Journal of Developmental Origins of Health and Disease (2013), **4**(3), 239–248.
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doi:10.1017/S2040174413000019

ORIGINAL ARTICLE

Mechanism of programmed obesity: altered central insulin sensitivity in growth-restricted juvenile female rats

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Intrauterine growth-restricted (IUGR) offspring are at increased risk of adult obesity, as a result of changes in energy balance mechanisms. We hypothesized that impairment of hypothalamic insulin signaling contributes to hyperphagia in IUGR offspring. Study pregnant dams were 50% food restricted from days 10 to 21 to create IUGR newborns. At 5 weeks of age, food intake was measured following intracerebroventricular (icv) injection of vehicle or insulin (10 mU) in control and IUGR pups. At 6 weeks of age, with pups in fed or fasted (48 h) states, pups received icv vehicle or insulin after which they were decapitated, and hypothalamic arcuate (ARC) nucleus dissected for RNA and protein expression. IUGR rats consumed more food than controls under basal conditions, consistent with upregulated ARC phospho AMP-activated protein kinase (pAMPK) and neuropeptide Y (NPY). Insulin acutely reduced food intake in both control and IUGR rats. Consistent with anorexigenic stimulation, central insulin decreased AMP-activated protein kinase and NPY mRNA expression and increased proopiomelanocortin mRNA expression and pAkt, with significantly reduced responses in IUGR as compared with controls. Despite feeding, IUGR offspring exhibit a persistent state of orexigenic stimulation in the ARC nucleus and relative resistance to the anorexigenic effects of icv insulin. These results suggest that impaired insulin signaling contributes to hyperphagia and obesity in IUGR offspring.

Received 11 August 2012; Revised 4 December 2012; Accepted 10 January 2013; First published online 21 February 2013

Key words: appetite, fetal programming, hyperphagia, intrauterine growth restriction, insulin resistance

Introduction

Obesity and its associated chronic conditions (e.g. diabetes, cardiovascular disease) continue to be a major public health concern throughout the United States and the world.[1] Although the putative etiologies of the obesity epidemic are uncertain, both genetic factors and/or environmental factors (e.g. Western high-fat diet) may contribute.[2] High birth weight is an important risk factor for subsequent obesity.[3] Moreover, epidemiological and experimental evidence have suggested that consequences of intrauterine undernutrition are closely associated with adult obesity,[4,5] giving rise to the concept of 'developmental origins of health and disease'.[4,6]

Intrauterine growth restriction (IUGR) or low birth weight may lead to permanent changes in energy balance that may confer a propensity to develop obesity.[7–9] Maternal food restriction leads to altered insulin sensitivity in the offspring.[10] Insulin-like growth factor (IGF)-1 gene is sensitive to the glucose level of the prenatal environment, with resultant alteration of IGF-1 mRNA expression and ultimately vulnerability to adult onset insulin resistance.[11] Although the nature of the maladaptations that underlie this long-term effect are poorly understood, mechanisms likely include enhanced appetite[12] and/or reduced satiety.[13,14] Appetite is primarily controlled by hypothalamic nuclei, which receive input from central and peripheral neural and hormonal signals. The hypothalamic arcuate (ARC) nucleus, a key and primary target of appetite regulatory factors, contains subsets of orexigenic [neuropeptide Y (NPY)] and anorexigenic [proopiomelanocortin (POMC)] neurons, which ultimately regulate appetite/ingestive behavior. Leptin, the adipocyte hormone, is well recognized as a satiety signal with potent actions at the ARC nucleus. Insulin is commonly associated with peripheral glucose homeostasis, it contributes to anorexigenic modulation, particularly following meals. Insulin is transported across the blood brain barrier via a saturable process,[15] and insulin receptors are widely expressed in the brain, expressed in relatively high content in areas implicated in the regulation of energy homeostasis (e.g. ARC) and localize to both NPY and POMC neurons.[16,17] The intracerebroventricular (icv) administration of insulin increases the expression of POMC[18] and decreases the expression of NPY in the ARC.[19] Indeed, exogenously administered insulin decreases appetite and food intake in rodents, nonhuman primates and humans.[20–23] Central insulin signaling deficiency is a cause of excessive food intake, which may be partially attenuated by peripheral insulin therapy.[24]

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https://doi.org/10.1017/S2040174413000019 Published online by Cambridge University Press

We have previously shown that maternal food restriction of rats during the second half of pregnancy results in IUGR newborns, which demonstrate excessive food intake, resulting in adult obesity and peripheral insulin resistance. These offspring demonstrate a programmed impaired anorexigenic behavioral response[12] and blunted hypothalamic leptin-specific (e.g. JAK/STAT) signaling to exogenous leptin.[13] In view of the development of peripheral insulin resistance, we sought to determine whether the programmed impaired anorexigenic function was specific to leptin or extended to insulin.

Methods

Maternal rat diets

Studies were approved by the Animal Research Committee of the Los Angeles BioMedical Research Institute at Harbor-UCLA Medical Center and were in accordance with the American Association for Accreditation of Laboratory Animal Care and National Institutes of Health guidelines. The rat model utilized for maternal food restriction during pregnancy has been previously described.[25–28] Briefly, first-time-pregnant Sprague–Dawley rats (Charles River Laboratories, Hollister, CA, USA) were housed in a facility with constant temperature (21.0 ± 2.0°C) and humidity and controlled 12-hour light (6:00 am–6:00 pm)/ and dark (6:00 pm–6:00 am) cycles. Pregnant rats were divided into two groups: Control dams had free access to standard laboratory chow (Lab Diet 5001, Brentwood, MO, USA; protein 23%; fat 4.5%; 3030 kcal/kg), whereas food-restricted dams were provided 50% of the Control food intake from day 10 of gestation to term (day 21) to produce IUGR offspring.

