Activation of AMPK in rat hypothalamus participates in cold-induced resistance to nutrient-dependent anorexigenic signals

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The exposure of homeothermic animals to a cold environment leads to a powerful activation of orexigenic signalling which is accompanied by molecular and functional resistance to insulin-induced inhibition of feeding. Recent evidence suggests that AMPK participates in nutrient-dependent control of satiety and adiposity. The objective of the present study was to evaluate the effect of cold exposure upon the molecular activation of AMPK signalling in the hypothalamus of rats. Immunoblotting demonstrated that cold exposure *per se* is sufficient for inducing, on a time-dependent basis, the molecular activation of the serine/threonine kinase AMP-activated protein kinase (AMPK) and inactivation of the acetyl-CoA carboxylase (ACC). These molecular phenomena were accompanied by resistance to nutrient-induced inactivation of AMPK and activation of ACC. Moreover, cold-exposure led to a partial inhibition of a feeding-induced anorexigenic response, which was paralleled by resistance to insulin-induced suppression of feeding. Finally, cold exposure significantly impaired insulin-induced inhibition of AMPK through a mechanism dependent on the molecular cross-talk between phosphatidylinositol-3(PI_3)-kinase/Akt and AMPK. In conclusion, increased feeding during cold exposure results, at least in part, from resistance to insulin- and nutrient-dependent anorexigenic signalling in the hypothalamus.

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During the last decade obesity has reached epidemic proportions in populations of several regions of the world (Friedman, 2000; Kopelman, 2000). The loss of a coordinated control between food intake and energy wastage is thought to play a pivotal role in the progressive gain of body weight and adiposity observed in overweight people (Flier, 2004). So far, leptin and insulin are known to be the most important adipostatic factors (Flier, 2004), controlling neurones of the arcuate nucleus that expresses orexigeninc (neuropeptide Y (NPY) and agouti-related protein homologue (AGRP)) and anorexigenic (α-melanocyte-stimulating hormone (MSH) and cocaine and amphetamine related transcript (CART)) neurotransmitters (Schwartz *et al.* 2000). The role played by the nutrients, *per se*, in the control of adiposity has been a question long debated (Jequier, 2002). Most data suggest that nutrients may have a role only as satiety signals (Blundell & MacDiarmid, 1997; Roberts, 2000). However, in a recent study, Minokoshi *et al.* (2004) showed that the serine/threonine kinase AMP-activated protein kinase (AMPK) participates as a molecular link between hormone and nutrient signals to hypothalamic neurones that control feeding and adiposity.

Homeothermic animals adapted to the cold environment provide a reproducible physiological model for studying several aspects of energy accumulation and expenditure (Vallerand *et al.* 1987; Gasparetti *et al.* 2003). During cold exposure, homeothermic animals become hyperphagic and resistant to the anorexigenic signals generated by insulin (Gasparetti *et al.* 2003; Pereira-Da-Silva *et al.* 2003; Torsoni *et al.* 2003). Considering that hyperphagia may provide the hypothalamus with a surplus of nutrient anorexigenic signals, we suspect that, in order to maintain continuous feeding, a mechanism of resistance to this signal may evolve in parallel with the already known resistance to hormonal inputs. Therefore, the objective of the present study was to evaluate the participation of AMPK in the control of feeding behaviour of rats exposed to a cold environment.

M. A. Torsoni and L. A. Velloso contributed equally as supervisors for this study.

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Methods

Antibodies, chemicals and buffers

Reagents for SDS-polyacrylamide gel electrophoresis and immunoblotting were from Bio-Rad (Richmond, CA, USA). Hepes, phenylmethylsulphonyl fluoride, aprotinin, dithiothreitol, Triton X-100, Tween 20, glycerol, bovine serum albumin (fraction V), citrate and LY-294002 were from Sigma (St Louis, MO, USA). Protein A-sepharose 6MB was from Pharmacia (Uppsala, Sweden),^{125}I-protein A was from ICN Biomedicals (Costa Mesa, CA, USA), and nitrocellulose paper (BA85, 0.2 μm) was from Amersham (Aylesbury, UK). Sodium thiopental and human recombinant insulin (Humulin R) were from Lilly (Indianapolis, IN, USA). Polyclonal anti-phosphotyrosine antibodies were raised in rabbits and affinity purified on phosphotyramine columns. Anti-phospho-[Ser^{473}] Akt (rabbit polyclonal, sc-7985-R) and anti-insulin receptor (IR) β (rabbit polyclonal, sc-711) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-phospho-[Ser^{79}] ACC (rabbit polyclonal, #07-184) was from Upstate Biotechnology (Charlottesville, VA, USA). Anti-ACC (goat polyclonal, sc-26816) was from Santa Cruz Biotechnology, Inc. Anti-phospho-[Thr^{172}] AMPKα (rabbit polyclonal, #2531) and anti-AMPKα (rabbit polyclonal, #2532) were from Cell Signalling Technology (Beverly, MA, USA). Insulin was determined by radioimmunoassay (RIA).

