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The Role of Gp91phox NADPH Oxidase during the Gestational Period of Mice

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Abstract

Gp91phox NADPH oxidase enzyme was established earlier to play its role in generating reactive oxygen species. In recent studies, gp91phox NADPH oxidase was reported to be involved in the regulation of ovulation and oestrial cycle. However, its mechanism of action during comparatively stressful gestational period 'gp' is not known. Current study was designed to investigate the role of gp91phox NADPH oxidase during 'gp' by using gp91phox deficient (gp91phox$^{-/-}$) mice whereas C57BL/6j mice in graviditas served as control. During 'gp' period the floating time (akinesia time) in control group was found to be highest on day 6, which gradually decreased towards parturition. Conversely, ingp91phox$^{-/-}$ mice, there was no such recognizable alteration during 'gp' as compared to control C57BL/6j mice. In addition, plasma level of adrenocorticotropic hormone (ACTH) was significantly higher on day 6 (gp) in the control mice. Similarly, the level of growth hormone (GH) was also found elevated during 'gp' in control mice. On the other side, in thegp91phox$^{-/-}$ mice, no such increase in ACTH or GH levels as compared to C57BL/6j mice was observed. It is worth mentioning that GH levels (gp) ingp91phox$^{-/-}$ mice were found ever lower than those of the control C57BL/6j mice. There were also fewer newborngp91phox$^{-/-}$ mice compared with C57BL/6j mice; interestingly, the number of fetalgp91phox$^{-/-}$ mice did not decrease until after gd 6. Furthermore, the average weight for the newborngp91phox$^{-/-}$ mice was significantly lower than the C57BL/6j mice. The results of the present study deonstrated that gp91phox NADPH oxidase is required during relatively stressful gestational period 'gp' and may play a role during fetal differentiation and growth.

Keywords: Gp91phox$^{-/-}$ (Gp91phox deficient mice); C57BL/6j mice (Control group); Gestational day 'gd'; Gestational period 'gp'; Forced swim test (FST); Adrenocorticotropic hormone (ACTH) plasma level; Growth hormone (GH) level

Introduction

Nicotinamide adenine dinucleotide phosphate oxidase (Nox) is a multicomponent enzyme complex originally described in phagocytes [1]. The phagocytic Nox consists of a catalytic subunit Nox2/gp91phox together with regulatory subunit p22phox located in membrane. Nox2/gp91phox enzyme is able to catalyze the reduction of molecular oxygen to superoxide, however, there are key differences in their activation, subunit composition, localization, and expression. Nox2/gp91phox enzyme complex is the major catalytic source of superoxide generation in several biological models [2]. We previously reported that the pigmentation induced by ultraviolet (UV) B eye irradiation did not occur in gp91phox deficient (gp91phox$^{-/-}$) mice [3].

In such mice, the reduction of the amount of α-melanocyte stimulating hormone (α-MSH) secreted from pituitary gland was seen by UVB eye irradiation. We therefore hypothesized that gp91phox might be involved in the hormonal secretion from pituitary gland. Recent it was suggested that the reactive oxygen species (ROS) released from gp91phox NADPH oxidase, expressed in neutrophils, (leads to) played a vital role in the regulation of ovulation and the oestrial cycle [4].

However, the role of gp91phox during 'gp' is not known. We herein examined the role of gp91phox during the stressful gestational period 'gp' and investigated the relation between gp91phox and hormones of pituitary gland origin.

