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Adrenal response of male rats exposed to prenatal stress and early postnatal stimulation

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Abstract

Stress in pregnant rats caused by chronic immobilization alters the pattern of secretion of corticosterone and modifies the hypothalamic-pituitary-adrenal axis (HPA) of the fetus. Early postnatal handling, however, may reverse the effects of increased secretion of corticosterone. We investigated the effects of prenatal stress and postnatal handling on the activity of the HPA axis of male offspring of stressed female rats. Male 90-day-old rats from four groups were investigated: prenatally stressed animals without postnatal handling, prenatally stressed animals with postnatal handling, unstressed control animals with postnatal handling, and unstressed control animals without postnatal handling. After sacrifice, the adrenal glands were weighed to determine the adrenal-somatic index. Apoptosis was evaluated by TUNEL assay and active caspase-3 expression. We found that the adrenal gland cortex:medulla ratio increased in animals with prenatal stress and that eventually the stress caused apoptosis. Handling newborns to simulate maternal activity ameliorated some of the negative effects of prenatal stress.

Key words: adrenal gland, apoptosis, handling, hypothalamic-pituitary-adrenal axis, immobilization, prenatal stress, rats

Stressful conditions during pregnancy can affect growth, behavior and physiological responses in the offspring including structural changes in the neuroendocrine system (Emack et al. 2008). Stress during pregnancy elevates basal corticosterone, decreases glucocorticoid receptor levels in the hippocampus and prefrontal cortex, and decreases tyrosine hydroxylase expression in noradrenergic neurons in the dorsal pons (Green et al. 2011).

García-Cáceres et al (2010) showed that prenatal stress reduces the body weight of rat pups and may cause growth retardation. Prenatal stress causes long-term effects on the endocrine system, but whether modifications to hypothalamic structures are involved remains largely unknown. Chronic prenatal stress causes hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis in adult male offspring under basal conditions, which is manifested by increased secretion of corticosterone by the adrenal gland (Mayer et al. 2011). Stress causes structural changes in adrenal gland proliferation and cell death; a balance between these two processes is required for gland integrity and function (Carsia et al. 1996). Apoptosis contributes to adrenocortical remodeling during embryonic and postnatal development (Yeastin et al. 1986, Ducsay et al. 1991).

Early postnatal stimulation by handling modifies the adverse effects that produce stress. Maternal behavior appears to contribute to changes in HPA axis activity of the pups; therefore the behavior of the mother can influence the response of HPA axis of offspring during adulthood (Liu et al. 1997). Handling decreases the stress-induced corticosterone secretion peak in the adult offspring (Maccari et al.

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1995). Increased frequency of arc-backed nursing, and licking and grooming affect the neurobiology and behavior of the offspring long term (Liu et al. 1997, Francis et al. 1999). Winkelmann-Duarte et al. (2011) demonstrated that neonates that were handled showed increased neuron and astroglia densities, and proliferation of hippocampal pyramidal cells compared to unhandled rats. Handling also protected against prenatal stress by decreasing the expression of glucocorticoid receptors in the prefrontal cortex (George et al. 2013).

As we demonstrated earlier, prenatal stress produced high levels of corticosteroid and caused hyperactivity of the HPA axis (Mayer et al. 2011). Early handling reduced the alteration of leukocyte distribution, mitogenic response of T lymphocytes (Liaudat et al. 2012) and testicular apoptosis evoked by prenatal stress (Chen et al. 2013). Therefore, we investigated the effects of handling newborns on the adrenal glands of prenatally stressed male rats.

Material and methods

Animals

Albino 200 − 250 g Wistar rats were obtained from the National University of Río Cuarto. We used five male and 10 female rats housed in plastic cages in groups of three under the following conditions: 12 h light/12 h dark, 22° C, constant humidity with water and food available *ad libitum*. Males and females (1:2) were mated overnight and the following morning a vaginal smear was obtained and examined microscopically. The first day of pregnancy was determined by the presence of sperm. The animals were maintained according to the Guide for Care and Use of Laboratory Animals and the experiments were approved by the local Institutional Animal Care Committee. All animals ultimately were sacrificed by decapitation.

