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ACTH Elevates Gene Expression for Catecholamine Biosynthesis in Rat Superior Cervical Ganglia and Locus Coeruleus by an Adrenal Independent Mechanism

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Abstract

Classically, upon hypothalamic stimulation, ACTH is released from the pituitary and acts on melanocortin 2 receptors (MC2R) in the adrenal cortex, stimulating glucocorticoid synthesis and release. Our earlier studies suggested that ACTH might have a direct effect on sympathetic ganglia. To analyze further the involvement of ACTH in regulation of gene expression of norepinephrine (NE) biosynthetic enzymes, we examined the effect of bilateral adrenalectomy (ADX) of Sprague Dawley male rats. Fourteen days post ADX, as expected, plasma ACTH was elevated, and levels of TH, DBH and MC2R mRNAs in superior cervical ganglia (SCG), and TH mRNA in locus coeruleus (LC) were increased compared to sham operated animals. To determine effect of pulsatile elevation of ACTH, corticosterone pellets were implanted to ADX rats. Similar to immobilization stress ACTH injections to these animals caused a rise in ACTH in plasma and triggered elevation of TH and DBH mRNAs in SCG and in LC with single and repeated daily injections, and MC2R mRNA in SCG with single injections. To study the effect of ACTH in isolated cells, primary cultures of rat SCG were transfected with TH and DBH promoter constructs and treated with ACTH. In agreement with the *in vivo* data, ACTH elevated their promoter activities similar to levels triggered by CPT-cAMP. ACTH in the human SK-N-SH neuroblastoma cells increased TH and DBH promoter activity and endogenous DBH mRNA levels. The results show that ACTH can have a direct effect on transcription and gene expression of NE biosynthetic enzymes even without contribution of adrenal hormones.

Keywords

ACTH; Superior cervical ganglia; Locus coeruleus; Tyrosine hydroxylase; Dopamine β -hydroxylase

Stress triggers important neuroendocrine modifications that enable the organism to survive and restore homeostasis. However, prolonged stress is a major contributor to the development of cardiovascular and neuropsychiatric disorders [reviewed in (McEwen 1998; Chrousos 2000)]. It also increases the body's susceptibility to infection, autoimmune diseases, chronic fatigue syndrome, and cancer and the propensity of an individual to self-administer drugs of abuse. Moreover, stress can influence the progression of chronic diseases such as diabetes. Key components mediating the broad physiological range of responses to stress include activation

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of the hypothalamic-pituitary-adrenocortical axis (HPA) and catecholaminergic systems, both central and peripheral.

Classically, the release of pituitary adrenocorticotrophic hormone (ACTH) under direction of the corticotropin releasing hormone (CRH) from the hypothalamus resulting in synthesis and release of glucocorticoids from the adrenal cortex and the subsequent feed back inhibition is a well characterized response to stress [reviewed in (Dallman 1993; Jacobson 2005)]. However, ACTH appears to have functions in stress that are independent of its effect on the adrenal glucocorticoids. The melanocortin 2 receptor (MC2R), responsive only to ACTH, is expressed not only in the adrenal cortex (Voisey et al. 2003), but also present in murine (not human) adipocytes (Boston and Cone 1996) and in skin, where it is believed to bind to ACTH, mediating DNA synthesis and cell proliferation of keratinocytes (Kapas et al. 1998). ACTH MC2Rs are also expressed in sympathetic ganglia (Nankova et al. 2003). In addition, the action of ACTH is not restricted to the MC2R as ACTH is also an agonist for other melanocortin receptor subtypes [reviewed in (Wikberg 1999; Voisey et al. 2003)].

Stress induced release of noradrenaline (NE) from postganglionic sympathetic neurons and epinephrine predominantly from the adrenal medulla. In the brain, the majority of the NE neurons activated by stress originate from the locus coeruleus (LC). A variety of stressors increase NE biosynthesis in sympathetic ganglia and the LC [reviewed in (Sabban and Kvetnansky 2001)]. TH and DBH enzymatic activity, mRNA levels, transcription of these genes and protein levels are elevated by stress [reviewed by (Kvetnansky and Sabban 1993; Sabban and Kvetnansky 2001; Sabban and Serova 2007; Wong and Tank 2007)]. The activation of the HPA axis by stress also appears to be involved in the regulation of the gene expression of catecholamine (CA) biosynthetic enzymes in the sympathoneuronal system. We have demonstrated that injections of ACTH to rats were as effective as immobilization (IMO) stress in eliciting elevation of TH and DBH mRNAs in superior cervical ganglia (SCG) (Nankova et al. 1996). Further exposure of ACTH treated animals to a single IMO stress did not elicit an additional increase.

