Corticotropin-Releasing Hormone and the Sympathoadrenal System Are Major Mediators in the Effects of Peripherally Administered Exendin-4 on the Hypothalamic-Pituitary-Adrenal Axis of Male Rats


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Glucagon-like peptide-1 (GLP-1) and the GLP-1 receptor agonist, exendin-4 (Ex-4), potently stimulate hypothalamic-pituitary-adrenal (HPA) axis activity after either central or peripheral administration. Because several GLP-1 derivative drugs, including synthetic Ex-4, are currently in use to treat patients with type II diabetes mellitus, the characterization of Ex-4 effects on the HPA axis is highly relevant. Herein, the roles of CRH and AVP on these effects were investigated by administering the antagonists astressin and d(CH2)5Tyr(Me)AVP, respectively. The role of the sympathoadrenal system (SAS) was explored in bilateral adrenal enucleated and guanethidine-treated rats, whereas primary pituitary cell cultures were used to study direct effects on the corticotropes. Astressin completely abrogated (P < .05) the effects of Ex-4 central administration on ACTH secretion but only slightly reduced (by 35%) the ACTH response to Ex-4 peripheral administration. Moreover, astressin significantly (P < .05) decreased the corticosterone response to centrally but not peripherally administered Ex-4, suggesting different mechanisms depending on the route of administration. Pretreatment with d(CH2)5Tyr(Me)AVP failed to diminish either the ACTH or corticosterone response to Ex-4 and no direct effect of Ex-4 or GLP-1 was observed on pituitary cell cultures. In contrast, a significant (P < .05) reduction in the corticosterone response elicited by Ex-4 peripheral administration was observed in enucleated and guanethidine-treated rats, indicating a role of the SAS in the glucocorticoid stimulatory effects of Ex-4. Our data demonstrate that the effects of Ex-4 on the HPA axis are partially mediated by CRH and the sympathoadrenal system, and stress the relevance of Ex-4 as a corticosterone secretagogue. (Endocrinology 155: 2511–2523, 2014)
Thus, central GLP-1 administration activates CRH-producing neurons in the parvicellular region of the paraventricular nucleus (PVN), leading to increases in plasma ACTH, vasopressin, and corticosterone levels (7). In addition, induction of visceral illness by peripheral injection of lithium chloride (LiCl) activates GLP-1 expressing neurons (8, 9). Moreover, the HPA response to LiCl and centrally administered GLP-1 is blocked by prior administration of a GLP-1 receptor antagonist, suggesting mediation by specific GLP-1 receptors in these effects (10). Although the involvement of circulating GLP-1 in HPA effects remains unclear, all of the above demonstrate a role for centrally produced GLP-1 in stress responses. Furthermore, we have shown that acute peripheral administration of both GLP-1 and Ex-4 potently stimulates HPA axis activity in rodents, the effects of Ex-4 being of much greater magnitude than those elicited by the native peptide (11), likely due to its longer life in plasma, because Ex-4 is considerably more resistant than native GLP-1 to cleavage by dipeptidylpeptidase-IV (12). Nevertheless, the stimulatory effects of GLP-1 on glucocorticoid secretion were also observed in control and diabetic humans after acute administration of relatively low doses of the peptide, which suggests that humans could be even more sensitive than rodents to the effects of circulating GLP-1 on HPA axis activity (11). Furthermore, we have recently demonstrated that prolonged peripheral Ex-4 administration induces a profound dysregulation of the HPA axis in rats, including disruption of glucocorticoid circadian secretion with elevated levels during the trough hours and hyper trophy of the adrenal gland (13). Since several GLP-1 derivative drugs, including synthetic Ex-4, are currently being used for the treatment of patients with type II diabetes (14, 15), these findings may have clinical implications. Therefore, the understanding of the mechanisms whereby peripherally administered Ex-4 stimulates HPA axis activity is highly relevant.

Herein, we investigated the role of CRH and vasopres sin, the two major ACTH secretagogues (16), in the stimulatory effects of Ex-4 on the HPA axis activity by administering astressin and d(CH2)5Tyr(Me)AVP, respectively. Astressin is a peptidic antagonist of CRH1 receptors that does not cross the blood-brain barrier (17), whereas d(CH2)5Tyr(Me)AVP is a peptidic antagonist of V1 receptors (either V1a and V1b types) that is commonly used to block the ACTH and glucocorticoid responses to vasopressin (18, 19). We also explored the possibility of a direct effect on the corticotrope cells by incubation of dispersed pituitary cells with both Ex-4 and the native peptide, GLP-1. Finally, because it is known that Ex-4 stimulates autonomic nervous system activity (20) and there is a well-established link between the sympathoadrenal system (SAS) and the adrenal gland (21), we studied the role of the SAS by administering Ex-4 to bilateral medullectomized and guanethidine-treated rats. Guanethidine is a sympatholytic drug known to induce a transitory depletion in the catecholamine content of peripheral sympathetic neurons (22).

