The relation between pregnancy and stress in rats: considering corticosterone level, hippocampal caspase-3 and MAPK activation

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Objectives: There are some evidences indicating that stress can affect hippocampal survival and function. During pregnancy mother is exposed to more stress and anxiety; also adrenal gland response to ACTH and glucocorticoid secretion is increased. Hence this study was done to assess the effect of restraint stress on corticosterone level, hippocampal caspase-3 and MAPK activation during pregnancy.

Study design: The restraint stress was applied in day 14 or days 14–20 (single and repeated stress) of rats’ pregnancy. The hippocampi were isolated after last stress episode and western blot analysis was done to assess caspase-3 and MAPK activation. Data were analyzed by one-way ANOVA followed by Student–Newman–Keuls for multiple comparison.

Results: Our study showed that single and repeated stress both increase corticosterone level compared to non-stressed pregnant rats, but do not induce hippocampal apoptosis. Single stress increases transient JNK activation but not P38 and ERK. Repeated stress activated none of the MAPKs.

Conclusion: It seems that pregnancy protects mother’s hippocampus against stress-induced damages.

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1. Introduction

Stress is a scientific term describing any significant distressing situation which demands physiologic and/or behavioral readjustment or adaptation. Exposure of an animal to a stressful situation is perceived through brain and thereby leads to the activation of two systems: the sympato-adrenomedullary system and hypothalamic–pituitary–adrenal (HPA) system. Activation of the former results in enhanced circulating adrenaline level. While the HPA axis, the levels of peptides such as corticotrophin releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) and glucocorticoids rise. Catecholamine actions are accomplished within seconds, while glucocorticoids slowly affect organ functions (usually taking >30–60 min) and give rise to long-lasting effects [1].

During stress response not only peripheral organ function are changed but also the brain. The hippocampus is a medial temporal structure crucial for declarative memory in humans [2] and spatial memory in rodents. Stress induces significant dendritic atrophy, neuronal loss [3,4], neurogenesis suppression [5] and memory impairment [6].

The term mitogen-activated protein kinases (MAPKs) are known as a family of signal transduction mediators which regulate a different array of cellular functions such as cell growth, differentiation, survival, apoptosis and cytokine production. In mammalian cells there are three well-characterized MAPK subfamilies, the extracellular signal-regulated kinases (ERKs), the P38 MAPK and the C-JUN NH2-terminal protein kinase (JNK) [7].

Studies on MAPK function in neurons have greatly accelerated recently as MAPKs are extensively distributed throughout the nervous system. There are some evidences indicating that multiple stressors can affect hippocampal MAPK activity [8–11]. Also in primary hippocampal cell culture, glucocorticoids can affect P38 and JNK activation [12].

During pregnancy the sensitivity to environmental stimuli is elevated as the adrenal gland responsiveness to ACTH and glucocorticoid secretion has been increased [13]. There are also some reports indicating that stress and anxiety rates are high during pregnancy (even comparing to post-partum period) [14]. During pregnancy the mother undergoes myriad changes in her physiology, including physical and neuroendocrine changes. HPA axis activity is altered and basal corticosterone level up regulated [15]. Estrogen and progesterone levels rise [16] and the cardiovascular system is remodeled [17]. There is much to suggest that many of these changes should protect mother’s brain from damages. For instance estrogen is known to have protective effects on the brain [18]. In spite of these,
there is no evidence assessing how pregnancy-period stress affects mother's hippocampus as the majority of pregnancy-period stress studies have been focused on fetus/offspring hippocampi than the mother's. Hence the aim of this study was to assess the effect of single and repeated restraint stress on circulating corticosterone level, hippocampal caspase-3 activation (as an apoptosis indicator) and MAPK activation in pregnant rats.

2. Materials and methods

2.1. Animals

Pregnant female albino Wistar rats (3–4 months of age) were obtained from the animal house of Neuroscience Research Center, Shahid Beheshti University (MC). The animals were maintained in a temperature and humidity-controlled room under a 12–12 h light/dark cycle with light off at 7 pm. Food and water provided ad libitum. All experiments were according to the NIH guide for the care and use of laboratory animals.

The animals were randomly assigned into 6 groups (7 in each): two control groups (day 14 and day 20 of pregnancy), single 1 h stress, single 3 h stress, repeated 1 h stress, and repeated 3 h stress.

2.2. Materials

Antibodies for western blotting (beta-actin, phosphorylated ERK, phosphorylated P38, phosphorylated JNK and secondary antibodies) were purchased from Cell Signaling Technology (Ozyme, France); sodium orthovanadate, aprotinin, and phenylmethylsulfonyl fluoride (PMSF) were from Sigma–Aldrich (Saint Quentin Fallavier). Other reagents were obtained from usual commercial sources.

2.3. Restraint stress

To induce stress, animals were immobilized in a Plexiglass rat restrainer for 1 or 3 h/day in day 14 or days 14–20 of pregnancy (single and repeated stress respectively) started at 11:00 [19].