The effect of ACTH on neurotransmitter gene expression in the sympathetic ganglia could be direct, or mediated by adrenal glucocorticoids or by other factors. Early studies showed that administration of a single dose of the synthetic glucocorticoid dexamethasone increases TH activity in sympathetic ganglia (Hanbauer et al. 1975) and enhances the response to cold stress (Otten and Thoenen 1975). However, several lines of evidence indicate that the effect of ACTH may be independent of adrenal glucocorticoids. This is based on results from infusion of cortisol as well as the effects of stress in adrenalectomized (ADX) animals. In contrast to the administration of ACTH, infusion of cortisol did not stimulate the expression of TH or DBH in sympathetic ganglia (Nankova et al. 1996). In addition, while ADX, as expected, eliminated circulating epinephrine and increased plasma ACTH, it also markedly increased plasma NE levels (Kvetnansky et al. 1995). Furthermore, ADX rats were found to display an exaggerated elevation of plasma NE and its metabolites in response to stress (Kvetnansky et al. 1993). These results are consistent with increased activity of the sympathoneuronal system in ADX rats, and with an increased concentration of NE at the sympathetic nerve endings of rats with elevated ACTH levels. An increased urinary output of NE after bilateral adrenalectomy was also observed in humans (Von Euler et al. 1954). Moreover, the MC2R mRNA is not only expressed in rat SCG and stellate ganglia (StG) but its levels were elevated upon exposure of rats to stress (Nankova et al. 2003). Thus, ACTH may be engaged in the up-regulation of gene expression of NE biosynthetic enzymes in sympathetic ganglia without the involvement of adrenal glucocorticoids.

In this study, we examined if ACTH had an effect, independent of adrenal glucocorticoids, on the regulation of TH and DBH gene expression in SCG and LC neurons *in vivo*, and in cell

cultures - both primary cultured rat SCG neurons and a human neuroblastoma cell line. *In vivo* experiments showed that elevated ACTH levels are associated with increased mRNA for NE biosynthetic enzymes in the absence of an adrenal. Experiments with cultures of SCG and neuroblastoma cells demonstrated that ACTH can have a direct effect on transcriptional activation of gene expression of NE biosynthetic enzymes.

EXPERIMENTAL PROCEDURES

Animals

All experiments were performed in accordance with the National Institute of Health Guide and Use of Laboratory Animals (NIH Publications No. 80-23) and were approved by the New York Medical College Animal Care and Use Committee. Adult Sprague Dawley male rats (230–250g) were obtained from Taconic farms (Germantown, NY). Rats were maintained four per cage under controlled conditions on 12 hours light/night cycle at $23 \pm 2^\circ\text{C}$ and provided water and food *ad libitum*.

Surgery

Bilateral ADX was performed under pentobarbital anesthesia. A 2 cm dorsal midline skin incision was made at the level of the 13th rib. The adrenals, located cranial and medial to the kidney were removed. The skin incisions were closed with wound clips. In some of the rats a 25 mg corticosterone 21 day release pellet (Innovative Research of America, Sarasota, FL) was implanted subcutaneously in the nape of the neck at the time of ADX. A solution of 0.9% sodium chloride was provided *ad libitum* to the ADX rats.

ACTH administration

ACTH (1–39, Sigma, St. Louis, MO) was freshly dissolved in saline and administered subcutaneously at 13 IU/kg once, or daily for 6 days at 9 AM. A control group of rats received the same volume of saline.

Immobilization stress

Immobilization stress was performed on metal platforms (Kvetnansky and Mikulaj 1970) exactly as in our previous studies (Serova et al. 1999; Serova et al. 2005). The head was placed within a loop to minimize movement and the forelimbs and hindlimbs secured with surgical tape to metal mounds attached to the platform.

Euthanasia

Rats were euthanized by decapitation. Blood was collected into 7.5% EDTA containing pre-chilled tubes. The SCG and LC were immediately dissected and frozen in liquid nitrogen, separately from each animal. For LC, the brain was dissected using a tissue slicer with digital micrometer (Stoelting Co, Wood Dale, IL) as described before (Serova et al. 1999). The frontal sections 9.2–10.4 mm from Bregma were placed in ice-cold saline, and the bilateral regions of the LC were punched out.

Determination of plasma ACTH levels

ACTH levels were determined by radioimmunoassay using the ^{125}I hACTH kit (ICN, Costa Mesa, CA) according to the manufacturer's protocol. ACTH concentration was analyzed in 0.1 ml of plasma, which was incubated with anti-serum and ^{125}I -ACTH in polystyrene tubes for 20 hours at 4°C . After centrifugation, precipitates were counted in a gamma counter. The standard curve was obtained from standards of ACTH in the range from 10 to 1000 pg/ml and used for quantification. The intra- and inter-assay coefficients of variation for ACTH and corticosterone assays were 9% and 11%, respectively.

