Expression and Function of Endocannabinoid Receptors in the Human Adrenal Cortex

Authors

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- anandamide
- CB1 antagonist
- cortisol
- aldosterone

Abstract

Endogenous cannabinoids are important signaling molecules in neuroendocrine control of homeostatic and reproductive functions including stress response and energy metabolism. The hypothalamic paraventricular and supraoptic nuclei have been shown to release endocannabinoids, which act as retrograde messengers to modulate the synaptic release of glutamate during stress response. This study endeavors to elucidate possible interaction of the endocannabinoid system with the regulation of adrenocortical function at the adrenal level. Human adrenocortical NCI-H295R cells and normal human adrenal glands were used to study the possible effects of anandamide and cannabinoid receptor 1 (CB1) antagonist SR141716A on aldosterone and cortisol secretion. Our data indicate the expression of CB1 in human adrenal cortex and adrenocortical NCI-H295R cells; CB2 was not expressed. Furthermore, anandamide inhibited basal release and stimulated release of adrenocortical steroids (corticosterone and aldosterone); this effect was reversed by CB1 antagonist (SR141716A). Therefore, the endocannabinoid system at the level of the adrenal, can directly influence adrenocortical steroidogenesis.

Introduction

The hypothalamic-pituitary-adrenal (HPA) axis is the major hormone system responsible for maintaining homeostatic balance in response to stressful stimuli [1]. Appropriate regulatory control of the HPA axis is critical for health and survival, and several limbic brain structures, such as the hippocampus, amygdala, and prefrontal cortex, are involved in the integration of the HPA hormonal response [2,3]. Activation of the stress system leads to behavioral and peripheral changes that improve the ability of the organism to adjust homeostasis and increase its chances for survival. The response of the endocrine system to stress is characterized by activation of the sympathetic adrenomedullary system with increased epinephrine secretion and by HPA axis activation with hypersecretion of adrenocortical hormones, particularly glucocorticoids. Aldosterone, the body's most potent mineralocorticoid, is secreted by the outer zone of the adrenal cortex, the zona glomerulosa. It plays an important role in blood pressure adjustment by regulating salt-water homeostasis [4].

The endocannabinoid system (ECS) is a recently identified neuromodulatory system involved in several physiological and pathopysiological processes. Endocannabinoids mimic some effects of marijuana by binding to cannabinoid receptors. Two cannabinoid receptors, CB1 and CB2, have been described with regard to their primary structure, ligand binding properties and signal transduction systems [5]. CB1 receptors are widely distributed throughout the brain and affect memory, pain perception and control of movement [6]. The highest densities of CB1 receptors are found in the basal ganglia, cerebellum, hippocampus and dentate gyrus. CB1 receptors are also found in amygdala and peripheral tissues such as spleen, tonsils, heart, reproductive organs, and adrenal [7]. CB2 receptors are found primarily in immune cells such as monocytes [8], but are also present in the brain stem and glia [9]. To date, the best-characterized endocannabinoids are arachidonoyl ethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG) [10].

Emerging evidence exists that the endocannabinoid system plays a role in endocrine regulation and energy balance including a modulation of

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HPA function [11]. Mice lacking cannabinoid receptor type 1 ($CB1^{-/-}$) displayed a dysregulation of the HPA axis with a central impairment of glucocorticoid feedback and an enhanced circadian HPA axis activity peak resulting in elevated plasma corticosterone levels at the onset of dark [12–14]. It is, however, unknown whether endocannabinoids influence adrenal glucocorticoid synthesis directly at the level of the adrenal in addition to their central effects. Furthermore, the CB1 receptor antagonist SR141716A has been shown to reduce blood pressure in obese patients; it is suggested that this is primarily mediated by the weight-reducing effect of the antagonist (for review [15]). A possible direct effect on aldosterone secretion at the adrenal level, however, has not yet been studied.

The present study, therefore, examines the expression of endocannabinoid receptors and possible effects of endocannabinoids on the release of cortisol and aldosterone in human adrenals and the human adrenocortical cell line NCI-H295R.

