

The Role of the Active Oxygen Produced from Gp91phox NADPH Oxidase on the Newborn Weight of Mouse Pups

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Summary

It is known that active oxygen plays an important role in a reproduction. However, no report has so far investigated the influence of active oxygen produced from gp91phox NADPH oxidase on newborns. In this study, we investigated the influence of active oxygen on the weight of newborns using graviditas gp91phox-knockout (gp91phox^{-/-}) mice. Gestational C57BL/6j (control), gp91phox^{-/-}, and insulin-like growth factor-1-knockout (IGF-1^{-/-}) mice were examined and the weight of the newborn mouse pups were analyzed. Gp91phox^{-/-} and IGF-1^{-/-} mouse pups had low weight compared with control mice. When the control mice were treated with an inhibitor of reactive oxygen species (ROS), the newborn weight decreased. Conversely, when the gp91phox^{-/-} mice were treated with an activator of ROS, the newborn weight increased, however, it remained low in the IGF-1^{-/-} mice. Moreover, there were decreased levels of IL-1 in the plasma of graviditas gp91phox^{-/-} mice compared with control and IGF-1^{-/-} mice. Treatment with an IL-1 receptor antagonist in the control mice resulted in a low newborn weight, similar to the gp91phox^{-/-} and IGF-1^{-/-} mice. Furthermore, the expression of NLRP3 and caspase-1 in the uterus of graviditas gp91phox^{-/-} mice was low compared with the control and IGF-1^{-/-} mice. These results clearly indicate that gp91phox NADPH oxidase produces ROS during graviditas. The ROS activate NLRP3, and NLRP3 leads to the production of caspase-1, which subsequently increases IL-1, thereby finally inducing IGF-1. Because the newborn weight is determined by IGF-1, gp91phox appears to be important for promoting fetal growth during graviditas.

Keywords: Gp91phox; Insulin-like growth factor-1 (IGF-1); Reactive oxygen species (ROS); Interleukin-1 β (IL-1 β); Caspase-1

Introduction

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) is a multicomponent enzyme complex originally decreased in phagocytes [1-4]. Nox consists of seven organization members (Nox1-5 and Duox1 and 2) and all the members produce reactive oxygen species (ROS) [5]. Nox2, also known as gp91phox, is mostly expressed in macrophages or neutrophils [6]. Gp91phox has been largely investigated due to its role in the production of ROS by p67 and the regular arrangement of collecting venules [6]. ROS, which are produced by gp91phox, plays an important role during biophylaxis by acting as a disinfectant [7]. However, ROS also cause oxidative stress. It has been suggested that ROS constitutes the pathogenesis of lifestyle-related disease, such as infection, inflammation, cancer, arteriosclerosis and diabetes mellitus, and various nervous system diseases such as Alzheimer's disease. Furthermore, according to recent research, ROS have been demonstrated to be important as a signal molecule. These studies show that ROS activates a specific signaling pathway, similar to other signal transducers, and may regulate cellular protection, cell differentiation propagation, and cell death [8-10].

In addition, it is reported that ROS are involved in sexual organ development or reproductive behavior. Recently, it was suggested that the ROS released from gp91phox NADPH oxidase, expressed in neutrophils, play a vital role in the regulation of ovulation and the estrous cycle [4]. Furthermore, we previously reported the decrement in the newborn weight using gp91phox-knockout (gp91phox^{-/-}) mice [11]. Moreover, gp91phox^{-/-} mouse pups also demonstrated decreased growth hormone levels [11]. Thus, although ROS has been shown to participate in ovulation or the estrous cycle, no study has investigated its effect on fetal growth.

We herein examined the role of gp91phox on the weight of newborn mouse pups and investigated the relationship between gp91phox and growth factors.

Materials and Methods

Animals

Female C57BL/6j mice (SLC, Hamamatsu, Shizuoka, Japan), C57BL/6j gp91phox^{-/-} mice (Jackson Laboratories, Bar Harbor, ME, USA) and C57BL/6j insulin-like growth factor-1-knockout (IGF-1^{-/-}) mice (Tokyo Metropolitan Institute of Gerontology, Itabashi, Tokyo, Japan) were used. The mice were kept on a 12-hour light/12-hour dark cycle at 23 \pm 1°C in SPF conditions. All animals had free access to water and laboratory chow diet (CE-2, Oriental Yeast Co., Tokyo, Japan) *ad libitum*. The animals were randomly allotted to different groups with six mice in each group. The weight of the newborn pups was recorded for the C57BL/6j, gp91phox^{-/-} and IGF-1^{-/-} mice. This study was conducted in accordance with 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 attempts were made to minimize suffering.

