J Occup Health 2007; 49: 431–442

Journal of Occupational Health

Effect of Chronic Topical Exposure to Low-Dose Noxious Chemicals and Stress on Skin Sensitivity in Mice

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Abstract: Effect of Chronic Topical Exposure to Low-Dose Noxious Chemicals and Stress on Skin Sensitivity in Mice: Yumiko NAKANO. Department of Environmental Health, Osaka Prefectural Institute of Public Health—It has been suggested that the recent increase in inflammatory diseases is related to an increase in environmental chemicals and psychiatric stress. To investigate the effect of chronic topical exposure to chemicals and isolation stress, low-dose formalin (a mild contact sensitizer and an irritant), 2,4,6-trinitrochlorobenzene (TNCB; a potent contact sensitizer) and sodium lauryl sulphate (SLS; an irritant) were applied to mouse ears at 7-d intervals under no-stress or stress conditions. Skin reactions (ear swelling) elicited by formalin and TNCB increased time dependently. At the chronic stage, a significant skin reaction peaking at 1 h after application was elicited on the formalin-treated sites, while a shift from a delayed-type hypersensitivity to an immediate-type response was observed on the TNCB-treated sites. At the formalin-treated sites, genes related to neurogenic inflammation, i.e., bradykinin (BK) B2 receptor, IL-6, and membrane metallo endopeptidase (NEP) mRNA were upregulated. In the TNCB-treated sites, marked upregulation of IFN-γ, IL-1β, IL-4, and IL-6 mRNA was observed in addition to B2 receptor mRNA. Pretreatment with HOE140, the B2 receptor antagonist suppressed these skin reactions. Increased skin sensitivity to an unrelated chemical, ethanol, and thermal stimuli were elicited in formalin and TNCB-treated mice. Cortisol levels in formalin-treated mice and IgE levels in TNCB-treated mice were elevated respectively. Stress markedly amplified the skin reactions and gene expression related to neurogenic inflammation. SLS did not induce any changes. It was concluded that chronic topical exposure to low-dose noxious chemicals and stress could easily induce skin sensitivity relating to the BK-B2 pathway and nociceptive sensitization reflecting neural sensitization. *(J Occup Health 2007; 49: 431–442)*

Key words: Noxious chemical, Formalin, Irritant, Contact sensitizer, Exposure, Stress, Neurogenic inflammation, Bradykinin, Skin sensitivity

Over the past few decades, skin sensitivity and inflammatory diseases including atopic dermatitis (AD), rhinitis, and bronchial asthma have exhibited increasing trends in many developed countries[1-3]. The prevalence of workplace exacerbation of skin sensitivity and inflammatory diseases has also been reported[4]. It has been suggested that these changes are related to an increase in the environmental chemicals and psychiatric stress, although disease-specific genes may play a considerable role[5].

Injury by noxious chemicals stimulates multiple mechanisms, such as neurogenic inflammation, time-dependent sensitization, and immune activation[6]. Nerve signals chronically arising from sites of tissue or nerve injury lead to long-term changes in the central nervous system and contribute to hyperalgesia[7,8]. It has been suggested that increased skin sensitivity is a manifestation of sensitization of central trigeminovascular neurons[9]. Neurogenic inflammation also plays a role in prototypic diseases such as AD, asthma, contact dermatitis (CD), and multiple chemical sensitivity (MCS)[10]. Patients with these diseases often have increased skin sensitivity and become overly sensitive to a wide variety of chemically unrelated compounds, which can include ethanol, caffeine and other psychotropic drugs[11,12]. Thus, the disorders induced by chemical exposure and inflammatory diseases seem to have common features.

It is well known that stress exacerbates various inflammatory diseases. Skin sensitivity in AD patients often worsens under stress. Recently, I showed in mice that chronic psychiatric stress enhances contact hypersensitivity (CHS) response and upregulates the levels of serum substance P (SP), the most impotent

Received Jan 31, 2007; Accepted Jul 4, 2007
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mediator of neurogenic inflammation[13]. Neuronal plasticity and increased neuro-immune interaction occur under stress and may alter inflammatory diseases in the skin where neurogenic inflammation plays a part[14]. Thus, a brain-skin connection may underlie inflammatory skin diseases triggered or aggravated by stress.

Chemical agents that are originally discharged as air pollutants may find their pathways to exposure through multiple routes, including dermal contact and ingestion, as well as direct inhalation[15]. Moreover, people have elevated risk of exposure to noxious chemicals via direct contact with the skin to not only from agricultural and industrial chemicals, but also from domestic products containing noxious chemicals such as preservatives[16–19]. Disinfectants cause adverse effects directly on the skin or systemically by permeating through the skin[20]. Although the exact incidence of textile CD is unknown, recent studies demonstrate that CD produced by allergic or irritant reactions to clothing not only more frequent than previously thought, but also increasing[21]. Thus, it is important to understand the percutaneous effect of noxious chemicals on the skin. However, few studies on the effects of chronic exposure to low-dose noxious chemicals on the skin have been performed.

In this study, to gain a better understanding of the contribution of chemicals and psychiatric stress to the pathogenesis of skin sensitivity and inflammatory diseases, I investigated the effects of chronic topical application of low-dose chemicals and stress on the skin in mice, focusing on neurogenic inflammation. Low-dose formalin (a mild contact sensitizer and an irritant), 2,4,6-trinitrochlorobenzene (TNCB; a potent contact sensitizer) and sodium lauryl sulphate (SLS; an irritant) were applied to mouse ears at 7-d intervals for 5–6 wk under no-stress or isolation stress. I report here for the first time that repeated topical applications of low-dose formalin or TNCB induced time-dependent increases in the skin sensitivity and hyperalgesia. These changes were based on neurogenic inflammation related to the bradykinin (BK)-B2 receptor pathway and were markedly augmented by psychiatric stress. Changes induced by chronic exposure to low-dose noxious chemicals and stress in mice may provide a possible model system for studying the developing mechanism of skin disorders in response to environmental factors.

Materials and Methods

Mice

Male BALB/c mice, 5–7 wk old, were used in all studies. Animal care and experimental procedures were performed according to the animal care guidelines of Osaka Prefectural Institute of Public Health. Each group consisted of 5 to 8 mice. Isolation stress was administered as mild psychiatric stress; the mice were housed singly until the end of the experiment[13]. Under this stress condition, mice had no social contact other than exposure to the noises and odors produced in the colony room. For enhancing the feeling of isolation, cages were blocked from each other by styrene foam boxes, and the bed volume in each cage for the isolated mice was reduced to one-tenth that in the control group (5 mice per cage).

