Adenosine regulates thrombomodulin and endothelial protein C receptor expression in folliculostellate cells of the pituitary gland

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Received: 24 June 2009 / Accepted: 8 October 2009 / Published online: 27 October 2009
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Abstract Adenosine stimulates the release of interleukin 6 (IL-6) and vascular endothelial growth factor from folliculostellate cells of the anterior pituitary gland indicating that such cells are also involved in the communication between the immune and endocrine systems during stress and inflammation. In order to understand the precise actions of adenosine on folliculostellate cells, DNA microarray analysis was used to determine global changes in gene expression. Hierarchical clusters revealed, of the genes that had altered expression, the majority were suppressed and many, such as B cell translocation gene 2 and cyclin-dependent kinase inhibitor 2b were related to cell cycle arrest or inhibition of proliferation. Several of the up-regulated genes were associated with cytokine signalling or membrane receptor activity. The most notable of these being IL-6, sulfiredoxin 1, endothelial protein C receptor (EPCR) and thrombomodulin (THBD) which can all play a role in controlling inflammation. The EPCR and THBD pathway is well known in anti-coagulation but also has anti-inflammatory and anti-apoptotic properties. Up-regulation of EPCR and THBD in folliculostellate cells was confirmed by qRT-PCR and western blotting analysis and their expression were also demonstrated in many of the hormone-secreting cells of the anterior pituitary gland. Our findings suggest that adenosine can stimulate expression of

stress and inflammation related genes from folliculostellate cells of the anterior pituitary gland. These genes include EPCR and THBD, neither of which has been previously identified in the pituitary gland.

Keywords Adenosine · Folliculostellate cells · Pituitary · Thrombomodulin · Endothelial protein C receptor

Introduction

Reciprocal interactions between the immune and neuroendocrine systems are of paramount importance in regulating the host's response to disturbances of homeostasis such as stress, infection and inflammation [1–3]. As part of the hypothalamic–pituitary–adrenal (HPA) axis, cells within the anterior pituitary gland play a key role in this process through generation of ACTH (adrenocorticotropin) which in turn stimulates production of adrenal-derived glucocorticoids. ACTH secretion is mainly regulated by direct activation of pituitary corticotropes via hypothalamic-derived secretagogues such as corticotropin-releasing hormone (CRH) and vasopressin [4, 5]. However, recent studies suggest that intra-pituitary cytokines are also important in this regulation [6]. For example, the endotoxin lipopolysaccharide can stimulate ACTH release from the anterior pituitary, but does so indirectly via interleukin-6 (IL-6) [7]. IL-6 is critically important for normal activation of the HPA axis in response to immune stimulation [8] and an important pituitary source of this cytokine seems to be the folliculostellate (FS) cell population [9].

FS cells form a three-dimensional network of cells in the anterior pituitary gland and are capable of synchronised excitability via gap junctions [10]. In addition to modulating hormone secretion in the anterior lobe [11, 12], these

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cells seem to play a central role in co-ordinating the interplay between the immune and endocrine systems [13]. The recent demonstration of macrophage migration inhibitory factor expression and secretion in cultured FS cells [14], and its modification by glucocorticoids, supports this contention. We have previously shown that adenosine can stimulate IL-6 production in cultured FS cells, an effect mediated via the A2b adenosine receptor subclass and involving activation of p38 MAP kinase [15, 16]. Adenosine exerts anti-inflammatory actions in a number of tissues [17] and is generated in high concentration under conditions of inflammation and anoxic stress [18, 19]. These properties have led to suggestions that it might offer therapeutic potential in inflammatory conditions such as sepsis [17, 20].

In light of these observations, we sought to determine the effects of adenosine receptor activation on the global gene expression profile in cultured pituitary FS cells. Our results firstly support an important role for FS cells in immune signalling and secondly demonstrate that activation of adenosine receptors in these cells can modify expression of numerous components of the immune system. Furthermore, novel targets for adenosine action have been identified, including two components of the activated protein C cascade, thrombomodulin (THBD) and the endothelial protein C receptor (EPCR), which were up-regulated following stimulation by an adenosine receptor agonist. Expression of THBD and EPCR was not confined to FS cells but also demonstrated within corticotrope, gonadotrope, somatotrope and lactotrope lineages.

