Effects of prolonged exendin-4 administration on hypothalamic-pituitary-adrenal axis activity and water balance

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Gil-Lozano M, Román-Pérez M, Outeiriño-Iglesias V, Vigo E, Brubaker PL, González-Maías LC, Mallo F. Effects of prolonged exendin-4 administration on hypothalamic-pituitary-adrenal axis activity and water balance. Am J Physiol Endocrinol Metab 304: E1105–E1117, 2013. First published March 26, 2013; doi:10.1152/ajpendo.00529.2012.—Exendin-4 (Ex-4) is a natural agonist of the glucagon-like peptide-1 (GLP-1) receptor, currently being used as a treatment for type 2 diabetes mellitus due to its insulinotropic properties. Previous studies have revealed that acute administration of both GLP-1 and, in particular, Ex-4 potently stimulates hypothalamic-pituitary-adrenal (HPA) axis activity. In this work, the effects of prolonged Ex-4 exposure on HPA function were explored. To this end, Sprague-Dawley rats were subjected to a daily regimen of two Ex-4 injections (5 μg/kg sc) for a minimum of 7 days. We found that subchronic Ex-4 administration produced a number of effects that resemble chronic stress situations, including hyperactivation of the HPA axis during the trough hours, disruption of glucocorticoid circadian secretion, hypertrophy of the adrenal gland, decreased adrenal gland sensitivity, impaired pituitary-adrenal stress responses, and reductions in both food intake and body weight. In addition, a threefold increase in diuresis was observed followed by a 1.5-fold increase in water intake; these latter effects were abolished by adrenalectomy. Together, these findings indicate that Ex-4 induces a profound dysregulation of HPA axis activity that may also affect renal function.

Exendin-4; adrenal; corticosterone; circadian; diuresis

EXENDIN-4 (EX-4) IS A 39-AMINO ACID PEPTIDE ISOLATED FROM THE SALIVARY SECRETIONS OF THE LIZARD Heloderma suspectum. Ex-4 shares a 53% amino acid sequence homology with the human incretin GLP-1, acting as a full agonist of the GLP-1 receptor (GLP-1R) (64). In addition, Ex-4 is considerably more resistant than native GLP-1 to cleavage by dipeptidyl peptidase-IV, thereby having a markedly extended half-life (59). Both peptides exert important effects in the pancreas, stimulating glucose-dependent insulin release, suppressing glucagon secretion, and increasing β-cell mass by promoting proliferation and differentiation of the cells. They also slow gastric emptying and diminish gastric acid secretion (9, 25, 29). Because of these properties, several Ex-4 and GLP-1 derivative drugs have been approved for the treatment of type 2 diabetes mellitus (10, 43).

The enteroendocrine L cells are the major source of circulating GLP-1 (29); however, it is also produced in the central nervous system (CNS), mainly in the nucleus of the solitary tract (NTS) (26, 33). Moreover, the GLP-1R is widely expressed in the CNS, particularly in the paraventricular nucleus (PVN) and arcuate nucleus (58) of the hypothalamus, and, complementarily, some clusters of NTS neurons project GLP-1-immunoreactive axons to the PVN, a key regulator of feeding and stress-related behaviors (56, 63, 69). As a consequence, it has been suggested that central GLP-1 could be serving as a neurotransmitter. Accordingly, GLP-1 has potent anorexigenic activity (61, 66), and central GLP-1R activates autonomic regulatory neurons, increasing blood pressure and heart rate in rats (3, 73). Intracerebroventricular (icv) administration of GLP-1 has been associated with improved learning as well as neuroprotection in rats (12). In addition, we and others (20, 30, 32, 34, 50) have previously reported that GLP-1 may be an important modulator of the hypothalamic-pituitary-adrenal (HPA) axis and stress responses. Thus, central GLP-1 administration activates CRH-producing neurons in the parvicellular region of the PVN, leading to increases in plasma ACTH and corticosterone levels (34). The induction of visceral illness by peripheral injection of lithium chloride (LiCl) activates GLP-1-expressing neurons in the NTS (32, 50), and the HPA response to LiCl is blocked by icv administration of a GLP-1R antagonist (30). Moreover, we have shown that acute peripheral administration of GLP-1 and Ex-4 potently stimulates HPA axis activity (20) independently of the metabolic state of the animals and the insulinoergic effects of these incretins, and it can be observed under both ad libitum and fasting conditions as well as in diabetic models. The effects of Ex-4 on HPA axis activity are of much greater magnitude than those elicited by the native peptide (20), likely because of its longer half-life in plasma. It is noteworthy that stimulatory effects of GLP-1 on glucocorticoid secretion are also observed in control and type 1 diabetic humans after acute administration of relatively low doses of the peptide (20).

