

“Green Odor” Inhalation Reduces the Skin-Barrier Disruption Induced by Chronic Restraint Stress in Rats: Physiological and Histological Examinations

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Abstract

We investigated whether inhalation of green odor (a mixture of equal amounts of *trans*-2-hexenal and *cis*-3-hexenol) prevents the skin-barrier disruption induced by chronic restraint stress in rats. To this end, transepidermal water loss (TEWL) was measured as an index of the disruption of skin-barrier function, whereas light- and electron-microscope examinations were performed to observe histological changes in the skin of the stressed animals. In addition, the effects on TEWL induced by chronic administration of a glucocorticoid, dexamethasone (DEX), were examined. Chronic restraint stress (8 h per day for 14 days) increased TEWL (vehicle + stress group). This effect (and the chronic stress-induced increase in adrenal weight) was prevented in rats that inhaled green odor at the beginning of each day's restraint (2 h each day for 14 days; green odor + stress group). Electron-microscope studies revealed that rats in the green odor + stress group possessed sufficient intercorneocyte lipids to create an effective skin barrier, although these had apparently been decreased in the vehicle + stress group. Daily administration of DEX for 14 days increased TEWL. The present results suggest that chronic stress-induced disruption of the skin barrier in rats can be reduced or prevented by green odor (possibly at least in part through an inhibitory effect on the stress-induced activation of the hypothalamo-pituitary-adrenocortical axis).

Key words: adrenal gland, dexamethasone, hypothalamo-pituitary-adrenocortical axis, stratum corneum, transepidermal water loss

Introduction

It is well known that an animal reacts to stressful stimuli with stereotyped responses that include increases in both blood pressure and the plasma concentration of adrenocorticotrophic hormone (ACTH) (Watanabe et al. 1998; Carrasco and van de Kar 2003). Such stress-induced activations of the sympathetic nervous system and hypothalamo-pituitary-adrenocortical (HPA) axis (DiMicco et al. 2006), if they continue for a while, can have deleterious effects on various organs, including the skin (Arck et al. 2006). Indeed, it has been shown in mice that chronic exposure to stress results in a disruption of the skin-barrier function, as evidenced by an increase in transepidermal water loss (TEWL; Aioi et al. 2001). The major component of this barrier is located in the outermost layer of the skin, the stratum corneum, which consists of corneocytes surrounded by lipid regions (Elias 2005). These intercorneocyte lipids

play a very important role in skin-barrier function (Rawlings and Harding 2004) and are reportedly reduced in chronically stressed animals (Aioi et al. 2001; Choi et al. 2005). It has been shown that recovery from the “acute,” “artificially” produced skin-barrier disruption induced by tape stripping is delayed by such forms of stress as immobilization or exposure to a novel environment, an effect of stress that can be blocked by sedative drugs and some odorants (Denda et al. 1998; Denda, Tsuchiya, Elias, Feingold 2000; Denda, Tsuchiya, Shoji, Tanida 2000). This raises the possibility that sedative drugs and/or odorants might also prevent chronic stress-induced disruption of the skin barrier, a type of disruption more likely to be encountered clinically.

Recently, Nakashima et al. (2004) described an attenuation by “green odor” of stress-induced elevations in plasma

ACTH in rats, indicating that this so-called green odor (extracted from green leaves) may have a relieving and sedative effect on animals exposed to acutely stressful situations. We hypothesized that green odor might prevent the skin-barrier disruption induced by chronic restraint stress in rats. To test this hypothesis, TEWL was measured as an index of such disruption, while light- and electron-microscope examinations were performed to observe histological changes in the skin of the stressed animals. In addition, because glucocorticoids are known to be secreted in large amounts under stressful conditions, we examined the effects of chronic administration of a glucocorticoid, dexamethasone (DEX), on TEWL. The present results showed that in rats inhalation of green odor (a mixture of equal amounts of *trans*-2-hexenal and *cis*-3-hexenol) prevents the chronic stress-induced disruption of skin barrier and also that chronic DEX treatment results in an increase in TEWL. These results are consistent with such disruption being prevented by green odor, an effect that it may possibly exert, at least in part, through an inhibitory effect on the HPA axis.