Experimental animals, intracerebroventricular (i.c.v.) cannulation and cold exposure protocols

Male Wistar rats (*Rattus norvegicus*) (8 weeks old/200–300 g) obtained from the University of Campinas Animal Breeding Center were used in the experiments. The investigation followed the University guidelines for the use of animals in experimental studies and conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication no. 85-23 revised 1996). The animals were maintained on 12 : 12 artificial light–dark cycles and housed in individual cages. After the acclimatizing period (3 days), the animals were stereotaxically instrumented under sodium thiopental anaesthesia (diluted in saline, 15–40 mg (kg body weight)$^{-1}$, intraperitoneal (I.P.)) with chronic unilateral 26-gauge stainless steel indwelling guide cannulas, aseptically placed into the lateral ventricle (0.2 mm posterior, 1.5 mm lateral and 4.2 mm ventral to bregma) as previously described (Michelotto *et al.* 2002). Post-operative analgesia was provided by the use of 0.05 mg kg^{-1} buprenorphine I. Cannula placement was confirmed by a positive drinking response after administration of angiotensin II (40 ng (2 μl)$^{-1}$). After a one-week recovery period, all rats were placed in individual cages and exposed either to 23 $\pm$ 1°C (control) or to 4 $\pm$ 1°C (*T*4°C) for three days, or for shorter periods of time, according to the description in the Results section. The animals were allowed free access to standard rodent chow and water *ad libitum*. For tissue extraction (at day 2 of the experimental protocol), rats were anaesthetized by I.P. injection of sodium thiopental (15–40 mg (kg body weight)$^{-1}$), and the experiments were performed after the loss of corneal and pedal reflexes. Following experimental procedures, the rats were killed under anaesthesia (thiopental > 200 mg kg^{-1}) following the recommendations of the NIH publication no. 85-23.

Metabolic, hormone and biochemical measurements

Measurements of food intake, rectal temperature and body weight (during the light cycle) were obtained at time 0 and 2 h from the beginning of the experimental period, and daily, during the three experimental days in control and cold-exposed rats. Rectal temperature was measured with a Thermistor high precision digital thermometer (Hanna Instruments, Inc., Woonsocket, RI, USA) inserted 1.5 cm from the anus. Blood samples were always obtained from rats fasted for 2 h on the morning of the second experimental day. Plasma glucose was measured by the glucose oxidase method in samples collected from the tail (Trinder, 1969). Insulin was detected by RIA, utilizing a guinea pig antirat insulin antibody and rat insulin as standard (Scott *et al.* 1981). Corticosterone and thyroid-stimulating hormone (TSH) were measured by RIA, according to the manufacturer's specifications.

Protocol for food ingestion determination

One week after cannula installation, the rats were randomly sorted into cold exposure or thermoneutrality protocols, in metabolic, individual cages. At 6 p.m. on the second experimental day (after 6 h of food deprivation), insulin (2 μl, 10^{-6} M), citrate (2 μl containing 200 pmol) or saline (2 μl) were injected into the cannula. Food ingestion was determined over the next 12 h.

Tissue extraction, immunoblotting and immunoprecipitation

Anaesthetized rats were acutely treated, or not, with insulin (2 μl, 10^{-6} M) or saline (2 μl) through the I.C.V. cannula. After different intervals (described in Results), the rats were submitted to decapitation and the hypothalamus was rapidly removed and immediately homogenized in solubilization buffer at 4°C (1% Triton X-100, 100 mM Tris-HCl (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA,

10 mM sodium orthovanadate, 2.0 mM PMSF and 0.1 mg aprotinin ml^{-1}) with a Polytron PTA 20S generator (model PT 10/35; Brinkmann Instruments, Westbury, NY, USA) operated at maximum speed for 30 s. Insoluble material was removed by centrifugation for 20 min at 9000 *g* in a 70.Ti rotor (Beckman) at 4°C. The protein concentration of the supernatant was determined by the Bradford dye-binding method. Aliquots of the resulting supernatants containing 2.0 mg of total protein were used for immunoprecipitation with antibodies against IR at 4°C overnight, followed by SDS/PAGE, transfer to nitrocellulose membranes and blotting with anti-phosphotyrosine or anti-IRβ antibodies. In direct immunoblot experiments, 0.2 mg of protein extracts obtained from each tissue were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted with anti-phospho-Akt, anti-phospho-AMPK, anti-AMPKα and anti-phospho-ACC antibodies, as described (Calegari *et al.* 2003). In some experiments, rats were pretreated (30 min in advance) with LY 294002 (2 μl, 50 μmol l^{-1}).

Data presentation and statistical analysis

All numerical results are expressed as the mean ± S.E.M. of the indicated number of experiments. The results of blots are presented as direct comparisons of bands in autoradiographs and quantified by densitometry using the ScionCorp software (Scion Image). Student's *t* test for unpaired samples and analysis of variance (ANOVA) for multiple comparisons were used for statistical analysis as appropriate. The *post hoc* test was employed when required. The level of significance was set at $P < 0.05$.

