Intracerebroventricular injection of citrate inhibits hypothalamic AMPK and modulates feeding behavior and peripheral insulin signaling

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Abstract

We hypothesized that citrate might modulate the AMP-activated protein kinase/acytetyl-CoA carboxylase (AMPK)/ACC pathway and participate in neuronal feeding control and glucose homeostasis. To address this issue, we injected citrate into the lateral ventricle of rats. Intracerebroventricular (ICV) injection of citrate diminished the phosphorylation of hypothalamic AMPK/ACC, increased the expression of anorexigenic neuropeptide (pro-opiomelanocortin and corticotropin-releasing hormone), elevated the level of malonyl-CoA in the hypothalamus, and reduced food intake. No change was observed in the concentration of blood insulin after the injection of citrate. With a euglycemic–hyperinsulinemic clamp, the glucose infusion rate was higher in the citrate group than in the control group (28.6 ± 0.8 vs 19.3 ± 0.2 mU/kg body weight/min respectively), and so was glucose uptake in skeletal muscle and the epididymal fat pad. Concordantly, insulin receptor (IR), IR substrate type 1 (IRS1), IRS2, and protein kinase B (AKT) phosphorylation in adipose tissue and skeletal muscle was improved by citrate ICV treatment. Moreover, the treatment with citrate for 7 days promoted body weight loss and decreased the adipose tissue. Our results suggest that citrate and glucose may serve as signals of energy and nutrient availability to hypothalamic cells.


Introduction

In the last decades, obesity and diabetes have become worldwide health problems to which effective treatments have yet to be found. Many researchers have attempted to understand the relationship between the increase in fat deposits and insulin resistance. Leptin and insulin, hormones related to the maintenance of fat deposits and glucose homeostasis, act through receptors located in two sub-populations of neurons in the hypothalamus arcuate nucleus. These hormones provide hypothalamic neurons with the most robust signals that coordinate food intake, energy expenditure, and peripheral metabolism (Friedman & Halaas 1998, Schwartz et al. 2000). However, a new hypothalamic regulatory mechanism based on an intermediate metabolic energy storage process has been identified recently. This mechanism involves malonyl-CoA and enzymes related to its production that serve as a cell energy sensors (Kahn et al. 2005, Lane et al. 2005, Wolfgang & Lane 2006).

AMP-activated protein kinase (AMPK) is an enzyme that senses the cell energy status and regulates fuel availability. It is expressed throughout several brain areas that control food intake and neuroendocrine functions. Physiologically, AMPK is modulated by the AMP:ATP ratio and upstream kinases, tumor suppressor kinase (LKB1) and calcium/calmodulin-dependent protein (CAMKK; Hawley et al. 2003). AMPK integrates hormonal and nutrient signals (Han et al. 2005) in order to regulate food intake and energy homeostasis through effects in the hypothalamus and peripheral tissues (Minokoshi et al. 2004, Kahn et al. 2005, Xue & Kahn 2006). AMPK phosphorylates a number of proteins, including acetyl-CoA carboxylase (ACC), and causes their inactivation and a decrease in the concentration of malonyl-CoA (Carling et al. 1987). Some conditions leading to AMPK activation in the hypothalamus also lead to phosphorylation and inactivation of ACC (Kim et al. 2004). Physiologically, the short-term control of ACC occurs largely through phosphorylation/inhibition by 5′-AMPK and by feed-forward allostERIC activation by citrate produced by ATP-citrate lyase (Loftus et al. 2000). Then, the presence of citrate activates ACC and promotes cellular accumulation of malonyl-CoA.

Citrater occupies a pivotal position in cellular metabolism. It is not only an intermediate in the citric acid cycle but also a source of cytosolic acetyl-CoA for the synthesis of fatty acids, isoprenoids, and cholesterol. Recently, a novel sodium-coupled citrate transporter (NaCT) expressed in most brain...
neurons has been described in mouse (Inoue et al. 2002). The transport of citrate into neuron allows it to participate in the generation of energy and the control of the ACC activity.

As cited above, the citrate produced by the mitochondrial metabolism has an important allosteric effect on ACC. The increase in cellular concentration of citrate occurs in parallel to the increase in nutrient availability. In the cytosolic metabolism, citrate is a known negative modulator of phosphofructokinase, a key enzyme of the glycolysis pathway. Thus, there is a link between the glycolytic pathway and the production of malonyl-CoA. Fluctuations in hypothalamic concentrations of malonyl-CoA during fasting and feeding cycles are caused by changes in the phosphorylation and the activity of ACC (Schwartz et al. 2000). In addition, even relatively small increases in glucose levels after refeeding may inhibit hypothalamic AMPK activity (Minokoshi et al. 2004), and consequently activate ACC.

