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RESEARCH ARTICLES

Capsaicin-induced transcriptional changes in hypothalamus and alterations in gut microbial count in high fat diet fed mice

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Abstract

Obesity is a global health problem and recently it has been seen as a growing concern for developing countries. Several bioactive dietary molecules have been associated with amelioration of obesity and associated complications and capsaicin is one among them. The present work is an attempt to understand and provide evidence for the novel mechanisms of anti-obesity activity of capsaicin in high fat diet (HFD)-fed mice. Swiss albino mice divided in three groups (n=8–10) i.e. control, HFD fed and capsaicin (2 mg/kg, po)+HFD fed were administered respective treatment for 3 months. After measuring phenotypic and serum related biochemical changes, effect of capsaicin on HFD-induced transcriptional changes in hypothalamus, white adipose tissue (WAT) (visceral and subcutaneous), brown adipose tissue (BAT) and gut microbial alterations was studied and quantified. Our results suggest that, in addition to its well-known effects, oral administration of capsaicin (a) modulates hypothalamic satiety associated genotype, (b) alters gut microbial composition, (c) induces "browning" genotype (BAT associated genes) in subcutaneous WAT and (d) increases expression of thermogenesis and mitochondrial biogenesis genes in BAT. The present study provides evidence for novel and interesting mechanisms to explain the anti-obesity effect of capsaicin.

Keywords: Capsaicin; Anti-obesity; TRPV1; Hypothalamus; Gut microflora

1. Introduction

Obesity is a global health problem and a growing concern for both developed and developing countries [1]. Current anti-obesity medications are associated with undesirable side effects upon chronic usage [2], leaving diet and physical exercise as the most effective means for obesity management. Several bioactive dietary molecules have been associated with amelioration of obesity and associated complications [3,4]. Capsaicin, the major capsaicinoids of red chili pepper, is well known for its potential anti-cancer,

Abbreviations: *ACTB*, actin, beta; *ACOX1*, acyl-CoA oxidase 1; *ADCYAP1R1*, adenylate cyclase activating polypeptide 1 receptor 1; *ADIPOQ*, adiponectin C1Q and collagen domain containing; *ADIPOR1*, adiponectin receptor 1; *ADIPOR2*, adiponectin receptor 2; *ADRA2B*, adrenergic receptor alpha 2b; *BDNF*, brain derived neurotrophic factor; *CARTPT*, CART prepropeptide; *CCK*, cholecystokinin; *CNR1*, cannabinoid receptor 1 (brain); *CPD*, carboxypeptidase D; *CIDEA*, cell death activator CIDE-A; *DRD1A*, dopamine receptor D1A; *DRD2*, dopamine receptor D2; *FASN*, fatty acid synthase; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *GALR1*, galanin receptor 1; *GHRL*, ghrelin; *GHSR*, growth hormone secretagogue receptor; *GPD1*, glycerol-3-phosphate dehydrogenase 1; *GRP*, gastrin releasing peptide; *GUSB*, glucuronidase beta; *HCRT*, hypocretin; *HCRTR1*, hypocretin (orexin) receptor 1; *HPRT*, hypoxanthine-guanine phosphoribosyl transferase; *HSP90AB1*, heat shock protein 90 alpha (cytosolic) class B member 1; *IAPP*, islet amyloid polypeptide; *IL1A*, interleukin 1 alpha; *IL1R1*, interleukin 1 receptor type I; *IL6RA*, interleukin 6 receptor alpha; *INSR*, insulin receptor; *LEP*, leptin; *MCHR1*, melanin-concentrating hormone receptor 1; *MAPK14*, mitogen-activated protein kinase 14; *NMB*, neuromedin B; *NPY*, neuropeptide Y; *NPY1R*, neuropeptide Y receptor Y1; *NR3C1*, nuclear receptor subfamily 3 group C, member 1; *NTRK2*, neurotrophic tyrosine kinase receptor, type 2; *NTSR1*, neurotensin receptor 1; *NRP1*, nuclear receptor-interacting protein 1; *NCOA1*, nuclear receptor coactivator 1; *PYY*, peptide YY; *PGC1α*, PPAR-gamma co-activator-1 alpha; *PPARα*, peroxisome proliferator-activated receptor alpha; *POMC*, pro-opiomelanocortin-alpha; *PTGS2*, prostaglandin-endoperoxide synthase 2; *RAMP3*, receptor (calcitonin) activity modifying protein 3; *SIRT1*, sirtuin 1; *SORT1*, sortilin 1; *UCP1*, uncoupling protein 1; *UCN*, urocortin.

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antioxidant, anti-inflammatory, antimicrobial and anti-obesity properties [5,6].

Capsaicin's role in enhancing energy expenditure (EE) and body weight regulation in both rodents and animals has been extensively studied. Among potential molecular mechanisms linked to anti-obesity action of capsaicin, capsaicin-induced TRPV1 activation appears to be critical. TRPV1 activation *via* capsaicin blocked *in vitro* adipogenesis as well as prevented high fat diet (HFD)-induced obesity [7]. It enhanced thermogenesis and heat diffusion *via* gastrointestinal TRPV1 activation in mice [8]. Similarly, capsaicin-induced TRPV1 activation in skeletal muscle leads to improved energy metabolism *via* activation of *PGC1α* and its target genes involved in fatty acid oxidation and mitochondrial biogenesis [9]. Capsaicin has been reported to increase satiety and reduce energy intake [10], whereas some authors found no effect of capsaicin on satiety although it boosted thermogenesis in humans [11]. In humans, a meta-analysis of 20 trials found a modest benefit of capsaicin related to body weight gain *via* increase in EE [12]. It also counteracted the effect of negative energy balance on EE and increased fat oxidation in Caucasian subjects [13]. Increased EE *via* capsaicin-induced TRPV1 activation can be a result of catecholamine release that activated the sympathetic nervous system *via* β-adrenoceptors [14]. Other reported effects of capsaicin include up-regulation of thermogenesis and lipid metabolism related proteins in white adipose tissue (WAT) of HFD-fed rats and enhanced EE in humans *via* brown adipose tissue (BAT) activation [5,15].

There is critical involvement of hypothalamus in energy intake and expenditure [16]. Recent reports have suggested the presence of TRPV1 in different regions of the hypothalamus including arcuate nucleus [17]. It is very well studied that TRPV1 containing neurons are co-expressed with multiple neuropeptides like CGRP, NPY and SP (neuropeptides with potential role in satiety and anxiety) [18]. Hence, it is worth studying the effect of capsaicin administration on TRPV1 expression and expression of several anorectic and orexigenic genes in HFD-induced obese animals.

