EFFECTS OF HYPOPHYSECTOMY AND *IN VIVO* ADMINISTRATION OF ACTH OR DEXAMETHASONE ON THE LEVEL OF ACTH RECEPTOR mRNA IN ADRENAL GLANDS AND ADIPOSE TISSUES OF MICE

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Objective. In rodents, ACTH induces steroidogenesis in the adrenal cortex and also lipolysis in adipose tissues via the sole specific receptor for ACTH. Up-regulation of the ACTH receptor (ACTH-R) mRNA by ACTH was found to be evident in adrenocortical cells. However, a role of *in vivo* ACTH on ACTH-R mRNA expression in the adrenal cortex is not well understood. In addition, so far less attention has been also paid to the regulation of ACTH-R expression in adipose tissues. We investigated the effects of hypophysectomy and *in vivo* administration of ACTH or dexamethasone on the level of ACTH-R mRNA in the adrenal gland and adipose tissues of mice.

Methods. ACTH mRNA in the adrenal glands and epididymal adipose tissues of mice sacrified 10 days after hypophysectomy or sham operation, and 24 hours after ACTH-Z (2 IU i.m.) or physiological saline solution (PSS) injection, and also 3-days after dexamethasone (300 μg/day i.m.) or PSS administration was analysed by Northern blot, and intensities of autoradiographic bands were quantified.

Results. In the adrenal gland, *in vivo* administration of ACTH-Z significantly increased the level of ACTH-R mRNA to 342 % of control values, but *in vivo* administration of dexamethasone or hypophysectomy induced no significant alteration of ACTH-R mRNA. In adipose tissues, these conditions did not significantly alter the level of ACTH-R mRNA.

Conclusion. Low circulating ACTH may not be a substantial factor for ACTH-R gene expression in the adrenal gland, but a high level of *in vivo* ACTH dose up-regulated the expression. A different regulation of ACTH-R gene expression exists in the adrenal cortex and adipose tissues of mice.

Key words: ACTH receptor - ACTH - Hypophysectomy - Dexamethasone - Adrenal - Adipose tissue

The main action of ACTH is to induce steroidogenesis in the adrenal cortex. It is also well known that an extra-adrenal action of ACTH is lipolysis in adipose tissues of rodents (WHITE and ENGEL 1958; RICHTER and SCHWANDT 1987). Binding studies of radiolabeled ACTH and ACTH analogues revealed the specific receptor for ACTH in membrane fractions of the adrenal gland (LEFKOWITZ et al. 1970; BUCKLEY and RAMACHANDRAN 1981) and adipose tissues (OELOFSEN and RAMACHANDRAN 1983). Functional studies of the ACTH receptor (ACTH-R) revealed ACTH-induced cAMP production in both tissues (GRAHAME-SMITH et al. 1967; BIRNBAUMER et al. 1969). After human and mouse ACTH-R genes were isolated in 1992 and 1995, respectively (MOUNTJOY et al. 1992; KUBO et al. 1995), the ACTH-R mRNA was noted to be exclusive in both tissues of mice (MOUNTJOY et al. 1992; BOSTON and CONE 1996).

Repeated ACTH administration leads to an increase in adrenocortical responsiveness to ACTH in normal subjects and in those lacking endogenous ACTH secretion (KOLANOWSKI et al. 1975, 1977). While this potentiating phenomenon was to a great extent attrib-

uted to stimulatory actions of ACTH on expression of steroidogenic enzymes (WATERMAN 1994), it may also be in part due to an ACTH-induced increase of the number of ACTH binding sites in the adrenal gland, as demonstrated in binding studies using *in vivo* rabbit adrenal glands (DURAND and LOCATELLI 1980), and cultured cells from bovine, ovine, and human adrenal glands (PENHOAT et al. 1989; RAINEY et al. 1989; LEBRETHON et al. 1994). MOUNTJOY et al. (1994) reported the up-regulation of ACTH-R mRNA expression by its ligand in a mouse adrenocortical cell line, Y-1, and a human adrenocortical carcinoma cell line, NCI-H295. LEBRETHON et al. (1994) found that ACTH induces an increase in the level of ACTH-R mRNA and the number of ACTH binding sites in primary cultured cells from the human adrenal cortex. On the other hand, we found only one report concerning regulation of ACTH-R in adipose tissues and these authors stated that there was no specific physiological regulation of ACTH-R by glucocorticoid (BEHRENS and RAMACHANDRAN 1981). However, several investigators reported alterations in ACTH-induced lipolytic sensitivity and/or adenylate cyclase activity by adrenalectomy, as based on data on isolated adipocytes (BRAUN and HECHTER 1970; ALLEN and BECK 1972; FERNANDEZ and SAGGERSON 1978). We report here effects of hypophysectomy and *in vivo* ACTH or dexamethasone administration on the level of ACTH-R mRNA in the adrenal gland and adipose tissues of mice.

