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Dietary ketosis actives the HPA axis via FGF21

Dietary manipulations that induce ketosis activate the HPA axis in male rats and mice: a potential role for fibroblast growth factor-21

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In response to an acute threat to homeostasis or well-being, the hypothalamic-pituitary-adrenocortical (HPA) axis is engaged. A major outcome of this HPA axis activation is the mobilization of stored energy, to fuel an appropriate behavioral and/ or physiological response to the perceived threat. Importantly, the extent of HPA axis activity is thought to be modulated by an individual's nutritional environment. In this study, we report that nutritional manipulations signaling a relative depletion of dietary carbohydrates, thereby inducing nutritional ketosis, acutely and chronically activate the HPA axis. Male rats and mice maintained on a low-carbohydrate high-fat ketogenic diet (KD) exhibited canonical markers of chronic stress including: increased basal and stress-evoked plasma corticosterone, increased adrenal sensitivity to ACTH, increased stress-evoked c-Fos immunolabeling in the paraventricular nucleus of the hypothalamus, and thymic atrophy, an indicator of chronic glucocorticoid exposure. Moreover, acutely feeding medium chain triglycerides (MCTs), to rapidly induce ketosis among chow-fed male rats and mice, also acutely increased HPA axis activity. Lastly, and consistent with a growing literature that characterizes the hepatokine fibroblast growth factor-21 (FGF21) as both a marker of the ketotic state and as a key metabolic stress hormone, the HPA response to both KD and MCTs was significantly blunted among mice lacking FGF21. We conclude that dietary manipulations that induce ketosis lead to increased HPA axis tone, and that the hepatokine FGF21 may play an important role to facilitate this effect.

Inducing dietary ketosis by two distinct mechanisms (one acute and one chronic) results in HPA axis activation, and these HPA effects are attenuated in mice that lack FGF21.

1. INTRODUCTION

In response to an acute threat to homeostasis or well-being, stress regulatory systems are engaged. One of the primary physiological responses to stress is activation of the hypothalamic-pituitary-adrenocortical (HPA) axis (1). In this system, information regarding the presence of a stressor is conveyed to hypophysiotropic neurons in the hypothalamic paraventricular nucleus (PVN). Activation of these neurons promotes the release of adrenocorticotropin hormone (ACTH) into systemic circulation, which acts on the adrenal cortex to release glucocorticoids (i.e., corticosterone in rodents and cortisol in humans). Glucocorticoids exert powerful effects throughout the body, including to enhance liver gluconeogenesis and mobilize stored energy,

providing fuel for behavioral and physiological responses that promote survival during acute stress exposure. During chronic stress, excessive, prolonged or repeated HPA axis activation results in marked changes in HPA axis tone (1). As a result, chronic stress typically leads to elevated basal/non-stress corticosterone secretion (which may occur despite normal non-stress ACTH secretion), habituated responses to homotypic stressors, facilitated responses to heterotypic stressors, overall increased adrenal responsivity to ACTH, and adrenal gland hypertrophy, in rats (2–16) (Though the extent to which each of these effects occur can vary among species and/or rodent strains (10)). Importantly, chronic elevations in HPA tone, as occurs during chronic stress, are often associated with negative side effects, including depression and anxiety disorders (17).

The extent of HPA axis activity is thought to be influenced by an individual's nutritional environment (18). For instance, when chow-fed rats were also provided free access to 30% sucrose drink for 10 days, both the ACTH and corticosterone response to an acute restraint stress were reduced (19). Similarly, free access to sucrose drink was sufficient to prevent the marked increase in central HPA axis tone that occurs following adrenalectomy. Whereas adrenalectomized control rats exhibited increased CRH mRNA expression in the PVN, resulting from lack of glucocorticoid negative feedback, this was abrogated among adrenalectomized counterparts consuming the 30% sucrose (19). Moreover, high dietary carbohydrates are associated with reduced plasma glucocorticoids in both people and experimental animals (20–22). Collectively, these findings indicate HPA axis tone may be inversely related to carbohydrate status, such that high glucose availability leads to decreased HPA activation.

Conversely, we reasoned that HPA axis tone may be increased during ketosis – a distinctive metabolic state that occurs when available glucose (the primary fuel of brain and body) is insufficient for metabolic needs. In response to this metabolic crisis, the liver converts fatty acids to ketone bodies providing an alternate fuel source for the brain and other organs. Consistent with this idea, prolonged fasting induces ketosis and is accompanied by elevated glucocorticoids (23). Ketosis can be induced even in the absence of a fast (i.e., when excess calories are available), for example by dramatically restricting carbohydrate consumption in favor of dietary fats. The implications of this 'dietary' ketosis for stress system function are unknown, despite that low-carbohydrate ketogenic diets (KD) are popular and effective for weight loss, and are thought to be useful for the treatment of various diseases (24,25). In this study, we tested the hypothesis that dietary manipulations that acutely and chronically induce ketosis lead to acute and chronic HPA activation, respectively.

Fibroblast growth factor-21 (FGF21) is an important stress-regulatory hormone that is produced by the liver during metabolic stressors including ketosis (26–30), and that crosses the blood-brain barrier to act on its receptors in HPA-regulatory brain regions, including the PVN (31,32). Recent evidence supports that FGF21 acts via the PVN to activate the HPA axis and increase corticosterone secretion *in vivo* (23). We therefore investigated the possibility that increased FGF21 signaling contributes to the HPA effects of dietary ketosis.

2. MATERIALS AND METHODS

2.1 Animals

Adult, male Long-Evans (~225-275 g) rats were purchased from Harlan. Adult, male C57BL/6J mice were bred in-house from breeders obtained from the Jackson Labs. Adult, male FGF21-deficient (KO) and wild-type (WT) control mice were bred in-house as previously described (33), and maintained in our facilities on a C57Bl/6J background. All animals were singly housed

on a 12-h light, 12-h dark cycle (lights typically on at 06:00 h) in a temperature (22ºC) and humidity-controlled vivarium with *ad libitum* access to food and water unless otherwise noted. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati and/or the University of California- Davis, and were consistent with the NIH Guide for the Care and Use of Laboratory Animals.

2.2 Diet treatments, food intake, body weight, and body composition

Normal chow (LM-485; Harlan Teklad) contained 5% fat, 19% protein and 44% carbohydrate by weight. Ketogenic diet (KD; F3666; Bio-Serv) contained 75% fat, 9% protein and 3% carbohydrates by weight. To acutely induce ketosis in chow-fed animals (34), we delivered medium chain triglycerides (MCT; Neobee 895; Stepan Lipid Nutrition) by orogastric gavage. In these studies, gavage of long chain triglycerides (LCT; pure corn oil) was used to control for the thermogenic effect of the triglycerides.