Recently the active role of human and rodent gastrointestinal tract microbes in the development of obesity has been suggested. Intestinal microbiota influences energy harvest, secretes lipopolysaccharide (LPS) and alters intestinal permeability [19]. Gut microflora also regulates brain function *via* CNS [20]. Very recently oral administration of live *Akkermansia muciniphila* has been shown to prevent diet-induced obesity by altering adipose tissue metabolism and gut permeability without affecting food intake [21]. It is well known that gut microbial communities can be manipulated by antibiotics, dietary fibers and other dietary molecules [22]. Intentional gut microbial manipulation can be a potential strategy to prevent obesity. Since chili peppers are common ingredient of the diet across the world, the effect of capsaicin on gut microflora may be of considerable interest. A recent *in vitro* study showed that red chili enhanced the production of L-lactate by increasing metabolic activity of *Lactobacillus acidophilus* [23]. However, the influence of capsaicin on gut microbes in normal and in HFD-induced obese rodents' models/humans has not yet been established.

Therefore, the present study is an attempt to understand the novel mechanisms of anti-obesity activity of capsaicin with special focus on nutrigenomic changes in hypothalamus, BAT and microbiome related changes in HFD-fed LACA mice.

2. Materials and methods

2.1. Materials

Regular rodent diet (D12450B) and HFD (D12492, 45% fat) were purchased from Research Diets (New Brunswick, NJ, USA). Capsaicin (≥95%) was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). All other reagents were of the highest commercially available grade.

2.2. Animal experiments

Male Swiss albino mice (5–6 weeks old; 25±3 g) were used in the study. Animals were housed in the Central Animal Facility of the National Institute of Pharmaceutical Education and Research (NIPER) under standard laboratory conditions (temperature, 22±2°C; humidity, 55±5%), having free access to food and water along with 12 h light and dark cycles. All experimental procedures were approved by Institutional Animal Ethical Committee, NIPER and conducted according to the Committee for the Purpose of Control and Supervision on Experiments on Animals guidelines on the use and care of experimental animals. After 1 week acclimatization, animals were randomly divided into three groups (n=8): normal diet (Ctrl) group, HFD group and HFD with capsaicin intervention (HFD+Cap) group. Each mice in HFD+Cap group orally received 2 mg/kg body weight of capsaicin (dissolved in 0.9% saline with 3% ethanol and 10% tween 80) on alternate days. Ctrl and HFD groups received the corresponding vehicle. Body weight and food intake were recorded every week. The study was conducted for a total of 12 weeks. At the end of the feeding period, blood was collected and animals were killed by cervical dislocation. Visceral white adipose tissue (vWAT), subcutaneous white adipose tissue (sWAT), BAT, hypothalamus, skeletal muscle and liver were isolated and samples were snap frozen and stored at −80°C for subsequent RNA isolation and gene expression analysis. The cecum was dissected and its content was isolated aseptically, weighed and snap frozen for extraction of bacterial DNA.

2.3. Blood analysis

Blood glucose levels were measured using glucometer (One Touch Ultra, LifeScan, West Chester, PA, USA). Serum was separated immediately by centrifugation of the blood samples (1013*g* for 15 min at 4°C). Serum leptin, adiponectin, TNFα and VEGF levels were determined by enzyme-linked immunosorbent assay (using commercial kits; Invitrogen, Camarillo, CA, USA) as per the manufacturer's instructions.

2.4. RNA isolation and quantification of genes

Total RNA was extracted from hypothalamus, vWAT, sWAT and BAT using ribopure RNA extraction kit (Invitrogen, USA) according to manufacturer's instructions. The quantification and qualitative ratiometric analysis of RNA was done using Infinite M200 ProNanoQuant (Tecan, Switzerland). Integrity of the RNA samples was evaluated using 1.4% agarose gel. Pure RNA was used for real-time polymerase chain reaction (RT-PCR) analysis.

One microgram of total RNA from hypothalamus, vWAT, sWAT and BAT was reverse transcribed using single strand cDNA synthesis kit (SABiosciences, Qiagen, USA) as per manufacturer's instructions. Relative expression of *TRPV1* gene (in hypothalamus), different obesity and thermogenic genes was determined by quantitative PCR (Applied Biosystems 7500 Fast Real-Time PCR machine) using SYBR green based custom designed mouse PCR array (CAPM 11044, PAMM-017ZA and CAPM 11784; Custom Mouse RT^2 profile PCR array, SABiosciences, Qiagen, USA). The conditions for RT-PCR were as follows: 95°C for 10 min, followed by 40 cycles of 95°C and 60°C for 1 min. Data were analyzed using $\Delta\Delta C_t$ method provided by SABiosciences, Qiagen, USA with normalization of obesity genes expression by geometric mean of five housekeeping (*GAPDH*, *ACTB*, *HSP90AB1*, *HPRT* and *GUSB*) genes, thermogenic genes expression by *18sRNA* and *TRPV1* gene expression by *GAPDH*.

2.5. DNA isolation from cecal contents and quantification of different microbial groups

About 100 mg of cecal content from each animal was taken and DNA was isolated using the QIAamp DNA Stool Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. DNA was quantified using Infinite M200 ProNanoQuant (Tecan, Switzerland). Integrity of the DNA samples was evaluated using 0.8% agarose gel. Real-time quantification of total bacteria, *Bacteroidetes* (BACT), *Firmicutes* (FIRM), *Lactobacillus* (LAB), Bifidobacteria (BIF), *Akkermansia* (AKK), *Enterobacteriaceae* (ENTB) and *Bacteroides-Prevotella* (BP), was performed as described above. Genus specific primer sets used for these groups are listed in Table 1. Total bacterial proportion was normalized and then the proportion of each bacterial group was compared with that from control which was kept as unity.

Table 1
List of primers used in the study

Gene name	Forward primer 5′–3′	Reverse primer 5′–3′
Lactobacillus (LAB)	CACCGCTACACATGGAG	AGCAGTAGGGAATCTTCCA
Bifidobacteria (BIF)	TCGCGTCYGGTGTGAAAG	CCACATCCAGCRTCCAC
Enterobacteriaceae (ENTB)	CATGACGTTACCCGCAGAAG	CTCTACGAGACTCAAGCTTG
Bacteroidetes (BACT)	ACGCTAGCTACAGGCTTAACA	ACGCTACTTGGCTGGTTCA
Firmicutes (FIRM)	GCGTGAGTGAAGAAGT	CTACGCTCCCTTTACAC
Bacteroides Prevotella (BP)	GGTGTCGGCTTAAGTGCCAT	CGGAYGTAAGGGCCGTGC
Akkermansia (AKK)	CAGCACGTGAAGGTGGGGAC	CCTTGCGGTTGGCTTCAGAT

2.6. Endogenous antioxidant status of liver and skeletal muscle

A 10% (w/v) each of liver and skeletal muscle homogenate was prepared in 0.1 M phosphate buffer (pH 7.4) and centrifuged at 10,000*g* for 15 min, and aliquots of supernatant was separated and used for estimating reduced glutathione (GSH), superoxide dismutase (SOD), catalase and nitrite activities [24].

2.7. Skeletal muscle — Mitochondrial complex activity

Mitochondrion-enriched supernatants were prepared from skeletal muscle. Different mitochondrial complex activities such as Complex I (NADH dehydrogenase), Complex II (succinate dehydrogenase), Complex III (MTT activity) and Complex IV (cytochrome oxidase) activities were estimated spectrophotometrically [24].

