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Agouti-related protein has an inhibitory paracrine role in the rat adrenal gland

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Abstract

α-Melanocyte-stimulating-hormone (α-MSH) is an agonist at the melanocortin 3 receptor (MC3-R) and melanocortin 4 receptor (MC4-R). α-MSH stimulates corticosterone release from rat adrenal glomerulosa cells in vitro. Agouti-related protein (AgRP) an endogenous antagonist at the MC3-R and MC4-R, is expressed in the adrenal gland. We investigated the expression of the MC3-R and MC4-R and the role of AgRP in the adrenal gland. MC3-R and MC4-R expression was detected in rat adrenal gland using RT-PCR. The effect of AgRP on α-MSH-induced corticosterone release was investigated using dispersed rat adrenal glomerulosa cells. AgRP administered alone did not affect corticosterone release, but co-administration of AgRP and α-MSH attenuated α-MSH-induced corticosterone release. To investigate glucocorticoid feedback, adrenal AgRP expression was compared in rats treated with dexamethasone to controls. AgRP mRNA was increased in rats treated with dexamethasone treatment compared to controls. Our findings demonstrate that adrenal AgRP mRNA is regulated by glucocorticoids. AgRP acting via the MC3-R or MC4-R may have an inhibitory paracrine role, blocking α-MSH-induced corticosterone secretion.

Keywords: AgRP; α-MSH; Rat; Adrenal; Corticosterone; MC3-R; MC4-R

Five melanocortin receptors (MC-Rs) have been cloned, all having differing affinity for pro-opiomelanocortin (POMC) products. α-MSH is derived from proteolytic processing of POMC and is a potent agonist at the MC1-R, MC3-R, and MC4-R [1]. α-MSH has been shown to stimulate corticosterone release from rat adrenal glomerulosa cells at concentrations of 0.1 nM [2,3]. Circulating levels of α-MSH in rat plasma are of the order of 0.2–0.3 nM, and hence, α-MSH may have a physiological role in the regulation of corticosterone release in normal rats. However, the receptor mediating the effects of α-MSH on the adrenal gland is not known since only the MC2-R and MC5-R have been reported in the rat adrenal gland [4,5] at which α-MSH is a low affinity agonist. Using Northern blot analysis and PCR, the presence of the MC3-R and MC4-R in the rat adrenal gland has not been detected [6–8]. However, a recent report has suggested expression of the MC3-R in the chicken adrenal [9].

AgRP is an endogenous competitive antagonist at the MC3-R and MC4-R. It is a weak antagonist at the MC5-R but does not bind to the MC1-R or MC2-R [10]. To date the majority of investigations have focused on AgRP's orexigenic role in the hypothalamus (reviewed in [11]). In humans and rats, the adrenal glands appear to be the site of the highest level of AgRP mRNA expression after the hypothalamus [10,12] and the rat adrenal contains approximately 16% of the AgRP protein observed in the hypothalamus [13]. AgRP-like immunoreactivity (AgRP-LI) has been shown to be present in the circulation of rats and humans [13,14]. However, bilateral adrenalectomy in rats did not change serum AgRP-LI. This suggests that the adrenal gland is not the

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source of circulating AgRP and the role of adrenal AgRP is likely to be local to the adrenal gland.

Recently, using in situ hybridisation, it has been demonstrated that AgRP is expressed in the adrenal cortex in rats and humans [8] and not in the adrenal medulla as previously reported [12]. AgRP mRNA expression is upregulated in the adrenal during fasting and in the contralateral adrenal gland following unilateral adrenalectomy but not during chronic stress [8]. The receptor through which AgRP may mediate its effects in the adrenal gland is not known, since AgRP has no antagonist properties towards the MC2-R and is only a weak antagonist to the MC5-R [10]. Thus the role of AgRP and the receptor at which it acts in the adrenal gland remains to be identified.

To establish the role of AgRP in the rat adrenal gland we investigated:

(1) If MC3-R and MC4-Rs are present in normal rat adrenal glands.
(2) The effect of AgRP on α-MSH-induced corticosterone release from dispersed rat adrenal glomerulosa cells.
(3) If adrenal AgRP mRNA was regulated by glucocorticoids.