Offspring

Following birth, at day 1 of age, pups were culled to eight per litter (four males and four females) to normalize rearing. Both Control and IUGR offspring were nursed (after cross-fostering the maternal food restricted or control pups) by *ad libitum*-fed dams.[25–29] At 3 weeks of age, all offspring were housed individually and weaned to *ad libitum* standard laboratory chow. For experiment 1, only Control ($n = 32$) female rats were used, and for experiment 2 Control ($n = 32$) and IUGR ($n = 32$) female rats were used, as males were sacrificed at the time of weaning for use in alternative studies unrelated to this topic.

Cannula implantation and icv injection protocol

At 4 weeks of age, a cannula was stereotaxically implanted into the third ventricle of all offspring as described previously.[30,31] Briefly, the animals were anesthetized with isoflurane and the fur at the top of the head was removed to expose the area to be incised. A hole was drilled at the intersection of bregma and the midsaggital sinus and the guide cannula (26 gauge stainless steel; Plastics One, Roanoke, VA, USA) was lowered using the following stereotaxic coordinates (level skull, anterior-posterior from bregma 0, medial-lateral from midsaggital sinus 0 and dorsal-ventral from the top of the skull −8.0 mm) targeted for placement just above the third ventricle. The guide cannula was secured to the skull using cyanoacrylate ester gel, 3/16 mm jeweler's screws and dental acrylic. A removable obturator sealed the opening in the guide cannula throughout the experiment except when it was removed for the injections. Rats were allowed 1 week to recover fully before starting any additional experimental procedures. The inner cannula (33 gauge stainless steel, Plastics One) extended 8.5 mm below the top of the skull and all injections were performed in 2.0 μl volume. After the experimental sessions, the position of the cannulas was carefully verified. The animals were sacrificed by ether inhalation and a third ventricle injection of Blue Evans dye was performed in order to confirm whether the tip of the cannula was in the proper place. Only the data from animals whose cannulas were strictly into the third ventricle were considered.

Measurement of food intake

At 5 weeks of age, food intake was measured as follows: two days before testing, rats were placed into the Comprehensive Laboratory Animal Monitoring System (CLAMS, Columbus Instruments, Columbus, OH, USA) for acclimation. Animals received access to their normal diet (in ground-up form) and water *ad libitum* in the CLAMS chambers. On the day of testing, animals had their food removed 6 h before treatment at 12:00 pm. Animals were lightly restrained by hand during the 30 s injection and the injection needle remained in place 30 s before withdrawal. All rats were injected with a 2.0 μl vehicle or insulin (doses detailed below) and food intake measurement was started at the beginning of the dark phase of the photoperiod (6:00 pm) for successive 12-h periods of 72 h post injection.

Food intake data were generated by automated monitoring of the food balance, with determinations of accumulated food intake, meal size and meal number. A meal was defined as an intake larger than 0.3 g that was sustained for a period longer than 13 s, and two distinct meals needed to be separated by >10 min.[32,33] Meal size was defined as cumulative food intake (g)/100 g body weight/meal number. Meal duration was defined as total meal time (min)/meal number. The end of a feeding bout (meal) was identified as the time when the balance had been stable for >10 s and a minimum of 0.1 g of food had been eaten.

Insulin effects on food intake in control offspring: determination of dose response

Sixteen Control female rats from four different litters were used in this experiment. At 4 weeks of age, rats had cannula

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stereotaxically implanted into the third ventricle, as described above and after 1 week recovery time (5 week of age), animals were randomly administered either vehicle (artificial cerebrospinal fluid) or insulin (source; human, Sigma-Aldrich, Woodlands, TX, USA) at 1, 10 or 100 mU icv ($n = 4$ per group). As noted above, food was removed 6 h before the injection and returned directly after the injection. Food intake was monitored before the onset of dark phase of the light cycle for 72 h post injection using the CLAMS apparatus as described above. Following 1 week of recovery after food intake measurements, these Control animals were again injected icv with either vehicle or 10 mU insulin to determine the time course of ARC responses. Thirty, 90 and 180 min ($n = 4$ per group) following icv injections, offspring were decapitated under isoflurane inhalation anesthesia, brains collected and hypothalamus dissected. ARC was obtained by dissecting the ventral part of the medial hypothalamus with anterior and dorsal margins (0.5 mm from the ventral surface of the medial hypothalamus) and posterior margin (border with mammillary body). Subsequently, ARC tissue was snap frozen for protein extraction and Western blot analysis for pAkt/Akt and pAMPK/AMPK.

