LESIONS OF AREA POSTREMA AND SUBFORNICAL ORGAN ALTER
EXENDIN-4-INDUCED BRAIN ACTIVATION WITHOUT PREVENTING THE
HYPOPHAGIC EFFECT OF THE GLP-1 RECEPTOR AGONIST

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Running head: Circumventricular organs and the central effects of Ex4

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ABSTRACT

The mechanism and route whereby glucagon-like peptide 1 (GLP-1) receptor agonists such as GLP-1 or Exendin-4 (Ex-4) access the central nervous system to exert their metabolic effects has yet to be clarified. The primary objective of the present study was to investigate the potential role played by two circumventricular organs (CVOs), the area postrema (AP) and subfornical organ (SFO), in mediating the metabolic effects and central nervous system (CNS) regions activated by Ex-4. We demonstrated that the electrolytic ablation of neither AP, SFO, nor AP and SFO acutely prevent the anorectic effects of Ex-4. Lesions of both the AP and SFO chronically decreased food intake and body weight and also modulated the effect of Ex-4 on the neuronal activation of brain structures involved in the hypothalamic-pituitary-adrenal axis and glucose metabolism. The results of the study also showed that CVO lesions blunted Ex-4–induced c-fos mRNA expression (a widely used neuronal activity marker) in (i) limbic structures [bed nucleus of the stria terminalis (BST) and central amygdala (CeA)], (ii) hypothalamus [paraventricular hypothalamic nucleus (PVH), supraoptic (SON) and arcuate nucleus (Arc)] and (iii) hindbrain [lateral and lateral-external parabrachial nucleus (PB), medial nucleus of the solitary tract (NTS) and ventrolateral medulla (VLM)]. In conclusion, while the present results do not support a role for the CVOs in the anorectic effect induced by a single injection of Ex-4, they do suggest that the CVOs do play important roles in mediating the actions of Ex-4 in the activation of CNS structures involved in homeostatic control.

Keywords: Exendin 4, circumventricular organs, food intake, energy expenditure, hypothalamic-pituitary-adrenal axis, glucose metabolism, c-fos.
INTRODUCTION

Exendin 4 (Ex-4) is a 39 amino-acid polypeptide originally isolated from the venom of the Gila monster (*Heloderma suspectum*) (18). It binds to the same receptor as native glucagon-like peptide-1 (GLP-1), with which it shares a 53% sequence homology (22). GLP-1 is a proglucagon-derived hormone secreted from enteroendocrine L cells, which are mainly found in the distal intestine. The best understood biological effects of GLP-1 and other GLP-1 receptor (GLP-1R) agonists relate to their actions in the regulation of blood glucose levels through the stimulation of insulin biosynthesis/secretion, inhibition of glucagon release and gastric emptying (15, 50, 64). Recently, GLP-1 was shown to inhibit food intake following both central and peripheral administration in a variety of different species (20, 60, 63).

In contrast to circulating GLP-1, which is rapidly degraded through the action of the enzyme dipeptidyl peptidase IV (DPP-IV) (14, 29), Ex-4 has a half-life of several hours in the circulation. Animal studies have shown anti-diabetic effects of Ex-4 (67) as well as reductions in food intake and body weight following either central (2, 47) or peripheral (47, 57) administration of the drug. Regardless of the route of administration, Ex-4 also induces a specific pattern of neuronal activation among brain structures involved in food intake regulation and associated behaviors (66). These structures include (i) hypothalamic centers [paraventricular hypothalamic nucleus (PVH), supraoptic nucleus (SON), arcuate nucleus (Arc)], (ii) hindbrain nuclei [parabrachial nucleus (PB), nucleus of the solitary tract (NTS) and area postrema (AP)] and (iii) limbic structures [bed nucleus of stria terminalis (BST), central nucleus of amygdala (CeA)](6, 66). Central injection of GLP-1 also stimulates *c-fos* expression in the PVH, specifically in
corticotrophin-releasing factor (CRF) neurons, suggesting that the GLP-1R mediates the central modulation of the hypothalamo-pituitary-adrenocortical (HPA) axis activity (33, 61, 62). Finally, it has been demonstrated that Arc neurons presenting GLP-1R also co-express glucokinase (GK) mRNA, a marker of neuronal glucose sensing (42).

The mechanism whereby peripherally administered GLP-1 or Ex-4 inhibits food intake remains largely unknown. The hypophagia induced by the peripheral injection of Ex-4 has been shown to be mediated by the GLP-1R as it is absent in GLP-1R-deficient mice (4). It is noteworthy that the anorectic effect of centrally injected Ex-4 is however insensitive to the GLP-1R antagonism (7). Furthermore, Ex-4-induced anorexia seems to be highly dependent on the peripheral GLP-1R, while ablation of sensory vagal pathways by systemic pretreatment with capsaicin prevents the ability of Ex-4 to suppress food intake in mice (59). More recently the circumventricular organs (CVOs), which are brain structures located on the peripheral side of the blood-brain barrier (BBB), have also been suggested as potential targets through which peripheral GLP-1R agonists may stimulate the brain. The AP and the subfornical organ (SFO) are two CVOs with both identified GLP-1 binding sites (23, 43) and GLP-1R mRNA expression (36). The AP has already been shown to be a key site for the central action of peripheral GLP-1R agonists (65). On the other hand, there is no data regarding the role of the SFO in mediating the central actions of GLP-1R agonists. Consequently, the present study aimed to explore the role played by both the AP and SFO in mediating the metabolic and brain-stimulating effects of Ex-4. Using three different types of animal models, created by electrolytic ablation of either AP, SFO, or AP plus SFO, we investigated the roles of the CVOs on Ex-4-induced
effects on food intake, energy expenditure, brain neuronal activation, and some aspects of the HPA axis activity and glucose metabolism.

METHODS

Animals and diet

Male Wistar rats weighing 250-300 g were purchased from the Charles River Canada (St-Constant, Canada). All rats were handled according to the Canadian Guide for the Care and Use of Laboratory Animals and our institutional animal care committee approved the present protocol. The animals were housed individually in wire-bottom cages suspended above absorbent paper. They were, unless otherwise specified, fed *ad libitum* with a stock diet (Rodent Laboratory CHOW Purina # 5075, Strathroy, ON, Canada). They were subjected to a 12:00:12:00 hour dark-light cycle and kept at an ambient temperature of 23 ± 1° C.

Electrolytic lesions

Groups of rats were subjected to electrolytic lesions, which were carried out under ketamine (60mg/kg) / xylazine (7.5mg/kg) anaesthesia. In sham-lesioned rats, electrodes were introduced within either the SFO or AP, or SFO and AP but no current was passed.