Chemicals

Formalin (Wako Pure Chemical Industries Ltd., Osaka, Japan), TNCB (Tokyo Kasei Co., Tokyo, Japan), 4-ethoxymethylene-2-phenyloxazol-5-one (Ox; Sigma, St. Louis, MO, USA), sodium lauryl sulphate (SLS; Wako), and HOE 140 (a BK antagonist; AnaSpec Inc., San Jose, CA, USA) were all obtained from the indicated manufacturers.

Sensitization and elicitation procedure

Mice were repeatedly painted on the ear with 20 μl of various concentrations of formalin, TNCB in ethanol, or SLS in phosphate buffered saline (PBS) at 7-d intervals for 5–6 wk. The ear thickness was measured using a dial thickness gauge (Ozaki Co., Tokyo, Japan) before and 1, 4 and 24 h after each application of chemical. The magnitude of the skin reactions was expressed as Δear swelling, given by the following formula: Δear swelling = (ear thickness after elicitation) – (ear thickness before elicitation). The degree of Δear swelling is given in units of 10^{-3} cm ± SEM.

RNA preparation

RNA was isolated according to the manufacturer's protocol. Briefly, 100 μg of pooled ears from 5 mice was suspended in 2 ml of Isogen (Nippongene, Toyama, Japan), and homogenized using a Polytron homogenizer (Kinematika, Steinhofhalde, Switzerland). RNA was extracted with chloroform, precipitated with isopropyl alcohol, washed once with 70% ethanol, air-dried, and suspended in RNase-free water. To remove genomic DNA, the RNA was treated with DNase-I (Roche Molecular Biochemicals, Mannheim, Germany) at 37°C for 30 min, and re-extracted with phenol-chloroform and ethanol precipitated.

Quantitative real-time RT-PCR

Total RNA was converted to cDNA using the Superscript FirstStrand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). PCR was performed using the LightCycler-FastStart DNA master^{Plus} SYBR Green I (Roche Molecular Biochemicals, Indianapolis, Ind, USA) that contained nucleotides, *Taq* DNA polymerase, reaction buffer, SYBR Green I dye, and 10 mM Mg^{2+}. The amplification of target genes in stimulated cells was calculated by first normalizing to the amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and then expressing the

Table 1. Primer sequences

Gene	Sequence (5'–3')	Sequence (3'–5)
IFN-γ	CATGGCTGTTTCTGGCTGTTAC	TTGCCAGTTCCTCCAGATATCC
IL-1β	GTCTTTCCCGTGGACCTTCC	AACGTCACACACCAGCAGGTT
IL-4	TCGGCATTTTGAACGAGGTC	ACCTTGGAAGCCCTACAGACG
IL-6	CCACTTCACAAGTCGGAGGCTTA	GCAAGTGCATCATCGTTGTTCATAC
NK-1R	GTCGGCCACTGCTACCAAAG	GGCCACTCAATCATGCACAC
BKR2	TCCTACGTGGCCTACAGCAA	TCTCCATCTGGACGGGTTCTC
NEP	CCATTGGACGGGTGTCTTGAG	AAGGCTAAGCATTCATGGAGCAAC

normalized values as fold increases over the value obtained with unstimulated control cells according to the manufacturer's protocols. The determinations were performed in triplicate. Nucleotide sequences for 5' and 3' primers for each primer pair are listed in Table 1.

Tail immersion test

Thermal hyperalgesia was evaluated by measuring response latencies in the warm water tail-immersion (tail-flick) assay[22]. Mice were immobilized in a tube restrainer and the tail flick response was measured when their tails were immersed in a water bath using a stimulus temperature of 50 degrees C.

Measurement of serum IgE and hormone levels

To guard against fluctuations in serum mediators due to circadian rhythm, blood samples were obtained at 10:00 a.m. on each day of assessment. Approximately 500 μl of whole blood was collected from each mouse by cardiac puncture under anesthesia with Nembutal (Abbott Laboratories, Abbott Park, IL, USA). Sera were stored at –30°C until each assay. Measurements of IgE, corticosterone, and β-estradiol levels were performed using an IgE EIA kit (Yamasa Co., Tokyo, Japan), corticosterone ELISA kit (IBL, Hamburg, Germany) and β-estradiol kit (Takeda Co., Tokyo, Japan) respectively, according to the manufacturer's instructions.

Statistical analysis

The results are shown as means ± SE. Comparisons between groups were performed with a two-tailed Student's t test, and $p<0.05$ was considered significant.

Results

Transversal observation of skin reactions induced by repeated topical application of chemicals

To investigate the chronic effect of chemicals, 20 μl of 10% formalin, 0.1% TNCB in ethanol, or 1% SLS in PBS were applied on the right ear of mice at 7-d intervals for 6 wk under no-stress or stress conditions, and the kinetics of the skin reactions (ear swelling) was measured on each application at several time points (0, 1, 4, 24 h). Skin reactions elicited by formalin application peaked at 1 h and increased time dependently, with the most prominent (significant) reaction being elicited in the 5th wk (Fig. 1). Marked amplification of the skin reactions was observed in the stressed mice. The repeated TNCB application also induced a time-dependent increase in the skin reactions and the reactions were amplified by stress administration. In the 6th wk, the skin reactions shifted from a delayed-type hypersensitivity to an immediate-type response, though the 24-h reaction was still strong. The repeated SLS application induced no significant reaction under neither the no-stress and nor the stress conditions. Vehicle (ethanol or PBS) treatment did not induce a significant skin reaction at any stage under the no-stress and stress conditions (data not shown).

It is possible that skin reactions elicited by repeated chemical application are associated with changes such as an increase in the expression of chemical receptors on the application sites.Therefore, the effect of the chemical application site on the elicitation of the skin reactions was investigated. Twenty microlitters of 10% formalin in ethanol were applied on the right or left ears of mice 4 times at 7-d intervals under no-stress or stress conditions. In the 5th wk, formalin was applied to the right ear of all groups and the reaction was measured. Significant reactions were elicited only when the sensitization site and the elicitation site were the same under the no-stress condition (Fig. 2A). Under the stress condition, a significant skin reaction was also elicited even when the sensitization site and the elicitation site were different (Fig. 2B). Thus, enhanced sensitivity to formalin was induced not only in the topical application sites but also systemically by the administration of stress.