Materials and Methods

Culture of NCI-H295R cells

As previously described [16], NCI-H295R cells were cultured in DMEM/F12 (containing 33 µM biotin, 17 µM calcium D-pantothenate, 15 mM HEPES, 14 mM $NaHCO_3$), supplemented with insulin (66 nM), transferrin (10 µg/ml), selenite (30 nM), penicillin (100 units/ml), streptomycin (100 µg/ml), and 2% FBS. NCI-H295R cells were grown in 75 cm^2 flasks (Becton Dickinson, Heidelberg, Germany) at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The medium was changed every 2 days, and cells were subcultured every 7 days using Accutase (PAA Laboratories, Pasching, Austria) for cell detachment. Cells used for experiments were subcultured from 70–80% confluent stock cultures in 48 or 96-well culture plates (Nunc) at a density of 140 000 cells per cm^2 for 96 or 120 h. H295R cells were stimulated with angiotensin II (Ang II; 10^{-7} M) or forskolin (FKS; 2×10^{-5} M) alone or in combination with anandamide (10^{-7}–10^{-9} M) or with AEA alone (10^{-7}–10^{-9} M). All stimulations were done for 3 h. The effect of AEA was antagonized using the CB1 antagonist SR141716A (SR; 10^{-6} M), kindly provided by Sanofi Aventis Deutschland, Berlin, Germany.

Aldosterone and cortisol measurement

Aldosterone and cortisol in the incubation medium were measured by direct specific radioimmune assays (DSL-8 600; Diagnostic Systems Laboratories, Webster, TX, USA) according to the manufacturer's protocol.

RT-PCR

Human adrenal tissue was obtained from nephrectomies where the ipsilateral adrenal was removed with the kidney. Total RNA from harvested NCI-H295R cells, human adrenal tissue and peripheral blood mononuclear cells (PBMC) was isolated using RNeasy Plus Mini kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription was performed with the reverse transcription buffer, RNase inhibitor, oligo(dT)15 primer, dNTP mix, and M-MLV reverse transcriptase at 37 °C for 60 min according to the manufacturer's specifications (Promega, Madison, WI, USA). The PCR reactions were carried out with initial denaturation at 94 °C for 4 min; 40 cycles amplification at 94 °C for 15 s, annealing for 15 s at 63 °C (CB1) or 65 °C (CB2), and elongation at 72 °C for 15 s, followed by final extension at 72 °C for 1 min.

PCR: Primer sequences (5–3')

Human CB1 (forward): ggacagaaaacaactggactcctca
(reverse):tagacaaaaatgacactccccagga
Human CB2: (forward): cgaagacatcaaaggagaatgagga
(reverse): ggaggacaggatcagatagagcaca

Statistics

Values are expressed as means ± SEM. One-way ANOVA was used to analyze the in vitro results followed by Tukey's multiple comparison test. A p-value of less than 0.05 was considered to be statistically significant. This study was approved by the ethical committee of the University of Dresden.

Results

RT-PCR data revealed that normal adrenal cells express the endocannabinoid receptor CB1 and to a lesser extent CB2 (**Fig. 1**). The tumorous human adrenocortical cell line (NCI-H295R) expressed CB1, but not CB2 (**Fig. 1**). Human peripheral blood monocytes (PBMC), known to express CB2, were used as positive control (**Fig. 1**). The presence of CB1 in human adrenal suggests an influence of CB1 agonists on adrenocortical function. We therefore tested whether the CB1 agonist anandamide (AEA) influences steroidogenesis in human adrenocortical cells. In NCI-H295R cells, a well-accepted model for human adrenocortical steroidogenesis, AEA significantly reduced basal aldosterone (**Fig. 2A;** $p < 0.01$) and cortisol secretion (3 h incubation) (**Fig. 3A**; $p < 0.01$). Furthermore, steroidogenesis was stimulated by angiotension II (Ang II 10^{-7} M; $p < 0.0001$) or forskolin (FSK 2×10^{-5} M; $p < 0.01$). Ang II and forskolin stimulate adrenocortical steroidogenesis via IP3 or PKA pathways, respectively. Ang II- (**Fig. 2B**) and forskolin-stimulated (**Fig. 2C**) secretion of aldosterone was inhibited significantly by AEA ($p < 0.01$ and $p < 0.05$, respectively). In addition, Ang II- (**Fig. 3B**) and forskolin-stimulated (**Fig. 3C**) secretion of cortisol was inhibited significantly by AEA ($p < 0.01$ and $p < 0.05$, respectively). In accordance with previous data [17, 18] the maximal effective dose of AEA was 10^{-9} M with no further increase at higher doses. This inhibition of steroidogenesis was reversed by the CB1 antagonist SR141716A (SR; 10^{-6} M) (**Fig. 2** and **3**).

