Developmental changes in the hypothalamic mRNA expression levels of PACAP and its receptor PAC1 and their sensitivity to fasting in male and female rats

Authors

Takeshi Iwasa, Toshiya Matsuzaki, Altankhuu Tungalagsuvd, Munkhsaikhan Munkhzaya, Maira Yiliyasi, Takeshi Kato, Akira Kuwahara, and Minoru Irahara

Department of Obstetrics and Gynecology, Institute of Biomedical Sciences, Tokushima University Graduate School, 3-18-15 Kuramoto-Cho, Tokushima 770-8503, Japan

Corresponding author

Takeshi Iwasa

Department of Obstetrics and Gynecology, Institute of Biomedical Sciences, Tokushima University Graduate School, 3-18-15 Kuramoto-Cho, Tokushima 770-8503, Japan

Phone number : +81−88−633−7177

e-mail: iwasa.takeshi@tokushima-u.ac.jp

Key words

PACAP, PAC1, leptin, hypothalamus

Abstract

The actions and responses of hypothalamic appetite regulatory and factors change markedly during the neonatal to pre-pubertal period. Pituitary adenylate cyclase-activating polypeptide (PACAP) has been found to play pivotal roles in the regulation of metabolic and nutritional status through its specific receptor PAC1. PACAP/PAC1 have anorectic roles, and their functions are regulated by leptin in adulthood. In the present study, we showed that hypothalamic PACAP mRNA expression decreases during the neonatal to pre-pubertal period (from postnatal day 10 to 30) in both male and female rats. During this period, hypothalamic PACAP mRNA expression was not affected by 24 h fasting in either sex, while the serum leptin levels (leptin is a positive regulator of hypothalamic PACAP expression in adulthood) of both sexes were decreased by fasting. On the other hand, hypothalamic PAC1 mRNA expression did not change during the neonatal to pre-pubertal period in either sex; however, its levels were consistently higher in males than in females. Hypothalamic PAC1 mRNA expression was decreased by 24 h fasting in males, but no such changes were observed in females. These results indicate while hypothalamic PACAP expression is sensitive to a negative energy state and the serum leptin level in adulthood, no such relationships are seen in the pre-pubertal period. In addition, we speculate that differences in the gonadal steroidal milieu might induce sexual dimorphism in the basal hypothalamic PAC1 mRNA level and its response to fasting. The mechanisms responsible for and the physiological effects of such changes in hypothalamic PACAP and PAC1 expression during the developmental period remain to be clarified.

1. Introduction

Many physiological functions develop rapidly during the neonatal to pre-pubertal period. In particular, the actions and responses of hypothalamic appetite-regulating factors change markedly during this period in order to maintain an appropriate metabolic and nutritional state (Iwasa et al., 2015). Our previous studies have shown that the basal expression levels of some hypothalamic orexigenic and anorexigenic factors and their responses to fasting change during the neonatal to pre-pubertal period and that these changes differ between males and females (Iwasa et al., 2014, 2015). These results indicate that the roles of such hypothalamic factors in the regulation of metabolic and nutritional status during the neonatal to juvenile period might differ between the sexes.

Pituitary adenylate cyclase-activating polypeptide (PACAP), which was originally identified in the ovine hypothalamus (Miyata et al., 1989), has been found to play pivotal roles in the regulation of metabolic and nutritional status through its specific receptor PAC1 (Morley et al., 1992; Chance et al., 1995; Moro & Lerner, 1997; Adams et al., 2008). The central injection of PACAP suppresses appetite and feeding (Morley et al., 1992; Chance et al., 1995), and increases core body temperature and locomotor activity (Resch et al., 2011). In addition, PACAP knockout mice exhibit marked reductions in core body temperature (Gray et al., 2002), and PAC1-deficient mice develop hyperinsulinemia in fed conditions (Jamen et al., 2000). The effects of PACAP on energy homeostasis are mainly mediated by two hypothalamic nuclei, the ventromedial nucleus (VMN) and the paraventricular nucleus (PVN) (Resch et al., 2013). Extremely high PACAP mRNA expression is seen in the hypothalamic VMN, and the PACAP-positive neurons in the VMN project into the PVN. PACAP-positive neurons from other brain sites, including the extrahypothalamic area, also project into the VMN and PVN; i.e., the VMN receives such projections from the medial amygdala and lateral parabrachial nucleus (LPB), whereas the PVN receives such projections from the bed nucleus of the stria terminalis and the LPB (Resch et al., 2013). Although the injection of PACAP into the PVN or VMN results in reduced overall food intake, meal patterns are affected by the injection of PACAP into the PVN, but not the VMN (Resch et al., 2013). On the other hand, the injection of PACAP into the VMN increases core body temperature and locomotor activity, but no such effects are seen after the injection of PACAP into

