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Endogenous glucocorticoids inhibit scratching behavior induced by the administration of compound 48/80 in mice

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Abstract

In this study, we investigated the effects of endogenous glucocorticoids on the compound 48/80 (a condensation product of *N*-methyl-*p*-methoxyphenethylamine with formaldehyde)-induced mouse scratching behavior using either RU-486 (mifepristone), a glucocorticoid receptor antagonist, or a surgical resection of the adrenal glands. Subcutaneous injection of compound 48/80 induced not only a corticosterone elevation in the plasma but also an enhanced expression of corticotropin releasing hormone (CRH) mRNA in the paraventricular nucleus, which thus suggests that hypothalamic–pituitary–adrenal axis is activated by the compound 48/80-induced cutaneous reaction. Inhibition of such an endogenous glucocorticoid activity by RU-486 significantly increased the degree of scratching behavior at not only the early-phase (<60 min) but also the late-phase (>60 min) time course after the injection of compound 48/80. Since the elevation of the histamine levels in the plasma in the RU-486-treated mice was no longer found in late-phase scratching behavior, these results thus indicate that histamine is a dominant mediator responsible for early-phase scratching behavior, while different mediators other than histamine may be also involved in the induction of late-phase scratching behavior. Moreover, surgical removal of adrenal glands also significantly increased the compound 48/80-induced scratching behavior without affecting anxiety and locomotor parameters, indicating that endogenous glucocorticoids exert their anti-pururitogenic effects independently of changes in behavioral performance. In conclusion, endogenous glucocorticoid activity was found to suppress the compound 48/80-induced scratching behavior in mice.

Keywords: Adrenectomy; Atopic dermatitis; Glucocorticoid; RU-486; Scratching behavior

1. Introduction

In pruritic cutaneous diseases such as atopic dermatitis and urticaria, it is critically important to control itching sensation because this uncomfortable sensation often elicits scratching behavior, which consequently induces an exacerbation of skin conditions. To mitigate this pruritus and improve skin lesions, glucocorticoid agents are generally used for topical and systemic therapeutic treatment (Rudikoff and Lebwohl, 1998). The therapeutic usefulness of exogenously administered glucocorticoid agents has been well established; however, whether or not the endogenous glucocorticoid activity may be responsible for an inhibition of the itching sensation accompanying pruritic cutaneous diseases remains to be clarified.

Recently, an animal model for evaluating the strength of itching was reported by Kuraishi et al. (1995). In this report, they showed that the subcutaneous injection of pruritogenic agents, such as compound 48/80 (a condensation product of *N*-methyl-*p*-methoxyphenethylamine with formaldehyde) and substance P, elicits scratching of the treated skin, while algesiogenic agents, such as capsaicin and formalin, are without any apparent behavioral effects. This experimental system using compound 48/80 is verified by several other researchers, and is now frequently used as a standard method for evaluating the effects of anti-allergic drugs

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(Sugimoto et al., 1998; Inagaki et al., 1999). Since the anaphylactoid reaction induced by the intravenous application of compound 48/80 has been reported to activate hypothalamic–pituitary–adrenal axis and thereby elevate glucocorticoid levels in the plasma (Földes et al., 2000), it is thus interesting to investigate whether or not this glucocorticoid elevation may be involved in the regulation for controlling itching sensation.

The purpose of this study is to elucidate the role of endogenous glucocorticoids in the compound 48/80-induced scratching behavior after either administrating RU-486 (mifepristone), a glucocorticoid receptor antagonist, or surgically removing the adrenal glands.

2. Materials and methods

2.1. Animals

Male BALB/c mice (6–8 weeks of age) were obtained from Charles River Japan (Shizuoka, Japan). All animals were maintained on a 12-h light/12-h dark cycle with food and water freely available. The mice were allowed to acclimate themselves to the colony for 7 days before the experiments began. The temperature of the colony room was maintained at 22–23 °C. This experiment was reviewed by the committee of Ethics on Animal Experiments in the Graduate School of Medical Sciences, Kyushu University, and was carried out under the control of the Guidelines for Animal Experiments of the Faculty of Medicine, Kyushu University and the Law (No. 105) and Notification (No. 6) of the Government.