Materials and methods

Animals

Male Wistar rats (11 weeks old) were housed in individual plastic cages (40 × 25 × 20 cm; length × width × depth) with woodchip bedding in a room maintained at 25 °C ± 1 °C, with the humidity set at 50%. They experienced a photoperiod of 12-h light:12-h dark (lights on at 0700). All had ad libitum access to drink and standard laboratory rat chow. The protocols were reviewed by the Committee on the Ethics of Animal Experiments in Tottori University Faculty of Medicine, and the experiments were carried out in accordance both with the Guidelines for Animal Experiments at Tottori University Faculty of Medicine and with the Federal Law (no. 221) and Notification (no. 6) issued by the Japanese Government.

This study comprised 2 experiments (Experiments 1 and 2), each rat taking part in only 1 experiment (Experiments 1 or 2).

Green odor and DEX

The green odor used was a mixture of equal amounts of *trans*-2-hexenal and *cis*-3-hexenol, diluted with triethyl citrate to 0.03% (w/w). This concentration of green odor has been reported to be effective in reducing the increase in the plasma concentration of ACTH induced by acute immobilization stress (Nakashima et al. 2004). Furthermore, a mixture of the 2 compounds is more potent than each compound alone (Sano et al. 2002). For the final part of this study, DEX was dissolved in sterile saline. The DEX used in our study was dexamethasone 21-phosphate disodium salt from Sigma (D1159-100MG, St. Louis, MO), a water-soluble prodrug that is converted to DEX in vivo.

Experimental protocols

Experiment 1: effects of green odor on chronic restraint stress-induced changes in rats

Experimental protocols for Experiment 1 are summarized schematically in Figure 1. Rats were restrained as required in a small cylindrical restrainer made of steel wire (7 × 22 cm [diameter × length]) for 8 h (0900 to 1700) on each of 14 successive days. Green odor or its vehicle (i.e., triethyl citrate) was administered for 2 h (0900 to 1100) on each of these 14 days. To this end, a cotton bowl (diameter = 15 mm) was impregnated with 0.2 ml of green odor or its vehicle and then held 3 cm from the nose of the rats. When green odor or its vehicle was administered to freely moving rats, the same type of bowl impregnated with 0.2 ml of green odor or its vehicle was held in the restrainer mentioned above, and this was placed in one corner of the rat's "home cage."

As shown in Figure 1, rats were divided into the following 5 groups. In the vehicle group, the vehicle for green odor was administered without imposing stress on the rats. In the vehicle + stress and green odor + stress groups, vehicle or green odor was administered during stress. In the green odor group, green odor was administered without imposing stress on the rats, whereas in the control group, nothing was administered at all to rats not subjected to restraint. Because the restrained rats were unable to reach the food and water, all groups were deprived of food and water from 0900 to 1700 each day for 14 days.

Measurements of TEWL and adrenal and total body weights. Total body weight was measured just before the start of any treatments (i.e., on the 1st day of the experimental period). In addition, immediately after the end of the protocols described above (i.e., on the 14th day of the experiments), back skin was shaved under general anesthesia (pentobarbitone sodium, 50 mg/kg, intraperitoneally) after measuring the rat's body weight. Thus, changes in total body weight during the

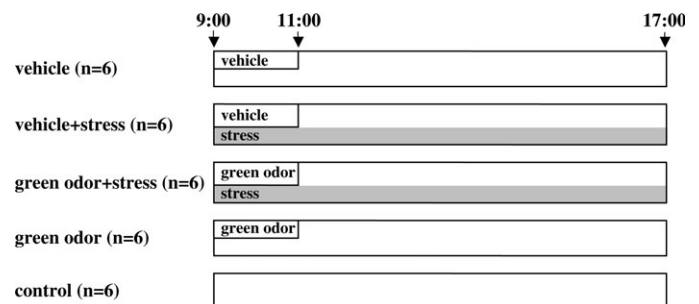


Figure 1 Time schedules for Experiment 1. Time of day is indicated together with period of exposure to stressor (restraint) and/or period for which green odor or its vehicle was administered. These protocols were repeated for 14 days. Measurements were made of TEWL and adrenal weight (each on the 15th day) and total body weight (on the 14th day), total body weight having previously been measured on the 1st day. Skin samples for histological examination were taken after measurement of TEWL.