Materials and methods

Experimental animals. Male and female Wistar rats (Specific pathogen free, Imperial College School of Medicine, London, UK) weighing 250–300 g were maintained in individual cages under controlled temperature (21–23 °C) and light (12 h light/dark cycle; lights on at 07:00) with ad libitum access to food (RM1 diet, SDS Ltd., Witham, UK) and water. Animal procedures were approved by the British Home Office Animals Scientific Procedures Act 1986 (Project License No. 90/1077).

Materials. α-MSH, AgRP(83–132), and ACTH(1–24) were purchased from Bachem (St. Helens, Merseyside, UK). Collagenase (Worthington type I) was from Lorne Laboratories Ltd. (Reading, UK). All radiolabels were purchased from Amersham Biosciences (Amersham, Buckinghamshire, UK). All other chemicals were obtained from Sigma (Poole, Dorset, UK).

Study 1: Investigation to determine the presence of MC3-R and MC4-R in normal rat adrenal glands. Male Wistar rats were killed and the adrenal glands and hypothalami were removed. Total RNA from the tissues was isolated using the Tri-reagent (Helena Biosciences, Sunderland, UK). RT-PCR was performed using nested primers to determine the presence of the MC3-R and the MC4-R in the adrenal glands and hypothalami (which were used as a positive control as the hypothalamus is known to contain the MC3-R and MC4-R).

RT-PCR analysis. RT-PCR was performed as previously described [15]. Briefly, twenty micrograms of total RNA was reverse transcribed using avian myoblastoma virus reverse transcriptase (RT) (Promega, Southampton, UK) in a reaction primed using oligod$T_{(12–18)}$. Half of the RT reaction was subjected to PCR using primers obtained from the published sequence of the rat MC3-R and the MC4-R (Accession Nos. X70667 and U67863, respectively). The primers were synthesised by Oswel DNA services (Southampton, UK). The primers used in the reaction for the MC3-R were cgtttctctctggagaaact and ggatgttttccatcagactg (corresponding to positions 163–182 and 451–470 of the GenBank nucleotide sequence) and for the MC4-R were atactaggtagagacctgc and aaggctctgatttctgctag (corresponding to 1162–1180 and 1481–1500 of the GenBank nucleotide sequence). The conditions used for the PCR were 95 °C for 45s, 58 °C for 30 s, and 72 °C for 60 s. Following PCR 1/20 of the reaction was subjected to a second round of PCR using internal primers for the MC3-R (atctagactggacagcatcc and acgatgcccagtgccaggaa, corresponding to positions 186–205 and 431–450 of the GenBank nucleotide sequence) and MC4-R (agaatttatcactcaggcac and gaaagaacagggttgactc, corresponding to positions 1181–1200 and 1457–1476 of the GenBank nucleotide sequence). Using these primers an amplified fragment of 264 bp for the MC3-R and 295 bp for the MC4-R would be expected. Controls treated in an identical way but with no reverse transcriptase added to the reverse transcription were conducted at the same time. All of the PCR were analysed on 1% agarose gels.

Study 2: To investigate if AgRP can antagonise α-MSH-induced stimulation of corticosterone release from dispersed rat adrenal glomerulosa cells. Rat adrenal glomerulosa cells were prepared using a method modified from that originally described by Vinson et al. [3]. Briefly, female Wistar rats were decapitated and adrenal glands were rapidly removed and cleared of adhering fat. Capsule fractions (containing mainly glomerulosa cells) were separated from inner adrenocortical tissue by pressure between glass plates. Adrenal glomerulosa cells were obtained by incubating the capsules in Krebs bicarbonate Ringer containing glucose (200 mg/100 ml), bovine serum albumin (200 mg/100 ml; BSA fraction V) (KRBGA), and 2 mg collagenase/ml for 1 h at 37 °C under 95% O_2 and 5% CO_2. Tissue was dispersed by repeated pipetting and filtered through nylon gauze. Suspensions were centrifuged at 1100 rpm and the supernatant was discarded. The pelleted cells were incubated in 1 ml KRBGA for 2 h under an atmosphere of 95% O_2 and 5% CO_2 in the presence or absence of stimulants. The final concentration of cells was $2–4 \times 10^5$ cells per tube.