Isolation of RNA and Quantitative RT-PCR

Total RNA was isolated and analyzed as previously described (Serova et al. 1999). Briefly, the tissue from each animal was homogenized in RNA-Stat-60 (Tel-Test, Inc., Friendswood, TX) and purified with RNAqueous-Micro RNA isolation kit (Ambion, Austin, TX) (Serova et al. 2004; Serova et al. 2005). To avoid DNA contamination, samples were incubated with DNase for 20 min at 37°C. After stopping the reaction and centrifugation, RNA was transferred to fresh RNase-free tubes. The concentration of total RNA from each sample was quantified using Ribo-Green fluorescent dye (Molecular Probes, Eugene, OR). The ratio of A260 and A280 was greater than 1.85. Quantitative analysis of TH and DBH mRNA levels was performed by Real-time RT-PCR with SYBR Green using LightCycler (Rocher Molecular Biochemicals, Indianapolis, Indiana) as previously described (Serova et al. 2004). RT reactions were performed separately with TH, DBH or MC2R specific primers:

5'-TCAGGCTCCTCTGACAG-3',

5'-GCACAGTAATCACCTTCC-3' and

5'-CTGCAATCACAGACAGGC-3', respectively.

The RT reactions were performed in 5 µl PCR mixture (1×AMV buffer, 10mM dNTP, 8 units RNase inhibitor, 1.25 units AMV, 10 µM reverse primer, and 1 µg of template RNA). PCR reactions were carried out in 20 µl with a final concentration of 1× LightCycler DNA Master SYBR Green, 0.5 µM of each of the forward and reverse primers, 3 mM MgCl₂ and 2 µl of the cDNA. A standard curve plotted using serial dilutions from 2 ng to 0.2 pg of TH, DBH or MC2R cDNA was used for the quantification by Fit Points Method. The following primers were used:

for TH, 5'-GTGAACCAATTCCCCATG-3', 5'-AGTACACCGTGGAGAG-3';

DBH, 5'-CACCACATCATCATGTATGAGG-3', 5'-CCTGTCTGTGCAGTAGCCAG-3';

MC2R 5'-CTGCTGGCTGTGATCAAAA-3', 5'-CCAGCAAAGAGAGGACGAAC-3'.

The presence of specific target sequences were confirmed with melting curve analysis by comparing its melting temperature to the melting temperatures of the standards as a positive control. The values for TH, DBH and MC2R mRNA were normalized to concentrations of total RNA in the sample.

Tissue culture

Primary SCG neurons—Sympathetic neuron cultures were prepared as described (Ferrari et al. 1995; Troy and Hart 1997). SCG, attached to the carotic artery were dissected under the EMZ-15 dissecting microscope (MEIJI Techno America, Santa Clara, CA) from 2–4 days old rat pups, cleaned and pooled together in 15 ml tube with RPMI 1640 medium (Gibco, Grand Island, NY), 10% horse serum (Gibco, Grand Island, NY) and mouse 100 ng/ml mNGF 2.5S (Roche, Indianapolis, IN.) on ice. Collected ganglia were incubated in Trypsin/EDTA solution [0.25% trypsin/2.21 mM EDTA in HBSS (Hank's Balanced Salt Solution, Mediatech Inc., Herndon, VA)] for 30 min. After centrifugation for 5 min (1000 rpm), cells were re-suspended in media, distributed into 24-well collagen-coated plates and incubated at 37°C in 7% CO₂ incubator. To prepare collagen plates 200 µl collagen solution [Stock collagen (Sigma, St. Louis, MO) was diluted 100 times with 0.1 N acetic acid], added in each well and left overnight under UV until dry. One day after plating, 10 µM uridine (Sigma, St. Louis, MO) and 10 µM 5-fluorodeoxyuridine (Sigma, St. Louis, MO) were added to the cultures and left for 3 days to eliminate non-neuronal cells, after which we did not detect any non-neuronal cells by visual observation.

Seven-ten days later, cells were transfected with plasmids in which firefly luciferase gene is controlled by the first 773 or 272 nucleotides of the rat TH promoter [p5'TH(-773/+27)/Luc] or [p5'TH(-272/+27)/Luc] (Nakashima et al. 2003) or the first 249 nucleotides of the rat DBH promoter [5'DBH(-249/+21)/Luc] (Serova et al. 2002) using SuperFect reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. Briefly, for each well 1.5 µl of lipofectamine was mixed with 1 µg of DNA and added into wells. After 6 hours, the complex was replaced with complete medium. Sixteen hours post transfection cells were treated with vehicle (water), 100 pM ACTH (1–39, Sigma, St. Louis, MO) or 200 µM 8-(4-Chlorophenyl-thio)adenosine 3',5'-cyclic monophosphate sodium salt (CPT-cAMP, Sigma, Saint Louis, MO) for an additional 24 hours. Cells were harvested after 5 min of incubation with Trypsin/EDTA solution (Mediatech Inc., Herndon, VA) at 37°C. Following the centrifugation the lysates were prepared by re-suspending the cell pellet in Passive Lysis Buffer (Promega, Madison, WI). For luciferase reporter assays, an aliquot of each lysate was mixed with 5 volumes of luciferase assay reagent (Promega, Madison, WI) and firefly luciferase activity was assayed within the linear range by immediate measuring in a luminometer (TD-20/20 Turner, Sunnyvale, CA).