Materials and Methods

Animals

Adult male Sprague-Dawley rats (275–350g) were purchased from the University of Santiago or Charles River Laboratories. They were accommodated to our facilities for several days before carrying out the experiments and handled daily to avoid experimental stress. The animals were maintained with free access to tap water and standard chow (A04, Panlab) under a 12:12 hours light-dark cycle with lights on at 9:00 AM and at a controlled room temperature (20–22°C).

All experimental procedures were conducted in accordance with the European Union and the Canadian Council on Animal Care guidelines regarding the use of animals for experimental purposes.

Drugs and peptides

Ex-4, ACTH, astressin, and d(CH2)5Tyr(Me)AVP were obtained from Bachem. CRH and arginine vasopressin (AVP) were supplied by Phoenix Europe. Guanethidine sulfate was provided by Santa Cruz Biotechnology, dexamethasone by Sandoz, and sodium pentobarbital by Sigma-Aldrich.

The day the experiments were conducted, the peptides were dissolved in water and diluted to desired concentrations using sterile NaCl 0.9%. CRH was reconstituted in an ascorbic acid solution (0.01%, pH 7.5) to protect the peptide from oxidation (23).

Experimental protocols

Role of CHR receptors in the effects of Ex-4 central administration on the HPA axis (experiment 1). A permanent polyethylene cannula (PE-50, Clay Adams) was implanted into the lateral ventricle of the animals while another was inserted into the jugular vein as previously described (11). The rats were allowed to recover for 7 days in individual cages with free access to food and water. On the day of the experiment, both guide cannulas were extended by inserting another 30 cm long polyethylene cannula (PE-50, Clay Adams) and the animals were left undisturbed for 30 minutes. A heparin bolus (0.3 ml, 1,000 IU/ml) was then administered and a basal blood sample (0.3 ml) taken. After that, either astressin (28 nmol/kg) or vehicle (0.9% NaCl) was infused by the iv cannula and, 15 to 20 minutes later, 10 μl of Ex-4 (0.5 nmol/rat) or vehicle (0.9% NaCl) were administered by the icv cannula using a Hamilton syringe (n = 6–7 rats/group). The doses of astressin and Ex-4 were selected based on previous reports (10, 17) and dose-response studies conducted in our laboratory. Blood samples (300 μl) were collected at 5, 15, 30, 60, and 120 minutes after Ex-4/vehicle central administration and the volume extracted was replaced with an equivalent volume of NaCl 0.9%. The experiment was con-
ducted between 9:00 and 12:00 PM. Only one negative control group (vehicle + vehicle) was included in the studies herein presented, since previous dose-response studies performed in our laboratory demonstrated no significant effect of any of the antagonists (astressin, d(CH$_2$)$_5$Tyr(Me)AVP or the combination of both) on the circulating levels of ACTH or corticosterone (data not show).

Peripheral administration of Ex-4 to anesthetized animals

Most of the studies were conducted in anesthetized animals to minimize experimental stress. This procedure was adopted based on our previous finding that there are no major differences in the Ex-4 effects on HPA axis activity between conscious and anesthetized animals (11). Briefly, a silastic cannula (Degania Silicone Ltd) was inserted into the right jugular vein of the rats under sodium pentobarbital anesthesia (50 mg/kg, ip) that was maintained until the end of the study. The experiments were conducted between 10:00 and 12:00 PM. The antagonists (astressin and d(CH$_2$)$_5$Tyr(Me)AVP) or vehicle were administered 15 to 20 minutes before the stimuli (Ex-4, CRH, AVP). A basal sample was always taken before the administration of any treatment (t = 0) and subsequent samples were collected at 5, 15, 30, and 60 minutes. The volume of blood extracted (300 µl) was replaced with an equivalent volume of NaCl 0.9%. The following studies were conducted:

Role of CRH receptors in the effects of peripheral Ex-4 administration on HPA axis activity (experiment 2). A stressin (28 nmol/kg, iv) or vehicle were administered before the iv infusion of CRH (4.2 nmol/kg), Ex-4 (1.2 nmol/kg), or vehicle (n = 6–7 rats/group). The CRH dose was selected based on previous dose-response studies performed in our laboratory (data not show). The experiment is represented in 2 different sets of panels to simplify the visualization but all groups were considered together during the statistical analysis.