experiment could be calculated. The next day (i.e., the 15th day from the start of the experiments), we measured TEWL through the back skin of conscious rats using a cyclone-type moisture-transpiration meter (Asahibiomed, Yokohama, Japan; AS-CT1). TEWL was measured 10 times in each animal, with average values being included in our data. During the measurement of TEWL, each rat was handled gently, with its eyes being covered by a hand wearing a glove. In this way, we could measure TEWL from quiet, conscious rats. After the TEWL measurements had been completed, rats were again anesthetized with pentobarbitone sodium (50 mg/kg, intraperitoneally) and a portion of the back skin from which TEWL measurements had been taken was removed for histological examination. In addition, both adrenal glands were removed for measurement of total adrenal weight. Afterward, animals were sacrificed by CO₂ stunning followed by decapitation.

Histological examination. The skin samples (see Experiment 1: effects of green odor on chronic restraint stress-induced changes in rats) were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.4) and then cut into small pieces and immersed in the same fixative for at least 2 h at 4 °C. The samples were postfixed in 2% osmium tetroxide for 2 h and stained with 1% uranyl acetate for 12 h at 4 °C. They were then dehydrated through a graded ethanol series and embedded in Epon. Vertical sections of the skin (0.5 μm thick) were stained with toluidin blue for light microscopy. Vertical thin sections of the skin (60 nm thick) were stained with uranyl acetate and lead citrate for examination under a Hitachi (Tokyo, Japan) H-7100 transmission electron microscope at 75 kV.

Experiment 2: DEX injection experiment

Each rat was given a single intraperitoneal injection of either DEX (1 mg/kg) (DEX group) or saline (saline group) at 0900 every day for 14 days. The procedures used for TEWL and total body weight measurement were essentially the same as those described for Experiment 1.

Statistical analysis

All results are expressed as mean ± standard error of the mean (SEM). Data for TEWL and adrenal and total body weights were analyzed for statistical significance by means of a one-way ANOVA, followed by Fisher's paired least significant difference test (post hoc test) (Macintosh, Cupertino, CA; Stat-View 4.0). Differences were considered significant at $P < 0.05$.

Results

Effects of green odor on chronic restraint stress-induced changes in rats

TEWL and adrenal and total body weights

As shown in Figure 2A, imposition of chronic stress (8 h per day for 14 days) resulted in a significant increase in TEWL