Materials and methods

Cell culture materials and reagents were obtained from Invitrogen (Paisley, UK), Autogen Bioclear (Calne, UK), Sarstedt Ltd (Leicester, UK) and Sigma-Aldrich (Poole, UK). Rat anterior pituitary tissue was taken from male Wistar rats (150–200 g) after cervical dislocation. Rodent pituitary GH3 (growth hormone (GH) and prolactin (PRL) secreting), MMQ (PRL secreting) [21], RC-4B/C (pituitary-adenoma-producing gonadotropin and PRL) [22–24] and AtT-20DV16 (ACTH secreting) cell lines are in-house. TtT/GF [25] and Tpit/F1 [26] FS cells were kindly provided by Professor Kinji Inoue (Department of Regulation Biology, Saitama University, Urawa, Japan). THBD antiserum was obtained from Santa Cruz Biotechnology (Santa Cruz, USA) and the EPCR antiserum was a kind gift from Dr. Charles Esmon (Howard Hughes Medical Institute, Oklahoma, USA). Molecular biology reagents, except TRIzol (from Invitrogen), were obtained from Promega (Southampton, UK). Western blotting reagents were from GE Healthcare (Chalfont St. Giles, UK).

Cell culture

TtT/GF and Tpit/F1 cells were cultured in 10% (v/v) heat-inactivated (HI) foetal bovine serum (FBS). GH3 and MMQ cells were cultured in Ham's F12, 7.5% (v/v) horse serum and 2.5% (v/v) FBS, AtT-20DV16 in DMEM 10% (v/v) FBS and RC-4B/C cells in DMEM/ α MEM (1:1) and 10% (v/v) HI FBS. All cells were cultured at 37°C in a 5% CO₂ environment.

Microarrays

Microarray analysis was performed on TtT/GF cells that were left untreated or treated with 10 μ M NECA (5'-N-ethylcarboxamidoadenosine), a stable adenosine receptor agonist, for 30 and 120 min. Briefly, 1×10^6 cells were cultured in 10 cm Petri dishes and allowed to reach 80% confluence; cultures were incubated in DMEM 2% HI FBS overnight prior to treatment with NECA in fresh media. Each time point was performed in triplicate. RNA was extracted using TriZol reagent. The integrity of the RNA was checked by spectrophotometry and gel electrophoresis. Ten micrograms of RNA was used to generate double-stranded cDNA by using a T7-linked oligo(dT) primer. Biotinylated cRNA was synthesised using the T7 RNA transcript labelling kit (Enzo Diagnostics, Farmingdale NY, USA), resulting in approximately 100-fold amplification of RNA. The biotinylated cRNA was alkaline treated to generate ~200 bp fragments which were then initially hybridised to a test chip as recommended by the manufacturers. Ten micrograms of fragmented cDNA was then hybridised overnight to the murine MG_U74A v2 GeneChip (Affymetrix, High Wycombe, UK) which contained 12,436 transcripts. Arrays were then washed and stained with streptavidin–phycoerythrin before being scanned on an Affymetrix GeneChip scanner.

Microarray data analysis

The resultant image data were analysed using the MAS 5.0 statistical algorithm to generate expression values for each probe set. In addition, detection calls (Present/Marginal/Absent) were calculated for each probe set. Subsequently, 405 probe sets (representing 374 unique genes) were identified as differentially expressed using ANOVA ($P < 0.05$). This gene list was taken forward for hierarchical clustering (using log-transformed, median-centred data) and the probe sets for each of the six distinct patterns identified were extracted by subsetting the gene dendrogram. Fold change values were calculated independently on the MAS 5.0 data.

