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Stress and Hypothermia in Mice in A Nose-Only Cigarette Smoke Exposure System

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In nose-only exposure systems, animals need to be restrained inside a tube, which leads to stress. Stress is known to cause hyperthermia in rodents. Chronically repeated episodes of hyperthermia could be detrimental to animal health and influence results of nose-only exposure studies. Therefore we investigated whether hyperthermia occurred in male C57BL/6J mice that were restrained for increasing lengths of time, using nosepieces held at room temperature, preheated at 37°C, or thermostat controlled at different temperatures, with and without exposure to different concentrations of cigarette smoke. Body temperature, body weight, plasma corticosterone levels, and adrenal weights were recorded. Restraint using nosepieces at room temperature caused a time-dependent decrease in body temperature, which could be reversed by preheating the nosepieces to 37°C. Cigarette smoke dose-dependently caused an additional decrease, which was counteracted by controlling nosepiece temperature at 38°C. During 3 mo of exposure using heated nosepieces, Δ body temperature remained constant. Body weight gain did not differ between smoke-exposed and room air-breathing animals exposed using either heated or room-temperature nosepieces, but both groups gained significantly less weight, while adrenal weights were significantly and similarly increased, when compared to unrestrained littermates. Plasma corticosterone levels did not differ between the three groups. In conclusion, during restraint in nose-only exposure tubes with room temperature metal nosepieces, mice suffer a pronounced hypothermia. Preventing this by heating the nosepieces does not reduce the stress experienced by the animals.

Nose-only exposure systems are widely used in inhalation studies on the effects of aerosols or gases on rodents. Cited ad-

vantages over whole-body exposure systems are more control over dosage, targeted delivery to the respiratory system only, and no soiling of fur (Cheng & Moss, 1989). However, this method of exposure also holds some intrinsic disadvantages. Animals are confined to a tube, sometimes for extended periods of time, and have to be restrained to ensure proper delivery of the agent of interest to the nasal region. This restraint inevitably causes stress, additional to that experienced because of exposure to a noxious substance, and could influence results. Also, confinement of the animal to an essentially closed container will have an effect on its ability to regulate body temperature (Pauluhn,

2003). We use a nose-only exposure system to deliver cigarette smoke to mice, to study the mechanisms of cigarette smoke-induced emphysema. Because restraint is a known and widely used stressor for mice (Johnson et al., 2000; Tuli et al., 1995; Marti et al., 1993; Chen & Herbert, 1995; Rybkin et al., 1997; Fernandez et al., 2000; Zelena et al., 2004), and different forms of stress, including restraint, are known to cause hyperthermia in these animals (Van der Heyden et al., 1997; Narciso et al., 2003), we wanted to investigate whether hyperthermia was also present in our experimental setup. For this, we measured body temperature before and after a gradually increasing period of restraint in the nose-only exposure tubes. Soon it became clear that the animals did not suffer from hyperthermia, but instead a quite severe hypothermia. Since this was at odds with textbook knowledge on the effects of stress on rodents, we tried to identify the cause of this phenomenon. The rather large, massive metal nosepieces of the nose-only exposure tubes that surrounded most of the mouse body during restraint were called into question. We therefore investigated the effect of regulating the temperature (heating) of the nosepieces on body temperature, and on weight gain, plasma corticosterone levels, and adrenal weight as systemic parameters of stress, in mice that were restrained only, or restrained and exposed to cigarette smoke.

MATERIALS AND METHODS

Animals

C57Bl/6J breeding pairs were obtained from Charles River (Maastricht, The Netherlands) and bred in the university breeding unit (Gemeenschappelijk Dieren Laboratorium, Universiteit Utrecht, Utrecht, The Netherlands). Male mice were transferred to the departmental animal unit at 7 wk of age and housed under a 12-h dark/12-h light cycle (lights on at 06.00), at 35–50% relative humidity and 20–22°C room temperature, in macrolon cages on wood-chip bedding, under filter tops, in groups of 6 animals. Experiments were started when animals were 11 wk old. Animals received water and feed (Research Diets Services, Wijk bij Duurstede, The Netherlands) ad libitum. All experiments were conducted in accordance with the Animal Care Committee of Utrecht University (DEC-DGK/FSB).

