HHS Public Access

Author manuscript

Int J Obes (Lond). Author manuscript; available in PMC 2021 March 19.

Published in final edited form as:

Int J Obes (Lond). 2021 February ; 45(2): 383–395. doi:10.1038/s41366-020-00688-z.

Metformin effectively restores the HPA axis function in diet-induced obese rats

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Abstract

Introduction—The hypothalamo-pituitary-adrenal (HPA) axis is perturbed in obesity. We previously reported presence of leptin resistance in the brainstem and uncoupling between central noradrenergic tone and the HPA axis in obesity-prone (DIO) rats. Metformin is shown to lower body weight and adiposity, but the underlying mechanism is unclear. We hypothesized that this is associated with restored HPA axis function.

Methods—Adult male DIO rats were placed on either a regular chow or HF diet for 7 weeks. Starting week 4, the animals were given either a low dose (60mg/kg) or high dose (300mg/kg) of metformin in drinking water. In addition to body weight and feeding, we examined different arms of the HPA axis to test if metformin can reinstate its function and coupling. To understand potential mechanisms, leptin signaling in the brainstem and circulating free fatty acid levels were also assessed.

Results—Metformin treatment lowered weight gain, fat mass, caloric intake, and serum leptin levels. HPA axis activity as determined by corticotropin-releasing hormone in the median eminence and serum corticosterone was decreased by metformin in a dose-dependent manner, and

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Competing Financial Interests

Authors declare no conflict of interest.

so was norepinephrine (NE) in the paraventricular nucleus. Importantly, metformin completely normalized the NE-HPA axis uncoupling. While brainstem pSTAT-3 and SOCS-3, key markers of leptin signaling, were not different between groups, circulating saturated and unsaturated free fatty acids were reduced in HF-fed, metformin-treated animals.

Conclusions—These findings suggest that oral metformin can successfully correct HPA axis dysfunction that is associated with lowered circulating free fatty acids in DIO rats, thereby uncovering a novel effect of metformin in the treatment of obesity.

Keywords

Diet-induced obesity; Norepinephrine; CRH; leptin; corticosterone

INTRODUCTION

Dysfunction of the hypothalamo-pituitary-adrenal (HPA) axis, or stress axis, is one of the pathophysiological hallmarks in obesity. Human studies have shown that urinary free cortisol levels are higher in patients with abdominal obesity[1–3], while there was no change in circulating morning cortisol levels in obese compared to lean individuals[4]. Obese subjects also have elevated cortisol secretion following stimulation with corticotrophin-releasing hormone (CRH) or adrenocorticotropic hormone (ACTH) analogue[1, 2], indicating an abnormally hypersensitive HPA axis. Consistent with this, diet-induced obese (DIO) rats also exhibit higher circulating corticosterone following physical and environmental stressors[5], making them an excellent model for investigating this phenomenon. While obesity-associated impairment in HPA axis sensitivity and function have been well appreciated over several decades, the mechanisms underlying the dysregulation are poorly understood. This is of clinical significance because impaired HPA axis activity can result in increased appetite and fat mass as well as impaired insulin sensitivity and glucose homeostasis[6] – symptoms commonly observed in obesity[7, 8]. Understanding this issue will therefore help us devise meaningful strategies to combat obesity.

Noradrenergic nuclei from the brainstem (A1, A2, A6) send out strong projections to CRH neurons in the paraventricular nucleus of the hypothalamus (PVN), and serve as a critical input for HPA axis activation. Supporting this notion are earlier findings that showed that direct norepinephrine (NE) injection into the PVN markedly elevates circulating corticosterone, while depleting PVN NE by chemical denervation of noradrenergic bundles or via pharmacological blockades leads to a significant reduction of both CRH release and corticosterone[9–11]. We have previously reported that the HPA axis is impaired in DIO rats[12, 13]. Through a careful examination of different arms of the HPA axis circuitry, we found that leptin, an adipocyte-derived hormone and an important regulator of various neuroendocrine functions including energy balance and the HPA axis, is unable to suppress noradrenergic output to the PVN, indicating a possible impairment of leptin signaling[12, 13]. Importantly, this was associated with uncoupling between the NE and the HPA axis especially on a high-fat (HF) diet[13]. It is thus tempting to speculate that correcting this crucial neuroendocrine function and the associated NE-HPA axis uncoupling would be a viable strategy to treat obesity.

Metformin is an oral biguanide, insulin-sensitizing agent that has been widely used to treat type 2 diabetes due to its ability to suppress hepatic glucose production and improve insulin sensitivity[14–16]. Apart from its anti-diabetic effects, recent clinical studies indicate that metfomin is also effective at lowering body weight and fat mass in obese children and adults[17–19]. Moreover, anti-diabetic, appetite-suppressing, and body weight-lowering effects by metformin were demonstrated in obese animals[20–22]. While the exact mechanism still remains unclear, along with our recent reports, these findings raise a possibility that the metabolic benefits accomplished by metformin, in particular the weight-reducing effects, may be partly mediated through restoration of the HPA function and/or integrity. The direct effect of metformin on the HPA axis seems plausible considering their ability to readily cross blood-brain-barrier[23, 24].

To explore this novel concept, selectively bred DIO rats placed on a regular chow or HF diet were treated with either a low-dose or high-dose metformin in drinking water for 4 weeks. Metabolic parameters including body weight and food intake and direct indices of HPA axis activation – NE in the PVN, CRH in the median eminence (ME), and serum corticosterone – were examined. Since circulating free fatty acids were found to be elevated in DIO rats in our previous study[13] that can potentially contribute to changes in HPA axis function, serum free fatty acids were measured at the end of metformin treatment. Metformin has been shown to improve leptin sensitivity in the hypothalamus and lower body weight in diet-induced obese rats and obese A^y agouti mice[25, 26]. This prompted us to assess pSTAT-3 and SOCS-3, key indicators of leptin signaling pathway, in brainstem noradrenergic nuclei to determine if metformin-induced changes in HPA axis function and NE-HPA axis coupling are associated with changes in leptin signaling.

METHODS

Animals

Selectively bred, DIO (i.e. obesity-prone) male Sprague-Dawley rats were raised in our colony using breeders obtained from Charles River Laboratories, Inc. (Wilimington, MA) as previously described[13]. These animals were used because their propensity to become obese is attributed to polygenic inheritance, thus closely mimicking the development of human obesity[27]. They were single-housed in the animal facility room with temperature of 23±2°C and 12:12-h light/dark cycle (lights on at 0700h; off at 1900h). Animals were provided with *ad libitum* access to regular chow diet (Teklad 8640 diet; 3.11kcal/g, 5% fat; Harlan, Indianapolis, IN) and water prior to experiments. All studies were conducted in accordance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* and the protocol was approved by the institutional animal care and use committee at Michigan State University.