The concentration of protein in the lysates was determined using Bio-Rad Protein Assay Reagent (Bio-Rad, Hercules, CA). Luciferase activity was normalized to the protein levels in each lysate and expressed relative to the respective control group (vehicle treatment). Each experimental group contained 5–6 replicates cell wells and each experiment was repeated at least twice.

SK-N-SH human neuroblastoma cells

SK-N-SH cells were purchased from ATCC, (The Global Biosource Center™, Manassas, VA). Cells were grown in Minimum Essential Medium Eagle (ATCC, Manassas, VA) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate and 10% fetal bovine serum (The Global Biosource Center™, Manassas, VA). Cells were transfected with p5'TH(-272/+27)/Luc or p5'DBH(-249/+21)/Luc reporter plasmids using SuperFect reagent (Qiagen, Valencia, CA) as previously described (Maharjan et al. 2005; Cheng et al. 2008). The transfection complex was formed according to the manufacturer's protocol with 1.5 µg of DNA, 90 µL DMEM without supplements and 6 µL SuperFect reagent for each well. After 10 min, the complex was diluted to 600 µl with DMEM media with supplement and gently added to each well. Cultures were incubated 3 hours at 37°C in 5% CO₂ incubator. Twenty four hours later, vehicle (water), 100 pM ACTH or 200 µM CPT cAMP were added into the media and incubated further for an additional 24 hours. Cells were harvested by centrifugation in 0.25% trypsin, 0.03% EDTA solution (Global Biosource Center™, Manassas, VA) and lysed in Passive Lysis Buffer (Promega, Madison, WI) and luciferase activity and protein concentration were determined as above. Each experimental group contained 5–6 replicate cell wells and each experiment was repeated at least twice.

To examine the response of endogenous human DBH gene to ACTH, cells were treated with 100 pM ACTH and harvested at different time points afterwards. Total RNA was isolated and RT-PCR was performed as described above using the following primers:

5'-GAAGTACTTCTGCAGGAAGCC-3' for RT and 5'-
CAGCCCTCACTTCCAGGAGA-3',
5'-ACAGCGCTCTTGCAGAGCTC-3' for PCR.

Statistical analyses

Data was analyzed using Prism 4 and In Stat (Graph Pad Software, Inc., San Diego, CA, USA). Results are presented as mean $\pm$ SEM. Statistical differences in experimental groups and controls were determined by one-way ANOVA followed by Fisher's post-hoc test. Levels of $P < 0.05$ were considered significant.

RESULTS

Effect of Adrenalectomy on TH, DBH and MC2R mRNA levels in SCG

To study whether endogenously elevated ACTH can alter gene expression of NE biosynthetic enzymes in SCG, rats were subjected to bilateral ADX or sham operated. Twelve days after surgery, levels of ACTH in plasma were about six fold higher in ADX rats compared to the sham operated controls (Fig. 1A). In SCG, both TH and DBH mRNA levels were significantly elevated more than 3-fold in ADX rats compared to levels in the sham operated control group (Fig. 1B). The levels MC2R mRNA were also significantly increased in the SCG of ADX rats.

Effects of ACTH injections on ADX rats with corticosterone pellets

In order to study the effect of ACTH in the regulation of gene expression of NE biosynthetic enzymes in SCG, it was necessary to keep corticosterone levels close to that in untreated animals. In this regard pellets with constant corticosterone release were implanted at the time of adrenalectomy, which is reported to maintain basal plasma ACTH levels in ADX rats similar to controls (Akana et al. 1988; Viau 2002). As shown in figure 2A, plasma ACTH levels in ADX rats with corticosterone pellets did not differ from levels in sham operated controls.

Since basal levels of ACTH were low, we could now examine the response to pulsatile increases in ACTH, which is similar to the response to stress. To evaluate if the previously used dose of ACTH (Nankova et al. 1996) mimics elevation of plasma ACTH induced by IMO stress, we measured changes of plasma ACTH in response to IMO and to subcutaneous injections of ACTH (1–39) (13 IU/kg) to naïve rats. The results are shown in figure 2B. Fifteen minutes from the beginning, levels of ACTH in plasma were robustly elevated to a similar extent in both groups of rats, reaching 1300 ± 100 pg/ml in ACTH injected and 1100 ± 130 pg/ml in immobilized rats. After 60 minutes, levels of ACTH were still high but reduced compare to the levels at 15 minutes. Although the decline of ACTH in plasma was more pronounced in ACTH treated compared to immobilized animals, the initial robust rise was very similar indicating that this can be an appropriate dose to study the role of ACTH in stress.