Restraint

Mice were restrained in nose-only exposure tubes (In-Tox Products, Inc., Albuquerque, NM). The exposure tubes consisted of a metal nosepiece connected to a translucent plastic tube by means of a bayonet closure, and a plastic cap with a restraint stopper to close the tube at the back (Figure 1). This stopper is used to move the mouse to the front side of the exposure tube, and secured by tightening a nut on the outside of the cap. The body of a restrained animal, especially at a young age, is almost entirely surrounded by the metal nosepiece. Nosepieces in this study were used at room temperature (approximately 22°C), preheated overnight in a 37°C stove, or connected to a thermostat-controlled heating device, as indicated.
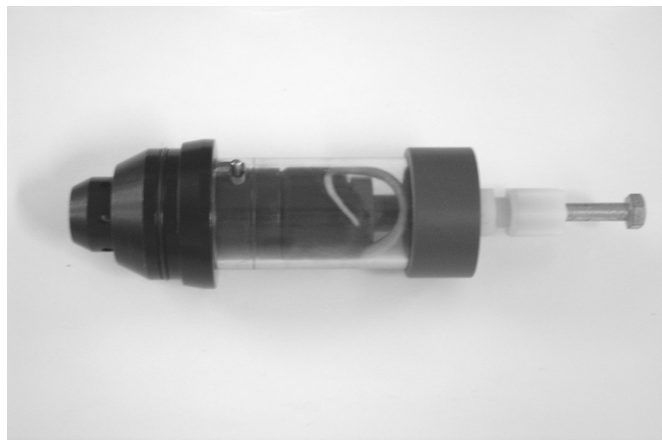

FIG. 1. Photograph depicting a mouse restrained inside a nose-only exposure tube.

Smoke Exposure

Cigarette smoke was generated by the burning of commercially available Lucky Strike cigarettes without filter (British-American Tobacco, Groningen, The Netherlands), using the TE-10z smoking machine (Teague Enterprises, Davis, CA), which is programmed to smoke cigarettes according to the Federal Trade Commission protocol (35-ml puff volume drawn for 2 s, once per minute). Mice were exposed nose-only to the diluted mainstream and sidestream smoke of a varying number of simultaneously burning cigarettes (1–3) during one run, using the In-Tox 24-port nose-only exposure chamber (In-Tox Products, Inc., Albuquerque, NM). In the long-term (3-mo) experiments, animals were exposed to two consecutive runs with three simultaneously burning cigarettes, every weekday. Previously, we determined a linear relationship between the number of cigarettes and the concentration of smoke in the exposure chamber, measured as total suspended particulate matter (data not shown). To maintain the nosepieces for the smoke-exposed mice at a stable temperature throughout the whole exposure period, the exposure chamber was fitted with a custom-built continuous thermostat-controlled heating mechanism. Nosepieces were left attached to the heated column to avoid delays in warming up. The temperature of the column, and thus the attached nosepieces, was adjusted to achieve the smallest possible changes in mouse body temperature during the exposure.

Study Outline

To study the effect of restraint on body temperature, mice were restrained for increasing periods of time (1–16 min) in exposure tubes with room temperature nosepieces, and with nosepieces preheated at 37°C. Next, the effect of exposure to the smoke of an increasing number of cigarettes (1–3) in tubes with thermostat-controlled nosepieces was determined. Finally, the effects of 3 mo of smoke exposure (31 min/day, 2 × 3 cigarettes/weekday) in exposure tubes with thermostat-controlled nosepieces on body temperature and parameters of

systemic stress were studied. Before starting the 3-mo experiment, mice were accustomed to the exposure tubes by gradually prolonging their stay over the course of 2 wk. Smoke exposure was started with 1 run of 1 cigarette, increasing the dosage to 2 runs of 3 cigarettes within 2 wk. Control mice were restrained in tubes with nosepieces pre-heated at 37°C. The tubes were placed on the bench next to the smoke-generating equipment, so the animals were exposed to the same level of noise as the smoke-exposed mice, and breathed room air. An additional control group consisted of mice that were left undisturbed in their cages, except for normal animal husbandry procedures. Body weight during the exposure period was monitored. Plasma corticosterone levels and adrenal weights were recorded at the end of the 3-mo exposure period. Finally, mortality rates were calculated and compared to data from previous experiments.