Prenatal stress

Chronic stress by immobilization was applied to the experimental group of pregnant females. Experimental rats were immobilized during the last two weeks of pregnancy (Bertuzzi et al. 2003) on a 20 x 20 cm wood board by taping their limbs to metal mounts as described by Michajloskij et al. (1988). Each female was subjected to 30 min stress three times each week at different times in the mornings of different days (PS). Control female rats were left undisturbed in their home cages (PC). The male pups from these two groups were used for subsequent experimentation.

Postnatal handling

Some offspring of stressed mothers and controls mothers were handled daily from postnatal day 1 until postnatal day 3 as described by Meaney et al. (1987). Briefly, the pups were picked up and transferred from their home cage to another one containing paper toweling. Separate cages were used for each litter throughout in the cage for 1 min (9 − 11 AM every day) in advance of being returned to their home cage. The mother was taken out of the home cage before the pups, kept alone in another cage for 1 min, then returned to the home cage with the pups. This period of separation stimulates maternal handling, which refers to arc-backed nursing, licking and grooming that rat pups receive from their mothers when they were together again in the same cage. All handling sessions were conducted in the same room and were supervised by the same researcher.

Experimental design

The handled pups comprised two groups: prenatal stressed and handled (PSH), and prenatal stressed and not handled (PSNH). The unstressed pups comprised two groups: handled prenatal control and (PCH) and not handled prenatal control (PCNH). Within 24 h of birth, the litters were culled to eight per mother to minimize the influence of number of offspring on the parameters measured. All groups were housed in the same animal room and all pups were kept with their mothers. Pups were weaned 21 days after birth and housed in groups of four males by litter and left undisturbed until testing. Two male siblings per litter from each group were tested when 90 days old (adults).

Blood samples

All determinations were performed in males at 90 days of age. Control, stressed and handled adult animals were sacrificed by decapitation. Blood samples were collected in heparinized tubes between 9 and 12 AM directly from the neck after decapitation. The blood was immediately centrifuged at 1000 × g for 15 min. Plasma was stored at −20° C and corticosteroid levels were measured in plasma samples.

Corticosterone assay

Plasma corticosterone levels were measured by radioimmunoassay (Krey et al. 1975) using a sheep antibody with high specificity (TECNOLAB). Corticosteriod standards were supplied by Sigma

Chemical Co. and the intra- and interassay coefficients of variation were 8.8 and 9%, respectively.

Adrenal-somatic index

Adrenal glands were excised from all pups and weighed to determine the adrenal-somatic index: adrenal-somatic index = (adrenal gland weight/ body weight) x 100.

Histology

The adrenal glands were fixed in buffered 10% formol for 8 – 10 h, then processed to paraffin using standard techniques. Sections 4 μm thick were cut, stained with hematoxylin and eosin (H & E) (Fischer 2008) and observed under a light microscope (Zeiss with VIDAS-KONTRON software, Augsburg, Germany). The cortex:medulla ratio was measured using the H & E stained sections.

TUNEL

Adrenal gland sections from all animal groups were processed using Apoptag Plus in Situ Apoptosis Peroxidase kit (Oncor, Gaithersburg, MD) according to the manufacturer's protocol. The sections were incubated with K-proteinase for 8 – 10 min in a humidified chamber. Endogenous peroxide was blocked using 3% hydrogen peroxide. The negative control lacked the terminal transferase desoxinucleotidyl transferase enzyme (TdT). Post-lactation mammary gland was used as the positive control. Normal nuclei were identified using 1% methyl green counterstain. Briefly, sections were stained in methyl green solution for 5 min at room temperature, then rinsed in distilled water. The samples were dehydrated quickly through 70, 90, 96 and 100% alcohols and cleared in xylol for 10 min.

Active caspase-3

Active caspase-3 (Chemicon International, Temecula, CA), was used to confirm apoptosis in the adrenal glands. The technique of Bullock and Petrusz (1983) was used to identify the cells that synthesized active caspase-3. The adrenal glands were fixed in 4% formaldehyde in phosphate buffer (pH 7.6) for 10 h. The glands were rinsed in PBS, dehydrated through an ascending series of alcohols, cleared in xylene and embedded in paraffin. Sections were deparaffinized as was described for H and E staining and treated with H_20_2 to block endogenous peroxidase. The sections for all experimental groups were treated with 2% horse serum in PBS and incubated overnight at 4° C with polyclonal rabbit anti-active caspase 3 antibodies. After washing, sections were exposed to the second biotinilate antibody (anti-rabbit developed in goat) (Vector Laboratories, Burlingame, CA) for 1 h at room temperature, rinsed again in PBS and stained using the avidin-biotin-immunoperoxidase kit (ABC) (Vectastain; Vector Laboratories). Finally, the sections were incubated with 3, 3'-diaminobenzidine in the presence of H_20_2 to reveal peroxidase expression. The reaction was stopped in distilled water.

