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Central inhibitory effects on feeding induced by the adipo-myokine irisin

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Abstract

Irisin, the soluble secreted form of fibronectin type III domain containing 5 (FNDC5)-cleaved product, is a recently identified adipo-myokine that has been indicated as a possible link between physical exercise and energetic homeostasis. The co-localization of irisin with neuropeptide Y in hypothalamic sections of paraventricular nucleus, which receives NPY/AgRP projections from the arcuate nucleus, suggests a possible role of irisin in the central regulation of energy balance. In this context, in the present work we studied the effects of intra-hypothalamic irisin (1 μl, 50-200 nmol/l) administration on feeding and orexigenic [agouti-related peptide (AgRP), neuropeptide Y (NPY) and orexin-A] and anorexigenic [cocaine and amphetamine-regulated transcript (CART) and proopiomelanocortin (POMC)] peptides in male Sprague-Dawley rats. Furthermore, we evaluated the effects of irisin on hypothalamic dopamine (DA), norepinephrine (NE) and serotonin (5-hydroxytryptamine, 5-HT) concentrations and plasma NE levels. Compared to vehicle, irisin injected rats showed decreased food intake, possibly mediated by stimulated CART and POMC and inhibited DA, NE and orexin-A, in the hypothalamus. We also found increased plasma NE levels, supporting a role for sympathetic nervous system stimulation in mediating increased oxygen consumption by irisin.

Keywords:

irisin; DA; NE; CART; orexin-A; POMC.

1. Introduction

In vertebrates feeding regulation and energy balance result from a complex interplay between neurotransmitters, neuropeptides and hormones, among which adipokines and gut-derived hormones play a key role in the integration of peripheral and central signaling (Kalra et al., 1999). In particular, gut peptides are mainly involved in short term control of food intake (Valassi et al., 2008), while adipokines signal energy availability and reserve in the long term (Jequier et al., 2002; Vásquez et al., 2008; Woods and Seeley, 2000). Being a region lacking a complete blood-brain barrier, the arcuate nucleus of the hypothalamus (ARC) is deeply involved in transducing circulating hormone signaling into the central nervous system (CNS) (Cone et al., 2001; Peruzzo et al., 2000), possibly through passive lipophilic diffusion or saturable transport systems (Banks et al., 1996; Kastin et al., 1999). In the ARC, neuropeptide Y (NPY) and agouti-related peptide (AgRP) co-expressing neurons mediate stimulation of food intake, and cocaine- and amphetamine-regulated transcript (CART) peptide and proopiomelanocortin (POMC) co-expressing neurons mediate inhibition of food intake (Cone et al., 2001). Both NPY/AgRP and POMC/CART neurons project to orexin neurons in the lateral hypothalamus, which downstream mediate increased feeding behavior (Schwartz et al., 2000). In the ARC, adipokine receptors could control energy expenditure through the modulation of sympathetic tone (Commins et al., 1999; Harlan et al., 2011; Wu et al., 2014). Moreover, biogenic amines such as dopamine (DA), norepinephrine (NE) and serotonin (5-hydroxytryptamine, 5-HT) also play a role in feeding regulation, by interacting with neuropeptides and hormones in the hypothalamus (Kalra et al., 1999).

Irisin, the proteolytic fibronectin type III domain containing 5 (FNDC5)-cleaved product, is an adipo-myokine that has been hypothesized as a possible link between physical exercise and energy homeostasis (Novelle et al., 2013). Originally identified as a myokine that is involved in browning of white adipose tissue, with consequent increase in energy expenditure and protection from high fat diet-induced obesity (Boström et al., 2012), irisin is also secreted by white adipose tissue, with increased levels found in obese animals (Roca-Rivada et al., 2013). A positive correlation was

observed between irisin and leptin levels (Palacios-González et al., 2015; Roberts et al., 2013), and it is well established that leptin plays a key role in energy balance signaling (Harvey and Ashford, 2003).

Similarly to leptin, irisin could play a role in the central nervous system. It has been reported a stimulatory effect on energy expenditure following central irisin injection (Zhang et al., 2015b), and irisin has been identified in rat and mice cerebellar Purkinje cells (Dun et al., 2013) and in different human brain areas, including hypothalamus (Piya et al., 2014; Roberts et al., 2013).