2.2. Experimental procedures

The observations of scratching behavior were carried out according to the previous method reported by Kuraishi et al. (1995). A total of 100 μg of compound 48/80 (Sigma, St. Louis, MO) was dissolved in physiological saline and injected subcutaneously into the rostral back in a volume of 50 μl. Immediately after the injection, each mouse was put into an individual cage. The scratching behavior was then recorded for 120 min using an 8-mm video camera (CCD-TRV86, Sony, Tokyo, Japan) under quiet conditions. The number of times that the injected site was scratched by the hind paws was counted, but any scratching of other sites was ignored. In general, the mice showed several scratches of the injected site for about 1 s and each time of such scratching behavior was counted as one incident of scratching at 10-min intervals. In a preliminary experiment, when the total number of scratches observed over 120-min observation period was plotted against the administered dose of compound 48/80, the effect was dose-dependent from 10 to 100 μg, although no such dose-dependency was found at doses higher than 100 μg. The total number of scratches induced by the injection of physiological saline alone was 20–30 times during 120 min of observation period, a similar degree that was spontaneously observed without any treatment. We thus chose 100 μg of compound 48/80 in this experiment because this dose induced the maximal scratching behavior but failed to elicit any systemic effects, such as a fall in blood pressure.

In some experiments, in order to examine the effect of RU-486 (Roussel-UCLAF, Romainville, France), a glucocorticoid receptor antagonist (Flint et al., 2001; Sudo et al., 1997, 2001), the mice were injected subcutaneouly with either RU-486 (10 mg/kg) or a corresponding vehicle (physiological saline containing 1% polyethylene glycol 400) 1 h prior to the injection of compound 48/80.

In analyzing the experimental data, any information as to which group was treated with drugs was blinded to the observer.

2.3. Adrenectomy and corticosterone replacement

In order to completely eliminate the endogenous glucocorticoid activity, a surgical removal of the adrenal glands was performed under anaesthesia with pentobarbital sodium (30 mg/kg i.p., Nembutal; Dainippon, Osaka, Japan). An adrenectomy was done by a laparotomy, as the back skin was left intact for experiments. The sham-operated controls ($n=5$) were similarly treated, but sutured without removing the adrenals. After surgery, adrenectomized animals ($n=5$) received 0.9% NaCl solution containing 1% polyethylene glycol 400 and 3 μg/ml corticosterone for 7 days. Corticosterone replacement was performed to prevent any weakness due to adrenal insufficiency. This replacement dose was chosen because it had no significant effects on the thymus weight and body weight (Häusler et al., 1992).

2.4. Determination of corticosterone and histamine levels in the plasma

The mice were sacrificed by a cervical dislocation to obtain plasma samples at the indicated time points. Whole blood was obtained by a cardiac puncture and then was collected in EDTA-coated sample tubes. The plasma obtained by centrifugation was stored at −80 °C for histamine and corticosterone determination later.

The plasma level of corticosterone was measured using commercially available radioimmunoassay kits (ICN Biomedicals, Costa Mesa, CA). The concentration of corticosterone in the serum samples was calculated from a standard curve and expressed in nanograms per milliliter. The detection limit of the assay was about 1 ng/ml.

The histamine concentration in the plasma was determined by ion-pair high-performance liquid chromatography (HPLC) coupled with post-column fluorescent derivatization, as described earlier (Itoh et al., 1992).

In the analysis of corticosterone and histamine, five mice per group were used for each time point (total 25 mice per each group).