Comparison effect of central insulin in control and IUGR offspring

Sixteen Control and sixteen IUGR female rats were utilized for these studies. At 4 weeks of age, rats had cannula stereotaxically implanted into the third ventricle, as described above and after 1 week recovery time, Control and IUGR animals were administered either vehicle or insulin (10 mU), icv ($n = 32$ per group) at 5:00 pm. As noted above, food was removed 6 h before the injection and returned directly after the injection. Food intake monitoring was initiated at the onset of dark phase of the light cycle (6:00 pm) for 72 h post injection using the CLAMS apparatus, as described above. Following 1 week of recovery after experiment 2, Control and IUGR female offspring were either fasted for 48 h or fed an *ad libitum* diet ($n = 16$ each group: Control/fed, Control/fasted, IUGR/fed, IUGR/fasted). Animals were then injected icv with either vehicle or 10 mU insulin ($n = 8$ per group). One hundred and eighty minutes following icv injections (based on experiment 1 results), blood was taken from the left ventricle of the heart (for measurement of insulin levels) and animals were rapidly decapitated, and their ARC were removed as described above and stored at $-80°C$ until RNA or protein extraction.

Quantitative reverse transcription polymerase chain reaction (RT-PCR)

RNA extraction and cDNA synthesis were performed using TRIzol and SuperScript III reverse transcriptase (both from Invitrogen), respectively, according to the manufacturer's protocols. Quantitative PCR (qPCR) was carried out as described previously[27,34] using the Sequence Detection System 7500 (Applied Biosystems, Foster City, CA, USA). In brief, qPCR was performed using the qPCR Master Mix Plus for SYBR green I (Eurogentec, San Diego, CA, USA) in a total volume of 25 μl consisting of 1× SYBR Master Mix, 200 nM of each primer, and 2 μl of cDNA sample. All reactions were performed with an initial denaturation of 2 min at 50°C, followed by 95°C for 10 min, followed by 40 PCR cycles of denaturing at 95°C for 15 s, and annealing/extension at 60°C for 1 min. Levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used to control the sample amount, and all assays were performed in triplicate. Detection of PCR product was accomplished by real-time detection of the increase in fluorescence of SYBR green caused by the primer extension. After each qPCR experiment, data were analyzed to select a threshold level of fluorescence that was in the linear phase of the PCR product accumulation. The cycle at which each reaction reached threshold fluorescence was defined as the threshold cycle (CT) for that reaction. The analysis was performed using the comparative CT method. The mRNA expression of the gene was determined using $2^{-\Delta\Delta C_T}$, and values expressed as a fold difference from the Control. The primers oligonucleotide sequences were obtained from Sigma-Aldrich (Woodlands, TX, USA).

Western blotting

Primary antibodies for Western blotting were Phospho (Thr^{172})-AMPKα (pAMPK) (Millipore, Lake Placid, NY, USA), AMPKα (AMPK) (Millipore), Phospho (Ser^{473})-Akt (pAkt) (Cell Signaling Technology Inc., Danvers, MA, USA), Akt (Cell Signaling), GAPDH (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Secondary horseradish peroxidase-conjugated antibody was anti-rabbit (Bio-Rad). All commercial antibodies were optimized for binding specificity. Protein was extracted in RIPA buffer containing protease and phosphatase inhibitors (HALT cocktail, Thermo Scientific, Rockford, IL, USA). Supernatants were obtained by microcentrifugation (12,000 × ***g***; 20 min) and protein concentration determined by BCA assay (Pierce). Protein expression was analyzed as previously conducted by our group.[14,27] Equal amounts of protein (20 μg) were mixed with a Criterion sodium dodecyl sulfate sample buffer (Bio-Rad, Hercules, CA, USA), boiled for 3 min, separated on a Criterion 4–12% Bis-Tris denaturing gel and transferred electrophoretically to a nitrocellulose membrane (Bio-Rad) for 2 h at 100 V. Nonspecific antibody binding was blocked by incubation overnight at 4°C with 5% nonfat dry milk or bovine serum albumin (BSA), in a Tris-buffered saline solution containing 0.05% Triton X-100 (TBSTx). The membrane was incubated with the appropriate primary antibody in TBSTx with 5% milk or BSA overnight, washed three times (for 10 min each) with TBSTx at room temperature, incubated with anti-rabbit secondary antibodies in TBSTx with 5% milk or BSA for 1 h at room temperature and washed

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three times for 10 min with TBSTx. HyGLO Quick Spray Chemiluminescent HRP Antibody Detection Reagent (Denville Scientific, Metuchen, NJ, USA) was used to detect the targeted protein. The band density on the X-ray film was optically scanned and quantitated using the Molecular Imager GS-800 Calibrated Densitometer and Quantity One (Bio-Rad). The blots were stripped with Restore stripping buffer (Pierce), reprobed and normalized to the reference protein (total protein of phosphorylation) and presented as fold change relative to the Control level.

Plasma insulin measurement

Plasma insulin levels were measured by Rat Insulin RIA kit (Millipore) following the manufacture's protocol.

Statistical analysis

All values are expressed as mean ± S.E. of the mean (S.E.M.). Statistical comparisons for quantitative data were made by using one-way, two-way or three-way analysis of variance (ANOVA) for single-, double- or multi-factors analysis with the Tukey test in multiple group experiments (Sigma Stat 3.5, SYSTAT, San Jose, CA, USA) with $P < 0.05$ required for statistical significance between the groups. Sample size estimates were based on a power of 80% to detect 30% changes between IUGR and control groups (assuming an expected standard deviation of 20% of mean values). This analysis results in a requirement for eight animals in each group. Differences between groups were determined using ANOVA with Dunnett's *post hoc* test with significance set at $P < 0.05$. Values are presented as the mean ± S.E.M.

