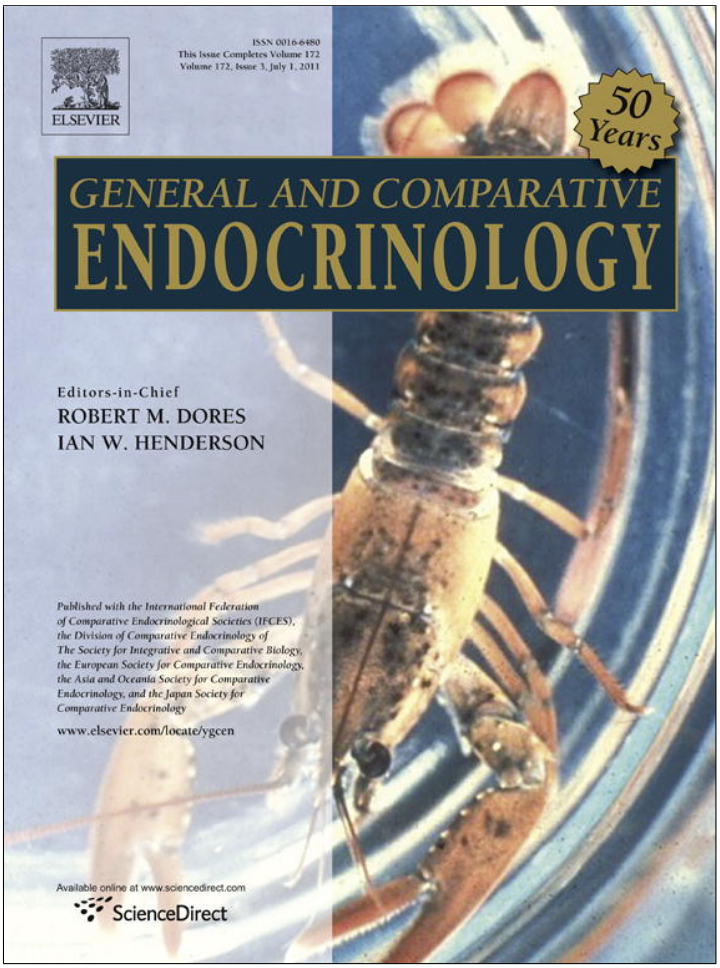

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Expression of urocortin and corticotropin-releasing hormone receptors in the bovine adrenal gland

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ABSTRACT

Urocortin (UCN), a 40 amino acid peptide, is a corticotrophin-releasing hormone (CRH)-related peptide. The biological actions of CRH family peptides are mediated via two types of G protein-coupled receptors, CRH type 1 receptor (CRHR1) and CRH type 2 receptor (CRHR2). The aim of the present study was to investigate the expression of UCN, CRHR1 and CRHR2 by immunohistochemistry, western blot and real-time RT-PCR in the bovine adrenal gland to clarify the mechanisms of the intra-adrenal CRH-based regulatory system. The results showed that UCN, CRHR1 and CRHR2 were expressed in both the cortex and medulla. Specifically, UCN-immunoreactivity (IR) was distributed in the outer part of the *zona fasciculata* and in the *zona reticularis* of the cortex and in the medulla. UCN and CRHR2 mRNA expression levels were higher in the cortex than in the medulla, while CRHR1 mRNA levels were undetectable in the cortex.

These results suggest that UCN, CRHR1 and CRHR2 are expressed in the bovine adrenal gland and that UCN might play a role in the intra-adrenal CRH-based regulatory system through an autocrine mechanism.

1. Introduction

Urocortin 1 or urocortin (UCN), a 40-amino acid peptide, is a corticotrophin-releasing hormone (CRH)-related peptide. UCN is the first CRH-related neuropeptide to be identified from a screen using an urotensin probe in a rat midbrain library and subsequently cloned in the human [40,9,11,7,42,43].

The first component of the name "Urocortin" is derived from the homology UCN exhibits to the teleost hormone urotensin I, while the second component stems from the homology to CRH. Indeed, UCN shares 63% identity to teleost fish urotensin I, 45% identity to rat/human CRH and 35% identity to a 40-amino acid non-mammalian CRH-like peptide, sauvagine. Like CRH, UCN can stimulate ACTH production from anterior pituitary corticotrophs *in vitro* and *in vivo* [1]. UCN is more potent than CRH with regard to other biological effects, including suppression of appetite, but is less potent in generating anxiety. A few years after the identification of UCN, two additional mammalian CRH-like peptides were isolated. Urocortin 2 (UCN2; stresscopin-related peptide) is a 38-amino acid peptide with an amidated C-terminus that was first predicted in mouse from a gene encoding a 112-amino acid protein [32,13,42]. Human urocortin 3 (UCN3; stresscopin) was identified later and found to have high identity (>80%) to UCN2 [20,37,33,16]. The biological effects of CRH and UCNs are mediated by two distinct receptors, CRH receptor type 1 (CRHR1) and 2 (CRHR2), which belong to the G protein-coupled receptor superfamily of brain-gut neuropeptides [28,41,5,29]. Two separate genes encode the CRH receptors [5]. CRHR1, a 415-amino acid protein, exhibits high affinity towards CRH and UCN, but low affinity towards UCN2 and no affinity towards UCN3. CRHR1 is primarily expressed in the CNS and the anterior pituitary. The CRHR2 receptor shares 70% sequence identity with CRHR1 and is expressed primarily in extra-CNS sites. The CRHR2 receptors exhibit high affinity towards UCNs and no affinity towards CRH. Activation of CRHR2 suppresses multiple metabolic functions, including feeding in fasted mice [2], heat-induced oedema and gastric emptying [19].