Role of V1 receptors in the effects of peripheral Ex-4 administration on HPA axis activity

1) d(CH$_2$)$_5$Tyr(Me)AVP (45 nmol/kg, iv) or vehicle were injected before the iv administration of AVP (9 nmol/kg) or vehicle in experiment 3, and before Ex-4 (1.2 nmol/kg) or vehicle in experiment 4 (n = 6 rats/group). The d(CH$_2$)$_5$Tyr(Me)AVP and the AVP doses were selected based on previous reports (18, 19) and dose-response studies conducted in our laboratory.

2) Experiment 5: A stressin (28 nmol/kg, iv) was infused alone or in combination with d(CH$_2$)$_5$Tyr(Me)AVP (45 nmol/kg, iv) prior to the iv administration of an Ex-4 (1.2 nmol/kg) bolus, to determine the consequence of blocking both V1 and CRH pituitary receptors in the effects of Ex-4 on HPA axis activity. Additionally, a vehicle plus Ex-4 group (positive control) and a vehicle plus vehicle group (negative control) were tested (n = 6 rats/group).

Study of the interaction of Ex-4 with CRH and vasopressin on HPA axis activity. Ex-4 (1.2 nmol/kg) was iv administered alone or in combination with vasopressin (0.5 nmol/kg, experiment 6) or CRH (4.2 nmol/kg, experiment 7). Additionally, a vasopressin or CRH treated group was respectively included as a positive control while a saline-treated group was tested as negative control (n = 5–7 rats/group).

Peripheral administration of Ex-4 to conscious animals

Effects of Ex-4 subcutaneous administration on HPA axis activity (experiment 8). Conscious rats were subcutaneously injected with Ex-4 (1.2 nmol/kg) or vehicle (n = 5–7 rats/group) and blood samples were extracted by tail-nicking at 0, 15, 30, 60, and 120 minutes as previously described (13).

Role of the sympathoadrenal system in the effects of Ex-4 on HPA axis activity

1) Ex-4 (1.2 nmol/kg) was subcutaneously administered to bilaterally enucleated (MEDX) or operated-control (SHAM) rats (experiment 9, n = 5–7 animals/group). Both MEDX and SHAM rats received a subcutaneous bolus of dexamethasone (0.65 µmol/kg) to block endogenous ACTH production and ACTH was administered (0.8 nmol/kg, SC) 3–4 hours later.

2) Rats were treated with guanethidine (30 mg/kg, ip) and 45 minutes later Ex-4 (1.2 nmol/kg) or vehicle was ip administered (experiment 11, n = 6–7 rats/group).

For the 3 experiments described above, blood samples were extracted by tail-nicking.

Sample processing

Blood samples were collected in Eppendorf tubes containing EDTA (0.05 M, 10 µl/tube) and placed on ice. Blood was centrifuged for 7 minutes at 3500 rpm and 4°C to separate the plasma that was stored at −20°C until hormone quantification.

Cell culture experiments

Pituitary glands were removed from male Sprague-Dawley rats under aseptic conditions and the anterior lobes were separated from the posterior and intermediate lobes and minced with a sterile surgical blade. The pituitary fragments were enzymatically dispersed using a 0.25% trypsin solution with EDTA (Gibco, Invitrogen). Dispersion of cells was increased by trituration of the fragments through a sterile pipette tip. Afterwards, the cells were pelleted by centrifugation (5 min, 2000 rpm) and washed three times in sterile Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich). The cells were then resuspended in DMEM (1000 mg/L of glucose) without phenol red, supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Invitrogen), 2% L-glutamine 200 mM (Gibco, Invitrogen), and 1% antibiotic cocktail containing penicillin (10000 IU/ml) and streptomycin (10000 µg/ml). Finally, they were plated at a den-
sity of 100000 cells/ml in 24-well plates (TPP, Biotech). Six pitu-
titary glands were used for every 24-well plate. Cell cultures
were incubated at 37°C in a humidified atmosphere of 5% CO₂
for 4–5 days.