Materials and Methods

Animals

Pregnant C57BL/6j mice (SLC, Hamamatsu, Aichi, Japan) and C57BL/6jgp91phox$^{-/-}$ mice (Jackson Laboratories, Bar Harbour, ME) were used. The difference in suppliers had no effect on the results. The animals were housed individually on gestational day (gd) 0 to 18. Pregnancy was determined by the observation of a vaginal plug. The plug date was considered to be gd 0 of gestation. The mice were kept on 12-hour light/12-hour dark cycle at 23 ± 1° C in SPF conditions. All animals had free access to water, and laboratory chow diet (CE-2, Oriental Yeast Co., Tokyo, Japan). The animals were randomly allotted to different groups havimg six mice each. The number of newborns and the newborn's weight was recorded by using (10 home bred, 'gp' mice) C57BL/6j and gp91phox$^{-/-}$ 'gp' mice, respectively. The number of fetal C57BL/6j and gp91phox$^{-/-}$ mice was recorded on days: gd 6 and gd 10. In all cases, the study was undertaken following the official Guide for the Care and Use of Laboratory Animals of the Suzuka University of Medical Science (Approval number: 34). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Forced swim test (FST, floating test)

The mice were introduced to a transparent pool (20x35x15 cm^3) filled with warm water (30° C, height 9.5 cm) and subjected to forced swimming for six minutes. A video camera recorded the experiment for six minutes. Next, we observed the behavior of the animals and measured the duration of complete immobility of the entire body for four minutes during the second half of the experiment [3].

Quantification of the levels of ACTH and GH in the plasma using an enzyme-linked immunosorbent assay (ELISA)

Blood samples were obtained from the heart on each day of the pregnancy, and the plasma samples were fractionated. The plasma levels of ACTH and GH were determined using commercial ELISA kits (ACTH; Phenix Pharmaceuticals Inc., CA; GH; Merck Millipore, Damstadt, Germany) according to the manufacturer's instructions.

Preparation and staining of the ovaries

The ovaries from the mice on each day of pregnancy. The ovary specimens were placed into phosphate-buffered paraformaldehyde (4%), embedded in frozen Tissue-Tek, OCT compound, and cut into 5-μm-thick sections. The sections of the ovaries were washed in PBS and then subsequently incubated overnight at 4° C with rabbit anti-gp91phox (1:100) polyclonal antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA) to determine the expression of gp91phox. The sections were then washed in PBS and incubated at room temperature for two hours with FITC-conjugated anti-rabbit immunoglobulin (1:30; Dako Cytomation, Glostrup, Denmark) and examined under a fluorescent microscope.

Statistical Analysis

All data are presented as mean ± SD of results derived from six animals. The results obtained from the animal groups were compared using either Student's t-test or ANOVA by using a standard computer software package. Differences were considered to be significant at $p<0.05$.

Results

Effects of pregnancy on the mouse behavior during the FST and on the expression of gp91phox

FST protocol was used to examination and measures a depressed state [5]. After being placed into the vessel containing water, the mice initially swim intensely to escape from the water, but then gradually give up and exhibit akinesia (immobility). In C57BL/6j mice, the duration of akinesia during the gestational period 'gp' was the longest on gd 6 (Figure 1A).

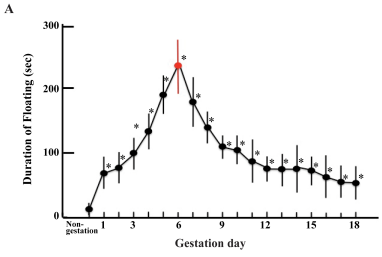

Figure 1A: Effects of pregnancy on the duration of akinesia in the FST and on the expression of gp91phox. The duration of akinesia in the gestational period of C57BLB/6j female mice

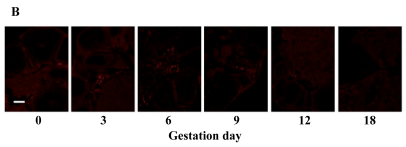

Figure 1B: Expression of gp91phox in the ovaries during the gestational period in C57BL/6j female mice was determined. The data show one typical experiment from six animals. The values are presented as the means ± SD derived from six animals. *P<0.05 in comparison to the non-pregnant mice. Scale bar = 100 μm

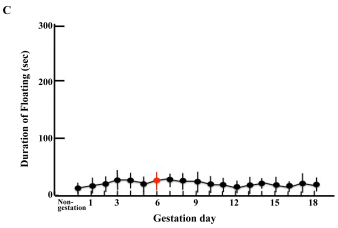

Figure 1c: The duration of akinesia in the gestational period of$gp91phox^{-/-}$ mice

However, in the$gp91phox^{-/-}$ mice, alteration of the akinesia temporal duration was not seen throughout 'gp' (Figure 1C). Moreover, in C57BL/6j mice, the expression of gp91phox in the ovaries during 'gp' was highest on gd 6 (Figure 1B).