We hypothesized that citrate might be a physiological modulator of feeding behavior and energy homeostasis through its effect on the activation of ACC and the formation of malonyl-CoA. To test this hypothesis, citrate was injected directly into the lateral ventricle for the evaluation of the phosphorylation of hypothalamic AMPK and ACC and the expression of neuropeptides. These effects were investigated in parallel to feeding behavior, body weight, glucose uptake, and insulin signaling in skeletal muscle and epididymal fat pad. Our results show that intracerebroventricular (ICV) injection of citrate diminishes the phosphorylation of hypothalamic ACC and AMPK. This effect is accompanied by reductions in food intake and body weight. Moreover, enhanced glucose uptake and improved insulin pathway activation in epididymal fat pad and skeletal muscle have been observed.

Materials and Methods

Experimental animals and surgical procedures

Male Wistar–Hannover rats (12 weeks old, 250–280 g) from Universidade Estadual de Campinas animal breeding center were used in all experiments. The rats were maintained at room temperature (25 °C) and in 12 h light:12 h darkness cycles with free access to water and chow, unless indicated otherwise. The rats were chronically instrumented with an ICV cannula and kept under controlled temperature and light–darkness conditions (0700–1900 h) in individual metabolic cages. Seven days after the ICV cannula installation, the rats were tested for cannula function and position and thereafter randomly selected for one of the experimental groups. The general guidelines established by the Brazilian College of Animal Experimentation were followed throughout the study. Briefly, the animals were anesthetized with 50 mg/kg ketamine and 5 mg/kg diazepam (i.p.) and positioned onto a Stoelting stereotactic apparatus after the loss of cornea and foot reflexes. A stainless steel 23 gauge guide cannula with indwelling 30 gauge obturator was stereotaxically implanted into the lateral cerebral ventricle at pre-established coordinates, anteroposterior, 0.2 mM from bregma; lateral, 1.5 mM; and vertical, 4.2 mM, according to a previously reported technique (Michelotto et al. 2002). Cannulas were considered patent and correctly positioned by dipsogenic response elicited after injection of angiotensin II (2 μl of 10⁻⁸ M solution; McKinley et al. 2001). All procedures were approved by the Ethical Committee of the Universidade Braz Cubas, Mogi das Cruzes.

Biochemical and hormonal measurements

Plasma insulin was determined by RIA according to a previously described method (Scott et al. 1981). Serum glucose was determined by the glucose oxidase method (Trinder et al. 1969) and plasma corticosterone was determined with a commercially available kit following the manufacturer’s (DSL Inc., Webster, TX, USA) instructions. All blood samples were collected from the tail vein.

Determination of hypothalamic malonyl-CoA

The tissue fragments were homogenized after the addition of trichloroacetic acid. Precipitated proteins were removed from the trichloroacetic acid extract by centrifugation and the supernatant was neutralized by extracting the acid five times with ether. Samples were lyophilized and stored at −70 °C until assayed (Corkey et al. 1981, 1988). Acid-soluble acyl-CoA compounds were determined by a modification of the reversed-phase HPLC method of Corkey et al. (1981, 1988). This involved the use of acetonitrile rather than methanol as the organic component of the mobile phase, a flow rate of 0.4 ml/min, and a shallow gradient from buffer to acetonitrile to separate malonyl-CoA from early eluting nucleotides. Malonyl-CoA standard was purchased from Sigma–Aldrich Corp.

Protocols for citrate, adenine 9-β-D-arabinofuranoside (ARA-A), and insulin treatments

Citrate and ARA-A (AMP analog adenine 9-β-D-arabinofuranoside) were administered through ICV injection in all the experiment. For the evaluation of the molecular events of the insulin signal transduction pathway in the epididymal fat pad and skeletal muscle, 0.2 ml saline (0.9% NaCl), either with or without insulin (10⁻⁴ M), was injected through the cava vein at 0800 h. In this protocol, the treatment with either ARA-A (2 nmol) or citrate (2·0 μl of 10⁻² M) occurred 30 min before insulin injection through the cava vein in fasted rats (food was withdrawn 10 h before the treatment). Citrate, ARA-A, and saline solution were buffered with 153 mM NaCl, 10⁻⁴ M sodium–phosphate buffer (pH 7.4) to guarantee the efficiency of the NaCT-mediated citrate uptake.

Intraperitoneal glucose tolerance test (i.p. GTT)

The i.p. GTT was performed after overnight fasting. The rats were anesthetized as described above. After collection of an unchallenged sample (time 0), a solution of 20% glucose
Radioactivity of 3-[3H]glucose in plasma was measured in the peritoneal cavity 30 min after ICV administration of citrate (20 nmol). Tail blood samples were collected at 5, 15, 30, 60, 90, and 120 min for the determination of glucose and insulin concentrations.