Materials and Methods

Animals and treatments. This study was carried out after permission from Committee of Animal Experimentation, Hokkaido University Graduate School of Medicine. Male Std:ddY mice aged 8 weeks were purchased from Japan SLC Inc., and maintained under 12-h light and 12-h dark conditions. Food and tap water were provided *ad libitum*. ACTH-Z (Cortrosyn-Z, Daiichi, Tokyo, Japan) in a dose of 2 IU was given intramuscularly at 8:00 pm. Dexamethasone (Decadron-A, Banyu, Tokyo, Japan) in a dose of 100 μg was given intramuscularly to other mice at 11:00 pm, and then repeated every 8 hours for 3 days. A similar injection of physiological saline solution was given to control mice. Three to five mice were in each group and three to four independent experiments were done. All mice were decapitated 24 hours after ACTH-Z administration or 4 hours after the last administration of dexamethasone. The adrenal glands and epididymal adipose tissues were rapidly excised and total RNA was extracted.

Hypophysectomy. Male mice aged 8 weeks were hypophysectomized (Hypox) or sham-operated. Hypophysectomy was performed by a transauricular approach (KOYAMA 1962). The mice were provided *ad libitum* with a commercial diet and a solution of physiological saline, including 0.5 % glucose to avoid hypoglycemia. The animals were weighed 7 days after the operation to ensure completeness of hypophysectomy as body weight correlates well with completeness of hypophysectomy (MAYBERRY et al. 1970). They were sacrificed at 10 days after the operation, the adrenal glands and the epididymal adipose tissues were rapidly excised and total RNA was isolated. Three mice were in each group and three independent experiments were done.

Northern blot analysis. Total RNA from adrenal glands and epididymal adipose tissues was isolated as described (CHOMCZYNSKI and SACCHI 1987). A DNA fragment consisting of the entire coding region of the mouse ACTH-R gene was [^{32}P]-labeled using a Random Primed DNA Labeling Kit (Boehringer Mannheim, Germany) and this served as a probe. Three μg of total RNA from adrenal glands of a mouse, and 20 μg from adipose tissues of a mouse were separated by electrophoresis on 1 % agarose gel containing formaldehyde, transferred to a nylon membrane (Biodyne B, PALL, USA), and hybridized overnight with the probe, under conditions reported elsewhere (SHIMIZU et al. 1997). For the final wash, we used a solution of 0.3XSSC (1XSSC = 16.65 mM NaCl, 16.65 mM Na-citrate) and 0.1 % SDS at 65 °C. The filter was exposed to an X-ray film with an intensifying screen. Intensities of autoradiographic bands were quantified using the BAS5000 image-analyzing system (Fuji Film, Japan). To monitor the amount and quality of the transferred RNA, the filters were rehybridized with a mouse β-actin cDNA probe. The mRNA concentrations are expressed as % of controls (= 100%) after correcting the signal of each sample for β-actin expression. In case of experiments on adrenal glands from the sham-operated or the Hypox mice, the filters were also rehybridized with mouse *CYP11A1* probe, a DNA fragment of the mouse cholesterol side-chain cleavage enzyme, prior to hybridization with the β-actin cDNA probe.

Statistical analysis. All values were expressed as the mean ± standard error (S.E.) as the index of dispersion. Mann-Whitney U-test was used to compare control and treated groups. Differences were considered significant at the $p<0.05$ level.