The finding that peripheral administration of Ex-4 increases HPA axis activity may have very relevant clinical implications, since therapies based on Ex-4 and other incretins are already in use for the treatment of patients with type 2 diabetes. Hyperactivation of the HPA axis in diabetic patients has been previously reported (5, 6, 52, 75), and further alterations to HPA function might result in harm to these patients. Thus, prolonged exposure to elevated glucocorticoid levels can lead to insulin resistance (2) and may also aggravate the metabolic status and the cognitive and affective disorders often associated with diabetes (37, 42). Since the impact of prolonged Ex-4 exposure on pituitary-adrenal function has not been clarified, we herein have examined the effects of subchronic (7–9 days) Ex-4 administration in the HPA axis activity and reported how it modifies the glucocorticoid circadian rhythm, basal and stress-induced ACTH and corticosterone levels and adrenal growth, and the role of the adrenal gland in mediating some of...
the effects of Ex-4 in body weight, food intake, and water balance.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (275–350 g) were purchased from the facilities at either the University of Santiago (Santiago de Compostela, Spain) or Charles River Laboratories (St. Constant, QC, Canada). They were acclimatized to our animal housing facility for several days before we carried out the experiments and were handled daily to avoid experimental stress. The animals were maintained with free access to tap water and standard chow (A04; Panlab, Barcelona, Spain) under a 12:12-h light-dark cycle with lights on at 0900 and at a controlled room temperature (20–22°C).

All experimental procedures were conducted in accordance with European Union guidelines regarding the use of animals for experimental purposes (Council Directive CEE 86/609).

Drugs and Peptides

Ex-4 and ACTH were obtained from Bachem (Bubendorf, Switzerland), pentobarbital sodium was provided by Sigma-Aldrich (Alcobendas, Spain), and dexamethasone by Sandoz (Boucherville, QC, Canada).

Adrenal Surgery

The animals were anesthetized with pentobarbital sodium (50 mg/kg ip), and a dorsal approach was performed to expose the adrenal glands. Briefly, a dorsal midline incision of the skin was followed by bilateral subcostal muscle penetrations. The adrenal glands were extracted (complete adrenal ablation, ADX) or visualized but not manipulated in the control animals (SHAM). Enucleation of the medullas of the adrenal glands (MEDX) was performed by cutting a nick in one of the poles of the gland with scissors for microsurgery, and the medulla was then extruded by gentle compression. The adrenals were returned to their usual location and the wounds were sutured with cotton thread. The rats usually became active within a few minutes after the operation. The materials used in surgery were sterilized in a chlorhexidine solution, and the animals were kept warm until fully ambulatory.

Experimental Protocols

All animals were individually housed in metabolic cages for at least 4 days before the beginning of the treatment, which consisted of a subcutaneous bolus of Ex-4 (5 µg/kg) or vehicle (NaCl 0.9%) twice daily (first administration between 1100 and 1200 and the second between 2100 and 2200) for 7–9 days. The Ex-4 dose was chosen on the basis of previous studies (20).

Effects of prolonged Ex-4 treatment in HPA axis basal activity. Four different experiments were performed. In the first, 26 rats were treated either with Ex-4 (n = 11) or vehicle (n = 15) twice daily for 8 days, as above. Every morning, between 1000 and 1200, a blood sample was taken (200 µl) immediately prior to the administration of the first daily injection. To avoid excessive loss of plasma volume, each animal was sampled only for 6 days, and a minimum of four Ex-4 and seven vehicle-treated rats were studied every day. Samples were drawn from a small cut in a lateral vein of the tail while the rats were restrained in clear tube restrainers (Panlab, Barcelona, Spain). The entire sampling procedure took less than 3 min, since it is known that longer times may induce a stress reaction and a rise in ACTH levels (68). In a second experiment, to study the circadian rhythm of corticosterone levels in our animals, tail venous samples were sequentially collected for 24 h at 1800, 2100, 0000, 0800, 1100, and 1600 from a group of eight animals that had received vehicle treatment. In a third experiment, the animals were treated with Ex-4 or vehicle (n = 8 rats/group) for 8 days and on the morning of the 9th day; then, tail venous samples were drawn at 1800, 2100, and 0000. In the fourth experiment, the animals received Ex-4 or vehicle as above for 7 days (n = 6 rats/group); on day 8, tail venous samples were extracted both in the morning and afternoon to measure aldosterone levels.

Study of the desensitization of Ex-4 effects in HPA axis. The rats were treated with Ex-4 (n = 6) or vehicle (n = 12) for 8 days, as above. At 0900 of day 9, a basal sample was taken, and the animals received an intraperitoneal injection of Ex-4 (5 µg/kg) or vehicle to test the ACTH and corticosterone responses (n = 6 rats/group). Blood samples were collected at 15, 30, 60, and 120 min by tail nicking as previously described. The vehicle + vehicle group was set as control; no Ex-4 + vehicle group was included in the study, since no changes in the HPA axis were observed after NaCl (0.9%) administration alone and previous studies performed in our laboratory did not show any response to vehicle in animals receiving prolonged Ex-4 treatment (data not shown).

Effects of prolonged Ex-4 treatment on adrenal gland sensitivity. Rats received Ex-4 or vehicle treatment for 9 days, as above (n = 6 rats/group). On day 10, the animals were administered a single dose of dexamethasone (250 µg/kg sc) to block endogenous ACTH secretion, and 3–4 h later they received a subcutaneous bolus of rat ACTH (4 µg/kg). Blood samples were extracted before ACTH stimulation and at times 15, 30, 60, and 120 min.

Effects of prolonged Ex-4 administration on the stress response. Twelve rats were administered Ex-4 or vehicle for 7 days, as above (n = 6 rats/group). On day 8, the animals were subjected to 20 min of restraint stress in a well-ventilated clear acrylic tube. A basal blood sample was taken at 0900, before start of the restraint, and subsequently at 20, 60, and 120 min. Samples were drawn by tail nicking at progressively more proximal points on the tail.