Aiming to further elucidate the role of irisin in the control of feeding and energy expenditures, in the present work we investigated, in Sprague-Dawley rats, the effects of acute intrahypothalamic injection of irisin, on food intake, the gene expression of the neuropeptides NPY, AgRP, CART, POMC, orexin-A, the concentrations of DA, NE and 5-HT in the hypothalamus, and plasma NE levels.

2. Materials and methods

2.1. Animals and drugs

48 male adult Sprague-Dawley rats (290-340 g) were housed in plexiglas cages (40 × 25 × 15 cm), one rat per cage, in climatized colony rooms (22±1°C; 60% humidity), on a 12 h/12 h light/dark cycle (light phase: 07:00 – 19:00 h), with free access to tap water and food, 24 h/day throughout the study. Rats were fed a standard laboratory diet (chow: 3.5% fat, 63% carbohydrate, 14% protein, 19.5% other components without caloric value; 3.20 kcal/g). Housing conditions and experimentation procedures were strictly in accordance with the European Union ethical regulations on the care of animals for scientific research. Human recombinant irisin (25 kDa; 100 μg/vial), was purchased from Vincibiochem [Vinci (FI), Italy], and diluted in saline at concentrations 50-200 nmol/L, as previously reported (Moon et al., 2013; Zhang et al., 2015b).

2.2. In vivo intrahypothalamic treatment

Rats were anesthetized by intraperitoneal injection with ketamine-xylazine (50 and 5 mg/kg, respectively), and subjected to stereotaxic procedure. After a midline incision, the skull was scraped clean, washed with sterile saline solution, and disinfected with 10% iodopovidone. A drop of lidocaine 2% solution was applied into the incision. Coordinates for placement of a cannula into the ARC were as follows: anteroposterior (AP), −3 mm (or +3 mm posterior to the bregma); mediolateral (ML), 0.2 mm; dorsoventral (DV), −10 mm (Paxinos and Watson, 2007). Small holes were drilled into the skull and stainless-steel screws were placed. A hole was drilled through the skull, in the marked site, for the placement of the 21-gauge sterilized stainless-steel cannula (1.7 cm long), which was implanted into the ARC. Once in place, the cannula was attached to the skull using dental cement (Formatray, Salerno, Italy). Sterile obturators were inserted into the cannulas to prevent them from clogging and to reduce the potential for brain infection. After 72 h recovery period, we did not also observe any significant alteration in basal food intake (Fig.1), in respect to six sham animals, not included in the present experimental paradigm [Food intake (g): 17±1.7]. Furthermore, preliminary measurements on spontaneous physical activity, recorded by a video camera (SSC-DC378P, Biosite, Stockholm, Sweden) positioned in the top-center of the cage and connected to a computer, indicated negligible alterations in respect to sham animals. Rats were randomized into two groups: 24 rats were fed ad libitum (fed rats) throughout the experimental paradigm; 24 rats were fasted for 24 h (fasted rats) before starting injections. As previously reported (Brunetti et al., 2011), at 9:00 a.m., rats (8 animals for each group) were unilaterally injected into the ARC with either vehicle (saline) or irisin (1 µl, 50-200 nmol/l), by connecting the cannula with a 5 µl syringe (Hamilton, Switzerland). Food intake intake was recorded at 1 h, 2 h, 4 h, 8 h and 24 h following injections, in both fed and fasted rats. Then, rats were anesthetized with ketamine-xylazine (50 and 5 mg/kg, respectively) and plasma was collected from femoral vein. Finally, animals were killed by CO_2 inhalation (100 % CO_2 at a flow rate of 20 % of the chamber volume per minute) at 8 h (*N=18*) and 24 h (*N=30*) following intrahypothalamic injection. In preliminary experiments performed in our laboratories on rats of the same age and weight, we have

confirmed the location of the cannula tip by the injection of dye (Evans blue 0.5% and Zelatin 5%) and histological examinations of frozen hypothalamic sections. Furthermore, after sacrifice we confirmed the cannula sites on the upper surface of the hypothalamus for each animal by comparison with mistargeted ones.