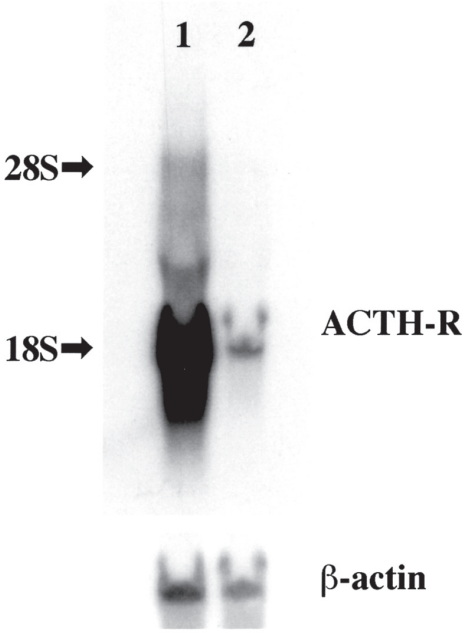

Fig. 1 Expression of ACTH-R gene in the mouse adrenal gland (lane 1) and adipose tissues (lane 2). Twenty μg of total RNA in both lanes were subjected to Northern blot analysis with [^{32}P]-labeled DNA fragment of the mouse ACTH-R coding region. The membrane was rehybridized with [^{32}P]-labeled mouse β-actin cDNA.

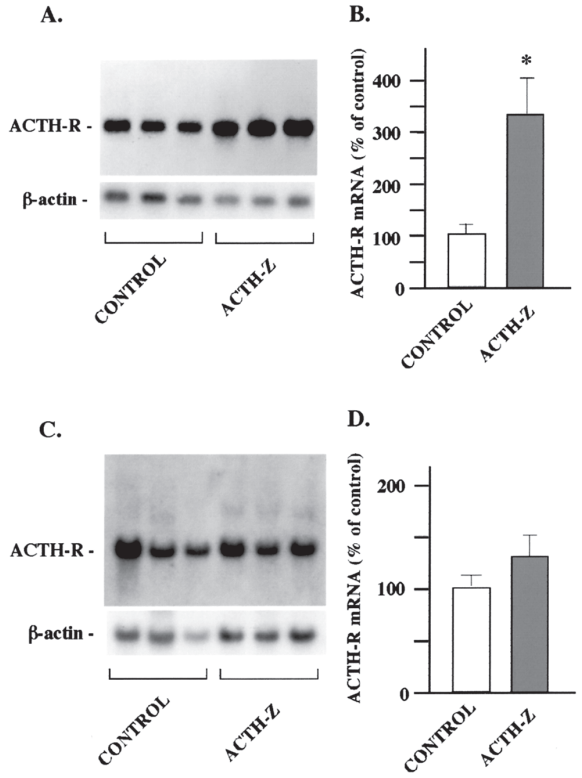

Fig 2 Effect of ACTH on the level of ACTH-R mRNA in the mouse adrenal gland and adipose tissues. Three to five mice were given ACTH-Z or physiological saline solution, then adrenals and epididymal adipose tissues were removed 24 hours after injection in an experiment. Three μg of total RNA from adrenal glands (*top*) and 20 μg of total RNA from adipose tissues (*bottom*) were subjected to Northern blot analysis. (A) and (C), Representatives of autoradiographs. (B) and (D), The intensity of ACTH-R mRNA was normalized for β-actin mRNA in each sample and expressed as a percent of the control value. The value is the mean ± S.E. from three independent experiments. In the adrenal gland, significant increase of ACTH-R mRNA was observed (p=0.002 vs. control), but the level of ACTH-R mRNA showed no significant change in adipose tissues (p=0.325 vs control).

Results

ACTH-R gene expression in adipose tissues. Northern blot analysis revealed a 1.8 kb ACTH-R mRNA in adipose tissues and in the adrenal gland (Fig. 1). The ACTH-R mRNA level in adipose tissues was approximately one-tenth of that found in the adrenal gland after correction for the intensity of β-actin.

Effect of ACTH on ACTH-R gene expression. Administration of ACTH-Z significantly increased the level of ACTH-R mRNA in the adrenal gland (Fig. 2A). The mean percentage increase over control after ACTH-Z treatment was 342.4±61.3 (p = 0.002), for three independent experiments (Fig. 2B). In adipose tissues, ACTH-Z administration led to no significant change in ACTH-R mRNA levels (Fig. 2C). The mean percentage over control was 131.8±20.2 (p=0.325), for four independent experiments (Fig. 2D).