Effects of prolonged Ex-4 treatment on HPA axis activity in unilateral enucleated rats. This study was developed to determine both the role of the adrenal medulla in Ex-4 effects on HPA axis and whether Ex-4 treatment could facilitate the recovery of normal steroidogenic function in a well-established model of adrenocortical regeneration, the unilateral enucleated (ULE) rat (14). Twelve animals underwent surgical enucleation of the left adrenal gland, while six were subjected to sham surgery of the left gland. All surgeries were accompanied by contralateral adrenalec- tomy of the right gland. The animals were left undisturbed for 3 days to recover from surgery, and then they received Ex-4 or vehicle treatment for 7 days (n = 6/group), as above. Blood samples were taken by tail nicking at 1800 on days 2 and 5 and at 1000 of day 7. On day 8, a restraint stress test was performed, as described above.

Role of the adrenal gland in the effects of prolonged Ex-4 treatment on animal weight, food intake, and water balance. Three different experiments were performed: one in nonoperated rats (n = 16), another in bilateral adrenalectomized rats (ADX; n = 10), and the last in rats that underwent unilateral adrenal enucleation (ULE) or sham surgery as above (n = 18). ADX and ULE rats were allowed to recover for 4 and 3 days, respectively, prior to the onset of the treatment. After surgery, saline solution (NaCl 0.9%) was given to the ADX rats for drinking to prevent sodium depletion caused by lack of aldosterone (49). Animal weight, food and water intake, and urine volume were monitored every day between 0900 and 1100. Ex-4 treatment was prolonged for 7 days in the nonoperated and ULE rats and for 4 days in ADX rats, as detailed above. At the end of the study, the rats were euthanized and the adrenal glands extracted.

Role of the adrenal gland in the effects of acute Ex-4 administration in urine excretion. Three groups of rats were used: animals that underwent bilateral adrenalec tomy (ADX), bilateral medullectomy (MEDX), and sham-operated controls (SHAM). Four days after surgery, the animals received two injections of Ex-4 (5 µg/kg sc) or vehicle (n = 5–8/group) at t = 0 and 12 h (t = 0 between 1100 and 1200). The volume of urine excreted was recorded at 4 (t4) and 24 h (t24) after the first injection.
**Samples Processing**

Blood samples were collected in Eppendorf tubes containing EDTA (0.05 M, 10 µl/tube) and placed on ice. Blood was centrifuged to separate the plasma, which was stored at −20°C until use.

**Adrenal Gland Weights and Total RNA Quantification**

The adrenal glands were cleaned of surrounding connective tissue and fat, weighed on a precision balance, and rapidly frozen at −80°C. To quantify total RNA, the tissue was homogenized in denaturant solution containing β-mercaptoethanol (7.5 µl/ml), and the RNA was extracted following the guanidinium thiocyanate method modified by Chomczynski and Sacchi (8). Total RNA was determined by spectrophotometric assay at 260 nm, and the purity was estimated by the ratio of the absorbance at 260 and 280 nm. Ratios above 1.8 were obtained for all samples.

**Hormone Determination**

Plasma ACTH (Phoenix Europe, Karlsruhe, Germany) and total corticosterone levels (DRG-Instruments, Marburg, Germany) were measured using RIA kits according to the manufacturer’s instructions. Aldosterone levels were determined using an ELISA kit (DRG-Instruments).

**Statistical Analysis**

Data are represented as means ± SE. Statistical analysis was performed using SPSS 14.0 software for Windows. Student’s t-test was conducted to compare two independent groups or one-way ANOVA.
followed by post hoc Tukey’s test to compare three or more groups. *P < 0.05 was set as the conventional criterion for statistical significance.

RESULTS

Effects on HPA Axis Basal Activity

Ex-4 treatment increased the corticosterone plasma levels in the morning (1000) from the first day and throughout the 10 days of treatment, reaching significance (P < 0.05) on days 3, 5, 6, and 9 (Fig. 1A) compared with control animals. On the contrary, ACTH plasma levels were not significantly affected by Ex-4 administration (Fig. 1B). In a closer examination of the variations in corticosterone levels (Fig. 1C), control rats exhibited, as expected, a marked circadian rhythm, with highest corticosterone levels during the hours immediately previous to the onset of the dark phase (1800–2100) and lowest levels during the morning (0800–1100). Based on these data, the time points 1800, 2100, and 0000 were selected to study the effects of prolonged Ex-4 administration on the zenith of corticosterone circadian levels. In contrast to the increased corticosterone levels during the nadir, the rats receiving Ex-4 presented significantly reduced corticosterone levels on the zenith after 9 days of treatment (P < 0.05; Fig. 1D). Furthermore, corticosterone levels at the nadir and the zenith were comparable in Ex-4-treated rats, within the range of average daily levels, indicating a disruption of the corticosterone circadian rhythm in these animals. Both AM and PM basal aldosterone levels were significantly increased (P < 0.05) in rats treated with Ex-4 for 7 days (Fig. 1E).