the PVN (Resch et al., 2013). PAC1 mRNA expression is widely distributed, with abundant expression seen in the hypothalamic nuclei, and the co-administration of a PAC1 antagonist into the PVN and VMN abrogated the effects of the injection of PACAP into these sites (Resch et al., 2013). These results indicate that PACAP signaling within the PVN induces hypophagia, while PACAP signaling within the VMN stimulates energy expenditure.

Recently, it has been reported that hypothalamic PACAP in the VMN is a target of central leptin signaling. For example, the anorectic actions of leptin are abolished in PAC1-deficient mice (Vu et al., 2015) and PACAP knockout mice (Tanida et al., 2013). In addition, PACAP mRNA expression in the VMN is reduced in leptin knockout mice and increased by the administration of leptin (Hawke et al., 2009). It has also been reported that PACAP mRNA expression fell during fasting, but rose after the administration of a high-fat diet (Takaki et al., 1992; Yokota et al., 1993). We speculate that, as has been found for other factors, the basal hypothalamic expression levels of PACAP and PAC1 and their responses to fasting change during development because the serum leptin level varies markedly during this period. In the present study, the developmental changes in hypothalamic PACAP and PAC1 mRNA expression and the serum leptin level were evaluated. In addition, the changes in the sensitivities of these factors to fasting that occur during the neonatal to pre-pubertal period were examined.

2. Materials and Methods

2.1. Animals

Pregnant Sprague-Dawley rats were purchased (Charles River Japan Inc., Tokyo, Japan) and housed under controlled lighting (14h light, 10h dark) and temperature (24°C) conditions. The day on which the pups were born was defined as postnatal day 1. Twelve pups were randomly assigned to each dam on postnatal day 2. The rats were weaned at postnatal day 21 and housed at three or four per cage. Rats of both sexes were randomly selected from each dam on postnatal days 10, 20, and 30, and divided into the fed and fasting groups (n = 8 per group). The rats in the fasting groups were subjected to 24 h maternal (postnatal day 10 and 20) or food (postnatal day 30) deprivation. Twenty-four hours later (between 0900 and 1000), the rats' brains and serum were collected by decapitation and stored at −80˚C and −20˚C, respectively. All

animal experiments were conducted in accordance with the ethical standards of the animal care and use committee of the University of Tokushima.