2.8. Statistical analysis

The data are expressed as mean±S.E.M. Statistical analysis was performed using Prism GraphPad software (GraphPad Software Inc., San Diego, CA, USA). Unless otherwise stated, one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was applied to check the level of significance. In all the tests, $P<.05$ was considered as statistically significant.

3. Results

3.1. Effects of capsaicin on body weight gain, food intake and blood glucose

HFD-fed mice showed significant increase in body weights at 12th week compared to mice fed with control diet (Fig. 1A). Significant body weight reduction was observed in the groups that fed with capsaicin along with HFD. HFD-fed mice co-treated with capsaicin had significantly inhibited this increase in body weight (Fig. 1A). In addition, capsaicin reduced epididymal fat in HFD-fed mice (data not shown). No significant difference was observed in fasting glucose levels as well as in daily food intake among all the three groups because of pair feeding (Fig. 1B and C).

3.2. Effect of capsaicin on serum adipokines

Leptin secretion was significantly increased in HFD-fed mice in comparison to control mice. However, co-administration of capsaicin with HFD significantly inhibited the leptin levels in comparison to HFD group. TNFα, a pro-inflammatory molecule, was significantly enhanced in HFD group as compared to control whereas its levels were significantly reduced in HFD mice fed with capsaicin (Fig. 1D). HFD significantly raised the adiponectin levels whereas capsaicin supplementation significantly down-regulated their levels. No change was observed in VEGF levels of all three groups (data not shown).

3.3. Effect of capsaicin on hypothalamic gene expression

Immunofluorescence imaging showed the widespread expression of TRPV1 immunoreactivity in hypothalamus and arcuate nucleus of mice (unpublished data from Dr. Premkumar laboratory). Further, effect of capsaicin on expression levels of *TRPV1* gene in hypothalamus of mice was studied. *TRPV1* was expressed in hypothalamus of control mice whereas its expression was significantly down-regulated in HFD-fed mice. However, capsaicin increased its expression levels comparable to control in HFD-fed mice (Fig. 2A). Further, to study the effect of capsaicin, various anorectic and orexigenic genes were evaluated in hypothalamus. Anorectic genes such as *UCN*, *PYY*, *RAMP3*, *GRP*, *BDNF* and *CARTPT* were significantly down-regulated in HFD-fed mice as compared to Ctrl group whereas their expression was significantly enhanced in HFD+Cap group (Fig. 2B). Expression

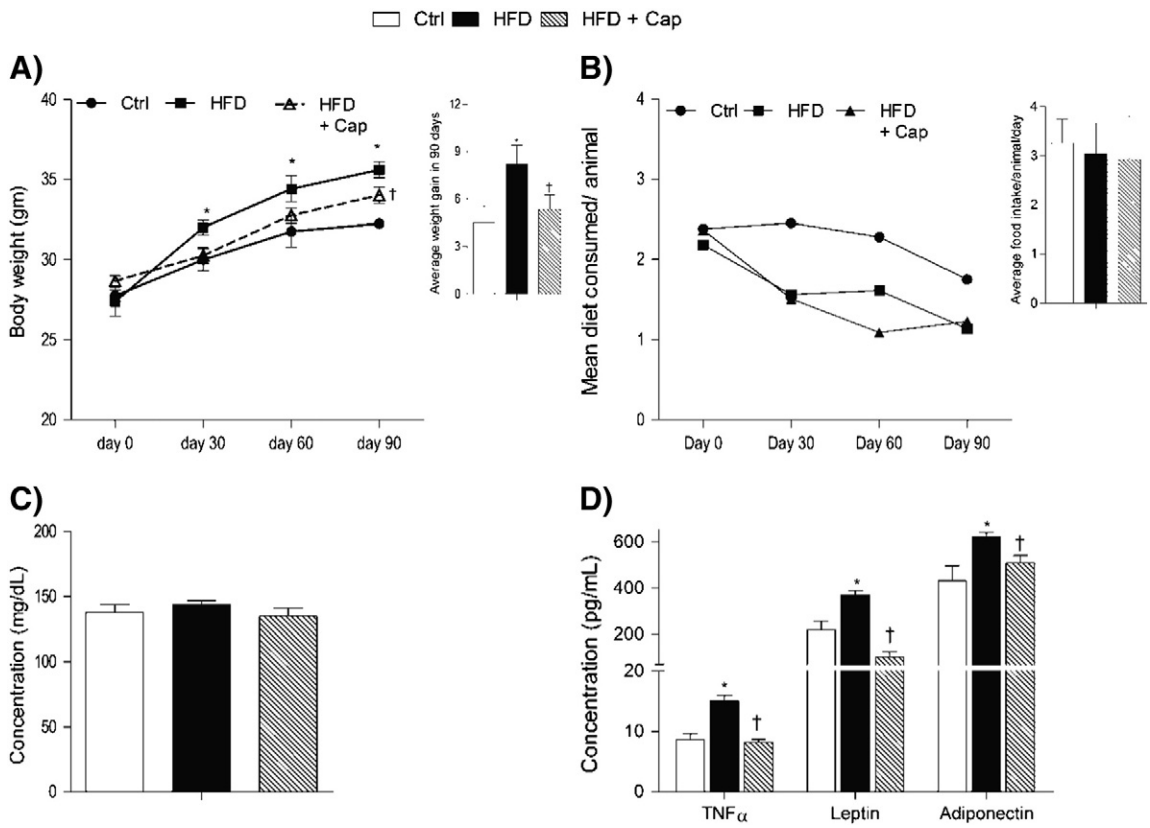

Fig. 1. Effect of capsaicin on HFD-induced phenotypic changes and blood biochemical parameters. (A and B) Effect of capsaicin on body weight and food intake of mice induced by 3 months of high-fat feeding. (C) Effect of capsaicin on blood glucose levels. (D) Changes in serum levels of TNFα, leptin and adiponectin. All values are expressed as mean±S.E.M. One-way ANOVA followed by Tukey's multiple comparison *post hoc* test was applied. *$P<.05$ versus control group, †$P<.05$ versus HFD group.