Temperature Measurement

Temperature measurements were always performed between 10.00 and 12.00, at the trough of the circadian rhythm, to minimize background variation in body temperature. Mouse body temperature was measured with an accuracy of 0.1°C using a digital thermometer (type 871A, Tegam, Geneva, OH; NiCr/NiAl thermocouple) before placement into (T1), and immediately after their removal from the exposure tubes (T2). The probe was lubricated with silicone oil and carefully inserted for a length of 2 cm into the rectum of the animal. After temperature readings were stable for 20 s, values were recorded and Δ body temperature (T2 − T1) was calculated. Room temperature in the laboratory was measured using the same thermometer, immediately prior to animal measurements.

Adrenal Function Measurements

Twenty-four hours after the last exposure, heparinized blood was obtained by heart puncture from animals anesthetized with 10% urethane (Sigma-Aldrich, Zwijndrecht, The Netherlands) in phosphate-buff (PBS) (0.4 ml ip) and centrifuged for 5 min at 14,000 rpm in a tabletop centrifuge to collect plasma. Mice were euthanized between 09.00 and 14.00, at the trough of the circadian rhythm, to minimize background variation in corticosterone levels. Furthermore, the time points at which euthanasia was performed were distributed evenly among the different treatment groups, and a correlation analysis between time of euthanasia and corticosterone level was performed. Corticosterone was measured in 10 μl plasma using the ImmuChem ^{125}I radioactive immunoassay kit (MP Biomedicals, Orangeburg, NY). Adrenals were removed from the same animals, dissected free from attached fat, dried overnight in a 37°C stove, and weighed on a microbalance.

Statistics

Values were compared between groups using one-way analysis of variance (ANOVA) followed by Bonferroni's correction for multiple comparisons and correlation analysis was performed using Pearson's method, in GraphPad Prism 4.03 for Windows. A confidence level of $p < .05$ was considered significant.

RESULTS

Effect of Restraint Duration and Nosepiece Temperature on Body Temperature

After a restraint period of 1 min inside exposure tubes with nosepieces at room temperature, the average body temperature of the mice had already decreased by 2.5°C (Figure 2). The drop in body temperature gradually increased with restraint time, to a maximum of 5.5°C at 12 min. Preheating the nosepieces at 37°C increasingly prevented the drop in body temperature to a great extent, leading to a small increase in body temperature after 16 min of restraint.

Effect of Smoke Exposure and Nosepiece Temperature on Body Temperature

The body temperature of mice restrained in exposure tubes with nosepieces thermostatically controlled at 36°C decreased after exposure to the smoke of 1 cigarette (Figure 3). This could be compensated for by increasing the nosepiece temperature to 37°C, but after exposure to the smoke of 2 cigarettes body temperature decreased again. Increasing the nosepiece temperature to 38°C showed that it more than compensated for the decline in temperature caused by 2 cigarettes and led to a minor decrease after exposure to the smoke of 3 cigarettes. For future studies, it was therefore decided to set the temperature of the nosepieces at 38°C for the smoke-exposed mice and at 37°C for the room-air-breathing controls.

Effect of Prolonged Exposure on Body Temperature

Weekly measurement of the body temperature of mice before and after restraint, during 3 mo of exposure in tubes with heated nosepieces (31 min/day, 2 × 3 cigarettes/weekday, after a 2-wk habituation period), showed that body temperature dropped on average 0.9°C in the smoke-exposed group and 1.5°C in the room air breathing controls (Figure 4). The observed changes in animal body temperature did not appear to be correlated to changes in room temperature (data not shown).