Stereological image analysis

Quantification of apoptotic and normal nuclei was performed using the System KS-300 v. 3.0 (Kontron/ Zeiss, Augsburg, Germany) and the program Image J (National Health Institute) was used for the analysis. Seven to ten images of adrenal fascicular zone were digitized using a Zeiss Axioscope microscope with a 3.2 Mpx Sony digital camera from each histological section for a total of 750 images.

Statistical analysis

Data were analyzed Statistical Software (Tulsa, OK). Differences between prenatally stressed offspring that were handled and not handled were analyzed using two-way 2 x 2 ANOVA: the factors were prenatal stress and postnatal handling. Post-hoc comparisons were made using Duncan's test. These values are expressed as means (± SEM); values for $p \leq 0.05$ were considered statistically significant.

Results

We demonstrated earlier that plasma corticosteroid levels in prenatally stressed animals (PSNH and PSH) were higher than in control (PCNH and PCH) groups (Chen et al. 2013). Prenatal stress did not modify the adrenal-somatic index compared to controls ($F_{1, 23} = 0.1570$, $p = 0.69$). Similarly, postnatal manipulation had no significant effect on the adrenal-somatic index ($F_{1, 23} = 0.2569$, $p = 0.81$) (Fig. 1). $F_{1,23}$ refers to an F-distribution with 1 degree of freedom (2 treatments - 1) and 23 degrees of freedom [27 (total number of samples) – 4 (quantity of groups evaluated).

Prenatal stress caused a significantly increased cortex:medulla ratio in PSNH animals ($F_{1, 33} = 17.67$, $p = 0.0059$) compared to PCNH animals (Fig. 2); the ratio was unaltered by postnatal stimulation ($F_{1, 33} = 23.27$, $p = 0.19$).

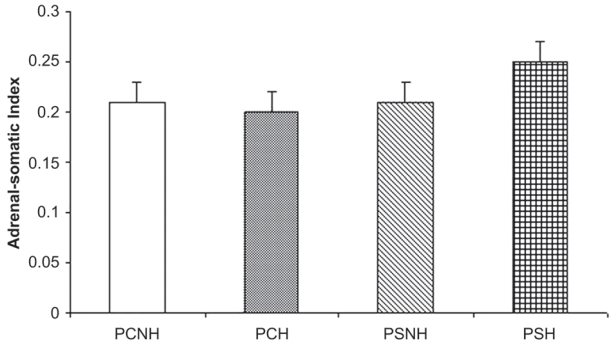

Fig 1. Effect of prenatal stress and postnatal handling on adult offspring adrenal-somatic index. Prenatally stressed rats without manipulation PSNH (n = 5), prenatally stressed offspring with manipulation PSH (n = 10), control adult male offspring PCNH (n = 5), control adult male offspring with manipulation PCH (n = 7). Bars represent mean ± SEM.

Figure 3A shows a section of adrenal cortex of a PSNH animal stained by the TUNEL technique; arrows show numerous apoptotic cells. The apoptotic index (Fig. 3B) of the fascicular layer showed significant effects of pre- and postnatal treatment ($F_{1, 38} = 4.57$; $p = 0.0079$). The apoptotic index of the adrenal gland of the PSNH group was significantly higher than the control (PCNH) group. PSH pups, however, showed no significant differences from the PCNH and PCH groups (Fig. 3).

We investigated also activated caspase-3 expression in the adrenal reticular zone to validate our TUNEL observations. Active caspase-3 activity was located in cells that were positive for TUNEL staining in PSNH animals (Fig. 4A, arrows). Handling of