Immunocytochemistry

Immunocytochemistry was carried out in paraformaldehyde-fixed rat pituitary tissue and acetone-fixed anterior pituitary

cell-line monolayers. Antigen retrieval was performed in the paraformaldehyde-fixed tissue sections by heating in a microwave for 30 min in 10 mM citrate buffer pH6. Incubations with specific antisera to THBD and EPCR (1:200) were carried out overnight at 4°C. Sections were then incubated for 90 min with the appropriate second antibody coupled to biotin and then visualised with streptavidin–fluorescein. Nuclei were stained with DAPI (4',6-diamidino-2-phenylindole). For negative controls, non-immune sera were used.

Conventional and qRT-PCR

Total cellular RNA, prepared using TriZol reagent, was treated with RQ1 ribonuclease-free DNase. RNA (0.2 µg) was reverse-transcribed using oligodeoxythymidilic acid [oligo(dT)₁₅] for 1 h at 37°C and the cDNA generated was subjected to PCR amplification using primers specific for rat or mouse THBD and EPCR. Primer sequences were designed using the Primer 3 software and gene sequences obtained from GenBank (Table 1). For the PCR reaction, 30–40 cycles were performed at 94, 60 and 72°C for, respectively, 30 s, 1 min, and 1 min followed by a final extension step of 72°C for 10 min. Amplified products were electrophoresed in 2% agarose and visualised with ethidium bromide.

Changes in THBD, EPCR, NR4A1 (nur77) and NR4A2 (nurr1) mRNA expression after NECA treatment were analysed by comparative QRT-PCR (using MX-3000 machine, Stratagene) with PGK-1 (phosphoglycerate kinase 1) or β-actin as the normaliser. Briefly, 80% confluent cultures were treated for 0, 0.5, 2, 4 and 8 h with 10 µM NECA, prior to RNA extraction and cDNA synthesis. The reaction mixture contained 1 µl (equivalent to 40 ng RNA) cDNA, 12.5 µl Platinum QPCR SYBR Green SuperMix

(Invitrogen) and 10 µM of each primer in a volume of 25 µl. The annealing temperature was 60°C. The ratios of THBD and EPCR to PGK-1 or β-actin in treated and untreated cells were compared and a ratio value of 1:1 was assigned for untreated cells.

Western blotting

TtT/GF cells (10⁶ cells/6 well plate) were cultured to 80% confluency and treated with 10 µM NECA in fresh DMEM containing 1% FCS for 0, 30 min, 1, 2, 4 and 8 h at 37°C. Cells were then washed 3× in ice-cold PBS containing 1 mM sodium ortho-vanadate followed by lysis in 0.2 ml electrophoresis sample (Laemmli) buffer. Aliquots of the samples (0.05 ml) were electrophoresed in 10% polyacrylamide and then electroblotted onto polyvinylidene fluoride (PVDF) membranes. Blots were exposed overnight at 4°C with antisera to THBD and EPCR (both 1:200, recommended). Secondary antisera conjugated to horseradish peroxidase, at 1:10,000, were applied for 1 h at RT and proteins were visualised with ECL Plus reagent. Blots were subsequently stripped and re-probed with antiserum to β-actin (1:1,000) for 1 h at 37°C to check for protein loading. Arbitrary densitometric ratios (integrated density values IDV) for THBD or EPCR and β-actin at the different time points were compared with the zero time point using the AlphaImager software (Alpha Innotech, San Leandro, California, USA).

Statistical analysis

QRT-PCR and western blotting experiments were performed at least three times. Results are expressed as means ± SEM and compared by ANOVA and the Tukey multiple comparison test. *P* < 0.05 is deemed to be significant.