2.2. Hormone assay and quantitative real-time polymerase chain reaction

The rats' serum leptin levels were measured using a 125I-radioimmunoassay (RIA) kit (multi-species leptin RIA kit, Linco Research Inc., MO, USA). Whole hypothalamic explants were dissected from the frozen brains, as described previously (Iwasa et al., 2015; Munkhzaya et al., 2015). Total RNA was isolated using a TRIzol® reagent kit (Invitrogen Co., Carlsbad, CA, USA) and an RNeasy mini kit® (Qiagen GmBh, Hilden, Germany). Five μg of total RNA were used for the cDNA synthesis. cDNA was synthesized with oligo (deoxythymidine) primers at 50 °C using the SuperScript III first-strand synthesis system for the real-time polymerase chain reaction (PCR; Invitrogen Co.). The PCR analysis was performed using the StepOnePlus™ real-time PCR system (PE Applied Biosystems, Foster City, CA, USA). Standard curves, which were generated by serially diluting an abundant sample at least 4 times, were used for the relative quantification of each mRNA expression level. The mRNA expression levels of PACAP and PAC1 were normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The following forward and reverse primers were used: PACAP: F: 5'- CAT GTG TAG CGG AGC AAG GTT -3', R: 5'- GTC TTG CAG CGG GTT TCC -3'; PAC1: F: 5'- GGT GCT TGA AGT CCA TAG TG -3', R: 5'- CTT GTA CAG AAG CTG CAG TC -3'; GAPDH: F: 5'- ATG GCA CAG TCA AGG CTG AGA -3', R: 5'- CGC TCC TGG AAG ATG GTG AT- 3'. The PCR cycling conditions were as follows: initial denaturation and enzyme activation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s; annealing at 56 °C for 30 s (PAC1), 64 °C for 30 s (PACAP), or 65 °C for 30 s (GAPDH); and extension at 72 °C for 1 min. There are three isoforms of PAC1 (Zhou et al., 1999), and the PAC1 primer used in this study was able to amplify all of these isoforms (Zhou et al., 1999; Basille et al., 2000). In this study, GAPDH mRNA expression was not affected by development or fasting in either sex. In addition, its expression did not differ between the sexes at any of the examined ages.

2.3. Statistical analyses

All data are presented as mean ± SEM values. The statistical analyses were performed using one-way or two-way analysis of variance (ANOVA), or the Kruskal-Wallis test together with the Tukey-Kramer or Steel-Dwass post-hoc test for

comparisons between the age groups. The Student's *t* test or Mann–Whitney U test were used for comparisons between the sexes, or between the rats kept under the fed and fasted conditions. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Developmental changes in hypothalamic PACAP and PAC1 mRNA expression and the serum leptin level under the fed conditions in male and female rats

Under the fed conditions, the hypothalamic PACAP mRNA expression level differed significantly among the examined age groups in both the male (one-way ANOVA; $F(3,47) = 32.9$, $P < 0.01$) and female rats (Kruskal-Wallis; df = 2, H = 18.5, $P < 0.01$) (Fig. 1A). Hypothalamic PACAP mRNA expression fell with aging in both the male and female rats, but no difference in hypothalamic PACAP mRNA expression was detected between the sexes in any age group. The hypothalamic PAC1 mRNA expression level differed significantly among the examined age groups in the male rats (Kruskal-Wallis; df = 2, H = 10.4, $P < 0.01$); however, post-hoc analyses did not find any significant differences among the age groups (Fig. 1B). On the other hand, hypothalamic PAC1 mRNA expression did not differ among the examined age groups in the females (Fig. 1B). Hypothalamic PAC1 mRNA expression was significantly higher in the males than in the females in all age groups (Fig. 1B). The serum leptin level differed significantly among the age groups in both the males (one-way ANOVA; $F(3,47) = 22.4$, $P < 0.01$) and females (one-way ANOVA; $F(3,47) = 13.4$, $P < 0.01$) (Fig. 1C). The serum leptin levels recorded on postnatal day 10 were higher than those seen on postnatal days 20 and 30 in both the males and females. No significant correlations were detected between the serum leptin level and the hypothalamic PACAP mRNA level on day 10, 20, or 30 in either sex. Similarly, there were no significant correlations between the serum leptin level and the hypothalamic PAC1 mRNA level in either sex.

3.2. Effects of 24 h fasting on hypothalamic PACAP and PAC1 mRNA expression in male and female rats

Hypothalamic PACAP mRNA expression did not differ between the fed and fasted groups at any age in the male or female rats (Figs. 2A and D). In the male rats, the hypothalamic PAC1 mRNA expression levels of the fasted groups were significantly lower than those of the fed groups at all examined ages (Fig. 2B). On the other hand, hypothalamic PAC1 mRNA expression did not differ between the fed and fasted groups at any age in the female rats (Fig. 2E). The serum leptin levels of the

fasted groups were significantly lower than those of the fed groups at all examined ages in both the males and females (Figs. 2C and F).

4. Discussion

In the present study, we showed that hypothalamic PACAP mRNA expression decreased during development in both male and female rats. Although the rats' serum leptin levels also tended to fall during the developmental period, the pattern of change in hypothalamic PACAP expression did not correspond with that of the serum leptin level. In addition, although fasting caused reductions in the serum leptin level in both sexes at all examined ages, it did not affect hypothalamic PACAP mRNA expression.