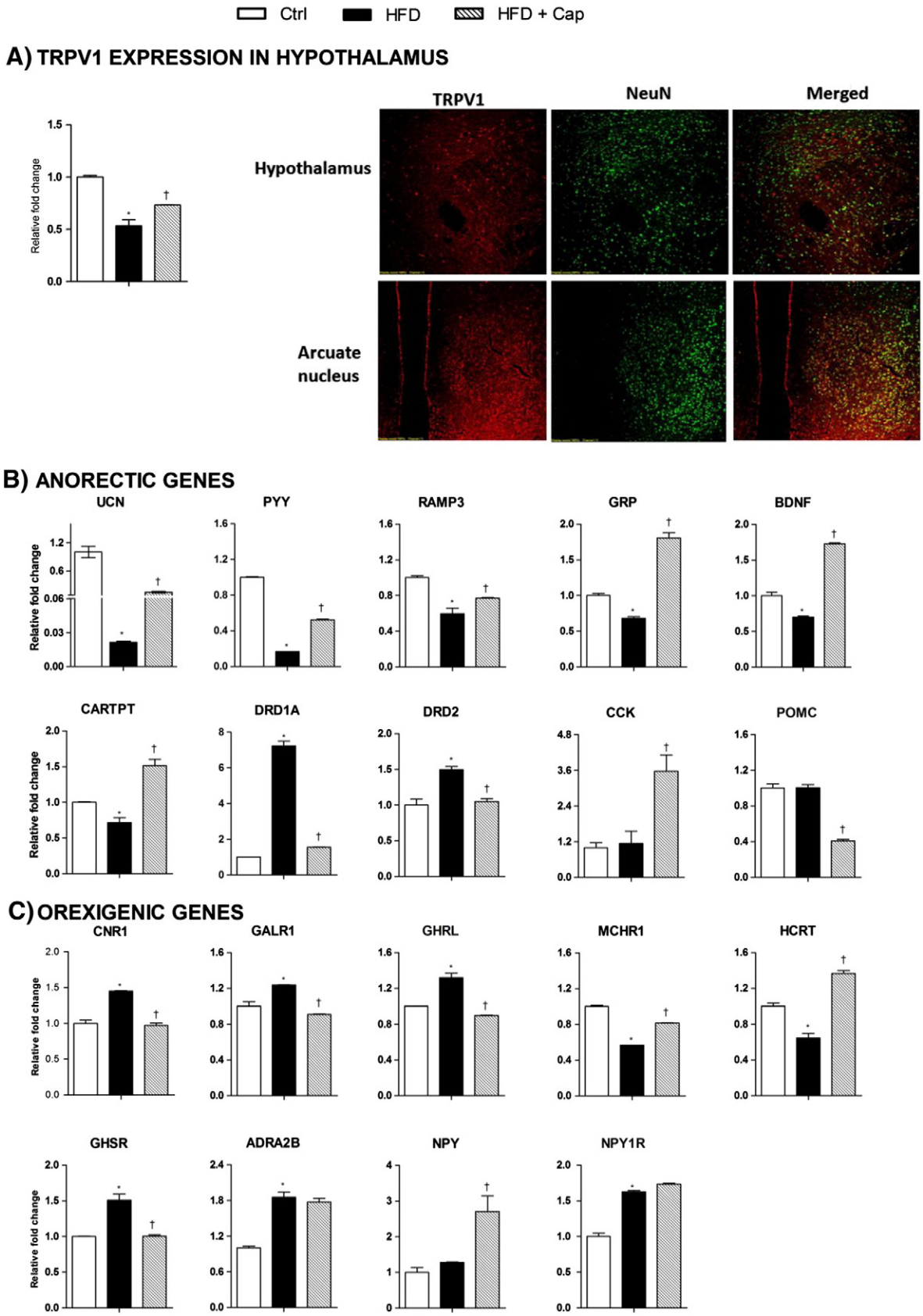

Fig. 2. Expression of TRPV1 in hypothalamus and capsaicin mediated transcriptional changes in TRPV1 expression and hypothalamus genotype. (A) RT-PCR analysis of TRPV1 mRNA in hypothalamus and co-immunofluorescence imaging of TRPV1 and NeuN in hypothalamus and arcuate nucleus. (B and C) Capsaicin-induced changes in expression levels of anorectic and orexigenic genes in hypothalamus of HFD-fed mice. All values are expressed as mean±S.E.M. One-way ANOVA followed by Tukey's multiple comparison *post hoc* test was applied. **P*<.05 versus control group, †*P*<.05 versus HFD group.

of *DRD1A* and *DRD2* was significantly enhanced in HFD group compared to Ctrl. However, capsaicin treatment lowered the expression levels significantly (Fig. 2B). No significant difference in the expression levels of *CCK* and *POMC* was observed in Ctrl and HFD group; however, HFD+Cap feeding significantly enhanced the levels of *CCK* and decreased the levels of *POMC* compared to HFD group.

HFD significantly increased the expression of orexigenic genes like *CNR1*, *GALR1*, *GHRL*, *ADRA2B*, *NPY1R* and *GHSR* (Fig. 2C). However, their expression, except for *ADRA2B* and *NPY1R*, was significantly lowered in HFD+Cap group (Fig. 2C). HFD induced significant decrease in *MCHR1* and *HCRT* that roused to levels comparable to control upon capsaicin administration (Fig. 2C). No change was observed in *NPY* expression levels in HFD group whereas its expression was significantly increased in HFD+Cap group (Fig. 2C).

3.4. Effect of capsaicin supplementation on "browning" genes in BAT, sWAT and vWAT

Capsaicin's effect on selected thermogenic and related genes such as *BDNF*, *UCP1*, *NRIP1*, *NCOA1*, *CIDEA*, *PPARα*, *PGC1α*, *MAPK14*, *SIRT1* and *PTGS2* in BAT, sWAT and vWAT was evaluated. In BAT, HFD significantly increased the expression of these genes as compared to control animals whereas their expression levels were further significantly up-regulated upon capsaicin administration in comparison to HFD group (Fig. 3). Similarly, in vWAT, these genes were significantly increased in HFD-fed mice but no significant difference was observed in capsaicin-treated group as compared to control group (Fig. 3). Different trend in expression levels of these genes was observed in sWAT. *BDNF*, *NCOA1*, *PPARα* and *PTGS2* showed similar pattern as that of BAT i.e. significant increase in expression in HFD group and further increase in HFD+Cap group (Fig. 3) whereas *UCP1*, *NRIP1*, *CIDEA*, *PGC1α*, *MPAK14* and *SIRT2* were significantly down-regulated in HFD group as compared to control whereas increased significantly in HFD+Cap group (Fig. 3).

3.5. Capsaicin supplementation on anorectic, orexigenic and EE related genes in WAT

Anorectic genes such as *BDNF*, *DRD1A*, *DRD2* and *PYY* were significantly up-regulated in HFD group as compared to control whereas significant reduction in expression level was observed in HFD+Cap group (Fig. 4A). On the other hand, HFD significantly down-regulated the expression level of other anorectic genes such as *IAPP*, *IL1A*, *IL1R1*, *IL6RA*, *INSR*, *LEP*, *NMB*, *NTRK2*, *NTSR1* and *SORT1* (Fig. 4A). Capsaicin administration significantly up-regulated their expression levels in comparison to HFD group, except for *NMB* and *SORT1* showing no change in their levels whereas NTSR1 level was significantly reduced further (Fig. 4A). HFD significantly up-regulated the expression levels of orexigenic genes *GALR1*, *HCRT* and *NR3C1* which upon capsaicin administration were significantly decreased as compared to HFD group (Fig. 4B). No significant change was observed in expression levels of *GHRL* and *HCRTR1* in HFD group as compared to control but they were significantly lowered in HFD+Cap group (Fig. 4B). EE related genes such as *ADCYAP1R1*, *ADIPOQ*, *ADIPOR1*, *ADIPOR2* and *CPD* were significantly reduced in HFD group as compared to Ctrl group whereas capsaicin administration significantly raised their levels (Fig. 4C).