The presence of UCN and CRH has previously been demonstrated in the rat adrenal gland [22,27]. Strong expression of UCN has been demonstrated throughout the mammalian adrenal medulla and in the cortical fasciculate and glomerulosa zones, but weak expression has been detected in the reticularis zone [8]. CRHR1 and CRHR2 were clearly expressed in the medulla, weakly expressed in the cortical fasciculate and glomerulosa zones and barely expressed in the reticularis zone [12,8]. These results suggest that UCNs may play important roles in physiological adrenal functions, particularly in the regulation of adrenal hormone synthesis.

In the bovine, CRH-IR has been demonstrated to be distributed in the adrenal medulla [23]. In addition, CRH binding sites have been demonstrated in cultured chromaffin cells [6]. From a

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physiological perspective, CRH has been proposed to enhance the steroidogenic response of the adrenal cortex to ACTH [10]. Moreover, exogenous CRH has been demonstrated to enhance adrenal steroidogenesis in hypophysectomised calves [18], implying that the effect of the CRH is direct and not via ACTH. To our knowledge, little or no data are available regarding the presence and putative roles of UCNs in the adrenal gland of the bovine.

The aim of the present study was to investigate the expression of UCN, CRHR1 and CRHR2 in the bovine adrenal gland to clarify the mechanisms of the intra-adrenal CRH-based regulatory system. For this purpose, we performed Western blot and immunohistochemistry to study the presence and distribution of these proteins and real-time RT-PCR to evaluate mRNA expression levels in both the cortex and medulla.

2. Methods

2.1. Animals and tissue collection

This study was performed using six adult (age ranged from 1.5 to 5 years) healthy animals of both sexes coming from the same farm located in Southern Italy. The animals were bred in natural environmental conditions and were fed hay and concentrate. Water was available continuously. The animals were sacrificed in a public slaughterhouse according to the current Italian animal care protocols. Bovine hypothalamus (as a positive control) and adrenal glands from these animals were collected in the same day. For western blot and RT-PCR analyses, adrenocortical tissue was dissected from medullary tissue and immediately frozen on dry ice and stored at −80 °C. For immunohistochemical studies, fresh segments of adrenal gland were immediately fixed.

2.2. Western blot analysis

Frozen tissues were homogenised in a homogenisation buffer (50 mM Tris HCl, pH 7.00; 150 mM NaCl; 2% Triton; 5 mM EDTA; 10 mg/ml leupeptin; 0.1 U/ml aprotinin; 1 mM PMSF) using an Ultra-Turrax homogeniser and centrifuged at 16,000*g* for 20 min at 4 °C. Aliquots of the supernatant were subjected to electrophoresis on 12% and 18% sodium dodecyl sulphate (SDS)–polyacrylamide (Bio-Rad, Hercules, CA, USA) gels. After electrophoresis, the gel was transferred to nitrocellulose using a semi-dry apparatus (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The membrane was incubated for 1 h at 42 °C in 5% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) in TBST (150 mM NaCl; 20 mM Tris HCl, pH 7.4; 0.3% Tween-20), washed with TBST and incubated for 2 h at RT in the antisera diluted 1:500 in TBST containing 1% BSA. The following antisera were used: polyclonal goat anti-urocortin (sc-1825, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-CRHR1 (sc-12383; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-CRHR2 (sc-1826; Santa Cruz Biotechnology, CA, USA). To confirm specificity, the membranes were also incubated in the presence of primary antibody pre-absorbed (pre-absorption control) with the specific immunising peptides. For pre-absorption, urocortin, CRHR1 and CRHR2 antibodies diluted 1:500 (0.4 μg/ml) were incubated with a 10-fold (by weight) excess of blocking peptide (UCN peptide sc-1825P, CRHR1 peptide sc-12383P and CRHR2 peptide sc-1826P; synthesised by Santa Cruz Biotechnology, Inc., 2145 Delaware Avenue Santa Cruz, CA 95060, USA; purity: >95%) in a small volume (500 μl) of TBS overnight at 4 °C before being applied to the blots instead of the primary antibody alone. The membrane was washed three times with TBST, incubated for 1 h with anti-goat IgG peroxidase conjugate (Vector Laboratories, Burlingame, CA, USA) diluted 1:2000 in TBST–1% BSA and washed three times with TBST. Proteins were visualised by an enhanced chemiluminescence kit (Amersham, Buckinghamshire, UK). Marker proteins (coloured protein molecular weight markers; Rainbow, Amersham) were used to estimate the molecular weight of each band.