Food intake and body weight were monitored regularly, as noted, and body composition was assessed by time domain NMR. Body length was measured along the dorsal body surface as the distance between the nose and the base of the tail.

2.3 Blood sample collection and plasma measures

For HPA axis assessments, tail blood samples (200 µl for rats; 20 µl for mice) were collected at the indicated time points into chilled tubes containing EDTA. For basal (pre-stress) measures, sample collection was completed in less than 3 min from first handling each rat's cage, thereby ensuring plasma ACTH and corticosterone levels that were reflective of the basal, unstressed state (35). Similarly, when we challenged rats and mice with 20-minutes of restraint in a well-ventilated Plexiglas (rats) or polyethelene (mice) tube, it took less than 3 min to collect each post-stress blood sample. Note that in all cases, the total collected blood volume represents ≤ 5% of the total blood volume, an amount that is well below the threshold for HPA activation (17,36). Blood samples were centrifuged (3000 x g, 15 min, 4ºC) and plasma was stored at -20ºC until measurement of immunoreactive ACTH and corticosterone by radioimmunoassay as described previously (37). For mice, the smaller blood collection volume precluded measurement of plasma ACTH. Integrated plasma corticosterone responses were calculated as the area-under-the-curve (AUC) of the corticosterone time course data. Rats were also perfused at 1 h after restraint stress onset, with collection of brains for cFos immunolabeling in the PVN (as described in Section 2.6). cFos is an immediate-early gene induced following membrane depolarization, and is often used as a general marker of neuronal activation (38). Elevated cFos immunolabeling is typically observed within 30-60 minutes after stress onset, and remains elevated through at least 2 h (38–40), and reportedly up to 4 h after stress onset (41). Rat adrenal and thymus glands were removed and weighed as indirect indices of chronic ACTH and glucocorticoid tone, respectively.

To assess adrenal responsivity to ACTH, rats were first given a maximal dose of dexamethasone (800 µg dexamethasone phosphate (Sigma-Aldrich) in 200 µl saline vehicle; sc) to block endogenous ACTH release. At 2 h after dexamethasone treatment, rats were given exogenous rat ACTH (150 ng/kg body weight; vehicle was 0.5% bovine serum albumin in phosphate-buffered saline (PBS), sc; Bachem). At 15 min after ACTH treatment, rats were killed by rapid decapitation with collection of trunk blood for measurement of plasma corticosterone, as described above.

Plasma β-hydroxybutyrate (the primary circulating ketone body) was measured to indicate the presence and degree of ketosis. For rats, tail blood samples were collected into chilled heparin-coated tubes as described above and plasma β-hydroxybutyrate was measured

using KetoSite test cards in the STAT-Site analyzer system (Stanbio Laboratory). For mice, tail blood samples were directly measured using blood ketone test strips in the Precision Xtra meter (Abbott) since this approach requires significantly less blood volume. Among mice that received MCT gavage, occasionally some were not in ketosis at the time of β-hydroxybutyrate measurement (which was performed as a positive control for the presence of ketosis), possibly due to the fact that the stomach contents of the non-fasted animals affected MCT dynamics. As such, mice that received MCT gavage with β-hydroxybutyrate levels below 1 mM were removed from the HPA assessment; this β-hydroxybutyrate threshold was set prior to measuring the corticosterone values, and these instances occurred regardless of genotype. Plasma FGF21 was measured by ELISA (Rat/Mouse FGF21 ELISA kit, Millipore).

2.4 Behavioral assays

Rats were tested in a standard test of behavioral anxiety (elevated plus-maze, EPM) as well as a standard test for depressive-like behavior (forced swim test, FST). For the EPM, rats were placed onto the maze apparatus for 5 minutes in a dimly-lit room. Video-recordings of the ensuing behavior were scored for the number of open arm entries as an index of behavioral anxiety, as well as for the total number of open plus closed arm entries as an index of locomotor activity (42). For the FST, rats were individually placed for 10 minutes into a cylinder filled with water sufficiently deep to prevent standing on the bottom. Video-recordings were scored for the amount of time spent immobile (defined as doing on the minimal movements necessary to prevent drowning) as an index of depressive-like behavioral despair (43,44). In all cases, behavior was scored by an individual unaware of group assignments.

2.5 Intracerebroventricular (icv) cannula implantation and infusions

Rats were outfitted with stainless-steel cannulas as described previously (45), with the exception that they were directed at the lateral ventricle (stereotaxic coordinates: 1.4 mm lateral from midline, 0.8 mm posterior from bregma, and 3.6 mm ventral from dura). Rats were regularly handled for habituation to cannula insertion and removal. Human recombinant FGF21 (ProSpec) was diluted into PBS vehicle for icv administration at a dose of 3 µg in 3 µl vehicle. The HPA axis response to acute icv FGF21 (vs. vehicle) was tested, with blood samples collected for measurement of plasma ACTH and corticosterone, as described in Section 2.3, just before (0 min) and at 1, 2 and 3 h after icv infusion. Several weeks later (to allow abundant washout time), rats again received acute icv FGF21 (vs. vehicle) and at 2 h after icv infusion, animals were perfused with collection of brains for PVN immunolabeling (as described in Section 2.6). The 2 h time point was selected to allow sufficient time for FGF21 to activate the PVN, and then initiate the expression of cFos protein which peaks at ~1-2 h after PVN activation(43,44). Brains were post-fixed for ~16 h at room temperature and then stored in sucrose (30% in PBS) at 4 ºC until microtome sectioning (see below). Cannula location was verified histologically; n= 4 rats with cannulas that missed the lateral ventricle were removed from all analyses. In addition, one rat had an abnormally short and stiff tail (possibly due to a developmental defect) precluding collection of tail blood samples for HPA assessment; though this rat could still be used for assessment of brain immunolabeling following icv infusion.