Effect of Prolonged Exposure and Nosepiece Temperature on Body Weight Gain

Body weight gain of mice, during 3 mo of exposure (from 3 to 6 mo of age, 31 min/day, 2 × 3 cigarettes/weekday, after a 2-wk habituation period) to smoke or room air in tubes with heated nosepieces, was similar (Figure 5A). Using nosepieces at room temperature instead of heated ones also did not affect body weight gain. However, the mice that were exposed to room air or cigarette smoke gained significantly less weight from weaning (3 wk of age) to the end of exposure (6 mo of age) than animals of similar age that had been left undisturbed in their cages (Figure 5B).
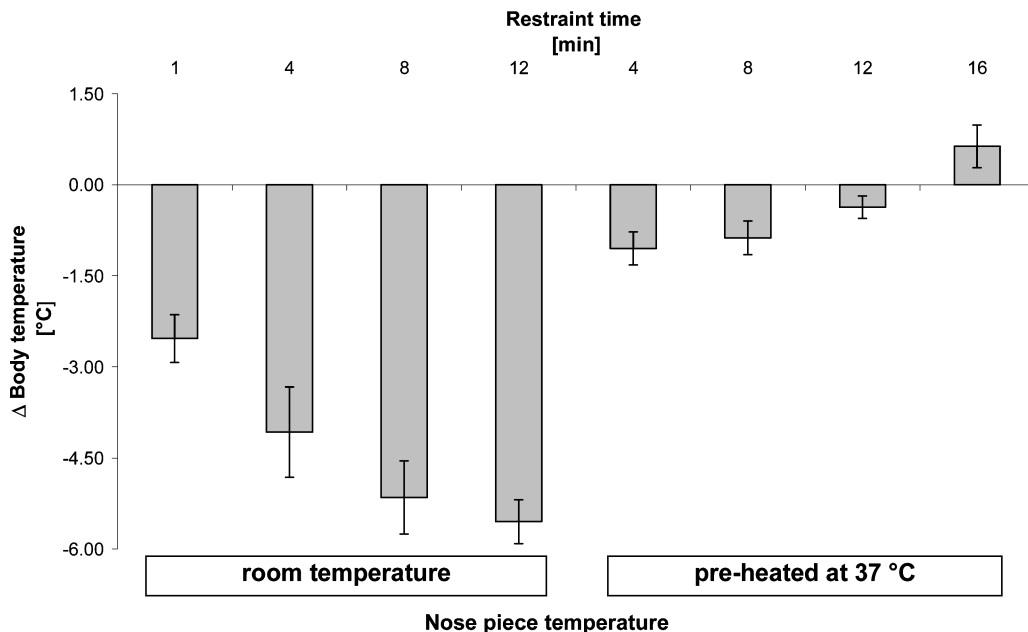
FIG. 2. Body temperature of mice as influenced by the time of restraining in exposure tubes with nosepieces at different temperatures. Male mice were restrained in exposure tubes with nosepieces at room temperature or preheated at 37°C. Body temperature was measured immediately before and after restraining to calculate Δ body temperature. Data are expressed as mean $\pm$ SEM of four animals.
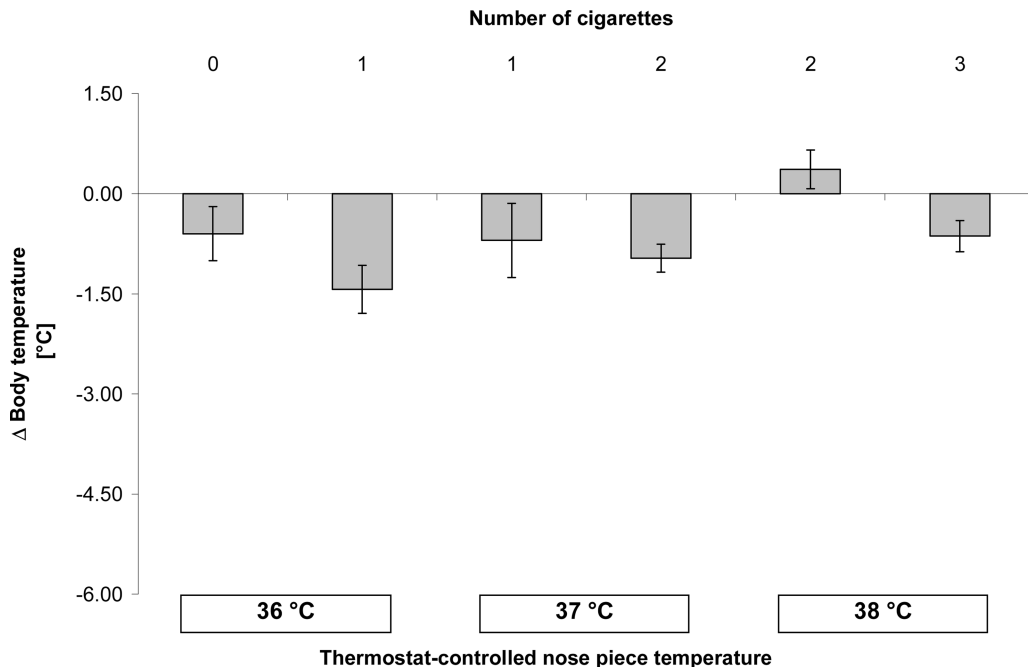