*AP lesion*

The rat was placed in a stereotaxic apparatus. Its head was flexed, and the cisterna magna was opened to permit access to the fourth ventricle. Rats were then subjected to the electrolytic lesion (Keithley Instruments 225 Current Source, 0.5 mA for 20 s) of the
AP using a monopolar parylene C-insulated tungsten electrode (Micro-Probe) with a tip exposure of 100 μm.

**SFO lesion**

The rat was placed in a stereotaxic apparatus and the head was horizontally fixed. A midline incision of the soft tissues above the skull was performed and a small hole was drilled in the cranium so that the electrode could be advanced into the region of SFO using the following stereotaxic coordinates: midline, 0.8 mm caudal to bregma (β), 4.5 mm below dura (58). Rats were then subjected to the electrolytic lesion as described above for the AP.

**Double AP/SFO lesion**

The rat underwent first the lesion of the SFO, then that of the AP.

**Histological analysis of AP and SFO lesions**

Validation of lesion sites was carried out by histological examination of coronal brain sections. Only animals having complete lesions were included in the statistical analyses. AP-lesioned groups excluded rats with any AP remaining or rats with damage to the adjacent NTS. In AP-sham-lesioned animals, no damage was present in the AP tissue or the surrounding NTS. The SFO lesion group included solely animals in which the structure was totally destroyed or rats with the rostral SFO and rostroventral stalk sufficiently damaged to disconnect the SFO from its targets in the ventral forebrain. Neuronal SFO projections to the preoptic region and hypothalamus leave the structure via its rostroventral stalk (37). Data from rats with partial damage to SFO without destruction of the rostroventral pole were not included in the analyses. SFO-sham-lesioned animals
had evidence of the electrode tracks into the hippocampal commissure, but no evidence of damage to the SFO.

Postoperative care

During the first two postoperative days, all rats were carefully followed and fed ad libitum with a ‘recovery’ diet (liquid chocolate flavoured Ensure and Pablum baby cereals, containing 10.45 kJ/g with 14.7% of energy as fat, 70.6% as carbohydrate and 14.4% as protein) and given antibiotic/anti-inflammatory treatment (Baytril 5mg/kg / Buprenorphine 0.5 mg/kg subcutaneously injected, daily). The ‘recovery’ diet was a palatable, well-balanced, diet which was provided to promote resumption of normal eating and drinking after the lesions. On the third post-operative day, animals were returned to a normal laboratory chow diet (containing 12.9 kJ/g with 4.5% of energy as fat, 77.5% as carbohydrate and 18.0% as protein). Rats were allowed 2 weeks to recover before any investigation.

Ex-4 injections

Ex-4 was intraperitoneally injected using a 1 ml syringe connected to a 25-gauge needle. The volume of injection was 1.1ml/kg in the feeding test and 1.25ml/kg in experiments examining c-fos activation. Ex-4 (Sigma-Aldrich Canada Ltd., Oakville, ON, CA) was dissolved in a solution of isotonic pyrogen-free saline and 0.1% BSA, which was also injected in vehicle control animals.

Body weight, food intake and body composition
Body weight and food intake were measured daily during the first 11 postoperative days and every second day during the rest of the experimental period. Food spillage was carefully accounted for in the measurement of food intake. Body composition was determined by Dual-Energy X-ray Absorptiometry (DEXA, PIXImus2, Lunar Corporation, Madison, WI, USA) on the surgery day and three weeks later. The PIXImus2, which was primarily designed for mice, does not allow for the assessment of the whole body composition in larger animals such as rats. However, we chose to measure the composition of a specific area, from which it is possible to infer on the whole body composition of the rat (40). During measurements, anaesthetized rats were lying in a prone position with the posterior legs maintained in external rotation. The hip, knee and ankle articulations were in 90° flexion. The scanned region was defined using the following anatomical reference points: frontally, the 1st lumbar vertebra; caudally, the caudal limit of the iliac bone; medially, a line splitting the backbone in half; laterally, right distal femoral metaphysis. The region of interest thus comprised the femoral region of the right hind leg and the ipsilateral half of the inferior abdomen. The machine was calibrated daily with the manufacturer’s phantom. The same region was scanned in all rats. The percentage of body fat as well as the fat free mass (FFM) and the fat mass (FM) were measured using this technique.

**Energy expenditure and food intake in metabolic chambers**

Between the 4th and 6th postoperative weeks, rats were housed in rat metabolic chambers in order to measure food intake and energy expenditure. The metabolic chambers consisted of air-proof cages, which were linked to an open-circuit indirect
calorimetry system (19, 54). Rats were put in metabolic cages 24 hours prior to the re-feeding test in order to acclimatize them to the new environment before measurements. After 20 hours of food deprivation, rats were intraperitoneally injected with saline or 2 µg/kg of Ex-4 and then given free access to food. The dose of Ex-4 used in this study has already shown to induce an anorectic effect without interfering with the general status and locomotor function of the rat (44). Food intake was measured at 20 minute intervals for 3 hours following injections, and O₂ and CO₂ consumption (VO₂ and VCO₂, respectively) was also recorded every 20 minutes. The average of values taken during the last 3 hours before the injection was used as the basal value, while for the post injection period, hourly values were obtained from the average of three consecutive 20 minute measurements. VO₂ was measured with an O₂ analyzer (model S-3A, Applied Electrochemistry inc., Sunnyvale, CA, USA) and VCO₂ was measured with a CO₂ analyzer (model CD-3A, Applied Electrochemistry inc., Sunnyvale, CA, USA). The respiratory quotient was calculated as VCO₂/VO₂.

**Brain in situ hybridization histochemistry**

At the end of the 6th postoperative week, after an overnight fast, all rats were intraperitoneally injected with Ex-4 (1.55 µg/kg, a dose based on a series of tests which demonstrated that this was the lowest dose that induces a complete pattern of neuronal activation), or saline and sacrificed 30 minutes later, between 08:30 and 11:30. Brains were collected and prepared (see below) to determine the expression of c-fos mRNA to estimate brain activation in response to various challenges (26, 48).
Rats were anaesthetized with a mixture of ketamine (60mg/kg) and xylazine (7.5mg/kg), given intraperitoneally and without delay, were intracardially perfused with 200 ml of ice-cold isotonic saline followed by 500 ml of paraformaldehyde (4%) solution. The brains were then removed and kept in paraformaldehyde (4%) for 7 days. They were then transferred to a solution containing paraformaldehyde (4%) and sucrose (10%) before being cut 12 hours later using a sliding microtome (Histoslide 2000, Reichert-Jung, Heidelberger, Germany). Brain sections (30µm) were taken from the olfactory bulb to the brain stem and stored at -30°C in a cold cryoprotecting solution containing sodium phosphate buffer (50 mM), ethylene glycol (30%), and glycerol (20%).