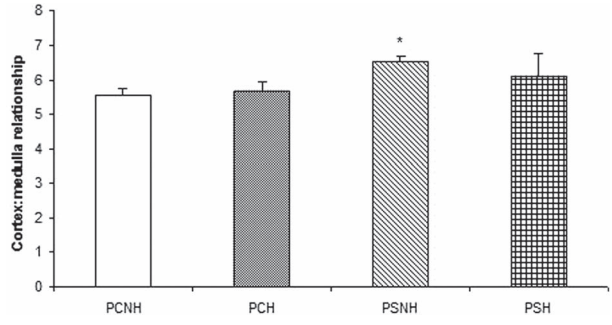

Fig 2. Effect of prenatal stress and postnatal handling in adult offspring adrenal gland cortex:medulla ratio. Prenatally stressed rats without manipulation PSNH (n = 10), prenatally stressed offspring with manipulation PSH (n = 9), control adult male offspring PCNH (n = 9), control adult male offspring with manipulation PCH (n = 9). Bars represent mean ± SEM. Difference between PSNH and PCNH, *$p < 0.05$.

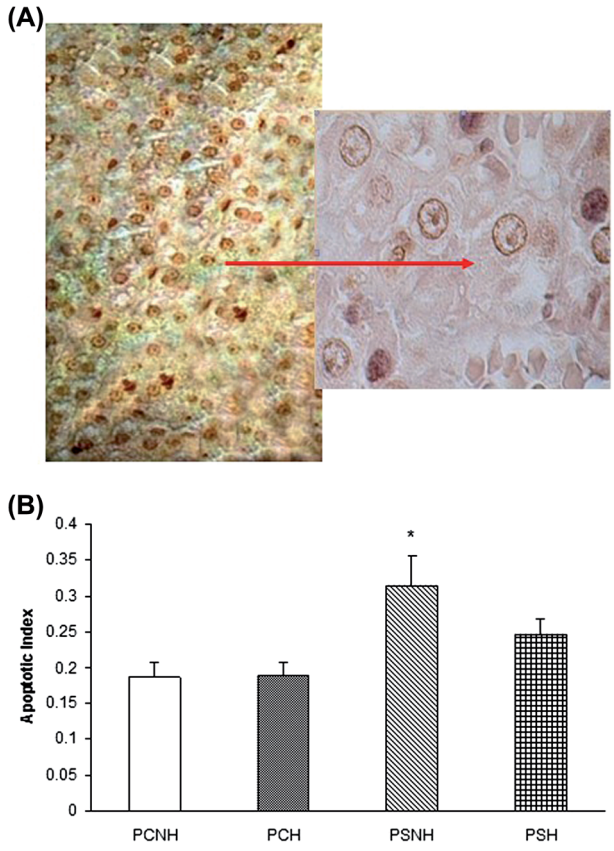

Fig 3. Histological sections of PSNH adrenal cortex zone. (A) Adrenal fascicular zone was stained by using the methyl green and TUNEL techniques. ×40. Arrows indicate TUNEL-positive nuclei. ×100. (B) Apoptotic index of adrenal gland fascicular layer of prenatal stressed pups without manipulation (PSNH; n = 8) and with postnatal manipulation (PSH; n = 13), control pups without manipulation (PCNH; n = 9) and with postnatal manipulation (PCH; n = 12).

prenatally stress newborns decreased the number of reticular cells that expressed caspase-3 (Fig. 4B). Handling did not alter caspase-3 expression in the PCH group compared to the PCNH group.

Discussion

Our findings suggest that the HPA axis is hyperactive in prenatally stressed rats, because the plasma corticosteroid levels were higher in these animals than in control group animals and are consistent with earlier reports (Darnaudéry et al. 2006, Szymańska et al. 2009). Consequently, high corticosteroid levels alter homeostasis during adult life (Van den Hove et al. 2014). We found no significant differences in the size of the adrenal glands, based on the adrenal-somatic index, among the different experimental groups; however, prenatal stress