FIG. 3. Body temperature of mice exposed to the smoke of different numbers of cigarettes when restrained in exposure tubes with nosepieces at different temperatures. Male mice were restrained in exposure tubes with nosepieces at different thermostat-controlled temperatures and exposed to the smoke of a different number of cigarettes for 20 min. Body temperature was measured immediately before and after restraining, to calculate Δ body temperature. Data are expressed as mean $\pm$ SEM of four animals.
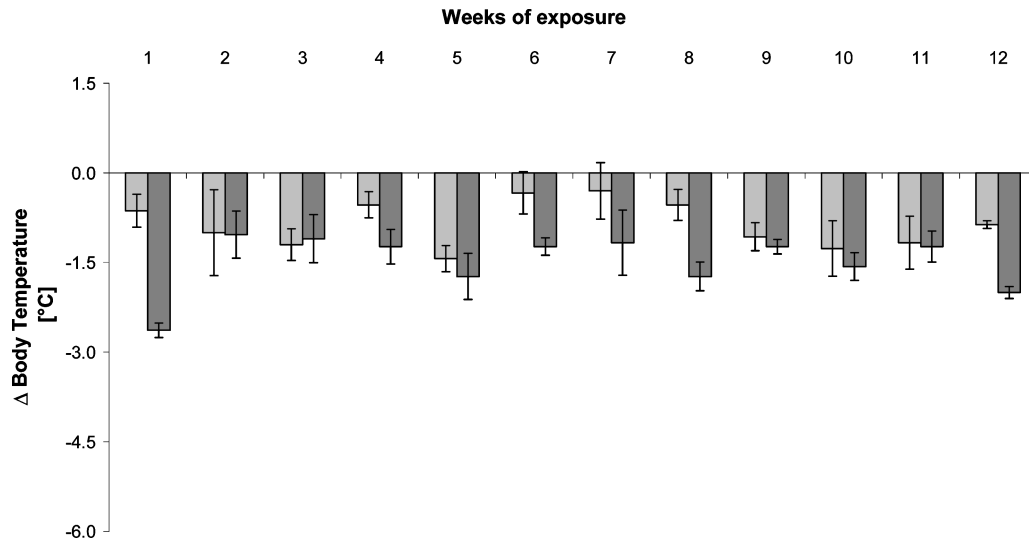
FIG. 4. Body temperature of mice exposed to cigarette smoke when restrained in exposure tubes with heated nosepieces. Male mice were restrained in exposure tubes with nosepieces preheated at 37°C and exposed to room air (light gray bars), or restrained in exposure tubes with nosepieces thermostatically controlled at 38°C and exposed to the smoke of 3 simultaneously burning cigarettes for 31 min (dark gray bars), every weekday for 3 mo. Body temperature was measured once weekly, immediately before and after restraining, to calculate Δ body temperature. Data are expressed as mean ± SEM of four animals.