All peptides were dissolved in KRBGA to the required concentrations and the following combinations of peptides were investigated: ACTH(1–24) 10 nM, AgRP(83–132) 1000 nM, α-MSH 10 nM, α-MSH 10 nM + AgRP(83–132) 10 nM, α-MSH 100 nM, α-MSH 100 nM + AgRP(83–132) 100 nM, α-MSH 1000 nM, and α-MSH 1000 nM + AgRP(83–132) 1000 nM. The doses of peptides chosen were based on previous work and demonstrating that α-MSH stimulates corticosterone release from rat glomerulosa cells with an EC_{50} of 5×10 nM, and ACTH(1–24) has a maximal effect at a dose of 10 nM [3]. AgRP has been previously shown to inhibit the effects of α-MSH-induced cAMP formation in vitro when the same concentrations are used [10]. After incubation, cells were sedimented by brief centrifugation. The cell-free media were decanted into Eppendorf tubes and the tubes were stored at −20 °C until the media were assayed for corticosterone by radioimmunassay.

Study 3: Measurement of adrenal AgRP mRNA in dexamethasone-treated animals compared to normal controls. Two groups of male Wistar rats were given ad libitum access to food and water ($n = 10$ per group). One group of rats received dexamethasone (1 mg/L) in their drinking water for 10 days. This dose of dexamethasone was chosen as it has previously been shown to cause suppression of HPA activity at all levels within the HPA axis in male rats [16]. Food intake and body weight of the animals were measured every day. After 10 days the rats were killed by decapitation and trunk blood was taken for corticosterone measurement using radioimmunoassay. The adrenal glands and hypothalami were removed and weighed and total RNA was isolated from the tissues using Tri-reagent (Helena Biosciences, Sunderland, UK). Rats treated with dexamethasone had smaller adrenal glands than untreated controls (dexamethasone-treated 28 ± 6 vs. controls 62 ± 4 mg, $p < 0.001$). As such the total amount of RNA extracted from adrenal glands of dexamethasone-treated rats was less than from controls (75 ± 4 vs. control 176 ± 23 μg, $p < 0.001$). To control for this, we used the same amount of RNA (50 μg) from each

group of rats for Northern blot analysis to quantify the amount of AgRP RNA expression.

Northern blot analysis. Northern blot analysis of RNA from adrenal glands and hypothalami of animals was performed as previously described [17]. Briefly, 50 μg total RNA was size separated on a denaturing MOPS [3-(*n*-morpholino)propane-sulphonic acid]/formaldehyde gel (1% agarose) and transferred to a Hybond-N membrane (Amersham Biosciences). The RNA was fixed by baking at 80 °C for 2 h before probing with a riboprobe corresponding to nucleotides 1–224 of the mouse AgRP cDNA sequence containing the coding region for AgRP [10]. The riboprobe was synthesised using [α-^{32}P]CTP using T_7 RNA polymerase (Promega). Hybridisation was carried out overnight at 55 °C in a mixture of 2.5 mM ethylenediaminetetraacetic acid (EDTA) (pH 8), 0.5% dried milk, 0.25 M sodium phosphate buffer (pH 7.2), 5% sodium dodecyl sulphate (SDS), and 25 μM aurin tricarboxylic acid. Non-specific hybridisation was removed by increasingly stringent washes, the final one being in 0.1× saline sodium citrate (SSC)/0.1% (w/v) SDS at 70 °C for 30 min. Autoradiography of the filter was carried out at −70 °C using Kodak X-OMAT (Eastman Kodak, NY, USA) and an intensifying screen for two days. Blots were reprobed with oligo$(dT)_{12-18}$ to enable differences in RNA loading to be corrected.

Radioimmunoassays. Plasma corticosterone was measured using a radioimmunoassay kit from ICN Biomedicals (Costa Mesa, CA, USA) as previously described [18]. The lower detection limit of the assay is 5 ng/ml; the inter- and intra-assay coefficients of variation were less than 7% and 10%, respectively.

Corticosterone was measured from aliquots of cell-free media from the dispersed adrenal glomerulosa cell experiments using an antiserum purchased from Sigma as previously described [19]. The lower detection limit of the assay was 19pg; inter- and intra-assay coefficients of variation were 10% and 6%, respectively.