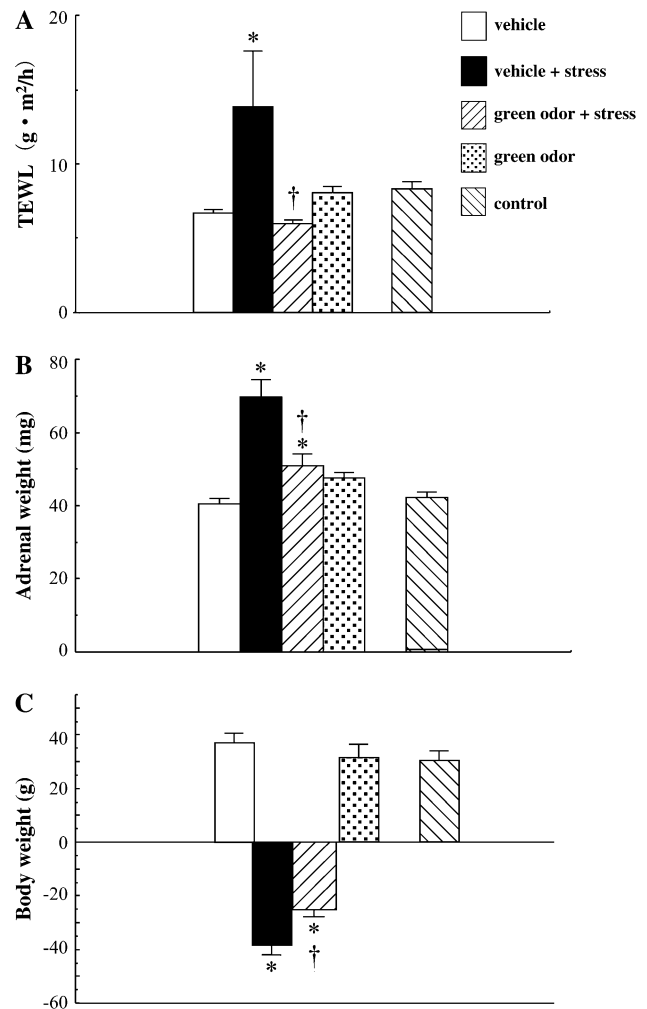


Figure 2 Effects of green odor on chronic restraint stress-induced changes in TEWL and adrenal and total body weights in rats. Mean values (\pm SEM) obtained for TEWL (A) and adrenal weight (B) and changes in total body weight during the experiment (C) in chronically stressed rats, in the absence (vehicle + stress group) or presence (green odor + stress group) of green odor inhalation. Effects of green odor (green odor group) and those of its vehicle (vehicle group) are also shown for nonstressed animals, as are the data for rats given no treatment at all (control group). * $P < 0.05$ versus vehicle, † $P < 0.05$ versus vehicle + stress.

(vehicle + stress group vs. vehicle group), an effect that was completely suppressed by green odor inhalation (green odor + stress group). As compared with the vehicle group, the vehicle + stress group showed a marked increase in adrenal weight (Figure 2B). This effect was significantly attenuated in the green odor + stress group. In the vehicle + stress group, body weight showed a marked decrease on the 14th day of the stress protocol while that of the vehicle group was increased (Figure 2C). The stress-induced body weight loss was significantly attenuated in the green odor + stress group.

Neither the vehicle (vehicle group) nor the green odor (green odor group) itself had any effect on TEWL (Figure 2A) or on adrenal gland weight (Figure 2B) or total body weight (Figure 2C) (vs. the control group).

Histological study

Figure 3 shows light-microscope observations of the skin in the 5 groups. There were no significant differences in light-microscope observations among the various groups, except that wrinkle formation was noticed in the vehicle + stress group.

Next, electron-microscope observations were carried out to explore any fine differences in the stratum corneum among the experimental groups. By comparison with the vehicle group, the vehicle + stress group showed an apparent decrease in intercorneocyte lipids (Figure 4). Also noticeable in Figure 4 are, in the vehicle + stress group, decreases in the thickness of the corneocytes in the lower stratum corneum and in the electron density of the corneocytes in the upper stratum. In contrast, in stressed rats allowed to inhale green odor for 2 h every day for 14 days, there appeared to be sufficient lipid between the corneocytes to provide a barrier function, and the cells had a normal thickness and a high electron density (as assessed on the 15th experimental day). There were no differences in electron-microscope observations among the vehicle, green odor, and control groups.

DEX injection experiment

Figure 5 shows that TEWL was significantly enhanced by daily intraperitoneal injections of DEX (vs. saline-injected rats). Moreover, such DEX treatment led to a significant decrease in body weight (measured on the 14th day).

Discussion

It is widely accepted that the skin, by acting as a barrier, plays important roles in the nonspecific defense mechanism and in the moisture-maintenance function (Elias and Choi

2005). Thus, a disruption of the skin barrier, if it occurs, can represent a serious problem. The present results show that although chronic stress exposure led to an increase in TEWL, this effect was completely inhibited by green odor inhalation. Thus, green odor has a potent ability to prevent chronic stress-induced disruption of the skin-barrier function. In addition, our histological studies revealed that whereas the green odor + stress group had sufficient intercorneocyte lipids to provide an effective barrier, these were apparently decreased in the vehicle + stress group. It seems likely that this decrease of lipids is responsible for the disruption of the skin-barrier function and that green odor is able to prevent this lipid decrease, leading to a prevention of such disruption. These results suggest that green odor inhalation may be therapeutically useful for the skin damage related to a disruption of skin-barrier function.

In this study, daily administration of DEX for 14 days resulted in an increase in TEWL. This finding is in good accordance with previous reports that glucocorticoid treatment had adverse effects on the skin barrier, as evidenced by a delay in barrier recovery after acute barrier-disruption had been induced by tape stripping (Denda, Tsuchiya, Elias, Feingold 2000; Kao et al. 2003). Because our chronically stressed rats also showed increases in TEWL, as mentioned above, an increased secretion of glucocorticoid during our “stress experiment” might possibly have been an important factor in the observed impairment of the skin barrier. Indeed, in our hands, chronic stress exposure led to a marked increase in adrenal weight, and this was attenuated by green odor inhalation. This effect of green odor inhalation (namely, partial inhibition of adrenal hypertrophy) indicates that the odor inhibited the chronic stimulation of the adrenal glands that would have been present in the stressed animals.