Effects of pregnancy on the plasma levels of ACTH and GH in the$gp91phox^{-/-}$ mice

The plasma levels of ACTH and GH during 'gp' were measured using standard procedure. ACTH levels fell gradually with the highest concentration seen on gd 6 in C57BL/6j mice. However, this change could not be seen in$gp91phox^{-/-}$ mice (Figure 2A).

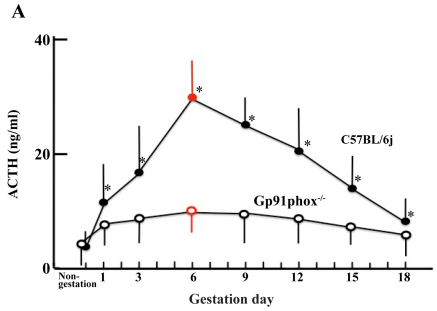

Figure 2A: Effects of pregnancy on the plasma levels of ACTH in the$gp91phox^{-/-}$ mice

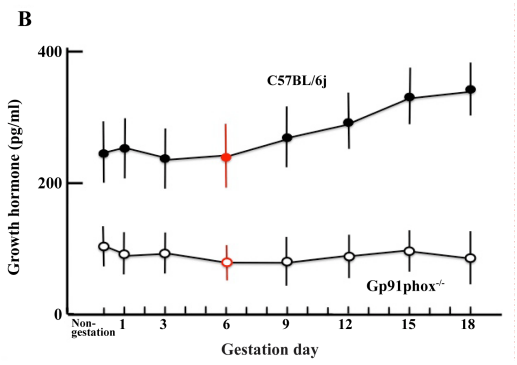

Figure 2B: Effects of pregnancy on the plasma levels of GH in thegp91phox$^{-/-}$ mice

The values are presented as the means ± SD derived from the results of six animals, $^{*}P<0.05$ in comparison to the non-pregnant mice.

Furthermore, GH levels were consistently high throughout 'gp' in C57BL/6j mice, whereas, in case ofgp91phox$^{-/-}$ mice lower GH levels were observed.

The number of fetalgp91phox $^{-/-}$ mice and the newborns' weights

The number of fetal C57BL/6j mice did not change during 'gp'. The number of fetalgp91phox$^{-/-}$ mice did not differ from C57BL/6j mice before gd 6, nevertheless, the number of fetalgp91phox$^{-/-}$ mice decreased from gd 6 to gd 10. Furthermore, the number of new born gp91phox$^{-/-}$ mice decreased significantly as compared to C57BL/6j mice (Figure 3A). The average newborn weight was lower ingp91phox$^{-/-}$ mice as compared with C57BL/6j mice (Figure 3B).

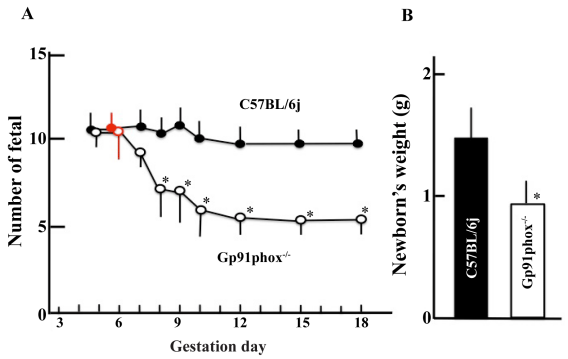

Figure 3: (A) Effects of gp91phox on the number of fetal mice (B) and the newborn mice's weight