**Hyperinsulinemic–euglycemic clamp procedures**

After 5-h fasting, the animals were anesthetized with 50 mg/kg ketamine and 5 mg/kg diazepam injection (i.p.), and catheters were placed into the left jugular vein (for tracer infusions) and the carotid artery (for blood sampling), as previously described (Prada et al. 2000). Each animal was monitored for food intake and weight gain for 5 days after surgery to ensure complete recovery. Food was removed 10 h before the beginning of the in vivo studies. A 120-min hyperinsulinemic–euglycemic clamp procedure was conducted in catheterized rats, as described previously (Combs et al. 2001), with prime continuous infusion of insulin at a rate of 3.5 mU/kg body weight per minute to raise plasma insulin concentration to ~800–900 pmol/l. Blood samples (20 ml) were collected at 5-min intervals for the immediate measurement of plasma glucose concentration, and 10% unlabeled glucose was infused at variable rates to maintain plasma glucose at fasting levels. Insulin-stimulated whole-body glucose flux was estimated using prime continuous infusion of HPLC-purified [3-3H]glucose (10 μCi bolus, 0.1 μCi/min) throughout the clamp procedure (Rossetti et al. 1997). Blood samples (10 μl) were collected before the start and at the end of the clamp procedure for measurement of plasma insulin concentrations. All infusions were performed using Harvard infusion pumps. At the end of the clamp procedure, the animals were killed by a sodium pentobarbital i.v. injection.

**Analytical procedures of hyperinsulinemic–euglycemic clamp**

Plasma glucose was measured using a glucometer (Advantage, Roche Molecular Biochemicals). Plasma tracer samples were deproteinized with equal volumes of barium hydroxide and zinc sulfate (0.015 M) and stored overnight at 4 °C. The radioactivity of 3-[3H]glucose in plasma was measured in Ba(OH)₂/ZnSO₄ precipitate supernatants after evaporation to dryness for the removal of tritiated water. Rates of whole-body glucose uptake and basal glucose turnover were determined as the ratio between the [3-3H]glucose infusion rate (disintegrations per minute) and the specific activity of plasma glucose (disintegrations per minute per milligram glucose) during the final 30 min of the respective experiments under steady-state conditions. Glucose transport activity in skeletal muscle and fat was calculated from plasma 2-[14C]DG concentrations. All infusions were performed using Harvard infusion pumps. At the end of the clamp procedure, the animals were killed by a sodium pentobarbital i.v. injection.

**Food intake and body weight measurements**

To measure individual food intake, the animals were divided into two groups: i) feeding rats, animals given free access to standard rodent chow and ii) fasted rats, animals fasted during light phase (8 h). Food intake was evaluated during the dark phase after ICV administration of either saline or citrate (20 nmol) at 1800 h.

In another experimental protocol, the rats were maintained in individual cages with free access to standard rodent chow and water for 2 days for adaptation. Later, the animals received ICV administration of either citrate or saline every day at 1800 h. Food intake measurements were performed in the next 12 h for 7 experimental days. To differentiate the effects of citrate administration per se versus effects induced by food intake inhibition, a citrate pair-fed group was included. Body weight, epididymal fat pad, and perirenal fat were obtained from control, citrate-treated rats, and the citrate pair-fed group after 9 days (2 days for adaptation plus 7 experimental days). Citrate was administered immediately before the beginning of the nocturnal cycle.

**RNA preparation for reverse transcription-PCR**

Total hypothalamic and hepatic RNA were extracted using Trizol (Life Technologies) reagent according to the manufacturer’s guidelines. Total RNA was rendered genomic DNA-free by digestion with RNase-free DNase (RQ1; Promega).

**Semi-quantitative reverse transcription-PCR**

Seven micrograms of total RNA were reverse transcribed with SuperScript reverse transcriptase (200 U/μl) using oligo (dT) (50 mmol/l) in 30 μl reaction volume (5 × RT buffer, 10 mmol/l dNTP, and 40 U/μl RNase-free inhibitor). The reverse transcriptions involved 50-min incubation at 42 °C and 15-min incubation at 70 °C. PCR products were submitted to 1.5% agarose gel electrophoresis containing ethidium bromide and visualized under u.v. light excitation. Photo-documentation was performed using the NucleoVision System (NucleoTech, San Mateo, CA, USA) and band quantification was performed using Gel Expert Software (NucleoTech). RPS-29 (ribosomal protein S29) of all samples was amplified and used as internal qualitative and quantitative controls. The semi-quantitative expression (SE) of genes of interest was calculated using the formula: SE = pixel area of product/pixel area of RPS-29×100. The primers used and the PCR conditions were: RPS-29 (NCBI: NM012876), sense: 5'-AGG CAA GAT GGG TCA CCA GC-3', antisense: 5'-AGT CGA ATC ATC CAT TCA GGT CG-3' (fragment: 202 bp; Tm: 57 °C; amplification: 27 cycles); POMC (proopiomelanocortin; NCBI: AF510391), sense: 5'-CTC CTG CTT CAG ACC TTC AT-3', antisense: 5'-TGG GAC GAC TAC TCC ACA GG-3' (fragment: 398 bp; Tm: 63 °C; amplification: 32 cycles); NPY (neuropeptide Y; NCBI: NM012614), sense: 5'-AGA GAT CCA GCC CTG AGA-3' (fragment: 398 bp; Tm: 63 °C; amplification: 32 cycles);