Effect of Prolonged Exposure on Adrenal Parameters

There was no correlation between the time of day the animals were euthanized and plasma corticosterone levels (data not shown). After 3 mo of exposure to smoke or room air in tubes with heated nosepieces (31 min/day, 2 × 3 cigarettes/weekday, after a 2-wk habituation period), the mean corticosterone level in plasma of the smoke-exposed mice tended to be higher than that of the controls, but not significantly (Figure 6). There was no difference between nonhandled and room-air-breathing control groups. Likewise, the mean dry adrenal weight, corrected for body weights, of smokers and room-air-breathing controls did not differ (Figure 7). However, relative adrenal dry weights of the two latter groups were almost twice as high as that of animals that had been left undisturbed in their cages.

Mortality in Current and Previous Experiments

Two room-air-breathing control mice died during the 3-mo exposure (31 min/day, 2 × 3 cigarettes/weekday, after a 2-wk habituation period) experiment with heated nosepieces, 1 in wk 6 and 1 in wk 7. One smoke-exposed animal died in wk 9, so the mortality amounted to 17% in the control group and 8% in the smoke-exposed group (Table 1). Mortality in previous experiments varied widely, from 0 to 24%.

DISCUSSION

In this study we investigated whether hyperthermia develops in mice that are inside nose-only exposure tubes with metal nosepieces during cigarette smoke exposure experiments. The observation that restraining mice for increasing periods of time in exposure tubes with nose-pieces at room temperature led to a substantial and proportional decrease in body temperature, while preheating the nosepieces to 37°C could prevent this, showed that the decrease in mice body temperature was linked to the nosepiece temperature. This was unexpected, since various forms of stress, including restraint in nose-only exposure tubes,

TABLE 1
Mortality in current and previous restraint/smoke exposure experiments

Heated	Smoke	Months	Sex	Start n	End n	Mortality (%)
+	+	3	M	12	11	8
+	−	3	M	12	10	17
−	+	3	M	16	14	13
−	−	3	M	16	15	6
−	+	3	F	24	24	0
−	−	3	F	24	23	4
−	+	6	F	21	18	14
−	−	6	F	21	16	24

Note. Male (Sex: M) and female (Sex: F) mice were exposed to restraint, with (Smoke: +) and without (Smoke: −) exposure to cigarette smoke, for 3 or 6 mo, in exposure tubes fitted with room-temperature (Heated: −) or 37°C preheated/38°C thermostat-controlled (Heated: +) nosepieces. Animal deaths were scored throughout the experiment and percentage mortality was calculated by dividing the number of animals just prior to sacrifice, at the end of the experiment (End n), by the number of animals at the beginning (Start n) and multiplying by 100.
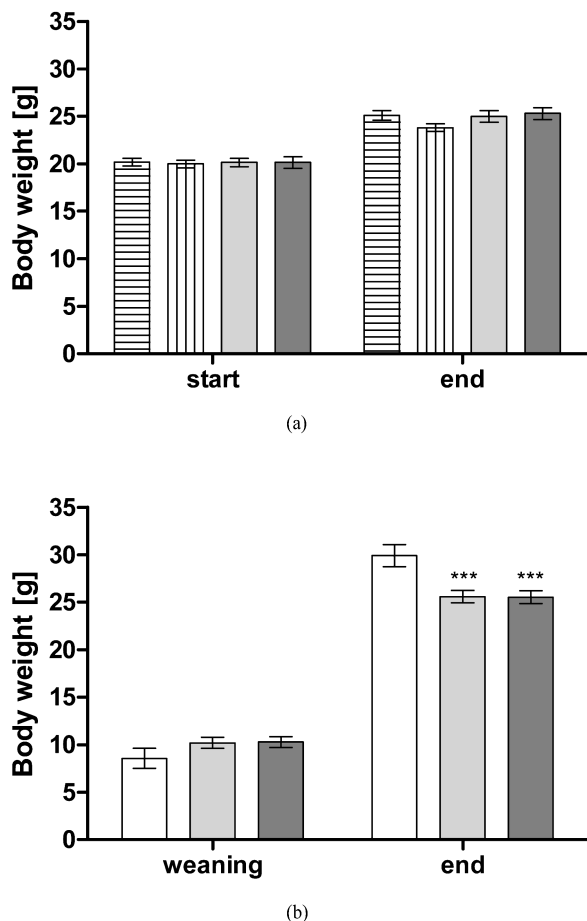
(a)

(b)