2.6 Brain immunolabeling

Brains were sectioned (25 µm) on a microtome, and the sections were stored in cryoprotectant (0.1 M PBS, 30% sucrose, 1% polyvinylpyrrolidone, and 30% ethylene glycol) at -20 ºC. Sections were immunolabeled (Table 1) with rabbit primary antiserum directed against cFos (1:5000; sc-52; Santa Cruz Biotechnology, Research Resource Identifier (RRID) AB_2106783)

via a standard immunolabeling procedure (46). Immunolabeling was not present when the primary antibody was omitted. Positive immunolabeling was detected by use of biotin-conjugated goat anti-rabbit secondary antibody (Vector Laboratories) followed by incubation with avidin-biotin-peroxidase (Vectastain ABC solution; Vector Laboratories) and reaction with 3,3'-diaminobenzidine. Immunolabeling was imaged using brightfield light microscopy (Zeiss Imager.M2 microscope with Apotome, AxioCam camera, Zen 2011 software; Carl Zeiss). Positive cells were counted from all available, intact sections at the mid-PVN rostral-caudal level (between approximately bregma -1.8 to -2.0 mm (47) using Image J (W. Rasband, National Institutes of Health) software. This typically resulted in bilateral quantification of 1-3 sections of PVN. These values were then averaged to obtain the representative cell count for each rat. Analyses were performed by personnel unaware of group assignments.

2.7 Statistical analyses

Data are shown as mean ± SEM. When comparing 2 treatment groups, data were analyzed by two-tailed t-test (for parametric) or Mann-Whitney test (for non-parametric). For multiple group comparisons, data were analyzed by ANOVA, with repeated measures when appropriate, followed by Neuman-Keuls or 2-tailed Dunnett's post-hoc analysis. For ANOVA, if the variance between treatment groups was not homogenous, analyses were performed following square-root transformation. Potential outliers were evaluated using 2 different standard outlier tests and were removed only if they failed both tests, as described previously (46). Statistical significance was taken as $p<0.05$.

3. RESULTS

3.1 Low-carbohydrate ketogenic diet activates the HPA axis

First, we maintained age-matched groups of adult male Long-Evans rats on a standard low-carbohydrate, high-fat KD or normal chow for 2-3 weeks. In agreement with a number of previous rodent studies (48–51), and with the widespread popularity of ketogenic diets for weight loss, KD-fed rats gained less body weight despite consuming more daily calories (Fig. 1A-B). KD lowered morning, post-prandial plasma glucose levels, consistent with the low carbohydrate content of the diet (Fig. 1C), and also modestly (~ 6%) reduced body length, consistent with the observation that ketogenic diets inhibit linear growth (Fig. 1D) (52–54). KD-fed rats were therefore smaller overall, but had equivalent total body composition (Fig. 1E).

Next we explicitly tested the hypothesis that, like other chronic stressors, KD facilitates HPA axis tone, resulting in elevated basal and stress-evoked HPA responses. We found KD-fed rats have increased morning basal (non-stress) plasma corticosterone compared to chow-fed controls, with unaltered plasma adrenocorticotropic hormone (ACTH; Fig. 2A-B). KD-fed rats also exhibited thymic atrophy (Fig. 2C), consistent with chronic exposure to elevated glucocorticoids, with no change in adrenal gland weight (Fig. 2D). Notably, this pattern of elevated morning non-stress corticosterone, normal resting plasma ACTH, thymic involution and normal adrenal weight is consistent with our previous studies in which Long-Evans rats were exposed to chronic variable stress (55–57), suggesting that chronic KD-feeding enhances basal HPA axis tone in a manner that resembles chronic stress exposure.

When challenged with a 20-min acute restraint stress, KD-fed rats exhibited an exaggerated plasma corticosterone response (Fig. 3A, C). The initiation of the plasma ACTH response to restraint was unaffected by KD, and ACTH levels returned to baseline more rapidly after the onset of restraint (Fig. 3B), suggesting the possibility that the history of elevated basal (non-stress) plasma corticosterone exerted negative feedback on the HPA axis to limit the extent and

duration of the ACTH response to acute restraint stress. This pattern of elevated basal and post-stress corticosterone levels, despite normal basal and post-stress ACTH, may implicate greater adrenal responsiveness to ACTH. We tested this directly, by first injecting rats with dexamethasone to block endogenous ACTH release, and then challenging them with exogenous ACTH. Rats maintained on a KD exhibited greater plasma corticosterone following the ACTH challenge, compared to chow-fed controls (Fig. 3D). Thus, we conclude that chronic consumption of KD is associated with greater adrenal responsivity to ACTH.

Because the brain plays a key role to initiate stress responses, and because chronic stress facilitates HPA responses to a novel stressor, we hypothesized that KD-fed rats would have greater restraint-induced neuronal activation in stress-regulatory brain regions compared to chow-fed controls. To test this, we sacrificed rats 1h following the restraint stress challenge, above, and collected their brains for cFos immunolabeling. Consistent with the increased corticosterone response, we observed a greater number of cell bodies expressing cFos in the paraventricular nucleus of the hypothalamus (PVN) of KD-fed rats (Fig. 3E-G).

Since elevated HPA axis tone is often accompanied by increased depressive-like and anxiety-related behavior, we reasoned that KD may impact these types of behavior. To test this possibility, a new cohort of rats was maintained on either KD or normal chow and their behavior was assessed in the EPM and FST. The EPM test was performed on day 29 of diet treatment. KD did not alter the number of open arm entries (Fig. 4A), nor the total number of arm entries (i.e., open + closed; Fig. 4B), indicating that KD did not alter either anxiety-related behavior or total locomotion in this test. Moreover, KD did not alter the percent time spent immobile the FST that was performed on day 32 of diet exposure (Fig. 4C). Collectively these behavioral data suggest that anxiety- and depressive-like behaviors are largely unaffected by KD, though it is still possible that KD could impact behavior in other types of tests.

3.2 Ketogenic medium-chain triglycerides activate the HPA axis

To acutely induce dietary ketosis without chronic alterations to macronutrient content of the diet, we administered medium-chain triglycerides (MCT) by oral gavage to adult chow-fed rats. Unlike long-chain triglycerides (LCT), dietary fatty acids from MCT (chain length of C_6 to C_{12}) are absorbed in the portal system and are carried directly to the liver where they are rapidly oxidized to ketone bodies (34,58,59). Age and weight-matched control groups were administered an equal volume of either LCT or water. As expected, rats receiving MCT gavage exhibited a rapid increase in the plasma ketone body β-hydroxybutyrate relative to both control groups (Fig. 5D). Concurrently, rats receiving MCT gavage exhibited robust activation of the HPA axis. In this case, both plasma ACTH (Fig. 5A) and corticosterone (Fig. 5B-C) were increased by the stress of handling and gavage, and this was significantly exaggerated among chow-fed MCT-treated rats relative to chow-fed LCT- and water-treated controls. Moreover, both the plasma ACTH (Fig. 5F) and corticosterone (Fig. 5G) responses were linearly related to the post-gavage levels of plasma β-hydroxybutyrate.