Metformin treatment

Nine-week-old male DIO rats were given 7 weeks of regular chow or high-fat (HF) diet (6 groups; n=9–10 each). The group size was based on our previous studies with these animals using similar protocols[12, 13]. The HF diet contained 45% fat with an energy density of 4.73kcal/g (D12451; Research Diets Inc., New Brunswick, NJ). At the end of the third week,

the animals received either a low dose (LD; 60mg/kg BW) or high dose (HD; 300mg/kg BW) of metformin (Spectrum Chemicals & Laboratory Products, Inc.; New Brunswick, NJ) dissolved in drinking water for 4 weeks. One group of animals were given normal drinking water without metformin to serve as a control group. The high dose was determined based on earlier studies[26, 28] and on its ability to achieve plasma metformin concentrations comparable to those in diabetic patients under metformin therapy[29]. The low dose of metformin was included to test its dose-dependent effect on body weight and the HPA axis. Water intake of each animal was monitored everyday to ensure that the animals were consuming the correct amount of metformin. Metformin concentrations in drinking water were readjusted to the body weight of animals once a week. A schematic of study design is shown in Fig. 1. Body weight and caloric intake were carefully measured every week throughout the experiment. At the end of 4-week metformin treatment, the animals were sacrificed by decapitation at 10am after 3h fast. Following blood glucose measurement through a hand-held glucometer (AlphaTrak, Zoetis) and trunk blood collection, serum was separated for detection of leptin and corticosterone by radioimmunoassay. The abdominal fat pads (epigonadal, perirenal, retroperitoneal) were harvested and weighed, and brains were collected, immediately frozen in dry ice, and stored at −70°C for analyses as described below.

Palkovits' microdissection

This procedure has been described earlier[12, 13]. Frozen brains were sectioned in 300μm increments using a cryostat at −10°C, and noradrenergic nuclei in the brainstem (A1, ventrolateral medulla; A2, nucleus tractus solitarius; A6, locus coeruleus), the paraventricular nucleus (PVN), lateral hypothalamus, and median eminence (ME) were dissected by Palkovits' microdissection with the help of a rat brain atlas[30]. The lateral hypothalamus/ME and brainstem punches were stored at −70°C for detecting orexin/CRH concentrations by ELISA and leptin signaling by western blots, respectively. The PVN was stored in 0.1M $HClO_4$ at −70°C for analysis of NE content using HPLC with electrochemical detection (EC).

NE concentrations in the PVN

Details of the HPLC-EC system employed here for detection of NE in the PVN punches have been described previously[31, 32]. Microdissected PVN samples were homogenized in 150μl of 0.1M $HClO_4$ and centrifuged for 10 min at 10,000 *g* (RCF). The lysates (120ul) were mixed with 30μl of the internal standard (0.05M dihydroxybenzylamine; DHBA) and 125μl of this mixture was injected into the HPLC system. The sensitivity for NE detection was <1pg, and NE concentrations were expressed as pg/μg protein.

Leptin signaling in the brainstem

Noradrenergic nuclei from the brainstem were homogenized in lysis buffer (Sigma Aldrich, St. Louis, MO) and protein concentrations were determined using BCA assay as previously described[12, 13]. 20μg of protein was loaded for each sample into SDS-PAGE gels (NuPAGE, Invitrogen, Carlsbad, CA). Proteins were then transferred onto PVDF membranes which were probed with antibodies including pSTAT-3 (1:1000; goat-polyclonal; Santa Cruz Biotechnology, Dallas, TX), SOCS-3 (1:1000; goat-polyclonal; Cell Signaling Technology,

Danvers, MA), and GAPDH antibody (1:2000; mouse-monoclonal; Sigma-Aldrich, St. Louis, MO). After washing 3 times of 10 min each, the membranes were incubated in blocking solution containing goat anti-rabbit DyLight 800 and goat anti-mouse DyLight 680 secondary antibodies (1:5000; Thermo Fisher Scientific; Waltham, MA). Bands were visualized and analyzed using an Odyssey imaging system (Li-COR biosciences, Lincoln, NE).

Serum leptin, brain CRH and orexin

A commercially available ELISA kit (TiterZyme Kits, Assay Design, Ann Arbor, MI) was used to detect leptin in serum according to the manufacturer's specifications. CRH in the ME was measured by CRH EIA kit (Phoenix Pharmaceuticals, Inc., Belmont, CA). The assay had a minimum sensitivity of 0.30 ng/ml and an inter-assay variability of <5%. Orexin in the lateral hypothalamus was measured using a commercially available radioimmunoassay (Phoenix Pharmaceuticals, Inc., Belmont, CA). A small aliquot was used for assessing protein levels using a Micro BCA protein assay kit (Pierce, Rockford, IL). Orexin was expressed as pg/μg protein.

Serum corticosterone

Radioimmunoassay for corticosterone was performed using a tracer and standards from EMD Millipore (Billerica, MA) and the primary and secondary antibodies generated in our lab, as described previously[12, 33]. Duplicates of samples were used to ensure reproducibility. The sensitivity and inter-assay variability were 0.2 ng/ml and less than 4%, respectively.

Circulating free fatty acids (FFAs)

Serum FFAs were determined as described before[13, 34]. Total lipids from serum were extracted using hexane:ethanol (1:1) solvent mix, after which the extracts were separated on a SP-2560 column (Supelco, Bellefonte, PA) and analyzed using a Clarus 500 gas chromatography (Perkin Elmer, Waltham, MA). >99% pure methylated esters were used as standards (Nu-Chek Prep, Elysian, MN). Concentrations of both saturated and unsaturated fatty acids were expressed as mg/dl.

Statistical analysis

NE, CRH, and orexin levels in the brain and serum leptin, corticosterone, and free fatty acids were analyzed by 2-way ANOVA with diet and metformin dose as two independent variables, followed by Bonferroni-adjusted multiple comparisons. Blood glucose and protein expression of leptin signaling markers (pSTAT3, SOCS-3), final body weight, weight gain, total caloric intake, feed efficiency, and fat mass were analyzed by 2-way ANOVA followed by Bonferroni-adjusted multiple comparisons. Weekly body weight and caloric intake differences were analyzed by 2-way repeated measures ANOVA (treatment as between-subject factor and time as within-subject factor) followed by Bonferroni *post hoc* test. Type II regression analysis was conducted to determine the association between NE, CRH, and corticosterone. Data from lean Sprague-Dawley rats and obesity-resistant (DR) rats were obtained from our previous studies[13, 33] to perform the regression analysis and compare to that in DIO rats in the present study. Samples affected by a significant hemolysis in serum or

insufficient amount of tissues were excluded. Data are expressed as Mean±SEM. Significant difference between groups was set at $p<0.05$.