It has been reported that leptin signaling via the leptin receptor (OBRb) is required for normal PACAP mRNA expression in the hypothalamus, and the central injection of an PACAP antagonist markedly reduced leptin-induced hypophagia and hyperthermia in adult male mice (Hawke et al., 2009). In addition, hypothalamic PACAP mRNA expression was reduced by 48 h food deprivation, but no such reduction was observed when leptin was simultaneously administered during food deprivation in adult male mice (Hawke et al., 2009). As hypothalamic PACAP functions as an anorexigenic and catabolic factor (Moro & Lerner, 1997), the reduction in PACAP activity induced by decreased leptin signaling during fasting might represent a compensatory response aimed at promoting feeding behavior and improving the energy balance in adulthood. On the other hand, in the present study although the serum leptin level was decreased by fasting in both sexes, hypothalamic PACAP mRNA expression was not affected by fasting in either sex. These results indicate that the relationship between the serum leptin level and hypothalamic PACAP expression observed in adulthood has not been established by the pre-pubertal period. It has been reported that during the pre-pubertal period leptin does not reduce food intake as markedly as it does in adulthood (Proulx et al., 2002). It is possible that during the pre-pubertal period the fact that PACAP is not very sensitive to the serum leptin level might blunt the effects of leptin on feeding behavior. This hypothesis could be tested by injecting leptin into animals during the pre-pubertal period and comparing their PACAP and PAC1 expression levels with those seen in adult animals. We speculate that the reduction in PACAP mRNA expression that occurred during development in both sexes, the fact that no fasting-induced changes in PACAP mRNA expression were observed in either sex, and the reduction in PAC1 mRNA expression

seen during fasting in male rats might be involved in the physiological mechanisms responsible for maintaining appetite and promoting growth in the pre-pubertal period. It has been reported that pituitary PACAP and PAC1 mRNA expression are regulated by gonadal steroids (Zheng et al., 2014). Thus, we also speculate that differences in the gonadal steroidal milieu might be responsible for the sexual dimorphism in hypothalamic PAC1 mRNA levels seen under basal and fasted conditions in the present study.

In the present study, hypothalamic PAC1 mRNA expression did not change during the developmental period in male or female rats; however, the male rats exhibited significantly higher hypothalamic PAC1 mRNA expression levels than the females at all examined ages. In addition, hypothalamic PAC1 mRNA expression was decreased by fasting in the male rats at all examined ages, whereas it was not affected by fasting at any of the examined ages in the female rats. Large variations in hypothalamic PAC1 mRNA expression were seen in the female rats under both the fed and fasted conditions. We could not identify the reason why such large variations were observed in the female rats in this study. As the basal serum leptin level and the fasting-induced changes in the serum leptin level did not differ between the sexes, the sex-related differences in hypothalamic PAC1 expression were probably not caused by dimorphism in the actions of leptin. Instead, we speculate that differences in the gonadal steroidal milieu might be responsible for the observed sexual dimorphism in the basal hypothalamic mRNA expression level of PAC1 and its response to fasting because the expression levels of some other appetite-regulating factors are regulated by gonadal steroids (Hirschberg, 2012). However, as far as we know, no previous study has examined the relationship between gonadal steroids and the hypothalamic PACAP/PAC1 system, and further examinations are needed to confirm our hypothesis.

The present study had several limitations. As whole hypothalamic blocks were used for the mRNA expression level measurements, the changes in mRNA expression seen in each hypothalamic nucleus; i.e., the VMN and PVN, could not be evaluated. Therefore, we could not clarify which site (or both) was responsible for the changes in PACAP/PAC1 mRNA expression observed during development and fasting in this study. Further examinations, for example, in situ hybridization, would be useful for identifying these specific sites.