In addition, the blood concentration of IGF-1 of the mother's body and the fetal weight tends to correlate, thus if the level of IGF-1 is high, then the fetal weight will increase [12-14]. Therefore, this study

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examined the change in the amount of IGF-1 in the tissue and blood of graviditas mice. Furthermore, the value of IGF-1 in the gestational age is high compared with after giving birth in C57BL/6j (control) mice (data not shown). In particular, the value of IGF-1 on the 18th (day 18) was the highest among the gestational age; therefore, we used the blood sample on day 18 for the examination.

N-acetyl-L-cysteine (NAC) treatment

Two hundred mg/kg of n-acetyl-L-cysteine (NAC) (ROS inhibitor; Nakarai Tesque, Kyoto, Japan) in 0.08% dimethyl sulfoxide (DMSO) was injected intraperitoneally for a total of 10 times, once a day, starting from the first day of graviditas; DMSO alone was injected into the graviditas control mice [15].

PAC-1 treatment

Ten mg/kg of the PAC-1 (ROS activator; Selleck Chemicals, Houston, TX, USA) in 0.08% DMSO was administrated orally a total of 10 times, once a day, starting from the first day of graviditas, while DMSO alone was administrated to the graviditas control mice [16].

Interleukin-1 (IL-1) β receptor antagonist (IL-1RA) treatment

Ten mg/kg of an IL-1 β receptor antagonist (IL-1RA; ATGen Ltd., Gyeonggi-do, South Korea) in saline was injected intraperitoneally into the mice throughout the graviditas period [17]. Saline alone was injected into the control mice.

Anti-tumor necrosis factor (TNF)- α treatment

Three μ g/kg of the anti-TNF- α antibody (R&D Systems, Minneapolis, MN, USA) in saline was injected intraperitoneally into the mice throughout the graviditas period [18]. Saline alone was injected into the control mice.

Caspase-1 inhibitor treatment

The mice were treated with the caspase-1 inhibitor, Ac-YVAD-CMK (10 mg/kg S.C.; Calbiochem, La Jolla, CA, USA), throughout the graviditas period [19]. Control mice were treated with vehicle only (1:1 v/v saline/polyethylene glycol 300).

Quantification of the levels of IGF-1, IL-1 β and TNF- α in the plasma using an enzyme-linked immunosorbent assay (ELISA)

Blood samples were obtained from the mice on day 18 of gestation, and the plasma samples were fractionated. The plasma levels of IGF-1, IL-1 β and TNF- α were determined using commercial ELISA kits (IGF-1; Assaypro LLC., St. Charles, MO, USA; IL-1 β and TNF- α ; R&D Systems) according to the manufacturer's instructions.

Measurement of plasma ROS concentration

The plasma ROS levels were determined using an OxiSelect™ *In Vitro* ROS/RNS Assay Kit (STA-347; Cell Biolabs, Inc., San Diego, CA, USA) according to the manufacturer's instructions.

Western blotting

Uterus samples were obtained from the mice on day 18 of gestation. Fixed whole uterus samples were homogenized in Lysis buffer (Kurabo, Osaka, Japan), and centrifuged at 8,000 x g for 10 min. The supernatant from each sample was then isolated and stored at -80°C until analysis. After thawing, the samples (amount of protein: 10 μ g/lane) were loaded onto a 4-12% BIS-TRIS Bolt gel (Life Technologies, Carlsbad CA,

USA) and electrophoresed at 165 V for 30 min. Following separation, proteins were transferred to a nitrocellulose membrane using an iBlot Western blotting system (Life Technologies, Carlsbad, CA, USA), which was subsequently blocked with 5% skim milk overnight at 4°C. After blocking, the membranes were incubated at 25°C for 1h with primary antibodies against nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP3) (1:1000; Abnova, Taipei, Taiwan), caspase-1 (1:1000; Epitomics, Burlingame, CA, USA), or β -actin (1:5000; Sigma-Aldrich, St. Louis, MO, USA). Immune complexes on the membranes were then visualized using a horseradish peroxidase-conjugated secondary antibody (Life Technologies, Frederick, MD, USA) and ImmunoStar Zeta (Wako, Osaka, Japan). Images were acquired using the Multi-Gauge software program (Fujifilm, Greenwood, SC, USA).