Effects on Adrenal Gland Growth

As shown in Table 1, long-term administration of Ex-4 significantly increased adrenal growth, as reflected by an increase in the absolute adrenal weight as well as the values of both weight (P < 0.01) and total RNA content (P < 0.01) of the left gland normalized for whole body weight.

Desensitization of Ex-4 Effects on HPA Axis

An acute Ex-4 challenge test (1.2 nmol/kg ip) was conducted in rats previously treated with Ex-4 or vehicle for 8 days (Fig. 2). The animals previously exposed to Ex-4 presented increased corticosterone basal levels (77.8 ± 11.6 vs. 62.9 ± 14.3 ng/ml), although this increment did not reach statistical significance. Saline-pretreated rats showed marked increases in both ACTH and corticosterone levels in response to acute Ex-4 injection (P < 0.001–0.01). These effects were significantly lower in Ex-4-pretreated animals, such that the ACTH response was significant only at 15 min (P < 0.01) with respect to control rats and with the corticosterone levels at that time point being significantly lower than the levels observed in the saline-pretreated animals that received Ex-4 (P < 0.001). The corticosterone responses showed similar peak levels in both groups of animals at 30 and 60 min but were significantly lower at 120 min in the rats previously treated with Ex-4 (P < 0.01), thus showing shortened responses. In examining the AUC of ACTH and corticosterone responses to Ex-4, we found that both were markedly reduced (P < 0.01) in the animals that had previously received Ex-4 for 8 days (AUC of ACTH response during the first 60 min = 11,415 ± 736 pg/ml in Ex-4-pretreated rats vs. 16,203 ± 799 in saline-pretreated rats, and AUC of corticosterone response = 63,883 ± 6,969 ng/ml in Ex-4-pretreated rats vs. 94,707 ± 3,306 in saline-pretreated rats.

Figure 2. Time course of effect of ip administration of Ex-4 (5 μg/kg) on ACTH and corticosterone levels in rats previously exposed to saline or Ex-4 for 8 days. Previous Ex-4 exposure greatly diminished ACTH (A) and corticosterone (B) responses to acute Ex-4 stimulation. Data are presented as means ± SE; n = 6 rats per group. ***P < 0.01, ###P < 0.001 vs. saline-treated rats; ####P < 0.01, ####P < 0.001 vs. exendin-treated rats.

Table 1. Trophic effects of Ex-4 treatment on left adrenal gland

<table>
<thead>
<tr>
<th></th>
<th>Adrenal Weight, mg</th>
<th>Adrenal Weight per Body Weight, mg/100 g</th>
<th>Total-RNA Content, μg</th>
<th>Total-RNA Content per Body Weight, μg/100 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (NaCl 0.9%)</td>
<td>25.37 ± 1.67</td>
<td>7.20 ± 0.47</td>
<td>44.47 ± 2.46</td>
<td>12.65 ± 0.86</td>
</tr>
<tr>
<td>Acute Ex-4</td>
<td>23.50 ± 0.97##</td>
<td>6.70 ± 0.41##</td>
<td>37.20 ± 5.47##</td>
<td>10.70 ± 1.90##</td>
</tr>
<tr>
<td>Prolonged Ex-4</td>
<td>33.42 ± 1.53**</td>
<td>10.34 ± 0.72**</td>
<td>65.44 ± 9.27*</td>
<td>20.00 ± 2.84*</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE; n = 6–8 rats per group. Prolonged but not acute exendin-4 (Ex-4) administration increased adrenal weight and total-RNA content in nonoperated rats. *P < 0.05, ##P < 0.01 vs. control group; ###P < 0.01 vs. exendin-treated rats.
animals). Together, these results suggest that the pituitary-adrenal responses to Ex-4 wane after prolonged exposure. However, the ACTH and corticosterone responses were still persistently higher in both groups treated with Ex-4 than in the control group (AUC of ACTH response during the first 60 min = 8,615 ± 623 pg/ml and AUC of corticosterone response = 18,721 ± 3,200 ng/ml).

Effects on Adrenal Gland Sensitivity

The corticosterone response to an exogenous bolus of ACTH was significantly attenuated \((P < 0.05)\) in rats previously exposed to Ex-4 treatment for 10 days, as observed for both the time course response and the AUC response (Fig. 3, A and B, respectively).

Effects on Restraint Stress Responses

Twenty minutes of restraint increased plasma levels of both ACTH and corticosterone in control rats \((P < 0.001\) at 20 min vs. basal values), whereas previous exposure to Ex-4 for 7 days had markedly diminished these responses (Fig. 3, C and D). Hence, plasma ACTH levels were significantly reduced \((P < 0.05)\) at 60 min after the onset of the stress test, while the attenuation of the corticosterone stress response was of great magnitude, reaching significantly lower plasma levels \((P < 0.01)\) at all the points sampled; in addition, poststress levels returned to baseline pre-stress levels faster in Ex-4-pretreated animals.