2.3. RNA extraction

Immediately after sacrifice, brains (N = 36) were rapidly removed, individual hypothalami were immediately dissected and stored in RNA*later* solution (Ambion, Austin, TX) at −20 ◦C until further processed. Total RNA was extracted from the hypothalamus using TRI Reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's protocol. Contaminating DNA was removed using 2 units of RNase-free DNase 1 (DNA-free kit, Ambion, Austin, TX), according to the manufacturer's instructions. The RNA solution was quantified at 260 nm by spectrophotometer reading (BioPhotometer, Eppendorf, Hamburg, Germany) and its purity was assessed by the ratio at 260 and 280 nm readings. The quality of the extracted RNA samples was also determined by electrophoresis through agarose gels and staining with ethidium bromide, under UV light.

2.4. Reverse transcription and real-time reverse transcription polymerase chain reaction (real-time RT PCR)

One μg of total RNA extracted from each sample in a 20 μl reaction volume was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's manual. Reactions were incubated in a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) initially at 25°C for 10 min, then at 37°C for 120 min, and finally at 85°C for 5 s. Gene expression was determined by quantitative real-time PCR using TaqMan probe-based chemistry (Applied Biosystems, Foster City, CA, USA). Reactions were performed in MicroAmp Fast Optic 96-well Reaction Plates (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 7900 HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). PCR primers and TaqMan probes were obtained from Applied Biosystems (Assays-on-Demand Gene Expression Products, Rn00567382_m1 for CART gene, Rn00595020_m1 for POMC

gene, Rn01431703_g1 for AgRP gene, Rn00561681_m1 for NPY gene, Rn00565995_m1 for orexin-A gene). β-actin (Applied Biosystems, Foster City, CA, USA, Part No. 4352340E) was used as the housekeeping gene. In accordance with the manufacturer's instructions, each amplification reaction was performed with 10 µl of TaqMan Fast Universal PCR Master Mix (2X), No AmpErase UNG (Applied Biosystems, Foster City, CA), 1 µl of primer probe mixture, 1 µl of cDNA, 8 µl of nuclease-free water. The thermal cycling conditions (fast operational mode) were: 95°C for 20 s, followed by 40 cycles of amplification at 95°C for 1 s and 60°C for 20 s. The real-time PCR was carried out in triplicate for each cDNA sample in relation to each of the investigated genes. In addition to samples, individual runs included no-template controls (one for each Assay-on-Demand Gene Expression Product) and a reverse transcriptase minus control (for AgRP gene Assay-on-Demand Gene Expression Product). Data were elaborated with the Sequence Detection System (SDS) software version 2.3 (Applied Biosystems, Foster City, CA, USA). The comparative $2^{-\Delta\Delta Ct}$ method was used to quantify the relative abundance of mRNA and then determine the relative changes in individual gene expression (relative quantification) (Livak and Schmittgen, 2001). This method uses a calibrator sample to enable a comparison of gene expression levels in different samples. The values obtained indicate the changes in gene expression in the sample of interest by comparison with the calibrator sample, after normalization to the housekeeping gene.

2.5. Neurotransmitter extraction and high performance liquid chromatography (HPLC) determination

Immediately after sacrifice, individual hypothalami and plasma samples (N = 12) were rapidly removed and subjected to biogenic amine extractive procedures. Briefly, samples were treated with 1 ml of 0.05 N perchloric acid containing 0.004% sodium EDTA and 0.010% sodium bisulfite, and centrifuged at 4,500 x g for 10 min. The supernatant was filtered on 0.45 µm PTFE sterile filters (Whatman) and directly injected for HPLC. Neurotransmitter recovery was satisfactory (≥ 90%) and reproducible, with percentage relative standard deviation ≤ 10%. The HPLC apparatus consisting of a Jasco (Tokyo, Japan) PU-2080 chromatographic pump and an ESA (Chelmsford,

MA, USA) Coulochem III coulometric detector, equipped with microdialysis cell (ESA-5014b) porous graphite working electrode and solid state palladium reference electrode. The analytical conditions for biogenic amine identification and quantification were selected as previously reported (Brunetti et al., 2013b).