RESULTS

Metformin supplementation lowers weight gain and caloric intake in DIO rats independent of diet

While final body weight was not statistically different among groups (Fig. 2A), as expected, weight gain was found to be significantly higher in HF-fed rats compared to that in chow-fed controls without metformin treatment (Fig. 2B). Interestingly, metformin dose-dependently reduced weight gain in both chow and HF-fed DIO rats (Fig. 2B). A close examination of weekly body weight change revealed a sharp deviation from the anticipated weight trajectory, with a significant decline in weight gain especially in animals supplemented with HD metformin (Fig. 2C). This was accompanied by reduced caloric intake in metformin-treated animals, especially in HF-fed rats within a week of metformin treatment (Fig. 2D, E). As a result, DIO rats treated with HD metformin had lower feed efficiency and were less energetically efficient to gain mass regardless of diet (Fig. 2F). Next, we sought to determine if metformin-induced resistance to weight gain is associated with lower adiposity. Indeed, HF-fed control group displayed a larger visceral fat mass compared to chow-fed controls (>100%), but oral metformin treatment significantly reduced it mainly in HF-fed animals (Fig. 3A, B). Leptin is an adipocyte-derived hormone whose circulating levels are generally proportional to body fat percentage. High circulating leptin levels are associated with leptin resistance that is thought to be one of the main drivers for obesity development[35]. Metformin supplementation decreased serum leptin levels in HF-fed DIO rats (Fig. 3C) most likely due to the reduction in fat mass. Metformin is also known to improve insulin sensitivity and glycemic control in obese or diabetic individuals[14, 36, 37], so it is reasonable to speculate that it would correct hyperglycemia in obesity-prone DIO rats. However, we did not observe any differences in blood glucose levels between chow and HF-fed DIO rats or following 4 weeks of metformin treatment (Fig. 3D). These results suggest that oral metformin supplementation lowers weight and fat gain that is at least partly due to reduced caloric intake.

Metformin suppresses HPA axis activity in HF-fed DIO rats in a dose-dependent manner

Hyperactivation of the HPA axis is associated with obesity[1–3, 5, 38]. Noradrenergic neurons located in A1, A2, and A6 brainstem regions project to the PVN and release NE to stimulate CRH neurons for stress axis activation[9–11]. HF feeding increased PVN NE concentrations compared to chow feeding by ~50% in control rats (Fig. 4A) which is consistent with our earlier report[13]. Interestingly, oral metformin supplementation was able to dose-dependently lower PVN NE concentrations, although the reduction was statistically significant only in HF-fed animals (Fig. 4A). Compared to HF-fed controls, HD metformin-treated animals with HF diet had 50% lower PVN NE levels. This was accompanied by a corresponding decrease in CRH levels in the ME (Fig. 4B) where PVN CRH neuropeptide is released into before stimulating ACTH secretion from the anterior pituitary. ACTH in turn enters the systemic circulation and stimulates glucocorticoid secretion from the adrenal gland. As expected, serum corticosterone in HF-fed, metformin-treated animals was significantly lower compared to that in HF controls (Fig. 4C). Next, orexin levels in the lateral

hypothalamus was determined because orexin-expressing neurons have reciprocal excitatory connections with CRH neurons in the PVN[39], and acute stimulation of these neurons has recently shown to activate the HPA axis[40]. Since metformin dramatically suppressed HPA axis activity, we anticipated orexin levels to be lower in the metformin-treated animals. In contrary to our expectation, orexin concentrations in the lateral hypothalamus was not different between groups (Fig. 4D), indicating that it may not play a major role in HF-induced activation of the HPA axis.

HPA axis circuitry is fully restored following metformin treatment

Type 2 regression analysis (Fig. 5) was used to demonstrate the lack of correlation between different arms of the HPA axis in DIO rats placed on a HF diet and the positive impact of metformin treatment. The analysis based on the data from different cohorts of normal Sprague-Dawley rats as well as obesity-resistant DR rats (from previous studies for comparison)[13, 33] shows a near perfect regression between PVN NE vs. corticosterone or ME CRH (R^2=0.92–0.99; Fig. 5A–C), whereas DIO rats show a clear disconnect between PVN NE vs. corticosterone (R^2=0.01; Fig. 5D) or ME CRH (R^2=0.18; Fig. 5E). Surprisingly, metformin treatment almost completely rescued the HPA axis integrity as shown by restored coupling between PVN NE vs. ME CRH (R^2=0.99) or serum corticosterone (R^2=0.89; Fig. 5D–E). These results suggest that oral metformin treatment is able to effectively reverse the HPA axis dysfunction induced by HF feeding in DIO rats.

Chronic metformin treatment fails to improve leptin signaling in noradrenergic neurons

Leptin is an important regulator of the HPA axis[31, 33, 41], and its receptors are expressed in noradrenergic neurons in the brainstem[42, 43]. Our previous reports revealed that unlike obesity-resistant DR rats, DIO rats have high circulating leptin but impaired brainstem leptin signaling, suggesting that this may be responsible for the observed failure to suppress NE in the PVN[13]. In the present study, metformin was able to effectively decrease PVN NE levels in HF-fed DIO rats, leading us to predict that this may be due to restored leptin signaling in noradrenergic nuclei in the brainstem. However, measurement of pSTAT-3 (marker of downstream leptin signaling) or SOCS-3 (negative feedback inhibitor of leptin signaling) expression were devoid of changes across all groups (Fig. 6A–F), suggesting that metformin-induced suppression of NE in the PVN is not likely a result of enhanced/restored leptin signaling in the brainstem.

Metformin decreases circulating free fatty acids in HF-fed DIO rats

Earlier studies have shown that free fatty acids (FFAs) can activate both the sympathetic outflow and the HPA axis and induce metabolic syndrome[44–46]. We speculated that metformin-induced suppression of stress axis activity and restored coupling within PVN NE-HPA axis in DIO rats may be associated with changes in circulating FFAs. Indeed, we observed that whereas HF-fed controls have significantly higher unsaturated fatty acids – oleic acid (300%; Fig. 7A), arachidonic acid (50%; Fig. 7C), linolenic acid (300%; Fig. 7F) – and saturated fatty acids like palmitic acid (200%; Fig. 7D) and stearic acid (250%; Fig. 7E) compared to chow-fed controls, treatment with LD metformin was able to nearly normalize the circulating levels of these FFAs (Fig. 7A–F). Interestingly, while serum docosahexanoic acid levels were not different between the groups, eicosapentanoic acid,

another active metabolite of linolenic acid, was significantly lower in HF-fed animals independent of the dose of metformin (Fig. 7G, H). Collectively, these results suggest that chronic metformin treatment can improve the circulating FFA profile in HF-fed DIO rats.

DISCUSSION

Metformin is generally regarded as the first-line medication for individuals with type 2 diabetes because of its consistent glucose-lowering and insulin-sensitizing effects, as well as for its higher safety threshold. Recent human and animal studies have shown that metformin can also effectively lower body weight and adiposity[17–20, 28, 47]. While the mechanisms underpinning its anti-diabetic effects such as its ability to suppress hepatic glucose production have been well described[14–16, 21], the potential neural, hormonal, and/or molecular pathways by which metformin successfully reduces body weight and fat mass remain unclear. We have previously shown that obesity-prone DIO rats have a dysfunctional HPA axis that is known to be strongly associated with obesity development[6, 8]. Here we tested if metformin could correct HPA axis function in DIO rats and help them lose weight when challenged with HF diet. Our current findings demonstrate that 4 weeks of oral metformin treatment renders them resistant to obesity by reducing body weight gain, caloric intake, and visceral fat depots. More importantly, these metabolic benefits are associated with normalization of NE levels in the PVN and HPA axis function that are independent of leptin signaling in brainstem noradrenergic neurons.