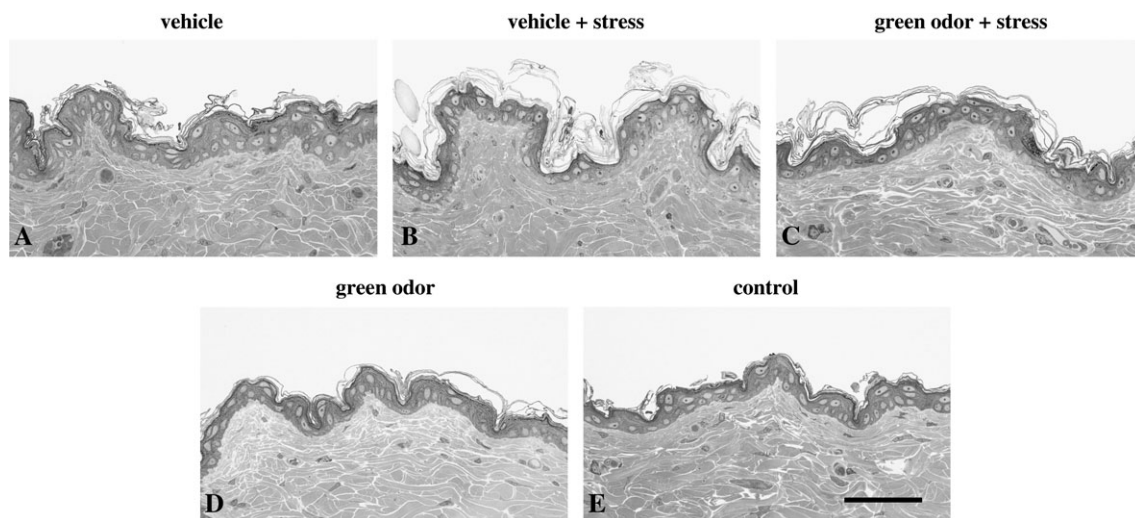


Figure 3 Effects of green odor on chronic restraint stress-induced changes in skin histology in rats (light-microscope examination). Light-microscope observations of the skin in chronically stressed rats in the absence (B; vehicle + stress group) or presence (C; green odor + stress group) of green odor. Effects of green odor (D; green odor group) and those of its vehicle (A; vehicle group) on skin histology are also shown for nonstressed animals, as is histology for rats given no treatment at all (E; control group). Scale bar = 50 μ m.