Gene expression in the chemical application sites

Expressions of cytokine- and neurogenic inflammation-related genes in the sites repeatedly applied with 20 μl of 10% formalin or 0.1% TNCB at 7-d intervals under no-stress and stress conditions were examined. RNA was isolated from the tissues obtained 1 h after the 5th application of the chemicals and the involvement of each mRNA was evaluated using the RT-PCR method. Among

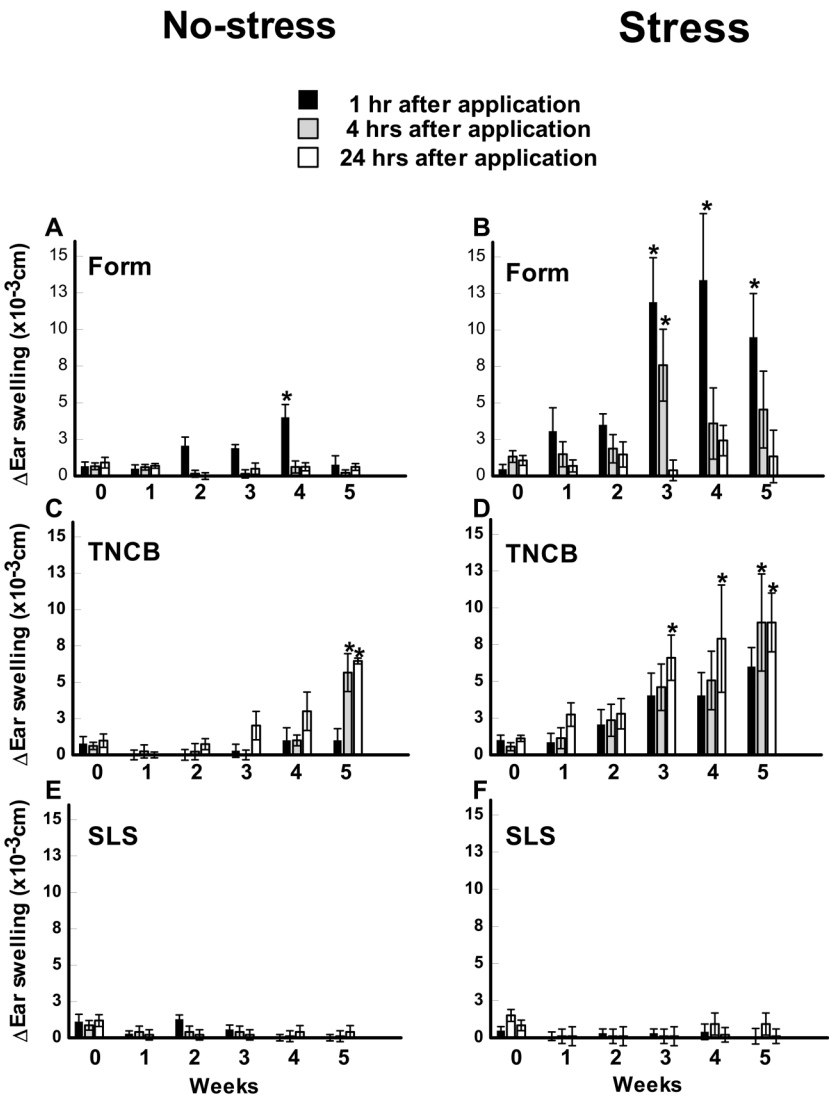

Fig. 1. Kinetics of skin reactions elicited by repeated topical application of chemicals in mice under no-stress and stress conditions. Formalin, TNCB, or SLS was applied on the right ear of mice at 7-d intervals for 6 wk under no-stress or isolation stress conditions, and the skin reactions were measured after each application at several time points (0, 1, 4, 24 h). Data are expressed as the mean Δear swelling ± SEM of 6 mice per group as indicated in Materials and Methods. *Significantly different from the ear swelling at 0 h after each application (p<0.05).

cytokine genes, IL-6 mRNA was up-regulated (≥2) in the formalin-treated sites, and the up-regulation was markedly enhanced by stress administration (Table 2). In the TNCB-treated sites, all the cytokine genes, i.e., IFN-γ, IL-1β, IL-4 and IL-6 mRNA were up-regulated drastically, and stress enhanced the expression of IL-1β and IL-6 mRNA, whereas it suppressed the expression of IFN-γ and IL-4 mRNA. Regarding neurotransmitter-related genes, marked up-regulation of BK B2 receptor (B2 receptor) and membrane metallo endopeptidase (NEP) mRNA was observed in the formalin-treated sites, and it was enhanced by stress administration. In contrast, neurokinin-1 (NK-1R, the receptor of SP) mRNA expression in both the formalin- and TNCB-treated sites was weak, and was further suppressed by stress administration.

Gene expression in the formalin-treated sites 1 h after each application was evaluated transversally. Mice for this experiments were bred under the no-stress condition. A time-dependent increase in the expression of B2 receptor mRNA was observed, while the expression of NK-1R mRNA peaked in the 3rd wk (Fig.3A and 3B).