Results

Body growth of offspring in control and IUGR rats

At birth, the pups from food-restricted dams (IUGR) had ~19% lower body weights compared with Control pups (6.12 ± 0.14 *v.* 7.57 ± 0.08 g). Body weights of IUGR rats remained significantly lower than Control rats (115.3 ± 1.7 *v.* 131.9 ± 2.6 g) at the time of food intake measurement (5 weeks of age) but caught up to Control rats (149.6 ± 3.5 *v.* 148.3 ± 2.0 g) by 6 weeks of age (Fig. 1).

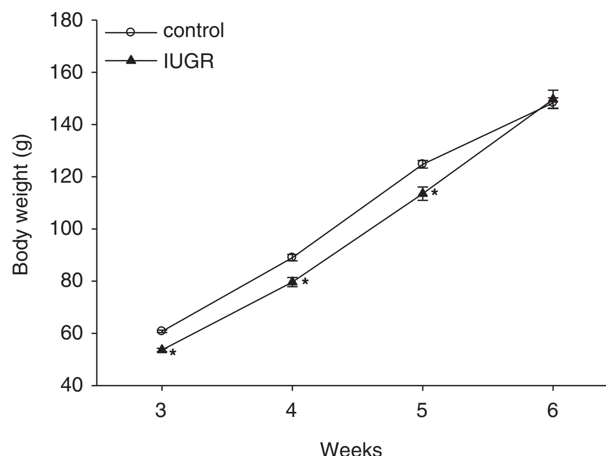

Fig. 1. Neonatal body weight change in Control (○) and intrauterine growth restriction (IUGR) (▲) group. Data are expressed as the mean ± S.E.M. $^*P < 0.05$ compared with Control rats.

Insulin effects on food intake in control offspring

Dose response

There was no significant effect of 1 mU icv insulin on food intake. However, 10 mU icv insulin decreased cumulative food intake at 24 h, and 100 mU icv insulin reduced food intake through 48 h (Fig. 2a and 2b) compared with icv vehicle treatments. Both 10 and 100 mU doses of insulin exhibited similar decreases in food intake through 6 h, though greater inhibition was observed in response to 100 mU, only at 48 h. Both 10 and 100 mU insulin significantly increased the length of time it took animals to start eating (meal latency; Fig. 2c) than icv vehicle and 1 mU insulin. As a result of these studies, we utilized a dose of 10 mU icv insulin to ensure detectable effects on food intake in comparing IUGR *v.* Control offspring.

ARC collection time after icv treatments determination in Control rats

To determine the appropriate time for ARC tissue collection, we examined the alterations of Akt and AMPK signaling. The relative band intensity of pAkt/Akt ratio was augmented by insulin (10 mU) treatment in a time-dependent manner from 30 to 180 min (data not shown), whereas the relative band intensity of pAMPK/AMPK ratio did not change (data not shown).

Effect of central insulin in control and IUGR offspring

CLAMS study

Food intake: IUGR offspring demonstrated significantly increased cumulative food intake at 24, 48 and 72 h compared with Control offspring (Fig. 3a and 3b). Icv insulin (10 mU) reduced cumulative food intake to a similar degree in both Control and IUGR offspring through 6 h, though Control offspring demonstrated greater suppression of food intake as compared with IUGR at 12 and 24 h (Fig. 3a and 3b).

Latency: In response to icv vehicle, there was a small but nonsignificant decrease in latency among IUGR as compared with Controls. In response to icv insulin, IUGR exhibited 27% shorter meal latency compared with Control rats (Fig. 3c).

Meal number: In response to vehicle treatments, IUGR rats demonstrated greater meal number compared with Control rats. In response to icv insulin, meal number was reduced in both IUGR and Control rats for the first 24 h, with a greater reduction observed in Control rats (Fig. 3d).

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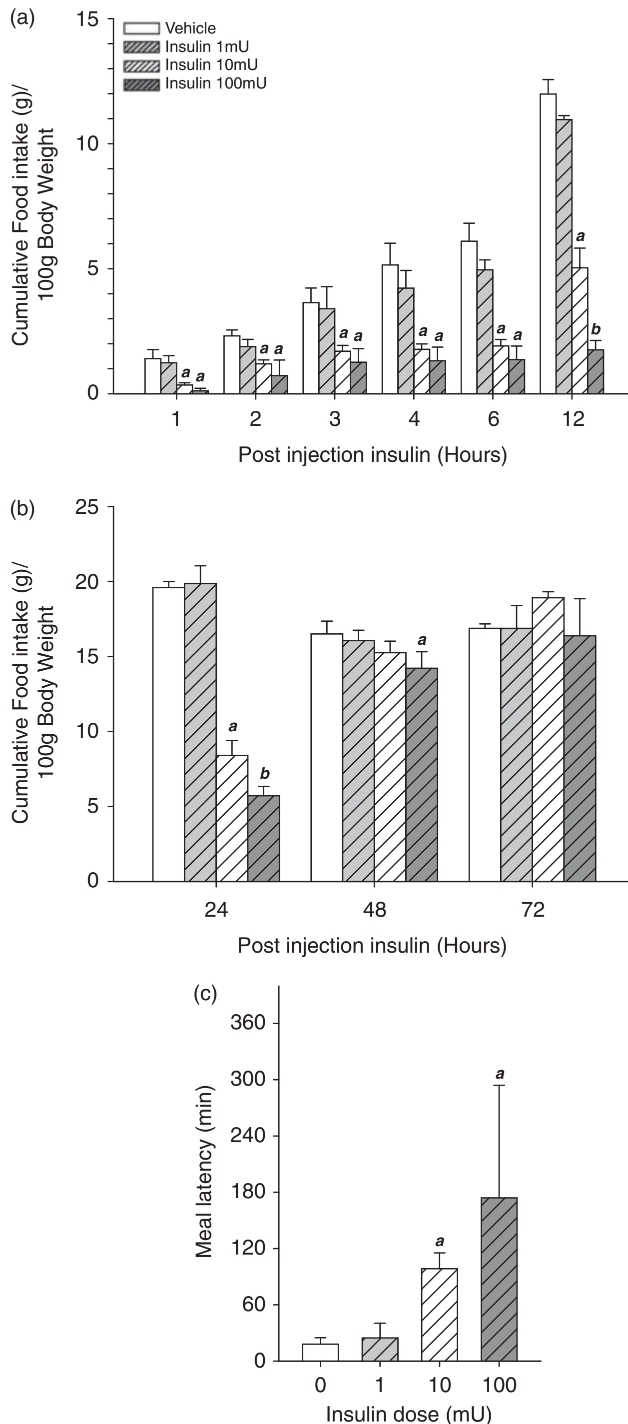