Effect of capsaicin supplementation in HFD-induced changes in expression levels of metabolic genes i.e. *FASN*, *GPD1* and *ACOX1* was evaluated in sWAT and vWAT. HFD significantly down-regulated the expression levels of all three genes in sWAT (Fig. 4D) as well as in vWAT except for *ACOX1* where no significant change was observed (Fig. 4D). Capsaicin supplementation significantly enhanced the expression levels of *GPD1* and *ACOX1* in sWAT and vWAT (Fig. 4D and E). *FASN* was significantly increased in HFD+Cap group in sWAT whereas no significant change was observed in vWAT (Fig. 4D and E).

3.6. Effect of capsaicin on different bacterial groups in cecum

Enterobacteriaceae and *Firmicutes* abundance was significantly higher in cecal contents of HFD-fed mice as compared to Ctrl whereas capsaicin supplementation significantly lowered their abundance (Fig. 5). Abundance of *Akkermansia*, *Bacteroidetes* and *Bacteroides-Prevotella* was significantly lowered in HFD group in comparison to Ctrl. Significant increase in their abundance was observed in HFD+Cap group (Fig. 5). Bifidobacteria and *Lactobacillus* abundance was significantly decreased in HFD group as compared to Ctrl. Bifidobacteria further showed significant decrease whereas *Lactobacillus* were found to be significantly higher in HFD+Cap group as compared to HFD group (Fig. 5).

3.7. Effect of capsaicin on endogenous antioxidant profile in liver and muscle

No significant difference was observed in GSH levels in liver of animals among three groups but was significantly reduced in skeletal muscle of HFD-fed group compared to control and capsaicin supplementation significantly restored its levels (Fig. 6A). No significant change in SOD in liver and skeletal muscle of animals in Ctrl and HFD group was observed. Capsaicin supplementation

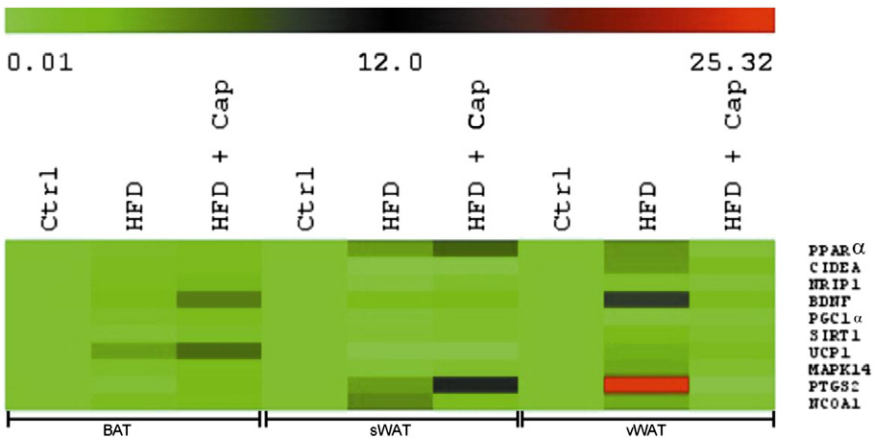

Fig. 3. Effect of capsaicin on HFD-induced changes in browning genes (mitochondrial biogenesis and thermogenesis related genes) in BAT, sWAT and vWAT.

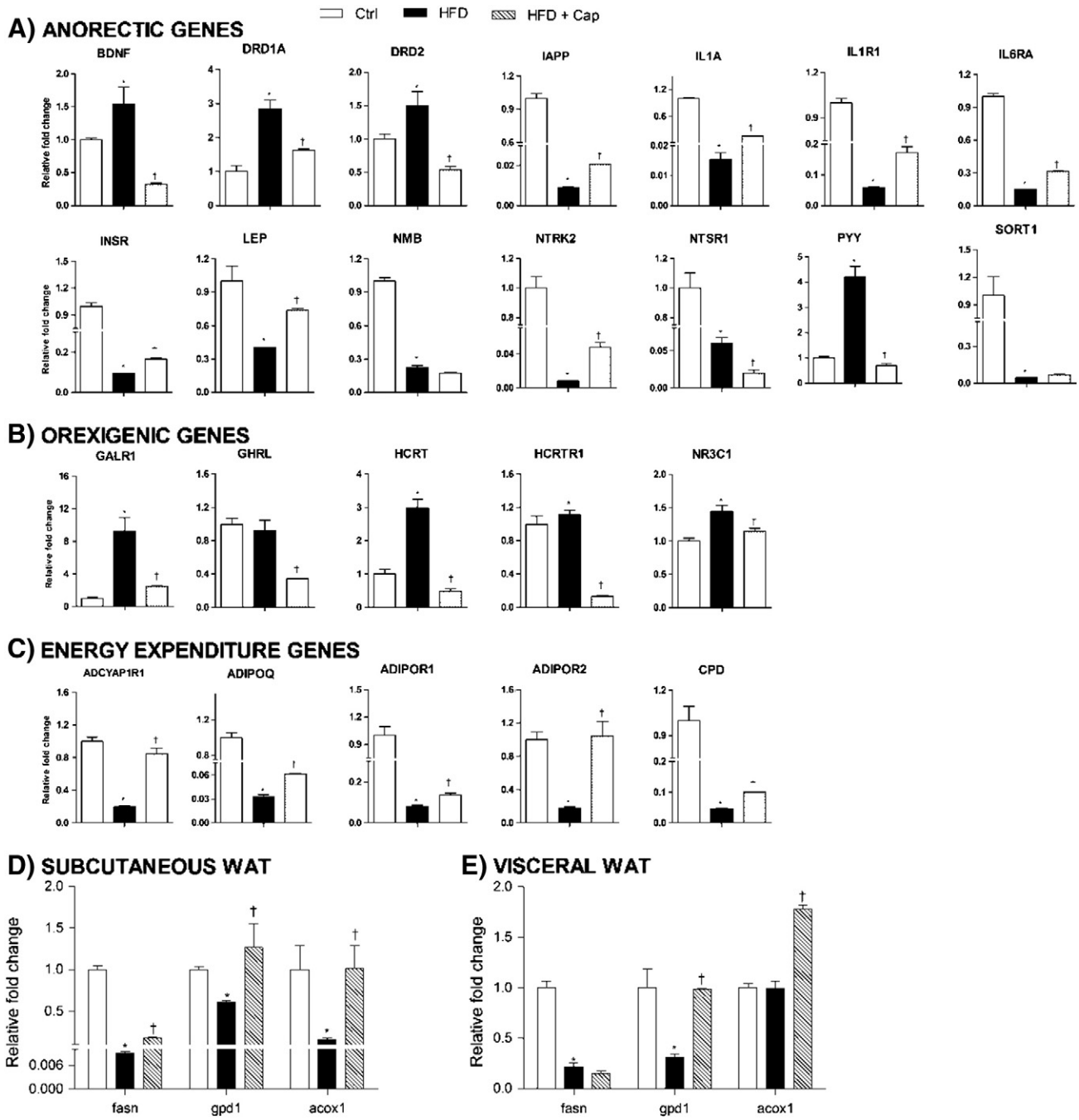

Fig. 4. Capsaicin-induced transcriptional changes in vWAT and sWAT of HFD-fed mice. (A–C) Effect of capsaicin on expression levels of anorectic, orexigenic and EE related genes in vWAT. (D and E) Effect of capsaicin on expression levels of genes, involved in lipid metabolism, in sWAT and vWAT. Data are shown mean±S.E.M. One-way ANOVA followed by Tukey's multiple comparison *post hoc* test was applied. *P<.05 versus control group, †P<.05 versus HFD group.

significantly improved its levels (Fig. 6B). Catalase activity in liver and skeletal muscle was significantly decreased in HFD-fed animals whereas capsaicin supplementation significantly improved its levels in both tissues (Fig. 6C).