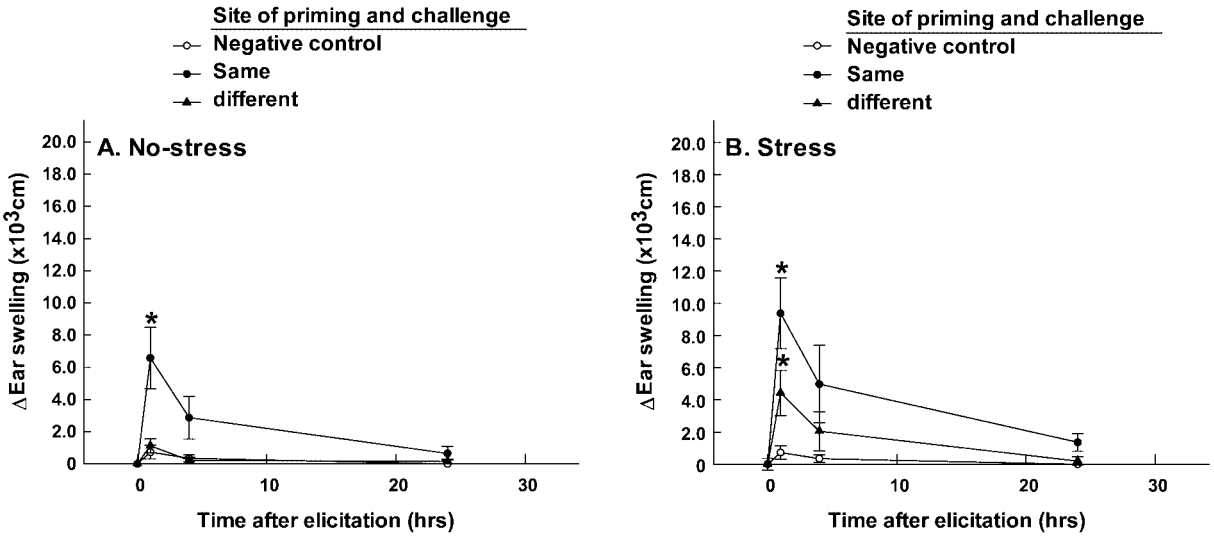

Fig. 2. Effect of application site on the elicitation of skin reactions. Formalin was applied topically at the same site of the right or left ear of mice at 7-d intervals for 4 wk under no stress (A) or stress conditions (B). Then, skin reactions to formalin were elicited on the right ear in all groups at 5th wk and measured at several time points (0, 1, 4, 24 h). Data are expressed as the mean Δear swelling ± SEM of 6 mice per group. Negative controls were treated with vehicle on the right ear in each experiment. *Significantly different from the mean Δear swelling of negative controls at each point (p<0.05).

Table 2. Gene expression in the elicited site of inflammation after repeated application of chemicals by RT-PCR*

Gene	Repeated application	Relative expression ratio No-stress	Stress
IFN-γ	Form	1.3 ± 0.03	2.0 ± 0.11
	TNCB	183.1 ± 0.21	132.2 ± 0.02
IL-1β	Form	1.9 ± 0.11	1.4 ± 0.22
	TNCB	20.1 ± 0.04	61.3 ± 0.21
IL-4	Form	1.5 ± 0.24	1.5 ± 0.00
	TNCB	88.0 ± 0.13	42.1 ± 0.06
IL-6	Form	2.2 ± 0.02	8.9 ± 0.16
	TNCB	21.3 ± 0.19	39.7 ± 0.45
B2 receptor	Form	6.6 ± 0.11	7.2 ± 0.04
	TNCB	2.7 ± 0.00	18.0 ± 0.09
NK-1R	Form	1.9 ± 0.22	1.0 ± 0.11
	TNCB	1.7 ± 0.11	0.5 ± 0.05
NEP	Form	7.4 ± 0.02	10.8 ± 0.33
	TNCB	1.7 ± 0.09	2.0 ± 0.09

* 20 μl of 10% formalin or 0.1% TNCB in ethanol were topically applied on mice ears 6 times at 7-d intervals under no-stress and stress conditions. Total RNA was extracted from the ears of mice 1 h after the 6th application and the expression of various cytokine and neurotransmitter receptor mRNA was estimated using the RT-PCR method. The amplification of target genes in stimulated cells was calculated by first normalizing to the amplification of GADPH, and then expressing the normalized values as fold increases over the value obtained from vehicle controls as indicated in Materials and Methods. Data are expressed as the mean relative expression ratio ± SEM of triplicate experiments.

The role of B2 receptor in the skin reactions induced by the repeated topical application of chemicals

B2 receptor mRNA was markedly expressed in the sites repeatedly treated with formalin. In order to confirm the role of B2 receptors in the skin reactions, the effect of HOE140 (a B2 receptor antagonist) was investigated. Mice were repeatedly treated with 20 μl of 10% formalin or 1.0% TNCB on the right ear at 7-d intervals under the no-stress or stress conditions. Two hundred micrograms of HOE140 were injected intraperitoneally just before the 5th application of chemicals, and the subsequent skin reactions were analyzed at several time points (0, 1, 4, 24 h). Control mice were injected with PBS. The results demonstrated that the pretreatment with HOE140 markedly reduced the skin reactions in the formalin-treated sites and partially reduced the reactions in the TNCB-treated sites in both the no-stress and stressed mice (Figs. 4A and 4B).

Elicitation of non-specific skin reactions in mice treated with chemicals topically

It is possible that the time-dependent increase in the skin reactions induced by repeated chemical application may be mediated by a mechanism similar to the time-dependent sensitization in MCS and that these mice may become sensitive to chemically unrelated compounds and ethanol[23]. Mice received repeated application of 20 μl of 10% formalin, 0.1% TNCB or 1% SLS at 7-d intervals under the no-stress or stress conditions, and skin reactions were elicited with 20 μl of 1.0% Ox or ethanol on the

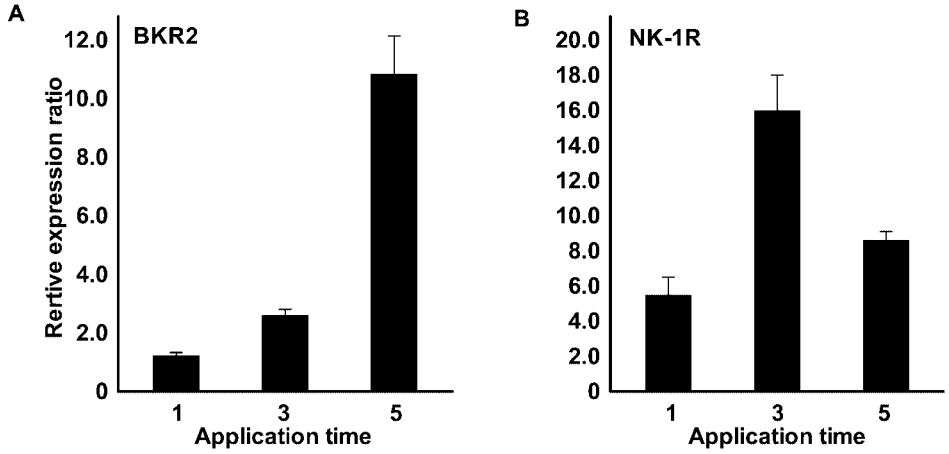

Fig. 3. Kinetics of B2 receptor and NK-1R mRNA expression in the local sites repeatedly treated with formalin. Twenty microlitters of 10% formalin in ethanol were repeatedly applied on the right ears of mice at 7-d intervals. Total RNA was obtained from the ear of 6 mice 1 h after each formalin application and mRNA expression was determined using RT-PCR as indicated in Materials and Methods. The levels of mRNA expression were normalized by GAPDH mRNA expression. (A) Time course of B2 receptor mRNA expression. (B) Time course of NK-1R mRNA expression. The results are shown as the mean relative expression ratio ± SEM of 3 independent experiments.