Results

Metabolic, hormonal and biochemical characterization of rats exposed to cold

As shown in Table 1, the exposure of Wistar rats to a cold environment for two days led to increased mean daily food intake, which was accompanied by weight loss, hypoinsulinaemia and hypoleptinaemia. No significant modulations of blood glucose, TSH and corticosterone levels were detected. Thus, the present model matches most of the previously published characterizations of rodents exposed to cold (Vallerand *et al.* 1987; Gasparetti *et al.* 2003).

Regulation of AMPK molecular activation by fasting and feeding

To evaluate the role of feeding upon the control of hypothalamic AMPK molecular activation, individually housed rats were randomly divided into two groups, fasting and feeding, and used for determination of [Thr^{172}] AMPK phosphorylation. The fasting group was food restricted (maintained only with water *ad libitum*) overnight for 12 h, while the feeding group received water and regular rodent chow *ad libitum* during the same period. As compared to fasted rats, feeding was accompanied by a reduction of 88% ($P < 0.05$) in [Thr^{172}] AMPK phosphorylation in the hypothalamus (Fig. 1*A*). Moreover, feeding was also accompanied by 79% ($P < 0.05$) reduction in [Ser^{79}] phosphorylation of ACC, a direct substrate of AMPK (Fig. 1*B*). There were no significant differences in the amounts of AMPK and ACC between fed and fasting groups.

Table 1. Metabolic, hormonal and biochemical characterization of Wistar rats exposed for two days to +4°C

Parameters	Control	*T*4°C
Mean food intake (g (24 h)$^{-1}$)	18.9 ± 1.3	27.2 ± 2.1*
Body temperature (°C)	36.4 ± 0.4	36.1 ± 0.4
Body weight variation (g)	+ 5.0 ± 0.6	−11.9 ± 0.8*
Glucose (mg dl^{-1})	92.4 ± 1.3	91.2 ± 2.0
Insulin (ng ml^{-1})	2.55 ± 0.34	1.32 ± 0.24*
Leptin (pg ml^{-1})	2435 ± 221	1503 ± 119*
TSH (ng ml^{-1})	13.6 ± 1.3	13.9 ± 2.0
Corticosterone (ng ml^{-1})	93.5 ± 3.6	98.3 ± 4.4

For all parameters; $n = 6$, $^*P < 0.05$.

Cold exposure induces the activation of AMPK in the hypothalamus

To evaluate the effect of cold exposure upon AMPK functional status, non-food-restricted Wistar rats were randomly divided into seven groups. Group 1 was the control, not submitted to cold. Rats in the remaining groups were exposed to cold for 2, 6, 12, 24, 48 and 72 h. After the respective periods of cold exposure, the rats were anaesthetized and the hypothalamus excised for total protein extract preparation. Samples were separated by SDS-PAGE, transferred to nitrocellulose membrane and blotted with anti-phospho-[Thr^{172}] AMPK. As shown in Fig. 2*A*, no [Thr^{172}] AMPK phosphorylation was observed in control rats. However, beginning at 2 h and reaching the highest levels at 48 h, cold exposure induced [Thr^{172}] AMPK phosphorylation in the hypothalamus. Moreover, 48 h cold exposure also induced a significant increase in [Ser^{79}] phosphorylation of ACC (Fig. 2*B*). It is important to notice that in this set of experiments the rats were not food restricted. Therefore, the hyperphagia induced by cold exposure was not sufficient to hamper the effect of cold upon this signalling system.

Cold exposure induces molecular and functional impairment of the anorexigenic signals through AMPK

To test the hypothesis that cold exposure impairs anorexigenic signalling through AMPK, rats were either

maintained at thermoneutrality or exposed to cold for two days. Rats from each group were then divided into fed and fasting (12 h fast). Animals were anaesthetized and the hypothalamus obtained for protein extraction, separation by SDS-PAGE, transfer to nitrocellulose membranes and blotting with anti-phospho-[Thr^{172}] AMPK or anti-phospho-[Ser^{79}] ACC. As depicted in Fig. 3*A*, cold-exposure led to phosphorylation of AMPK and ACC even in fed rats, suggesting that a molecular pro-orexigenic signal is continuously maintained in