The day of the experiment the cells were washed and incu-
bated in serum-free DMEM for 2 hours. Afterwards, the serum-
free DMEM was replaced by complete DMEM containing the
different treatments and ascorbic acid (0.1 mg/L) that was added
to protect CRH from oxidation (23). Three different studies were
conducted: 1) vehicle, CRH 10^{-9} M and GLP-1 10^{-9},10^{-8},10^{-7},
and 10^{-6} M were tested; 2) vehicle, CRH 10^{-9} M, Ex-4 10^{-9},10^{-7},
10^{-6} M, and CRH 10^{-5} M in combination with Ex-4 10^{-9} M; and
3) vehicle, CRH 10^{-9} M and Ex-4 10^{-12},10^{-11},10^{-10},10^{-9} M (n =
8 wells/treatment). A sample of medium (100 μL) was removed
at time 3 hours and a second sample at time 6 hours, the aliquots
were centrifuged to pellet any cellular material and then stored
at −20°C.

Hormone determinations

Plasma ACTH (Phoenix Europe) and total corticosterone lev-
els (DRG-Instruments) were measured using a specific RIA kit
according to the manufacturer’s indications.

Statistical analysis

Data are represented as mean ± standard error of the Δ values
(expressed as the difference with the respective time 0 value).
Student’s t test was conducted to compare two independent
groups and 1-way ANOVA followed by post hoc Tukey test to
compare three or more groups. P < .05 was set as the criterion
for statistical significance.

Results

Role of CRH receptors in the effects of Ex-4 central
administration on HPA axis activity

Central administration of Ex-4 (0.5 nmol/rat, icv) po-
tently stimulated ACTH and corticosterone secretion as
shown in Figure 1, A and B, respectively. Ex-4 significantly
increased ACTH circulating levels from 5 minutes (P <
.05) to the end of the study (P < .01); a rapid rise in
corticosterone levels was also observed, becoming signif-
icient from 15 (P < .05) to 60 minutes (P < .01). Pretreat-
ment with astressin (28 nmol/kg, iv) completely sup-
pressed the Ex-4 effects on ACTH
secretion during the entire experi-
ment and significantly (P < .05) pre-
vented the increment in corticoste-
rone levels during the first phase of
the study (15 minutes). However,
corticosterone circulating levels be-
gan to rise at 30 minutes and this el-
evation became significant at time 60
minutes (P < .05). Similar effects can
be appreciated by examining the
AUC of the ACTH (Figure 1C) and
corticosterone (Figure 1D) re-
sponses, such that astressin pretreat-
ment completely abolished the ef-
teffects of Ex-4 on ACTH circulating
levels and decreased the rise in cor-
icosterone levels by 48%, although
this reduction did not reach signifi-
cance (P = .076).

Role of CRH receptors in the effects of peripheral Ex-4
administration on HPA axis activity

Peripheral administration of
CRH (4.2 nmol/kg, iv) significantly
induced ACTH secretion at 15 (P <
.001) and 30 minutes (P < .05) and
robustly increased corticosterone
levels from 15 minutes (P < .001) to
the end of the assay at 60 minutes.

Figure 1. Role of CRH peripheral receptors in the effects of centrally administered Ex-4 on
pituitary-adrenal axis. Plasma ACTH (A) and corticosterone (B) responses elicited by icv
administration of Ex-4 (0.5 nmol/rat) in freely moving rats were significantly attenuated after
blocking CRH peripheral receptors by astressin administration (28 nmol/kg, iv). Time-course
responses are expressed as the difference from the respective basal levels shown at the top of
each graph. The area under the curve (AUC) profiles of both responses are shown in panels C
and D respectively. Data represented as mean ± SE (6–7 animals/group; **, P < .01, *, P < .05
vs vehicle-treated group; ##, P < .01, #, P < .05 vs group receiving Ex-4 alone).
The effects promoted by CRH on ACTH release were completely abrogated following astressin pretreatment (28 nmol/kg, iv), whereas the effects on corticosterone levels were significantly attenuated ($P < .01$ at time 15 and 30 minutes vs CRH-treated group). In the same experiment, a potent ACTH secretory response was also observed after the iv administration of Ex-4 (1.2 nmol/kg), reaching significantly elevated ACTH levels at 15 and 60 minutes ($P < .01$ at both time points). In addition, the effect of Ex-4 on corticosterone secretion was comparable to that elicited by a 3.5-fold dose of CRH ($\text{AUC} \Delta \text{corticosterone response} = 17762 \pm 1691 \text{ng/ml} \times 60$ minutes in the CRH-treated group vs 15866 $\pm$ 1734 in the Ex-4-treated group). The ACTH response induced by Ex-4 peripheral injection was notably reduced after astressin administration ($\text{AUC} \Delta \text{ACTH response} = 1059 \text{pg/ml} \times 60$ minutes in the Ex-4-treated group vs 5260 $\pm$ 810 in the rats receiving astressin plus Ex-4, a 35% reduction), although it continued to be significant at time 15 minutes ($P < .05$) as compared to control animals. In contrast, astressin did not affect the potent corticosterone response promoted by Ex-4 administration.