2.3. Immunohistochemistry

Fresh segments of adrenal gland were fixed by immersion in Bouin's fixative (6–24 h), processed for paraffin embedding in a vacuum and cut at a thickness of 5–7 μm. The avidin–biotin–peroxidase complex (ABC) method was performed using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). Paraffin sections were deparaffinised in xylene and hydrated in a graded series of ethanol solutions. After the quenching of endogenous peroxidase activity in water containing 3% hydrogen peroxide for 30 min, nonspecific binding was blocked by treatment with 1.5% normal rabbit serum (Vector) in 0.01 M phosphate-buffered saline (PBS; pH 7.2) for 30 min. Polyclonal goat anti-urocortin (sc-1825), anti-CRHR1 (sc-12383) and anti-CRHR2 (sc-1826) were singularly applied to the sections at a dilution of 1:500, and each specimen was incubated in a moist chamber overnight at 4 °C. After the sections had been washed three times in PBS, biotinylated anti-rabbit IgG (Vector) was applied at a dilution of 1:200. The sections were again incubated for 30 min at room temperature. Freshly prepared ABC reagent (Vector) was applied and incubated for 30 min after three washes in PBS. The localisation of the immunoreactions was visualised by incubating the sections for 5 min in freshly prepared diaminobenzidine–nickel solution (Vector). The specificity of the immunoreactions was tested by replacing the primary antibody with buffer. No immunoreaction was detected in control tests. The specificity of the primary antibodies was tested by absorption with excess homologous antigen peptide as described above for the Western blot (UCN peptide sc-1825P, CRHR1 peptide sc-12383P and CRHR2 peptide sc-1826P; Santa Cruz Biotechnol-

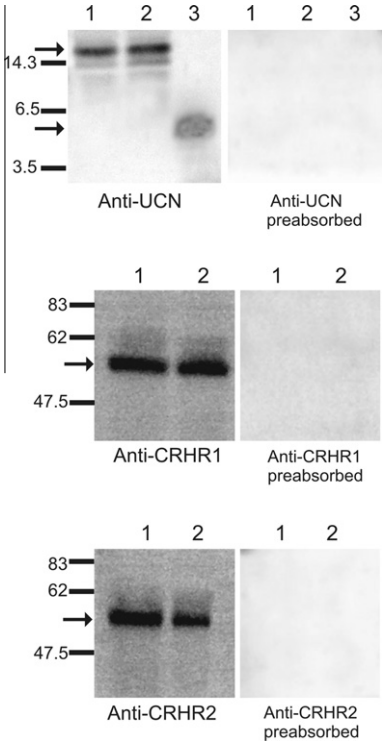

Fig. 1. UCN, CRHR1 and CRHR2 expression by western blot analysis. Lane 1: adrenal cortex tissue; lane 2: adrenal medulla tissue; lane 3: UCN synthetic peptide (100 ng/lane); molecular weight markers are expressed in kDa. No immunoreaction was observed in the blots tested with pre-adsorbed antibodies.