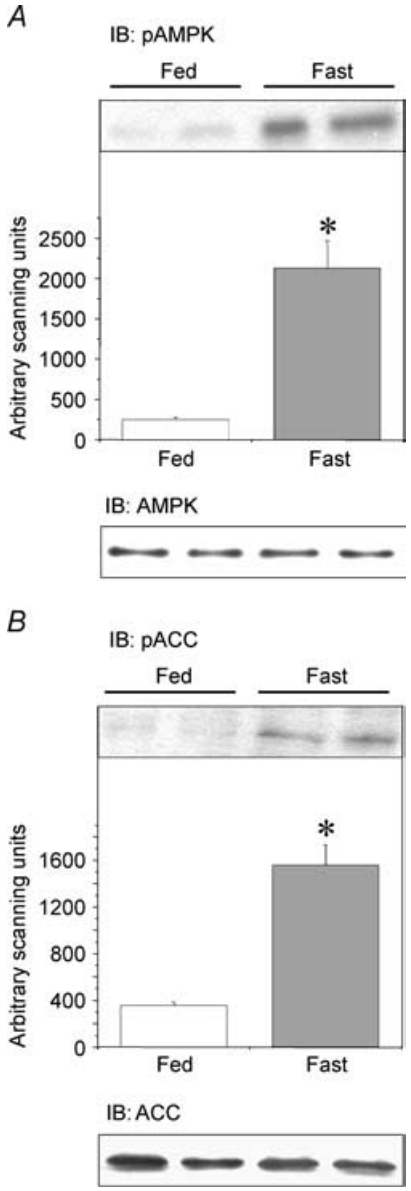


Figure 1. Effect of fasting and feeding upon the expression and molecular functional status of AMPK and ACC in the hypothalamus
The hypothalami of anaesthetized rats maintained on an unrestricted offer of diet (Fed), or food restricted for 12 h (Fast) were homogenized on ice-cold extraction buffer. Aliquots of protein extracts containing 0.2 mg protein were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted (IB) with anti-phospho-[Thr^{172}] AMPK (pAMPK) (*A*, upper blot) or anti-AMPK (*A*, lower blot), or anti-phospho-[Ser^{79}] ACC (pACC) (*B*, upper blot) or anti-ACC (*B*, lower blot). Specific bands were quantified by scanning densitometry and means ± S.E.M. are plotted as arbitrary scanning units. In all experiments, $n = 5$, $^*P < 0.05$ *versus* Fed.

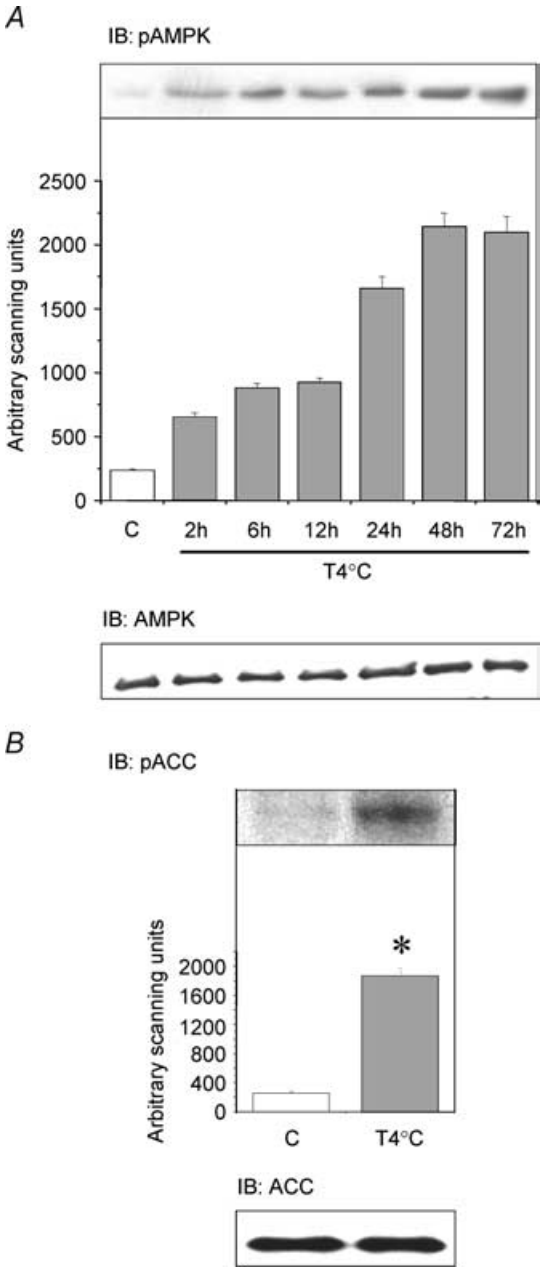


Figure 2. Effect of cold exposure upon the molecular activity of AMPK
A, rats were maintained at thermoneutrality (C) or exposed to a cold environment (T4°C) for progressively longer time-frames (as depicted in the figure). After anaesthesia, hypothalami were obtained for protein extraction. Aliquots of protein extracts containing 0.2 mg protein were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted (IB) with anti-phospho-[Thr^{172}] AMPK (pAMPK) (*A*, upper blot) or anti-AMPK (*A*, lower blot), and anti-phospho-[Ser^{79}] ACC (pACC) (*B*, upper blot) or anti-ACC (*B*, lower blot). In *B*, rats were exposed to cold for 48 h. Specific bands were quantified by scanning densitometry and means ± S.E.M. are plotted as arbitrary scanning units. In all experiments, $n = 5$, $^*P < 0.05$ *versus* C.

cold-exposed rats independently of their nutritional state. To determine whether cold exposure would functionally impair nutrient-induced anorexigenic response, control and cold-exposed rats were I.C.V. cannulated and treated with saline, insulin or citrate (an activator of ACC) and evaluated for 12 h for spontaneous food intake. As depicted in Fig. 3*B*, similarly to the impairment of the insulin-induced anorexigenic molecular signal, there was a significant impairment of the citrate-induced anorexigenic signal ($35 \pm 11\%$ *versus* $9 \pm 3\%$ reduction of food intake ($P < 0.05$)) demonstrating that at both the molecular and functional level, cold exposure promotes an impairment of the nutrient-induced anorexigenic signal.