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CA-3', antisense: 5'-AAC GAC AAC AAG GGA AAT GG-3' (fragment: 236 bp; Tm: 62 °C; amplification: 31 cycles); CRH (corticotropin-releasing hormone; NCBI: NM031019), sense: 5'-ATC CGC ATG GGT GAA GAAT-3', antisense: 5'-AAG CGC AAC ATT TCA TTT CC-3' (fragment: 408 bp; Tm: 62 °C; amplification: 31 cycles).

Tissue extraction, immunoprecipitation, and immunoblotting
The rats were anesthetized after specific treatments and tissues samples were obtained and homogenized in freshly prepared ice-cold buffer (1% Triton X-100, 100 mM TRIS, pH 7-4, 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM phenylmethylsulphonyl fluoride (PMSF), and 0.01 mg aprotinin/ml). The insoluble material was removed by centrifugation (10 000 g for 25 min at 4 °C). Aliquots of the resulting supernatants containing 2 mg total protein were used for immunoprecipitation with either specific antibodies (anti-IR (rabbit; sc-711), or anti-IR substrate-1 (IRS1; rabbit; sc-559), or anti-IRS2 (goat; sc-1555)), all from Santa Cruz Biotechnology (Santa Cruz, CA, USA) at 4 °C overnight, followed by the addition of protein A–Sepharose (Pharmacia) for 15 min at 4 °C. The pellets were washed three times in ice-cold buffer (0-5% Triton X-100, 100 mM Tris, pH 7–4, 10 mM EDTA, and 2 mM sodium vanadate), resuspended in Laemmli sample buffer, and boiled for 5 min before separation in SDS-PAGE using a miniature slab gel apparatus (Bio–Rad). Electrotransfer of proteins from the gel to nitrocellulose was performed for 90 min at 120 V (constant). The nitrocellulose transfers were probed with specific antibodies. The blots were subsequently incubated with 125I-labeled protein A (Amersham). For direct immunoblot analysis, 0-2 mg protein from tissue extracts was separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with specific antibodies, anti-phospho-AMPK, and anti-phospho-ACC (Cell Signaling Technology, Danvers, MA, USA). Subsequently, the blots were incubated with 125I-labeled protein A (Amersham). The results were visualized by autoradiography with pre-flashed Kodak XAR film. Band intensities were quantified by optical densitometry of developed autoradiographs (Scion Image software; Scion-Corp, Frederick, MD, USA).

Data presentation and statistical analysis
All numerical results are expressed as means ± S.E.M. of the indicated number of experiments. Blot results are presented as direct band comparisons in autoradiographs and quantified by densitometry using the Scion Image software (ScionCorp). Student’s t-tests of unpaired samples and ANOVA for multiple comparisons were used as appropriate. Post hoc test (Tukey) was employed when required. The level of significance was set at P<0.05.

Results
Citrate is an important positive allosteric modulator of ACC and a source of cytosolic acetyl-CoA. The formation of ACC activity and malonyl-CoA is controlled by AMPK. The acetyl-CoA present in the cytoplasm originates from the citrate produced within mitochondria. However, Inoue et al. (2002) identified NaCT located at the plasma membrane, suggesting that circulating citrate may also be an important source of cytoplasmic citrate. Since NaCT is expressed in most of the brain neurons, it may play an important role in supplying citrate to these cells as a metabolic precursor of ATP.