The ability of metformin to decrease weight gain, fat mass and food consumption in DIO rats is supported by other studies[17, 20, 28, 47]. Caloric intake declined sharply in rats as soon as metformin treatment was started and rose modestly the following week and remained at that level for the rest of the observation period. This suggests an acute and persistent effect, perhaps through downregulation of orexigenic NPY in the hypothalamus[48]. Another possibility is that metformin is able to reduce weight gain by increasing energy expenditure. This notion is supported by a recent study that showed higher energy expenditure following metformin treatment in HF-fed mice[49]. It is not clear if a similar mechanism is in operation in DIO rats in the present study. Contrary to other studies, metformin treatment failed to lower blood glucose in DIO rats. While the reasons are not clear, it is possible that 3h fasting instead of an overnight fasting (a known stressor that can lower circulating leptin and activate the HPA axis) before the sacrifice and glucose measurement potentially diminished the differences in blood glucose between the diet control groups and metformin-treated animals. Furthermore, improved glucose homeostasis is typically observed following modest weight loss[50, 51]. In the present study, metformin treatment only resulted in less weight gain and not weight loss from baseline. Likewise, the fat mass in HF-fed, metformin-treated animals did not drop to the degree seen in chow-fed animals which is twice as that in lean DR rats[13]. The absence of body weight and fat loss following metformin treatment may have led to the failure of metformin to lower blood glucose. These findings are consistent with earlier findings by Kim and colleagues[26].

Leptin suppresses NE release in the PVN and also lowers circulating corticosterone in lean rats[32, 33, 41, 52, 53], pointing to an inverse relationship between the satiety hormone and the HPA axis. Previously, we observed that when DIO rats are placed on a HF diet, they display

higher NE levels in the PVN in spite of elevated circulating leptin, indicative of possible leptin resistance in brainstem noradrenergic nuclei[13]. Since noradrenergic innervation to the PVN is critically involved in stimulating feeding[54, 55], higher PVN NE observed in DIO rats may be one of the drivers that contribute to increased feeding and the development of obesity. In the present study, metformin was able to effectively suppress NE levels in the PVN and this was associated with lower weight gain. Decreased NE is most likely not a result of high circulating leptin since metformin also reduced it. The leptin-lowering effects of metformin and its ability to counter weight gain is consistent with findings from a recent study that showed resistance to diet-induced obesity via reducing leptin production[56]. While blocking the synthesis led to lower circulating leptin, surprisingly the study found restored leptin sensitivity in hypothalamic neurons that play an important role in energy balance. Hence, we hypothesized that metformin may lower PVN NE in DIO rats possibly by restoring leptin signaling in extra-hypothalamic areas also such as the brainstem noradrenergic nuclei. This is based on the previous findings from our group[13] as well as others[57, 58] that showed impaired brain leptin signaling in obese animals compared to lean animals even at baseline without stimulation with leptin. Interestingly, our data show that pSTAT-3 and SOCS-3, markers of leptin signaling, were not different between the control and metformin-treated groups. Along with our previous findings on leptin responsiveness in DIO rats[13], these results support the concept that the reduction in PVN NE and HPA axis activity caused by metformin is not likely mediated by leptin signaling in the brainstem. Metformin could directly affect noradrenergic neurons to decrease NE synthesis, a possibility that needs to be investigated in the future. Similar to leptin, insulin is another nutrient-related hormone that is involved in neuroendocrine regulation of feeding and body weight[59, 60]. Interestingly, insulin can also exert sympathoexcitatory actions through the arcuate nucleus and downstream activation of glutamate receptors and MC3/4 receptors in the PVN[61–63], and insulin during hyperinsulinemic euglycemic clamps has been shown to increase HPA axis activity (i.e. increased CRH in the PVN and plasma ACTH and corticosterone)[64]. Thus, it is possible that metformin treatment lowers both the sympathetic and HPA axis activation in part by decreasing insulin signaling in the arcuate nucleus and/or the PVN in our animals. If this is true, then whether this phenomenon is brain region-specific and how this may interact with insulin's anorexigenic action through the hypothalamus are interesting questions that warrant a detailed investigation.

On the other hand, our results clearly show that metformin treatment can lower circulating FFAs. Since FFAs are known activators of sympathetic outflow[44], these findings are in line with the ability of metformin to inhibit sympathoactivation[65, 66] possibly through reduction in PVN NE. Reduction in serum FFAs by metformin could be simply because the metformin-treated animals ate less food. Since HF diet contains 45% of calories as fat, less fat consumption may have partially corrected the dyslipidemia found in DIO rats. Alternatively, metformin has been shown to increase lipid uptake and induce lipolytic and thermogenic programs in brown adipose tissue[67, 68]. The fat-burning properties of metformin may have led to efficient utilization of fatty acids, thereby reducing circulating FFA levels.

Another interesting outcome of this study was the complete reinstatement of NE-HPA axis coupling following metformin treatment in HF-fed DIO rats. This is clinically significant in

light of our previous findings[13] that showed a disconnect between PVN NE and the HPA axis, leading to dysregulation of the stress axis activity that has been shown to promote obesity and other metabolic disorders. The restored coupling between PVN NE and HPA axis indices in HF-fed DIO rats in this study was similar to what we observed in lean Sprague-Dawley rats and obesity-resistant DR rats. We cannot rule out the possibility that the restored NE-HPA axis coupling may be a secondary effect of metformin-induced resistance to weight gain. However, it is important to note that the observation of significantly decreased PVN NE and HPA axis activity in the absence of differences in weight gain points to weight-independent effects of metformin on NE-HPA axis circuitry. Nonetheless, comparing the effects of metformin to pair-fed or weight-matched animals will be necessary to clearly address this question.

Collectively, our findings indicate that oral metformin treatment in obesity-prone DIO rats lowers body weight gain and caloric intake that is associated with effective suppression of the HPA axis. More importantly, this study showed that metformin can completely rescue the impaired coupling between PVN NE and the HPA axis present in DIO rats, thus revealing metformin's novel mechanism of action in the treatment of obesity. Although not completely clear, decreased circulating FFAs may play a role in this phenomenon. Further studies are needed to understand direct effects of metformin on brainstem noradrenergic neurons.

Acknowledgements

We would like to thank Ms. Katrina Linning for her excellent technical assistance and for maintaining the rat colony.

Funding Sources

This study was supported in part by NIH grant R01 AG027697 to PSM and NSF grant IBN0236385 to SMJM and PSM. ACS was supported by Biomedical Health Research Initiative grant, MSU.