Effects of Prolonged Ex-4 Treatment on HPA Axis Activity on ULE Rats

As shown in Fig. 4, ULE rats showed markedly reduced glucocorticoid levels at the zenith 5 days after surgery \((P < 0.01)\). By that point, the rats had received Ex-4 or vehicle treatment for 36 h, as shown in Fig. 4A. However, normal basal corticosterone levels were restored at the zenith on day 8 after surgery (5 days after onset of treatment) in ULE vehicle-treated rats. This recovery was impaired by Ex-4 administration, as ULE exendin-treated rats continued to show significantly lower corticosterone levels at that point. In contrast, ULE exendin-treated rats showed significantly higher corticosterone levels \((P < 0.05)\) during the nadir of the 10th day after surgery (e.g., 7th day of treatment), suggesting that the disruption of the corticosterone secretion pattern observed with prolonged Ex-4 treatment in nonoperated control rats (Fig. 1) also occurred in adrenal enucleated animals. Finally, the corticosterone stress responses to restraint were markedly impaired in both groups of ULE rats at the zenith on day 8 of treatment. However, this reduction was particularly marked in the exendin-treated rats.
in which the corticosterone response was almost completely abolished, as can be seen in Fig. 4B. Since the prestress basal corticosterone levels were significantly different (P < 0.05) among groups (32.9 ± 14.9 ng/ml in SHAM rats vs. 53.3 ± 7.1 ng/ml in ULE vehicle-treated and 71.6 ± 9 ng/ml in ULE exendin-treated rats), the time course of the increments in corticosterone levels is presented instead of the absolute values.

Mediation of Adrenal Gland in Effects of Prolonged Ex-4 Administration in Body Weight, Food Intake, and Water Balance

Baseline body weight, food and water intake, and diuresis were determined in control rats for 5 days, and then either vehicle or Ex-4 was administered for 8 days, as presented in Fig. 5. Ex-4 markedly reduced body weight and food intake within 24 h, and the daily values of both parameters remained significantly diminished until the end of the study (Fig. 5, A and B). Ex-4 also significantly reduced water intake during the first 24 h (P < 0.05). However, a daily water intake increase was observed from the 3rd day of treatment, reaching significantly diminished until the end of the study (Fig. 5, C and D). Ex-4 administration also potently increased urine excretion, doubling to tripling basal values during the study (P < 0.001–0.01; Fig. 5D).

The effects of Ex-4 treatment in the same parameters were studied in ADX rats, as shown in Fig. 6. Body weight slightly, and food intake markedly, dropped for 2 days after surgery, but thereafter both parameters gradually increased to reach baseline values. Administration of Ex-4 dramatically reduced food intake, almost completely suppressing appetite during the first day of treatment. This drop in food intake was reflected in reduced body weights of the animals onward (P < 0.05; Fig. 6A). Ex-4 treatment also significantly reduced water intake during the first 2 days (P < 0.05 and P < 0.001, respectively; Fig. 6C), although this returned to the baseline values observed in vehicle-treated ADX animals thereafter. Diuresis increased after surgery in all ADX rats, and it was not affected by Ex-4 administration; the values were, therefore, similar in both groups throughout the study (Fig. 6D).

As shown in Fig. 7, food intake was the only parameter that significantly changed after surgery in ULE rats, dropping during the first 3 days following surgery but remaining stable thereafter in the ULE vehicle-treated rats. Body weight, food and water intake, and urine excretion responses to Ex-4 administration in ULE rats were very similar to those described in nonoperated control animals (Fig. 5), although the effects on body weight and food intake appeared to wane with time (Fig. 7, A and B). Interestingly, the effects of Ex-4 in diuresis and water intake, nearly abolished with ADX, were completely preserved in ULE rats (Fig. 7D). To determine whether enucleation could have any impact on any of the parameters studied, a SHAM control group was included in the study, although it is not shown in the time course graphs to simplify visualization. However, when the AUC of the responses of these parameters was examined, comparable profiles were observed for both the SHAM and the ULE vehicle-treated rats, as depicted in Fig. 7E. Moreover, no significant differences were detected at any single time point in the study between these two groups on any of the four parameters (data not shown), including an initial decrease in food consumption also observed in the SHAM animals, suggesting that it may be a secondary effect derived from the trauma of the surgery.

Role of the Adrenal Gland in Effects of Acute Ex-4 Administration on Urine Excretion

As shown in Table 2, acute Ex-4 treatment increased 4-h urine volume in all the groups studied, reaching a three- (ADX, P < 0.001) to fourfold increment (SHAM, P < 0.01; MEDX, P < 0.001) compared with control vehicle-treated animals. This effect was reproduced for the 24-h interval in SHAM (P < 0.05) and MEDX (P < 0.001) but not in ADX rats.

DISCUSSION

Previous studies have shown that the incretin hormone GLP-1 plays a role in HPA axis function and in the responses to stressors (20, 30, 32, 34, 50). Notably, we have observed that when present in the peripheral circulation GLP-1 and the related peptide Ex-4 strongly stimulate HPA axis activity in both healthy and diabetic humans and rodents (20). Since a therapy based on Ex-4 is currently being used by type 2 diabetic patients (10, 43)
and it is established that repeated exposure to a hormonal secretagogue (51, 60) as well as to stressful stimuli (15, 45) may affect the basic mechanisms for the maintenance of HPA axis homeostasis, the effect of prolonged exposure to Ex-4 on the HPA axis is highly relevant. Herein, we show that sub-chronic Ex-4 administration produces a number of typical signs of persistent HPA stimulation, including disrupted circadian pattern of glucocorticoid levels, increased basal values of corticosterone, decreased adrenal gland sensitivity, impaired hormonal response to stress, and hypertrophy of the adrenal gland.