2.5. Polymerase chain reaction analysis of mRNAs corticotropin releasing hormone

The total RNA was extracted from the paraventricular nucleus in the mouse brain using a commercially available kit (Sepasol-RNA II, Nacalai Tesque, Kyoto, Japan). Paraventricular nucleus was identified according to the illustrated atlas, ''Mouse Brain in Stereotaxic Coordinates'' (Paxinos and Franklin, 2001). Total RNA (1 μg) diluted into 10 μl double-distillated water was converted to cDNA with a 0.1 μg random hexamer. Following 10 min of incubation at 75 °C, 4 μl of 5 × polymerase chain reaction (PCR) buffer, 0.4 mM dNTP mix and 1 μl MMLV reverse transcriptase (all purchased from Toyobo, Osaka, Japan) were added and incubated first at 42 °C for 11 min, and then at 95 °C for 10 min. The levels of mRNAs for corticotropin releasing hormone (CRH) and 3-phosphate dehydrogenase (GAPDH) were evaluated by the semiquantitative RT-PCR method. The specific primer for GAPDH was designed: sense primer, 5′-TCCTGCACCACCAACTGCTTAG-3′: antisense primer, 5′-TCTTACTCCTTGGAGGCCATGT-3′. The primers for CRH were classified as follows: sense primer, 5′-AACTCAGAGCCCAAGTACGTTGAG-3′: antisense primer, 5′-TCACCCATGCGGATCAGAATC-3′. The amplified products were 560 bp for GAPDH mRNA and 355 bp for CRH mRNA. To normalize the signals from different RNA samples, GAPDH mRNA was co-amplified as an internal standard. The amplification of CRH and GAPDH was stopped within the linear range (CRH, 33 cycles; GAPDH, 25 cycles). The PCR reaction mixtures (25 μl) contained 2.5 μl 10 × PCR buffer, 2.5 μl dNTP mix (2 mM), 1.5 μl $MgCl_2$ (25 mM), 0.125 *Taq* polymerase (5 U/ml), respectively, along with 1.25 μl sense and antisense primer. For the amplification of GAPDH cDNA, the PCR cycle was 95 °C for 40 s; 60 °C for 40 s; 72 °C for 20 s and the final extension was 72 °C for 1 min. For CRH cDNA, the PCR cycle was 95 °C for 60 s; 60 °C for 60 s; 72 °C for 60 s and the final extension was 72 °C for 2 min. The PCR products were separated by electrophoresis on 1.5% agarose gel and then were visualized by ethidium bromide staining and UV light.

2.6. Behavioral analysis

The anxiety levels of mice were measured according to the previously reported method using elevated pluz-maze (Rodgers and Johnson, 1995). The maze made of wood was elevated to a height of 38.5 cm with two open (30 × 5 cm) and two enclosed arms (30 × 5 cm), arranged so that the arms of the same type were opposite each other, connected by an open central area (5 × 5 cm). To prevent the mice from falling off, a rim made of transparent acryl clear Plexiglas (15 cm high) surrounded the perimeter of the open arms. At the beginning of the experiment, a mouse was placed in the center of the maze, facing one of the enclosed arms, and observed for 5 min. Time spent in open arms, number of open arm entries and number of closed arms entries (defined as entry of all four limbs into an arm of the maze) were recorded. The results were expressed as the mean ratio of time spent in open arms to total time spent in both open and closed arms, mean ratio entries into open arms to total entries into both open and closed arms, mean total number of both closed and open arm entries.

Locomotor activity was analyzed by the previous method reported by Takahashi et al. (1999), with some modifications. The movement of mice was recorded for 5 min using an 8-mm video camera (CCD-TRV86, Sofny, Tokyo, Japan) under quiet conditions in a gray wooden box (50 × 50 × 50 cm). Video images were captured on a personal computer at 15 frames/s. All such data were analyzed by our analytic programs with the use of the public domain software NIH image (http://www.rsb.info.nih.gov/nih-image).

2.7. Data analysis

All data are expressed as the mean ± S.E. Statistical differences between the groups were determined by a repeated measures analysis of variance followed by the unpaired *t*-test.