Fig. 2. The effect of intracerebroventricular (icv) insulin injection on cumulative food intake (g)/100 g body weight in Control rats at 1, 2, 3, 4, 6 and 12 h (*a*) and 1, 2 and 3 days (*b*) post icv vehicle or insulin. The effect of icv insulin injection on meal latency (*c*) in each icv Control rats. Rats were injected icv with vehicle (☐), 1 mU (▨), 10 mU (▨) or 100 mU (▨) insulin in a repeated-measures design. [a]$P < 0.05$ compared with icv vehicle and insulin 1 mU treatments, [b]$P < 0.05$ compared with icv vehicle, insulin 1 and 10 mU treatments.

Meal size and duration: There was no effect of vehicle or icv insulin on meal size (Fig. 3e) or duration (Fig. 3f). IUGR offspring demonstrated small but nonsignificant increases in meal duration under basal and insulin-treated conditions.

AMPK and Akt ratio

Fast v. fed: Among icv vehicle-treated Control offspring, fasting induced a significant increase in pAMPK/AMPK ratio (1.5-fold; Fig. 4a) and a nearly 50% decrease in pAkt/Akt ratio (Fig. 4b) compared with Control fed rats. However, fasting did not change AMPK or Akt ratios in IUGR rats. Rather IUGR offspring in the fed state demonstrated an elevated AMPK ratio and reduced Akt ratio as compared with fed Controls. In response to icv insulin, Control offspring markedly suppressed AMPK ratio and increased Akt ratio in both fed and fasting states. Icv insulin administration to IUGR offspring induced less suppression of AMPK ratio in both fed and fasting states, and less stimulation of Akt ratio in the fed state as compared with Controls.

Orexigenic (NPY) and anorexigenic (POMC) peptide mRNA expression

Fast v. fed: Both Control and IUGR vehicle-treated offspring demonstrated increased NPY in response to fasting, whereas Control, though not IUGR offspring, exhibited suppressed POMC mRNA expression (Fig. 4c and 4d). In the fed state, NPY levels were significantly greater and POMC significantly less in IUGR *v.* Controls. In the fasting state, NPY levels were less in IUGR offspring, though POMC levels were similar in both groups. Icv insulin markedly suppressed NPY mRNA in both fed and fasted Control and IUGR, though the IUGR offspring demonstrated increased NPY compared with control rats fasting in response to insulin. Icv insulin increased POMC in fed and fasted Control and IUGR, though there was a reduced POMC response among the IUGR offspring. In our study, GAPDH mRNA level was not changed in Control and IUGR rats.

Plasma insulin concentration

Plasma insulin measured at 6 week of age. In vehicle treatment studies, fasting induced a significant decrease in plasma insulin levels in both IUGR (0.81 ± 0.01 to 0.61 ± 0.01 ng/ml) and Control (0.49 ± 0.01 to 0.22 ± 0.01 ng/ml) rats (Fig. 5). IUGR showed significantly increased plasma insulin concentrations compared with Control rats in both fed and fasting states. Icv insulin had no effect on plasma insulin levels in either group.

Discussion

Ingestive behavior and energy balance are regulated in a complex manner by networks of neurons with numerous appetite-related molecules.[35–37] We have previously