To examine the effect of ACTH, ADX rats with corticosterone pellets were given single or repeated injections once daily for six consecutive days of ACTH 1–39 (13 IU/kg) or saline. Levels of TH, DBH and MC2R mRNA were determined in the SCG. A single injection of ACTH triggered increased TH and DBH mRNA levels (Fig. 3). Changes in mRNA levels in the response to repeated injections of ACTH were similar to those with single injection (Fig. 3). The levels of mRNA in ACTH treated animals were about double that of control levels. The mRNA levels for MC2R were also elevated about 60–70% above control levels with the single dose of ACTH administration. However, no changes were found after six daily injections of ACTH (Fig. 3).

Effects of ADX and ACTH administration on NE biosynthetic enzymes in LC

The effect of ACTH was also examined in the LC, the major NE nucleus. Adrenalectomy doubled TH, but did not change DBH mRNA levels (Fig. 4A). A single injection of ACTH to ADX rats with corticosterone pellets triggered about 2-fold induction of both TH and DBH mRNAs compared to sham operated rats (Fig. 4B). Repeated daily injections of ACTH elicited an even higher rise in mRNAs levels in these animals (Fig. 4B). TH and DBH mRNAs were

about 4 fold above the control group. Thus, ACTH triggered significant changes in TH and DBH mRNA levels also in the LC.

ACTH elicited activation of TH and DBH promoters in primary cultured SCG neurons and SK-N-SH neuroblastoma cell line

To further evaluate if ACTH can directly regulate TH promoter activity we performed several experiments on SCG neurons from newborn rats. SCG neurons from 2–3 days old rat pups were removed and cultured during 6–7 days. To eliminate non-neuronal cells, antimetabolic agents uridine and 5-fluorodeoxyuridine were added to the cultures one day after plating and left for 3 days. This treatment was sufficient to prevent non-neuronal cell proliferation from dissociated SCG neurons. Representative primary culture of ten days old SCG neurons are shown in figure 5A. Neurons were transfected with TH promoter constructs in which the first 773 or 272 nucleotides upstream of the TH, (Fig. 5B) or 249 nucleotides upstream of the DBH (Fig. 5C), transcription start sites control expression of the luciferase gene. Treatment with 100 pM ACTH elicited robust, 12–13-fold, increase in reporter activity with both TH constructs 24 hours later. It also triggered significant elevation of DBH promoter driven luciferase activity evident after 12 and 24 hours. In addition, when 200 μ M of non-hydrolysable cAMP analog CPT-cAMP, was added to the cultures, the responses of the TH and DBH promoters was similar to those attained with ACTH.

The ability of ACTH to regulate TH and DBH gene transcription was further examined on SK-N-SH neuroblastoma cells, which originated from human sympathetic nervous system. Similar to results obtained for TH on cultured rat SCG neurons, treatment with CPT-cAMP or ACTH for 24 hours triggered activation of TH promoter reporter activity (Fig. 6). The response of DBH promoter activity was also examined. When cells were transfected with DBH (–249/+21)/Luc construct, luciferase activity was significantly increased in response to CPT-cAMP stimulation after 24 hours. A similar response was observed after incubation with ACTH at 12 hours, but reduced by 24 (Fig. 6).

ACTH triggered increase human DBH mRNA levels in SK-N-SH neuroblastoma cell line

In order to further confirm an adrenal independent effect of ACTH on CA gene expression we measured response of endogenous human DBH gene to ACTH. Human SK-N-SH neuroblastoma cells were treated with 100 pM ACTH for different times and mRNA levels for DBH were determined (Fig. 7). Incubation with ACTH during 12 and 24 hours led to significant elevation of DBH mRNA levels compared to a vehicle treated group.

DISCUSSION

The results of this study demonstrate that ACTH can regulate gene expression of NE biosynthetic enzymes in the SCG and LC by an adrenal independent mechanism. This was demonstrated in the SCG by both *in vivo* and *in vitro* experiments. TH and DBH mRNA levels were increased in SCG of rats with high levels of ACTH in plasma induced by adrenalectomy and by injections of ACTH to ADX rats, in which corticosterone pellets maintained basal ACTH levels. Levels of MC2R mRNA in SCG of these animals were also elevated. Moreover, ACTH was effective in increasing TH and DBH promoter driven reporter activity in primary SCG neurons and human neuroblastoma cells. The study revealed, for the first time, that ACTH could have a direct effect on the regulation TH and DBH gene expression in sympathetic ganglia.