2.4. Corticosterone assay

At the complement of restraint stress period (single and repeated), the animals were anesthetized through CO2 inhalation, decapitated and blood samples were collected into tubes containing 5% EDTA and centrifuged at 2500 rpm for 10 min at 4 °C. Plasma was removed and kept at −70 °C to quantify corticosterone levels using a commercial rat corticosterone radioimmunoassay kit (DRG, Germany). Intra-assay and inter-assay coefficient of variations for corticosterone measurements were less than 10%.

2.5. Western blot analysis

Western blot analysis was performed as mentioned before [20,21]. Briefly the hippocampi were homogenized on ice in cold lysis buffer containing 50 mM Tris/HCl (pH 7.5), 2 mM phenylmethylsulfonyl fluoride (PMSF), 100 μM sodium orthovanadate, 10 μg/mL aprotinin and 10 μg/mL leupeptin. The lysates were centrifuged at 13,000 μg for 15 min at 4 °C to remove debris. Samples with equal amounts of protein were then separated by 12% polyacrylamide gel electrophoresis, and transferred to PVDF membranes. After blocking in 2% ECL advanced blocking reagent kit, the membranes were probed with primary antibodies (caspase-3, phospho-JNK, phospho-ERK, phospho-P38 and β-actin) overnight in 4 °C. After washing, the membranes were incubated for 2 h at room temperature with horseradish peroxidase-conjugated anti-rabbit antibody. All antibodies were diluted by 2% blocking reagent in TBST. The immune complexes were visualized by ECL advanced chemiluminescence method. The relative expression of protein bands was quantified using NIH Image J 1.3.1-03 software (National Institutes of Health, Bethesda, MD).

2.6. Data analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls for multiple comparison. All results have been shown as means ± SEM. In all statistical comparisons, p < 0.05 was considered as significant difference.

3. Results

3.1. Plasma corticosterone levels following single and repeated restraint stress

Single stress (both 1 and 3 h) elevated plasma corticosterone levels (p < 0.01 and p < 0.05, Fig. 1A). Repeated restraint stress during days 14–20 of pregnancy increased corticosterone levels both after 1 h and after 3 h stress periods (p < 0.001, Fig. 1B).

![Fig. 1. The effect of restraint stress on plasma corticosterone levels. (A) Plasma corticosterone levels after 1 and 3 h single stress episode. (B) Plasma corticosterone levels after 1 and 3 h repeated stress episode. Data are represented as mean ± SEM. ***p < 0.001, **p < 0.01 and *p < 0.05 represent the difference between control and stress groups.](image-url)
3.2. The effect of single and repeated restraint stress on hippocampal caspase-3 activation

Western blot analysis showing the effects of single or repeated stress exposure on activated caspase-3 protein in the hippocampi is depicted in Fig. 2. Antibody against activated (cleaved) caspase-3, as an indicator of apoptosis, detected two bands at 19 and 17 kDa. This study showed neither single nor repeated stress exposure induced caspase-3 activation.

3.3. The effect of single and repeated restraint stress on hippocampal JNK activation

Western blot analysis on phosphorylated (activated) JNK was conducted on the hippocampi of stressed rats. Significant increase in phospho-JNK appeared after 1 h stress exposure (p < 0.01, Fig. 3A), while after 3 h it decreased to control level. Repeated stress exposure during days 14–20 did not affect JNK activation (Fig. 3B).

3.4. The effect of single and repeated restraint stress on hippocampal ERK 1/2 activation

Western blot analysis on phosphorylated (activated) ERK 1/2 was conducted on the hippocampi of stressed rats (Fig. 4). Single restraint stress exposure (in 1 or 3 h episode) did not affect ERK 1/2 activation. Also repeated stress exposure (both in 1 and in 3 h episodes) did not affect ERK 1/2 activation.

3.5. The effect of single and repeated restraint stress on hippocampal P38 activation

Fig. 5 shows the result of western blot analysis on hippocampal phosphorylated (activated) P38 of stressed rats. Single restraint stress exposure (in 1 or 3 h episode) did not affect P38 activation. Similarly repeated stress exposure (both in 1 and in 3 h episodes) did not induce P38 activation.

4. Comment

This study assessed the corticosterone level, hippocampal caspase-3 activation and MAPK phosphorylation in response to restraint stress during pregnancy. Single and repeated restraint stress (in 1 and 3 h episodes) both elevated circulating corticosterone levels. Single restraint stress exposure activated JNK while after 3 h stress exposure, its level decreased to control level. Repeated restraint stress exposure (in 1 and 3 h episodes) did not
pregnancy of swimming and stressors are considered as control group as pregnancy itself makes considerable changes in mother's physiology. Additionally pregnancy makes a system with more stable hormonal levels as in non-pregnant rats gonadal hormones fluctuate across the estrous cycle. Even during one phase such as proestrous, estrogen levels are high in the morning but decrease to low levels in the afternoon.