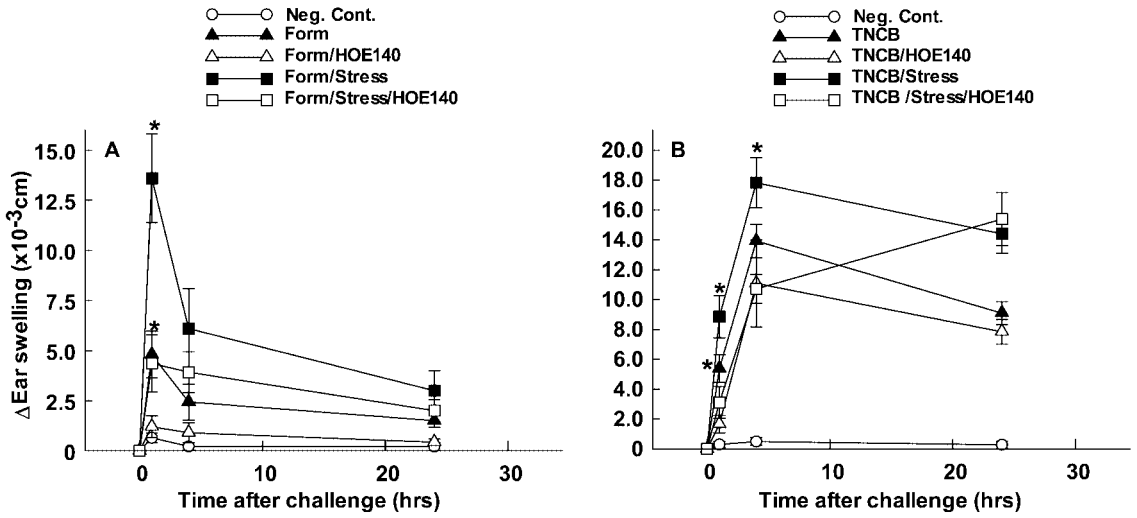

Fig. 4. Effect of HOE140 on the elicitation of skin reactions in mice repeatedly treated with chemicals. Formalin (A) or TNCB (B) in ethanol was topically applied on the ear of mice at 7-d intervals for 4 wk under no-stress or stress conditions. At the 5th wk, mice were injected with HOE140 (B2 receptor antagonist) just before chemical application, and the subsequent skin reactions were measured at several time points (0, 1, 4, 24 h). Data are expressed as the mean Δear swelling ± SEM of 6 mice per group. Negative controls were treated with vehicle only. *p<0.05 as compared with the HOE140-pretreated groups.

same sites of the ear in the 6th wk. The results showed that Ox and ethanol elicited the significant skin reactions in the formalin- and TNCB-treated mice, and the reactions were enhanced by stress administration (Figs. 5A, 5B, 5D and 5E respectively). In contrast, neither non-specific skin reaction nor reaction to ethanol was elicited in mice repeatedly treated with SLS, even when stress was administered (Figs. 5C and 5F). The kinetics of the skin sensitivity to ethanol in mice treated with 20 μl of 10% formalin at 7-d intervals were investigated transversally. Elicitation with ethanol was performed 1 wk after each formalin application. Skin reactions to ethanol increased