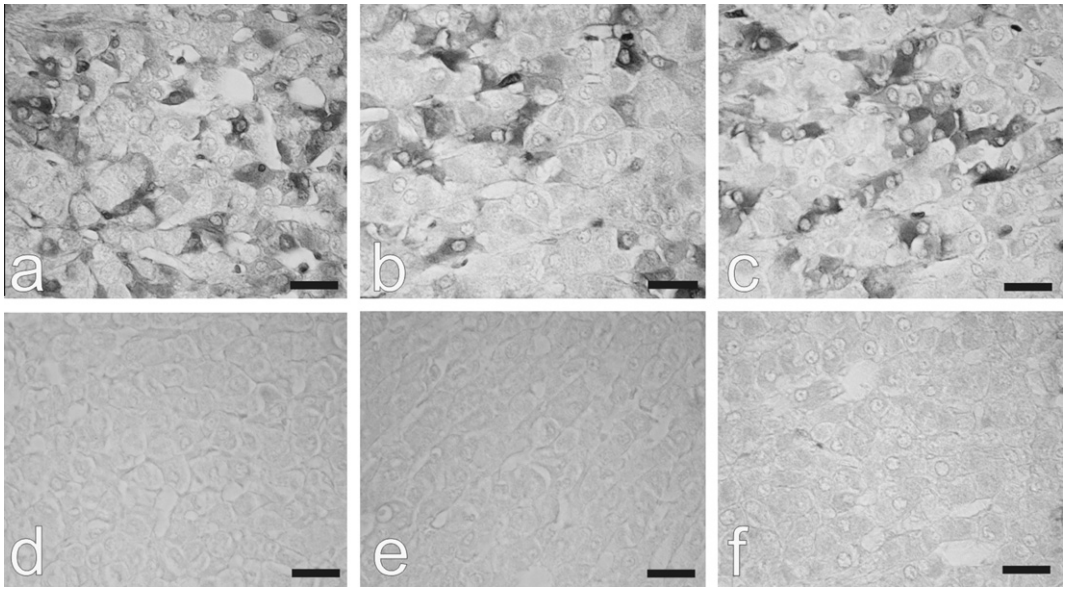

Fig. 2. Representative controls for immunohistochemistry experiments in the bovine adrenal gland. (a–f) Sections of the *zona fasciculata* of the adrenal cortex. (a) UCN-IR; (b) CRHR1-IR; (c) CRHR2-IR. The staining disappeared when the primary antibodies against (d) UCN, (e) CRHR1 and (f) CRHR2 were pre-adsorbed with synthetic peptides. Bar = 50 micron.

ogy) (Fig. 2). The specificity of the anti-CRHR1 and anti-CRHR2 antisera was tested in dot-blot assays. To perform dot-blot analysis, we spotted a serial dilution of tissue homogenate onto a nitrocellulose membrane and allowed it to air dry. The membrane was then treated in a manner similar to that used in the Western blot analysis.

The slides were observed using a Leica DMRA2 microscope (Leica Microsystems, Wetzlar, Germany).

2.4. RNA extraction, cDNA synthesis, RT-PCR and sequencing

Samples of the adrenal cortex and medulla were individually homogenised in ice-cold TRI-Reagent (Sigma, St. Louis, MO, USA) using an Ultra-Turrax homogeniser. After chloroform extraction and isopropyl alcohol precipitation, RNA was dissolved in RNAase-free DEPC water. Total RNA was measured with an Eppendorf Biophotometer (Eppendorf AG, Basel, Switzerland). For cDNA synthesis, 1 μg of total RNA was retro-transcribed with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) and random hexamers as primers. For PCR reactions, specific primers for bovine urocortin and CRH-R1 were designed from the published gene sequences (*Bos taurus* urocortin mRNA, Genbank accession number NM_001032301; *B. taurus* CRHR1, mRNA, Genbank, NM_174287) using the Primer Express™ software (PE Applied Biosystems). Specific primers for bovine CRH-R2 were designed generating a multiple sequence alignment based on the published sequences in GenBank from *Mus musculus* CRHR2 mRNA Genbank accession number NM_009953, *Homo sapiens* CRHR2, mRNA Genbank accession number NM_001883, *Rattus norvegicus* CRHR2, mRNA Genbank accession number NM_022714 and primers were designed in conserved regions of the nucleotide sequence. The sense and anti-sense urocortin primers used were 5′-CGA CCC TCC CCT GTC CAT-3′ and 5′-TTC CTG TCG CTC ACG TCT CA-3′, respectively, which amplify a 200-bp fragment; the sense and anti-sense CRH-R1 primers were 5′-TAC GAC AAT GAG AAG TGC TGG TTT-3′ and 5′-GGC ACG GAT TGA GTG CTT GT-3′, respectively, which amplify a 409-bp fragment; the sense and anti-sense CRH-R2 primers were 5′-GCT GTC TGC GGA ACG TGA TT-3′ and 5′-CGA AGT AGT TGA AGA TGG TGG TGA T-3′, respectively, which amplify a 200-bp fragment.

The PCR cycle conditions were as follows: 94 °C (30 s), 60 °C (30 s), 72 °C (1 min) for 35 cycles; 72 °C (5 min). The PCR products of bovine urocortin, CRHR1 and CRHR2 were purified using GFX PCR DNA and Gel Purification Kit (code 28-9034-70, GE Healthcare) and sequenced.

2.5. Real-time RT-PCR

The sense and anti-sense urocortin primers used were 5′-CGA CCC TCC CCT GTC CAT-3′ and 5′-TTC CTG TCG CTC ACG TCT CA-3′, respectively, which amplify a 200-bp fragment. The sense and anti-sense CRH-R2 primers were 5′-GCT GTC TGC GGA ACG TGA TT-3′ and 5′-CGA AGT AGT TGA AGA TGG TGG TGA T-3′, respectively, which amplify a 200-bp fragment. The sense and anti-sense beta-Actin primers were 5′-CAG CTC CTC CCT GGA GAA GA-3′ and 5′-CTG CTT GCT GAT CCA CAT CTG-3′, which amplify a 398-bp fragment.