The hippocampus plays an important role in hippocampal-dependent learning and memory and is markedly susceptible to stress [28]. It is demonstrated that stress decreases dentate gyrus (DG) neurogenesis and reduces hippocampal volume [29]. There are some reports indicating that stress can induce apoptotic cell death in the hippocampus [30,31]. Jalalvand et al. showed that 7 day restraint stress in Wistar rats causes hippocampal apoptosis [19]. Our study, however, showed that this model of stress applied for 7 days does not induce caspase-3 activation and therefore apoptosis, although corticosterone level was significantly higher than in non-stressed dams. The steroid glucocorticoid hormones (GCs) are principal effectors of the stress response [32]. The effects of corticosterone are mediated by two intracellular receptor types: the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). The hippocampus expresses high levels of both the receptor types and is therefore considered as a major target for corticosterone action [33]. Cortisol/corticosterone bind both MR and GR albeit with differing affinities: whereas GR becomes occupied by high levels of corticosterone, MR appears to be tonically active under basal levels of corticosterone. The activity of these two receptors is coordinated to maintain balance [33]. It is documented that MR activity leads to protection but GR activity leads to apoptosis; additionally activation of MR counteracts deleterious effects of GR activation by corticosteroid on neuronal survival [34]. It is known that GR availability significantly decreases but MR activity increases in the hippocampus during pregnancy [35]. These changes in the GR and MR activities during pregnancy period may contribute to protect the hippocampus from stress adverse effects. Additionally some studies suggest that progesterone is a potent anticoercitor substance during pregnancy [35]. In rat progesterone has 3-times affinity of that aldosterone to MR and acts as an antagonist to corticosterone induced GR activation [36]. It is possible that high progesterone level during pregnancy is another agent counteracting corticosterone and thereby prevents hippocampal apoptosis.

There are some reports indicating that stress can affect MAPK signaling. Shen et al. reported that acute swim stress increases phosphorylated JNK level in the hippocampus [11]. Also Liu et al. showed that JNK signaling pathway can be activated following both acute restraint stress and forced swim stress in mice [8]. Our study showed that JNK activated in response to stress after 1 h but not after 3 h. Also repeated daily stress for 7 consecutive days did not affect JNK activation, although corticosterone level was higher than in non-stressed group. It seems that JNK activation in response to restraint stress is transient rather than persistent. While sustained JNK activation contributes to apoptosis progression, early transient JNK activation is known to rescue cells from apoptosis [37,38]. It is suggested that JNK activation is a prerequisite for antiapoptotic function of nerve growth factor (NGF) [39]. Additionally JNK is reported to be neuroprotective against platinum induced neuronal apoptosis [40]. There is no other study to assess the time course of rat hippocampal JNK activation as both studies mentioned above just assessed JNK activation immediately after a single stress exposure. By the way as

**Fig. 4.** Western blot analysis showing the effect of single (A) and repeated (B) restraint stress for 1 or 3 h episode on ERK 1/2 activation. Antibody against phosphorylated ERK 1/2 detected two bands at 44 kDa (ERK 1) and 42 kDa (ERK 2). Data are represented as mean ± SEM.

A Phospho-ERK 1/2

β-Actin

Control 1h 3h

Single stress

B Phospho-ERK 1/2

β-Actin

Control 1h 3h

Repeated stress

**Fig. 5.** Western blot analysis showing the effect of single (A) and repeated (B) restraint stress for 1 or 3 h episode on P38 activation. Antibody against phosphorylated P38 detected a weak band at 43 kDa. Data are represented as mean ± SEM.

A Phospho-P38

β-Actin

Control 1h 3h

Single stress

B Phospho-P38

β-Actin

Control 1h 3h

Repeated stress
this stress paradigm did not induce hippocampal apoptosis after repeated stress during rat pregnancy, it is suggested that transient JNK activation may contribute to mother's neuroprotection against stress-induced hippocampal apoptosis.

Restraint stress in this study failed to induce P38 and ERK 1/2 MAPKs. These results suggest that although JNK signaling pathway may play a role in stress response, P38 or ERK is not involved. Although one study showed that restraint stress increases ERK, but not JNK or P38 [9], some other studies are consistent with our finding showing that restraint stress mainly activates JNK, but not P38 and ERK [8,11]. P38 is mainly activated by growth factors, cytokines and inflammatory mediators in central nervous system [41]. ERK signaling is mostly involved in rapid signaling functional events such as neurotransmission [42].

During pregnancy many women experience stress-related mood disorders. It is valuable to know how stress affects mothers' neurobiology. This study showed for the first time that although stress elevates corticosterone level in pregnant dams, it does not induce hippocampal apoptosis. This work is consistent with some studies suggesting that pregnancy and reproduction may confer some beneficial changes to human mothers in terms of lowering behavioral anxiety/stress response and enhancing certain aspects of memory [43].

In conclusion this study showed that single and repeated restraint stress in pregnant rats significantly elevate corticosterone levels but do not induce apoptosis. Also restraint stress induced a transient JNK activation which may be involved in this neuroprotection. This study is valuable in understanding how physiologic changes during pregnancy might protect mother from stress-induced damages. Understanding how stress affects mothers during pregnancy will aid in improving the health and wellbeing of the mother and child.

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