FIG. 5. Body weight gain of mice exposed to cigarette smoke when restrained in exposure tubes with nosepieces at different temperatures. (A) Three-month-old male mice were restrained in exposure tubes with nosepieces preheated at 37°C (light gray bar; $n = 11$) or at room temperature (horizontally hatched bar, $n = 15$) and exposed to room air for 31 min, every weekday for 3 mo, or restrained in exposure tubes with nosepieces thermostatically controlled at 38°C (dark gray bar; $n = 10$) or room temperature (vertically hatched bar; $n = 11$) and exposed to the smoke of 3 simultaneously burning cigarettes, for 31 min, every weekday for 3 mo. Body weight was record at the start (3 mo of age) and the end of the 3-mo exposure (6 mo of age). (B) Three-month-old male mice were restrained in exposure tubes with nosepieces preheated at 37°C and exposed to room air (light gray bar; $n = 11$), or restrained in exposure tubes with nosepieces thermostatically controlled at 38°C and exposed to the smoke of 3 simultaneously burning cigarettes (dark gray bar; $n = 10$), for 31 min, every weekday for 3 mo. Mice that were not handled were left undisturbed in their cages for 3 mo ($n = 6$). Body weight was recorded at weaning (3 wk of age) and at the end of the experiment (6 mo of age). Data are expressed as mean ± SEM. Triple asterisk indicates significance at $p < .001$ compared to not handled group.
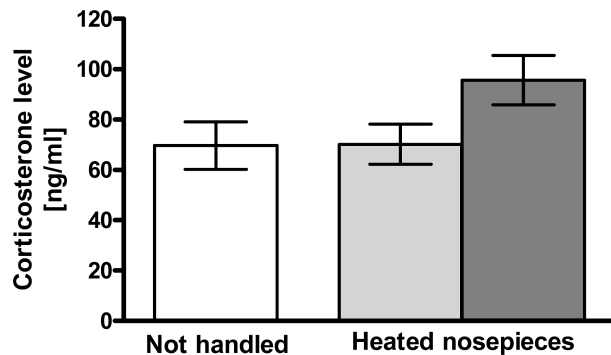

FIG. 6. Plasma corticosterone levels of mice exposed to cigarette smoke for 3 mo when restrained in exposure tubes with heated nosepieces. Three-month-old male mice were restrained in exposure tubes with nosepieces preheated at 37°C and exposed to room air (light gray bar; $n = 9$), or restrained in exposure tubes with nosepieces thermostatically controlled at 38°C and exposed to the smoke of 3 simultaneously burning cigarettes (dark gray bar; $n = 11$), for 31 min, every weekday for 3 mo. Mice that were not handled were left undisturbed in their cages for 3 mo (white bar; $n = 6$). Twenty-four hours after the last smoke exposure, blood was obtained to assess plasma corticosterone levels. Data are expressed as mean ± SEM.
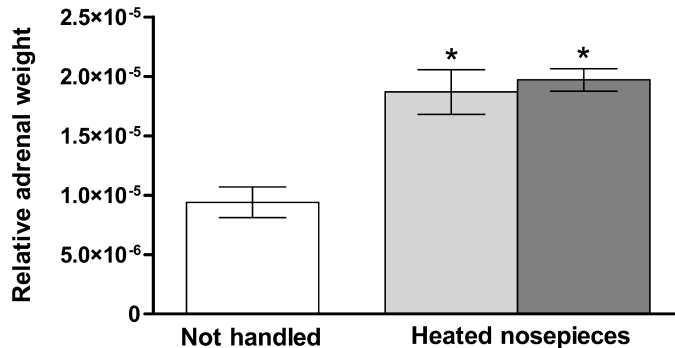

FIG. 7. Relative adrenal weight of mice exposed to cigarette smoke for 3 mo when restrained in exposure tubes with heated nosepieces, corrected for body weight. Three-month-old male mice were restrained in exposure tubes with nosepieces preheated at 37°C and exposed to room air (light gray bar; $n = 11$), or restrained in exposure tubes with nosepieces thermostatically controlled at 38°C and exposed to the smoke of 3 simultaneously burning cigarettes (dark gray bar; $n = 10$), for 31 min, every weekday for 3 mo. Mice that were not handled were left undisturbed in their cages for 3 mo (white bar; $n = 6$). Twenty-four hours after the last exposure, adrenals were removed, dissected free from attached fat, dried, and weighed. To correct for differences in body weight, values were divided by animal body weight. Data are expressed as mean ± SEM. Asterisk indicates significance to $p < .05$ compared to not handled group.