Table 1 Primer sequences for EPCR, THBD, NR4A1 and NR4A2

Target		Primer sequence 5'–3'	Amplicon (bp)	Acc. no
Mouse THBD	F	GGCTGG AGATGACCCAGATA	175	NW009378
	R	GAAACAACGGTAAGGGCAAA		
Rat THBD	F	ATGCTTGGGGTTTCTCTCT	188	NM031771
	R	CGCACTGTCATCAAATGTCC		
Mouse PROCR	F	GACGAAGTTTCTGCCGCTAC	199	NM01117
	R	CTGGAGGATGGTGACGTTT		
Rat PROCR	F	ACCTGGAGAACCACATCACC	223	NM001025733
	R	TTGGCTTCATTTGACCTTCC		
Mouse NR4A1	F	CCTAAGGCTTCTTCAGGG	109	NM010444
	R	GTGGTGGGAACAATTCA		
Rat NR4A1	F	TGTTGCTAGAGTCCGCCTTT	231	NM024388
	R	CAGTGATGAGGACCAGAGCA		
Mouse NR4A2	F	AGTCTGATCAGTGCCCTCGT	149	NM013613
	R	CCGGTCAGGAGATCGTAGAA		
Rat NR4A2	F	CATTACGGTGTTCGCACTTG	151	NM019328
	R	GGCACTTCTGAAACCGACAG		

Table 2 Clusters of genes that were induced (on) or suppressed (off) in TtT/GF cells after treatment with NECA

Time	0	0.5 h	2 h
Cluster 1	Off	Off	On
Cluster 2	Off	On	On
Cluster 3	On	On	Off
Cluster 4	On	Off	Off
Cluster 5	Off	On	Off
Cluster 6	On	Off	On

Results

Analysis of gene expression profile changes in NECA-treated TtT/GF cells

Of the 12,436 transcripts present on the gene chip, 6,553 were called as having expression “Present” in at least one of the time points, i.e. untreated and 0.5 and 2-h treatments with 10 μ M NECA. The complete data set is available

(accession number GSE15564) on the NCBI Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/projects/geo>). The concentration of NECA and the time points chosen were based on our earlier experiments where we reported that adenosine agonists stimulated IL-6 secretion in TtT/GF cells [15]. Differentially expressed genes were identified using ANOVA (see Table 2), and a subsequent hierarchical clustering and heatmap visualisation (Fig. 1) identified six patterns in changes of expression between sample groups. The majority of these genes were suppressed (clusters 3 and 4) whereas many fewer genes were switched on (clusters 1 and 2) and even fewer genes showed a biphasic change of expression (clusters 5 and 6).

Examples of each cluster based on $P < 0.01$ are shown in Table 3, together with fold change at 0.5 and 2 h relative to the untreated samples; using these strict criteria, no genes were found in cluster 6 (i.e. genes that were switched off at 0.5 h and switched on again at 2 h). Those genes that are up-regulated contain several members that are associated with cytokine synthesis and immune function and membrane receptor activity and those that were inactivated were associated with anti-proliferation and transcription. IL-6

Fig. 1 Heatmap of hierarchical clusters of genes in TtT/GF cells that were induced (red) or suppressed (green) by NECA treatment for 0.5 and 2 h (three replicates were performed for each time point)

Table 3 Representative genes in clusters 1–5 showing fold changes at 0.5 and 2 h in TtT/GF cells treated with NECA in comparison to untreated cells

Probe ID	Gene symbol	Fold change (0.5 hr)	Fold change (2 hrs)
Cluster 1			
94747_at	CSF2RB2	0.90	22.2
92232_at	SOCS3	2.87	15.7
99964_at	VDR	0.99	10.18
160526_s_at	CREM	1.69	6.66
100019_at	SRXN1	1.20	4.00
102780_at	PROCR (EPCR)	1.81	4.14
94375_at	HK2	1.37	3.10
100277_at	INHBA	1.25	2.99
100109_s_at	VCAN	0.90	2.76
95016_at	NRP	1.11	2.56
99847_at	ST3GAL1	1.07	2.17
99649_at	GCLC	1.06	2.06
161980_f_at	BAG3	1.20	2.05
161040_at	FZD1	1.05	1.83
99864_at	A2BR	1.20	1.60
101805_f_at	NFIL3	0.96	1.47
Cluster 2			
102218_at	IL6	30.9	107
93430_at	CXCR7	6.08	15.87
97844_at	RGS2	5.83	13.57
102239_at	BCL3	3.38	7.99
92820_at	USP2	2.77	5.31
104601_at	THBD	1.98	3.64
160894_at	CEBPD	1.98	3.28
160084_at	ODC1	2.16	3.18
Cluster 3			
93294_at	CTGF	0.64	0.004
98579_at	EGR1	1.34	0.07
102661_at	EGR2	1.09	0.01
99109_at	IER2	1.68	0.12
101583_at	BTG2	1.25	0.17
94147_at	SERPINE1	0.96	0.18
102292_at	GADD45A	0.90	0.20
101900_at	CDKN2B	1.04	0.22
101538_i_at	CES3	0.75	0.24
160547_s_at	TXNIP	0.70	0.25
93956_at	IFIT3	0.67	0.28
103639_at	IFIT2	0.61	0.29
98083_at	KLF6	0.73	0.29
160747_at	RGS3	0.73	0.31
96825_at	TENC1	1.20	0.34
100016_at	MMP11	0.92	0.34
102362_i_at	JUNB	2.20	0.38
102798_at	ADM	1.61	0.39
92738_at	GDNF	1.45	0.40