The PCR conditions were 50 °C for 2 min and 94 °C for 10 min, followed by 40 cycles of 94 °C for 15 s and 60 °C for 1 min. The PCR reactions contained 1 μl cDNA (40 ng/well) and 24 μl of SYBR Green Master Mix (Applied Biosystems) containing specific primers. A sample without cDNA template was used to verify that the master mix was free from contaminants. The samples were analysed in triplicate.

The β-Actin gene was also amplified in separate tubes under the same conditions to serve as an active endogenous reference to normalise quantification of the mRNA target. Real-time PCR reactions for the target and reference genes were run in the same RT reaction. Real-time detection was performed on an ABIPRISM 7300 Sequence Detection System (Applied Biosystems), and data from SYBR Green I PCR amplicons were collected with ABI 7300 System SDS Software. The fluorescence baseline signal and threshold were set manually for each detector (UCN, CRHR2, beta-Actin), generating a threshold cycle (C_t) for each sample. An amplification plot graphically displayed the fluorescence detected over the number of cycles that were performed.

Standard curves for both targets and the endogenous reference gene, created on the basis of the linear relationship between the C_t value and the logarithm of the starting amount of cDNA, showed acceptable slope values (between −3.8 and −3.3). Standard curves

were obtained by using serial dilutions of sample cDNA (1:2, 1:4, 1:8, 1:16 and 1:32).

The relative quantification method 2-[Delta][Delta] C_t (2-delta delta C_t) (DDC_t) was used for normalisation of gene expression [21,14,44]. Before using the DDC_t method for relative quantification (comparative method), a validation experiment was required to demonstrate that the efficiencies of the target amplification and of the reference amplification were almost equal. All PCR efficiencies were measured and found to be adequate (slope < 0.1). The DDC_t method used in this study has previously been described [36]. Briefly, the difference between C_t values was calculated for each mRNA by taking the mean C_t of triplicate reactions and subtracting the mean C_t of triplicate reactions for the reference RNA measured in an aliquot from the same RT reaction ($DC_t = C_t$ (target gene) − C_t (reference gene)). All samples were then normalised to the DC_t value of a calibrator sample to obtain a DDC_t value (DC_t (target) − DC_t (calibrator)). Adrenal cortex was used as the calibrator sample in the comparison with adrenal medulla. Dissociation melt curves confirmed the specific amplification of the cDNA target and the absence of nonspecific products. The results are represented as mean (±SEM) of triplicate determinations for each tissue and from each experimental animal. For statistical analyses, the data were expressed as the mean ± standard error. Significant differences in UCN and CRHR2 mRNA levels between the calibrator sample (adrenal cortex) vs. adrenal medulla were determined using Student's *t*-test for independent samples. The level of statistical significance was set at $p < 0.05$ for all.

3. Results

3.1. Western blot

The results of the western blot analysis are shown in Fig. 1. Tissue extracts of the cortex and medulla of the bovine adrenal gland reacted with the anti-UCN, -CRHR1 and -CRHR2 antibodies. The cortex and medulla tissue extracts and the synthetic peptide UCN reacted with the anti-UCN antibody (Fig. 1). The antiserum recognised one major protein band of approximately 16 kDa from homogenates and one protein band of approximately 6.5 kDa from the synthetic peptide. The cortex and medulla tissue extracts also reacted with the anti-CRHR1 and anti-CRHR2 antibodies (Fig. 1). The antisera recognised one protein band measuring approximately 55 kDa from the homogenates.

3.2. Immunohistochemistry

In the bovine adrenal gland, UCN-immunoreactivity (IR) was found both in the cortex and in the medulla (Fig. 3a). In the cortex, UCN-IR was distributed in the *zona fasciculata* (Fig. 3b) and in the *zona reticularis* (Fig. 3e). In the *zona fasciculata*, immunoreactivity was primarily detected in the fasciculate cells located in the outer portion underneath the *zona glomerulosa*. In the *zona reticularis*, only some scattered cells were immunoreactive. In the adrenal medulla, almost all of the medullary cells were positive (Fig. 3h). The distribution of CRHR1- (Fig. 3c, f and i) and CRHR2- IR (Fig. 3d, g and l) was similar to that of UCN-IR.

3.3. RT-PCR and real time RT-PCR

RT-PCR experiments demonstrated that UCN and CRHR2 were expressed in both the cortex and medulla, while CRHR1 was expressed only in the medulla (Fig. 4a). Real-time RT-PCR experiments confirmed that UCN and CRHR2 were expressed in both the bovine adrenal cortex and medulla. The levels of UCN and CRHR2 mRNA expression were higher in the cortex than in the medulla (Fig. 4b).