Statistical analysis. Results are presented as means ± SEM. Results from adrenal cell dispersion experiments are expressed as effect of peptide administered as a percentage of basal values ± SEM because while quantitative reproducibility of results is good within a single experiment based on a uniform cell crop, there may be variation between experiments in, for example, the basal (unstimulated) or the maximal steroid output in dispersed adrenal glomerulosa cells [2]. For each peptide administered, seven wells per experiment were used and the experiment was repeated. Data from adrenal cell dispersion experiments were analysed by ANOVA followed by post hoc. Least Significant Difference (Systat, Evanston, IL, USA). For the adrenal AgRP mRNA experiment 10 animals per group were used and results were compared by unpaired *t* test. In all cases $p < 0.05$ was considered to be statistically significant.

Results

Study 1: To determine the presence of MC3-R and MC4-R in normal rat adrenal glands

RT-PCR of normal rat adrenal glands demonstrated the presence of both the MC3-R and MC4-R. RT-PCR of rat hypothalami was performed as a positive control and demonstrated the presence of the MC3-R and MC4-R. To check for possible artefacts generated by amplification of remnants of genomic DNA, control RT-PCRs were performed and treated in an identical way but with no reverse transcriptase added to the RT-reaction mixture. No MC3-R and MC4-R were detected in these reactions (Fig. 1).

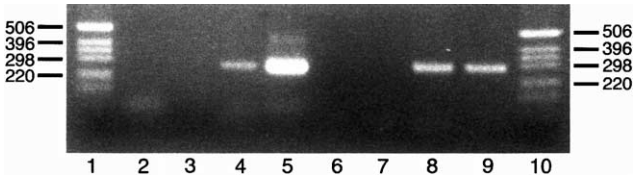

Fig. 1. MC3-R and MC4-R RT-PCR products of normal rat adrenal glands and hypothalami. Lanes 1, 1 kb ladder; 2, water + RT; 3, adrenal gland mRNA–RT; 4, adrenal gland RNA + RT; 5, hypothalamic mRNA + RT; 6, water+RT; 7, adrenal gland mRNA−RT; 8, adrenal gland mRNA + RT; 9, hypothalamic mRNA + RT; and 10, 1 kb ladder (+ or − indicates the presence or absence of reverse transcriptase enzyme (RT) in the reaction). For lanes 1–5 MC4-R primers were used and for lanes 6–10 MC3-R primers were used. The size of the expected RT-PCR product for MC3-R was 264 base pairs (bp) and for the MC4-R 295 bp.

Study 2: To investigate if AgRP can antagonise α-MSH-induced stimulation of corticosterone from rat adrenal glomerulosa cells

ACTH (10 nM) resulted in an 11-fold rise in corticosterone release compared to controls, indicating that the dispersed adrenal glomerulosa cells were viable (corticosterone release: basal 100 ± 4%, ACTH (10 nM) 1156 ± 116%, $p < 0.0001$ vs. basal). α-MSH caused a dose-dependent increase in corticosterone release from adrenal glomerulosa cells in agreement with previous observations [2,3] (Fig. 2). AgRP(83–132) administered alone did not affect corticosterone release from glomerulosa cells. However, co-administration of AgRP (83–132) and α-MSH attenuated α-MSH-induced stimulation of corticosterone release from glomerulosa cells (Fig. 2).