**Role of AVP in the effects of Ex-4 peripheral administration on HPA axis activity**

A robust ACTH response was triggered by the iv administration of AVP (9 nmol/kg), reaching significantly elevated ACTH levels at 5 ($P < .001$), 15 ($P < .001$) and 30 minutes ($P < .01$, Figure 3A). This remarkable increment in ACTH levels was accomplished by significantly elevated corticosterone levels from time 15 ($P < .001$) to 60 minutes ($P < .01$, Figure 3B). The effect of AVP on ACTH secretion was completely abolished following pretreatment with the V1 receptor antagonist $d(\text{CH}_{2})_{5}\text{Tyr(Me)}\text{AVP}$ (45 nmol/kg, iv) as can be seen in Figure 3A. Similarly, the effect on corticosterone levels was significantly reduced ($P < .01$ at 15 minutes; $P < .001$ at 30 minutes; and $P < .01$ at 60 minutes) although not completely abrogated in the animals pretreated with the antagonist ($P < .01$ at 15 minutes; and $P < .01$ at 30 minutes vs vehicle-treated animals, Figure 3B).

The consequences of blocking V1 receptors on the stimulatory action of Ex-4 on HPA axis activity were studied in a separate group of rats. Intravenously administered Ex-4 (1.2 nmol/kg) produced the expected stimulatory responses observed on ACTH and corticosterone secretion as shown in Figure 3, C and D, respectively. These responses were not attenuated by the previous administration of the V1 receptor antagonist at any of the time points sampled.

To determine whether $d(\text{CH}_{2})_{5}\text{Tyr(Me)}\text{AVP}$ administration could potentiate the attenuation elicited by
astressin in the Ex-4 stimulatory effects on HPA axis, both antagonists were simultaneously infused at the doses previously studied (Figure 4). The robust ACTH response promoted by the peripheral administration of Ex-4 (Figure 4A) was significantly reduced by astressin pretreatment by almost 50% (Figure 4C) although it continued to be significant as compared to control animals. As previously shown, pretreatment with astressin did not significantly affect the potent corticosterone response elicited by Ex-4 peripheral administration (Figure 4, B and D). Importantly, the infusion of the V1 antagonist in combination with astressin did not produce any further attenuation in the response of either of the two hormones studied.

Study of the interaction of Ex-4 with CRH and AVP on pituitary-adrenal activity

Because a reciprocal potentiation of the effects of the most important corticotropin secretagogues, CRH and AVP, on pituitary-adrenal axis activity is well documented (23, 25), the present study was designed to test whether Ex-4 could also potentiate the effects of these two peptides. To this end, Ex-4 was iv administered in combination with CRH or AVP. Interestingly, an additive effect was found when Ex-4 and AVP were administered together, such that the addition of Ex-4 significantly increased ACTH levels at 15 minutes (P < .01, Figure 5A) and corticosterone levels at 5 minutes (P < .05, Figure 5B) as compared to the group receiving AVP only. The group treated with Ex-4 plus AVP also showed significantly elevated levels of ACTH and corticosterone at 5 minutes (P < .01 and P < .05, respectively) when compared to the group receiving Ex-4 alone. The same conclusions were reached by examining the AUC of the ACTH and corticosterone responses (Figure 5, C and D). In contrast, no additive effect on either ACTH or corticosterone secretion was found when Ex-4 was administered in combination with CRH (Supplemental Figure 1).

Effects of Ex-4 sc administration on HPA axis activity

Because Ex-4 is sc administered to patients, we tested whether this route of administration also stimulates HPA activity in rats. Both ACTH and corticosterone plasma levels significantly (P < .05-P < .001) increased in response to a sc bolus of Ex-4 (1.2 nmol/kg) as compared to vehicle-treated animals. The elevation in ACTH and corticosterone levels was observed from the first time point studied, 15 minutes, and persisted up to the last time point sampled, 60 and 120 minutes respectively (Figure 6).