Again, because the brain plays a key role to initiate stress responses, we hypothesized that MCT-fed rats would have greater neuronal activation in stress-regulatory brain regions compared to LCT and water controls. To test this, we sacrificed a new cohort of rats at 2h following MCT, LCT, or water gavage and collected their brains for cFos immunolabeling. Consistent with the increased corticosterone and ACTH response, we observed a greater number of cell bodies expressing cFos in the PVN (Fig. 5E). Thus, acute dietary (MCT) ketosis elicited an acute stress response, characterized by elevated ACTH and corticosterone. Chronic dietary (KD) ketosis, on the other hand, increased adrenal responsivity to ACTH, resulting in a larger corticosterone

response to stress despite an equivalent ACTH response. Such a pattern of elevated adrenal responsivity and disproportionate glucocorticoid-to-ACTH responses resembles that which occurs during chronic stress (60–66). Accordingly, inducing dietary ketosis by two distinct mechanisms (one acute and one chronic) resulted in typical patterns of HPA axis activation by acute and chronic stress, respectively (Table 2).

3.3 FGF21 contributes to HPA activation by low-carbohydrate ketogenic diet and by ketogenic medium chain triglycerides

During ketosis the liver produces the hormone FGF21, an endocrine member of the FGF superfamily (67). Importantly, FGF21 crosses the blood brain barrier (31) and is thought to activate the HPA axis by stimulating CRH neurons in the PVN (23,68,69). Consistent with this, when we delivered either FGF21 (3 μg) or its vehicle acutely to the lateral ventricle of male rats, we observed significantly increased immunolabeling for cFos (Fig. 6A) in the PVN, together with greater circulating ACTH and corticosterone (Fig. 6B-D). In light of this, we hypothesized that FGF21 contributes to HPA activation following dietary ketosis. Consistent with this possibility, plasma FGF21 was greater among both rats (Fig. 7A) and mice (Fig. 7B) maintained on KD compared to chow-fed controls. Likewise, plasma FGF21 was greater among both rats (Fig. 7C) and mice (Fig. 7D) receiving MCT gavage compared to LCT and water control groups.

To test the potential contribution of FGF21 to mediate the HPA response to dietary ketosis, we compared the effects of KD on HPA activity in FGF21-deficient (KO) mice and wild-type (WT) controls. Three weeks of KD-feeding resulted in equivalent levels of blood β-hydroxybutyrate in KO and WT mice (Fig. 7G). Nonetheless, although the plasma corticosterone response to restraint stress was clearly enhanced by KD in both genotypes, this was significantly attenuated in FGF21-deficient mice (Fig. 7E, F). Similarly, we compared the effects of MCT gavage in FGF21-KO and WT control mice. Plasma corticosterone was increased after MCT (vs. LCT) in both genotypes, and this was significantly reduced in FGF21-deficient mice (Fig. 7H-I), despite equivalent levels of ketosis (Fig. 7J). These data suggest that FGF21 contributes to a portion of the robust increase in glucocorticoids that occur during ketosis.

4. DISCUSSION

4.1 Impact of dietary ketosis on the HPA axis and stress-related behavior

The present work tested the hypothesis that dietary manipulations that acutely and chronically induce ketosis lead to indices of acute and chronic HPA activation, respectively. Acute dietary ketosis, induced by MCT gavage in chow-fed rats, elevated plasma ACTH and corticosterone and increased cFos-positive cells in the PVN compared to both LCT and water gavage controls. The pattern and time course of these effects parallel those that occur following a variety of other acute stressors, including restraint, footshock, and loud noise (56,70–73), consistent with the idea that acute dietary ketosis is a metabolic stressor that rapidly and transiently activates the HPA axis (Table 2).

When longer-term feeding with a low-carbohydrate, high-fat diet was used to chronically induce dietary ketosis, body weight gain and final body length were attenuated despite greater caloric intake, consistent with prior findings using ketogenic diets (52,53). Ketogenic diet feeding was then used to ask whether this alters HPA axis tone in a manner similar to chronic stress. Chronic stress enhances basal HPA tone, and also facilitates responses to a novel acute stressor (though the precise pattern can vary somewhat between species and strains)(10). More specifically, these effects of chronic stress include increased basal/non-stress plasma

corticosterone near the nadir of the circadian rhythm (which may occur despite normal non-stress plasma ACTH), reduced thymus weight, increased adrenal weight, enhanced adrenal responsivity to ACTH, and elevated plasma corticosterone and PVN cFos responses to a novel stressor (2,4,7,8,12–16,56). Importantly, the HPA effects of ketogenic diet closely resemble this pattern (Table 2), with the only exception that ketogenic diet did not affect adrenal weight – a finding that is not surprising since in our hands Long-Evans rats typically demonstrate little-to-no increase in adrenal weight following chronic stress (55–57). The ability of both chronic stress and ketogenic diet to increase adrenal responsivity to ACTH is particularly noteworthy, as it suggests that the adrenal secretes a greater amount of glucocorticoid for a given amount of ACTH hormone. This effect may explain, at least in part, ketogenic diet-induced elevations in basal and post-restraint plasma corticosterone that occurred without concomitant ketogenic diet-induced increases in plasma ACTH.

Since elevated HPA axis tone is often accompanied by anxiety- and depressive-like behaviors(17), the behavioral impact of longer-term ketogenic diet was tested in EPM and FST. Collectively, these data suggest that ketogenic diet does not markedly alter anxiety- and depressive-like behaviors in either of these tests, indicating that ketogenic diet does not uniformly alter all stress-related end points. The findings also underscore the fact that stress-related behaviors like anxiety and depression are controlled by complex and partially-overlapping networks of brain circuitry whose function is not governed exclusively by glucocorticoid levels (74,75).

The precise mechanisms by which chronic stressors facilitate basal and HPA axis tone are not fully known, but likely involve both central (brain) and peripheral (adrenal) mechanisms. A number of stress-regulatory brain regions have been implicated in regulating the HPA response to chronic stress (1). These regions may influence HPA axis tone centrally, for example by directly or indirectly modulating PVN hypophysiotropic neuron activity. Stress-regulatory brain regions may also act in the periphery, for example by modulating adrenal responsivity to ACTH via the adrenal sympathetic innervation and/or via the trophic effects of prior ACTH secretion (64,66,76,77). Moreover, a host of peripheral actors, such as circulating hormones and metabolites, could act directly on the adrenal gland to modulate its responsivity (64,78,79). Finally, signals from the periphery (either hormonal or neural sensory signals) could act directly or indirectly on brain stress-regulatory regions to alter HPA activity during chronic stress (80). Given this wide scope of potential mediators, the present work explored the potential role of hormonal FGF21, as it is elevated during ketosis in rodents and is linked with HPA axis activation (as detailed in Section 4.2).