Table 3 (continued)

Probe ID	Gene symbol	Fold change (0.5 hr)	Fold change (2 hrs)
94530_at	EIF2B2	0.78	0.41
97740_at	DUSP16	0.83	0.42
92310_at	PLK2	1.00	0.42
162125_f_at	UBB OR UBC	1.05	0.43
Cluster 4			
99380_at	KCNA2	0.58	0.27
160747_at	RGS3	0.73	0.31
161425_r_at	DW4SU132E	0.68	0.47
97206_at	SPINT1	0.73	0.49
97406_at	CBFA2T2	0.77	0.5
102200_at	AQP8	0.56	0.51
92747_at	NKX2-2	0.69	0.52
94470_i_at	SLC30A5	0.78	0.52
101290_at	GNRH1	0.82	0.55
94946_at	MAP3K3	0.77	0.58
101810_at	FSHR	0.82	0.58
103838_at	SYPL2	0.83	0.60
161557_at	MNAT1	0.88	0.61
103670_at	CYP2A12	0.75	0.62
100890_at	CHAF1B	0.83	0.65
161573_at	SLC4A7	0.77	0.68
161655_at	DAD1	1.05	0.82
Cluster 5			
102371_at	NR4A1	15.7	1.24
92248_at	NR4A2	5.63	2.01
104701_at	BHLHB2	2.25	1.14

Genes shown are those showing the highest induction or suppression; gene levels, at 0.5 and 2 h in clusters 3 and 4, are expressed as a fraction of those in untreated cells

mRNA expression increased at both time points, (0.5 (30-fold) and 2 h (100-fold), consistent with our previous data on secretion [16]. Three transcription factors, NR4A1, NR4A2 and BHLHB2 (basic helix loop–helix domain containing class B2; cluster 5) were also transiently switched on at the 30-min time point before falling back to basal levels at 2 h. Of particular interest was increased expression of two related genes THBD and EPCR which are usually associated with endothelial cells and blood coagulation but have also been linked with anti-inflammation and anti-apoptosis. THBD and EPCR have not been previously described in the pituitary gland and this prompted us to examine their expression further.

QRT-PCR was used to validate the affymetrix data for EPCR, THBD, NR4A1 and NR4A2; Table 4 shows the fold change for each gene (relative to PGK-1) at both time points and is consistent with that seen with the chip analysis. Western blotting analysis was used to determine if changes in EPCR and THBD gene expression could be translated into protein.

Table 4 QRT-PCR showing fold changes of EPCR, THBD, NR4A1 and NR4A2 in TtT/GF cells treated with NECA in comparison to untreated cells

mRNA	0.5 h	2 h
NR4A1 (Nur77)	16±5**	4.9±2*
NR4A2 (Nurr1)	13±3**	2±3
THBD	2±0.5	8±2.5*
EPCR	1±0.2	4±0.6*

Changes were normalised to the invariant house-keeping gene, PGK-1 and values are means ± SEM of three experiments with three replicates for each time point

* $P<0.05$, ** $P<0.01$

Densitometric analysis, relative to β -actin, showed that 10 μ M NECA stimulated a rapid increase in EPCR (Fig. 2); increases of one- and threefold were seen after 2 and 8 h, respectively. Putative changes in THBD protein expression were however less convincing and variable.