Statistical Analysis

The results obtained from the animal groups were compared using either ANOVA or Student's *t*-test using an ANOVA software program (XHL STAT, Artwork Conversion Software Inc., Santa Cruz, CA, USA). First, we analyzed all data by an ANOVA, and only items with significant differences were further evaluated using the *t*-test. All data are expressed as the means \pm standard deviation, and significance was set at $P < 0.05$.

Results

The plasma levels of ROS and IGF-1 and newborn weight in gp91phox^{-/-} and IGF-1^{-/-} mice

The newborns weight of gp91phox^{-/-} and IGF-1^{-/-} mice decreased compared with the control mice (Figure 1A). Additionally, the plasma ROS level was decreased in graviditas gp91phox^{-/-} mice (Figure 1B). On the other hand, the plasma IGF-1 level was low in both gp91phox^{-/-} and IGF-1^{-/-} graviditas mice compared with control mice (Figure 1C).

Effect of NAC or PAC-1 administration on the plasma IGF-1 and ROS levels, and newborn weight in graviditas mice

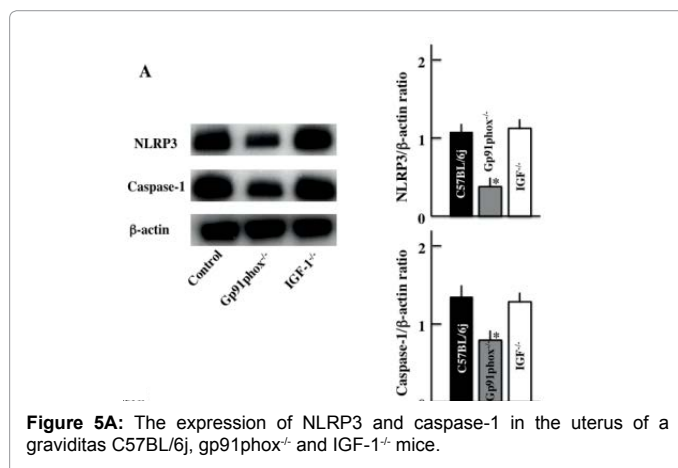
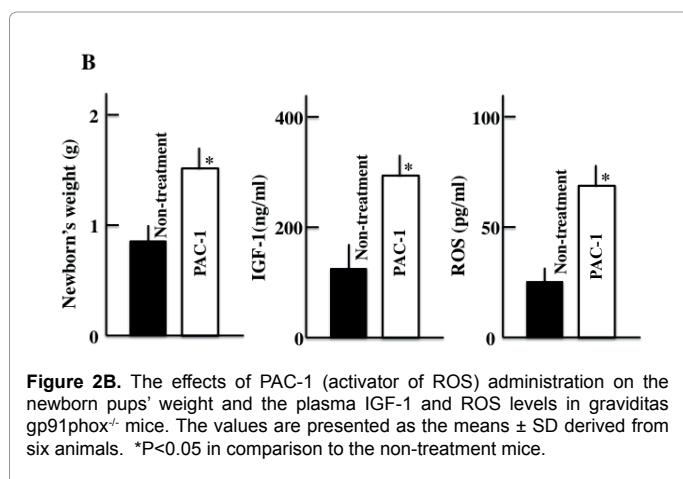
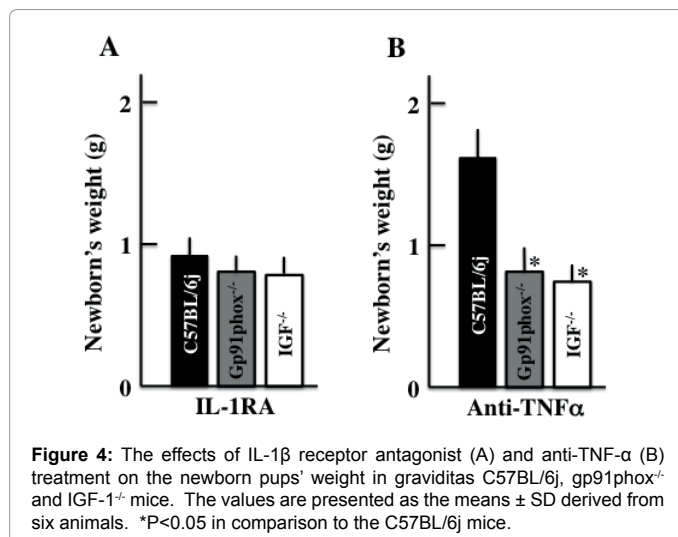
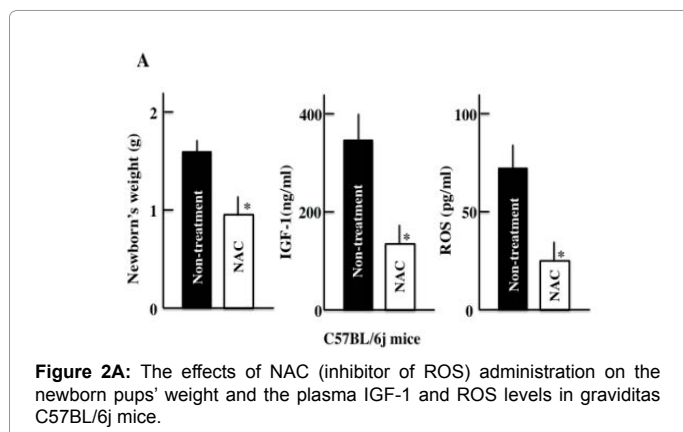
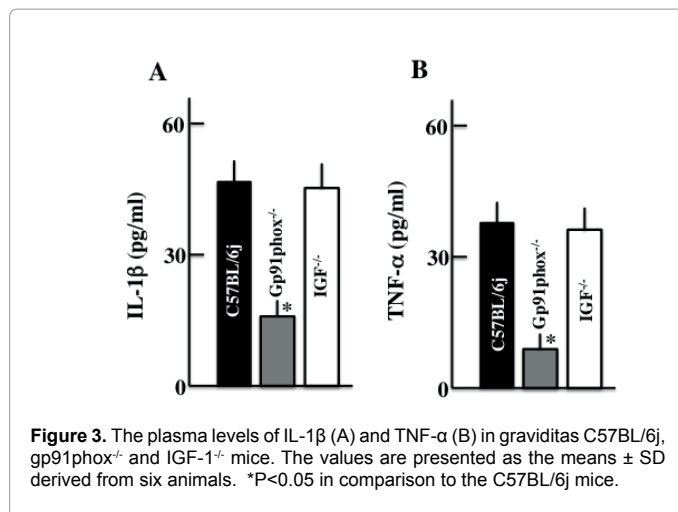
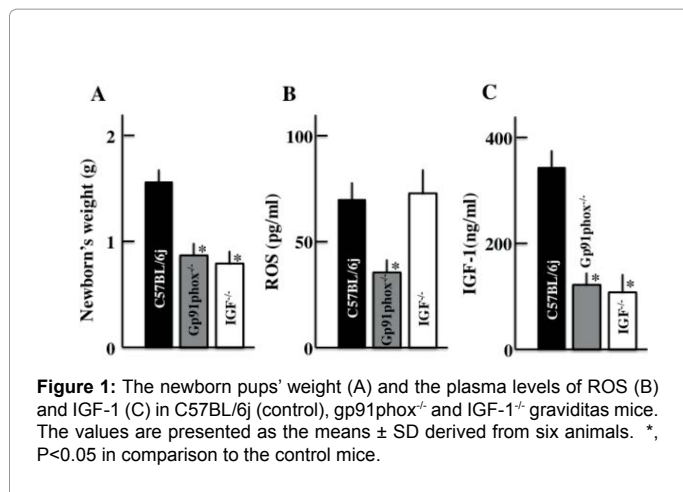
The newborn weight and the plasma IGF-1 and ROS levels in graviditas mice decreased in the control mice (C57BL/6j) following treatment with NAC (an inhibitor of ROS; Figure 2A). Conversely, graviditas gp91phox^{-/-} mice treated with PAC-1 (an activator of ROS), showed increased newborn weight and plasma IGF-1 and ROS levels, similar to the control mice (C57BL/6j) (Figure 2B).