4.2 Contribution of FGF21 to ketosis-induced HPA axis activation

The present work investigated the possibility that increased FGF21 signaling contributes to the HPA effects of dietary ketosis. Both MCT gavage and ketogenic diet increased plasma FGF21, consistent with prior observations that hormonal fibroblast growth factor-21 (FGF21) is produced by the liver during a variety of metabolic stressors, including ketosis. Moreover, FGF21 can cross the blood-brain barrier and its receptors are expressed in the PVN, suggesting the FGF21 is well-positioned to alter PVN activity (23,31). Consistent with this idea, previous reports convincingly demonstrate that direct icv infusion of FGF21 activates the HPA axis in a CRH-dependent manner (23). The present data corroborate these findings, as icv administration of FGF21 increased HPA axis activation relative to vehicle controls, and also increased cFos immunolabeling in the PVN. It should be noted that the density of cFos-positive cells in the PVN varied markedly between experiments depending on the particulars of each experimental

design. The highest cFos density was observed after restraint stress (Fig. 3E), more moderate levels occurred after gavage (Fig. 5E), and the lowest levels occurred after icv drug administration in rats that were pre-habituated to cannula manipulation (Fig. 6A). The differing extent of cFos responses likely reflect the relative intensity of stress associated with each experimental procedure (70). Moreover, while icv FGF21 significantly increased the number of cFos-positive neurons in the PVN, the relatively low density of these neurons may indicate that central FGF21 signaling alone is a modest regulator of PVN activation. Alternatively, FGF21 receptors may act primarily via intracellular signaling pathways that are not strongly coupled to cFos expression.

To more directly test the potential role of FGF21 in mediating the HPA response to dietary ketosis, we compared the effects of KD and MCT gavage on HPA activity in FGF21-deficient (KO) mice and wild-type (WT) controls. Genetic FGF21 deficiency significantly blunted acute MCT-induced HPA activation, as well as longer-term KD-induced facilitation of the HPA response to restraint stress. These results demonstrate that endogenous FGF21 contributes to ketosis-induced HPA activation. However, as FGF21-deficiency only partially abrogated the HPA effects of dietary ketosis, future work will be directed towards identifying additional factors that contribute to ketosis-induced HPA activation. This FGF21-independent regulation of corticosterone or other counter-regulatory hormones (e.g., glucagon, epinephrine) is likely sufficient to maintain glycemia during nutritional ketosis, since we previously reported that FGF21 KO mice are normoglycemic during KD-feeding (54). Future studies can also determine the extent to which the HPA-modulatory effects of FGF21 during ketosis occur directly via FGF21 signaling in the PVN, indirectly via FGF21 signaling in other HPA-regulatory brain regions, or indirectly via the peripheral consequences of whole-body loss of FGF21 actions.

Of note, when maintained on a chow diet, FGF21-deficiency did not alter basal (pre-stress) plasma corticosterone, nor the corticosterone response to an acute restraint stress (Fig. 7E, F). Likewise, neither basal nor post-LCT gavage corticosterone levels were impacted by loss of FGF21 (Fig.7H, I). These results suggest that endogenous FGF21 does not contribute to HPA responsivity when an individual is in a normal (e.g., non-ketogenic) metabolic state. Moreover, consistent with our previous report (54) the ability of MCT gavage and ketogenic diet to induce ketosis, as indicated by circulating β-hydroxybutyrate levels, was not impacted by FGF21 ablation.

4.3 Potential clinical implications

Collectively these findings may have important clinical implications. Chronic consumption of low-carbohydrate KDs have been popular and effective for weight loss (81,82), and there is a growing interest in acutely boosting ketosis by consuming MCTs as a dietary supplement. Given the broad systemic effects of chronic glucocorticoid exposure, it will be important to interrogate the translational value of these findings. Indeed, limited clinical evidence already links low-carbohydrate KDs with increased HPA axis activity in humans. Langfort and colleagues, for example, reported higher pre and post-exercise levels of circulating corticosterone among untrained volunteers consuming a KD compared to controls (83). Likewise a recent study of the effects of dietary composition during weight loss maintenance observed higher urinary cortisol among participants consuming a very low-carbohydrate diet, compared to those consuming low-glycemic index or low-fat diets (84,85).

Low-carbohydrate KDs and consumption of MCTs are thought to be useful for the treatment of several diseases, including cancer, rheumatoid arthritis, various neurological disorders, and pharmacotherapy-resistant epilepsy (86–89), but underlying physiological mechanisms have

remained elusive. Several studies report increased plasma cortisol among patient populations receiving KDs as putative therapies. For example, when cortisol was measured in plasma from epileptic children collected before and after KD therapy, elevated cortisol was observed in all individuals (90). Likewise plasma cortisol was increased with KD among adult patients with rheumatoid arthritis (91). To the extent that dietary ketosis increases HPA axis activity in humans, the present work supports the possibility that diet-induced increases in circulating glucocorticoids may contribute to these therapeutic effects.

Importantly, this work further identifies FGF21 signaling as a key downstream mechanism contributing to HPA axis activation during dietary ketosis. Plasma FGF21 was increased following both KD and MCT gavage in rats and mice. Moreover, and consistent with previous reports (23,68), icv administration of recombinant FGF21 acutely increased plasma ACTH and corticosterone (Fig. 5). Lastly, KD and MCT-induced increases in circulating corticosterone were significantly attenuated among mice lacking FGF21 (Fig. 6). Taken together, these data are consistent with the hypothesis that this hepatokine acts as a metabolic stress hormone (92), and draw additional attention to potential stress-regulatory side effects of FGF21-based therapeutics currently in the pipeline for treatment of metabolic diseases (93). They also highlight the need for additional clinical research delineating the relationships among dietary ketosis, FGF21 and stress system function in human subjects.