Expression of NR4A1, NR4A2, THBD and EPCR in anterior pituitary gland cells

Transcription profiling was performed using FS cells, which comprise about 10% of the cells in the anterior pituitary gland; the remaining cells are comprised of classical hormone-secreting cells. Established cell lines were used to investigate the extent of THBD and EPCR expression in the different cell types within the anterior pituitary gland. Simple RT-PCR showed that both genes were detectable in rat anterior pituitary tissue and in all of the cell types investigated (Fig. 3); the band for EPCR in AtT-20DV-16 cells however was particularly faint. Sequence analysis confirmed that the bands were either EPCR or THBD as appropriate. Immunocytochemistry data (Fig. 4) showed that the proteins for THBD and EPCR were also expressed in rat anterior pituitary tissue and the GH3 and AtT-20 cell lines, consistent with the mRNA findings (Fig. 3).

We also used QRT-PCR to assess whether NR4A1, NR4A2, THBD and EPCR were regulated by 10 μ M NECA in Tpit-F1, GH3 and RC-4B/C cells. Figure 5 shows that THBD and NR4A1 mRNA expression are stimulated, respectively, five- and threefold after incubation with 10 μ M NECA in Tpit-F1 FS cells. THBD mRNA stimulation persisted up to 8 h whereas that for NR4A1 peaked after 30 min; these data were consistent with those observed in the TtT/GF cell line. On the other hand, in contrast to that seen in TtT/GF cells, NECA had no effect on NR4A2 or EPCR mRNA expression in Tpit/F1 cells (Fig. 5). NR4A1, NR4A2, THBD and EPCR mRNA were, however, not affected by NECA (up to 8 h treatment) in GH3 cells (Fig. 6) or in RC-4B/C cells (data not shown).

Discussion

Previous data indicates that adenosine has an important regulatory role in FS cells of the pituitary gland and one of these roles is the regulation of inflammatory processes. Our earlier experiments showed that FS cells respond to complement molecules [27, 28] and to lipopolysaccharide [7] clearly indicating a role in inflammation processes. We thus set out to address the question of how adenosine, an endogenous molecule that fluctuates during cellular metab-

Fig. 2 Western blotting analysis of EPCR (a) and THBD (b) in TtT/GF cells after treatment with NECA (a) EPCR or THBD and (b) β -actin are representative gels. Arbitrary densitometric values (IDV) (c) are means ± SEM of three experiments; IDV values are the ratio of EPCR or THBD to β -actin, with the zero time point assigned as 100%. * $P<0.05$, ** $P<0.01$

Fig. 3 RT-PCR showing expression of EPCR and THBD in anterior pituitary tissue and anterior pituitary cell lines

olism, influences signalling in the FS cell line, TtT/GF. To do this, we performed DNA microarray analysis using NECA as the universal adenosine receptor agonist. We used NECA as opposed to adenosine because of the latter's very short half life; treatment times were based on our earlier findings on stimulated IL-6 secretion [15]. Overall ~50 genes were switched on and ~300 genes switched off. A closer inspection of these two groups showed that of the genes that had the highest activation, several were associated with receptor activity or cytokine signalling. These included IL-6, chemokine receptor 7 (CXCR7), regulator of G protein signalling 2 (RGS-2), β subunit of

Fig. 4 Immunocytochemistry of EPCR and THBD in anterior rat pituitary tissue (a–b) and GH3 (c–d) and AtT-20DV-16 (e–f) cells (a, c and e—EPCR; b, d and f—THBD)

Fig. 5 QRT-PCR of time-related changes in **a** NR4A1, **b** NR4A2, **c** EPCR and **d** THBD in Tpit/F1 cells (changes were normalised to the invariant house-keeping gene, PGK-1 and values are means $\pm$ SEM of three experiments with three replicates for each time point. * $P < 0.05$, ** $P < 0.01$. The ratio of each gene to PGK-1 (relative quantity dR) in untreated cells (zero time point) is arbitrarily set at 1)

IL-5 receptor (CSF2RB2), suppressor of cytokine signalling 3 (SOCS 3), vitamin D receptor (VDR), neuropilin (NRP- vascular endothelial growth factor-165 receptor) and sulfiredoxin 1 (SRXN-1).