In situ hybridization histochemistry was used to localize c-fos mRNA, CRF heteronuclear RNA (hnRNA), and GK mRNA on brain sections. The protocol was adapted from the technique described by Simmons et al. (53). Briefly, brain sections (one out of every six brain slices) were mounted onto poly-L-lysine coated slides and allowed to dessicate overnight under vacuum. The sections were then successively fixed for 20 minutes in paraformaldehyde (4%), digested for 30 minutes at 37°C with proteinase K (10 µg/ml in 100 mM Tris-HCl containing 50 mM EDTA, pH 8.0), acetylated with acetic anhydride (0.25% in 0.1 M trietholamine, pH 8.0), and dehydrated through graded concentrations (50, 70, 95, and 100%) of alcohol. After vacuum drying for at least 2 hours, 90 µl of the hybridization mixture, which contains an antisense 35S labeled cRNA probe (10^7 cpm/ml), were spotted on each slide. The slides were coverslipped and incubated overnight at 60°C in a slide warmer. The next day, coverslips were removed
and the slides rinsed 4 times with 4x saline-sodium citrate (SSC) (0.6 M NaCl, 60 mM trisodium citrate buffer, pH 7.0) containing 1mM 1,4-dithiothreitol (DTT), digested for 30 minutes at 37°C with RNAse-A (10mg/ml, Roche Diagnostics GmbH, IN, USA), washed in descending concentrations of SSC (2x, 10 minutes; 1x, 5 minutes; 0.5x, 5 minutes; 0.1x, 30 minute at 60°C) containing 1mM DTT and dehydrated through graded concentrations of alcohol. After a 2 hour period of vacuum drying, the slides were exposed on X-ray film (Eastman Kodak, Rochester, NY) for 24h. Once removed from the autoradiography cassettes, the slides were defatted in toluene and dipped in NTB2 nuclear emulsion (Kodak). The slides were exposed for 7 days, before being developed in D19 developer (Kodak) for 3.5 minutes at 14-15°C and fixed in rapid fixer (Kodak) for 5 minutes. Finally, tissues were rinsed in running distilled water for 1 to 2 hours, counterstained with thionin (0.25%), dehydrated through graded concentrations of alcohol, cleared in toluene, and coverslipped with dibutylphthalate-xylol (DPX) mounting medium.

**Antisense 35S-labeled riboprobes**

Complementary RNA probes was generated from rat cDNA fragments for c-fos (Dr. I. Verma, The Salk Institute, La Jolla, CA, USA), GK (Dr. J. Buteau, Institut universitaire de cardiologie et de pneumologie de Québec, QC, Canada) and the 530bp genomic fragment of the CRF intron 1 for hnCRF (Dr. S. Watson, University of Michigan, Ann Arbor, MI, USA). Radioactive riboprobes were synthesized by incubation of 250 ng linearized plasmid in 10 mM NaCl, 10 mM dithiothreitol, 6 mM MgCl, 40 mM Tris (pH 7.9), 0.2 mM ATP/GTP/CTP, 100 μCi α-35S-UTP (Perkin Elmer), 40 U RNase
inhibitor (Roche Diagnostics GmbH, IN, USA), and 20 U of RNA polymerase (T7 or T3 for respectively antisense and sense probes for c-fos mRNA and GK mRNA and T7 or SP6 for respectively antisense and sense probes for CRH hnRNA) for 60 minutes at 37°C. The DNA templates were treated with 100 μl of DNase solution containing 0.1 U/ml DNase (Roche Diagnostics GmbH, IN, USA), 0.25 mg/ml tRNA and 50 mM Tris/10 mM MgCl2. The riboprobes were purified using RNeasy Mini Kit (Qiagen Inc, Mississauga, ON, CA), eluted in 150 μl of 10 mM Tris/1 mM EDTA buffer and incorporated in a hybridization solution containing (per ml) 10⁷ cpm of 35S probe, 52% formamide, 330 mM NaCl, 10 mM Tris pH 8, 1 mM EDTA pH 8, Denhart’s solution 1X, 10% dextran sulphate, 0.5 mg/ml tRNA, 10mM DTT, diethyl pyrocarbonate (DEPC) water. This solution was mixed and heated at 65°C before being spotted on slides. The specificity of the probe was confirmed by the absence of positive signal in sections hybridized with sense probe.

Quantitative analysis of the hybridization signals

The hybridization signals revealed on NTB2 dipped nuclear emulsion slides were examined under dark-field microscopy using an Olympus BX51 microscope (Olympus America, Melville, NY). Images were acquired with an Evolution QEi camera and analyzed with ImagePro plus version 5.0.1.11 (MediaCybernetics, Silver Spring, MD). Saturation of the hybridization signal was avoided by adjusting the exposure time for the image with the strongest hybridization signal sampled for each region in every series. The luminosity of the system was set to the maximum and the saturation warning option was used to visualize saturated regions in the image preview. Thereafter, according to the
pixels distribution histogram, the exposure time was adjusted in order to reduce to zero the number of saturated (pure white) pixels. The same exposure time was conserved throughout the analysis of the entire series.

The oval part of the bed nucleus of stria terminalis [(BSTov) (0.10 to 0.26 mm caudal to β)], the supraoptic nucleus [(SON) (1.10 to 1.50 mm caudal to β)], the parvocellular and magnocellular subnuclei of the paraventricular hypothalamic nucleus [(PVHp and PVHm respectively) (1.80 to 2.00 mm caudal to β)], the central nucleus of amygdala [(CeA) (2.00 to 2.45 mm caudal to the β)], the arcuate nucleus [(Arc) (2.54 to 3.25 mm caudal to β)], the parabrachial nuclei [lateral (PBl) and lateral-external (PBle) (9.16 to 9.25 mm caudal to β)], the medial part of the nucleus of the solitary tract [(NTSm), at the level at which the NTS contacts the fourth ventricle (13.28 to 13.60 mm caudal to β)], the ventrolateral medulla [(VLM) (13.76 to 14.16 mm caudal to β)] were outlined and measurements of the pixel density of the hybridization signal were performed on both hemispheres of 2 to 4 brain sections for each animal assigned to a given treatment. When no hybridization signal was visible under darkfield illumination, the brain structures of interest were outlined under brightfield illumination and then subjected to densitometric analysis under darkfield illumination. The pixel density for each specific region was corrected by subtracting background readings taken from areas immediately surrounding the analysed region. The brain sections from the different groups of rats were matched for rostrocaudal levels as closely as possible.