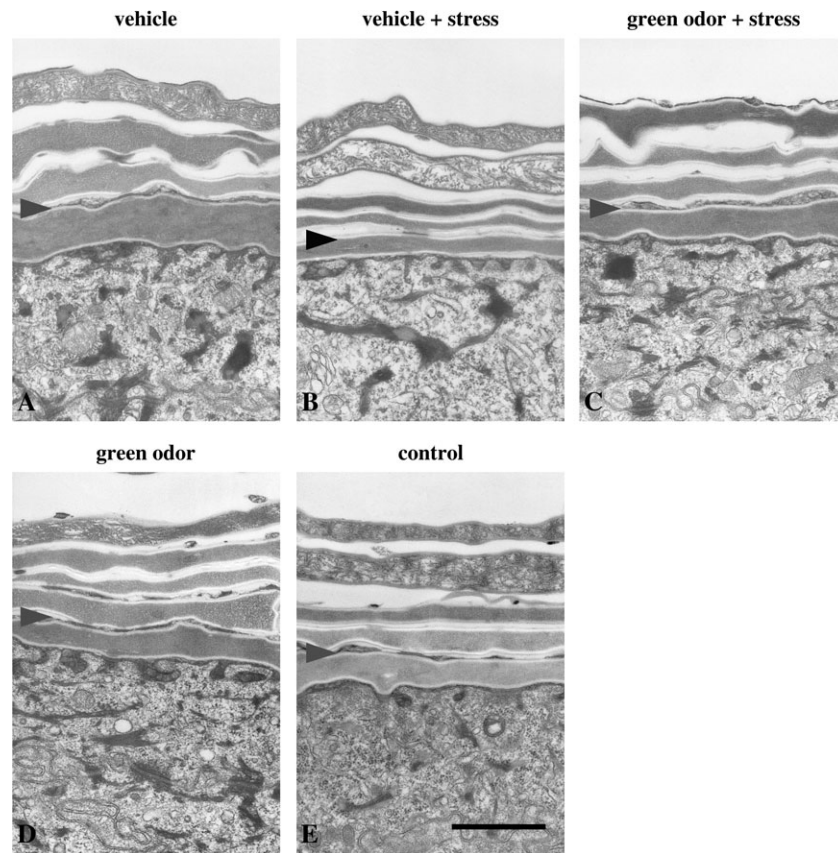


Figure 4 Effects of green odor on chronic restraint stress-induced changes in skin histology in rats (electron-microscope examination). Electron-microscope observations of the skin in chronically stressed rats in the absence (**B**; vehicle + stress group) or presence (**C**; green odor + stress group) of green odor. Effects of green odor (**D**; green odor group) and those of its vehicle (**A**; vehicle group) on skin histology are also shown for nonstressed animals, as is histology for rats given no treatment at all (**E**; control group). Gray arrowheads indicate sufficient intercorneocyte lipids and black one their apparent decrease. Scale bar = 1 μm .

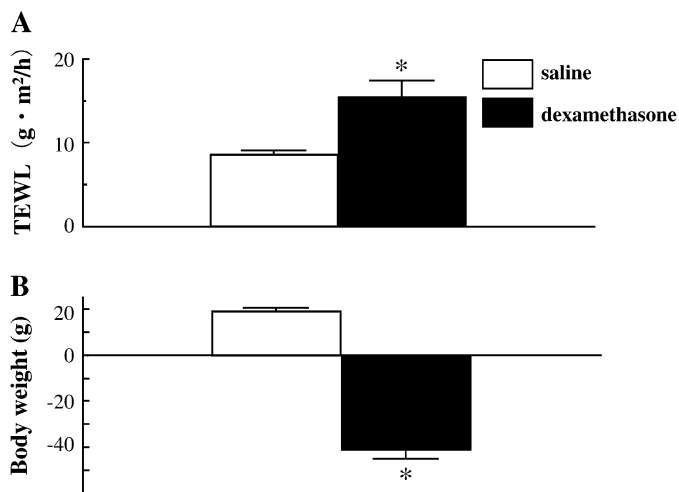


Figure 5 Effects of chronic DEX treatment on TEWL and body weight in rats. Mean values (\pm SEM) obtained for TEWL (**A**) and change in body weight (**B**) in rats given a daily intraperitoneal injection of DEX (1 mg/kg; DEX group) or saline (saline group) for 14 days. TEWL and changes in body weight during the experiment were measured on the 15th day and 14th day, respectively, in both saline- and DEX-injected rats. * $P < 0.05$ versus saline.

It is likely, therefore, that chronic stress-induced glucocorticoid secretion would also be found to be inhibited by such odor inhalation. Actually, Nakashima et al. (2004) found that an immobilization stress-induced increase in plasma ACTH was inhibited by the inhalation of green odor. Therefore, it is likely that this odor is somehow able to suppress the activity of the HPA axis. Taken together, the above results suggest that in our experiment, an increased secretion of glucocorticoid may have occurred in response to stress exposure and that this was attenuated by green odor inhalation, leading to at least a partial prevention of the skin-barrier disruption induced by such chronic stress. Such a relieving effect of green odor is supported by our finding that the chronic stress-induced body weight loss was attenuated by the same inhalation. Because DEX treatment, too, led to weight loss, this inhibition by green odor of stress-induced weight loss may likewise be attributable to an inhibitory effect of the odor on glucocorticoid secretion. However, we must keep in mind the possibility that the body weight loss caused by other factors (not typical stressors) might induce skin damage as well and that the odor might block the skin damage reaction by decreasing body weight loss. To examine this

possibility, the effects of body weight loss per se on skin function should be determined in the near future.