5. CONCLUSIONS

The present data clearly demonstrate that two dietary manipulations that induce ketosis despite the availability of excess calories, one acute and one chronic, significantly increase activity of the HPA axis. This is mediated in part by associated increases in the hepatokine FGF21, since FGF21 KO mice exhibited a blunted HPA response to these dietary interventions relative to WT controls. These findings may have important clinical implications for individuals using ketogenic diets for weight loss and as nutritional therapy for the treatment of diseases.

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AUTHOR CONTRIBUTIONS

KKR, AEBP, JS, SMF, AMKT, KL, KMH, KS, DP-T, RJS, and YMU planned experiments. KKR, AEBP, KRL, JS, SMF, AMKT, KL, KMH, KS, and YMU acquired data. KKR, KMH,

NI, DP-T, MT, RJS, and YMU interpreted data. KKR and YMU wrote the manuscript, which was read, edited and approved by the other authors.

Disclosure summary:

RJS is a consultant for Ethicon Endo-Surgery/Johnson & Johnson, Orexigen, Novo Nordisk, Daiichi Sankyo, Janssen/Johnson & Johnson, Novartis, Paul Hastings Law Firm, Zafgen, Takeda, and Boehringer-Ingelheim, and receives research support from Ethicon Endo-Surgery/Johnson & Johnson, Novo Nordisk, Janssen/Johnson & Johnson, MedImmune, Boehringer-Ingelheim, and Sanofi. The other authors have no conflicts of interest to disclose.

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Figure 1. Low-carbohydrate, ketogenic diet (KD) reduced body size despite adequate caloric intake. (A) Body weight (2-way ANOVA), (B) caloric intake (2-way ANOVA), (C) morning post-prandial plasma glucose (2-tailed t-test), and (D) final body length (2-tailed t-test), of rats given KD (vs. normal chow) for 23 days. n=12-13/group. (D) Final percent body fat (*left*; 2-tailed t-test) and lean (*right*; 2-tailed t-test) of rats given KD (vs. normal chow) for 14 days. n=10-11/group. *p<0.05 vs. Chow.

Figure 2. KD increased resting plasma corticosterone in the morning. Basal, unstressed plasma (A) corticosterone (2-tailed Mann-Whitney test) and (B) ACTH (2-tailed Mann-Whitney test) sampled near the circadian nadir after 19-23 days of KD-feeding (vs. normal chow). n=24-26/group. (C) Thymus (2-tailed Mann-Whitney test) and (D) adrenal (2-tailed Mann-Whitney

test) weights normalized to body weight for rats given KD for 23 days. n= 12-13/group. *p<0.05 vs. Chow.

Figure 3. KD increased the plasma corticosterone response to an acute psychological stressor. Time course of the plasma (A) corticosterone and (B) ACTH response to a 20-min restraint stress in rats consuming KD (vs. normal chow) for 23 days (two-way ANOVA). (C) The area-under-the-curve (AUC) of the corticosterone response shown in panel A (2-tailed t-test). n=12-13/group. (D) Plasma corticosterone response to ACTH treatment (150 ng/kg body weight) in dexamethasone-blocked rats (2-tailed t-test) after 15 days on KD. n=5-6/group. (E) Activation of cFos-positive cells in the paraventricular nucleus of the hypothalamus at 1 hr after the onset of a 20-min restraint stress in rats consuming KD for 23 days (2-tailed t-test). n=12/group. *p<0.05 vs. Chow. Representative images of cFos-positive cells in the PVN of (F) chow-fed, and (G) KD-fed rats at 1 hr after the onset of a 20-min restraint stress. The outline denotes the border of the PVN. Scale bar = 100 μm.

Figure 4. KD had little impact on anxiety- and depression-related behaviors in elevated plus-maze (EPM) and forced swim test (FST), respectively. (A) Number of open arm (2-tailed t-test) and (B) number of total (open plus closed) arm entries in the EPM on day 29 of KD (vs. normal chow) consumption. (C) Percent time spent immobile in the FST (2-tailed t-test) on day 32 of KD (vs. normal chow) treatment. n=11-13/group. *p<0.05 vs. Chow.

Figure 5. Ketogenic medium-chain triglycerides (MCT) activated the HPA axis. Time course of the plasma (A) ACTH, and (B) corticosterone response to gavage of 3 ml MCT, long-chain triglycerides (LCT) or water in chow-fed rats (two-way ANOVA). (C) The area-under-the-curve (AUC) of the corticosterone response shown in panel A (one-way ANOVA). n=8-12/group. (D) Plasma ß-hydroxybutyrate in rats at 1 hr after gavage of MCT, LCT or water in chow-fed rats (one-way ANOVA). (E) Activation of cFos-positive cells in the paraventricular nucleus (PVN) of the hypothalamus at 2 hr after gavage of MCT, LCT or water in chow-fed rats (one-way ANOVA). n=9-11/group. *p<0.05 vs. both LCT and Water. (F) Relationship between plasma ACTH versus plasma ß-hydroxybutyrate at 1 hr after gavage of MCT, LCT or water in chow-fed rats (linear regression, p<0.01). (G) Relationship between the AUC of the corticosterone response versus plasma ß-hydroxybutyrate at 1 hr after gavage of MCT, LCT or water in chow-fed rats (linear regression, p<0.01).

Figure 6. Central FGF21 administration activated the HPA axis. (A) cFos-positive (2-tailed t-test) cells in the paraventricular nucleus of the hypothalamus at 2 hr after icv infusion of 3 μg FGF21 (vs. vehicle (Veh)-treated controls). n=8-10/group. Time course of the plasma (B) ACTH, and (C) corticosterone response to icv infusion of 3 μg FGF21 or vehicle (two-way ANOVA). (D) The area-under-the-curve (AUC) of the corticosterone response shown in panel D (2-tailed t-test). n=7-10/group. *p<0.05 vs. Veh.