An acute ICV injection of 20 nmol of citrate in fed animals elicited a decrease (35%) in food intake (Fig. 1A). A similar effect was observed in animals ICV injected a competitive inhibitor of AMPK (ARA-A). In fasted animals, the citrate effect was similar to that observed in fed animals (Fig. 1B). Food intake was evaluated at early time points (10 and 30 min after the exogenous administration of citrate), but the food intake measured value in this period was very small. Since the AMPK/ACC pathway plays an important role in the feeding behavior, we evaluated the phosphorylation of hypothalamic AMPK and ACC 30 min after the ICV administration of citrate to fed (Fig. 1C and D) and fasted rats at different times after the exogenous administration of citrate (Fig. 1E). AMPK phosphorylation was reduced significantly in both groups (fed and fasted animals) after ICV administration of citrate (54 and 62% respectively) comparatively with values in animals ICV-administered saline. The evaluation of the hypothalamic phosphorylation of AMPK at 5, 10, and 15 min after the ICV administration of citrate resulted in an effect on the level of phosphorylation of AMPK similar to that observed at 30 min (Fig. 1E). Similarly to citrate, ICV administration of a competitive AMPK inhibitor (ARA-A) also reduced AMPK phosphorylation and ACC phosphorylation in the hypothalamus (Fig. 1C and D). Interestingly, hypothalamic phosphorylation of ACC was reduced when either citrate or ARA-A (71 and 52% respectively) was administered to fed rats, showing that citrate can reduce the kinase activity of hypothalamic AMPK. To evaluate the potential mechanism involved in the effects on the food intake, we quantified the level of malonyl-CoA in the hypothalamic extract. Consistent with its effect on AMPK/ACC, exogenous administration of citrate increased malonyl-CoA in the hypothalamus when compared with the results of animals that received saline (7.4±1.0 vs 5.2±0.9 pmol/mg protein respectively). Consistent with a previous report (Kalra et al., 1991), fasting increased the expression of NPY mRNA markedly. On the other hand, POMC and CRH mRNA levels diminished in fasted animals. ICV administration of citrate prevented the fasting-induced expression of NPY. Data quantification shows that the NPY mRNA level diminished by 40% (Fig. 2). By contrast, citrate caused a substantial increase in CRH and POMC mRNA levels in fasted rats (Fig. 2).
Blood insulin and corticosterone levels were quantified in fasted rats to eliminate the secondary effect of these hormones on the parameters evaluated. Blood samples were obtained from rat tails before (basal) and after ICV injection of citrate (10, 30, and 60 min). Blood insulin did not change significantly after ICV injection of citrate (basal: 8.5 ± 2.0, 10 min: 9.3 ± 1.3, 30 min: 8.7 ± 2.5, 60 min: 8.8 ± 1.9 pmol/l), in contrast to blood corticosterone level, which diminished by 42% (10 min; basal: 197.2 ± 45.2, 10 min: 115.8 ± 39.6, 30 min: 112.2 ± 38.0, 60 min: 118.6 ± 45.8 ng/ml).

Food intake was evaluated after single daily ICV injection of either citrate or saline vehicle (control) just before the darkness cycle onset (1800 h) for 7 days. Food intake was 25% lower in the citrate ICV group, whereas no significant change in food intake was observed in the saline ICV group during the experimental period (7 days; Fig. 3A). At the end of the experimental protocol, the cumulative body weight was positive in the saline group (35 g), whereas a negative body weight variation was detected in animals treated with citrate (5 g). Interestingly, the citrate pair-fed group showed a positive body weight variation (23 g) significantly different from that of the citrate group (Fig. 3B). Concordantly, the epididymal fat pad (1.7 g) and perirenal fat (1.4 g) were significantly reduced after 7 days of treatment with citrate. However, in the citrate pair-fed group only the epididymal fat pad was significantly reduced (Fig. 3C and D) when compared with the control group result.

An intraperitoneal glucose tolerance test was performed in fasted rats administered a single citrate dose 30 min before the beginning of the test. There was no difference in basal blood glucose between the saline and citrate ICV groups. Thereafter, a solution of 20% glucose (2.0 g/kg body weight) was administered into the peritoneal cavity of both groups (saline...
and citrate ICV). Tail blood samples were collected for the analysis of blood glucose disappearance. As observed in Fig. 4A, citrate ICV injection improved glucose uptake. The area under the curve was 50% smaller than that of the saline group. Additionally, the samples collected during i.p. GTT presented significantly lower insulin levels than those of the control group (Fig. 4B).

A hyperinsulinemic–euglycemic clamp procedure was performed to evaluate the effect of citrate ICV injection on the metabolism of glucose in skeletal muscle and adipose tissue (Fig. 5). The glucose infusion rate needed to clamp glycemia at fasting levels under constant insulin infusion was 48% increased in citrate-treated animals comparatively with the control group (Fig. 5A). Accordingly, the insulin-stimulated whole-body glucose disposal rate was also significantly increased in citrate-treated animals when compared with that of the control group (39.5±3.1 vs 29.2±2.2 mg/kg per min respectively; Fig. 5B). Insulin-stimulated glucose uptake in skeletal muscle and adipose tissue was quantified using 2DG uptake analysis (Fig. 5C and D). As shown, glucose uptake was significantly increased in citrate-treated rats compared with their respective control values. Glucose uptake in skeletal muscle increased from 250±23 nmol/g per min to 402±15 nmol/g per min (Fig. 5C). In adipose tissue, previous treatment with citrate also induced a significant increase in glucose uptake (from 160±10 to 220±12 nmol/g per min; Fig. 5D).

