).

Clearly, such signalling could have the capacity to confer on adrenocortical cells, and on the cords they form, the polarity...
that we postulate is required for an understanding of the regulation of adrenocortical zonation (Vinson & Ho 1998, Vinson 2003).

The present real-time PCR results show clearly that mRNA coding for a wide range of EphRs are transcribed in the rat adrenal gland (Fig. 1). To determine which of these are relevant to zonation, glands were taken from animals subjected to different treatments known to affect specifically the glomerulosa/fasciculata phenotypes in the rat. Thus, a low-sodium diet, which provokes angiotensin II generation, is widely used to stimulate the zona glomerulosa and aldosterone secretion (Aguilera & Cath 1983, Lehoux et al. 1997, Raza et al. 2005), while the ACE inhibitor captopril suppresses it (McEwan et al. 1996, van Kats et al. 2005). Similarly, ACTH treatment suppresses aldosterone secretion, and quickly initiates the transformation of glomerulosa into fasciculata cells (Aguilera et al. 1981, Pudney et al. 1984, Abayasekara et al. 1989): prolonged treatment may result in the elimination of the glomerulosa altogether (Vinson 2003). Betamethasone, by contrast, suppresses circulating ACTH, and hence fasciculata function and glucocorticoid secretion (Buckingham & Hodges 1976, Cam & Bassett 1983, Raza et al. 2005).

A clear relationship to the expected phenotypic changes in adrenocortical cells that are brought about by the various treatments was revealed by EphA2 receptor mRNA, which ISH showed was exclusively limited to the zona glomerulosa, as also is EphA receptor protein (Fig. 2). The transcription of EphA2 receptor mRNA closely followed the expected glomerulosa phenotype: it was enhanced by a low-sodium diet, but reduced by captopril treatment (Fig. 4). Furthermore, treatment with ACTH or betamethasone also induced changes in EphA2 expression consistent with the view that EphA2 reflects the expression of the zona glomerulosa phenotype and function: ACTH treatment, which decreases the glomerulosa phenotype, also decreased EphA2 expression whereas betamethasone, which specifically affects the fasciculata/reticularis, but not the glomerulosa, had no effect on EphA2. That these EphRs are indeed functional is suggested by the further finding that mRNA coding for ephrin A1, considered to be the most important ligand for the EphA2 receptor, is also transcribed in the rat adrenal, although in this case its transcription, though affected by the various treatments, is enigmatic. Its lack of response to betamethasone and its decrease following ACTH treatment reflects the changes seen with EphA2, but in contrast to the decreases in response to both a low-sodium diet and captopril were not clearly related to physiology (Fig. 5). Failure to respond to glomerulosa-specific treatments in a predictable manner clearly does not exclude other EphR subtypes from a glomerulosa site, and the transcription of mRNA coding for the A3 subtype also mostly occurs in the glomerulosa (Fig. 3), although unaffected by a low-sodium diet or captopril (data not shown).
It is paradoxical that, although morphological zonation in the rat adrenal seems clearest in the distinction between glomerulosa and fasciculata, the complete role of the glomerulosa is in reality not easy to define. Although it is absolutely clear that CYP11B2 (aldosterone synthase) is located only in zona glomerulosa cells (Mitani et al. 1994, Halder et al. 1998, Peters et al. 2007) and thus the glomerulosa is undeniably the source of secreted aldosterone, the other steroidogenic enzymes required for aldosterone synthesis are sparse at best in comparison (Vinson 2004), and in any case even in glomerulosa cells, only the outermost contain either CYP11B2 or even StAR (and then only following induction by nephrectomy or high potassium) (Peters et al. 1998, 2006). The rest of the glomerulosa is enigmatic, though because of its lack of significant steroidogenic function it has been postulated to be a stem cell population for both glomerulosa and fasciculata cells (Mitani et al. 1996, Miyamoto et al. 1999, 2001). Here, proof has been elusive, since mitoses can occur throughout the gland (Wright 1971, Horiba et al. 1987, McNicol & Duffy 1987, Clark et al. 1992, Basile & Holzwarth 1993, Holzwarth 1995, Zielenski et al. 1995, McEwan et al. 1996, 1999, Ennen et al. 2005), including in the subcapsular region and in the capsule itself (McNeill et al. 2005). The possible function of the major, silent, part of the glomerulosa has been discussed elsewhere (Vinson 2003, 2004), but taking this evidence at face value, the hypothesis emerges that the glomerulosa is probably not a stem cell population, but may be considered an essentially undifferentiated tissue on to which evolution has grafted some minimal (though crucial) steroidogenic function, in the form of CYP 11B2. Its main role is to act as a cellular reserve that can be diverted either to secretion of aldosterone or glucocorticoid, depending on physiological demand. The mechanism remains obscure, but clearly EphR/ephrin signalling may be involved. Thus, it is evident that recruitment to aldosterone secretion, stimulated for example by a low-sodium diet, is initiated in the glomerulosa cells immediately below the existing CYP11B2 expressing population (Vinson & Ho 1998). Conversely, the recruitment of undifferentiated glomerulosa cells to glucocorticoid secretion evidently starts in cells at the innermost border of the glomerulosa, adjacent to the fasciculata (Pudney et al. 1984). Forward/reverse signalling, as achievable through EphR/ephrin interaction, could be part of the mechanism that identifies the next cells in line for differentiation, at whatever locus.

Declaration of Interest

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