3.8. Mitochondrial complex activities in skeletal muscle

HFD significantly impaired all the mitochondrial enzyme activities (complex I, II, III and IV) compared to control group. Capsaicin supplementation along with HFD significantly restored the activities of complex I and IV (Fig. 6D). Complex II activity did not showed significant change in animals fed with HFD and HFD+Cap, whereas complex III activity was significantly lowered in HFD+Cap group compared to HFD group (Fig. 6D).

4. Discussion

In the present study, we provide a novel anti-obesity action of orally administered capsaicin. Oral administration of capsaicin (a) modulates hypothalamic gene pattern, (b) induces “browning” genotype (BAT associated genes) in sWAT, (c) increases expression

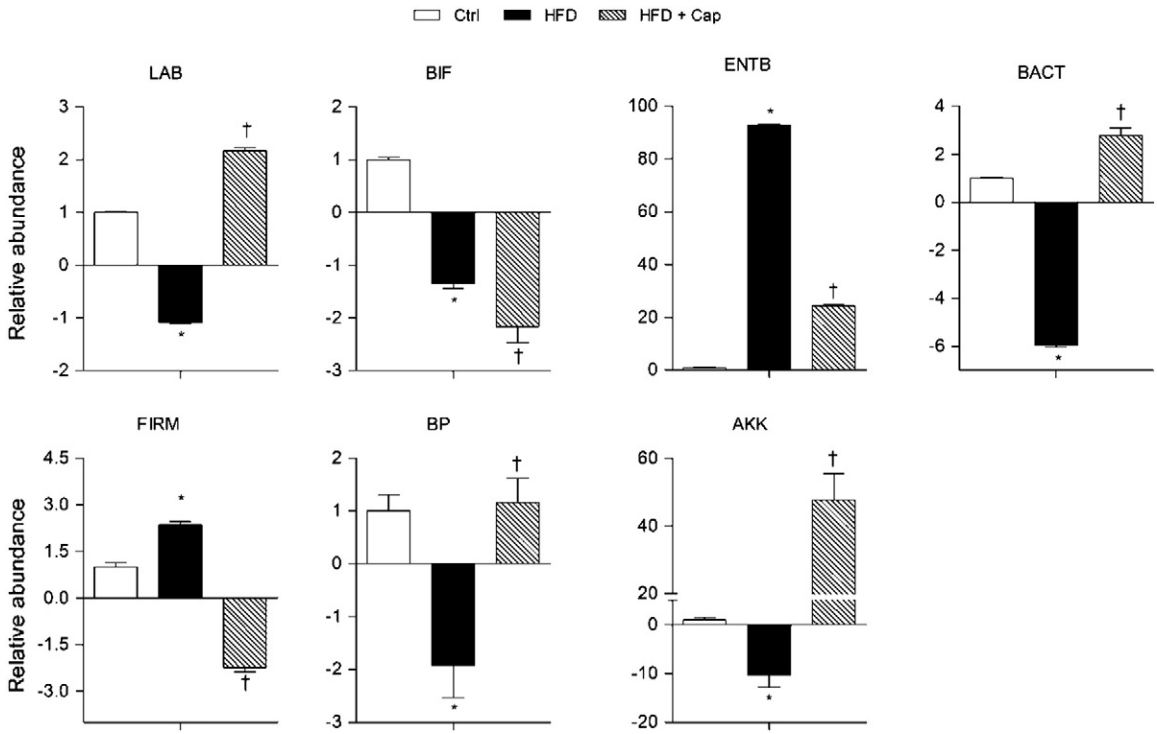

Fig. 5. Capsaicin reversed HFD-induced alteration in the microbial composition in cecal contents. Data are shown mean±S.E.M. One-way ANOVA followed by Tukey's multiple comparison *post hoc* test was applied. *P<.05 versus control group, †P<.05 versus HFD group.

level of thermogenesis and mitochondrial biogenesis related genes in BAT and (d) alters gut microbial composition.

Presence of TRPV1 in adipocytes and its resultant role in mediating the anti-obesity effect of capsaicin is still not conclusive. Zhang *et al.* demonstrated functional presence and role of TRPV1 in rodent and human adipocytes. In the same study, using 3T3-L1 cell lines, authors have shown that expression levels of TRPV1 were declined during the differentiation of preadipocytes into adipocytes [7]. In our *in vitro* studies, *TRPV1* expression significantly decreased over the course of differentiation with minimal expression in differentiated adipocytes (unpublished data). In rodent visceral adipocytes, we did not detect significant TRPV1 expression. This is in accordance with other studies (including studies with knockout animals), which were unable to detect role of TRPV1 convincingly neither in rodent nor in humans [25]. Multiple modes of anti-obesity action of capsaicin have been reported. Luo *et al.* suggested the role of capsaicin-induced TRPV1 activation in up-regulating *PGC1α* in skeletal muscle, improving energy metabolism and exercise endurance [9]. Induction of diet-induced thermogenesis, fat oxidation and decrease in appetite and triglyceride level in wild and genetically obese mice was reported by others [5,26,27]. Topical application of capsaicin has been shown to increase the expression of adiponectin and other adipokines, thus reducing fat accumulation in adipose tissue of obese mice [28]. Dietary capsaicin reduced obesity-induced glucose intolerance by suppressing inflammatory responses and enhancing fatty acid oxidation in adipose tissue and/or liver [28–30]. These studies established the anti-obesity effect of capsaicin *via* either TRPV1 dependent or independent. Our study corroborated previous findings that oral administration of capsaicin caused weight reduction in HFD-fed animals, improved oxidative stress in liver, increased mitochondrial efficacy in muscle, improved inflammatory make up of HFD-fed animal and gene related changes in visceral adipose tissue. More importantly, in the present manuscript, we have tried to establish novel mechanisms for the putative anti-obesity effect of capsaicin involving gut-brain and brain-adipose tissue axis.