Figure 7. FGF21 contributed to HPA activation by low-carbohydrate ketogenic diet (KD) and by ketogenic medium chain triglycerides (MCT). Plasma FGF21 in (A) rats after 7 days on KD vs. chow (2-tailed Mann Whitney test n=7-8/group, *p<0.05 vs. Chow), (B) mice after 7 days on KD vs. chow (2-tailed Mann Whitney test, n=11/group, *p<0.05 vs. Chow), (C) rats at 1 hr after gavage of 3 ml MCT, long-chain triglycerides (LCT) or water (one-way ANOVA, n=9-11/group, *p<0.05 vs. both LCT and water), and (D) mice at 2 hr after gavage (200 μl) of MCT, LCT or

water (one-way ANOVA, n=4-6/group, *p<0.05 vs. both LCT and water). (E) Time course (three-way ANOVA), and (F) area-under-the-curve (AUC; two-way ANOVA) of the plasma corticosterone response to a 20-min restraint stress in wild-type (WT) and FGF21-deficient (KO) mice consuming KD (vs. normal chow) for 20 days prior (*p<0.05 vs. KD WT, #p<0.05 vs. respective Chow group). (G) Blood β-hydroxybutyrate after three weeks of KD-feeding in KO and WT mice (2-tailed t-test, p=0.86). n=6-11/group. (H) Time course (three-way ANOVA), and (I) AUC (two-way ANOVA) of the plasma corticosterone response to gavage of 200 µl MCT or LCT in chow-fed WT and KO mice (*p<0.05 vs. MCT WT, #p<0.05 vs. respective LCT-treated group). (J) Blood β-hydroxybutyrate at 1 hr after gavage of MCT in chow-fed WT and KO mice (2-tailed t-test, p=0.42). n=6-12/group.

Table 1. Antibody used for immunolabeling.

Peptide/protein target	Antigen sequence (if known)	Name of Antibody	Manufacturer, catalog #, and/or name of individual providing the antibody	Species raised in; monoclonal or polyclonal	Dilution used	RRID (required in revised MSs)
cFos		Anti-cFos	Santa Cruz Biotechnology, sc-52	Rabbit Polyclonal	1:5000	AB_2106783

Table 2. Summary of the HPA effects of acute (MCT) and chronic (ketogenic diet) dietary ketosis and their resemblance to typical acute vs. chronic stress responses, respectively.

HPA axis response to acute stress (70,71)**:**	**HPA axis response to acute ketosis:**
- Increased PVN cFos	- Increased PVN cFos
- Increased plasma ACTH	- Increased plasma ACTH
- Increased plasma corticosterone	- Increased plasma corticosterone
HPA axis response to chronic stress:	**HPA axis response to chronic ketosis:**
- Increased morning basal plasma corticosterone which may occur despite normal ACTH (7,8,10,12–15,56)	- Increased morning basal plasma corticosterone despite normal ACTH
- Reduced thymus weight (11,12,56,66)	- Reduced thymus weight
- Increased adrenal weight (2,10–16,66)	- No change in adrenal weight
- Increased adrenal responsivity to ACTH (16,66)	- Increased adrenal responsivity to ACTH
- Facilitated corticosterone responses to a heterotypic (e.g., restraint) stress (4,8,16,56)	- Facilitated corticosterone responses to a heterotypic (e.g., restraint) stress