In summary, we showed that hypothalamic PACAP mRNA expression decreased

during the neonatal to pre-pubertal period in both male and female rats. However, hypothalamic PACAP mRNA expression was not affected by 24 h fasting in either sex during the study period. In adulthood, hypothalamic PACAP mRNA expression is sensitive to a negative energy status and the serum leptin level, but it appears that no such relationship exists in the pre-pubertal period. Hypothalamic PAC1 mRNA expression did not change during the neonatal to pre-pubertal period in either sex; however, it was consistently higher in males than in females. The hypothalamic PAC1 mRNA expression level fell after 24 h fasting in male rats, but no such changes were observed in the females. Differences in the gonadal steroidal milieu might be responsible for this sexual dimorphism. The mechanisms responsible for and the physiological effects of the changes in hypothalamic PACAP and PAC1 mRNA expression in the developmental period remain to be clarified.

References

Adams, B.A., Gray, S.L., Isaac, E.R., Bianco, A.C., Vidal-Puig, A.J., Sherwood, N.M., 2008. Feeding and metabolism in mice lacking pituitary adenylate cyclase-activating polypeptide. Endocrinology 149, 1571-1580.

Basille, M, Vaudry, D., Coulouarn, Y., Jegou, S., Lihrmann, I., Fournier, A., Vaudry, H., Gonzalez, B., 2000. Comparative distribution of pituitary adenylate cyclase-activating polypeptide (PACAP) binding sites and PACAP receptor mRNAs in the rat brain during development. J. Compl. Neurol. 425, 495-509.

Chance, W.T., Thompson, H., Thomas, I., Fischer, J.E., 1995. Anorectic and neurochemical effects of pituitary adenylate cyclase activating polypeptide in rats. Peptide 16, 1511-1516.

Gray, S.L., Yamaguchi, N., Vencova, P., Sherwood, N.M., 2002. Temperature sensitive phenotype in mice lacking pituitary adenylate cyclase-activating polypeptide. 143, 3946-3954.

Hawke, Z, Ivanov, T.R., Bechtold, D.A., Dhillon, H., Lowell, B.B., Luckman, S.M., 2009. PACAP neurons in the hypothalamic ventromedial nucleus are targets of central leptin signaling. J. Neurosci. 29, 14828-14835.

Hirschberg, A.L., 2012. Sex hormones, appetite and eating behavior in women. Maturitas 71, 248-256.

Iwasa, T., Matsuzaki, T., Munkhzaya, M., Tungalagsuvd, A., Kawami, T., Murakami, M.,

Yamasaki, M., Kato, T., Kuwahara, A., Yasui, T., Irahara, M., 2014. Changes in the responsiveness of hypothalamic prokineticin 2 mRNA expression to food deprivation in developing female rats. Int. J. Dev. Neurosci. 34, 76-78.

Iwasa, T., Matsuzaki, T., Munkhzaya, M., Tungalagsuvd, A., Kuwahara, A., Yasui, T., Irahara, M., 2015. Developmental changes in the hypothalamic mRNA levels of prepro-orexin and orexin receptors and their sensitivities to fasting in male and female rats. Int. J. Dev. Neurosci. 46, 51-54.

James, F., Persson, K., Bertrand, G., Rodriguez-Henche, N., Puech, R., Bockaert, J., Ahren, B., Brabet, P., 2000. PAC1 receptor-deficient mice display impaired insulinotropic response to glucose and reduced glucose tolerance. J. Clin. Invest. 105, 1307-1315.

Miyata, A., Arimura, A., Dahl, R.R., Minamino, N., Uehara, A., Jiang, L., Culler, M.D., Coy, D.H., 1989. Isolation of a novel 38-residue hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. Biochem. Biophys. Res. Commun. 164, 567-574.

Morley, J.E., Horowitz, M., Morley, P.M., Flood, J.F., 1992. Pituitary adenylate cyclase activating polypeptide (PACAP) reduces food intake in mice. Peptide 13, 1133-1135.

Moro, O., Lerner, E.A., 1997. Maxadilan, the vasodilator from sand flies, is a specific pituitary adenylate cyclase-activating peptide type I receptor agonist. J.Biol. Chem. 272, 966-970.

Proulx, K., Richard, D., Walker, C.D., 2002. Leptin regulates appetite-related neuropeptides in the hypothalamus of developing rats without affecting food intake. Endocrinology 143, 4683-4692.