Effect of dexamethasone on ACTH-R gene expression. Dexamethasone administration decreased the level of ACTH-R mRNA in the adrenal gland (Fig. 3A). The mean percentage decrease over control after dexamethasone treatment was 69.4±10.3 (p = 0.086), for four independent experiments (Fig. 3B). In adipose tissues, dexamethasone treatment led to no significant change in ACTH-R mRNA levels (Fig. 3C). The mean percentage over control was 103.4±9.7 (p=0.728), for four independent experiments (Fig. 3D).

Effect of hypophysectomy on body weight. The mean preoperative body weight of sham-operated and Hypox mice was 32.4g and 34.1g, respectively. After hypophysectomy growth virtually ceased and body

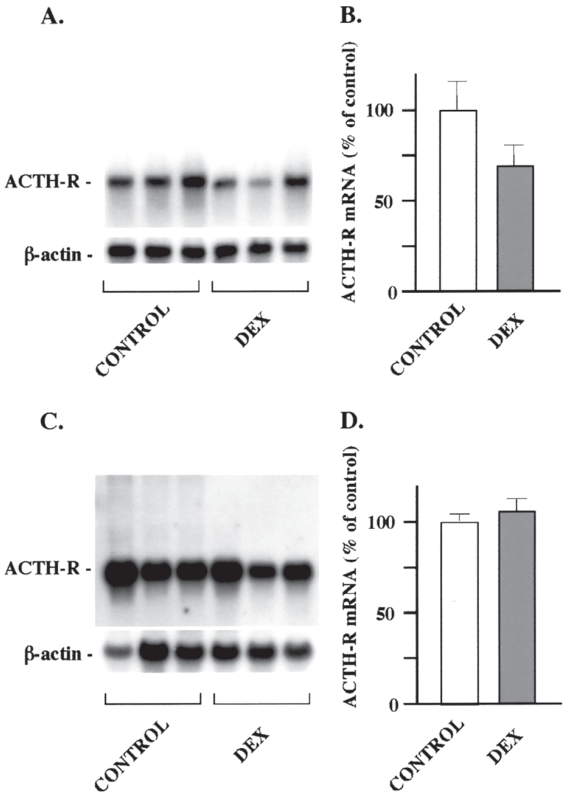

Fig. 3 Effect of dexamethasone (DEX) on the level of ACTH-R mRNA in the mouse adrenal gland and adipose tissues. Three to five mice were given dexamethasone (100 μg x3/day) or physiological saline solution for 3 days, then adrenals and epididymal adipose tissues were removed 4 hours after the last injection in each experiment. Three mg of total RNA from adrenal glands (*top*) and 20 μg of total RNA from adipose tissues (*bottom*) were subjected to Northern blot analysis. (A) and (C), The representatives of autoradiographs. (B) and (D), The intensity of ACTH-R mRNA was normalized for β-actin mRNA in each sample and expressed as a percent of the control value. The value is the mean ± S.E. from four independent experiments. The level of ACTH-R mRNA showed no significant changes in both adrenal glands (p=0.086 vs control) and adipose tissues (p=0.728 vs control).