Figure 1

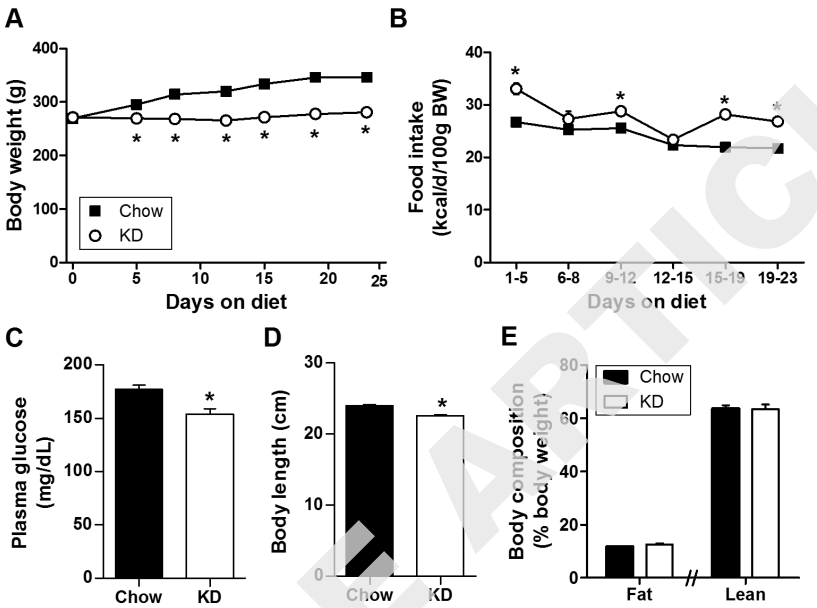

Figure 2

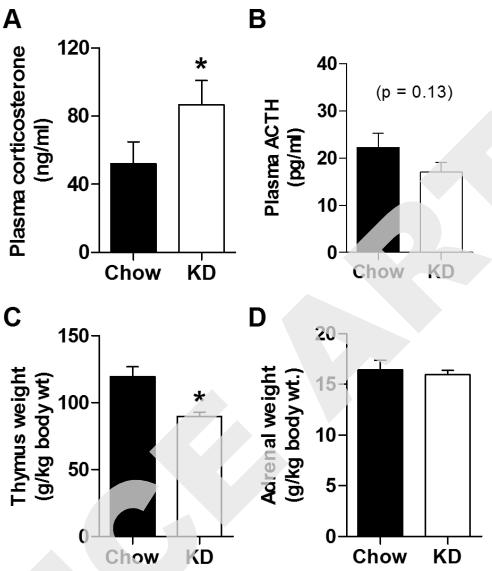

Figure 3

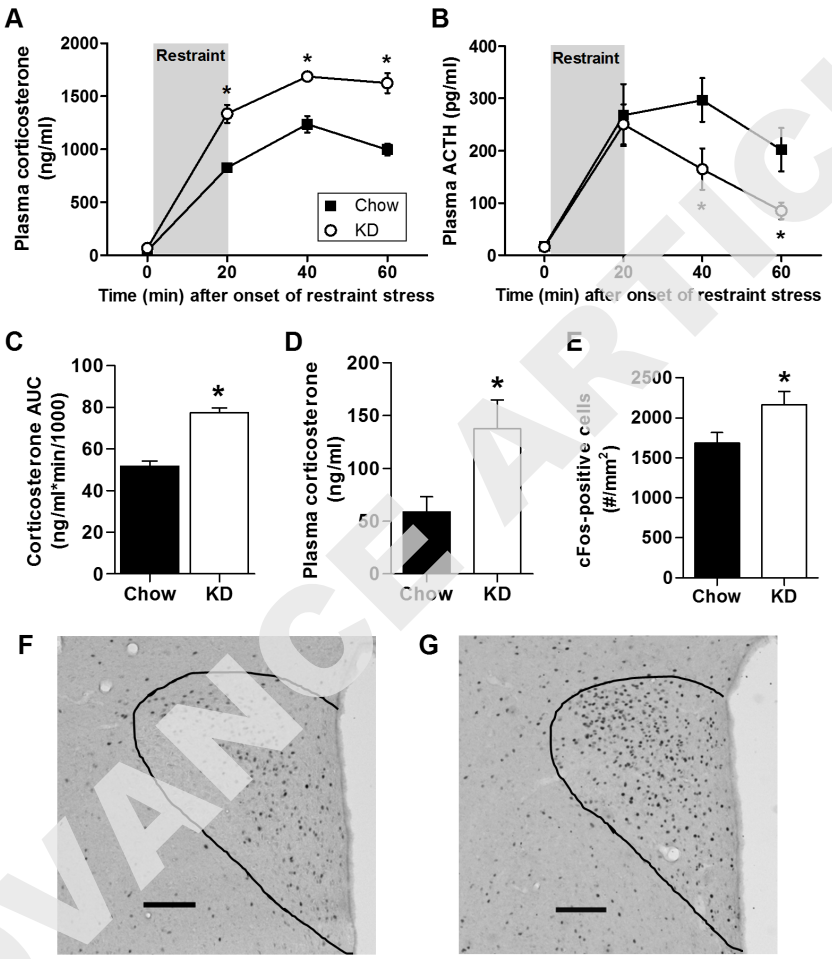

Figure 4

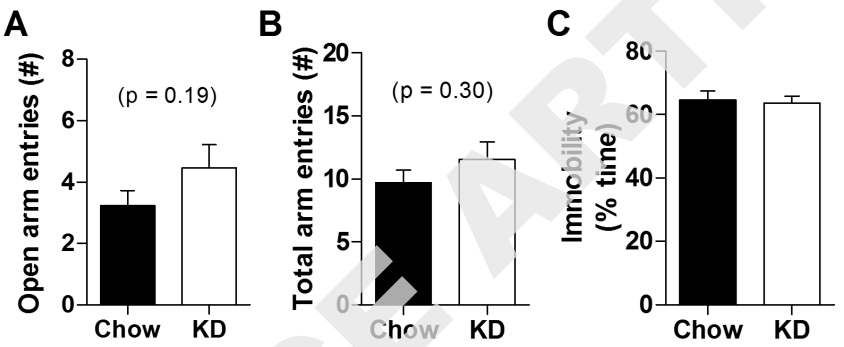

Figure 5

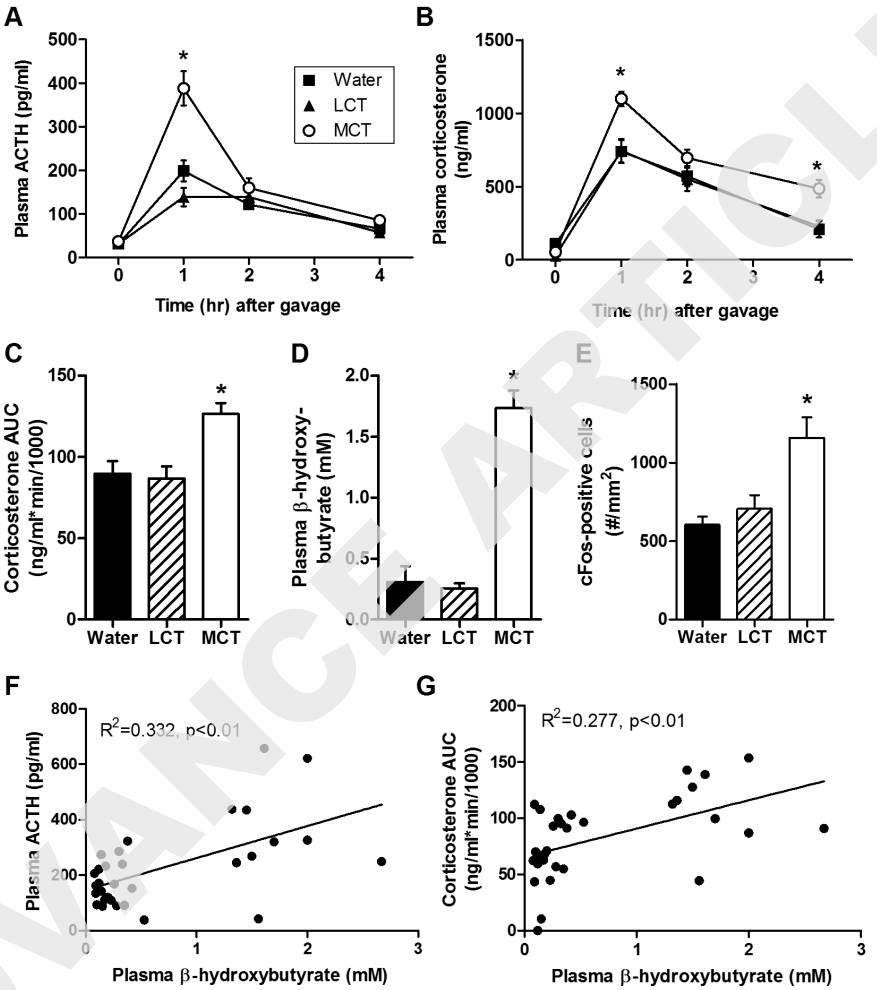

Figure 6

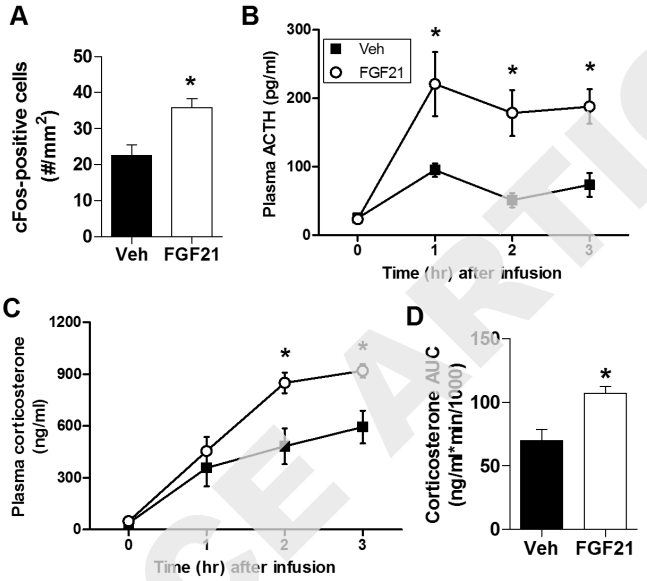

Figure 7