Role of the sympathoadrenal system in the effects on HPA activity produced by Ex-4

Basal ACTH and corticosterone levels in bilateral enucleated animals (MEDX) were not significantly different
from the basal values in SHAM-operated rats (Figure 7, A and B). The robust corticosterone response elicited by the sc-administration of Ex-4 (1.2 nmol/kg, iv) peripheral administration. Time-course profiles are represented as the difference from the respective basal levels shown at the top of each graph. Panels C and D show, respectively, the AUC profiles of ACTH and corticosterone responses. Data are expressed as mean ± SE (n = 6 rats/group; ***, P < .001, **, P < .01, *, P < .05 vs control group; #, P < .05 vs animals receiving Ex-4 alone).

Figure 4. Effects of pretreatment with astressin (28 nmol/kg, iv) and d(CH2)5Tyr(Me)AVP (45 nmol/kg, iv) on ACTH (A) and corticosterone (B) time-course secretory responses induced by Ex-4 (1.2 nmol/kg, iv) peripheral administration. Time-course profiles are represented as the difference from the respective basal levels shown at the top of each graph. Panels C and D show, respectively, the AUC profiles of ACTH and corticosterone responses. Data are expressed as mean ± SE (n = 6 rats/group; ***, P < .001, **, P < .01, *, P < .05 vs control group; #, P < .05 vs animals receiving Ex-4 alone).

from the basal values in SHAM-operated rats (Figure 7, A and B). The robust corticosterone response elicited by the sc-administration of Ex-4 (1.2 nmol/kg) was significantly (P < .05) attenuated at 30 and 60 minutes in the MEDX rats (Figure 7B). In addition, the AUC of the corticosterone response throughout the first 60 minutes of the study was significantly (P < .05) reduced by 35% in the MEDX rats (Figure 7C). In contrast, a similar time-course response of ACTH was observed in both groups of animals (Figure 7A). An ACTH stimulation test carried on the SHAM and MEDX rats confirmed that the adrenal cortex of the MEDX rats was functionally recovered by the time the study was conducted, such that a comparable corticosterone response to exogenous ACTH was observed in both groups of animals (Figure 7D). The role of the sympathoadrenal system in the effects of Ex-4 on corticosterone secretion was further demonstrated when the peptide was administered to rats pretreated with guanethidine, a blocker of the action of adrenergic neurons (22). Although a comparable ACTH response to Ex-4 stimulation was observed in the rats previously exposed to either vehicle or guanethidine (Figure 7E), the corticosterone response was significantly diminished in the animals pretreated with guanethidine (Figure 7F).

Effects of GLP-1 (7–36)amide and Ex-4 on ACTH release in dispersed pituitary cells

Primary cultures of pituitary glands were incubated with GLP-1 (7–36)amide and Ex-4 to determine whether there is a direct effect of these peptides on corticotrope cells (Figure 8). Incubation with CRH (10⁻⁹ M, used as positive control) significantly (P < .001) increased ACTH release at 3 and 6 hours, as expected. On the contrary, and despite the wide range of doses of both GLP-1 (from 10⁻⁹ to 10⁻⁶ M) and Ex-4 (from 10⁻¹² to 10⁻⁶ M) tested, no significant effect on ACTH release was observed in the wells incubated with these peptides. Finally, a group of wells was incubated with Ex-4 plus CRH to establish whether there is any synergistic or inhibitory effect between the two peptides, but Ex-4 did not significantly affect the response of the cells to CRH incubation at either of the time points studied.

Discussion

Previous studies have shown that the incretin hormone GLP-1 plays a role in HPA axis function and in the responses to stressors (7–11). Thus, it has been demonstrated that intracerebroventricular (icv) administration of this peptide increases the circulating levels of ACTH and corticosterone (7). The NTS is the major site of GLP-1 expression in the CNS (5, 6). Because it is known that NTS neurons innervate CRH-producing neurons of the PVN and that these latter neurons are activated by GLP-1 central administration, CRH has been proposed as the principal mediator of GLP-1 effects on the adrenal axis (7, 26–28). Accordingly, the rise in ACTH and corticosterone levels elicited by icv administration of GLP-1 is completely abolished in rats pretreated with astressin, a peptidic agonist of the CRH type 1 receptor that does not cross the blood-brain barrier (10). However, the effects of centrally
administered Ex-4 on HPA axis have been demonstrated to be much more potent than those elicited by GLP-1 (11), and the role of CRH in these effects has not been investigated. Herein, we show that the increase in ACTH levels observed after Ex-4 central administration is completely suppressed by pretreatment with astressin; surprisingly, the corticosterone response, although reduced, was still significant. Importantly, considering the therapeutic use of several GLP-1 derivative drugs for the treatment of patients with type II diabetes, we also investigated the mechanisms underlying the effects on HPA axis of peripherally administered Ex-4.