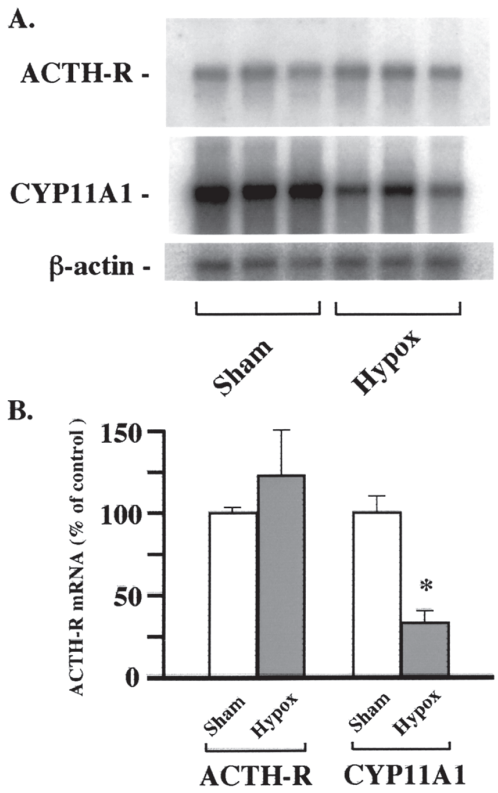

Fig. 4 Effect of hypophysectomy on the level of ACTH-R and CYP11A1 mRNA in mouse adrenal gland. Three mice were subjected to sham-operation (Sham) or hypophysectomy (Hypox) and 10 days later the adrenals were removed in an experiment. Three μg of total RNA from adrenal glands were subjected to Northern blot analysis with [^{32}P]-labeled mouse ACTH-R coding region cDNA (A, *top*). The membrane was rehybridized with [^{32}P]-labeled mouse *CYP11A1* (A, *middle*) and a mouse β-actin cDNA (A, *bottom*) probes. (A), Representatives of autoradiographs. (B), The intensity of ACTH-R and CYP11A1 mRNA were normalized for β-actin mRNA in each sample and expressed as a percent of the control value. The value is the mean ±S.E. from three independent experiments. The level of ACTH-R mRNA in the adrenal gland showed no significant difference in hypophysectomized mice, as compared to sham-operated control mice (p=0.895), but the level of CYP11A1 mRNA showed significant decrease in Hypox mice (p<0.001 vs. sham-operated control mice).

weight of all Hypox mice 7 days after the operation was decreased. On the other hand, the sham-operated mice gained weight steadily so that by the 7th postoperative day they were 6.4g heavier than the Hypox mice (p<0.001).

Effect of hypophysectomy on ACTH-R and CYP11A1 gene expression. The level of ACTH-R mRNA in adrenal glands showed no significant difference in Hypox mice, as compared to sham-operated control mice (Fig. 4A, *top*). The mean percentage over control was 125.0 ± 27.1 (p=0.895), for three independent experiments (Fig. 4B, *left*). Northern blot analysis revealed the mRNA for CYP11A1 as a single band of approximately 1.8kb. The level of CYP11A1 mRNA showed significant decrease in Hypox mice, as compared to control values (Fig. 4A, *middle*). The mean

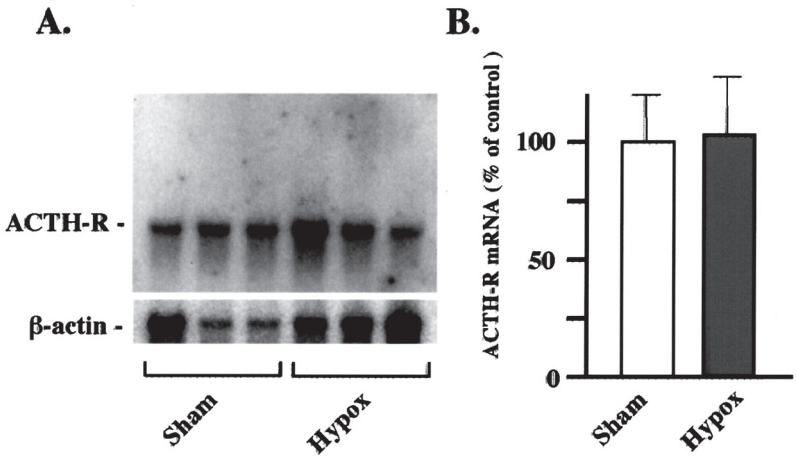

Fig. 5 Effect of hypophysectomy on the level of ACTH-R mRNA in mouse adipose tissues. Epididymal adipose tissues were removed simultaneously for experiments described in Fig. 4. Twenty μg of total RNA from adipose tissues were subjected to Northern blot analysis. (A), A representative of autoradiographs. (B), The intensity of ACTH-R mRNA was normalized for β-actin mRNA in each sample and expressed as a percent of the control value. The value is the mean ± S.E. from three independent experiments. The level of ACTH-R mRNA showed no significant change in adipose tissues (p=0.873 vs control).

percentage over control was 34.0±4.5 ($p<0.001$), for three independent experiments (Fig. 4B, *right*).

In adipose tissues, hypophysectomy led to no significant change in ACTH-R mRNA levels (Fig. 5A). The mean percentage over control was 102.1±23.7 ($p=0.872$), for three independent experiments (Fig. 5B).