Plasma analyses and tissue weights
At time of sacrifice, blood was collected by cardiac puncture, centrifuged (1500 g, 15 minutes at 4 °C) and the separated plasma was stored at -20 °C until later biochemical measurements. Plasma glucose concentrations were determined using an automated glucose analyzer YSI 2300 Stat Plus (YSI Incorporated, Yellow Springs, OH). Commercially available radioimmunoassay kits were used for the measurement of plasma insulin (Linco Research, St. Charles, MO) and plasma corticosterone (MP Biomedicals, Toronto, ON) levels.

Interscapular brown adipose tissue (BAT), inguinal subcutaneous white adipose tissue (IWAT), epididimal subcutaneous white adipose tissue (EWAT), retroperitoneal white adipose tissue (RWAT) and soleus muscle were sampled and weighted.

**Statistical analysis**

Results are presented as mean values ± standard errors of the means (SE). Statistical differences in cumulative weight gain and food intake between sham and lesioned rats were determined within each type of lesion (AP, SFO or double lesion AP/SFO) using repeated-measure analysis of variance (RM-ANOVA) in a mixed model analysis with a heterogeneous autoregressive covariance structure. Multivariate normality was verified with the Mardia’s test. For all the other variables, statistical differences within each type of lesion were determined by one or two-way analysis of variance (ANOVA). Fisher’s PLSD or Bonferroni-Dunn *post hoc* tests were used to identify the intergroup differences in cases of significant interactions. When necessary, data were log-transformed in order to satisfy the variance normality criterion. Results were considered as being significant with *p* values < 0.05. The correlation between the levels of
expression of NPY mRNA in the Arc and plasma levels of corticosterone was estimated using the Pearson correlation analysis. RM-ANOVA were performed using the SAS v9.1.3 software package (SAS Institute, Cary, NC), whereas all other statistical analyses used StatView v5.0 software (SAS Institute Inc).

RESULTS

Histological verification of electrolytic lesions

A total of 72 animals were used in the present study, which following histological evaluation of AP and SFO, were assigned to one of 6 experimental groups as follows: AP lesion (n=14), SFO lesion (n=7), double AP/SFO lesion (n=7), AP sham-lesion (n=9), SFO sham-lesion (n=13) and double AP/SFO sham-lesion (n=11). Only animals with complete lesions (as described in METHODS, see Fig. 1) were included for further evaluation. The remaining 11 animals were excluded from analyses due to incomplete lesions or excessive lesions that damaged or destroyed adjacent structures (see METHODS).

Body weight gain, food intake, and body composition in sham and lesioned rats

Body weight gain and food intake (normalized for body weight) during the 40 days of the study are presented in Fig. 2A-C. On the day of surgery, there was no significant difference in body weight between groups (Fig. 2A). During the first 2 post-surgical days, when rats were nourished with the ‘recovery’ diet, AP- and AP/SFO-lesioned rats presented a significant reduction in food intake compared to their sham controls (32.5% - 52% of controls, p<0.05 - Fig. 2B). Afterwards, daily food intake in
AP-lesioned rats was restored to the sham level while food intake in the AP/SFO-lesioned rats remained less than that of controls (65.5% - 80% of respective sham, \( p < 0.05 \)) during the next 10 days (Fig. 2B). Cumulative food intake in AP/SFO-lesioned rats remains lower than that of their shams (71.7% - 92.3% of controls, \( p < 0.05 \) - Fig. 2C). Also, AP/SFO-lesioned rats gained less body weight during the first 32 days of experiment (see Fig. 2A for body weight, 90.9% - 94.5% of controls, \( p < 0.05 \), Table 1 for body weight composition measured by DEXA at the end of the 3rd postoperative week). Reduced body weight gain of the AP/SFO-lesioned rats was associated with a smaller gain in fat mass (17.9% - 52.4% of controls, \( p < 0.05 \), Table 1). However, at the end of the experimental period, the AP/SFO-lesioned rats showed a trend to regain weight and no statistical significant difference was observed in body weight (Fig. 2A) and adipose tissue mass (data not shown) in double-lesioned compared to sham rats. Rats presenting a single lesion of AP or SFO did not show any significant difference in body weight gain compared to their respective shams.

In SFO-lesioned rats, a reduction in fat-free mass gain was detected by DEXA after 3 postoperative weeks (Table 1). This seems to be a transitory effect of the SFO lesion as the weight of muscle tissue sampled immediately after sacrifice was similar between groups (data not shown).

Indirect calorimetry performed during the 4th to 6th postoperative weeks in fasting state showed that rats having SFO lesion (alone or in combination with AP lesion) presented a lower respiratory quotient (VCO\(_2\)/VO\(_2\)) than shams (0.707 ± 0.010 vs. 0.743 ± 0.009, \( p = 0.02 \) for SFO-lesioned vs. sham and 0.708 ± 0.016 vs. 0.754 ± 0.011, \( p = 0.01 \) for AP/SFO-lesioned vs. sham).
Energy expenditure and food intake during re-feeding in sham and lesioned rats intraperitoneally injected with Ex-4

Food intake and oxygen consumption (VO₂) during the first hour of re-feeding following food deprivation was significantly reduced in Ex-4-injected rats (food intake: 28 - 51% of saline, VO₂: 80 - 95% of saline, \( p < 0.0001 \) for the main effect of drug in ANOVAs, including all groups) (Fig. 3A-B). During this period food intake and VO₂ (raw data and data normalized for body weight) showed no difference in either AP-, SFO- or AP/SFO-lesioned rats versus their shams (Fig. 3), a trend which was continued for the next 2 hours of re-feeding (data not shown).

c-fos mRNA in brain of sham and lesioned rats following intraperitoneal administration of Ex-4