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Discussion

The present of the present study demonstrated that the duration of akinesia and FST was longest at gd 6 in the C57BL/6j mice, however, in thegp91phox$^{-/-}$ mice, the alteration of akinesia was not seen during 'gp'. Furthermore, the number of newborns and the average newborn weight was decreased ingp91phox$^{-/-}$mice as compared with C57BL/6j mice. In addition, the plasma levels of ACTH and GH were lower ingp91phox$^{-/-}$ mice when compared with C57BL/6j mice, and the alteration of these hormones during 'gp' was not seen in thegp91phox$^{-/-}$ mice.

It is well documented that ACTH is important for embryonic differentiation [6]. In the placentas, MC2R (ACTH-R) is known to be high, and a fetal animal is directly affected by ACTH level [7]. Earlier reports also suggested that the high levels of plasma ACTH raised the levels of leukemia inhibitory factor (LIF) in the fetal cerebrospinal fluid (CSF). It is well established that LIF has an important role during embryonic cerebral differentiation [8-10]. In the present study, the maximal level of ACTH was secreted at gd 6 in the control C57BL/6j mice. At gd 6, the increase observed in the secretion of ACTH confirmed the stage of active differentiation to be taking place in the fetal mice. Our results are full in agreement with the earlier reports. It is worth mentioning that no such increase in ACTH could be observed ingp91phox$^{-/-}$mice (Figure 1C). Moreover, the number of fetal mice decreased between gd 6 and gd 10. For this reason, we speculate that LIF was not secreted, and due to a deficit of ACTH from the placenta, differentiation of the systemic nervosum did not progress [8-10].

In the present study, the secretion of GH was also lower in thegp91phox$^{-/-}$ mice as compared to C57BL/6j mice. Although it is well known that gp91phox NADPH oxidase induces ROS [11]; it is still not clear if any relationship between ROS and GH secretion. GH is known to be a hormone of pituitary gland origin. In our earlier reports we demonstrated that UV stimulus to mice caused increases in the secretion of propiomelanocortin (POMC) systemic hormones such as: ACTH, α-melanocyte stimulating hormone (α-MSH), and α-endorphin. It is noteworthy that all of them have the same pituitary gland origin like GH [3,5,12]. The increase in secretion of POMC-origin hormones was not induced in thegp91phox$^{-/-}$ mice. Although, ACTH increased during the gestational period in C57BL/6j mice, the increase in ACTH was not seen in thegp91phox$^{-/-}$ mice. Gp91phox NADPH oxidase acts on the hypothalamus-pituitary gland system by psychical stresses, such as a gravidities, UV exposure, and the oxidase induced the increase in GH secretion. Therefore, it was suggested that NADPH oxidase is required for the increase in GH during the gestational period. However, the exact mechanisms are unknown and further studies are warranted.

In the present study, gp91phox NADPH oxidase was found to play an important role in the secretion of ACTH and GH during 'gp'. Ingp91phox$^{-/-}$ mice, a deficit of ACTH during 'gp' inhibited embryonic cerebral differentiation and induced a decrease in the number of fetal mice. In addition, deficient levels of GH induced a depression of the newborn's weight. A gravidities was again noticed to be a big psychical stress. Thegp91phox$^{-/-}$ mouse is considered to be less susceptible to psychical stress than to changes in ACTH concentration. These results revealed that gp91phox NADPH oxidase was induced the psychical stress in 'gp'. However, this psychical stress induction by gp91phox NADPH oxidase plays the important role during fetal's differentiation and growth.

Conflict of Interests

The authors declare no conflicts of interest (COI).

Authors' Contributions

KH wrote the article and designed the research, KH and YY analyzed and interpreted the data, and EFS contributed the essential reagents and tools.

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