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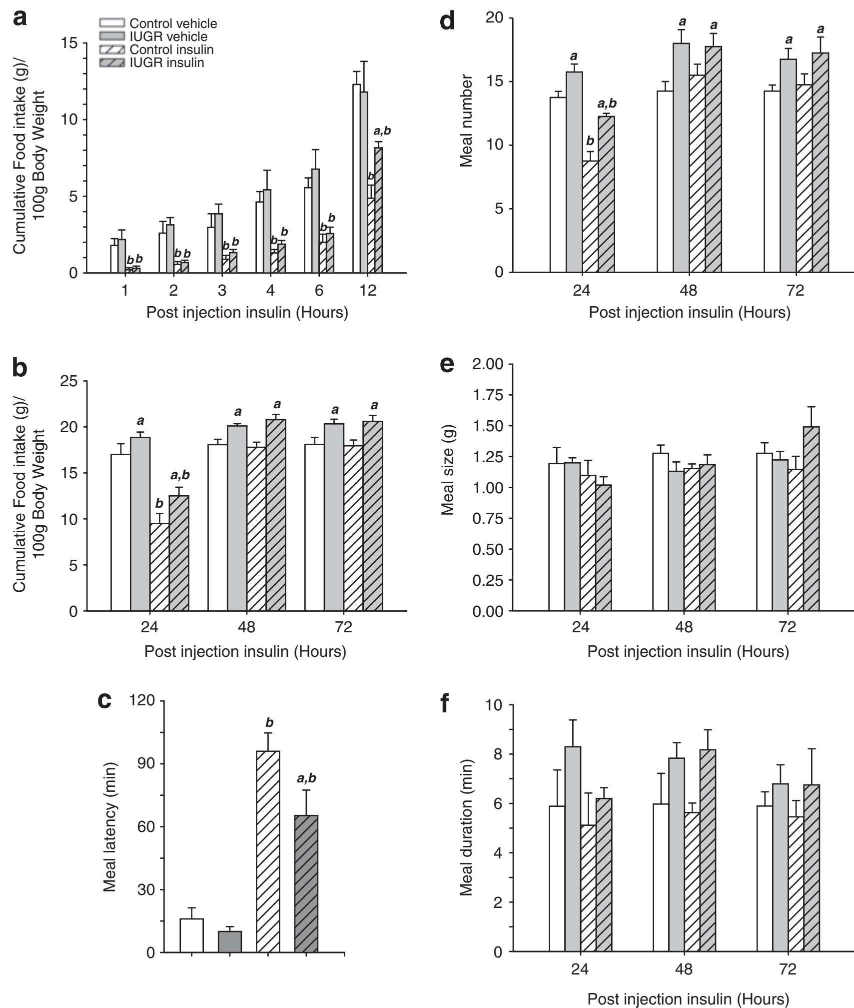

Fig. 3. The effect of intracerebroventricular (icv) insulin injection on cumulative food intake (g)/100 g body weight in Control and IUGR rats at 1, 2, 3, 4, 6 and 12 h (*a*) and 1, 2 and 3 days (*b*) post icv vehicle or insulin. The effect of icv insulin injection on meal latency (*c*), meal number (*d*), size (*e*) and duration (*f*) in Control and IUGR rats. Rats were injected icv with vehicle or 10 mU insulin in a repeated-measures design. $^{a}P < 0.05$ comparisons of IUGR *v.* Control rats, $^{b}P < 0.05$ comparisons of icv insulin *v.* vehicle treatments. Control vehicle (□), IUGR vehicle (▒), Control insulin (▨) and IUGR insulin (▨).

demonstrated that the adult obesity exhibited by IUGR offspring is mediated, in part, via increased food intake and impaired ARC anorexigenic signaling responses to systemic leptin.[13] Consistent with our[13,27] and other[29] previous findings, in the vehicle treatment study IUGR rats consumed more food per gram body weight, greater meal number compared and nonsignificant increases in meal size and duration as compared with Controls. Thus, the increased food intake is primarily a result of increased meal number, consistent with results in human overconsumption.[38]

IUGR rats displayed increased ARC NPY and decreased POMC mRNA expression during the fed state, indicative of a heightened 'orexigenic state'. The lack of change in IUGR ARC expression between fed and fasting states indicates a persistent upregulation of orexigenic drive in programmed obese offspring. Insulin-induced suppression of firing in controls, whereas in IUGR rats inhibition was significantly reduced and activation increased. Satiety signal insulin on ARC neurons is reduced in neonatal overfed IUGR rats. This can be regarded as insulin resistance, which is induced during early development and persists in later life.[39] Of note, food was provided *ad libitum* to both adult IUGR and Controls. Thus, the IUGR fed state findings are not a result of a more rapid return to an orexigenic state following earlier feeding, but suggest a persistent state of 'hunger'. Similarly, the impaired POMC expression suggests a failed 'satiety' response to feeding. The increased plasma insulin in fed IUGR suggests that reduced central insulin is likely not responsible for the enhanced orexigenic state, though central insulin levels were not measured. Of note, we previously demonstrated that fasted (overnight) IUGR adults have elevated plasma leptin, and 3-week-old IUGR offspring have decreased mRNA expression of hypothalamic leptin receptor.[40] It is likely that reduced leptin and/or insulin-induced ARC signaling responses contribute to upregulation of NPY.