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA). Data reported in Figs 1A, 1B, 2, 3A, 3B and 5 were analyzed through analysis of variance (ANOVA) followed by Newman-Keuls *post-hoc* test. Data reported in Fig. 4 were analyzed through unpaired T-test. For the gene expression analysis (relative quantification method), 1.00±0.00 (calibrator sample) was considered the theoretical mean for the comparison, as previously described (Livak and Schmittgen, 2001). Gaussian distribution of data was assessed by D'Agostino and Pearson omnibus normality test. Statistical significance was set at $P < 0.05$. As regards to the animals randomized for each experimental group, the number was calculated on the basis of the "Resource Equation" $N=(E+T)/T$ ($10 \leq E \leq 20$), where E, N and T represent the numbers of degrees of freedom in an ANOVA, animals and treatment, respectively. In our experiments E was 20, for behavioral evaluation. On the other hand, E was 10, for biochemical evaluation. Any sample size, which keeps E between 10 and 20, should be considered as an adequate (Charan and Kantharia, 2013). Considering that T value was 3, as regards to behavioral evaluations, N value was 8, in both fed and fasted rats. In agreement with the recognized principles of "Replacement, Reduction and Refinement of Animals in Research", N value was 4 for biochemical analyses, in order to investigate the effects of irisin injection on different hypothalamic neuromodulators (neurotransmitter and neuropeptide activity), requiring different experimental paradigms of extraction and quantification, without adding more animals to the experimental procedures.

3. Results

Fig. 1A shows that intrahypothalamic irisin (50-200 nmol/l) administration dose-independently reduced food intake, in fed rats, only 24 h following administration. The observed inhibition of feeding induced by irisin could be related to the neuromodulatory effects induced by the peptide. Actually, we observed a significant reduction of hypothalamic orexin-A mRNA levels, in a dose-independent manner, and a dose-dependent increase of CART and POMC gene expression, 24 h following irisin injection. On the other hand, NPY and AgRP gene expression was not affected by irisin (Fig. 2).

In addition, we also investigated the gene expression of AgRP and POMC in fed and fasted rats at 8 h and 24 h following irisin administration. At 8 h following injection, we found a null effect on POMC and AgRP gene expression, in both irisin injected fasted and fed rats (Figs 3A-3B). Furthermore, we found a null effect on AgRP and POMC in fasted rats, at 24 h following irisin injection (Fig. 3B). On the other hand, we observed an increase in AgRP gene expression, in vehicle-treated fasted rats at both 8 and 24 h following injection, paralleled by no significant alteration of POMC gene expression, compared to fed vehicle group (Fig. 4). This different pattern of gene expression regulation observed between AgRP and POMC, at 8 and 24 h post injection, is consistent with the findings of Palou and Collegues, that suggested the induction of hypothalamic AgRP gene expression as an early event in fasting (Palou et al., 2009). Furthermore, the same authors observed that the elevation in AgRP gene expression was not reverted with refeeding.

Finally, irisin treatment significantly reduced hypothalamic DA level, in a dose-independent manner. In addition irisin dose-dependently reduced NE concentration, while it did not modify 5-HT (Fig. 5). On the other hand, irisin administration dose-dependently increased plasma NE level [(Vehicle: 2.48±0.99 ng/ml; irisin 50 nmol/l: 21.37±3.93* ng/ml; irisin 200 nmol/l: 39.04±5.81* ng/ml) ANOVA, P<0.01, F=13.66, df=10; *post-hoc*, *P<0.05 vs. vehicle].

4. Discussion

In the present work we show that intrahypothalamic administration of irisin inhibits food intake in rats, only 24 h after treatment , in both fed and fasted rats (Fig. 1). Previous studies have shown that