4. Discussion

The results of the present study revealed the presence of UCN, CRHR1 and CRHR2 mRNA and protein in the bovine adrenal gland using different experimental approaches. Specifically, western blot analysis was used to study the presence of UCN and its receptors in the bovine adrenal cortex and medulla. Immunohistochemical studies were performed to evaluate the distribution and localisation of these proteins throughout the bovine adrenal gland, and RT-PCR and real-time RT-PCR analyses were performed to reveal and quantify the mRNA levels of UCN, CRHR1 and CRHR2. The utilisation of multiple experimental approaches allowed us to obtain complementary information regarding the expression and the putative roles of UCN, CRHR1 and CRHR2 in the bovine adrenal gland.

Western blot analysis showed that the anti-mammalian UCN antiserum used in the present study recognised a protein band with a molecular weight of approximately 16 kDa in the bovine adrenal gland. This protein is comparable to the mammalian UCN precursor, which is a 122-amino acid protein [40,9]. In addition, the antisera used against CRHR1 and CRHR2 recognised protein bands with molecular weights of approximately 55 kDa. These proteins are comparable to the mammalian CRH-Receptors [29,5].

The results of the present study showed that in the bovine adrenal cortex, UCN-, CRHR1- and CRHR2-IRs were distributed in the *zona fasciculata* and *zona reticularis*, but not in the *zona glomerulosa*. These results agree only in part with those reported in human and rat adrenal cortex, in which immunoreactivity to these proteins is expressed in all three adrenocortical zones, with a stronger intensity in the *zona glomerulosa* and *zona fasciculata* than in the *zona reticularis* [7,23,11]. These discrepancies may reflect interspecies variations in the distribution and consequently, the function, of the UCN/CRHRs system throughout the mammalian adrenal cortex.

Our findings suggest that UCN affects steroid secretion via CRHRs preferentially in an autocrine manner. Several reports have indicated that CRH and its related peptides play a role in the regulation of adrenal hormone synthesis [7,11] and affect the adrenal glands directly as local modulators of adrenal functions [38,39]. CRH and UCN stimulate cortisol production by human foetal adrenocortical cells *in vitro* [34,35] and cortisol and aldosterone secretion in human adrenal H395R cells [17]. In the bovine, exogenous CRH has been reported to enhance adrenal steroidogenesis in hypophysectomised calves [16], implying that the effect of the CRH is direct and does not act via ACTH. Because the immunoreactivity to UCN and its receptors was not detected in the *zona glomerulosa*, one can hypothesise that the UCN system influences cortisol [34] and sexual steroid [30,31,34,35] rather than aldosterone synthesis in the bovine adrenal gland.

The results regarding the distribution of UCN-, CRHR1- and CRHR2-IRs in the medulla are in accordance with previous reports that have described the expression of UCN-, CRHR1- and CRHR2-IRs in the mouse, human and rat adrenal glands [7,23,11,38].

CRH peptides also affect *de novo* catecholamine synthesis. More specifically, UCN induces tyrosine hydroxylase (TH) expression via cAMP in rat adrenomedullary PC12 cells [24], while UCN2 induces TH phosphorylation via the PKA-Erk1/2 pathway [25,26]. Both CRHR1 and CRHR2 agonists have been observed to induce *de novo* catecholamine synthesis via induction of TH [8]. Thus, prolonged exposure of the adrenal glands to stress peptides (a condition that occurs during chronic stress) may result in increased TH expression levels, leading to the elevation of stored adrenaline in the secretory vesicles of adrenal medullary cells, thus augmenting the magnitude of the catecholaminergic response to stressors [8].