The hybridization signal for c-fos mRNA at 30 minutes after the intraperitoneal injection of Ex-4 was quantified in sham and lesioned rats, in brain nuclei that are likely involved in the control of food intake and associated behaviors: BSTov, CeA, PVHp, PVHm, SON, Arc, PBI, PBle, NTSm and VLM (Fig. 4 and 5A for pixel density and Fig. 5B-R for darkfield photomicrographs). In saline-injected rats, no difference was observed between sham and lesioned groups, for any type of surgery (data not shown). In agreement with previous data [our previous results and (5, 6, 66)], c-fos mRNA expression was markedly increased after the injection of Ex-4 in all nuclei (\( p \) ranged between \(<0.0001\) and 0.01 vs. saline, Fig. 4). In Ex-4-treated rats, the AP lesion blunted the neuronal activation in the hypothalamic Arc nucleus and in the brainstem nuclei such
as the PBl, PBle, NTSm and VLM ($p<0.05$, Fig. 5A for pixel density and 5C, 5G, 5K, 5O for darkfield microphotographs). In SFO-lesioned rats, Ex-4-induced neuronal activation was lower (compared to sham) in the PVHm, Arc, PBle and VLM ($p<0.05$, Fig. 5A for pixel density and 5D, 5H, 5L, 5P for darkfield microphotographs). SFO ablation led to a tendency for a decrease in c-fos mRNA expression in the SON, but this effect was not statistically significant. The double AP/SFO lesion significantly reduced c-fos mRNA expression in almost all regions of interest: the limbic structures (BSTov and CeA), the parvocellular (PVHp) and magnocellular (PVHm and SON) nuclei and the brainstem regions (NTSm and VLM) ($p<0.05$, Fig. 5A for pixel density and 5E, 5I, 5M, 5R for darkfield microphotographs). In contrast to single lesions, ablation of both AP and SFO did not reduce the neuronal activation in the Arc of Ex-4-treated rats.

**NPY and POMC mRNA in the Arc of sham and lesioned rats following intraperitoneal administration of Ex-4**

Expression of NPY mRNA in the Arc was slightly increased following the intraperitoneal injection of Ex-4 in sham-operated rats ($p<0.05$ vs. saline, Fig. 6A for pixel density and 6C for darkfield microphotographs). In contrast, POMC mRNA expression in the Arc was not significantly changed 30 minutes after Ex-4 administration (Fig. 6D, 6F). However, lesions of AP and SFO (alone or in combination) did not alter the expression of NPY and POMC transcripts in the Arc of either saline- (data not shown) or Ex-4-treated rats (Fig. 6B, 6E). A positive significant correlation was found between NPY mRNA levels and plasma corticosterone ($r=0.412$, $p=0.018$), but not between NPY mRNA and plasma insulin and glucose.
GK mRNA in the Arc in sham and lesioned rats following intraperitoneal administration of Ex-4

The expression of GK mRNA in the Arc was slightly increased following the intraperitoneal injection of Ex-4 in sham-operated rats (p=0.06 vs. saline, Fig. 7A for pixel density and 7C for x-ray film autoradiograms). Double lesion of AP and SFO prevented this increase (p<0.05 vs. sham operated rats), whereas individual ablation of AP or SFO had no effect (Fig. 7B for pixel density and 7D for darkfield microphotographs). In control, saline-injected rats, the absence of the two CVOs, AP and SFO, did not alter GK mRNA levels in the Arc (data not shown).

CRF hnRNA in the PVHp in sham and lesioned rats following intraperitoneal administration of Ex-4

Examination of the PVHp revealed a significant increase in CRF hnRNA expression following Ex-4 injection in sham-operated rats (p<0.0001 vs. saline, Fig. 8A for pixel density and 8C for darkfield microphotographs). This effect was prevented in AP/SFO-lesioned (p<0.05 vs. sham-operated rats) animals (Fig. 8B, 8C) but not in rats with single lesions (Fig. 8B). In saline-injected rats, ablations of AP or SFO did not induce any significant modification in CRF hnRNA expression (data not shown).

Plasma corticosterone, insulin and glucose in sham and lesioned rats following intraperitoneal administration of Ex-4
Intraperitoneal injection of Ex-4 led to a significant elevation in plasma corticosterone (by 2 - 5 fold, \( p<0.0001 \)), insulin (by 2 - 3.5 fold, \( p<0.001 \)) and glucose (by 1.2 - 1.3 fold, \( p<0.0001 \)) as compared to saline (Table 2). In contrast to the hnCRF expression in the PVHp, plasma corticosterone levels were not influenced by the absence of one or both CVOs in either saline- or Ex-4-injected rats. Likewise, plasma insulin levels were not affected by AP and SFO ablation. However, the elevation of plasma glucose was prevented by AP/SFO lesion in Ex-4-treated rats (\( p<0.05 \) for AP/SFO lesion vs. AP/SFO sham, in Ex-4-treated rats) (Table 2).

**DISCUSSION**

The primary objective of the present study was to investigate the role played by the AP and SFO in mediating the CNS actions of Ex-4 in the activation of central autonomic control centers and metabolic function. The results demonstrated that the absence of both AP and SFO did not significantly attenuate the inhibitory effects of Ex-4 on food intake. However, it was observed that lesions of both AP and SFO altered Ex-4-induced c-fos expression in specific brain structures potentially related to the regulation of energy balance and associated behaviors. In addition, absence of both AP and SFO alters the expression of genes likely involved in the central modulation of the HPA axis and glucose metabolism. This study also demonstrated that the concomitant ablation of both the AP and SFO could significantly influence long-term energy homeostasis.

The present results confirm the ability of the GLP-1R agonist, Ex-4, to reduce food intake (5) and to decrease oxygen consumption. Similar reductions in energy expenditure generated by administration of GLP-1R agonists in laboratory rodents and
humans have already been demonstrated (4, 21, 32). Studies on the effects of GLP-1R agonists in rats (32) and humans (21) and our unpublished data (using Ex-4 in rats) suggest that the anorectic effect of Ex-4 could be the main cause for the Ex-4-induced reduction in energy expenditure. Unexpectedly, our results did not support a role for either the AP or SFO in the anorectic effects of Ex-4, which suggests that these CVOs, even though they express the GLP-1R (23, 36, 43), are not essential to the inhibitory effects of GLP-1R agonists on food intake in the re-feeding model that was used in the present study. The reasons for this lack of effect of CVOs lesion on Ex-4-induced anorexia are not known and suggest the involvement of other mechanisms. Previous studies, showing that vagal neurons express GLP-1R mRNA (41) and that vagotomy prevents the anorectic effect of peripheral GLP-1 (1) and Ex-4 (59), suggest a role for the vagus nerve. Also, the hypothesis that Ex-4-induced hypophagia could be mediated by central receptors others than GLP-1R cannot be excluded.