The effect of insulin infusion through the cava vein on elements of the insulin–signaling pathway was investigated in epididymal fat pad (Fig. 6) and skeletal muscle (Fig. 7) of animals previously treated with either saline, or citrate, or ARA-A by ICV injection. Thereafter, the animals received either saline (open bars) or insulin (closed bars) through the cava vein (endovenous, EV). Protein expression was evaluated...
insulin-stimulated phosphorylation of IR, IRS1, IRS2, and AKT was powered in animals previously treated with either citrate (citrate ICV) or ARA-A (ARA-A ICV) ICV injection.

Discussion

A number of recent studies demonstrate that hypothalamic AMPK activity regulates food intake (Minokoshi et al. 2004, Xue & Kahn 2006). Fasting results in increased AMPK activity in multiple hypothalamic regions, whereas refeeding inhibits it. The activation of hypothalamic AMPK is sufficient to increase food intake and body weight, and its suppression is sufficient to decrease these parameters (Minokoshi et al. 2004). In addition, increased food intake is observed by lowering malonyl-CoA with an ACC inhibitor or by ectopic expression of malonyl-CoA decarboxylase in the hypothalamus (Wolfgang & Lane 2006). Therefore, conditions that increase hypothalamic malonyl-CoA lead to the suppression of food intake, showing that the formation of malonyl-CoA plays an important role in energy homeostasis. Interestingly, studies on soleus muscle of rats showed that the increase in malonyl-CoA caused by insulin and glucose is promoted by an increase in the cytosolic concentration of citrate, an allosteric activator of ACC (Elmquist 2001). Consistent with this report, our results demonstrate that central administration of citrate increases the level of malonyl-CoA in the hypothalamus. Since citrate is produced in nutrient availability conditions, we hypothesized that citrate might act as a hypothalamic anorexigenic signal and modulate the AMPK/ACC pathway and peripheral glucose homeostasis. This hypothesis is strengthened by the presence of a plasma membrane transporter with preference for citrate as a substrate. Recently, a plasma membrane transporter capable of citrate transport was described in mouse brain (Inoue et al. 2002). This transporter is located in most brain neurons and may contribute to pool cytosolic citrate. Following cytoplasm entry, citrate is cleaved by ATP-citrate lyase to generate acetyl-CoA, but the citrate pool may also serve as an energy substrate. Recently, a plasma membrane transporter with preference for citrate as a substrate. Furthermore, citrate transport was described in mouse brain (Inoue et al. 2002). This transporter is located in most brain neurons and may contribute to pool cytosolic citrate. Following cytoplasm entry, citrate is cleaved by ATP-citrate lyase to generate acetyl-CoA, but the citrate pool may also serve as an energy substrate.

In our protocol, citrate was prepared at pH 7.4 and in 153 mM NaCl, pH 7.4) without citrate presented no effects, as expected. These results suggest that citrate can modulate the AMPK/ACC pathway and indicate energy availability in neurons. Several studies have shown that AMPK inhibitor can reduce food intake and body weight (Minokoshi et al. 2004, Xue & Kahn 2006). Consistent with its effect on hypothalamic AMPK, citrate ICV injection produced 35% reduction in food intake, showing that the formation of malonyl-CoA plays an important role in energy homeostasis. Interestingly, studies on soleus muscle of rats showed that the increase in malonyl-CoA caused by insulin and glucose is promoted by an increase in the cytosolic concentration of citrate, an allosteric activator of ACC (Elmquist 2001). Consistent with this report, our results demonstrate that central administration of citrate increases the level of malonyl-CoA in the hypothalamus. Since citrate is produced in nutrient availability conditions, we hypothesized that citrate might act as a hypothalamic anorexigenic signal and modulate the AMPK/ACC pathway and peripheral glucose homeostasis. This hypothesis is strengthened by the presence of a plasma membrane transporter with preference for citrate as a substrate. Recently, a plasma membrane transporter capable of citrate transport was described in mouse brain (Inoue et al. 2002). This transporter is located in most brain neurons and may contribute to pool cytosolic citrate. Following cytoplasm entry, citrate is cleaved by ATP-citrate lyase to generate acetyl-CoA, but the citrate pool may also serve as an energy substrate. Recently, a plasma membrane transporter with preference for citrate as a substrate.
citrate or ARA-A ICV injection, the animals presented reduced hypothalamic phosphorylation of AMPK and ACC and low food intake comparatively with the control group. Together, these results suggest that citrate inhibits AMPK and activates ACC to elevate the malonyl-CoA level and reduce food intake. Thus, AMPK/ACC enzymes may respond to cytoplasmic citrate, resulting in changes similar to those observed after ICV injection of other AMPK inhibitors such as...
insulin, leptin, and glucose in rats (Friedman & Halaas 1998, Schwartz et al. 2000, Andersson et al. 2004, Minokoshi et al. 2004). Moreover, leptin action in the hypothalamus results in thermogenesis and energy expenditure (Elmquist 2001) and the activation of ACC appears to be important to leptin-mediated anorectic action (Gao et al. 2007). Therefore, since citrate treatment reduced food intake and body weight significantly, but the variation in body weight and final epididymal fat pad mass in the citrate pair-fed group was smaller than those of the citrate group were, we may speculate that the citrate treatment increased energy expenditure. This makes sense considering that the inhibition of hypothalamic