Discussion

We confirmed the expression of a 1.8 kb ACTH-R mRNA in mouse adipose tissues. The level of ACTH-R mRNA in epididymal adipose tissues was approximately one-tenth of that in the adrenal gland, in accord with the results of the binding study indicating that the number of ACTH binding sites in rat adipocytes was 521-841 per cell (Oelofsen and Ramachandran 1983) compared to 3840 ± 1045 per cell in the rat adrenal cortex (Buckley and Ramachandran 1981).

In vivo administration of ACTH increased the level of ACTH-R mRNA in the mouse adrenal gland, consistent with previous studies done using the adrenal gland of other species. Durand and Locatelli (1980) reported that *in vivo* administration of ACTH increased the number of ACTH binding sites in the adrenal gland of normal and Hypox rabbits. Matsuyama et al. (1995) found an increase in ACTH-R mRNA expression by following *in vivo* administration of ACTH in adrenal glands of dexamethasone-treated rats. The *in vivo* effect of ACTH on the ACTH-R mRNA level in the adrenal gland, including our present finding, is consistent with *in vitro* effect of ACTH in Y-1 cells (Mountjoy et al. 1994), and primary cultured adrenocortical cells from ovine, bovine, and human (Picard-Hagen et al. 1997; Penhoat et al. 1994; Lebrethon et al. 1994).

In vivo administration of dexamethasone for 3 days tended to decrease the level of ACTH-R mRNA in the mouse adrenal gland, but as the level was insignificant, the low circulating ACTH level in serum does not substantially regulate ACTH-R expression. Contrary to our finding, Matsuyama et al. (1995) reported that *in vivo* dexamethasone administration for 5 days decreased ACTH-R mRNA to an undetectable level in the rat adrenal gland. Thus, direct and/or indirect effects other than ACTH suppression of glucocorticoid on ACTH-R mRNA expression in the adrenal gland have to be considered. Whether or not there is a direct effect of glucocorticoid on ACTH-R and steroidogenesis in the adrenal gland has been controversial. Using primary cultured ovine adrenocortical cells, Darbeida and Durand (1987, 1988) reported that chronic exposure (2-3 days) with dexamethasone enhanced ACTH-induced cAMP formation and steroidogenesis, an event associated with an increase of the number of ACTH

binding sites (Darbeida and Durand 1990) and the ACTH-R mRNA level (Pichard-Hagen et al. 1997). On the other hand, Latner et al. (1977) reported that cortisol decreased the binding of ACTH to bovine adrenocortical plasma membranes. Netchitailo et al. (1984) found no direct effect of dexamethasone on adrenal steroid secretion in the amphibian, using a perfusion system. In Y-1 cells, alteration in the number and affinity of the ACTH-R by corticosterone was nil, while corticosterone inhibited ACTH-induced steroidogenesis in the cells (Saito et al. 1979). Regulations of ACTH-R gene expression in the adrenal by glucocorticoids seem to differ among various species and/or experimental conditions.

We also investigated changes in ACTH-R mRNA in adrenals between Hypox and sham-operated mice. We found that significant decline in body weight and a remarkable reduction of adrenal CYP11A1 mRNA level in Hypox mice, consistent with previous reports (Vernikos-Danellis 1968; Kimura 1969; Imai et al. 1990) indicating the completeness of the hypophysectomy. The levels of adrenal ACTH-R mRNA in Hypox mice showed no significant decrease. These findings are in accord with our data on *in vivo* administration of dexamethasone. Thus, low circulating endogenous ACTH may not alter adrenal ACTH-R gene expression and the decline in steroidogenic enzyme gene expression may be attributed to an impaired sensitivity of adrenocortical steroidogenesis to ACTH.