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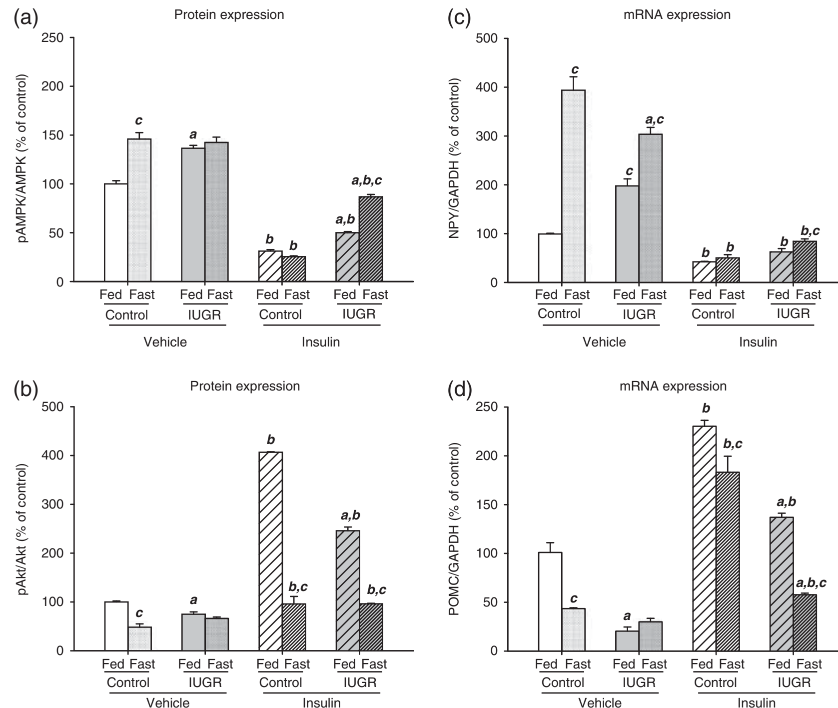

Fig. 4. The effect of intracerebroventricular (icv) insulin injection on hypothalamic protein phosphorylation ratio and mRNA expression in Control and IUGR rats injected icv with either vehicle or 10 mU insulin. Hypothalamic (*a*) AMP-activated protein kinase (AMPK) and (*b*) Akt ratio by Western blotting quantifications and (*c*) neuropeptide Y (NPY) and (*d*) proopiomelanocortin (POMC) mRNA expression by quantitative reverse transcription polymerase chain reaction (RT-PCR) were measured at 180 min post injection insulin or vehicle. Control icv vehicle and fed status was defined as 100%. Means were transformed into fold changes, and S.E.M. was expressed as a percentage proportional to the original mean and S.E.M. $^{a}P < 0.05$ comparisons of fast *v.* fed status, $^{b}P < 0.05$ comparisons of IUGR *v.* Control rats, $^{c}P < 0.05$ comparisons of icv insulin *v.* vehicle treatments. Fed (☐) and Fast (▩).

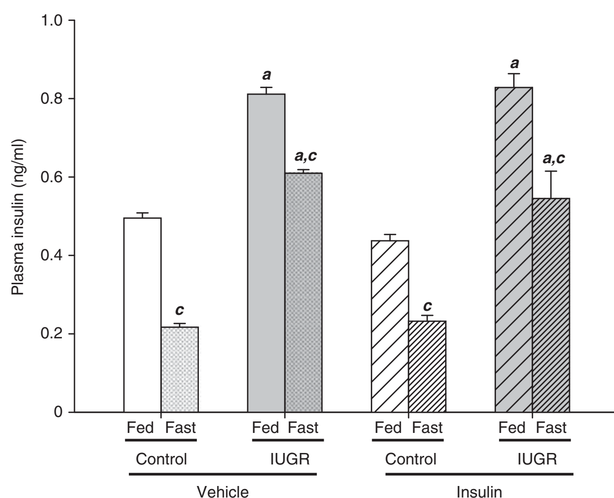

Fig. 5. Plasma insulin levels at 180 min following intracerebroventricular (icv) treatment (vehicle or insulin). Data are expressed as the mean ± S.E.M. $^{a}P < 0.05$ comparisons of fast *v.* fed status, $^{b}P < 0.05$ comparisons of intrauterine growth restriction (IUGR) *v.* Control rats, $^{c}P < 0.05$ comparisons of icv insulin *v.* vehicle treatments. Fed (☐) and Fast (▩).

AMPK is a fuel-sensing enzyme that is activated by physiological and pathological metabolic stresses, which alter cellular energy status.[41–44] Akt is critical to the insulin signaling pathway and is required to induce glucose transport. Both AMPK and Akt act as a molecular links between hormone/nutrient signals (e.g. leptin, insulin) and cellular metabolism by ATP generating pathways. Hypothalamic AMPK, which is modulated by fasting and feeding, responds differently as compared with peripheral AMPK. Whereas AMPK activation in the hypothalamus promotes energy intake, AMPK promotes energy consumption in the peripheral tissues (liver, skeleton muscle).[45] Accordingly, the anorexigenic hormone leptin, central insulin, melanocortin 3 and 4 receptor agonists and re-feeding[46] also inactivate hypothalamic AMPK and reduce food intake.[47] Conversely, orexigenic factors, including 5-aminoimidazole-4-carboxamide riboside, ghrelin[48] and cannabinoids[49] activate (phosphorylate) hypothalamic AMPK. AMPK and Akt act in concert to mediate ingestive behavior, with AMPK stimulating orexigenic drive and Akt stimulating anorexigenic drive.[50] In the present study, during the fed state, IUGR rats demonstrated markedly increased pAMPK/AMPK ratio and a failure to suppress the pAkt/Akt ratio suggesting a primary dysfunction in upstream regulatory factors, which target orexigenic/anorexigenic peptides.