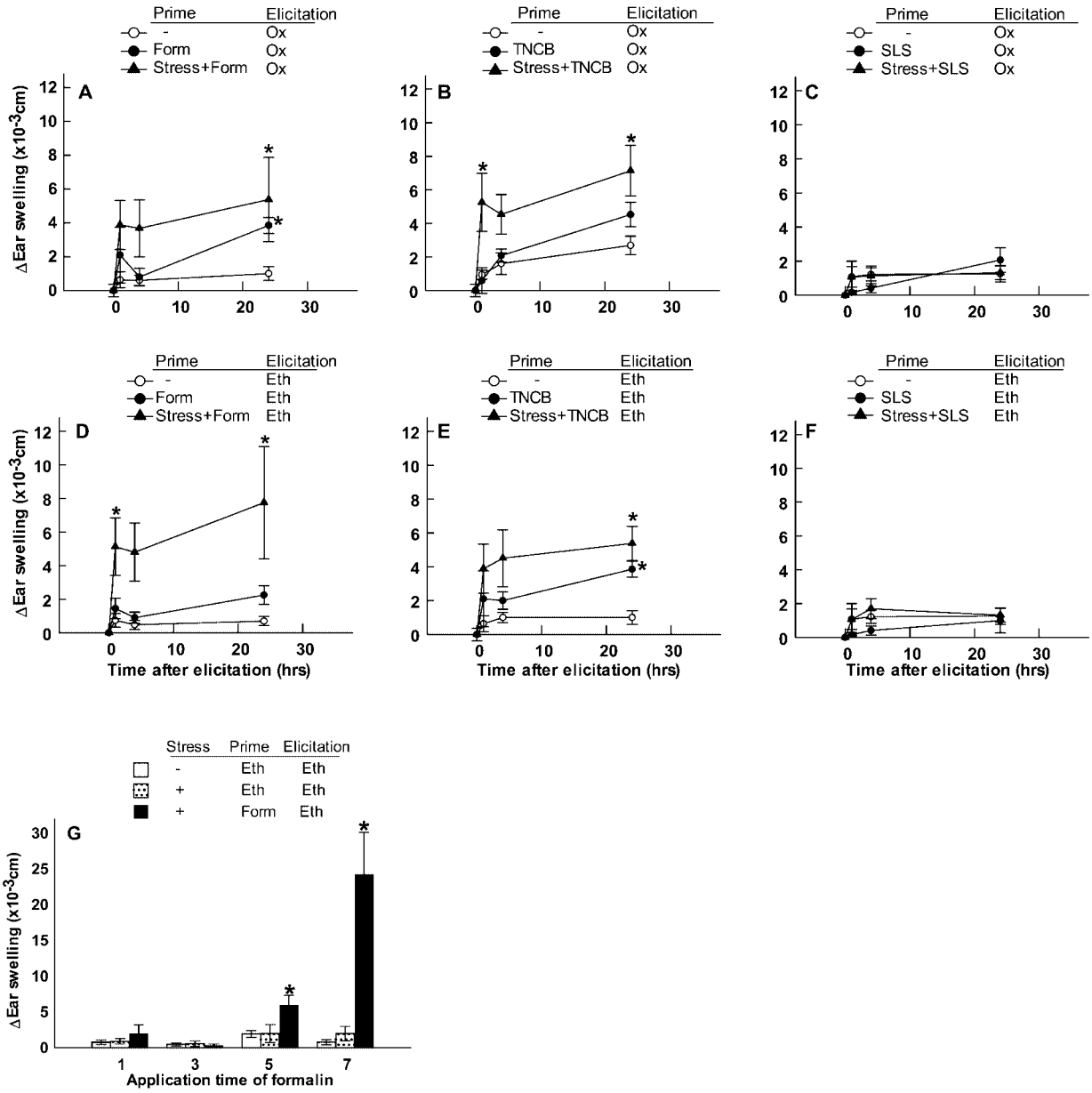

Fig. 5. Skin reactions elicited by a non-specific antigen and ethanol in mice repeatedly treated with chemicals. Formalin, TNCB, or SLS were topically applied on mice ears at 7-d intervals for 5 wk under no-stress or stress conditions, and reactions were elicited with Ox (A, B, C respectively) or ethanol (D, E, F respectively) on the same sites of the ear at the 6th wk. Formalin was also topically applied on mice ears at 7-d intervals and the skin reactions to ethanol were elicited 1 wk after each formalin application on the same sites under the stress condition (G). Data are expressed as the mean Δear swelling ± SEM of 6 mice per group (In experiments A, B, C, D, E and F, Δear swelling 0, 1, 4, and 24 h after chemical application. In experiment G, Δear swelling 4 h after each applicaton.). Negative controls were treated with vehicle only. *Significantly different from the mean swelling of negative controls ($p<0.05$).

time dependently, and a drastic skin reaction to ethanol was elicited in mice after receiving the 7th formalin application (Fig. 5G).

Behavioral changes caused by repeated exposure to chemicals

It was examined whether mice acquired nociceptive sensitization by the repeated topical application of chemicals. Various concentrations of chemicals were applied on the ears of mice 5 times at 7-d intervals under

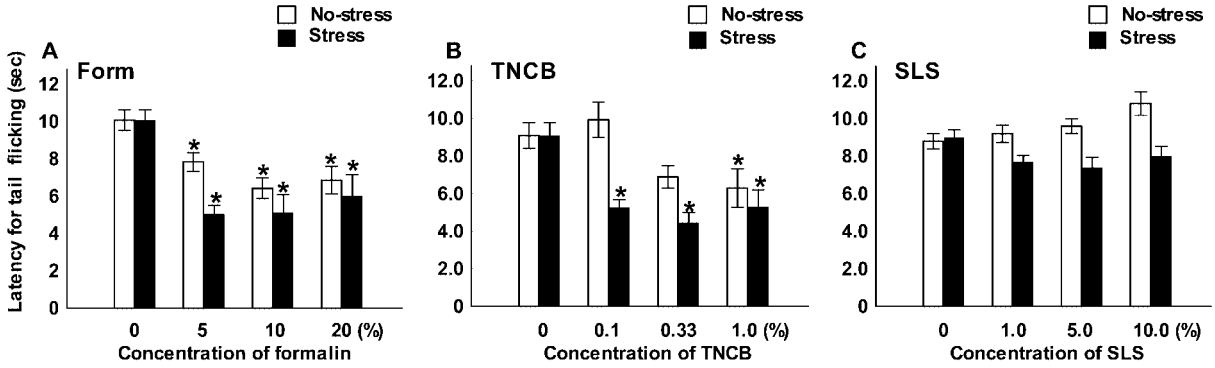

Fig. 6. Tail immersion test using mice repeatedly treated with chemicals. Mice were topically treated with various concentrations of formalin, TNCB or SLS 5 times at 7-d intervals under no-stress or stress conditions. Latency periods of tail flick after immersion in a water bath were measured using a stimulus temperature of 50 degrees C at the 6th wk as indicated in Materials and Methods. Data are expressed as the mean latency period (sec) ± SEM of 6 mice per group. *Significantly different from latency periods of tail flick in vehicle controls using a stimulus temperature of 50 degrees C ($p<0.05$).

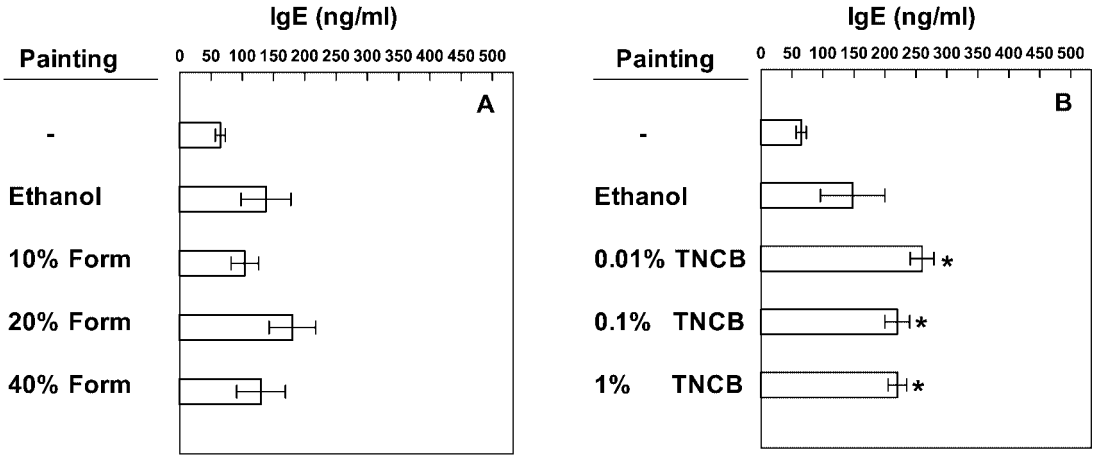

Fig. 7. Serum IgE levels in mice repeatedly treated with chemicals. Mice were topically treated with various concentrations of formalin or TNCB in ethanol 6 times at 7-d intervals, and serum IgE levels 1 h after the final application were determined using ELISA. Data are expressed as the mean IgE levels (ng/m*l*) ± SEM of 6 mice per group. *Significantly different from untreated controls ($p<0.05$).