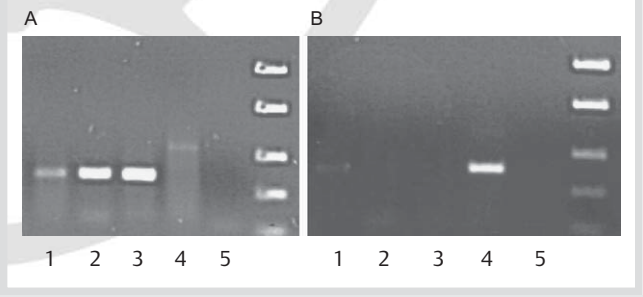

Fig. 1 RT-PCR for human CB1 **(A)** and human CB2 **(B)** receptors. The PCR products for CB1 and CB2 have the expected length of 259bp and 295bp, respectively. CB1 **(A)** is expressed in human adrenal (1) and NCI-H295R cells (2 and 3) while CB2 **(B)** is not expressed in NCI-H295R cells (2 and 3) and only weakly in the human adrenal (1). PBMC (4) show a strong expression of CB2. Lanes 5 in **A** and **B** are the respective negative controls.

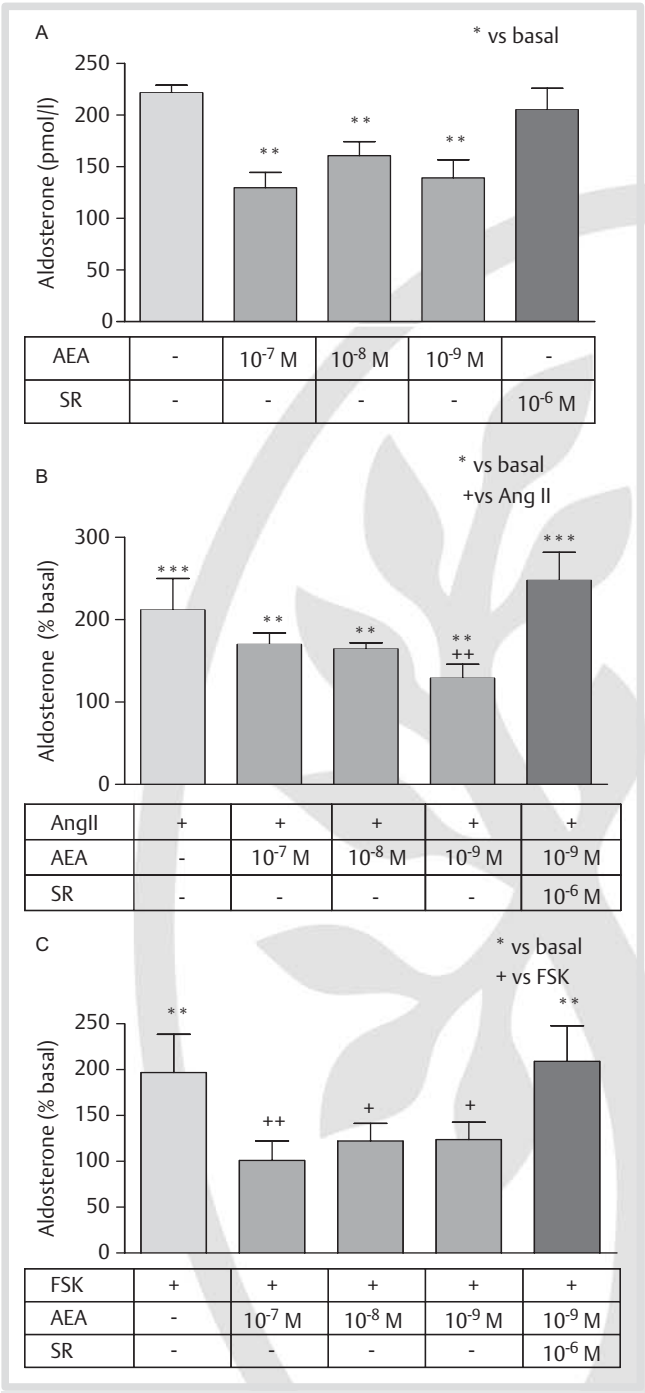

Fig. 2 Effect of anandamide (AEA) and SR141716A on aldosterone secretion after 3-hour incubation. **(A)** Basal, **(B)** angiotensin II (Ang II, 10^{-7}M), and (C) forskolin (FSK, 2×10^{-5}M) induced aldosterone secretion was significantly reduced by AEA 10^{-7}–10^{-9}M. This effect was reversed by concomitant incubation with SR141716A (SR, 10^{-6}M) (n = 3, mean ± SEM; *** p < 0.0001; ** and ++ p < 0.01; + p < 0.05).

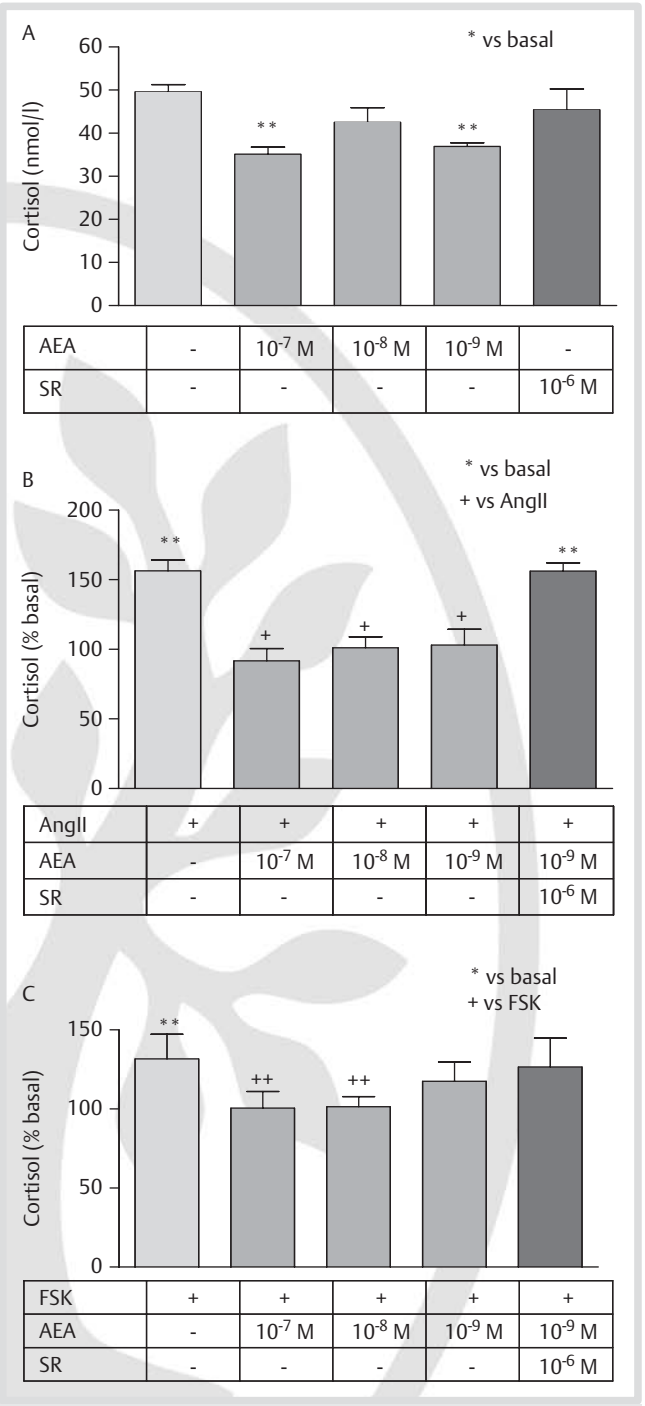

Fig. 3 Effect of anandamide (AEA) and SR141716A on cortisol secretion after 3-hour incubation. **(A)** Basal, **(B)** angiotensin II (Ang II, 10^{-7}M), and **(C)** forskolin (FSK, 2×10^{-5}M) induced cortisol secretion was significantly reduced by AEA 10^{-7}–10^{-9}M. This effect was reversed by concomitant incubation with SR141716A (SR, 10^{-6}M) (n = 3, mean ± SEM; ** p < 0.01; + p < 0.05).