3. Results

3.1. Subcutaneous injection of compound 48/80 activates hypothalamic–pituitary–adrenal axis

In order to clarify whether or not our experimental protocol substantially induces hypothalamic–pituitary–adrenal axis activation, we first examined the kinetics of both CRH expression levels in the paraventricular nucleus and corticosterone levels in the plasma.

As shown in Fig. 1, the mRNA expression level of CRH in the paraventricular nucleus was enhanced at 15 min after the injection of compound 48/80. Similarly, the plasma corticosterone levels also elevated at 30 min after such an

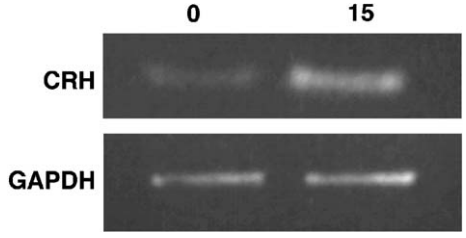

Fig. 1. Enhancement of CRH gene expression in paraventricular nucleus after the injection of compound 48/80. Total RNA was prepared from paraventricular nucleus before (0 min) and at 15 min after the injection of compound 48/80 as described in Materials and methods. The results shown are representative of three such experiments. GAPDH was used as a control to monitor the amount of mRNA in the sample. The sizes are 355 bp for CRH and 560 bp for GAPDH.

injection, although these levels remained sufficiently high even at 120 min after the injection (Fig. 2).

3.2. RU-486 enhances the scratching behavior induced by compound 48/80

To elucidate the involvement of such elevated glucocorticoids in itching sensation, we next tested the effects of RU-486, a glucocorticoid receptor antagonist, on the compound 48/80-induced scratching behavior.

As shown in Fig. 3, a repeated measured analysis of variance revealed a significant group difference between the RU-486-treated and vehicle-treated animals. This group difference was also confirmed by analyses of both the total number of scratches (RU-486 487 ± 54.2 times, vehicle 244 ± 41.8 times; $P<0.01$) and the value estimated by the area under the time–scratch curve (RU-486 10,019 ± 1123 times/120 min, vehicle 5037 ± 915.9 times/120 min; $P<0.01$). Interestingly, the significant enhancement of scratching numbers in the RU-486-treated mice was found at not only the early-phase (<60 min) but also the late-phase (60 min <) time course after treatment with compound 48/80.

3.3. Effect of RU-486 on plasma histamine levels

A previous study (Sugimoto et al., 1998) of this experimental system using compound 48/80 showed histamine to be mainly responsible for the induction of scratching behavior. Therefore, we analyzed the time course changes in the plasma histamine levels after the injection of compound 48/80. As a result, the plasma histamine level in the RU-486 treatment group significantly increased at 30 min after the injection, although its level rapidly returned to the basal at 60 min after the injection (Fig. 4).

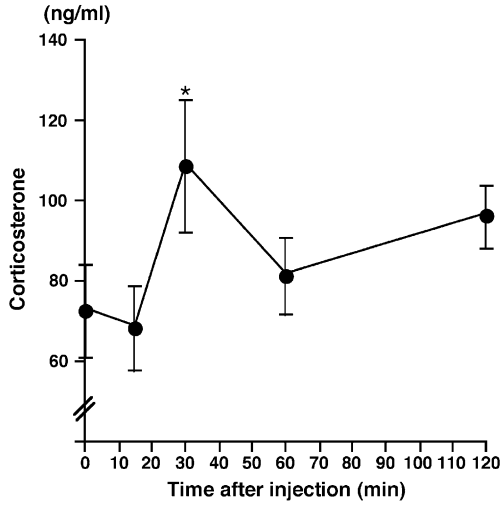

Fig. 2. Kinetics of the plasma corticosterone levels. Plasma corticosterone levels were measured as described in Materials and methods. All data are expressed as the mean ± S.E. *$P<0.05$ was considered to be significantly different from the corresponding basal value.