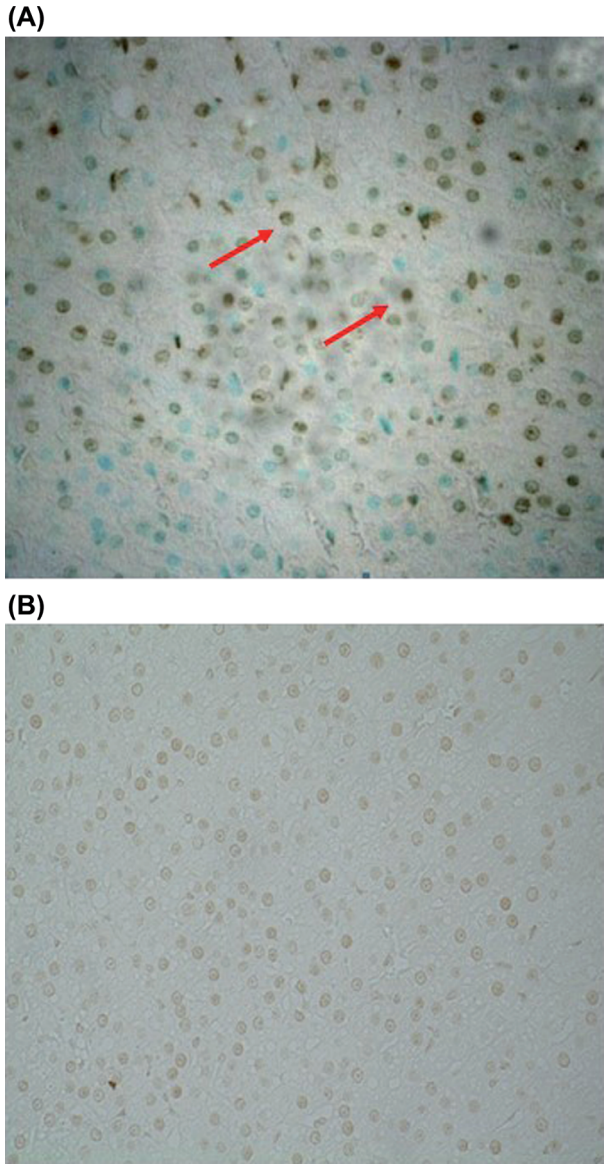

Fig 4. Histological sections of PSNH (A) and PSH (B) adrenal fascicular zones. Activated caspase-3 counterstained with methyl green. ×400.

increased the cortex:medulla ratio of adrenal gland. Both the high plasma corticosteroid levels and the cortex:medulla ratio increased in PSNH animals, which suggests that the hyperactivity of the HPA axis was produced by prenatal stress.

HPA hyperactivity is produced, in part, by maternal glucocorticoids that cross the placenta and alter the development of various fetal organs including the adrenal glands (Klemcke et al. 1995, Mayer et al. 2011, Chen et al. 2013). Excess glucocorticoids during pregnancy alter neural morphogenesis and disrupt the fetal circadian clock (De Kloet et al. 1988, Zarrow et al. 1970). Prenatal stress decreased the number of mineralocorticoid receptors in the hippocampus of the offspring (Tamura et al. 2011, Maccari et al. 2003), which are the main targets of negative feedback control of adrenal glucocorticoid secretion under normal conditions (De Kloet et al. 1987).

In an earlier study, we observed only a downward trend in the plasma corticosteroid levels when prenatally stressed pups were handled (Liaudat et al. 2012). In addition, the apoptotic index of the adrenal fascicular zone was lower in prenatally stressed animals after handling. It is possible that rapid release of ACTH stimulates synthesis of glucocorticoids in the fascicular area of the adrenal cortex (Carsia et al. 1998).

Handling of pups (PCH) alone did not cause changes in the apoptotic index in adrenal glands; however, the increased apoptosis observed in the PSNH group were at control levels after the animals were handled.

The cortex:medulla ratio, apoptotic index and caspase 3 expression returned to control levels when the prenatally stress animals were handled. These effects could be due to modification of some aspects of the mother's behavior including care of pups (Levin and Stern 1975, Maccari et al. 1995, Fenoglio et al. 2005), duration of offspring licking (Barbazanges et al. 1996) and frequency of arc-backed nursing and grooming. Maternal manipulations produce long-term effects on neural circuits, behavior and response to infection (Liu et al. 1997, Francis et al. 1999, Staci et al. 2007). Also, infants who received greater amounts of arc-backed nursing, licking and grooming showed increased hippocampal synaptogenesis, which improved learning, memory and reduced anxiety in adulthood (Liu et al. 2000, Fish et al. 2004).

Our findings indicate that the decreased levels of apoptosis in the fascicular zone cannot be attributed to corticosteroid, which remained at high levels in PSH animals. Consequently, the apoptosis in adrenal gland cells may be regulated by the catecholaminergic system (Pallarés and Antonelli 2015, Masserano et al. 2000).

Prenatal stress modifies the HPA axis in offspring and produces alterations in adrenal gland physiology. Early postnatal handling could be an effective treatment for attenuating or reversing these negative effects in adult life.

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