Cold exposure induces molecular and functional resistance to insulin action in hypothalamus

Together with leptin, insulin provides the most robust and reproductive anorexigenic signal to the hypothalamus. To evaluate the effect of cold exposure upon insulin action in the hypothalamus, I.C.V. cannulated rats were exposed or not to a cold environment for four days. On the second day the rats were fasted for 6 h and then acutely treated with saline (2 μl) or insulin (2 μl, 10^{-6} M) I.C.V. Chow was reintroduced, and 12 h food intake was determined. As depicted in Fig. 4*A*, cold exposure significantly impaired insulin-induced suppression of food ingestion (reduction of $55 \pm 6\%$ *versus* $11 \pm 4\%$ ($P < 0.05$), for

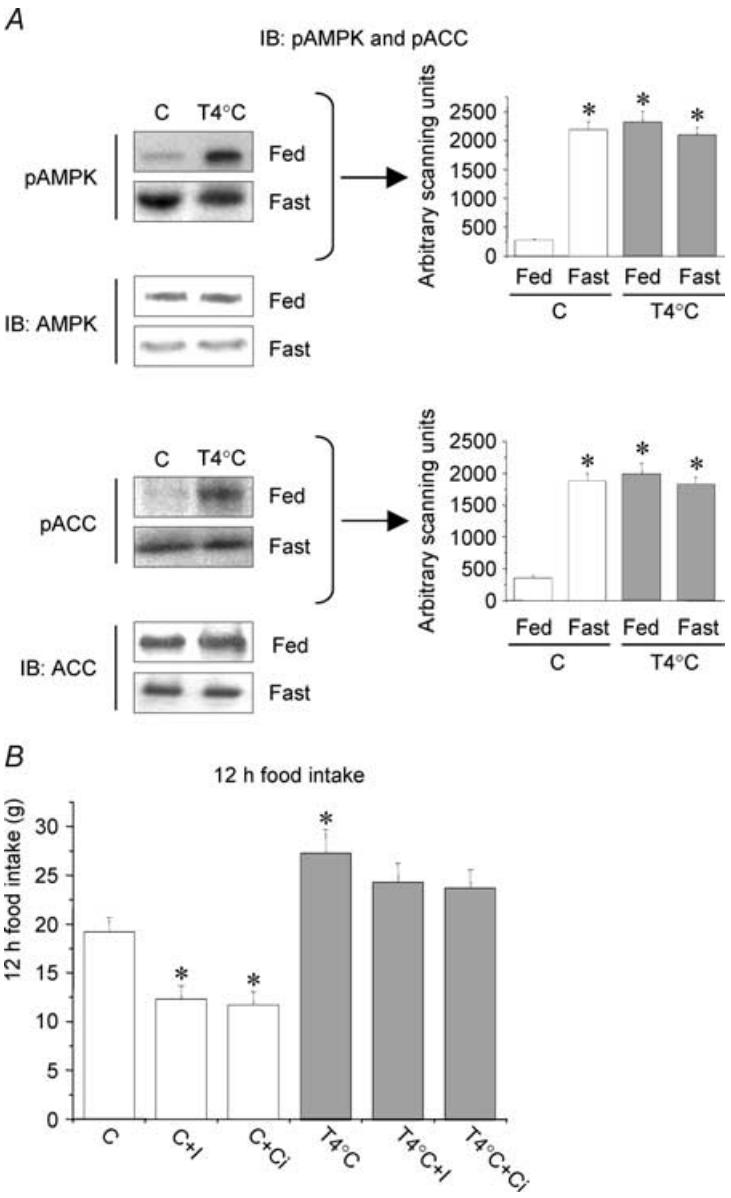
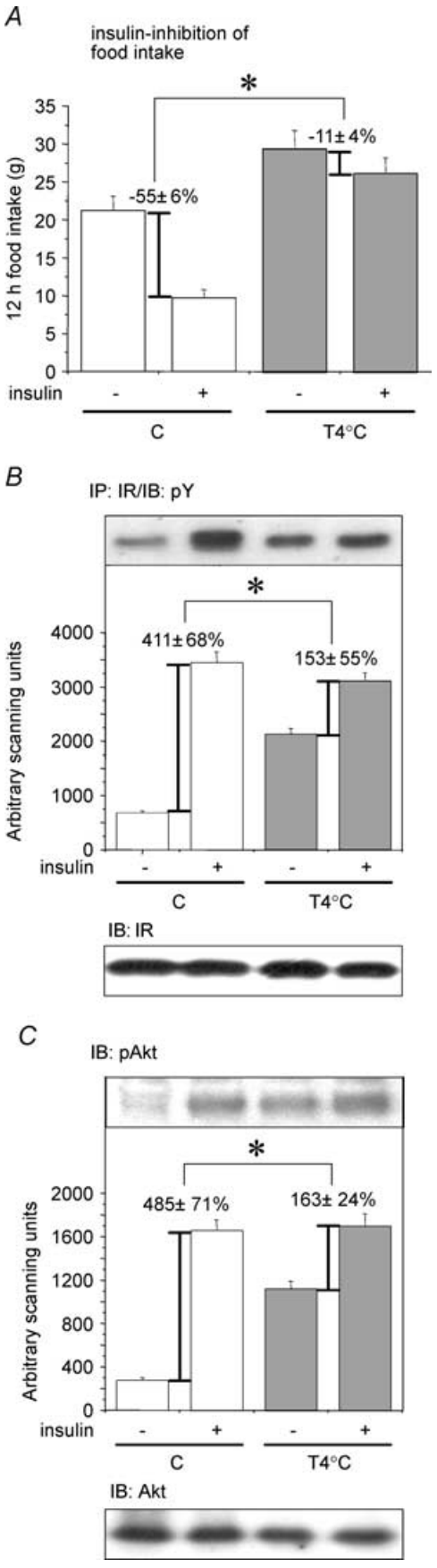