Herein, we show that astressin pretreatment significantly reduced the ACTH response elicited by the peripheral administration of Ex-4. In contrast, a previous study suggests that the effects of peripheral Ex-4 on HPA axis might be totally CRH-independent (29). This apparent contradiction may be explained by the different doses of Ex-4 used (10- to 20-fold higher in the previous study), the single time point sampled (at 1 hour) in the first study and, most importantly, by the different antagonist utilized. Thus, in order to enable a direct comparison with preexisting data regarding the role of CRH in GLP-1 effects and with our own observations about the role of CRH in the effects of centrally administered Ex-4, astressin was the antagonist utilized in the present study. In contrast, α-helical-CRH was the antagonist used in the previous study, an antagonist that has been reported to suffer from limited solubility and persistent intrinsic activity as well as weak potency blocking the pituitary effects of CRH (17). Nevertheless, in agreement with the previous study, antagonism of CRH receptors failed to affect the corticosterone response to peripheral Ex-4. Since the ACTH response was reduced but not totally prevented in these animals, it could be argued that the remaining elevation of ACTH levels might be enough to induce a full adrenal response. However, a persistent corticosterone response to centrally administered Ex-4 was also observed in astressin-pretreated rats, even though the ACTH response was...
totally abrogated in these animals. These findings suggest that factors in addition to CRH may be contributing to the corticoadrenal effects of Ex-4.

The link between SAS and adrenocortical function is well-established (21). Thus, it has been demonstrated that catecholamines can stimulate adrenal steroidogenesis (30, 31) and that sympathetic innervation of the gland can modulate the adrenocortical response to ACTH (32). Because Ex-4 has been reported to stimulate the activity of catecholamine neurons in the brain (20), leading to an activation of the sympathetic tone that results in increments of blood pressure and heart rate (20, 33, 34), we hypothesized that the effects of Ex-4 on glucocorticoid secretion may be partially mediated by the SAS. This hypothesis is supported by our finding that the robust corticosterone response elicited by peripherally administered Ex-4 was significantly attenuated in bilateral enucleated rats and in animals previously treated with guanethidine. In contrast, neither of the two approaches had a significant effect on the ACTH response to Ex-4. Because it is known that after the adrenal enucleation procedure the adrenal cortex is damaged and does not completely regenerate for approximately 4 weeks (24), our study involving enucleated animals was conducted 5 weeks after surgery to ensure a full recovery of cortical function, which was further confirmed by an ACTH stimulation test. Furthermore, the studies designed to investigate the role of SAS were conducted on conscious animals in order to avoid any potential effect of the anesthetic on the sympathetic tone (35).

Vasopressin is a major modulator of ACTH secretion (36) whose circulating levels have been reported to be elevated in response to central and peripheral administration of GLP-1 (7, 34). Herein, we tested the role of vasopressin in the effects of Ex-4 on HPA axis by administering d(CH2)5Tyr(Me)AVP, a nonselective antagonist with a high affinity for the two types of V1 receptors (V1a and V1b) commonly used to block the pituitary-adrenal effects of vasopressin (18, 19). This antagonist effectively blocked the adrenocortical responses promoted by vasopressin, but failed to either reduce the responses exerted by Ex-4 or to further potentiate the attenuation of these responses induced by astressin; thus, a role for vasopressin as a mediator of Ex-4 effects on adrenal axis seems unlikely. Similarly, since previous studies have detected expression of