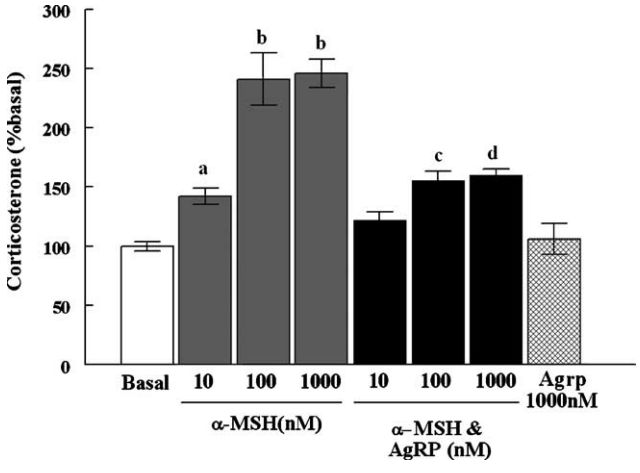

Fig. 2. Effect of α-MSH, AgRP, and the combination of α-MSH and AgRP on the release of corticosterone from dispersed adrenal glomerulosa cells. Results are expressed as mean effect of peptide administered as a percentage of basal values. (a) $p < 0.01$ vs. basal, (b) $p < 0.001$ vs. basal, (c) $p < 0.001$ vs. α-MSH 100 nM, and (d) $p < 0.001$ vs. α-MSH 1000 nM.

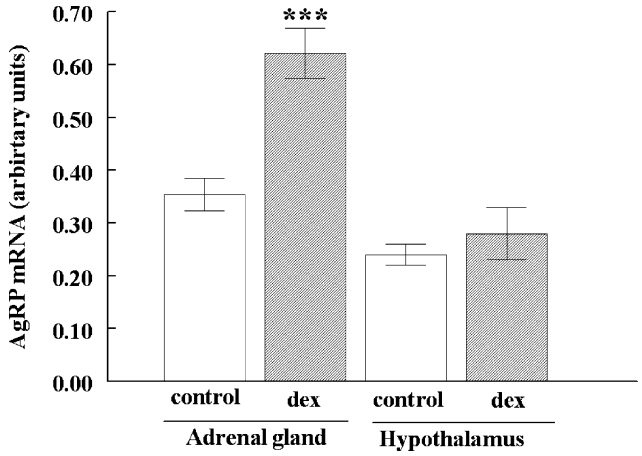

Fig. 3. Adrenal and hypothalamic AgRP mRNA levels measured by Northern blot analysis in rats treated with dexamethasone (1 mg/ml) in their drinking water for 10 days and untreated controls. Both groups of animals had ad libitum access to food and water. ***$p = 0.0001$ vs. untreated controls. dex, dexamethasone-treated animals.

Study 3: Measurement of adrenal AgRP mRNA in dexamethasone-treated animals compared to normal controls

Rats receiving dexamethasone in their drinking water had plasma corticosterone levels below the detection limit of the assay (<5 ng/ml), whereas untreated controls had normal levels (40 ± 7 ng/ml). Dexamethasone treatment also resulted in a lower cumulative food intake (246 ± 9 g vs. controls 312 ± 4 g, $p < 0.001$) and body weight (166 ± 9 g vs. controls 313 ± 5 g, $p < 0.001$) after 10 days. AgRP mRNA in the adrenal glands of dexamethasone-treated rats was increased to $176 \pm 8\%$ of levels observed in rats receiving normal drinking water (AgRP mRNA (arbitrary units): dexamethasone-treatment 0.62 ± 0.05 vs. control 0.35 ± 0.03, $p = 0.0001$). Hypothalamic AgRP mRNA levels were not significantly different in the two groups (AgRP mRNA (arbitrary units): dexamethasone-treated rats 0.28 ± 0.05 vs. controls 0.24 ± 0.02, $p = 0.4$) (Fig. 3).

Discussion

To date, only the MC2-R and MC5-R have been reported in the adrenal gland [4,5]. Using Northern blot analysis and PCR, the presence of the MC3-R and MC4-R in the rat adrenal gland has not been previously detected [6–8]. Our RT-PCR results demonstrate that both the MC3-R and the MC4-R exist in the normal rat adrenal gland. We have used RT-PCR using nested primers which increases the sensitivity of this technique, and this may account for why previous groups have not reported the presence of these receptors in the adrenal gland.

α-MSH has been reported to stimulate corticosterone release from rat adrenal glomerulosa cells [2,3], but the receptor mediating this effect is not known. Since α-MSH is a high affinity agonist at the MC3-R and the MC4-R it is possible that the effect of α-MSH on corticosterone release from the adrenal gland is mediated via these receptors. It has also been shown that α-MSH stimulates cAMP release from rat adrenal glomerulosa cells, whereas agouti protein (an antagonist at the MC1-R, MC2-R, and MC4-R) [20] has no effect. However, in α-MSH-stimulated rat adrenal glomerulosa cells, agouti significantly inhibited the cAMP response to α-MSH stimulation [21], suggesting that this effect may be mediated via the MC4-R. Our PCR results demonstrate the presence of the MC4-R in the adrenal gland and hence the effect of agouti on α-MSH-induced cAMP release may indeed be mediated via the MC4-R.