Figure 3. Effect of nutrients on AMPK activity and suppression of feeding
A, rats were divided into two groups, control (C), which were maintained at thermoneutrality; and cold-exposed (T4°C). Some animals were food restricted for 12 h (Fast), while others were not food restricted (Fed). After anaesthesia, hypothalami were obtained for protein extraction. Aliquots of protein extracts containing 0.2 mg protein were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted (IB) with anti-phospho-[Thr^{172}] AMPK (pAMPK) or anti-AMPK; and anti-phospho-[Ser^{79}] ACC (pACC) or anti-ACC (as depicted in the figure). Specific bands were quantified by scanning densitometry and means ± S.E.M. are plotted as arbitrary scanning units. *B*, I.C.V. cannulated rats were maintained at thermoneutrality (C) or exposed to cold (T4°C). Six hours food restricted rats were treated with an I.C.V. dose of insulin (C + I) 2 μl, 10^{-6} M, citrate (C + Ci) 2 μl containing 200 pmol or saline (2 μl) and spontaneous food ingestion was determined over the next 12 h. In all experiments, $n = 5$; in *A*, $^*P < 0.05$ *versus* C/Fed; in *B*, $^*P < 0.05$ *versus* C.



control and cold-exposed, respectively). In a parallel series of experiments, four-day cold-exposed or control I.C.V. cannulated rats were fasted for 6 h, anaesthetized and acutely treated with insulin (2 μl, 10^{-6} M, I.C.V.) or saline (2 μl, I.C.V.). Insulin-induced engagement of IR and Akt were determined by immunoprecipitation and immunoblot experiments. As shown in Fig. 4*B*, cold exposure significantly reduced insulin-induced tyrosine phosphorylation of IR, as measured by the difference in tyrosine phosphorylation levels between basal and insulin-stimulated ($411 \pm 68\%$ *versus* $153 \pm 55\%$ ($P < 0.05$), for control and cold-exposed, respectively). In addition, cold exposure also led to a significant reduction in insulin-stimulated [Ser^{473}] phosphorylation of Akt, which was measured as the difference in [Ser^{473}] phosphorylation levels between basal and insulin-stimulated ($485 \pm 71\%$ *versus* $163 \pm 24\%$ ($P < 0.05$), for control and cold-exposed, respectively; Fig. 4*C*).

Cold exposure impairs the cross-talk between PI_3-kinase/Akt and AMPK signalling pathways

Acting through the phosphatidylinositol (PI_3)-kinase/Akt pathway, insulin exerts a negative control upon AMPK (Kovacic *et al.* 2003). To evaluate if the cross-talk between PI_3-kinase/Akt and AMPK signalling pathways plays a role in cold-induced impairment of nutrient-dependent inactivation of AMPK, cannulated, fasting, non-cold-exposed; and fed, cold-exposed rats were treated with the PI_3-kinase inhibitor, LY-294002, and evaluated for insulin-induced inactivation of AMPK. LY-294002 treatment significantly inhibited insulin-induced [Ser^{473}]-Akt phosphorylation both in control and cold-exposed rats (Fig. 5*A*). These effects

Figure 4. Effect of cold exposure upon insulin-induced inhibition of food intake and insulin signal transduction in the hypothalamus
A, I.C.V. cannulated control (maintained at thermoneutrality) (C) or cold-exposed (T4°C) rats were food restricted for 6 h (from 12 to 18 h). At 18 h, rats received an I.C.V. dose of insulin (+) (2 μl, 10^{-6} M), or saline (−) (2 μl). Food was reintroduced and total consumption (g) was evaluated during the next 12 h. For evaluation of insulin signalling, I.C.V. cannulated, control and cold-exposed rats were treated with an I.C.V. dose of insulin (+) (2 μl, 10^{-6} M), or saline (−) (2 μl). Hypothalami were obtained for protein extraction. Aliquots containing 2.0 mg total protein were used in immunoprecipitation (IP) experiments with anti-insulin receptor (IR) antibodies (*B*). Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted (IB) with antiphosophotyrosine (pY) antibodies. In addition (*C*) aliquots containing 0.2 mg total protein were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted (IB) with anti-phospho-[Ser^{473}] Akt (pAkt) antibodies. Specific bands were quantified by scanning densitometry and means ± S.E.M. are plotted as arbitrary scanning units. In all experiments, $n = 5$; $^*P < 0.05$.