The results obtained in the ADX rats, demonstrated that endogenously increased concentrations of ACTH in plasma are associated with elevated TH, DBH and MC2R mRNA levels in SCG. The levels of ACTH in ADX rats are lower than attained with IMO stress, yet are sustained

for a prolonged period of time compared to the transient rise reached with stress. However, the absence of adrenal hormones, especially glucocorticoids, and prolonged elevated blood ACTH induced by removal of feedback inhibitory mechanisms, may significantly influence the sensitivity of melanocortical and glucocorticoid receptors and/or employ an additional signaling mechanism not normally involved in regulation of NE biosynthetic enzymes in SCG.

Administration of ACTH into rats with close to basal plasma ACTH levels, due to corticosterone pellets, would prevent most of these complications. ACTH given in the dose of 13 IU/kg mimicked IMO stress induced plasma ACTH elevation: robust but transient. In these experimental conditions, mRNA levels for TH and DBH and MC2R were also found to be elevated in the SCG, indicating that induction of gene expression of NE biosynthetic enzymes might be due to a direct effect of ACTH and is not mediated by adrenal glucocorticoids. In this regard, infusion of cortisol did not stimulate the expression of these genes in sympathetic ganglia (Nankova et al. 1996). However, the synthetic steroid dexamethasone, but not three other glucocorticoids (corticosterone, hydrocortisone and triamcinolone), was effective in increasing TH activity in SCG (Sze and Hedrick 1983). This effect of dexamethasone appears to be at the pre-ganglionic cholinergic terminals (Sze and Hedrick 1983).

The results found in this study are in agreement with our previous experiments, where ACTH (1–39) administered by injections elicited elevation of gene expression of NE biosynthetic enzymes in SCG of intact rats (Nankova et al. 1996). However, the magnitude of response to ACTH injections observed here in ADX rats is lower than the previously reported experiments in intact rats. This discrepancy might be due to variations among experiments performed at different times with different animals. However, we cannot rule out the possibility that there is also a partial contribution from adrenal factors. Data obtained here support the hypothesis that ACTH has a direct effect on the regulation of gene expression of NE enzymes in SCG, although they did not eliminate the possibility that other factors originating from adrenals might be also important for this regulation.

The importance of trans-synaptic nerve stimulation for regulation of gene expression of CA biosynthetic enzymes in the sympathoneural system has been well demonstrated. Pre-ganglionic nerve stimulation elicits increases in TH and DBH activities and elevated TH protein and mRNA levels in rat SCG (Zigmond 1980; Ip et al. 1983; Biguet et al. 1989). Denervation of pre-ganglionic inputs prevents the elevation of TH in response to reserpine. However, contribution of trans-synaptic inputs to the stress triggered changes is less certain. Decentralization of SCG in young rabbits, greatly reduced basal TH activity, however, exposure of rabbits to cold stress triggered a large induction in TH activity even following decentralization (Andrews et al. 1993). Thus, their study shows that TH activity was induced in the SCG in the absence of pre-ganglionic input demonstrating a non-synaptic regulatory mechanism. Based on our results we can propose that one of the mechanisms for stress-elicited regulation of gene expression of NE enzymes in SCG is activation by ACTH. The results of our studies clearly demonstrate heightened levels of MC2R mRNA in SCG of rats with elevated ACTH levels induced by ADX, ACTH injections to ADX rats with corticosterone pellets or IMO stress (Nankova et al. 2003). Significant increase of MC2R mRNA was observed after ACTH administration in rat adrenals and dose-dependent increase in ACTH binding was shown in response to ACTH pretreatment in bovine adrenal cells, suggesting that ACTH is one of the few polypeptide hormones having a positive trophic effect on its own receptors and target-cell responsiveness (Penhoat et al. 1989; Morita et al. 1995).

ACTH probably can also act on the terminal post-ganglionic sympathetic axons affecting transmitter release since its perfusion into isolated rabbit hearts enhanced electrically stimulated neuronal NA release (Szabo et al. 1988). The findings suggested that under certain conditions presynaptic receptors may be activated *in vivo* by circulating ACTH.

We can speculate that effect of ACTH on gene expression of NE biosynthetic enzymes in SCG is mediated by MC2Rs, since mRNA levels for MC2Rs were enhanced in SCG in response to ACTH. However, we cannot rule out the possibility that ACTH might act through other melanocortin receptors. The five MCRs have high sequence homologies, ranging from 60% identity between MC2R and MC5R, to 38% identity between MC2R and MC4R [rev. in (Catania et al. 2004)]. ACTH has similar affinity as α or γ melanocyte-stimulating hormone (MSH) to MC1Rs, MC3Rs, MC4Rs and MC5Rs (Chhajlani and Wikberg 1992; Roselli-Reh fuss et al. 1993; Suzuki et al. 1996). What specific MCRs can contribute in regulation of gene expression of NE biosynthetic enzymes in SCG remains to be determined.