Resch, J.M., Boisvert, J.P., Hourigan, A.E., Mueller, C.R., Yi, S.S., Choi, S., 2011. Stimulation of the hypothalamic ventromedial nuclei by pituitary adenylate cyclase-activating olypeptide induces hypophagia and thermogenesis. Am. J. Physiol. Regul. Integr. Comp. Physiol. 301, R1625-R1634.

Resch, J.M., Maunze, B., Gerhardt, A.K., Magnuson, S.K., Phillips, K.A., Choi, S., 2013. Intrahypothalamic pituitary adenylate cyclase-activating polypeptide regulates energy balance via site-specific actions on feeding and metabolism. Am. J. Physiol. Regul. Integr. Comp. Physiol. 305, E1452-E1463.

Takaki, A., Aou, S., Oomura, Y., Okada, E., Hori, T., 1992. Feeding suppression elicited

by electrical and chemical stimulations of monkey hypothalamus. Am. J. Physiol. Regul. Integr. Comp. Physiol. 262, R586-R594.

Tanida, M., Hayata, A., Shintani, N., Yamamoto, N., Kurata, Y., Shibamoto, T., Morgan, D.A., Rahmouni, K., Hashimoto, H., 2013. Central PACAP mediates the sympathetic effects of leptin in a tissue-specific manner. Neuroscience 238, 297-304.

Vu, J.P., Goyal, D., Luong, L., Oh, S., Sandhu, R., Norris, J., Parsons, W., Pisegna, J.R., Germano, P.M., 2015. PACAP intraperitoneal treatment suppresses appetite and food intake via PAC1 receptor in mice by inhibiting ghrelin and increasing GLP-1 and leptin. Am. J. Physiol. Gastrointest. Liver. Physiol. 309, G816-825.

Yokota, C., Kawai, K., Ohashi, S., Watanabe, Y., Suzuki, S., Yamashita, K., 1993. Stimulatory effects of pituitary adenylate cyclase-activating polypeptide (PACAP) on insulin and glucagon release from the isolated perfused rat pancreas. Acta Endocrinol. (Copenh) 129, 473-479.

Zheng, W., Grafer, C.M., Halvorson, L.M., 2014. Interaction of gonadal steroids and gonadotropin-releasing hormone on pituitary adenylate cyclase-activating polypeptide (PACAP) and PACAP receptor expression in cultured rat anterior pituitary cell. Reprod. Sci. 21, 41-51.

Zhou, D., Frendewey, D., Lobo Ruppert, S.M., 1999. Pac1p, an RNase III homolog, is required for formation of the 3’ end of U2 sn RNA in Schizosaccharomyces pombe. RNA 5, 1083-1098.

Figure legends

Fig. 1.

Hypothalamic mRNA expression levels of PACAP (A) and PAC1 (B) and the serum leptin levels (C) observed under the fed conditions on postnatal days 10, 20, and 30 in male (■) and female (□) rats (n = 8 per group). Data are expressed as mean ± SE values. All mRNA expression levels were normalized to the mRNA expression level of GAPDH, and the values seen on postnatal day 10 male were defined as 1.0. Values with different letters (a-c) are significantly different (P <0.05). ** P <0.01 vs. each other.

Fig. 2.

Effects of 24 h maternal or food deprivation on the hypothalamic mRNA expression levels of PACAP (A, D) and PAC1 (B, E) and the serum leptin level (C, F) on postnatal days 10, 20, and 30 in male and female rats (□ fed, ■ fast, n = 8 per group). Data are expressed as mean ± SE values. All mRNA expression levels were normalized to the mRNA expression level of GAPDH, and the values seen in the fed groups on each day were defined as 1.0. * P <0.05, ** P <0.01 vs. each other.