were accompanied by inhibition of the insulin-induced reduction of [Thr^{172}] AMPK phosphorylation and [Ser^{79}] ACC phosphorylation (Fig. 5*B*). However, this effect was significantly more pronounced in control, fasting rats than in cold-exposed, fed rats ($865 \pm 55\%$ *versus* $235 \pm 32\%$ increase from insulin alone to insulin + LY294002 ($P < 0.05$), for AMPK phosphorylation in control, fasting and T4°C, fed, respectively; and $543 \pm 67\%$ *versus* $167 \pm 19\%$ increase from insulin alone to insulin + LY294002 ($P < 0.05$), for ACC phosphorylation in control – fasting and T4°C – fed, respectively), suggesting that cold-induced impairment of anorexigenic signals is affected by the cross-talk between PI_3-kinase/Akt and AMPK signalling pathways.

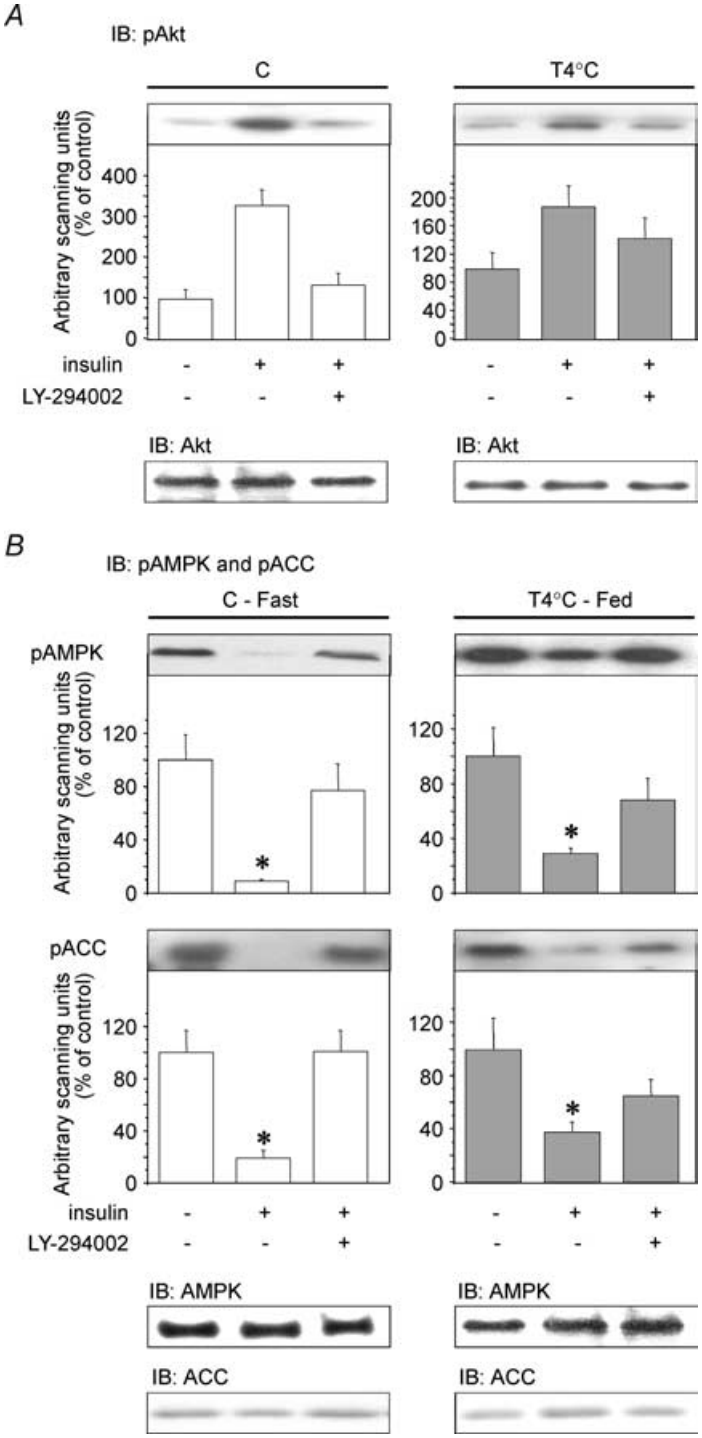


Figure 5. Effect of inhibition of PI_3-kinase on AMPK molecular activation
I.C.V. cannulated rats were anaesthetized and treated with saline (–), insulin (2 μl, 10^{-6} M) and/or LY-294002 (30 min in advance, 2 μl, 50 μmol l^{-1}). Hypothalami were obtained for protein extraction. Aliquots of protein extracts containing 0.2 mg protein were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted (IB) with anti-phospho-[Ser^{473}] Akt antibodies (pAkt) (*A*, upper blots) or anti-Akt (*A*, lower blots); anti-phospho-[Thr^{172}] AMPK (pAMPK) (*B*, the first two blots), anti-phospho-[Ser^{79}] ACC (pACC) (*B*, the second two blots), anti-AMPK (*B*, the third two blots) and anti-ACC (*B*, the fourth two blots). Specific bands were quantified by scanning densitometry and means ± S.E.M. were used for comparison. In all experiments $n = 5$; $^*P < 0.05$ *versus* the respective control –/– (insulin/LY-294002).