irisin levels are inversely correlated to 24 h food intake in humans (Schögl et al., 2015), and a thermogenic effect of irisin has been observed by Boström et al. (2012), supporting a role for this peptide in signalling the obese phenotype to the CNS, and activating an homeostatic response involving decreased feeding and increased energy expenditure. Since irisin levels have been found to be positively correlated to adiposity and leptin levels (Palacios-González et al., 2015; Roberts et al., 2013), both peptides could represent adipose tissue signals to the CNS triggering increased energy expenditure and decreased food intake. On the other hand, plasma irisin levels are inversely correlated with omentin-1 (Panagiotou et al., 2014), and we demonstrated stimulated food intake in rats following omentin-1 administration (Brunetti et al., 2013a). Furthermore, we observed that omentin-1 may possibly act on specific hypothalamic neuronal pathways in an opposite way compared with leptin (Brunetti et al., 2013a). In this context, we can speculate an antagonistic interplay between orexigenic and anorexigenic adipokines, in the hypothalamus. As regards to increased thermogenesis induced by irisin, sympathetic NE release has long been considered to play a key role in stimulating thermogenesis (Cannon et al., 2004), synergizing with leptin to induce uncoupling protein 1 (UCP-1) in brown adipose tissue (Commins et al., 1999). Similarly to leptin which activates in the ARC increased sympathetic (Harlan et al., 2011), our findings of increased plasma NE levels following central irisin administration support a role for sympathetic nervous system stimulation in mediating increased locomotor activity, oxygen consumption, body temperature and brain UCP4 and UCP5 gene expression by central irisin administration (Erden et al., 2016; Zhang et al., 2015b).

Feeding modulatory effects induced by adipokines primarily relay in the hypothalamus upon peptidergic neurons which downstream control hunger and satiety. We found a significant stimulation of CART and POMC gene expression, paralleled by inhibition of orexin-A gene expression (Fig. 2). The stimulated POMC gene expression following irisin administration adds to previous studies showing irisin involvement in exercise-induced expression of brain derived-neurotrophic factor (BDNF) (Wrann et al., 2013), which has been shown to be modulated by

melanocortin signaling (Xu et al., 2013). The anorexigenic role of CART and POMC peptides paralleled by the food intake stimulatory effects of orexin-A are well established pathways in feeding control (Kalra et al., 1999; Schwartz et al., 2000), and previous works showed that the feeding-suppressant effects induced by the adipokines leptin, chemerin and vaspin, could be related to inhibited orexin-A and stimulated CART and POMC gene expression (Brunetti et al., 2011; Brunetti et al., 2014; Dridi et al., 2005). On the other hand, irisin administration did not modify NPY and AgRP gene expression (Fig. 2), in fed rats, despite the co-localization of irisin with neuropeptide Y in human hypothalamic sections of the paraventricular nucleus, which receives NPY/AgRP projections from the ARC (Ganguly et al., 2010; Piya et al., 2014). Actually, the selective stimulation of CART and POMC gene expression following irisin administration could be explained by a possible up-regulation of phosphorylated STAT3 protein activity (Moon et al., 2013), which has been shown to regulate POMC expression (Dey et al., 2016). This is consistent with the direct hormone injection in the ARC; by contrast, we cannot exclude that irisin reached neighbor hypothalamic areas such as ventromedial and lateral hypothalamus, both involved in the control of food intake. Nevertheless, intracerebroventricular irisin administration was able to stimulate c-fos activity in the paraventricular hypothalamus, without any effect on ARC, ventromedial, dorsomedial and lateral nuclei (Zhang et al., 2015a).

In addition, considering that NPY/AgRP neurons are more sensitive than POMC/CART neurons to food deprivation (Henry et al., 2015; Palou et al., 2009), we also investigated the gene expression of AgRP and POMC in fed and fasted rats at 8 h and 24 h following irisin injection. The elevation in AgRP gene expression in fasted rats was not reverted by peptide injection (Figs 3A-3B). This result was paralleled by a null effect on POMC gene expression in fed and fasted animals (Figs 3A-3B), and corroborated the null effect on food intake in fasted rats, at 1, 2, 4, 8 and 24 h following injection (Figs 1A-1B).

We further evaluated the possible involvement of DA, NE and 5-HT in irisin signaling in the hypothalamus. DA and NE levels were decreased after irisin administration, while 5-HT was not

affected (Fig. 5). Hypothalamic catecholamines have long been known to be involved in feeding control (Kalra et al., 1999; Valassi et al., 2008). Several studies have shown that anorexigenic adipokines, such as leptin and resistin, have an inhibitory effect on DA and NE synthesis and release, in the hypothalamus, which could be related to the feeding inhibitory effects induced by these adipokines (Brunetti et al., 1999; Brunetti et al., 2004; Clark et al., 2006; Kutlu et al., 2010). Finally, as regards to the dose-response relationship related to irisin administration, we found a dose-independent inhibitory effect on food intake, in fed rats. The same dose-response relationship was observed for dopamine levels and orexin A gene expression, both involved in appetite control (Cason et al., 2010; Yang and Meguid, 1995). On one side, this could depend on the narrow dosage range that, consistently with the work performed by Moon and colleagues (2013), is about 5-10 fold higher compared to plasma irisin levels. On the other hand, the observed dose-dependent effects in irisin-treated fed rats, as regards to POMC/CART and sympathetic activity, support a major involvement of irisin in the central control of energy expenditure.