In animals and humans, resting plasma concentrations of glucocorticoids show major diurnal variations, with peak levels toward the beginning of the active periods and a nadir at the onset of the inactive periods (70). In rats, animals with nocturnal habits, the zenith corresponds to the late afternoon and the nadir with the first hours in the morning (72). This diurnal rhythm is of high amplitude, with a 5- to 10-fold increase from trough to peak (67). In this study, the expected circadian rhythm of corticosterone was observed in control rats, and Ex-4 administration significantly increased corticosterone plasma levels in the resting hours of the morning, indicating adrenal cortex hyperactivation. High glucocorticoid levels during the nadir are also found in response to the prolonged administration of other HPA axis stimulators, such as CRH and ACTH (51), and are also commonly linked to pathologies that imply a chronic stress status, including diabetes (5, 6, 52, 75) and depression (7, 24, 53). Conversely, resting ACTH plasma levels during the nadir were not significantly affected by Ex-4 administration. In agreement with this, normal or slightly elevated ACTH levels in addition to high glucocorticoid levels are often found in stress-related disorders (19, 45, 53). It must be noted that the ACTH circadian rhythm is of low amplitude and frequently not significant throughout the day (67), suggesting that non-ACTH factors may contribute to the circadian rhythm in plasma corticosterone levels. Complementarily to the significant increase in circulating corticosterone levels during the morning, we observed greatly diminished corticosterone levels during the peak hours in rats that received Ex-4 treatment for 9 days. As a whole, the circadian rhythm is clearly altered with subchronic Ex-4 administration, showing similar circulating corticosterone levels during the nadir and the peak hours. This suggests that prolonged Ex-4 exposure induces a particularly marked dysregulation of HPA axis activity that results in complete disruption of the corticosterone circadian rhythm, thereby maintaining corticosterone circulating levels constantly around daily average levels, as has been observed in rats implanted with a low- to medium-dose corticosterone pellet (1, 55). The reduced corticosterone levels during the zenith observed in the present study may be the result of compensatory responses in the HPA axis triggered as an attempt to adjust to the persistent elevations of corticoste-
rone levels during the acrophase, as has been previously proposed (1). A previous report (35) has also shown that Ex-4 exposure for 7 days significantly increases corticosterone circulating levels in both control nondiabetic rats and in streptozotocin-treated rats without affecting ACTH levels in the nondiabetic rats, in agreement with our observations. However, the fact that all hormonal measurements in that study were made on samples extracted 3 h after the last Ex-4 injection demonstrates an acute effect of Ex-4 administration more than an effect on corticosterone circadian rhythm as we are showing herein.

Two main factors contribute to the circadian rhythm in corticosterone secretion, the rhythm in ACTH secretion and the daily variation in the adrenal gland’s responsiveness to ACTH. Thus, it is known that the adrenal sensitivity to ACTH is higher during the peak hours of corticosterone secretion, the splanchnic innervation of the adrenal gland being one of the key components contributing to this rhythm (67). Interestingly, Ex-4 potently activates the autonomic nervous system (ANS) (46, 73), and this may enhance its acute effects on corticosterone release (20). Nevertheless, mediation by the ANS appears not to be essential in the effects of Ex-4 on corticosterone circadian rhythm, since ULE rats receiving Ex-4 treatment also presented a disrupted corticosterone rhythm. It is known that adrenal enucleation impairs normal cortical function and that the adrenal cortex does not completely regenerate for approximately 4 weeks (14, 71). However, previous studies have shown that rat cortical function, although not fully restored, is greatly recovered 7 days after adrenal enucleation and significant AM-PM differences in plasma corticosterone concentrations are found (71). Consistent with this, the ULE vehicle-treated rats of our study presented significantly reduced basal corticosterone after enucleation that returned to normal basal values 8 days later, while the corticosterone circadian rhythm also appeared to be restored, since PM levels were significantly higher than AM levels. Conversely, no corticosterone rhythm was found in the ULE Ex-4-treated rats that continued to show comparable corticosterone levels at peak and trough hours, suggesting that the adrenal medulla and adrenal innervation (which is also damaged after enucleation), are not essential for the effects of Ex-4 on corticosterone circadian rhythm.

It is known that continuous exposure to a secretagogue (51, 60) or to a repeated stressful stimulus (15, 31, 45, 47) induces desensitization and reduces the responsiveness of both the HPA axis and the sympathetic-adrenal medullary system. Herein, we show that repeated administration of Ex-4 significantly decreased adrenal gland sensitivity, reducing corticosterone response to a corticotropin stimulation test. We also observed that animals previously exposed to Ex-4 for 7 days exhibited a greatly dampened corticosterone response to restraint, which is considered as a paradigm of psychogenic stressor of great intensity (47), along with a minor reduction in