Despite absence of action on Ex-4-induced feeding behaviour, AP and SFO lesions were found to have significant effects on the pattern of Ex-4-induced neuronal activation shown using brain c-fos expression as a marker of neuronal activation (26, 48). AP lesion impaired Ex-4-induced neuronal activation in a number of hindbrain nuclei including the PBI and PBle, NTSm and VLM, which are all structures connected to the AP (24, 52). In addition, AP ablation resulted in reduced c-fos mRNA expression in response to Ex-4 in the hypothalamic Arc nucleus, which maintains polysynaptic connections with the AP principally via synapses in the NTS and PBI (9, 11). SFO ablation blunted Ex-4-induced activation of the PVHm, which is known as a major site receiving SFO projections (37). In addition, c-fos expression in both hypothalamic Arc
and brainstem regions (such as the PBle and VLM), was reduced by SFO lesions. The SFO neurons could activate those regions via the limbic system, which receives a extensive inputs from the SFO (55, 56). It is indeed well known that neurons of the limbic BST and CeA project to the hypothalamic Arc, pontine PB and brainstem VLM (11, 12, 38). Combined lesions of both the AP and SFO reduced c-fos mRNA expression in limbic structures (BSTov and CeA), in the PVHp, PVHm and SON, and in the brainstem regions (NTSm and VLM). In contrast to single AP or SFO lesions, ablation of both AP and SFO did not prevent Ex-4-induced neuronal activation in the Arc. This lack of effect may be explained by the activation of the Arc neurons as part of a central compensatory mechanism aimed at limiting the negative energy balance induced by AP/SFO lesion. Reasons as to why CVO lesions had a suppressive effect on Ex-4-induced brain c-fos expression but not on Ex-4-induced hypophagia are not known, although this finding suggests that CVO-mediated central effects of Ex-4 are not directly linked to the effects on feeding and metabolism associated with this behavioural paradigm. The possibility that c-fos might not be a good marker for Ex-4 induced anorexia cannot be excluded.

The attenuation of Ex-4-induced neuronal activation of the PVHp, produced by the ablation of both AP and SFO, was accompanied by a reduction in the CRF hnRNA levels in the medio-dorsal PVHp. The hnCRF is the primary transcript in the CRF gene expression process and is not constitutively present in CRF-expressing brain structures (31, 46). Measurement of its expression has proved to be useful in detecting rapid changes in CRF expression in the PVH, where changes in CRF synthesis cannot easily be measured due to the high density of the CRF message (25). It is noteworthy that the
inhibitory effect of AP/SFO lesions on hnCRF expression in response to Ex-4 was not paralleled by a decrease in the corticosterone levels. This observation was surprising in light of the recognized importance of PVHp CRF neurons in governing the pituitary-adrenal axis (39). However, there is evidence that Ex-4 injected peripherally may also stimulate corticosteroid release through activating GLP-1Rs located on pituitary and adrenal glands (28).

Elevation in plasma corticosterone could be partially responsible for Arc NPY mRNA expression induced by Ex-4. It is well known that Arc NPY neurons present glucocorticoid receptors and there is evidence of a positive association between NPY and glucocorticoids (8). A recent study showed the lack of co-expression for GLP-1R and NPY mRNA in Arc neurons (49). It is therefore unlikely that the enhanced NPY expression that we found following Ex-4 injection was due to a direct neuronal activation of the Arc. On the other hand, the same study showed that 68% of GLP-1R positive neurons co-express POMC mRNA (49). Moreover, it has been demonstrated that POMC mRNA hypothalamic expression is increased 2 hours after central administration of GLP-1 in fasted rats (51). We did not find a significant increase in Arc POMC mRNA expression after intraperitoneal injection of Ex-4, likely because our rats were killed 30 minutes after Ex-4, before such changes occur. Our data suggest that different mechanisms may be responsible for central regulation of Ex-4-induced anorexia, and also suggest that AP and SFO are not involved in immediate effects of peripheral Ex-4 on NPY and POMC mRNA expression in Arc.

The results of the present study show that the AP/SFO lesion reduced body weight gain and daily food intake. The reduction in weight gain in AP/SFO-lesioned rats was
accompanied by a reduction in fat gain, which was significant for the first part of the study (up until the 3rd postoperative week) but which tended to normalize beyond that time point. At the end of the experiment, AP/SFO-lesioned rats showed a tendency to regain weight, an effect which possibly resulted from compensatory homeostatic processes aimed at counteracting the negative energy balance status created by the AP/SFO lesion. The effect of single ablations of the AP and SFO on body weight, food and water intake has already been reported (27, 30, 35, 58), but the impact of the absence of both CVOs on feeding and energy homeostasis had not been examined prior to the present investigation. Ablation of the AP and the adjacent tissue of the caudal-medial portion of NTS has been reported to cause physiological changes such as transient hypophagia and body weight loss when the animals are fed normal laboratory chow (13, 17, 27, 30). We observed similar effects on the ingestive behavior following the AP ablation even when the animals were placed immediately after the surgery on a diet apparently more palatable than chow. Some studies have highlighted the preference in AP-ablated rats for palatable foods but this effect was observed between 2 weeks and 1 month after surgery (16, 30, 45). Confirming previous data (10, 16, 27, 30), we also observed that in the second part of the experimental period, rats with AP lesions exhibited energy intakes similar to control rats. In addition, body weight gain of AP-lesioned rats was slightly smaller than sham animals during the first post-lesion week but was not significantly different from the respective shams afterwards. As mentioned above, AP lesions were restricted to the AP region and all rats presenting damage of surrounding NTS tissue were excluded. Similar results have also been obtained by other groups using same criteria of exclusion (10). With regard to body weight and food intake, rats
presenting lesions of the SFO did not differ from sham-operated animals, confirming previous data (35, 58).

Ex-4-induced elevation of insulin was not affected by the AP or SFO ablation. However, the elevation of plasma glucose induced by Ex-4 was prevented by AP/SFO lesions. Hyperglycaemia following Ex-4 injection in fasted non-diabetic rats has been reported before (3, 34). The mechanism underlying this phenomenon would seem to involve the sympathoadrenal system. Ex-4 has been shown to activate neurons in brain sites implicated in the autonomic control of the adrenal medulla (66). The prevention of this hyperglycaemic effect by the ablation of both AP and SFO suggests the presence of central mechanism involving GLP-1R in the control of glucose metabolism. It is noteworthy that the expression of GK mRNA in the Arc neurons was increased following the intraperitoneal injection of Ex-4 in sham-operated rats and that the double lesion of AP and SFO prevented the increase in GK mRNA, emphasizing a potential role of these two CVOs in the central regulation of glucose homeostasis.