Figure 7. Role of the sympathoadrenal system in Ex-4 effects on corticosterone secretion. Stimulatory effect of Ex-4 (1.2 nmol/kg, sc) on ACTH (A) and corticosterone (B) secretion in bilateral enucleated (MEDX) and SHAM-operated rats. Time-course responses are expressed as the difference from the respective basal levels shown at the top of each graph. C, AUC profile of the corticosterone response in the same animals during the first 60 minutes. D, Corticosterone plasma levels following an ACTH stimulation test (0.8 nmol/kg, sc) in dexamethasone-blocked rats. Effects of guanethidine pretreatment in the ACTH (E) and corticosterone (F) responses elicited by ip injection of Ex-4 (1.2 nmol/kg) in conscious rats. Data are expressed as the mean ± SE (n = 5–7 rats/group); *, P < .05 vs SHAM rats or rats pretreated with vehicle).
GLP-1r in the anterior pituitary by in situ hybridization (37) and GLP-1 has been shown to stimulate secretion of TSH from pituitary cells in culture (38), we also investigated a possible direct effect of Ex-4 on the pituitary corticotropes. Nevertheless, no effects on ACTH release were found for either GLP-1 or Ex-4 in pituitary primary cultures, suggesting that a direct effect of Ex-4 on the pituitary to regulate ACTH secretion is also unlikely.

Figure 8. ACTH release from dispersed rat pituitary cells incubated either with GLP-1 (7–36)amide, Ex-4, or CRH used as a positive control; after 3 (A, C, E) and 6 hours (B, D, F) of incubation. Values are represented as the mean ± SE from either two (A-D), three (F), or four (E) separate experiments, each performed in quadruplicate (***, P < .001, **, P < .01 vs vehicle treated-group).
An additive effect on corticosterone secretion at early time points was observed when Ex-4 was administered in combination with vasopressin and a similar trend was found for ACTH secretion. This observation may be explained by the activation of CRH-producing neurons induced by Ex-4, as the CRH released by these neurons may, ultimately, interact at the pituitary level with vasopressin, potentiating ACTH secretion (19). In contrast, no additive effects were found when Ex-4 was given in combination with CRH, although more complex studies involving different doses (39) would be necessary in order to definitely discard a potential interaction between CRH and Ex-4.

Based on our data, we hypothesize that the effects of centrally administered Ex-4 on ACTH secretion are predominantly mediated by CRH since they are totally abrogated by the administration of astressin. Thus, Ex-4 might be directly acting through GLP-1 receptors in CRH-producing neurons that are known to possess high density of GLP-1 binding sites (7). A direct activation of these neurons could also occur after peripheral administration of the peptide since Ex-4 has been demonstrated to readily cross the blood-brain barrier (40). Nevertheless, alternative, not exclusive pathways can be postulated for the actions of circulating Ex-4, as it has been reported that GLP-1 receptors are densely expressed in circumventricular organs, such as the area postrema (41) and in sensory afferent fibers of the nodose ganglion (42). These areas are activated following the peripheral administration of Ex-4 and, in turn, activate a number of brain regions, including the NTS and PVN (41, 42). As a result, not only the same CRH pathways activated by centrally administered Ex-4, but also complimentary pathways, not identified in the present study, and perhaps responsible for the persistent elevation in ACTH levels in astressin pretreated animals, could be stimulated in response to peripheral Ex-4. In addition, several autonomic control sites are also activated by both central and peripheral administration of Ex-4 (20), which, likely, may be responsible for the role of SAS in corticosterone secretion.

Herein, we show the potent stimulatory effects on HPA axis promoted by central (i.c.v) and peripheral (via different routes: iv, sc, and ip) administration of Ex-4 in rats. Previous studies have shown that the acute iv administration of a GLP-1 bolus (11), as well as the iv infusion of both GLP-1 and Ex-4 (43, 44), can significantly elevate cortisol levels in humans. Whether the long-term subcutaneous administration of exenatide and other GLP-1 derivative drugs currently used for the treatment of type II diabetic patients has any effect on the HPA axis of these patients remains elusive because, as far as we know, no specific studies have been designed to address this question. However, a previous study found that sc injection of Ex-4 before moderate exercise significantly elevated postexercise cortisol levels in healthy volunteers (45), suggesting that the acute subcutaneous administration of Ex-4 may also stimulate the HPA axis in humans. However, further studies are required to address the relevance of Ex-4 effects on the corticoadrenal axis in humans and the mechanisms underlying these effects.

In summary, our data demonstrate the essential role of both CRH and the SAS in the stimulatory effects of Ex-4 on the HPA axis, whereas they exclude both a role for vasopressin and a direct effect of Ex-4 on the pituitary corticotropes in these effects. Ultimately, these findings indicate the relevance of Ex-4 as a corticosterone secretagogue, particularly when considering the clinical use of this insulinotropic peptide. Excitingly, they open the window to a potential novel clinical use of Ex-4 in medical conditions characterized by a transitory impairment in the steroidogenic activity of the adrenal gland, such as secondary adrenal insufficiency due to exogenous glucocorticoid therapy (46).

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