Figure 7 Insulin signaling in skeletal muscle of saline control animals, citrate ICV rats (20 nmol), and ARA-A ICV rats (2 nmol). The saline, citrate, and ARA-A ICV injections were performed 30 min before either the acute treatment with saline (saline EV, open bars) or insulin (insulin EV, closed bars) through the cava vein (EV; 0 μl, 10−6 M). (A) Immunoprecipitation (IP) with anti-IR antibody and immunoblotting (IB) with anti-αPy antibody, (B) IP with anti-IRS1 antibody and IB with anti-αPy antibody, (C) IP with anti-IRS2 antibody and IB with anti-αPy antibody, and (D) IB with anti-p-AKT. The expression of IR, IRS1, IRS2, and AKT was determined in the protein extract separated by SDS-PAGE and transferred to nitrocellulose directly. The membranes were blotted with anti-IR, anti-IRS1, anti-IRS2, and anti-AKT antibodies to evaluate the total expression of protein. *P ≤ 0.05 for saline ICV/insulin EV versus saline ICV/saline EV, **citrate ICV/insulin EV versus citrate ICV/saline EV, ***ARA-A ICV/insulin EV versus ARA-A ICV/saline EV, +ARA-A ICV/insulin EV versus saline ICV/insulin EV. All results were considered significantly different at P ≤ 0.05.
AMPK is an anorexigenic signal (Minokoshi et al. 2004). Recently, it has been shown that adiponectin-deficient mice present decreased AMPK phosphorylation and food intake and increased energy expenditure (Kubota et al. 2007). Therefore, hypothalamic AMPK seems to play an important role in energy expenditure control. It is worth noting that this effect was obtained under fasting as well. In this condition, the counter-regulatory hormones are increased and promote energy intake (Kubota et al. 2007). Therefore, citrate might be acting as a cell signal and informing about the energy availability level. Probably, the effect of citrate on the phosphorylation of hypothalamic AMPK would be indirect and originate from the production of ATP from the entry of citrate into the cell.

The anorexigenic effect of citrate is corroborated by data obtained after evaluation of hypothalamic NPY, POMC, and CRH mRNA levels. In our study, citrate could also promote the reduction of NPY mRNA expression in the hypothalamus. Additionally, POMC and CRH expressions were increased in fasted rats after citrate ICV treatment. In another study, AMPK inhibition in the hypothalamus also was sufficient to decrease food intake and suppress mRNA expression of NPY (Minokoshi et al. 2004). Additionally, the plasma corticosterone level in fasted rats (basal level) was markedly diminished (42%) by citrate ICV injection. Other authors showed that the blood corticosterone level presented a prandial fall subsequent to feeding (Homma et al. 1986, Jahng et al. 2005). Therefore, we can admit that citrate ICV administration changed the feeding behavior and the molecular characteristic of the hypothalamus despite the effect of fasting in rats.

In addition to the effects on food intake and neuropeptide expression in the hypothalamus, hypothalamic AMPK seems to play an important role in the central control of the peripheral metabolism. Hypothalamic AMPK modulation mediates glycogen synthesis in muscle (Perrin et al. 2004) and fatty acid oxidation in muscle is induced by early activation of AMPK by leptin acting directly on muscle, whereas later activation depends on leptin functioning through the hypothalamic–sympathetic nervous system axis (Minokoshi et al. 2002). Furthermore, AMPK knockout mice presented impaired glucose homeostasis (Vioillet et al. 2003). Although the mechanisms are not completely known yet, these studies established an important correlation between AMPK inhibition and glucose homeostasis. In accordance to this possibility, our results showed that rats treated with citrate by ICV infusion display improved glucose homeostasis as evaluated by GTT and euglycemic–hyperinsulinemic clamp. The possibility of citrate itself inducing insulin secretion was investigated, but it was not confirmed, since the blood insulin level did not change after citrate ICV administration (up to 60 min) in fasted rats. Therefore, we suspected that an improvement in glucose homeostasis could be caused by a more efficient stimulus of the elements of the insulin signaling pathway.