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Fig. 6. Effect of prolonged Ex-4 treatment (administered sc twice daily for 4 days) on daily body weight (A), food intake (B), water intake (C), and urine excretion (D) in adrenalectomized (ADX) rats. Data are expressed as means ± SE; n = 8 rats per group. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control group.
the ACTH response, supporting a loss of adrenal gland sensitivity. The attenuation of the corticosterone stress response induced by prolonged Ex-4 administration was even more pronounced in adrenal enucleated animals. Consistently with what we show herein, it is known that adrenal responses are greatly diminished in unilateral adrenalectomy rats (our control group) and further decreased in ULE animals (14). However, we demonstrate that the corticosterone response to stress was almost completely abolished in ULE rats receiving Ex-4 treatment, indicating that the effect of Ex-4 on gland sensitivity is not mediated by the adrenal medulla. Interestingly, it has been previously suggested that disruption of the corticosterone rhythm and maintenance of constant glucocorticoid levels, as described in the present study, may result in loss of adrenal gland sensitivity and attenuation of HPA axis responsiveness (1, 39, 55). We also observed that animals previously exposed to Ex-4 for 9 days presented greatly reduced ACTH and corticosterone responses to a further acute challenge test with Ex-4, indicating that the pituitary-adrenal responses to Ex-4 wane with time. Nevertheless, both the ACTH and corticosterone responses, although reduced, continued to be robust and significant compared with control animals. Interestingly, the ACTH response being much more attenuated than that of corticosterone suggests that Ex-4 may exert direct effects on

Fig. 7. Effect of prolonged Ex-4 treatment (administered subcutaneously twice daily during 7 days) on daily body weight (A), food intake (B), water intake (C) and urine excretion (D) in unilateral enucleated rats. (E) AUC of the ∆ body weight, and absolute food intake, water intake and urine volume responses during the 7 days of treatment in the SHAM animals (white bars), and the ULE rats receiving vehicle (light grey bars) or Ex-4 (dark grey bars). The data are expressed as mean ± SE (n = 6 rats per group, *P < 0.05, **P < 0.01 vs. ULE saline-treated rats).
Table 2. Effect of acute Ex-4 administration on urine volume excretion in SHAM, ADX, and MEDX rats

<table>
<thead>
<tr>
<th></th>
<th>4-h Urine Volume</th>
<th>24-h Urine Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (NaCl 0.9%)</td>
<td>3.37 ± 0.50</td>
<td>16.00 ± 2.51</td>
</tr>
<tr>
<td>Ex-4</td>
<td>14.87 ± 2.12***</td>
<td>26.37 ± 2.60*</td>
</tr>
<tr>
<td>MEDX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.67 ± 0.24</td>
<td>11.67 ± 1.80</td>
</tr>
<tr>
<td>Ex-4</td>
<td>12.22 ± 1.58***</td>
<td>25.22 ± 2.38***</td>
</tr>
<tr>
<td>ADX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.00 ± 1.32</td>
<td>19.20 ± 1.95</td>
</tr>
<tr>
<td>Ex-4</td>
<td>13.00 ± 1.06***</td>
<td>22.60 ± 1.15</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE; n = 5–8 rats per group. SHAM, sham operated; MEDX, bilaterally medulectomized; ADX, adrenalectomized.

Ex-4 administration (1.2 nmol/kg sc) was injected at times 0 and 12 h. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control group.

The adrenal. Accordingly, it has been shown that Ex-4 stimulates glucocorticoid release from dispersed adenocortical cells in vitro (36) and that Ex-4 binds with high affinity to the adrenals (21), although expression of the GLP-1r has not been detected in the gland to date (11). However, considering that Ex-4 can cross the blood-brain barrier (BBB) (27) and bind to BBB-free areas of the CNS such as the area postrema (44), the major effects of peripherally administered Ex-4 on HPA axis function may be exerted at the CNS level.

Adrenal gland hypertrophy is a typical sign of hyperactivation of the HPA axis (40). The enlargement of the adrenal gland volume is apparently limited to the zona fasciculata of the adrenal cortex, as confirmed by post mortem studies in experimental animals (40) and by nuclear magnetic resonance imaging in human subjects (53). Here, we show that prolonged exposure to Ex-4 generates a marked enlargement of adrenal weight in addition to an increase in the total RNA content of the gland, which represents an indirect index of increased functional adrenal tissue (13). In contrast, a previous study failed to find an effect of subchronic Ex-4 exposure on adrenal weight (35). This apparent contradiction may be explained by a different grade of exposure to Ex-4 (once daily vs. twice daily injections in our study) or a sex-dependent sensitivity to Ex-4 effects, since the previous study was conducted on female rats.

All of the above demonstrate that long-term Ex-4 administration has a profound impact on normal HPA axis function. In contrast, a previous study had shown no effects on corticosterone and ACTH basal levels or on HPA axis responses to stress in animals receiving intracerebroventricular GLP-1 treatment for 1 week (62). These differences are likely attributable to the larger half-life and consequently greater effects of Ex-4 compared with that of the native peptide, as we previously demonstrated (20), and also to the route of administration (central vs. peripheral). The present study was designed to mimic the administration of Ex-4 in humans (i.e., sc injection any time within the 60 min before the first and last meal of the day; 10, 43). However, dose translation from animals to humans and vice versa is not always a simple task. Following the body surface area approach (48), the dose of Ex-4 here administered to rats is approximately five times the dose administered to humans (5–10 μg). Nevertheless, other factors such as the sensitivity to the drug should be considered. Importantly, our previous studies provided evidence that the human body could be more sensitive to the effects of circulating GLP-1 on HPA axis than rats (20). In addition, it is known that the Ex-4 elimination rate is approximately five times greater in rats than in humans (16). Therefore, the fact that the effects here observed in rats may also take place in patients receiving Ex-4 treatment is a possibility to be considered, although further studies are required to clarify the issue.