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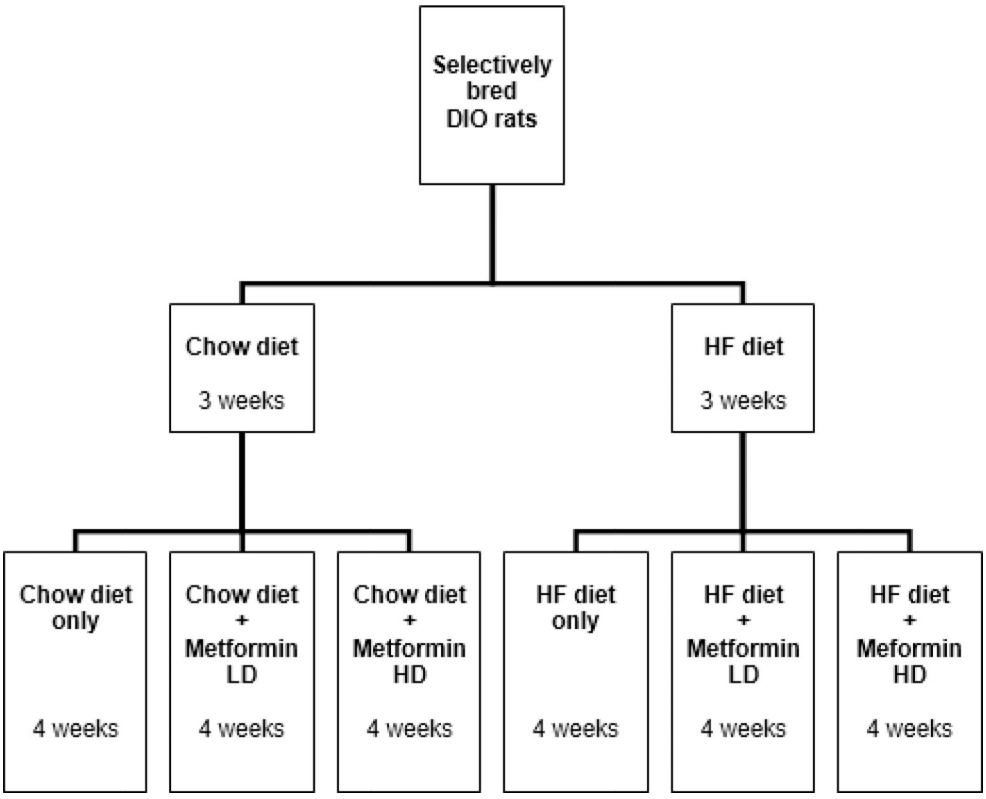

Fig. 1. Study design.

Selectively bred, obesity-prone DIO rats were initially placed on a regular chow or high-fat (HF) diet for 3 weeks. The animals were treated with either a low dose (LD; 60mg/kg) or high dose (HD; 300mg/kg) of metformin in drinking water from week 4–7 while remaining on the same diet. Groups without metformin supplementation were included as controls.

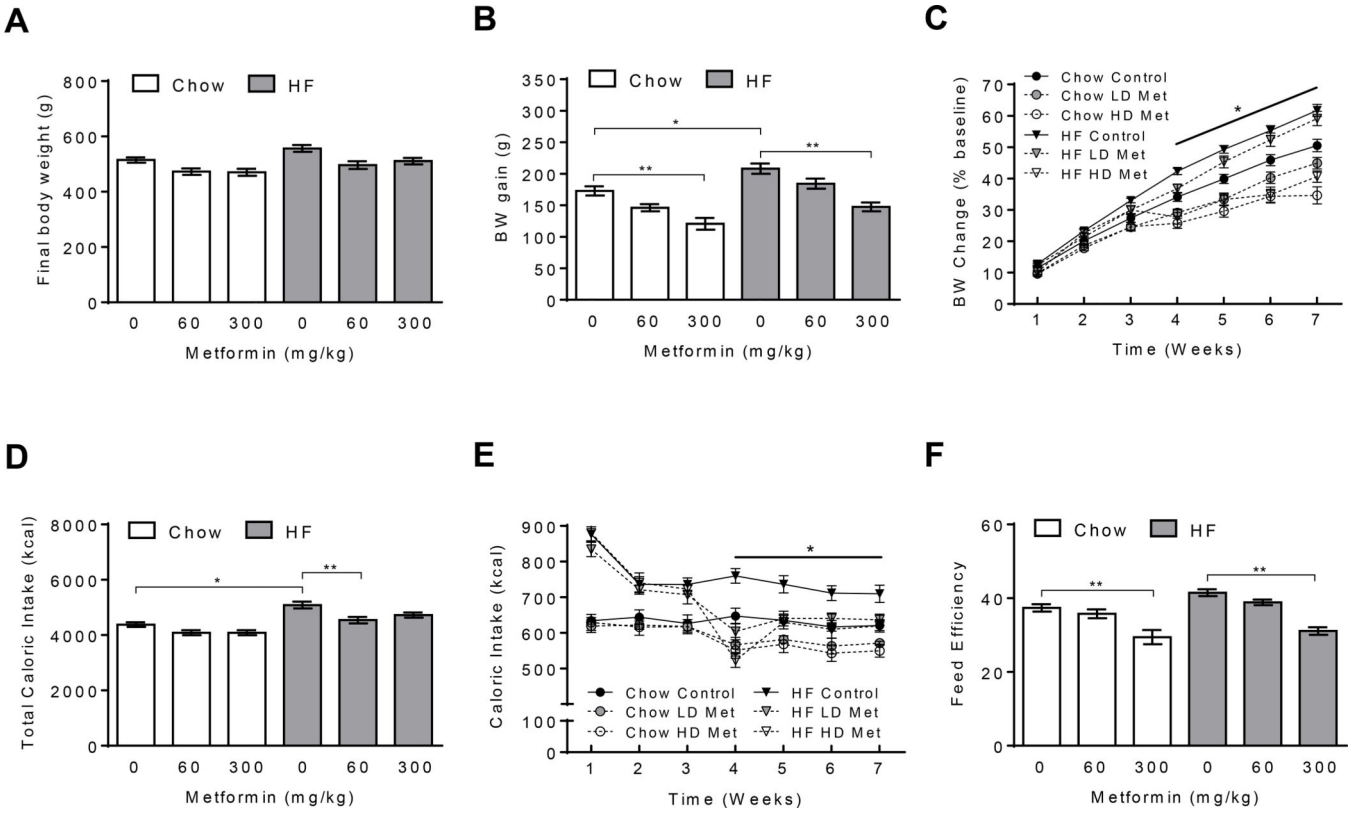

Fig. 2. BW, caloric intake, and feed efficiency.

The effects of metformin treatment on **A)** Final body weight (BW), **B)** BW gain, **C)** Weekly BW change, **D)** Total caloric intake, **E)** Weekly caloric intake, and **F)** Feed efficiency (BW gain/total caloric intake x 1000). n=5–9 per group. * $p<0.05$; ** $p<0.01$. For **C)** and **E)**, * indicates statistically significant difference between HD metformin-treated groups and their respective control groups.