no-stress or stress conditions, and thermal hyperalgesia was estimated by the tail immersion test using warm water (50°C) in the 6th wk as indicated in Materials and Methods. Formalin-treated mice exhibited significantly shorter periods for the latency of tail flicking than the vehicle controls at every dose of formalin (Fig. 6A). Hyperalgesia was amplified although not caused by stress administration. The TNCB-treated mice also showed significantly shorter periods for the latency of tail flicking compared to the vehicle controls, and stress amplified this tendency (Fig. 6B). In contrast, treatment with SLS did not affect the latency of tail flicking even under the stress condition (Fig. 6C).

Serum IgE and hormone levels in mice treated with chemicals repeatedly

To determine the systemic effect of chronic chemical application, serum IgE and hormone levels in mice were evaluated using ELISA. Mice were repeatedly treated with 20 μl of 10% formalin under the no-stress and stress conditions, and serum IgE levels 1 h after the final application were determined. Since chronic alcohol consumption is associated with an increase in serum IgE, untreated mice were used as controls[24]. As shown in Fig. 7A, formalin application did not elevate serum IgE levels compared to the controls at any concentration. In contrast, IgE levels were significantly elevated in the TNCB-treated mice compared with the control group (Fig. 7B).

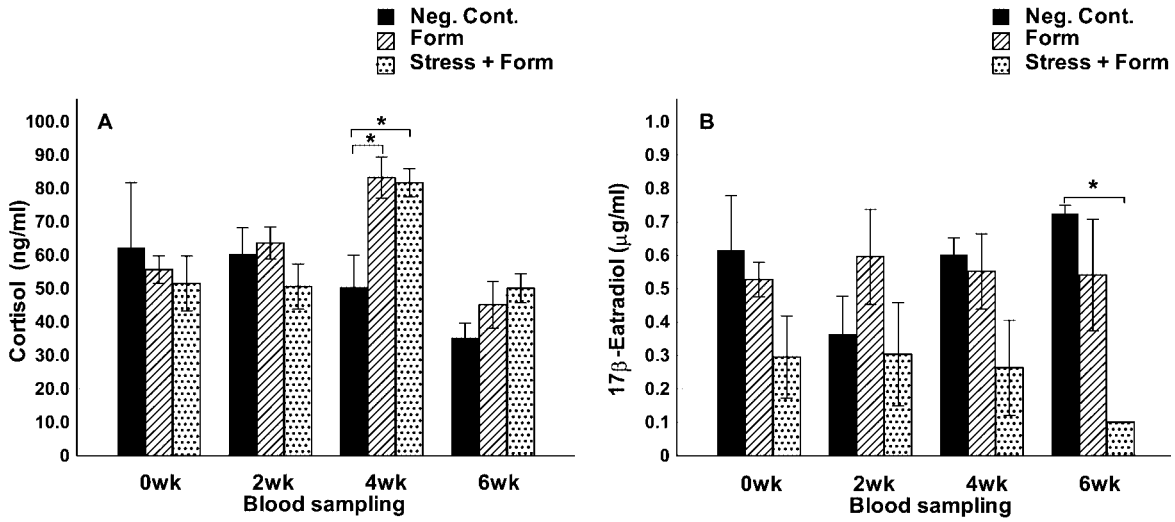

Fig. 8. Serum hormone levels of mice repeatedly treated with formalin. Mice were topically treated with 20 μl of 10% formalin in ethanol at 7-d intervals under no-stress and stress conditions and blood samples were obtained 1 h after each formalin application (at 10:00 a.m.). Serum cortisol (A) and 17-βestradiol levels (B) were determined using ELISA. Data are expressed as the mean hormone levels (ng/m*l*) ± SEM of 6 mice per group. Negative controls were treated with vehicle only. *Significantly different from vehicle controls (p<0.05).

Mice were treated with various concentrations of formalin or TNCB, and 1 h after each application serum cortisol and 17β-estradiol levels were determined transversally under the no-stress or stress conditions. Cortisol levels were slightly but significantly increased by the 5th application (4th wk) (Fig. 8A). Stress did not affect the cortisol levels. In contrast, stress suppressed 17β-estradiol levels significantly after the 7th application in the formalin-treated mice, though they were not changed by the formalin application in the no-stress mice (Fig. 8B).

Discussion

In this study, I demonstrated that the repeated topical application of low-dose formalin and TNCB was shown to induce time-dependent increases in skin reactions to specific chemicals, skin sensitivity to an unrelated antigen and ethanol, and thermal hyperalgesia. These changes were markedly amplified by stress administration. As previously reported, skin reactions induced by repeated TNCB application involved the shift from a delayed type hypersensitivity to an early type hypersensitivity accompanied by marked IFN-γ and IL-4 mRNA expression in the local sites and the elevation of serum IgE levels[25]. In contrast, skin reactions elicited by formalin were the immediate type from the relatively early stages and the treatment did not elevate IFN-γ and IL-4 mRNA expression or the serum IgE levels at the chronic stage. Thus, the changes induced by the repeated topical application of low-dose noxious chemicals cannot be fully explained by an immunological basia. It is reported that long-term cumulative skin treatment with SLS induces irritant CD, and an increase in skin blood flow after thermal stimulus dose dependently in humans[26,27]. However, SLS treatment induced neither chronic skin inflammation nor skin sensitivity in the present system, even under high concentration and stress conditions. It is possible that the sensitivity of mice to SLS may be lower than that of humans.