In summary, the present study provides evidence for a role of AP and SFO in mediating the central effects of Ex-4. Ablation of these CVOs significantly alters brain neuronal activation but not hypophagia induced by intraperitoneally injected Ex-4. However, both AP and SFO seem to be synergistically involved in central GLP-1R-mediated activation of PVH neurons governing the regulation of HPA axis and the Arc neurons involved in the glucose metabolism.

**Perspectives and Significance**
The mechanisms underlying of the anorexigenic effect of Ex-4 remain poorly understood. The present data shows that the CVOs (AP and SFO) do not seem to be directly implicated in hypophagia, but do play important roles in mediating the activation of a number of autonomic control centres in response to peripherally injected Ex-4. AP and SFO also seem to be synergistically involved in the central GLP-1R-mediated regulation of HPA axis and glucose metabolism. These findings open new insights for the central pathways underlying the stress-related effects of the GLP-1R signalling system. It should be recognized, however, that the functional effects of these alterations in neuronal activity induced by lesions of the CVOs in response to Ex-4 have not been described by the present studies, but do represent intriguing targets for future investigation.
ACKNOWLEDGMENTS

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FIGURE LEGENDS

Figure 1. Photomicrographs of complete lesions of subfornical organ (SFO) and area postrema (AP) vs. respective sham operation. Bregma levels are -0.8 mm for SFO and -13.7 mm for AP. Scale bars correspond to 200µm.

Figure 2. Body weight (A), and daily (B) and cumulative (C) food intake in sham-operated and lesioned rats. Measurements were taken during 41 post-operative days. Data represents means ± SE. * Significant effect of lesion as assessed by repeated measures ANOVA, p<0.05.

Figure 3. Effect of Ex-4 on energy expenditure (A) and food intake (B) in sham-operated and lesioned rats at the end of 1 hour re-feeding following 20 hours food deprivation. Rats were intraperitoneally injected with Ex-4 or saline, 2 hours before the measurements. Data represents means ± SE. * Significant main effect of drug as assessed by two-way ANOVA, p<0.01.

Figure 4. C-fos mRNA expression in brain of fasted sham-operated rats, 30 minutes after intraperitoneal injection of Ex-4. Pixel density of the hybridization signal was determined in the oval part of the bed nucleus of stria terminalis (BSTov), central nucleus of amygdala (CeA), paraventricular hypothalamic nucleus, parvocellular (PVHp) and magnocellular part (PVHm), supraoptic nucleus (SON), arcuate hypothalamic nucleus (Arc), parabrachial nucleus, lateral (PBl) and lateral-external part (PBlE), nucleus of the
solitary tract, medial part (NTSm), ventrolateral medulla (VLM). Values represent means ± SE. Significant effect of drug as assessed by one-way ANOVA, * $p<0.05$, ** $p<0.001$, *** $p<0.0001$.

**Figure 5.** C-fos mRNA expression in brain of fasted rats, 30 minutes after intraperitoneal injection of Ex-4. (A) Pixel density of the hybridization signal in lesioned rats vs. respective sham (% of control) in the oval part of the bed nucleus of stria terminalis (BSTov), central nucleus of amygdala (CeA), paraventricular hypothalamic nucleus, parvocellular (PVHp) and magnocellular part (PVHm), supraoptic nucleus (SON), arcuate hypothalamic nucleus (Arc), parabrachial nucleus, lateral (PBl) and lateral-external part (PBle), nucleus of the solitary tract, medial part (NTSm), ventrolateral medulla (VLM). Values represent means ± SE. * Significant effect of lesion as assessed by two-way ANOVA, $p<0.05$. (B-R) Darkfield photomicrographs demonstrating positive hybridization signal for c-fos mRNA in the parvocellular part (PVHp) and magnocellular part (PVHm) of the paraventricular hypothalamic nucleus (B-E), supraoptic nucleus (SON) (F-I), arcuate nucleus (Arc) (J-M) and medial nucleus of the solitary tract (NTSm) (N-R), in non-lesioned (B, F, J, N), AP-lesioned (C, G, K, O), SFO-lesioned (D, H, L, P) and AP/SFO-lesioned rats (E, I, M, R). V3 - third ventricle. Bregma levels are -1.80 mm for PVH, -1.30 mm for SON, -2.60 mm for Arc and -13.30 mm for NTS. Scale bars correspond to 200 μm.

**Figure 6.** NPY and POMC mRNA expression in arcuate nucleus (Arc), 30 minutes after intraperitoneal injection of Ex-4 in sham-operated or lesioned rats. Pixel density of the
hybridization signal for NPY mRNA following: (A) Ex-4 or saline injection in fasted sham-operated rats, effect of drug as assessed by t-test, \( p<0.05 \); (B) Ex-4 injection in fasted AP- and/or SFO-lesioned vs. sham-operated rats (% of saline). (C) Darkfield photomicrographs demonstrating positive hybridization signal for NPY mRNA in the Arc in sham-operated rats treated with Ex-4 or saline. Pixel density of the hybridization signal for POMC mRNA following: (D) Ex-4 or saline injection in fasted sham-operated rats; (E) Ex-4 injection in fasted AP- and/or SFO-lesioned vs. sham-operated rats (% of saline). (F) Darkfield photomicrographs demonstrating positive hybridization signal for POMC mRNA in the Arc in sham-operated rats treated with Ex-4 or saline.

**Figure 7.** GK mRNA expression in the arcuate nucleus (Arc), 30 minutes after the intraperitoneal injection of Ex-4 in sham-operated or lesioned rats. Pixel density of the hybridization signal for GK mRNA following: (A) Ex-4 or saline injection in fasted sham-operated rats, effect of drug as assessed by t-test, \( p=0.06 \); (B) Ex-4 injection in fasted AP- and/or SFO-lesioned vs. sham-operated rats (% of saline), * significant effect of lesion as assessed by two-way ANOVA, \( p<0.05 \). Data represents means ± SE. (C) X-ray film autoradiograms of coronal brain sections depicting the positive hybridization signal for GK mRNA, bregma level -3.15 to -3.25mm. (D) Darkfield photomicrographs demonstrating positive hybridization signal for GK mRNA in the Arc in sham-operated or AP/SFO-lesioned rats treated with Ex-4 or saline. V3 - third ventricle. Bregma level -3.15 mm. Scale bar corresponds to 200 \( \mu \text{m} \).
Figure 8. CRH hnRNA expression in the parvocellular part of the paraventricular hypothalamic nucleus (PVHp), 30 minutes after the intraperitoneal injection of Ex-4 in sham-operated or lesioned rats. Pixel density of the hybridization signal for CRH hnRNA following: (A) Ex-4 or saline injection in fasted sham-operated rats, *** significant effect of drug as assessed by $t$-test, $p<0.0001$; (B) Ex-4 injection in fasted AP- and/or SFO-lesioned rats (% of saline), * significant effect of lesion as assessed by two-way ANOVA, $p<0.05$. Data represents means ± SE. (C) Darkfield photomicrographs demonstrating positive hybridization signal for CRH hnRNA in the PVHp in sham-operated or AP/SFO-lesioned rats treated with Ex-4 or saline. V3 - third ventricle. Bregma level -1.80 mm. Scale bar corresponds to 200 μm.
Figure 1.
Figure 2.