In conclusion, our data point to a role for irisin in inhibiting food intake, which could be related to stimulated CART and POMC gene expression and decreased orexin-A, DA and NE levels in the hypothalamus. In addition, the increased hypothalamic CART/POMC and sympathetic activity induced by irisin is consistent with the previously reported energy expenditure-stimulating effect induced by the peptide. In this context, irisin agonists could represent a future target for the pharmacological treatment of obesity.

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Legends to figures

Fig. 1. Time course of food intake (in grams) in fed (1A) and fasted (1B) rats treated with either vehicle (*N* = 24) or irisin (1 µl, 50-200 nmol/l) (*N* = 24). Vehicle or irisin were administered by intrahypothalamic injections, during the light phase, at 9:00 a.m.. Food intake was recorded 1, 2, 4, 8 and 24 h following irisin injection. Values represent the means ± SEM. Compared to vehicle, irisin significantly inhibited food intake 24 h following administration, in fed rats. (ANOVA, $P<$ 0.0001, F=96.67, df=20; *post-hoc*, ***P<0.001 vs vehicle)

Fig. 2. Relative gene expression of hypothalamic neuropeptides at 24 h following intrahypothalamic injection, as determined by real-time RT-PCR, in fed rats treated with irisin (1 µl, 50-200 nmol/l). Data were calculated using the $2^{-\Delta\Delta Ct}$ method; they were normalized to β-actin mRNA levels and then expressed as relative to vehicle (calibrator sample, defined as 1.00±0.00). Irisin administration significantly increased POMC (ANOVA, $P<$ 0.001, F=29.98, df=10; *post-hoc*, **P<0.01, ***P<0.001 vs vehicle) and CART (ANOVA, $P<$ 0.001, F=25.46, df=10; *post-hoc*, **P<0.01 vs vehicle:) gene expression, and decreased orexin A gene expression (ANOVA, $P<$ 0.01, F=12.25, df=10; *post-hoc*, **P<0.01 vs vehicle), compared with vehicle group.

Fig. 3. Relative gene expression of hypothalamic AgRP and POMC neuropeptides at 8 and 24 h following intrahypothalamic treatment with vehicle or irisin (1 µl, 50-200 nmol/l), as determined by real-time RT-PCR, in both fed (3A) and fasted (3B) rats. Data were calculated using the $2^{-\Delta\Delta Ct}$ method; they were normalized to β-actin mRNA levels and then expressed as relative to respective vehicle group (calibrator sample, defined as 1.00±0.00).

Fig. 4. Effects of fasting on basal AgRP and POMC gene expression at 8 and 24 h following intrahypothalamic vehicle injection. Gene expression analyses was performed on both fed and fasted vehicle-treated rats. Data were calculated using the $2^{-\Delta\Delta Ct}$ method; they were normalized to β-actin mRNA levels and then expressed as relative to fed vehicle (calibrator sample, defined as 1.00±0.00). Compared with fed vehicle group, fasting induced AgRP gene expression at 8 and 24 h after injection (Unpaired T-test, ***P<0.001 Vs. respective Fed Vehicle group, df=6).

Fig. 5. Hypothalamic dopamine (DA), norepinephrine (NE) and serotonin (5-HT) steady state concentrations (ng/mg wet tissue) 24 h after intrahypothalamic injection, as determined by HPLC, in fed rats treated with irisin (1 µl, 50-200 nmol/l). Compared to vehicle, irisin significantly decreased DA (ANOVA, $P< 0.0001$, $F=79.68$, df=10; *post-hoc*, ***$P<0.001$ vs vehicle) and NE (ANOVA, $P< 0.0001$, $F=150.4$, df=10; *post-hoc*, ***$P<0.001$ vs vehicle) steady state concentrations, in fed rats.