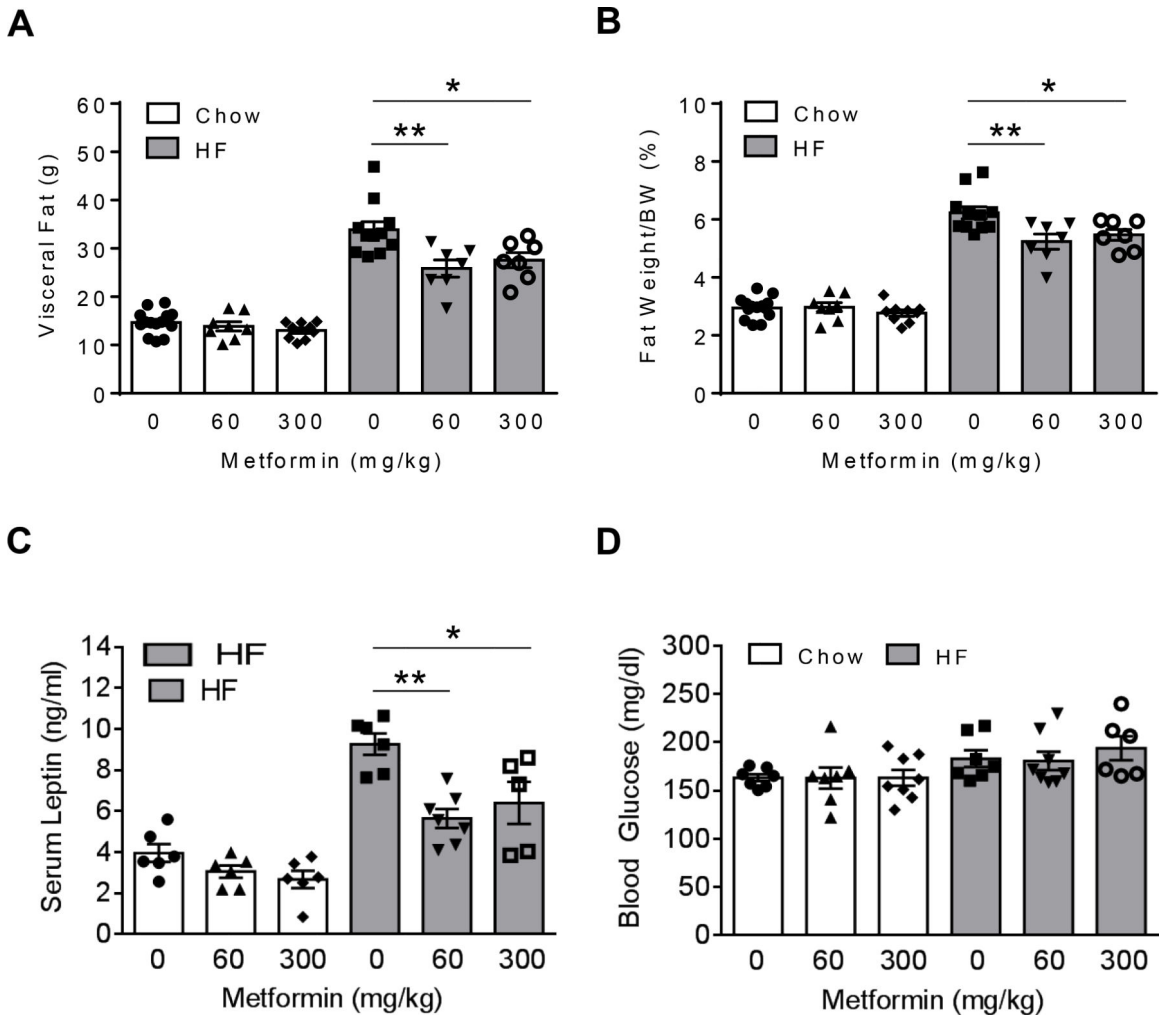

Fig. 3. Oral metformin treatment decreases fat mass and circulating leptin in HF-fed DIO rats.

A) Visceral fat mass (epigonadal, retroperitoneal, perirenal), **B)** Relative fat weight (Fat weight/BW (%), **C)** Serum leptin levels, and **D)** Blood glucose at sacrifice after 3h fasting. n=5–9 per group. * $p<0.05$; ** $p<0.01$.

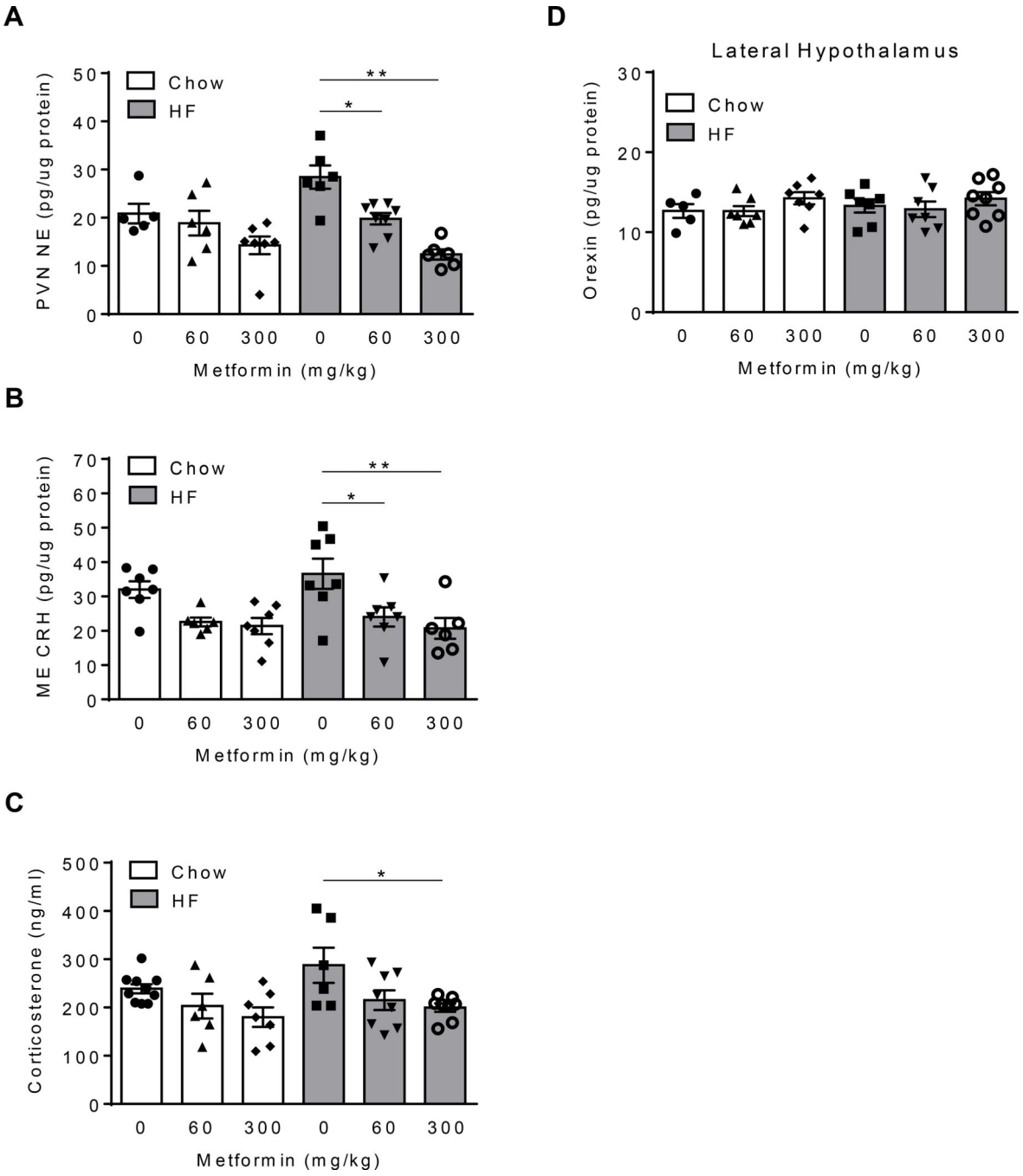

Fig. 4. Metformin dose-dependently lowers PVN NE and suppresses the HPA axis.