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This finding is consistent with our previous study demonstrating that despite obesity and *ad libitum* food availability, fed IUGR female adults exhibit upregulated ARC AMPK activity, reduced Akt activity and altered mRNA expression of downstream appetite stimulatory neuropeptides, similar to that exhibited by fasting conditions in control adults.[12]

Among Control offspring, central insulin treatment decreased food intake by altering ingestive behavior patterns, increasing meal latency and decreasing both meal size and number of feeding bouts in a time-dependent manner. Concomitant with anorexigenic effects, icv insulin also altered hypothalamic appetite regulatory neuropeptide mRNA expression, decreasing the orexigenic neuropeptide (NPY) and increasing the anorexigenic neuropeptide (POMC), which likely contributed to reductions in cumulative food intake.

In addition to examining ingestive behavior, we compared the effects of central insulin treatment on hypothalamic neuropeptide mRNA and signaling protein expression in Control and IUGR offspring. Noteworthy, the alterations in neuropeptide mRNA expression were correlated with meal number and latency (without changes in meal size/duration) in both offspring groups and under basal or insulin treatments. The observed increases in NPY and decreases in POMC in IUGR offspring are consistent with the increased food intake and the insulin-induced reduction in NPY and increases in POMC are consistent with suppression of food intake in both groups. Icv insulin treatments markedly suppressed NPY mRNA in both fed and fasted Control and IUGR, though the IUGR offspring demonstrated a greater NPY level compared with control rats fasting in response to insulin. Icv insulin increased POMC in fed and fasted Control and IUGR, though there was a reduced POMC response among the IUGR offspring.

IUGR rats exhibited a significant increase in basal insulin levels in both fed and fasting states compared with Control rats, as previously demonstrated.[13] There were no significant increases in plasma insulin levels at 180 min following icv insulin in either group. Previous reports have demonstrated that icv insulin resulted in a significant increase in plasma insulin.[51,52] As plasma insulin half-life is only 5 to 7 min,[53] a potential plasma increase may have not been detected at 180 min. We have previously reported about leptin and glucose levels in this model.[14,25,26]

In this study, we did not control for the effects of estrus cycle, which may somewhat modulate appetite. Menarche occurs around 6 weeks of age in Sprague–Dawley female rat.[54] Rats typically have 4-day estrus cycle (proestrus, estrus, metestrus and diestrus) with estrogen peaking during proestrus. Twenty-four-hour food intake decreases during estrous phase compared with other phases[55,56] secondary to the inhibitory effect of estrogen on feeding. Consistent with this physiologic response, icv injection of estrogen acutely decreases AMPK activity,[57] whereas central or peripheral estrogen decreases NPY mRNA.[58,59] Pre-proNPY mRNA levels were measured by *in situ* hybridization in the rat ARC during estrus were only 25–30% lower than other periods.[60] In the present study, NPY mRNA levels increased in Control fasting (fourfold) and IUGR fed (twofold) states in comparison with Control fed rats. Although study days were arbitrarily selected in both groups, the magnitude of the changes makes it unlikely that our findings are a result of random estrus phase differences.

In summary, the present studies demonstrate that fed IUGR female rats exhibit a persistent state of orexigenic drive: upregulated ARC pAMPK activity, reduced pAkt activity and altered mRNA expression of downstream hypothalamic appetite stimulatory neuropeptides, similar to that exhibited by fasting conditions in Control rats. Consistent with the role of insulin as a critical satiety stimulant following meals, icv insulin treatment reduced food intake and induced an anorexigenic shift in hypothalamic peptides in Control rats. In contrast, IUGR rats demonstrated relative central insulin resistance with regard to both food intake and hypothalamic signaling. If extrapolated to humans, the proposal of frequent small meals for weight loss programs may represent an adaptive behavioral therapy to programmed obese humans that do not have an effective satiety response to meals.

Acknowledgments

The authors acknowledge Linda Day and Stacy Behare for assistance with animals. This work was supported by National Institute of Health Grants R01HD054751, R01DK081756 and R03HD060241. Fukami is a recipient of (1) Grant of Clinical Research Foundation (Fukuoka, Japan), (2) Nakayama Foundation of Human Science (Tokyo, Japan), (3) International Research Fund for Subsidy of Kyushu University Alumni (Fukuoka, Japan), (4) Fukuoka University School of Medicine Eboshi Association, (5) Young Investigator Research Award from the Fukuoka University School of Medicine Eboshi Association (Fukuoka, Japan) and (6) Kanzawa Medical Research Foundation (Matsumoto, Japan).

Financial Support

This work was supported by National Institute of Health Grants R01HD054751, R01DK081756 and R03HD060241. Fukami is a recipient of (1) Grant of Clinical Research Foundation (Fukuoka, Japan), (2) Nakayama Foundation of Human Science (Tokyo, Japan), (3) International Research Fund for Subsidy of Kyushu University Alumni (Fukuoka, Japan), (4) Fukuoka University School of Medicine Eboshi Association, (5) Young Investigator Research Award from the Fukuoka University School of Medicine Eboshi Association (Fukuoka, Japan) and (6) Kanzawa Medical Research Foundation (Matsumoto, Japan).

Conflicts of Interest

None

https://doi.org/10.1017/S2040174413000019 Published online by Cambridge University Press

Ethical Standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guides on the care and use of laboratory animals (the American Association for Accreditation of Laboratory Animal Care and National Institutes of Health guidelines) and has been approved by the institutional committee (the Animal Research Committee of the Los Angeles BioMedical Research Institute at Harbor-UCLA Medical Center).

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