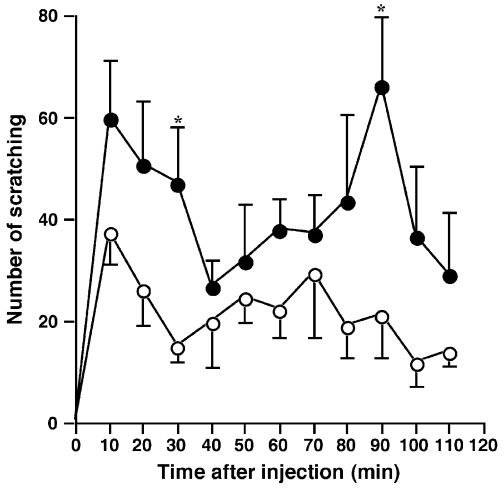

Fig. 3. Effect of RU-486 on the compound 48/80-induced scratching behavior. The mice were administered RU-486 (closed circle) or a corresponding vehicle (open circle) 1 h prior to the injection of compound 48/80. All data are expressed the mean ± S.E. ($n=5$). The results shown are representative of three such experiments. Similar results were obtained from several independent experiments. *$P<0.05$ was considered to be significantly different from the corresponding values in the vehicle group.

3.4. Surgical resection of adrenal glands increases the number of scratching behavior without affecting the behavioral status

To further confirm such a suppressive effect of endogenous glucocorticoid on the itching sensation, we studied the effect of adrenectomy on the compound 48/80-induced scratching behavior.

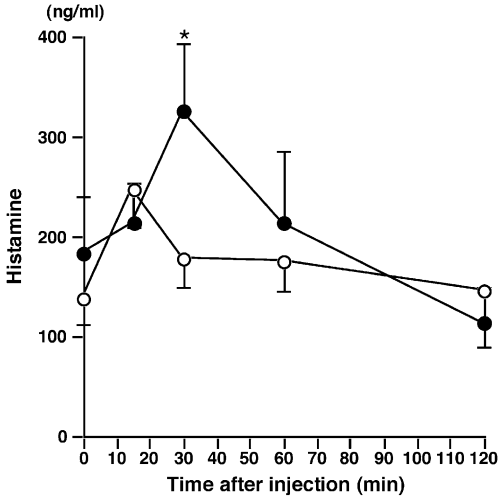

Fig. 4. Kinetics of the plasma histamine levels in the RU-486-treated mice. The mice were administered RU-486 (closed circle) or a corresponding vehicle (open circle) 1 h prior to the injection of compound 48/80. Plasma histamine levels were measured as described in Materials and methods. All data are expressed as the mean ± S.E. *$P<0.05$ was considered to be significantly different from the corresponding values in the vehicle group.

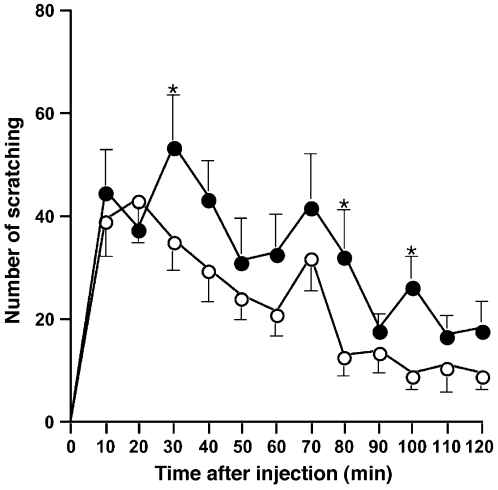

Fig. 5. Effect of adrenectomy on the compound 48/80-induced scratching behavior. The adrenectomized-(closed circle) or sham-operated (open circle) mice received the injection of compound 48/80. All data are expressed as the mean ± S.E. ($n=5$). *$P<0.05$ was considered to be significantly different from the corresponding values in the vehicle group.