VDR mediates inhibition of NF κ B (nuclear factor kappa B) signalling and has been associated with improved survival in patients with chronic kidney disease [29]. IL-6 is a mediator of responses to somatic stress [30] and SRXN-1 contributes to oxidative stress resistance by reducing cysteine–sulfinic acid that is formed during exposure to oxidants. IL-6 and SRXN-1 alongside EPCR and THBD, which were also significantly up-regulated, can all play a role in anti-inflammatory processes. EPCR is mainly found in endothelial cells where its main role is as an anti-coagulant; in addition, EPCR is a key molecule in the control of inflammation via the protein C pathway. The primary components of this pathway are protein C itself, its receptor EPCR, thrombin, THBD and protein S [31, 32]. The binding of thrombin to THBD stimulates protein C activation (together with its cofactor, protein S), which, on binding to EPCR, enhances further its activity [31]. Activated protein C (APC) has anti-inflammatory as well as anti-coagulant properties. Targeted disruption of the protein C gene in animals leads to an increase in expression of pro-inflammatory cytokines and inflammatory responses [33]. APC inhibits the expression of cytokine-like molecules by monocytes and macrophages which may be related to the proteolytic cleavage of protease-activated receptor1 [34]. EPCR itself may also have anti-inflammatory properties; in its membrane-bound form, it is involved in activation of protein C whereas the soluble form (i.e. the extracellular domain) interferes with neutrophil adhesion

and anti-coagulation [34]. THBD, which also exists as membrane-bound and soluble forms, possess anti-inflammatory properties which are probably mediated via its lectin domain or the EGF-like repeats domain [34]. The lectin domain appears to down-regulate NF κ B and MAP kinases leading to a decrease in leukocyte adhesion and extravasation. The soluble forms of EPCR and THBD can also move to sites of inflammation where they interact with APC to dampen down inflammatory processes [34].

Although THBD and EPCR are primarily involved in the anti-coagulation pathway and associated with the endothelium, their expression [34] also occurs in other cell types. For example, THBD and EPCR are both expressed in keratinocytes where their anti-inflammatory roles are particularly relevant to the wound-healing process [35–37]. The anti-inflammatory properties of EPCR have also been demonstrated in lung pneumocytes [38], intestinal dendritic cells [39], and in neutrophils [40]. Our findings, as far as we are aware, are the first report of the existence of THBD and EPCR in the anterior pituitary gland. We have shown that their expression is not confined to FS cells but also evident in the hormone-secreting cells. However, in contrast to FS cells, THBD and EPCR expression in hormone-secreting cells does not appear to be regulated by adenosine. This, however, may relate to the differential expression of adenosine receptors in the different cell types [16].

NECA also stimulated a transient increase in two orphan receptors, NR4A1 and NR4A2. Both of these receptors in the pituitary gland have been associated with the release of CRH and proopiomelanocortin genes by interacting with a specific *cis*-acting sequence in their proximal promoter regions [41–43]. This indicates that these orphan receptors are likely to

Fig. 6 QRT-PCR of time-related changes in **a** NR4A1, **b** NR4A2, **c** EPCR and **d** THBD in GH3 cells (changes were normalised to the invariant house-keeping gene, PGK-1 and values are means $\pm$ SEM of three experiments with three replicates for each time point. The ratio of each gene to PGK-1 (relative quantity dR) in untreated cells (zero time point) is arbitrarily set at 1)

have a role in modulating stress and inflammation. In support of this is the expression of both NR4A1 and NR4A2 in human atherosclerotic lesion macrophages where they play a protective role by reducing inflammation [44]. Adenosine, acting in particular through the A2a receptor and NR4A2 has also been shown to mediate the anti-inflammatory effects of methotrexate in rheumatoid arthritis [45]. NR4A2 also mediates the repression of matrix metalloproteinases during inflammation [46].