Recent reports have confirmed that TRPV1 is not only expressed in nociceptors in primary sensory ganglia but also in other brain areas including hypothalamus (caudal) and arcuate nucleus [17]. Immunohistological results showed TRPV1 immunoreactivity in mouse hypothalamus and arcuate nucleus (ARC), which are major brain components involved in food regulation and EE (unpublished data from Dr. Premkumar laboratory). Our RT-PCR analysis showed expression of TRPV1 in murine hypothalamus that was down-regulated upon HFD feeding. Interestingly, it was restored upon capsaicin supplementation (2 mg/kg for 3 months). It is possible that TRPV1 activation in hypothalamus can be a result of signal transmission by capsaicin-sensitive neurons in the gut to hypothalamus in brain. Further capsaicin administration significantly altered the gene expression profile of hypothalamus in HFD-fed animals. Expression of anorectic genes like *UCN*, *PYY*, *RAMP3*, *GRP*, *BDNF*, *CARTPT* and so on was significantly decreased whereas capsaicin supplementation significantly increased the expression of orexigenic genes like *CNR1*, *GALR1*, *GHRL*, *ADRA2B* and *GHSR*. Expression of some of the anorectic and orexigenic genes remains unchanged (*CCK*, *POMC* and *NPY*) or opposite effect (*MCHRI*, *HCRT* and *NPY1R*). As there are more than 30 neuropeptides and their respective receptors present in hypothalamus and there is interdependency of these on each other, changes may have occurred in a few as a compensatory response to change in others due to common activating factor and mechanism. HFD induced significant increase in dopamine receptor gene expression (*DRD1A* and *DRD2*), which may be related to compensation related to anorexia and may be seen as defensive control on increase in dopaminergic activity in response to a positive energy balance in order to prevent addiction like behavior. Oral administration of capsaicin showed interesting changes in hypothalamic gene pattern like significant reversal of HFD-induced decrease in anorectic neuropeptide genes especially *UCN*, *BDNF*, *GRP*, *CARTPT* and *CCK*. Hypothalamic urocortin has a known role in inhibition of feeding behavior [31–33]. Hypothalamic BDNF has a prominent role in food intake and energy regulation *via* mechanisms such as induction of

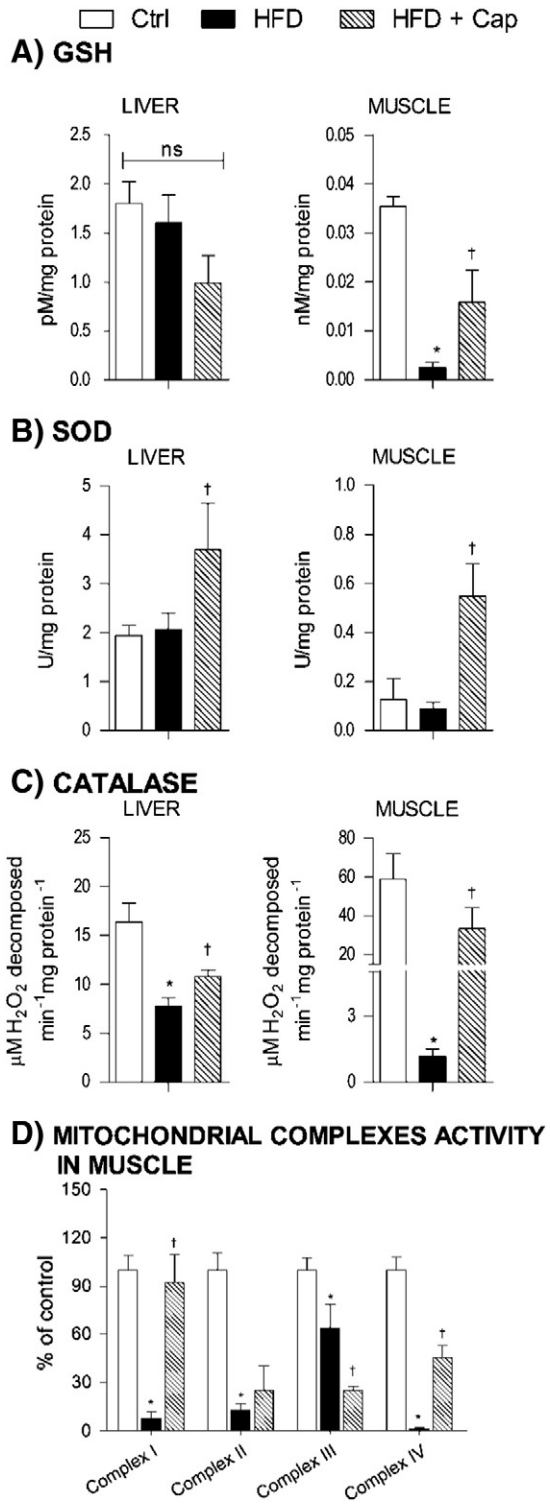

Fig. 6. Capsaicin-induced changes in endogenous antioxidants and mitochondrial complexes activity in liver and muscle in HFD-fed mice. (A–C) Effect of capsaicin on GSH, SOD and catalase in liver and muscle in HFD-fed mice. (D) Effect of capsaicin on mitochondrial complexes activity in muscle in HFD-fed mice. All values are expressed in mean±S.E.M. One-way ANOVA followed by Tukey's *post hoc* test for multiple comparison was applied. *$P<.05$ versus control group, †$P<.05$ versus HFD group.

"browning" genotype in rodents. Both global and selective depletion of BDNF causes increase in food intake and weight gain [34–36]. Also, the co-expression of nerve growth factor (NGF) receptors with TRPV1 and associated release pattern of NGF during activation of TRPV1 suggests that TRPV1 activation by capsaicin changes *BDNF* gene expression. Other than this, capsaicin also increased the expression of other key anorectic neuropeptides like *CARTPT*, *GRP* and *CCK* (there is known interaction between *CCK* and *TRPV1*) [37]. Capsaicin also significantly reversed the HFD-induced increased expression of several orexigenic genes like *CNR1*, *GALR1*, *GHRL* and *GHSR*. Here, its effect on *CNR1* is interesting as *CB1* receptors have been co-expressed with TRPV1 and *CB1* receptor has ability to suppress the TRPV1 expression and *vice versa*. In HFD animals, increase in *CB1* receptor gene expression may be linked to orexigenic effect as well as decrease in *TRPV1* expression, which has been restored by capsaicin. HFD also increased the expression of ghrelin gene that, in turn, stimulates growth hormone with capsaicin reversing the effect. Although genotypic changes in hypothalamus are interesting, it may be because of the resultant effect of the phenotypic changes that occurred due to peripheral effects of capsaicin. This can be answered by looking at genotypic changes after intra-hypothalamic administration of lower doses of capsaicin (TRPV1 activating). Overall, considering our results and linking it with available literature about presence and significance of TRPV1 immunoreactivity in sensory neurons innervating the gastrointestinal tract and vagus nerve [8,38], we hypothesize that oral administration of capsaicin activated TRPV1 present at vagal nerve or capsaicin-sensitive afferent nerves present in gastrointestinal tract, which in turn activates TRPV1 neurons at arcuate nucleus and hypothalamus causing change in hypothalamic genotype (Fig. 7).