Discussion

Physiological regulation of feeding behaviour is, certainly, one of the most important phenomena developed during evolution to warrant organism survival. As in other vital physiological events, here, redundancy and multifactoriality are *sine qua non* requirements for avoiding life-threatening failures in the appropriate response to a lack of nutrient. In this multiplex regulation, the satietogenic effects of nutrients such as glucose, other carbohydrates, fat and aminoacids, and the short-term regulation by gut hormones have been known for quite some time (Blundell & MacDiarmid, 1997; Bray, 2000; Roberts, 2000; Schwartz *et al.* 2000). These mechanisms play an important role in feeding initiation and termination; however, their effects upon long-term feeding control and body adiposity were thought to be scarce.

During the last decade, impressive progress has been made in the characterization of adipostatic factors (Friedman, 2000; Schwartz *et al.* 2000; Flier, 2004). Insulin and leptin have been characterized as the mediators responsible for delivering the most robust signals informing the central nervous system about peripheral energy stores. This allows for an appropriate adjustment of feeding behaviour and thermogenesis (Schwartz *et al.* 2000; Zabeau *et al.* 2003; Flier, 2004). In addition, a recent study by Minokoshi *et al.* (2004) and a subsequent study by Andersson *et al.* (2004) have established a new paradigm in nutrient-dependent control of feeding and adiposity. AMPK is a component of a protein kinase cascade that acts as an intracellular energy sensor (Hardie *et al.* 2003). In the hypothalamus, AMPK is inactivated by both hormonal and nutrient anorexigenic signals and also by the exogenous stimulation of the receptor for melanocortin, a potent anorexigenic neurotransmitter (Minokoshi *et al.* 2004). Moreover, the genetic manipulation of AMPK in the hypothalamus is sufficient to control not only feeding behaviour, but also body weight. Therefore, AMPK seems to act as a link between hormonal and nutritional controllers of feeding and adiposity, and for this reason it has become an attractive potential target for therapeutics in obesity (Minokoshi *et al.* 2004).

In the present study, we have explored the hypothesis that during cold exposure, when there is a higher requirement for energy in order to maintain body temperature, an impairment of the anorexigenic stimulus generated by feeding, would be dependent on AMPK regulation. In the first part of the study, it was confirmed that hypothalamic AMPK activity is modulated by the nutritional status (Minokoshi *et al.* 2004), in such a way that during fasting AMPK is active and delivers a negative signal to ACC. In contrast, after feeding AMPK is rapidly inactivated and ACC activity is restored. In the second part of the study, cold exposure was shown to be capable, in a time-dependent fashion, of increasing [Thr^{172}] AMPK phosphorylation. This effect was accompanied by increased [Ser^{79}] ACC phosphorylation. According to the studies by Minokoshi *et al.* (2004), hypothalamic AMPK may participate in the control of neurotransmitter production in at least two subpopulations of neurones; first, in arcuate nucleus NPY/AGRPergic neurones, increasing the production of these orexigenic signallers; and second, in MC4R-expressing neurones of the paraventricular nucleus, negatively controlling the production of putative anorexigenic neurotransmitters. Since the requirement for energy is increased during cold exposure, we suspected that the increased activation of AMPK would play a role in the maintenance of higher feeding even under an unrestricted offer of nutrient. This suspicion was confirmed by the evaluation of the effect of an exogenous inhibitor of AMPK signalling upon food consumption. During this test, it was demonstrated that cold exposure leads to resistance to citrate inhibition of feeding. This phenomenon is paralleled by functional resistance to the anorexigenic effect of insulin, which has been previously reported (Torsoni *et al.* 2003).

In the final part of the study the signal transduction through the PI_3-kinase/Akt pathway was inhibited using the compound, LY-294002, as an attempt to evaluate whether the cross-talk between hormone-dependent and nutrient-dependent anorexigenic signal transducing pathways is affected by cold exposure. Both in control and cold-exposed rats, the inhibition of PI_3-kinase promoted restoration of the insulin-induced inactivation of AMPK. However, the effect was significantly more pronounced in control rats, suggesting that cold exposure may impose a negative control upon the cross-talk between both pathways.

Thus, it is concluded that, during cold exposure, in spite of the reduced blood levels of insulin, there is functional and molecular resistance to nutrient-dependent anorexigenic signal transduction in the hypothalamus of rats. This phenomenon is paralleled by resistance to insulin signal transduction through the PI_3-kinase/Akt signalling pathway, and may participate in the complex mechanism of adjustments of feeding behaviour that might have been developed to warrant increased feeding during the exposure to cold. Moreover, the confirmation of a molecular cross-talk between PI_3-kinase/Akt and AMPK reinforces the importance of AMPK as a potential target for therapeutics in obesity and related diseases.

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