Fig. 1

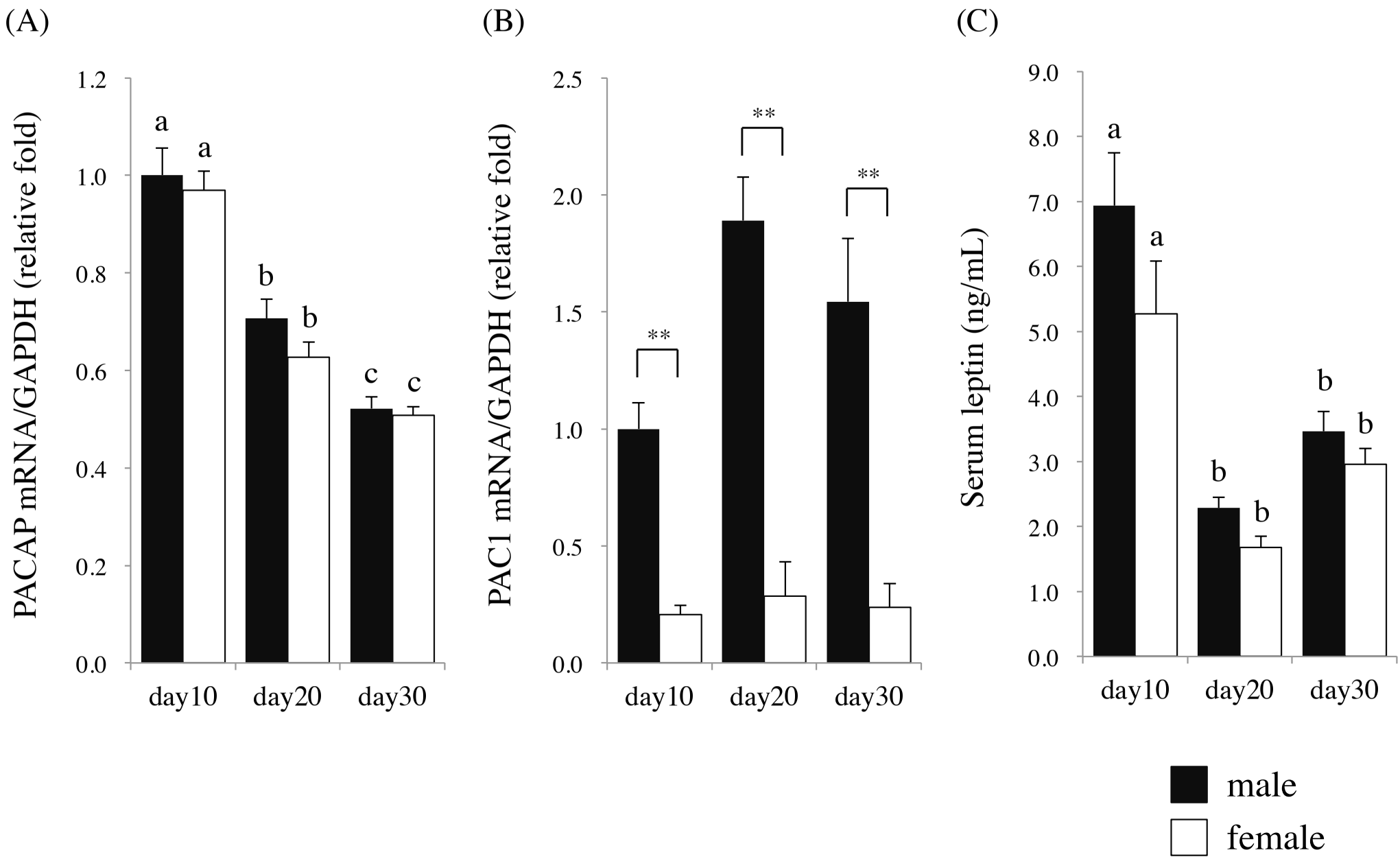
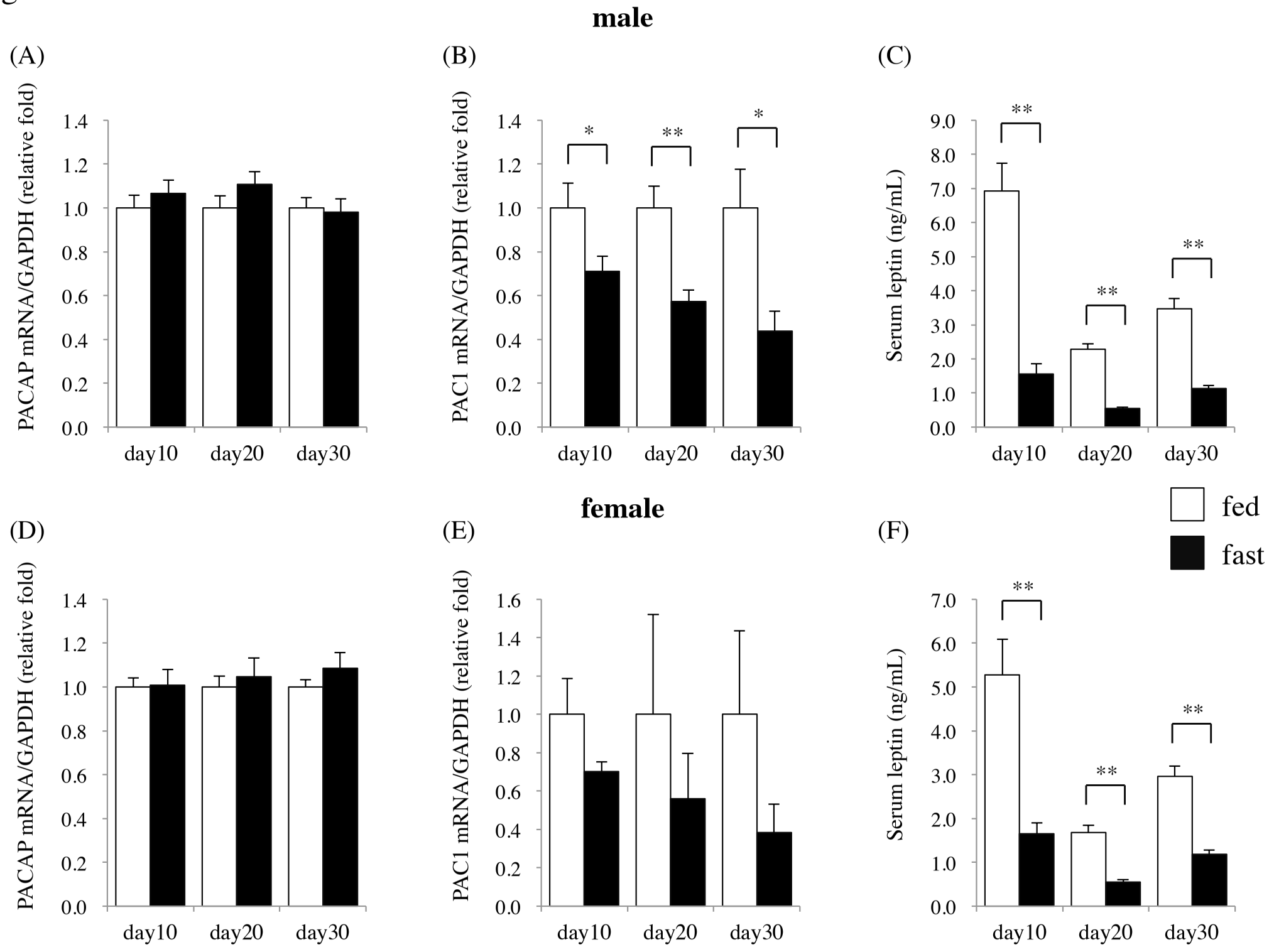

(A)
PACAP mRNA/GAPDH (relative fold)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
a
b
b
c
c
day10
day20
day30
(B)
PAC1 mRNA/GAPDH (relative fold)
2.5
2.0
1.5
1.0
0.5
0.0
**
**
**
day10
day20
day30
(C)
Serum leptin (ng/mL)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
a
a
b
b
b
b
day10
day20
day30
male
female

Fig. 2


male
female
(A)
(B)
(C)
(D)
(E)
(F)
PACAP mRNA/GAPDH (relative fold)
PAC1 mRNA/GAPDH (relative fold)
Serum leptin (ng/mL)
day10
day20
day30
fed
fast
*
**