Finally, with regard to the citrate ICV effect on protein phosphorylation stimulated by insulin, we evaluated the phosphorylation level of IR, IRS1, IRS2, and AKT. Interestingly, the citrate treatment (ICV) promoted an improvement in the insulin-stimulated phosphorylation of IR, IRS1, and AKT proteins in the epididymal fat pad. On the other hand, in skeletal muscle, IR, IRS2, and AKT proteins presented an increase in insulin-stimulated phosphorylation after citrate administration. These effects were also obtained when the AMP analog, ARA-A, an AMPK competitive inhibitor, was ICV injected. Moreover, skeletal muscle of rats treated with citrate for 7 days presented a glycogen level (59%) higher the control group (data not shown) did. Since citrate improved insulin signaling, a more robust control of the glycogen synthesis activation and inhibition of glycogen breakdown seem to have occurred during the experimental protocol (7 days). Thus, the inhibition of hypothalamic AMPK seems to have particular importance for events observed in peripheral tissues. In contrast to our findings, studies revealed that total α2AMPK knockout mice display insulin resistance, impaired AICAR and glucose tolerance, impaired glucose-stimulated insulin secretion, reduced insulin-stimulated whole-body glucose utilization and skeletal muscle glycogen synthesis, and elevated catecholamine excretion in urine (Viollet et al. 2003). Nevertheless, in our study, only hypothalamic AMPK was inhibited by treatment. Thus, we believe that the peripheral tissue effects are secondary to the inhibition of hypothalamic AMPK. We cannot disregard the involvement of hypothalamic ACC in these effects, particularly because ACC is responsible for the synthesis of malonyl-CoA, which has been attributed an important role in the energy homeostasis (Wolfgang & Lane 2006). However, the mechanisms of such effects are unclear, but activation of the hypothalamic–sympathetic nervous system is likely.

Although several possibilities may be discussed to try to elucidate the mechanisms involved in the inactivation of hypothalamic AMPK and its effects on glucose homeostasis, a new component that may enhance the insulin signaling pathway protein should be considered. Heterotrimeric G-proteins have been recognized as important points of convergence of signaling from G-protein-linked pathways and tyrosine kinase-mediated pathways (Morris & Malbon 1999). The expression of a constitutively active form of G-protein, Gα12, leads to enhanced glucose tolerance (Chen et al. 1997) and glucose transporter type 4 (GLUT4) localization at the plasma membrane in the absence of insulin (Song et al. 2001). In addition, when Gα12 is overexpressed in vivo, enhanced insulin signaling is observed, probably via suppression of protein, tyrosine phosphatase 1B (Tao et al. 2001). Thus, we can speculate that central administration of citrate might activate Gα12 in the epididymal fat pad and in skeletal muscle and improve insulin signaling.

The biochemical events related to the presence of citrate in the cytoplasm are well known. Citrate is an important negative modulator of phosphofructokinase (PFK) and would decrease the glycolytic flux and the ATP level, leading to AMPK activation. Thus, the exogenous administration of citrate would be expected to activate AMPK. Later, citrate would be transferred to the mitochondria, and thus the
activation of AMPK would be reduced. However, AMPK is regulated by fasting and feeding in such a way that during fasting, AMPK is active and imposes a negative regulation on ACC. By contrast, after feeding, AMPK is rapidly inactivated and ACC activity is restored, as previously demonstrated by our group (Roman et al. 2005). The data shown in the present paper were obtained from fasted animals that presented increased phosphorylation of hypothalamic AMPK. The reduced phosphorylation of hypothalamic AMPK for all the times after exogenous citrate administration investigated suggests that, like glucose, citrate could elevate the ATP level in the cytoplasm very fast. Consistent with the important effect attributed to citrate administered exogenously, food intake stimulated by fasting was diminished by citrate ICV injection in fasted animals.

In conclusion, the increase in the concentration of cytoplasmic citrate in neurons may indicate the nutrient availability to the central nervous system and modulate glucose homeostasis and energy intake. A point of note, the signal is strong enough to change feeding behavior and glucose homeostasis even in fasted animals. Taken together, these results provide a new target for the study of the central regulatory mechanism of body energy disposal. In addition to enzymes AMPK and ACC, the enzymes involved in the citrate metabolism are potential targets of studies of therapeutic agents for diabetes control and body weight reduction.

Declaration of Interest

The authors declare that there is no conflict of interest that would prevent the impartiality of this scientific work.

Funding

This study was supported by grants from Fundação de Amparo à Pesquisa do Estado de São PauloFAPESP (03/127382) and CNPq.

Acknowledgements

We thank Mr Luiz Janeri, Mr Józimo Ferreria, and Mr Marcio Alves da Cruz for their technical assistance and Mr Laerte J Silva for the English language editing.

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Received in final form 5 May 2008
Accepted 8 May 2008
Made available online as an Accepted Preprint 9 May 2008