Pro-opiomelanocortin (POMC) gene-knockout (KO) mice revealed that the adrenal gland was aplastic and hypoplastic on the 129 and C57BL/6 genetic background, respectively (Yaswen et al. 1999; Smart and Low 2003), indicating the incapability for deducing the regulation of ACTH-R expression by ACTH. Allen et al. (1995) used transgenic mice harboring a fusion gene composed of the pituitary specific promoter region of the POMC gene to drive the herpes simplex virus-1 thymidine kinase. A targeted ablation of pituitary POMC gene expression by the 12-days administration of ganciclovir led to decrease of ACTH-R mRNA levels in the adrenal cortex. The reason for the difference on ACTH-R mRNA levels between targeted POMC gene-KO and Hypox mice is obscure, but pituitary hormones other than POMC-derived peptides may influence ACTH-R gene expression. Reincke et al. (1997) asked if circulating physiological ACTH levels would influence adrenal ACTH-R mRNA expression *in vivo*. For this they studied 42 patients with adrenal diseases and found that plasma ACTH concentrations seemed to have no major influence on the ACTH-R mRNA levels, as ACTH-R mRNA levels were neither increased in adrenal hyperplasia in case of Cushing's disease nor reduced in adrenal Cushing's syndrome with suppressed plasma ACTH. They also reported that ACTH-R mRNA expression did not differ between cortisol-secreting adenoma and adjacent normal adrenal cortex as determined by *in situ* hybridization (Allolio and Reincke 1997). Conversely, Matsuyama et al. (1995) noted a decreased level of ACTH-R mRNA in the adjacent normal adrenal cortex compared to that in cortisol-secreting adenoma. Thus, the pathophysiological role of ACTH on ACTH-R gene expression requires further study.

In mouse adipose tissues, neither hypophysectomy nor *in viv*o ACTH or dexamethasone administration altered the level of ACTH-R mRNA. Although we found no report concerning the number of ACTH binding sites in adipose tissues after such experimental conditions, Behrens and Ramachandran (1981) analyzed [^{3}H]ACTH binding to isolated adipocytes from normal, adrenalectomized and adrenalectomized dexamethasone-treated rats, and found no difference in the magnitude or the affinity of the binding among the three groups. Their results and our obsevations indicated that ACTH and glucocorticoid, even in the pharmacological dose we gave do not play a role in regulating ACTH-R expression in adipose tissues of mice. However, the possibility that *in vivo* complex events elicited by ACTH or dexamethasone administration led to unchangeable ACTH-R expression in adipose tissues was not ruled out and further studies using cultured adipocytes are mandatory. *In vitro* studies using isolated fat cells in normal and adrenalectomized rats revealed that adrenalectomy reduced ACTH-induced lipolysis (Behrens and Ramachandran 1981; Allen and Beck 1972; Exton et al. 1972), and glucocorticoids increased ACTH-induced cAMP production (Behrens and Ramachandran 1981, Braun and Hechter 1970). These effects of adrenalectomy and glucocorticoids on ACTH-induced lipolysis are not related to effects on the number of ACTH-R but rather to post-receptor events.

The different regulation of ACTH-R gene expression between the adrenal gland and adipose tissues is likely due to the usage of the different promoter in the mouse ACTH-R gene. Recently, we found the novel first exon (exon 1f) of the ACTH-R gene specific for mouse adipose tissues by 5' RNA ligase-mediated rapid amplification of cDNA ends which is located ap-

proximately 1.4 kb downstream of previously reported exon 1 (exon 1a) of the gene in the adrenal gland (KUBO et al. 2004). The promoter region of exon1f contained no consensus CRE (cAMP responsive element) sequence nor steroidogenic factor (SF)-1 binding sequence. Cyclic AMP and/or forskolin mimick the up-regulatory action of ACTH in Y-1 cells (MOUNTJOY et al. 1994; SCHIMMER et al. 1995) and NCI-H295 cells (MOUNTJOY et al. 1994), indicating that the A-kinase pathway has a pivotal role in this phenomenon. There are SF-1 binding sites in the promoter region of human exon 1 and mouse exon 1a of the ACTH-R gene but not the typical CRE sequence. It was reported that SF-1 binding sites were required for cAMP-stimulated regulation of human ACTH-R gene expression (NAVILLE et al. 1999; SARKAR et al. 2000). SF-1 is not expressed in mouse breast adipose tissues (CLYNE et al, 2002), and we confirmed no signals of SF-1 mRNA in mouse epididymal adipose tissues by Northern hybridization (data not shown).

In conclusion, a high level of *in vivo* ACTH is a major factor regulating ACTH-R gene expression in the adrenal, while low circulating ACTH may not be, and a different regulation of ACTH-R gene expression exists in the adrenal cortex and adipose tissues.

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