A

Body weight (g)

Time (days)

B

Daily food intake (kJ/kg BW)

Time (days)

C

Cumulative food intake (kJ/kg BW)

Time (days)
Figure 3.

### ANOVA Results

**Figure A**
- **ANOVA**
  - Region (R): 0.816
  - Lesion (L): 0.614
  - Drug (D): <0.0001
- **Interaction**
  - Region x Lesion (R x L): 0.566
  - Region x Drug (R x D): 0.507
  - Lesion x Drug (L x D): 0.188

**Figure B**
- **ANOVA**
  - Region (R): 0.510
  - Lesion (L): 0.328
  - Drug (D): <0.0001
- **Interaction**
  - Region x Lesion (R x L): 0.548
  - Region x Drug (R x D): 0.911
  - Lesion x Drug (L x D): 0.357
Figure 4.

C-fos mRNA pixel density

saline  Ex-4

BSTov  CeA  PVHp  PVHm  SON  Arc  PBI  PBle  NTSm  VLM

***  **  ***  **  **  **  ***  ***  **  **
Figure 5.
Figure 6.

A

NPY mRNA pixel density (% of control)

Saline  Ex-4

B

NPY mRNA pixel density (% of control)

AP lesion  SFO lesion  AP/SFO lesion

C

Sham - Saline

V3  Arc

Sham - Ex-4

V3  Arc

D

POMC mRNA pixel density

Saline  Ex-4

E

POMC mRNA pixel density (% of control)

AP lesion  SFO lesion  AP/SFO lesion

F

Sham - Saline

V3  Arc

Sham - Ex-4

V3  Arc
Figure 7.

A

GK mRNA pixel density

Saline Ex-4

p = 0.06

B

GK mRNA pixel density (% of control)

AP lesion SFO lesion AP/SFO lesion

C

Saline Ex-4

D

Sham - Saline AP/SFO lesion - Saline

Sham - Ex-4 AP/SFO lesion - Ex-4
Figure 8.
Table 1. Body composition in sham-operated and lesioned rats at the end of 3rd postoperative week. BW, body weight; FM, fat mass; FFM, free fat mass. Data represents means ± SE. § Significant effect of lesion, p<0.05, as assessed by two-way ANOVA followed by post-hoc tests.

<table>
<thead>
<tr>
<th></th>
<th>Sham AP</th>
<th>Sham SFO</th>
<th>Sham AP%/SFO</th>
<th>Lesion AP</th>
<th>Lesion SFO</th>
<th>Lesion AP%/SFO</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW gain (g)</td>
<td>35.76 ± 6.37</td>
<td>34.63 ± 3.32</td>
<td>37.77 ± 5.00</td>
<td>39.23 ± 6.97</td>
<td>49.60 ± 6.98</td>
<td>6.24 ± 6.17 §</td>
</tr>
<tr>
<td>FM gain (g)</td>
<td>5.94 ± 1.28</td>
<td>4.70 ± 0.99</td>
<td>5.97 ± 0.85</td>
<td>4.02 ± 0.98</td>
<td>7.72 ± 1.35</td>
<td>2.10 ± 1.03 §</td>
</tr>
<tr>
<td>FFM gain (g)</td>
<td>1.31 ± 2.10</td>
<td>3.51 ± 1.42</td>
<td>0.26 ± 1.67</td>
<td>6.56 ± 2.10</td>
<td>-4.75 ± 2.49</td>
<td>-4.35 ± 1.87 §</td>
</tr>
</tbody>
</table>
Table 2. Plasma corticosterone, insulin and glucose in sham-operated and lesioned rats
30 minutes after intraperitoneal injection of Ex-4 (1.55 µg/kg) or saline. Data represents
means ± SE. * Significant main effect of drug, p<0.001, as assessed by two-way ANOVA
determined within each type of lesion, and § significant effect of lesion, p<0.001, as
assessed by Fisher’s PLSD and Bonferroni-Dunn post hoc tests.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Sham AP</th>
<th>Sham SFO</th>
<th>Sham APSFO</th>
<th>Lesion AP</th>
<th>Lesion SFO</th>
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<tr>
<td>Corticosterone (nmol/l)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Saline</td>
<td>568.01 ± 211.97</td>
<td>318.42 ± 108.79</td>
<td>577.99 ± 188.75</td>
<td>532.17 ± 144.00</td>
<td>501.41 ± 236.68</td>
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<td>Ex-4</td>
<td>1347.36 ± 409.58 *</td>
<td>1520.75 ± 264.06 *</td>
<td>1654.79 ± 179.84 *</td>
<td>1805.48 ± 111.96 *</td>
<td>1878.52 ± 144.23 *</td>
<td>1521.20 ± 223.93 *</td>
</tr>
<tr>
<td>Insulin (pmol/l)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Saline</td>
<td>49 ± 11</td>
<td>64 ± 7</td>
<td>128 ± 33</td>
<td>80 ± 26</td>
<td>50 ± 4</td>
<td>59 ± 7</td>
</tr>
<tr>
<td>Ex-4</td>
<td>167 ± 63 *</td>
<td>230 ± 67 *</td>
<td>218 ± 89</td>
<td>168 ± 43</td>
<td>151 ± 28</td>
<td>138 ± 15</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Saline</td>
<td>9.05 ± 0.41</td>
<td>8.96 ± 0.14</td>
<td>8.84 ± 0.34</td>
<td>7.80 ± 0.83</td>
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<td>Ex-4</td>
<td>10.48 ± 1.27 *</td>
<td>11.56 ± 0.49 *</td>
<td>11.10 ± 0.64 *</td>
<td>9.82 ± 0.28 *</td>
<td>11.88 ± 0.54 *</td>
<td>8.17 ± 0.26 *</td>
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