Discussion

These data show that adrenocortical steroidogenesis within the human adrenal is directly influenced by the endocannabinoid system via CB1 receptors. Until a few years ago, the impact of cannabinoids on the HPA axis was considered to be an exception. Whereas the commonly accepted view attributes the cannabinoid system as having a general inhibitory role on neuroendocrine functions, it has been suggested that cannabinoids are, on the contrary, able to stimulate the HPA axis (for review [11]). This concept was recently challenged, however, by several reports showing a different action of endocannabinoids on the HPA axis. Specifically, it has been demonstrated repeatedly that acute treatment with the CB1 receptor antagonist SR141716A results in enhanced neuronal activation within the PVN and increased basal and stress-induced corticosterone secretion [19–21] as well as anxiety-like behavior [22]. Other

data indicate that the ECS might function as a gatekeeper of the HPA axis, exhibiting tonically high activity to suppress a persistent drive of the stress axis [23]. These investigations have demonstrated that endocannabinoids negatively modulate HPA axis activity in a context-dependent manner, thus reinforcing the idea initially proposed by Manzanares et al [24] that there is an endocannabinoid tone which exerts an inhibitory action over HPA axis activation [24,25]. It is suggested that upon exposure to stress, endocannabinoid levels rapidly decline through an undetermined mechanism, resulting in a disinhibition of glutamatergic projections to the PVN and allowing activation of the HPA axis [25].

Consistent with the assumption that endogenous cannabinoids inhibit the HPA axis, several independent reports employing transgenic mice lacking the CB1 receptor ($CB1^{-/-}$) have shown that these animals exhibit an enhanced HPA axis drive with an impaired glucocorticoid feedback [12–14]. Pharmacological studies support this hypothesis and indicate that the changes in HPA axis activation in $CB1^{-/-}$ mice are not a result of developmental compensation for the loss of the receptor. Interestingly, the in vitro release of ACTH from anterior pituitary fragments is similar in $CB1^{-/-}$ and wild-type (WT) mice [13] indicating interactions of the endocannabinoids with the HPA axis at other levels than the pituitary.

Previous studies focused on the interplay between the endocannabinoid system with the HPA axis at the central level. Our data indicate that, in addition to the central regulation of the HPA axis, endocannaboids via CB1 receptors directly inhibit adrenocortical steroidogenesis at the level of the adrenal. Human adrenal cortex expressed predominantly CB1 receptors. Similar to the normal human adrenal, the human adrenocortical cell line NCI-H295R expressed CB1 but not CB2 receptors. NCI-H295R cells are a widely accepted model for human adrenocortical studies and represent the only cell line able to produce all adrenocortical steroids and to express the three major pathways of adrenal steroidogenesis including the main steroidogenic enzymes. They have been used extensively to characterize the regulation of steroidogenesis including the regulation of aldosterone synthesis [26]. In the present study, these cells were used to analyze the effects of AEA and SR141716A on cortisol and aldosterone release. The CB1 agonist anandamide reduced basal as well as angiotensin II- and forskolin-stimulated cortisol and aldosterone secretion. Ang II stimulates adrenocortical steroidogenesis via inositol-1,3,4-phosphate [IP(3)] formation and subsequent Ca^{2+} mobilization while forskolin is an activator of the PKA pathway. The inhibition of Ang II- and forskolin-induced steroidogenesis by AEA indicates an effect further downstream in steroid synthesis. The inhibitory effect of AEA was reversed by the concomitant incubation with the CB1 receptor antagonist SR141716A.

In conclusion, our data contributes further in vitro evidence for an interaction of the endocannabinoid system with adrenal steroidogenesis by inhibiting adrenal function directly at the level of the adrenal. This effect might, in addition to a central influence of endocannabinoids on HPA axis function, be relevant in glucocorticoid homeostasis. Furthermore, endocannabinoids might, in addition, influence blood pressure regulation by reducing aldosterone secretion from the adrenal.

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Conflict of Interest

This study was supported by a research grant from Sanofi-Aventis, Germany.

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