The molecular mechanisms underlying neurogenic inflammation evoked by noxious stimuli are orchestrated by a large number of chemical substances such as BK released from the bloodstream and various inflammatory cells into the local sites[28]. The stimulation of chemical receptors on the chemosensitive sensory nerve fibers induces inflammation, triggering the release of endogenous mediators, such as SP and calcitonin gene-related peptide[29]. Moreover, the release of neurotransmitters results in the activation and upregulation of various neuronal receptors such as NK-1R and B2 receptor, and the production of proinflammatory cytokines (IL-1, IL6 etc.) and neurotrophins (nerve growth factor, glial cell line-derived neurotrophic factor, etc.) in the tissues[30–32]. B2 receptor-mediated BK sensitivity of rat cutaneous C-fiber nociceptors is enhanced during persistent inflammation[33]. Increases in the expression of B2 receptor mRNA parallel to the skin reactions elicited by repeated formalin application and the abrogation of skin reactions by HOE140 pretreatment indicate that the inflammation

induced by chronic formalin application is mainly caused by the amplification of neurogenic inflammation based on the BK-B2 pathway. Nerve growth factor (NSF) is an important factor responsible for upregulating B2 receptor expression after nerve injury *in vivo*[34]. It is possible that NGF may activate BK-B2 activation by chemicals as described above.

In patients with allergic dermatitis, SP and vasoactive intestinal polypeptide (VIP) are secreted to the surrounding tissues and increased levels of these neurohormones in the blood serum are observed[35]. It is reasonable to assume that neurogenic inflammation is also induced by the chronic application of TNCB.

BK is one of the mediators of pruritus (itch) and pain activating histamine-sensitive C-fibers[36]. Recently it has become apparent that BK can turn into a potent and histamine-independent pruritogen in AD[37]. Thus, it is possible that chronic topical exposure to low-dose noxious chemicals directly induces or exacerbates the disorders implicated in the interface between the internal milieu and the external environment by activation of the BK-B2 pathway.

In the sites repeatedly treated with formalin, NK-1R mRNA expression peaked at the 3rd wk and declined thereafter. Thus, it seems that the NK system, one of the typical pathways of neurogenic inflammation, is regulated at the chronic stage of chemical application. NEP is bound to the membranes of various cells that have receptors for tachykinins and limits the degree of neurogenic inflammation[38]. Marked upregulation of NEP mRNA at the 6th application of formalin supports the possibility that local reactions begin to be regulated by the enzyme at this stage.

Patients with AD often have increased skin sensitivity and the symptoms often worsen under stress[39]. It has been suggested that neurogenic inflammation may have a pathocausal role in stress conditions. Acute or chronic psychogenic stress produces chronic inflammatory changes, i.e., neurogenic inflammation in various organs[40]. Chronic isolation stress induces neurogenic inflammation that is evaluated by enhanced serum SP levels in mice[13]. In the present study, skin reactions to specific and non-specific chemicals, and hyperalgesia were amplified by psychiatric isolation stress, though stress itself did not evoke a reaction. Enhancement of IL-6, B2 receptor and NEP mRNA expression by stress in both formalin- and TNCB-treated sites indicates that these stress-enhanced of these phenomena may also be mediated by neurogenic inflammation related to BK-B2 pathway. Suppression of NK-1R mRNA expression in both the formalin- and TNCB-treated sites by stress suggests that the combination of chemical and psychiatric stress may accelerate the cascade of the inflammatory process. Stress weakens the barrier function of the skin by suppressing spontaneous proliferation and E-cadherin (homophilic adhesion molecule) expression of keratinocytes[13]. It is possible that stress may also amplify skin sensitization by allowing penetration of chemicals through the impaired skin barrier.

In the present study, elicitation of skin reactions by a non-specific chemical, Ox, and ethanol on the local sites suggests that multiple allergy-like skin sensitivity was induced by the repeated application of low-dose noxious chemicals. Multiple allergies are found in patients experiencing irritant and allergic CD and are suggested to involve the BK-B2 pathway[41]. Since HOE140 pretreatment inhibited skin reactions elicited by ethanol in mice repeatedly treated with formalin, skin reactivity to ethanol may also be attributable to neurogenic inflammation via activation of the BK-B2 pathway (data not shown).

Rats repeatedly exposed to formaldehyde express behavioral changes such as increased anxiety, cocaine-induced locomotor activity and hyperalgesia[42]. It has been suggested that the acquisition of nociceptive sensitization reflects not only a reduction in the threshold and an increase in the responsiveness of the peripheral terminals of nociceptor neurons, but also neural sensitization occurring in the central nervous system, though the mechanism is only partially been understood[23]. Expression of thermal hyperalgesia in the present study suggests that neuronal sensitization is induced in the central nervous system by the chronic topical application of low-dose noxious chemicals in mice.

Repeated exposure to low-dose formaldehyde increased basal corticosterone levels and produced stress-like alterations in hippocampal neurogenesis in rats[43]. In the present study, significant elevation of serum cortisol levels was observed at the 5th formalin application when the magnitude of skin reactions to formalin was most prominent. It is possible that the repeated topical application of low-dose formalin might also result in reaching the hypothalamus and alteration of the hypothalamic-pituitary-adrenal (HPA) axis functioning. On the other hand, isolation stress did not change the cortisol levels in the formalin-treated mice. Recently, I reported that serum cortisol levels are enhanced in the acute stage of isolation stress, while they return to normal levels at the chronic stage of stress in mice[13]. It is possible that the repeated application of formalin may stimulate the HPA axis as chemical stress by a pathway different from chronic psychiatric stress.

The serum 17β-estradiol level did not change throughout the formalin application, though stress showed a tendency to suppress its level in the formalin-treated mice and significantly suppressed it at the chronic stage. It was previously reported that stress suppresses hormone levels in the rat[44]. Further studies may be required for understanding the complex mechanism of this phenomenon.

The results of the present study and those discussed above clearly indicate that chronic topical exposure to low-dose noxious chemicals induces specific and non-specific skin sensitivity that can be evaluated by skin reactions, and that stress markedly enhances these phenomena. Thus, the skin can be regarded as a "signal organ" for putative noxious environmental influences and can be used as predictor of general skin sensitivity. In fact, the application of various volatile organic compounds (VOCs) such as toluene, p-dichlorobenzene and phenol also induced time dependent increases in skin reactions and hyperalgesia under the stress condition (data not shown).

Acknowledgments: This work was supported by a grant from the Long-range Research Initiative of the Japan Chemical Industry Association.

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