As shown in Fig. 5, a repeated measured analysis of variance revealed the significant difference in the number of scratching between the adrenectomized- and sham-operated mice. This group difference was also confirmed by analyses of both the total number of scratches (adrenectomy 434 ± 54.4 times, sham 276 ± 23.9 times; $P<0.05$) and the value estimated by the area under the time–scratch curve (adrenectomy 8999 ± 1150 times/120 min, sham 5683 ± 508.1 times/120 min; $P<0.05$). The number of scratches in the adrenectomized mice was significantly higher than the corresponding sham values at 30, 80, and 100 min after the injection.

Since behavioral profiles, especially such as anxiety and locomotor activity, are generally considered to profoundly affect scratching behavior, one possible explanation for this enhanced scratching is that the adrenectomized mice may show the enhanced scratching behavior because of change in their behavioral performance. Therefore, in order to rule out this possibility, we actually examined both the anxiety and locomotor behaviors in the adrenectomized- and sham-operated-mice.

As summarized in Tables 1 and 2, no significant difference in the anxiety and locomotor activities was seen between the adrenectomized and sham-operated mice. These results were also reproduced by the other repeated investigations performed in the same experimental settings.

Table 1
Effect of adrenectomy on anxiety status[a]

	Control (%)	Adrenectomy (%)
Time spent in open arms	55.2 ± 14.4	66.8 ± 14.2
Entries into open arms	64.4 ± 9.7	70.4 ± 7.9

[a] Anxiety levels in the sham-operated (control) or adrenectomized mice were measured by elevated pluz-maze as described in Materials and methods. All data are expressed as mean ± S.E. ($n=5$).

Table 2
Effect of adrenectomy on motor activity[a]

	Control (cm)	Adrenectomy (cm)
From 0 to 1 min[b]	122.8 ± 27.7	150.2 ± 24.6
From 1 to 2 min	131.0 ± 52.1	86.9 ± 23.0
From 2 to 3 min	91.1 ± 22.9	104.0 ± 38.7
From 3 to 4 min	84.6 ± 21.9	119.0 ± 43.0
From 4 to 5 min	84.8 ± 29.1	144.4 ± 39.5
Total movement	514.6 ± 107.8	604.8 ± 152.7

[a] Motor activities in the sham-operated (control) or adrenectomized mice were measured by the method described in Materials and methods. All data are expressed as mean ± S.E. ($n=5$).

[b] A construct like 'from 0 to 1 min' means total distance of movement during this period.

These results thus suggest that the enhanced scratching in the adrenectomized mice is not ascribed to the alteration of behavioral profiles.

4. Discussion

In this study, the treatment with compound 48/80 induced not only an elevation of the plasma corticosterone levels but also enhanced the mRNA expression level of CRH in the paraventricular nucleus, which thus suggest that the cutaneous reaction induced by compound 48/80 activates the hypothalamic-pituitary-adrenal axis. These results are consistent with a previous study (Földes et al., 2000) in which the intravenous injection of compound 48/80 was reported to cause an adrenocorticotropic hormone and corticosterone elevation in the plasma in Wistar rats. The precise mechanisms whereby the local signals produced by the cutaneous reaction can be transmitted to the central nervous system and thereby activate hypothalamic-pituitary-adrenal axis still remain to be elucidated. However, since it has now been well-established that close interaction does exist between mast cells and the peripheral sensory nerve, one such sensory nerves, which are referred to as histamine specific C-fibers (Schmelz et al., 1997) may play an important role in this transmission to the paraventricular nucleus.

It has been speculated that emotional reactions in patients with atopic dermatitis, such as anxiety, may lead to an altered autonomic activity which results in peripheral vascular changes and a lowering of itch thresholds (Faulstich and Williamson, 1985). Consequently, an itch-scratch vicious cycle would be easily initiated, allowing skin lesions to be developed. Indeed, it was reported that patients with atopic dermatitis develop conditioned scratch responses much sooner than healthy volunteers (Jordan and Whitelock, 1974). Therefore, it is possible that the present findings, in which adrenalectomized mice scratched more frequently than control mice, may be due to changes in the anxiety status. However, this possibility seems unlikely because the anxiety levels in the adrenalectomized mice were almost identical with those in the control mice.