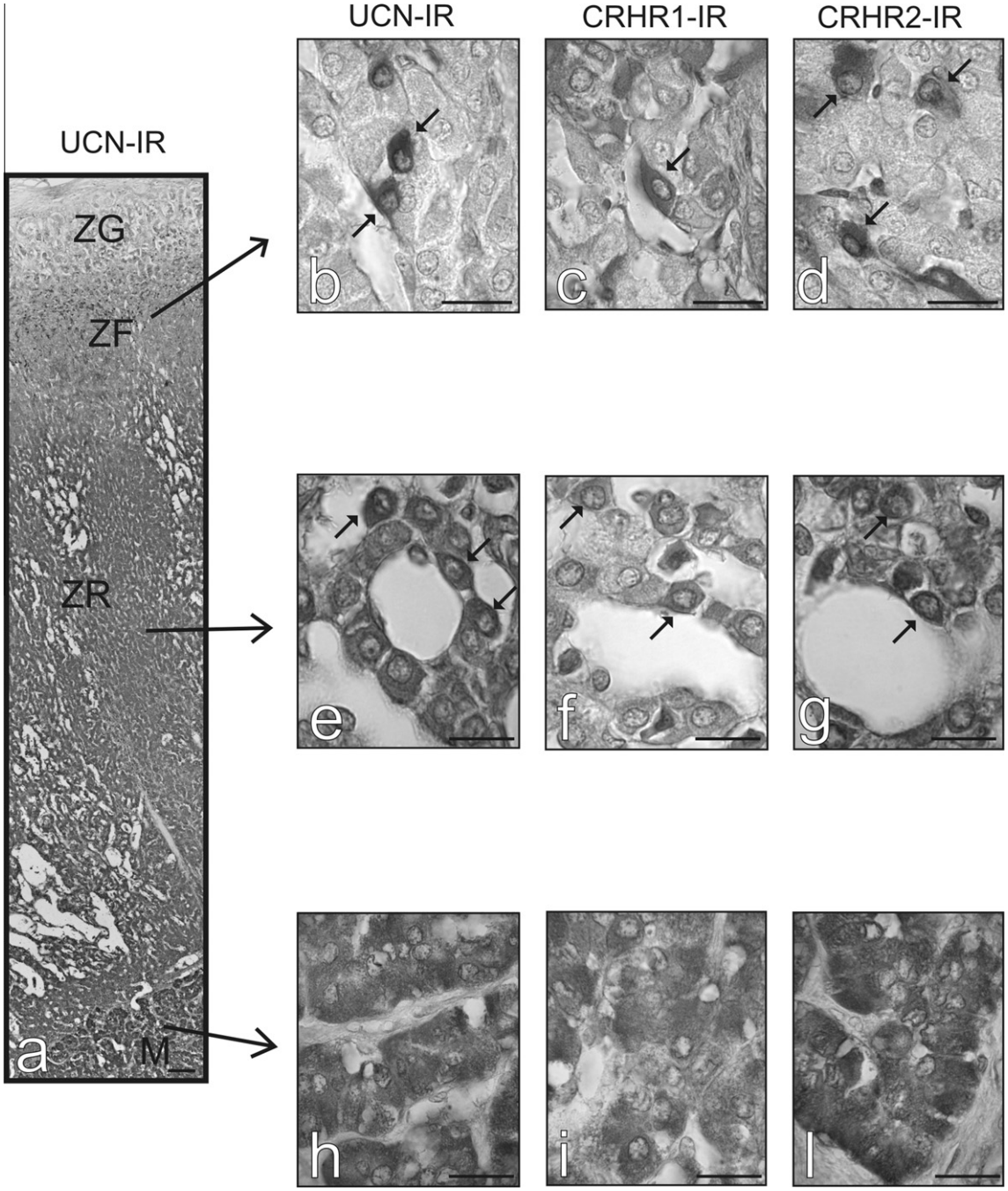

Fig. 3. Distribution of the UCN-, CRHR1- and CRHR2-immunoreactivities in the bovine adrenal gland. (a) UCN-IR was distributed in the *zona fasciculata* and *zona reticularis* of the adrenal cortex and in the medulla. ZG: *zona glomerulosa*; ZF: *zona fasciculata*; ZR: *zona reticularis*; M: medulla; bar = 100 μm. (b) Higher magnification of the *zona fasciculata* shows UCN-immunoreactive cells in this zone (arrows); bar = 25 μm. (c) Higher magnification of the *zona fasciculata* shows CRHR1-immunoreactive cells in this zone (arrows); bar = 25 μm. (d) Higher magnification of the *zona fasciculata* shows CRHR2-immunoreactive cells in this zone; bar = 25 μm. (e) Higher magnification of the *zona reticularis* shows UCN-immunoreactive cells in this zone (arrows); bar = 25 μm. (f) Higher magnification of the *zona reticularis* shows CRHR1-immunoreactive cells in this zone (arrows); bar = 25 μm. (g) Higher magnification of the *zona reticularis* shows CRHR2-immunoreactive cells in this zone (arrows); bar = 25 μm. (h) Higher magnification of the medulla shows that the great majority of the cells were UCN-positive in this zone; bar = 25 μm. (i) Higher magnification of the medulla shows that the great majority of the cells were CRHR1-positive in this zone; bar = 25 μm. (l) Higher magnification of the medulla shows that the great majority of the cells were UCN-positive in this zone; bar = 25 μm.

RT-PCR experiments revealed that UCN, CRHR1 and CRHR2 mRNAs were expressed in the bovine adrenal gland, thus confirming the immunohistochemical staining data. These results were in accordance with previous studies in which UCN, CRHR1 and CRHR2 mRNAs were found in both the human and murine adrenal glands [24,12]. However, in the present study, RT-PCR analysis revealed that CRHR1 mRNA was present only in the medulla, while immunohistochemistry and western blot analysis demonstrated that CRHR1 protein was present in both the cortex and medulla. This discrepancy is probably due to the very low levels of CRHR1 mRNA expression.