In line with a previous report showing wrinkle formation of the skin in stressed mice (Aioi et al. 2001), our light-microscope examination revealed wrinkle formation in the vehicle + stress group. This effect was not present in the green odor + stress group, which may be another example of a relieving effect of green odor inhalation. Moreover, our electron-microscope study revealed a decrease in the thickness of the corneocytes in the lower stratum corneum in the vehicle + stress group. This effect may have been responsible for the decrease in electron density (i.e., keratin content) observed in the corneocytes in the upper stratum in the same group of rats (if the decrease in thickness was due to a decrease in the total keratin content of the cells in the lower stratum prior to their movement to the upper stratum). In the present study, inhalation of green odor prevented both the decrease in corneocyte thickness and the decrease in their electron density that were observed in the vehicle + stress group. It would be interesting to clarify the mechanisms underlying these effects of green odor. Corneocytes contain hygroscopic and hydrosoluble substances called natural moisturizing factors (NMFs) (Marty 2002; Rawlings and Harding 2004). These are formed during epidermal differentiation and may represent up to 10% of the corneocyte mass. The principal ones are amino acids, carboxylic pyrrolidone acid, lactic acid, urea, glucose, and mineral ions. Because keratinization plays an important part in the formation of NMFs (Marty 2002), it is possible that a reduction occurs in the amount of NMFs present within corneocytes, as well as in their keratin content, when the animal undergoes chronic stress exposure, and that this contributes to barrier disruption. These possibilities will require testing in the near future.

In summary, the present results represent the first evidence that in rats, inhalation of green odor reduces or prevents the chronic stress-induced disruption of skin-barrier function, an effect probably due to a green odor-induced prevention of the decrease in intercorneocyte lipids. These findings are supported by the previous observations that in rats and mice, acute stress exposure delays recovery from the skin-barrier disruption artificially and acutely induced by tape stripping, and that this delay can be blocked by administration of sedative drugs or by inhalation of an odorant with a sedative action (Denda et al. 1998; Denda, Tsuchiya, Elias, Feingold 2000; Denda, Tsuchiya, Shoji, Tanida 2000). However, it seems likely that the precise mechanisms underlying the adverse effects of acute and chronic stress on the skin barrier are not the same. It has been reported that baseline TEWL did not change in rats that underwent 3 days of immobilization stress (6 h per day), in comparison with the untreated controls (Denda et al. 1998). Therefore, imposing stress on more than 3 days (e.g., the 14 days adopted in our study) may be necessary to evoke an increase in TEWL reflecting a disruption of the skin barrier. The present results show that green odor inhalation reduced such stress-induced

disruption of the skin barrier and also the changes in adrenal and total body weights. However, it remains unknown whether the odor suppresses other stress reactions such as increases in blood pressure and heart rate, a possibility that it would be interesting to test in the near future. Be that as it may, our data may provide a pointer toward a future treatment for skin diseases such as atopic dermatitis, in which the skin barrier is often disrupted in chronically stressed patients (Taieb 1999; Strid and Strobel 2005; Wright et al. 2005; Segre 2006). For that reason, the therapeutic significance of green odor inhalation should be verified in the not-too-distant future. However, we should bear in mind the possibility that any of a number of treatments causing relaxation (suppression of HPA-axis activation) may block the skin impairment induced by stress. Indeed, in our hands, DEX induced the same reactions as those induced by chronic stress, whereas green odor inhibited not only those stress reactions but also the stress-induced increase in adrenal weight. Moreover, the exact mechanisms underlying the effects exerted by green odor remain to be elucidated. Although we have suggested here that an inhibition of the HPA axis may be (possibly and at least in part) responsible for the observed effects, it remains unknown how this might be induced by odor stimulation (and indeed what happens within the brain in rats inhaling green odor). It is possible that a perfume component of green odor is absorbed via the membrane of the nose or lung and that this absorbed component triggers a certain reaction in the skin that serves to prevent barrier disruption. These issues should be tested in the near future.

Acknowledgements

We are grateful to Dr Robert J. Timms for a critical reading of the English manuscript. We would like to express our gratitude to Mss Kaori Hayano and Yuka Terasaki for their valuable contributions to this study.

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Accepted April 5, 2007