Of the genes that were repressed, several were related to cell cycle arrest and transcription. For example, B cell translocation gene 2 (*Btg2*) and cyclin-dependent kinase inhibitor 2B (*Cdkn2b*) are anti-proliferative molecules. Growth arrest and DNA damage-inducible 45 α (*Gadd45a*) is also involved in cell cycle arrest and dual-specificity phosphatase 16 (*DUSP16*), and mitogen-activated protein kinase kinase kinase 3 (*MAP3K*) are associated with the MAPK pathway. Genes that have a transcription role include thioredoxin-interacting protein (*Txnip*), kruppel-like factor 6 (*Klf6*) core-binding factor, runt domain, alpha subunit 2; translocated to, 2 (*CBFA2T2*) and NK2 transcription factor related, locus 2 (*NKX2-2*). A repression of growth-inhibitory genes is consistent with our previous data where we showed that adenosine acting through A2 receptors stimulated the proliferation of TtT/GF cells [16].

Our findings may also be important in the context of sepsis as there are dramatic changes in the HPA axis during the development of the disease. During inflammation and stress, the pituitary gland responds both directly and indirectly (via the hypothalamus) to peripheral inflammatory signals that arise from immune cells. The increased secretion of ACTH, GH and prolactin, and inhibited

secretion of leuteinizing hormone and thyroid-stimulating hormone during sepsis [47] may be due to increased expression of peripheral and central anaphylatoxins and other cytokines [27]. Such molecules, including C5a, IL-6, IL-1 β and TNF α [48] are also elevated in the pituitary gland during sepsis and are responsible for the breakdown of the blood–brain barrier. Activation of EPCR and THBD in the pituitary gland will have a role in inhibiting these inflammatory molecules and other inflammatory responses, thereby modulating the progress of sepsis. EPCR and THBD are necessary for the generation of APC from inactive protein C and addition of exogenous APC has been shown to significantly reduce mortality in patients with severe sepsis [49, 50].

Adenosine receptors themselves are also strongly associated with inflammation and sepsis and several agonist and antagonist compounds are currently in clinical trials for treating inflammatory diseases [51, 52]. Mediation through the A2a receptor has been reported to be beneficial in wound healing, inflammatory bowel disease, ischaemia, arthritis and chronic obstructive pulmonary disease. A2b receptor actions are generally thought to be pro-inflammatory although anti-inflammatory properties have also been reported [53–55].

In summary, DNA microarray data have identified many genes that are up- or down-regulated by adenosine in FS cells of the pituitary gland. Several of these genes are associated with limiting inflammatory processes; we chose to investigate further the expression of the two functionally related molecules EPCR and THBD, neither of which has been previously described in the pituitary gland. Our data shows that EPCR and THBD are expressed in many cell

types throughout the anterior pituitary gland and adenosine stimulates expression of these two molecules and activation of the protein C pathway. Activated protein C has a protective role in patients with severe sepsis. How adenosine actually influences the protein C pathway in the pituitary gland during inflammation is unknown. In this study, we have not attempted to identify the specific adenosine receptor(s) involved although previous studies indicate that the A2b subtype is highly expressed in FS cells [15, 16]. The A2b receptor has been shown to play a significant role in inflammatory processes [55]. Whole animal experiments will, however, be needed to understand the precise role of adenosine signalling in the control of inflammation.

Acknowledgements This work was supported by a Society for Endocrinology, Clinical Endocrinology Trust training fellowship to DAR. The authors acknowledge the technical expertise of Dr. S. Forbes-Robertson (now at the School of Medicine, Swansea University, Singleton Park, Swansea, SA2 8PP, UK).

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