Real-time RT-PCR experiments revealed that UCN and CRHR2 mRNA levels were higher in the cortex than in the medulla. Until now, no data have been available regarding the quantitative distribution of UCN and CRHR2 transcripts in the mammalian adrenal gland. This result suggests that the activity of the urocortinergic

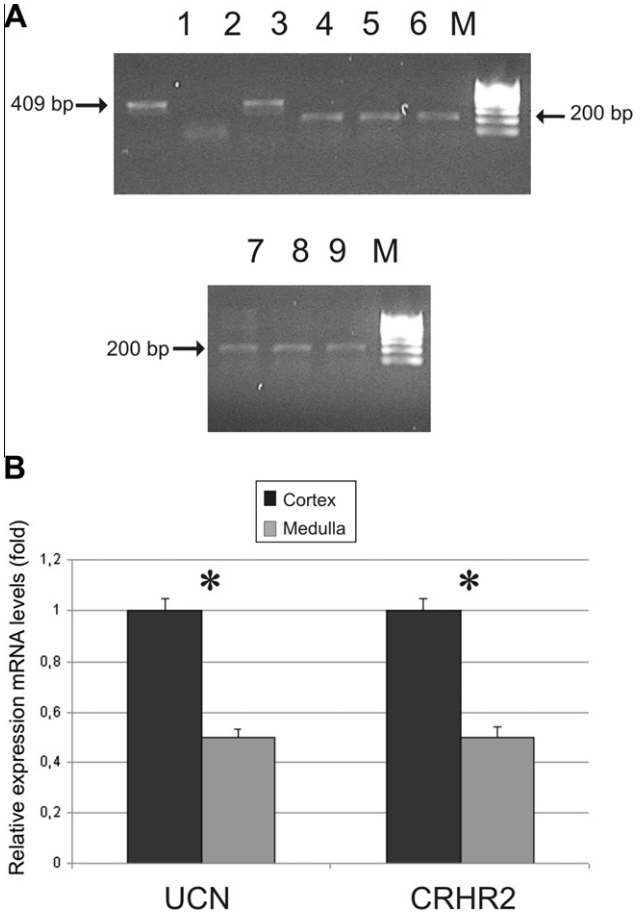

Fig. 4. UCN, CRHR1 and CRHR2 expression by RT-PCR (A) and real-time RT-PCR (B). (A) CRH-R1 (409-bp fragment) was expressed in the bovine hypothalamus (lane 1) and in the adrenal medulla (lane 3), but not in the adrenal cortex (lane 2); CRH-R2 (200-bp fragment) was expressed in the bovine hypothalamus (lane 4), adrenal cortex (lane 4) and medulla (lane 6); UCN (200-bp fragment) was expressed in the bovine hypothalamus (lane 7), adrenal cortex (lane 8) and medulla (lane 9); M: molecular markers of 100-bp ladder. (B) Real-time RT-PCR of UCN and CRH-R2 mRNA expression in the bovine adrenal gland. The calibrator sample was adrenal cortex vs. adrenal medulla. Significant differences in CRHR2 and UCN mRNA levels between the calibrator sample (adrenal cortex) vs. adrenal medulla were determined using Student's *t*-test for independent samples. The level of statistical significance was set at $p < 0.05$ for all. $^{*}p < 0.05$;

system in the adrenal gland is higher in the cortex than in the medulla.

CRH peptides in the adrenals may affect catecholamine and steroid synthesis under a complex mechanism that depends on the ligand concentration and receptor availability. The concentration of bioavailable CRH peptides is known to be modulated by the CRH-binding protein (CRH-BP), a 37 kDa protein first isolated in the human plasma [3] and subsequently discovered in the brain [30] and in several other tissues, including the adrenal gland [4]. CRH-BP binds to both CRH and UCN with high affinity and to UCN2 with intermediate affinity, but does not appreciably bind to UCN3 [15]. CRH-BP has higher affinity for CRF and UCN than CRHR2, thus CRH-BP is generally considered an antagonist of this receptor. CRH-BP seems to limit the activity of UCN [30,34] and, moreover, to increase the half-life of CRH or other CRH-like ligands by protecting them from degradation and delivering the ligand to receptors in the target tissue (thus enhancing activity). Other CRHR2 ligands, such as UCN2 and UCN3, probably interact with this receptor to regulate adrenal functions. In addition to UCN1, UCN2 and UCN3 have also been demonstrated to be involved in the intra-adrenal CRH-based regulatory system [8,12,38]. A complex intramedullary UCN1–UCN2/CRH receptor system exists in the human and rat adrenal gland that controls catecholamine synthesis and secretion [8]. In addition, UCN3 is expressed in the human adrenal gland, where it may play a role by binding to CRHR2 in the regulation of the adrenal steroidogenesis and may therefore exert vasodilator effects on the adrenal circulation [12,37,38].

In conclusion, these results suggest that UCN, CRHR1 and CRHR2 are expressed in the bovine adrenal gland. The levels of CRHR2 and UCN mRNAs are higher in the adrenal cortex. These data suggest that UCN plays a major role in the intra-adrenal CRH-based regulatory system through an autocrine mechanism that preferentially acts via CRHR2.

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