The effects of oral metformin treatment on **A)** NE concentrations in the PVN, **B)** CRH levels in the ME, and **C)** serum corticosterone, and **D)** orexin levels in the lateral hypothalamus. n=6–9 per group. * $p<0.05$; ** $p<0.01$.

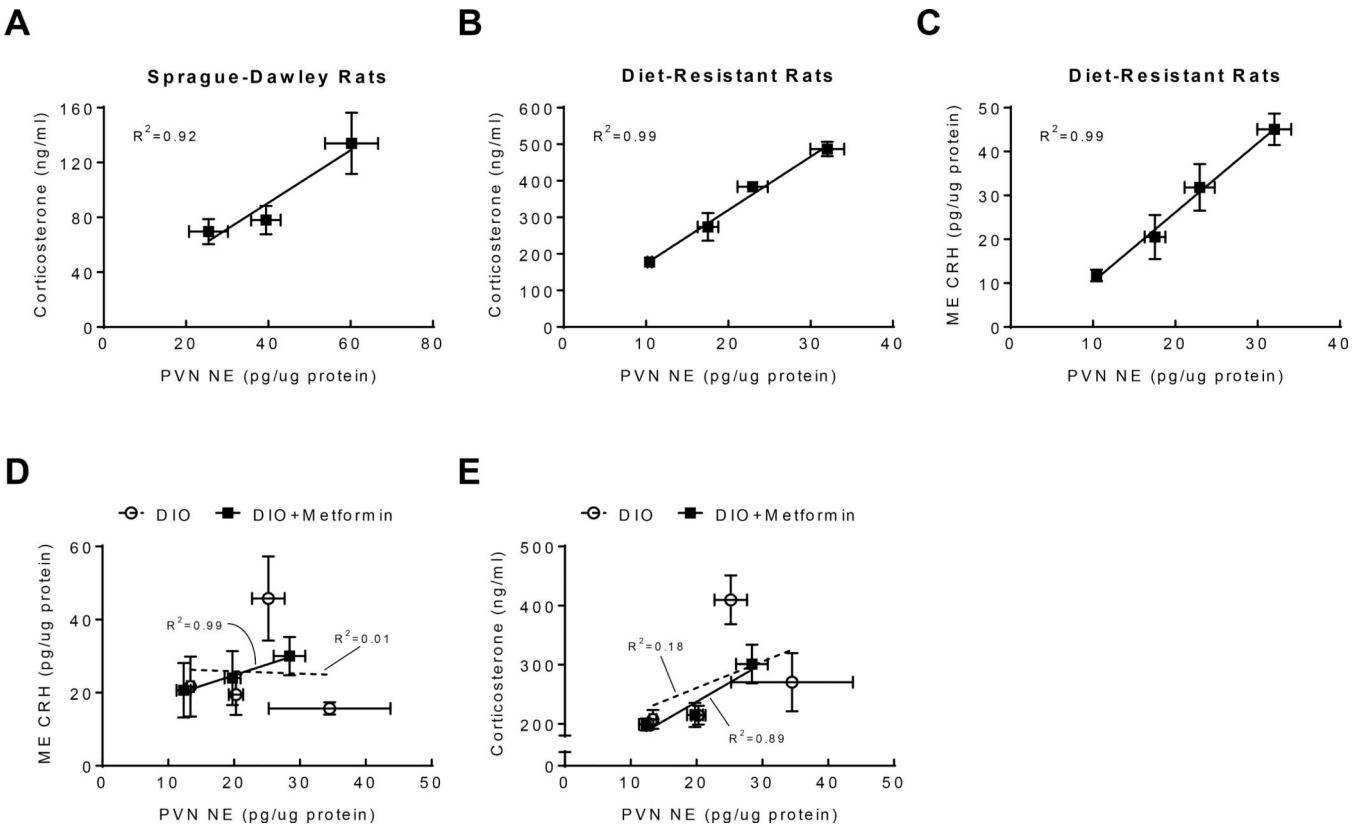

Fig. 5. Metformin effectively rescues the coupling between PVN NE and the HPA axis in DIO rats.

Type II regression analysis between **A)** PVN NE vs. serum corticosterone in lean Sprague-Dawley rats, **B)** PVN NE vs. serum corticosterone and **C)** PVN NE vs. ME CRH in obesity-resistant DR rats, **D)** PVN NE vs. ME CRH and **E)** PVN NE vs. serum corticosterone in DIO rats and DIO rats treated with metformin. Lines of best fit and R^2 are indicated. n=4–7 per group.