In humans and rats, the adrenal glands appear to be the site of the highest level of AgRP mRNA expression after the hypothalamus [10,12], but the role of adrenal AgRP is not known. The results from our dispersed adrenal cell experiments demonstrate that AgRP attenuates α-MSH-induced corticosterone release, but AgRP has no effect when administered alone. This suggests that α-MSH-induced corticosterone release is mediated via the MC3-R and/or the MC4-R and that AgRP in the adrenal gland may have an inhibitory paracrine role on corticosterone release. α-MSH has also been shown to be an agonist at the MC5-R. However, its agonist potency at the MC5-R compared to the MC3-R and MC4-R is at least 12-fold lower [1] and therefore it is unlikely that any of the effects of α-MSH seen in our study are mediated through the MC5-R.

We also found that AgRP mRNA in the adrenal glands of rats treated with dexamethasone is upregulated compared to normal controls, but there was no difference in hypothalamic AgRP mRNA levels. This is also in keeping with an inhibitory paracrine role for adrenal AgRP. The cumulative food intake and body weight in dexamethasone-treated animals was less than in normal controls following 10 days of treatment. However, both groups of rats had ad libitum access to food and water and therefore were not deprived of food. The dose of dexamethasone chosen for our study was based on the dose used in a previous study which demonstrated that this dose of dexamethasone was required to suppress the HPA axis at all levels. In this study, Nicholson et al. [16] also noted a fall in body weight in animals following treatment with dexamethasone compared to controls. It is unlikely that the difference in the food intake and body weight of dexamethasone-treated animals compared to controls influenced the changes in adrenal AgRP mRNA levels seen in our study since fasting increases both adrenal and hypothalamic AgRP mRNA levels [8,13]. In our

study, there was no difference in hypothalamic AgRP mRNA levels between rats treated with dexamethasone compared to controls. This would suggest that the increase in adrenal AgRP mRNA in dexamethasone-treated rats is a specific effect.

In human adrenocortical cells desacetyl-α-MSH (thought to be the major form of the peptide in the human pituitary and in circulating plasma) has been shown to cause a significant stimulation of steroidogenesis [22]. Desacetyl-α-MSH is also present in the human circulation [23]. Thus, it is plausible that desacetyl-α-MSH may have a role in the control of adrenal function in normal human. Indeed, it has been demonstrated that pituitary levels of immunoreactive desacetyl-α-MSH are elevated in patients with Addison's disease [24] and pituitary and plasma desacetyl-α-MSH levels are elevated in the majority of patients with Cushing's disease [25]. It would be interesting to investigate if adrenal AgRP mRNA levels are altered in patients with Addison's disease or Cushing's disease.

It has previously been demonstrated that pro-γ-$MSH_{1–77}$ and $Lys^1\gamma_3$MSH enhance the steroidogenic response of adrenal cells to ACTH [26,27]. The receptor through which pro-γ-$MSH_{1–77}$ and $Lys^1\gamma_3$MSH potentiate ACTH-induced steroidogenesis is not known. The potentiation properties of pro-γ-$MSH_{1–77}$ are thought to reside in the C-terminally located γ-MSH portion of the molecule [27,28]. Binding sites with typical receptor characteristics have been shown to exist for γ-MSH on rat adrenocortical cells, suggesting the existence of a separate receptor distinct from the ACTH receptor [29]. The MC3-R has a high affinity for γ-MSH [6] and thus could be the receptor that evokes the effects of γ-MSH in the adrenal gland. If this is the case AgRP in the adrenal gland may also inhibit γ-MSH-induced potentiation of ACTH-induced steroidogenesis. However, further studies are required to investigate this hypothesis.

In summary, AgRP expression in the rat adrenal gland is regulated by glucocorticoids. AgRP in the adrenal gland may via the MC3-R or MC4-R have an inhibitory paracrine role, blocking α-MSH-induced corticosterone release.

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