The elevation of TH mRNAs was not only detected in SCG, but also in LC of ADX rats. These results are consistent with the observation of increased TH activity in LC in ADX mice (Markey and Sze 1984). It remains to be determined whether or not this is a direct effect of ACTH, which can cross the blood brain barrier to some extent (Banks and Kastin 1995). It could also be mediated by CRH. CRH is regulated by glucocorticoids which affect CRH synthesis and release (Paull and Gibbs 1983; Plotsky and Sawchenko 1987; Akana et al. 1992). Adrenalectomy enhances CRH synthesis in the paraventricular hypothalamic neurons, and its release into the portal system (Sawchenko et al. 1984; Jingami et al. 1985; Plotsky and Sawchenko 1987; Imaki et al. 1991). Thus, the elevation of TH mRNA in the LC might be mediated by increased CRH action as a neurotransmitter in the LC. In this regard, ADX rats had higher basal LC discharge rates compared to sham operated controls, and had displaced dose response curve, suggesting that a proportion of the CRH receptors in the LC were already occupied (Pavcovich and Valentino 1997). However, it is interesting that even when ACTH levels were kept at basal conditions, administration of ACTH triggered a marked induction of TH mRNA in the LC and also induced DBH mRNA. These effects are unlikely mediated by CRH. In this regard, chronic i. p. injections of ACTH or its analogs to mice effectively increased TH activity in LC, suggesting that ACTH might have a more direct role previously recognized in the regulation of the LC with stress (Markey and Sze 1984). Moreover, iontophoretically administered ACTH to the LC increased the firing rate of noradrenergic neurons from male rats both *in vivo* (Olpe and Jones 1982) and *in vitro* (Olpe et al. 1987).

A direct effect of ACTH on regulation of gene expression of NE biosynthetic enzymes was further confirmed in experiments in tissue culture. Both SCG primary cultures and neuroblastoma cells enabled us to show that ACTH can cause not only elevation of TH and DBH mRNA levels but also activate transcription of these genes. The data revealed robust rise in luciferase activity driven by TH and DBH promoters in primary cultured neurons from 2–3 days old rat pups in response to ACTH. Moreover, ACTH is also effective in regulating transcription of TH and DBH genes and DBH mRNA levels in human neuroblastoma cells.

Signaling pathway-mediating effect of ACTH through MC2Rs, which are G protein-coupled receptors, involve stimulation of cAMP-dependent protein kinase A (PKA). In the adrenal cortex binding of ACTH to its receptor stimulates adenylyl cyclase and induces an increase in cAMP; this leads to activation of PKA (Gorostizaga et al. 2007). As mentioned above, MC2R expression in adrenal cells is up-regulated by its ligand ACTH (Mountjoy et al. 1994). Activation of PKA has been shown previously to be a potent stimulator of TH transcription (Kim et al. 1994; Lewis-Tuffin et al. 2004). It also activates DBH transcription (McMahon and Sabban 1992; Shaskus et al. 1992).

However, the kinetics, and probably the mechanisms that underlie the regulation of NE enzyme gene expression are tissue specific [rev. in (Sabban and Kvetnansky 2001)]. For example, IMO, cold stress, hypoxia 2-deoxyglucose and insulin elicited much slower response of TH and DBH genes in SCG compared to rapid elevation in adrenal medulla. Constant light for 20 days reduces TH and DBH mRNA levels in SCG but increases them in adrenal medulla (Gallara et

al. 2004). ACTH administration increases mRNA levels for these genes in SCG but not in adrenal medulla (Nankova et al. 1996). The differential interplay between positive and negative transcription factors and their kinetics of action may be responsible for tissue specific long-term TH regulation (Trocme et al. 2001). It has been reported that cAMP-responsive element on the proximal TH promoter binds different transcription factors in adrenal medulla and the SCG and reserpine treatment enhances expression and binding of the inducible cyclic AMP early repressor (ICER) in the adrenal medulla, whereas in the SCG it enhances the binding of cAMP response element-modulator protein (CREM) (Trocme et al. 2001).

The study suggests that ACTH may directly contribute to the stress triggered activation of gene expression of NE biosynthetic enzymes in SCG. It remains to be determined if other sympathetic ganglia, such as the stellate, which innervates the heart also responds similarly. This is likely, as we have previously observed the expression of MC2R mRNA in both SCG and stellate ganglia from adult rats (Nankova et al. 2003).

The results of this study might have important clinical implications since ACTH has a variety of cardiovascular effects in various species depending on its site of action (Whitworth et al. 1990; Versteeg et al. 1998; Brown et al. 2006; Kawabe et al. 2006). In this regard, about 80% of Cushing Disease patients with ectopic ACTH secretion display elevated blood pressure (Torpy et al. 2002). We have shown that ACTH given in the same dose as here produced pressor responses to the similar extent as IMO stress (Sabban et al. 2007). Thus, in addition to contributing to the response to stress, ACTH and related compounds have pharmacological potential for regulating the cardiovascular system by controlling NE biosynthesis in the SCG and in the LC. It is especially important since the adrenal medulla does not alter TH or DBH gene expression to ACTH (Nankova et al. 1996) in contrast to the SCG and LC which allows selective modulation of NE biosynthesis.