have been reported to induce hyperthermia in mice (Van der Heyden et al., 1997; Narciso et al., 2003). We think that the massive metal nosepiece, when at room temperature, functions as a very efficient heat sink because of the close contact of the mouse body with it, so rapidly draining heat from the mouse body. Wetting of the fur by trapped sweat facilitates this further. Previously, it was found in rats that restrained animals have both a greater effective body surface exposure and a greater rate of heat loss per unit of effective body surface exposure, compared to unrestrained animals (Bartlett & Quimby, 1958). Our observation that smoke exposure caused a dose-dependent further decrease in body temperature indicates that the inhalation of cigarette smoke decreases body temperature as such. This has previously been observed in rats (Altland et al., 1978) and is in accordance with observations on the hypothermic effects of inhalation of carbon monoxide and/or nicotine (Altland & Rattner, 1979; Lopez et al., 2003). In fact, inhalation of a wide range of xenobiotic substances such as ozone, acrylates, and formaldehyde (Watkinson et al., 2001; Jaeger & Gearhart, 1982; Silver et al., 1981) elicits a hypothermic response in rodents. The magnitude of this response is determined by the concentration of the xenobiotic, the mass of the animal, and environmental stress associated with, for example, restraint (Watkinson et al., 2003). Increasing the nosepiece temperature to just over animal body temperature could counteract the body-temperature-decreasing effect of the smoke of three cigarettes. Prolonged exposure (3 mo) to cigarette smoke or room air in tubes with heated nosepieces resulted in minor decreases in body temperature without a clear trend, indicating that there was no habituation or sensitization of animals to restraint or smoke regarding this parameter. The observations that nosepiece temperature did not affect body weight gain after 3 mo of restraint, irrespective of smoke exposure, and that the weight gain of mice exposed to smoke or room air was less than that of littermates that had been left undisturbed in their cages show that restraint, but not smoke exposure or hypothermia, reduced body weight gain. This suggests that repeated restraint of mice in our exposure tubes is a major cause of chronic stress. It agrees with data from literature, since it has previously been shown that restraint of mice represents psychological and physical stress, and that restraint-stressed mice lose weight (Hotchkiss et al., 2004), possibly due to a lowered activation threshold of the adrenal gland (Harris et al., 2004). The reduced weight gain would be the consequence of the persistent action of adrenal-derived corticosterone, which in the absence of insulin inhibits or even depletes fat storage (Dalllman et al., 2004). This is supported by our observation that restraint, but not smoking or hypothermia, increased relative adrenal weights. Our failure to observe an increase in plasma corticosterone levels in animals that had been restrained in heated tubes for 3 mo, even though a pronounced adrenal hypertrophy was present, could be due to the fact that the mice experienced a moderately severe novel stress just prior to euthanasia (i.e., the ip injection of an overdose of urethane). This has previously been shown to lead to a blunted corticosterone response in repeatedly restrained mice (Harris

et al., 2004). From this we conclude that decapitation would have been a better method to terminate the animals. Since 4 animals died prematurely during the 3-mo exposure experiment of the present study with heated nosepieces, we conclude that preventing the hypothermia does not prevent deaths from occurring. As compared to previous experiments using room-temperature nosepieces, mortality was not even appreciably reduced. Summarizing, we showed that mice experienced a substantial drop in body temperature and a decrease in weight gain when restrained and/or exposed to cigarette smoke in nose-only exposure tubes with metal nosepieces at room temperature. While heating the nosepieces greatly diminished the drop in body temperature, animals still gained significantly less weight and displayed a pronounced adrenal hypertrophy after 3 mo of restraint on weekdays. This shows they suffered chronic stress, which could be a confounding factor in any long-term nose-only exposure study using a similar experimental setup.

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