It is well established that GLP-1 and Ex-4 markedly reduce body weight and food intake (61, 66), although the majority of the long-term studies were conducted in diabetic subjects (18, 74). Here, we show that prolonged Ex-4 administration significantly diminished body weight and food intake in nondiabetic rats from the first day of treatment and up to the end of the study. The anorexigenic effect of Ex-4 was even greater in the absence of the adrenal glands, such that food intake dramatically dropped in the adrenalectomized animals receiving Ex-4 therapy, being almost completely abolished during the first day of treatment. It is known that glucocorticoids stimulate appetite over days in rats and that adrenalectomy decreases feeding and food-seeking behavior, which is reversed by glucocorticoid administration (54). So, in the absence of the stimulatory effect of Ex-4 on corticosterone release, the anorexigenic effect of this peptide may be more pronounced. By contrast, in unilateral enucleated animals that maintained basal corticosterone secretion, body weight and food intake responses were comparable to those observed in the nonoperated rats, precluding a role for the adrenal medulla in these effects.

Previous reports have shown that GLP-1 administration acutely inhibits water intake in both rodents (38, 61) and humans (22, 23). The effects on water intake of GLP-1 and GLP-1r agonists are partially independent of their effects on food intake, since they have been observed even when food was unavailable (38), and it has been proposed that different neuronal pathways may be involved in these two effects (61). To our knowledge, the hypodipsic effects of GLP-1 have only been demonstrated for periods of 24 h or shorter. Consistent with these data, we observed that Ex-4 reduced water intake within the first 24 h of the treatment. However, this effect was reversed from the 3rd day of treatment, when Ex-4 produced a significant increase in water intake that remained until the end of the study, likely to compensate for the remarkable loss of fluid due to the increase in diuresis induced by Ex-4 from the onset of the treatment. In support of this, Ex-4 administration failed to increase water intake when no increments in diuresis were detected, as observed in ADX animals. Previous studies have reported an effect of GLP-1 to increase urinary excretion (22, 23, 41), although the time frame was shorter and the effects not as pronounced as in the current study. The effects of GLP-1 on renal function are not fully established yet, but they have been associated with an increase in glomerular filtration rate and an inhibition of Na+ reabsorption, and it has been suggested that these might be mediated by either the SNS (41) or a direct effect of the peptide on the kidney (57). Here, we show that the potent effect of Ex-4 on diuresis observed throughout the entire duration of the study both in control nonoperated and MEDX rats was completely abolished in ADX animals. That may be interpreted as a ceiling effect due to an increased basal diuresis produced by the lack of aldosterone. However, the fact that basal diuresis continuously decreased from the beginning of the treatment and that control nonoperated and MEDX rats receiving Ex-4 showed a twofold...
increment in diuresis compared with ADX animals preclude that possibility and suggest a role for the adrenal cortex in the long-term renal effects of Ex-4. However, it must be noted that the short-term effects may not be affected by this mechanism, since ADX animals also increased diuresis in response to acute Ex-4 administration. A role of aldosterone in these effects seems unlikely, since Ex-4-treated animals showed significantly elevated levels at both peak and trough hours. Whether the rise in aldosterone levels is a direct effect of Ex-4 administration or a compensatory mechanism in response to the massive fluid loss remains unknown. The disruption of corticosterone rhythm may also play a role in the long-term Ex-4 renal effects, since it is known that glucocorticoids affect renal function (4, 65) and modulate both atrial natriuretic polypeptide (17) and arginine vasopressin secretion (54), while constant levels of corticosterone have been associated with attenuated glucocorticoid and mineralocorticoid receptor activity (55).

Taken together, our results demonstrate that prolonged exposure to Ex-4 can lead to profound dysregulation of HPA axis functions, as observed with repeated administration of other secretagogues such as CRH and ACTH or in response to continuous exposure to stressful stimulus. These alterations include disruption of the corticosterone circadian rhythm (elevated corticosterone levels during the trough period and reduced levels during the peak hours), hypertrophy of the adrenal gland, decreased adrenal gland sensitivity, impaired pituitary-adrenal stress responses, and reductions in food intake and body weight. Although it has been previously established that Ex-4 stimulates adrenal medulla activity, the medulla appears not to be instrumental in these effects. Persistent Ex-4 exposure may also induce dysregulation of renal activity, leading to a robust increase in diuresis, likely due to its effects on the adrenal cortex. Although the relevance of these effects in humans is still unknown, these results may have clinical implications since Ex-4 is currently being used therapeutically in patients with type 2 diabetes. Moreover, the deleterious effects of Ex-4 on HPA axis homeostasis may even be magnified in these patients, since in diabetes there is a preexistent hyperactivation of this hormonal axis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.G.-L., L.C.G.-M., and F.M. conception and design of research; M.G.-L., M.R.-P., V.O.-L., and E.V. performed experiments; M.G.-L. analyzed data; M.G.-L. and F.M. interpreted results of experiments; M.G.-L. prepared figures; M.G.-L. drafted manuscript; M.G.-L., P.L.B., and F.M. approved final version of manuscript.

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