Nonetheless, it should be noted that a clear interpretation of this data is difficult because of the small sample size used in the present experiments. A new project in our laboratory is now in progress to further clarify the effect of behavioral parameters on mouse scratching behavior.

In the next series of experiments, we observed an enhancement of scratching behavior in the RU-486-treated mice not only in the early-phase (0–60 min) but also in the late-phase (60–120 min) time course after the compound 48/80 injection. Furthermore, this tendency was also confirmed in the adrenalectomized mice. Together with the data that the plasma histamine level in the RU-486 treatment group significantly increased at 30 min after the injection, while rapidly decreasing to the basal level at 60 min after the injection, these results thus indicate that histamine is a dominant mediator responsible for early-phase scratching behavior, although different mediators other than histamine are also involved in the induction of late-phase scratching behavior. The precise reason for this enhancement of late-phase scratching behavior has not definitely been answered at present; however, substance P released from the sensory C-fiber nerve-ending following compound 48/80-triggered mast cell activation may be involved in this phenomen, because glucocorticoid has been reported to inhibit substance P-induced neurogenic edema formation partly through a mechanism dependent upon lipocortin (Ahluwalia et al., 1995). Further studies are still called for to elucidate the contribution of tachykinin to this compound 48/80-induced late cutaneous reaction.

The present findings that RU-486 treatment enhanced the elevation of the plasma histamine levels suggest that endogenous glucocorticoids may suppress histamine release from cutaneous mouse mast cells. This is consistent with the previous reports describing that dexamethasone can inhibit not only immunoglobulin E-mediated histamine release from mouse peritoneal mast cells (Daeron et al., 1982), mouse bone marrow-derived mast cells (Robin et al., 1985), and rat peritoneal mast cells (Lewis and Whittle, 1977; Heiman and Crews, 1984), but also compound 48/80-induced degranulation from rat peritoneal mast cells (Taylor et al., 1996).

Some recent clinical reports suggest that a reduced hypothalamic–pituitary–adrenal axis activity may be involved in the maintenance and exacerbation of atopy (Buske-Kirschbaum et al., 1997, 2002; Rupprecht et al., 1995). In fact, this suggestion is supported by the observation in patients with atopic dermatitis that diurnal plasma cortisol variations are closely associated with the diurnal variation of atopy-relevant inflammatory parameters such as basophils or eosinophils as well as the severity of allergic symptomatology (Herrscher et al., 1992). Similarly, such a reduced hypothalamic–pituitary–adrenal axis response was also found in children with persistent asthma attacks (Nomura et al., 1997). More importantly, the pathogenic significance of a dysfunctional hypothalamic–pituitary–adrenal axis in skin atopy is further underlined by an incidental observation by Laue et al. (1990). They reported that after treatment with the glucocorticoid receptor antagonist RU-486, healthy volunteers showed atopy-like symptoms such as erythema and eczematous skin. None of the subjects had a prior history of atopy. Together with the present results, these findings thus indicate that an impaired hypothalamic–pituitary–adrenal response in patients with atopic dermatitis may elicit an excerbation of skin lesions through aggravating itching sensation.

In conclusion, endogenous glucocorticoid was found to be responsible for the suppression of the compound 48/80-induced scratching behavior in mice. This conclusion is supported by the present data that the inhibition of such an endogenous glucocorticoid activity by RU-486 significantly increased the degree of scratching behavior at not only the early-phase but also the late-phase time course after the injection of compound 48/80. Moreover, the surgical removal of adrenal glands also significantly increased the compound 48/80-induced scratching behavior without affecting anxiety and locomotor parameters. These results taken together suggest that endogenous glucocorticoid activities may play an important role in protecting against the aggravation of an itch-associated response.

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