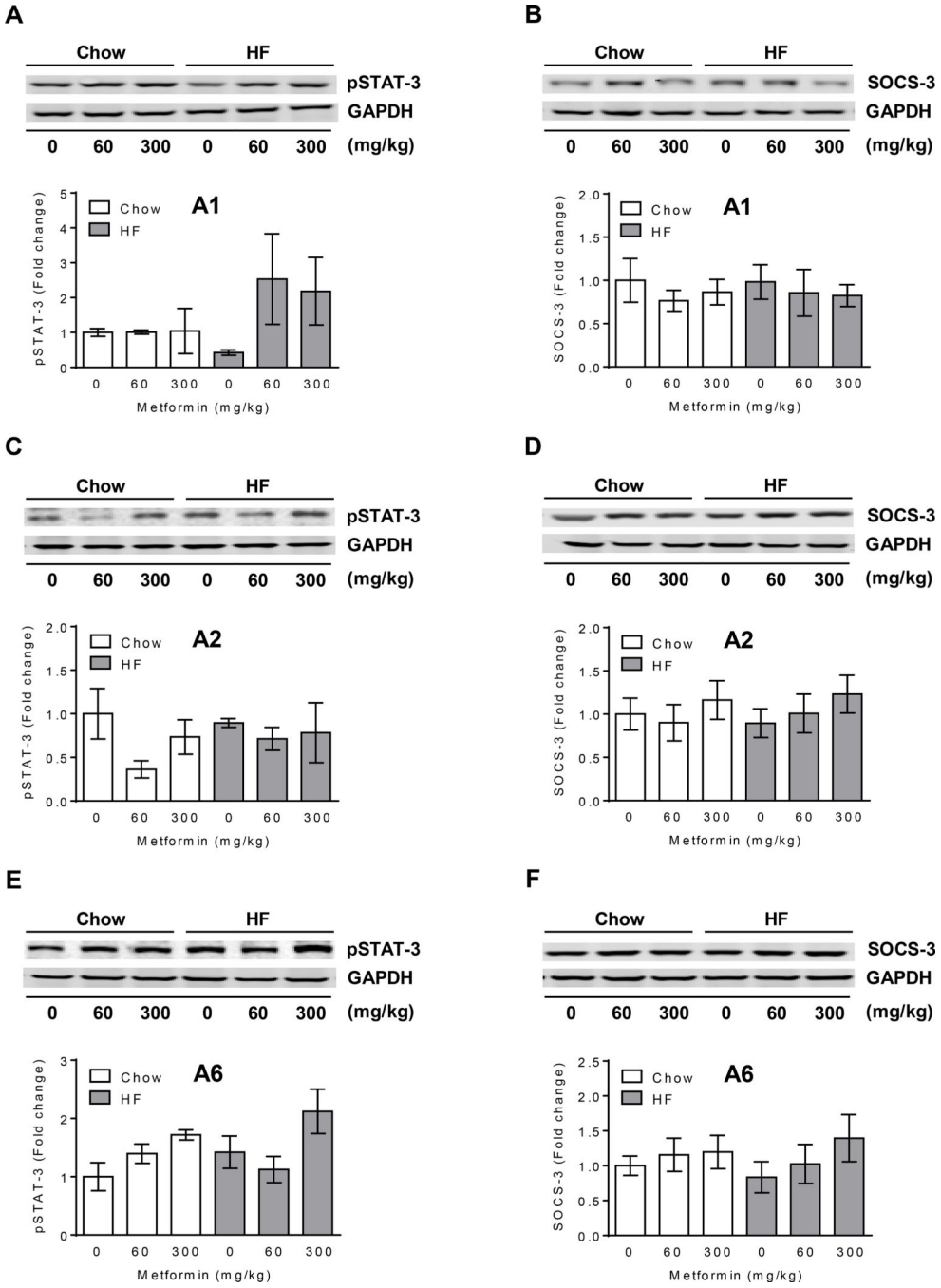

Fig. 6. No change in brainstem leptin signaling following metformin supplementation.

Western blot results of pSTAT-3 and SOCS-3 in brainstem noradrenergic nuclei (A1, A2, A6) in control or metformin-treated DIO rats on either chow or HF diet. **A, B)** pSTAT-3 and SOCS-3 expression in the A1 region, **C, D)** A2 region, and **E, F)** A6 region. Data are normalized to GAPDH and expressed as fold change from regular chow-fed DIO group. n=4–5 per group.

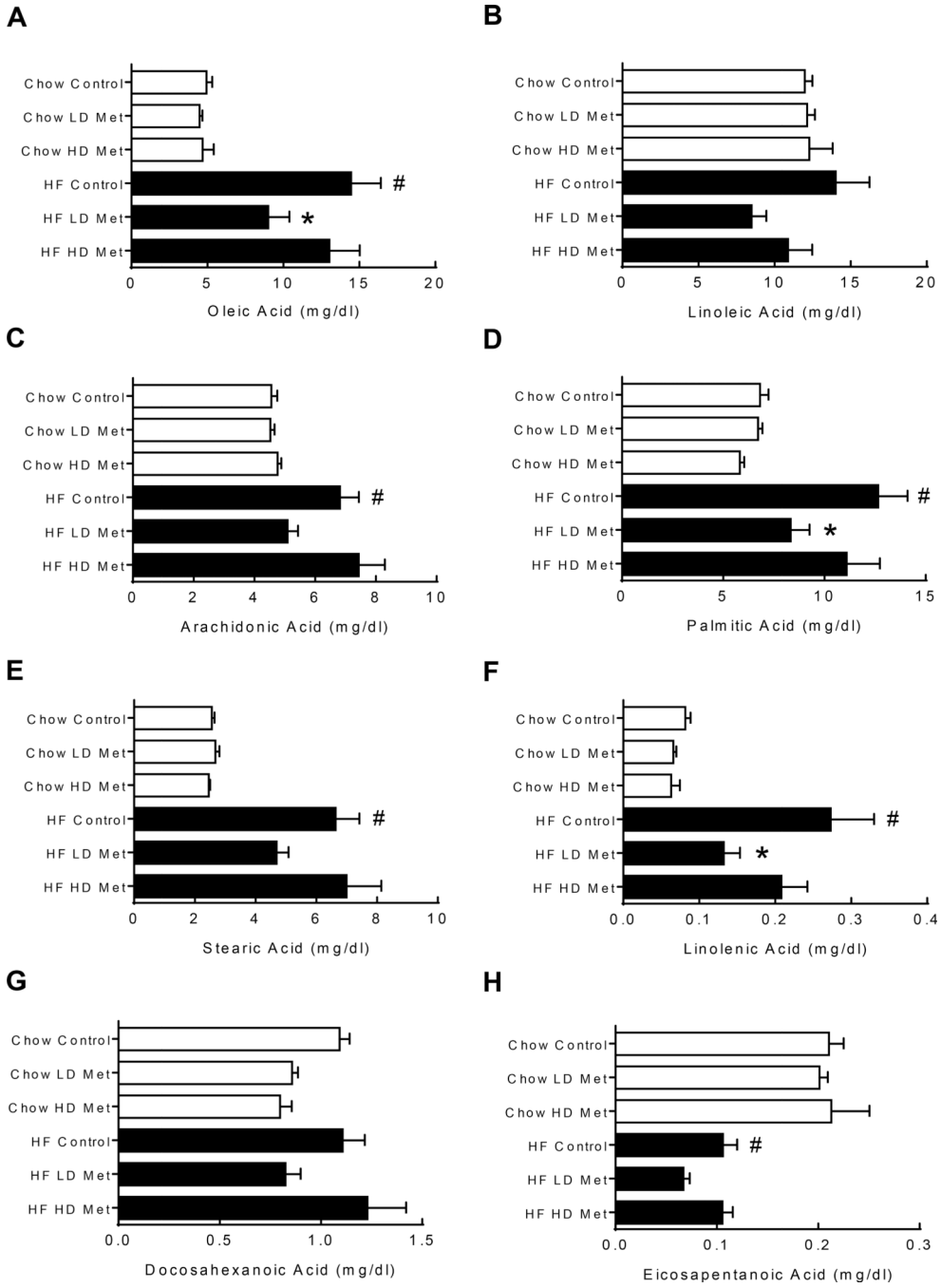

Fig. 7. Metformin treatment reduces circulating free fatty acids in HF-fed DIO rats.

At the end of metformin treatment, both unsaturated and saturated serum free fatty acids from DIO rats on regular chow or HF diet were analyzed by gas chromatography. **A)** oleic acid, **B)** linoleic acid, **C)** arachidonic acid, **D)** palmitic acid, **E)** stearic acid, **F)** linolenic acid, **G)** docosahexanoic acid, and **H)** eicosapentanoic acid. n=6–9 per group. * indicates statistically significant difference compared to HF controls. # indicates significant difference compared to all chow-fed groups.