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ABBREVIATIONS

ADX, adrenalectomy
 ACTH, adrenocorticotrophic hormone
 CA, catecholamine
 cAMP, adenosine 3', 5'-cyclic monophosphate
 CPT-cAMP, 8-(chlorophenylthio) adenosine 3', 5'-cyclic monophosphate
 CRH, corticotrophin releasing hormone
 DBH, dopamine β -hydroxylase
 HPA, hypothalamic-pituitary-adrenocortical axis
 IMO, immobilization stress
 LC, locus coeruleus
 MC2R, melanocortin 2 receptor
 NE, norepinephrine
 SCG, superior cervical ganglia
 StG, stellate ganglia

TH, tyrosine hydroxylase

Fig. 1.

Effect of adrenalectomy on plasma ACTH levels (A) and TH, DBH and MC2R mRNA levels (B) in SCG. Rats were ADX or sham operated and decapitated twelve days later. Plasma ACTH levels were determined by radioimmunoassay. Total mRNA from SCG was isolated and analyzed by quantitative RT-PCR. Data are expressed as mean $\pm$ S.E.M. of 6–8 samples each from an individual animal, and normalized to the mean of the sham operated group taken as 1. * $P < 0.05$, ** $P < 0.01$ versus sham operated group.

Fig. 2.

Changes in plasma ACTH levels. Plasma ACTH levels determined in sham operated and in ADX rats with and without corticosterone pellets seven days after surgery is shown in A. * $P < 0.05$, versus sham operated group. Plasma ACTH levels in rats with IMO stress or following ACTH injections are shown in B. ** $P < 0.01$, versus 15 minutes, *** $P < 0.001$ versus 0 minutes.

Fig. 3.

ACTH injections induce TH, DBH and MC2R mRNA levels in SCG of ADX rats with corticosterone pellets. Seven days after surgery rats received injections for one or six consecutive days of saline (0) or ACTH. Rats were euthanized three hours after the last injection. RNA from SCG was isolated and analyzed by RT-PCR. Data are expressed as mean $\pm$ S.E.M. of 6–8 samples, each from individual animal and normalized to the mean of the saline treated group taken as 1. * $P < 0.05$ versus saline treated group.

Fig. 4. Levels of TH and DBH mRNA in locus coeruleus of ADX rats (A) and after ACTH injections to ADX rats with corticosterone pellets (B). The experimental details are the same as in fig. 1 and fig. 3. LC was dissected from brain slices, total RNA was isolated and analyzed by quantitative RT-PCR. Data are expressed as mean $\pm$ S.E.M. of 6–8 samples, each from an individual animal, and normalized to the mean of sham operated (A) or the saline treated group (B) taken as 1. * $P < 0.05$, ** $P < 0.01$ versus respective controls.

Fig. 5.

ACTH increases luciferase reporter activity under control of TH promoter in SCG cultured neurons from newborn rats. Cultured SCG neurons are shown in A. Summary data of ACTH induced changes in TH (B) or DBH (C) reporter activity are shown. SCG neurons were dissected from 2–4 days old rat pups and cultured during 7 days. Transfection of different length of TH [pTH(-773/+27)/Luc or pTH(-272/+27)/Luc] or pDBH(-249/+21)/Luc promoter controlled the luciferase gene was performed using Superfect. Sixteen hours after transfection cells were treated with vehicle (water, 0), CPT cAMP (200 μ M) or ACTH (100 pM) for an additional 24 hrs. Relative luciferase activities normalized to the protein concentrations are shown. Values are presented as mean $\pm$ SEM. Mean in control groups (0) are taken as 1. ** $P < 0.01$, *** $P < 0.001$ versus (0).

Fig. 6.

ACTH triggers elevation of luciferase reporter activity under control of TH and DBH promoters in SK-N-SH neuroblastoma cells. Cells were transfected with pTH(-272/+27)/Luc or pDBH (-249/+21)/Luc, incubated with vehicle (water, 0), CPT cAMP (200 µM) or ACTH (100 pM) and harvested 12 or 24 hour later. Relative luciferase activities normalized to the protein concentrations, are shown. Values are presented as mean $\pm$ SEM. Mean in control groups (0) are taken as 1. ***P < 0.001 versus (0)

Fig. 7.

ACTH elicited induction of DBH mRNA levels in SK-N-SH neuroblastoma cells. Cells were treated with vehicle (0) or ACTH (100 pM) for different times. Total RNA was isolated and DBH mRNA was measured by quantitative RT-PCR. Values are presented as mean $\pm$ SEM. Mean in control groups (0) are taken as 1. * $P \leq 0.05$ versus (0)