HFD induces "browning" — through increased expression of BAT specific genes in multiple adipose tissues [39]. This may well be related to a compensatory enhancement in thermogenesis in response to intake of HFD to counter energy storage properties of HFD [40]. We suggest it only as compensatory mechanism as it has no positive correlation with body weight gain, as it is a polygenic multi tissue involving complex phenomenon. Capsaicin administration further increased the expression of "browning" related thermogenesis, mitochondrial biogenesis and EE genes many fold as compared to HFD group. This is in corroboration with multiple preclinical and clinical studies where investigators have linked TRPV1 activation and specifically capsaicin administration with induction of "browning" [41–45]. Capsaicin increased the expression of several "browning" related genes like *CIDEA*, *PGC1α*, *UCP1*, *BDNF* and *PPARα* in both BAT and sWAT but not in vWAT suggesting an increase in their thermogenic ability. The differential effect in two types of WAT may be because brown-like transformation is more prominent in sWAT, whereas vWAT is less susceptible to browning [46]. Recent reports also suggested that brite/beige cells ("browning" cells) are found more sporadically in sWAT that makes them more susceptible to "browning" in comparison to vWAT [47].

Recent reports suggest that gut microflora regulates host metabolism and energy harvest and contributes for obesity development [48]. Dysbiosis in gut microflora decreases *Bacteroidetes* and enhanced *Firmicutes* and LPS secreting gram-negative pathogens as observed in the gut of obese humans and animals [49–51]. Higher body weight and type 1 diabetes have been shown to cause gut microbial dysbiosis leading to inflammation and altered gut permeability causing metabolic endotoxemia and inflammation [52,53]. Very recently, these changes have been attributed to a significant reduction in *A. muciniphila* [21], a mucin-degrading bacterium in the intestinal mucus layer [54]. *A. muciniphila* supplementation reversed the complications associated with HFD-induced metabolic disorders in mice [21]. Molecules such as oligofructose and pharmacological agents such as metformin significantly increased the abundance of *A. muciniphila* [55,56]. Different dietary components effect the microflora composition [57]. Recently, Sharma and colleagues reported that capsaicin or red chili extract enhanced lactic acid production by *L. acidophilus* [23]. This suggests that capsaicin may induce the release of low molecular weight metabolites (short chain fatty acids) from

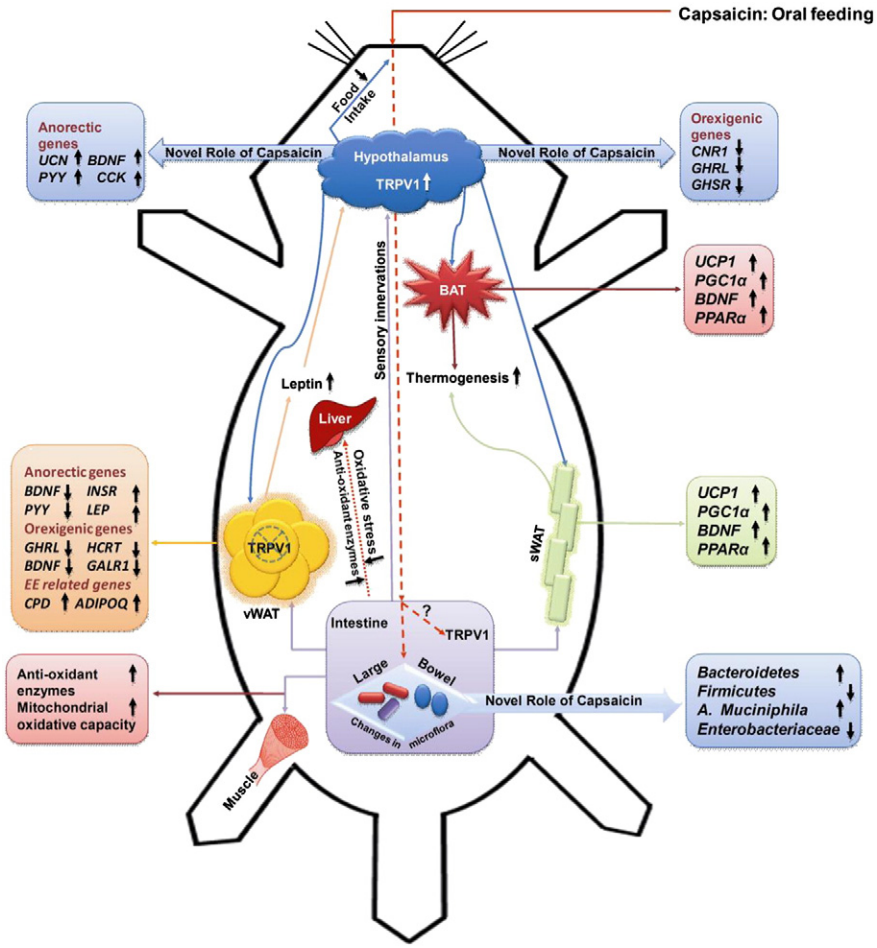

Fig. 7. Schematic diagram that summarizes the proposed mode of action of capsaicin in HFD-induced obese mice. Capsaicin supplementation *via* gastrointestinal TRPV1 activation or vagal afferent activation induces hypothalamic TRPV1. In hypothalamus, it alters anorectic and orexigenic genes. Induced hypothalamic activity induces SNS activation that might be responsible for increased thermogenesis in BAT and sWAT and decrease in obesity markers in vWAT. Capsaicin can directly affect the gene changes in vWAT and sWAT *via* direct absorption into blood. Unabsorbed capsaicin lead to beneficial alteration in gut microbial population.

the gut microflora that may activate certain gut receptors or translocate into the circulation and ameliorate the obesity effects. Therefore, we hypothesized that unabsorbed capsaicin may be affecting the gut microflora composition in large intestine (Fig. 7). We have followed the changes in selected bacterial groups in the cecal contents upon dietary intervention with HFD and HFD+Cap. Total bacteria were not changed in the tested groups; however, significant changes in the *Enterobacteriaceae* and *A. muciniphila* were observed. *Enterobacteriaceae* proportions were significantly high in HFD group as previously reported [58] and their levels were significantly decreased upon capsaicin treatment. Reduced *Enterobacteriaceae* abundance in the HFD+Cap group might be due to the antimicrobial action of capsaicin against *Enterobacteriaceae* members. We have noticed a significant reduction in *A. muciniphila* abundance in the HFD-induced obese mice, which is in agreement with earlier observations [21]. Interestingly, capsaicin intervention significantly enhanced *A. muciniphila* abundance in the cecal contents. Either capsaicin treatment might enhance mucus secretion, in turn, leading to enhanced *A. muciniphila* abundance, or that capsaicin *per se* promoted the growth of *A. muciniphila*. One cannot rule out the role of (i) TRPV1-induced mucin production or (ii) TRPV1 expressing afferent neurons in large intestine, which warrants more thorough investigations.

We propose that oral capsaicin decreases adiposity *via* global nutrigenomic changes in different tissues (Fig. 7). Further, in addition to its well-known effect on WATs and liver and muscle associated change in gene expression, oxidative stress and inflammation, the present study also provides an evidence for novel mechanisms to explain the anti-obesity effect of capsaicin *via* modulation of hypothalamic anorectic genotype, induction of "browning" genotype and increased thermogenic and/or mitochondrial biogenesis potential of BAT and beneficial alteration in gut microbial population.

Conflict of Interest

There is no conflict of interest among authors of this manuscript.

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