The plasma IL-1 β and TNF- α levels in graviditas mice

We measured the plasma IL-1 β (Figure 3A) and TNF- α (Figure 3B) levels in graviditas C57BL/6j, gp91phox^{-/-} and IGF-1^{-/-} mice. The IL-1 β and TNF- α levels were decreased in the graviditas gp91phox^{-/-} mice compared with the control mice, whereas the levels remained unchanged in the graviditas C57BL/6j and IGF-1^{-/-} mice.

Effect of IL-1RA and anti-TNF- α treatment on the newborn weight in graviditas mice

In the C57BL/6j, gp91phox^{-/-} and IGF-1^{-/-} graviditas mice, the influence of an IL-1RA on the newborn weight is shown in Figure 4A, and the influence of anti-TNF- α treatment is shown in Figure 4B. Following the IL-1RA treatment, the newborn weight was decreased in C57BL/6j, gp91phox^{-/-} and IGF-1^{-/-} mice. Following the anti-TNF- α treatment, the newborn weight was decreased in gp91phox^{-/-} and IGF-1^{-/-} mice compared with C57BL/6j mice.



Expression of NLRP3 and caspase-1 in the uterus of graviditas mice, and the influence of caspase-1 inhibitor treatment on the newborn weight

In gp91phox^{-/-} mice, there was little expression of NLRP3 and caspase-1 (Figure 5A) in the uterus compared with the C57BL/6j and IGF-1^{-/-} mice. The newborn weight was low in all groups following caspase-1 treatment (Figure 5B).

Discussion

In this study, a decrement in the newborn weight was seen in gp91phox^{-/-} and IGF-1^{-/-} mice compared with the C57BL/6j mice. The IGF-1 level in the blood of the graviditas gp91phox^{-/-} and IGF-1^{-/-} mice was also lower compared with the control mice, whereas the ROS level was only low in the gp91phox^{-/-} mice. Moreover, the plasma levels of IL-1β and TNF-α were low in graviditas gp91phox^{-/-} mice, and the

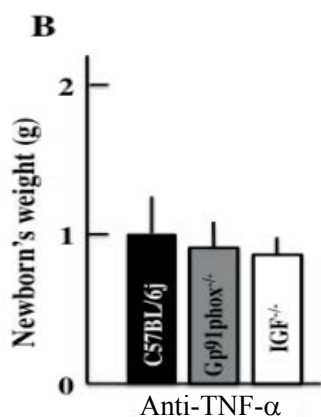


Figure 5B: The effects of caspase-1 inhibitor administration on the newborn pups' weight in graviditas C57BL/6j, gp91phox^{-/-} and IGF-1^{-/-} mice. The data show the results from a representative experiment involving six animals. The values are presented as the means ± SD derived from six animals. *, P<0.05 in comparison to the C57BL/6j mice.

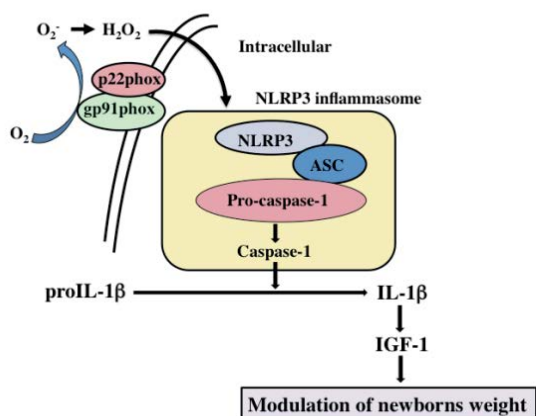


Figure 6: Mechanism of activation of the inflammasome and secretion IGF-1 in graviditas mice.

expression of NLRP3 and caspase-1 in the uterus were also low in the graviditas gp91phox^{-/-} mice. Furthermore, following treatment with an inhibitor of IL-1β receptor or caspase-1, the newborn weight was decreased in all groups.

Gp91phox (Nox2) is a main component of NADPH oxidase, and if gp91phox is stimulated, then O₂⁻ (active oxygen) will be generated [3]. This active oxygen controls the NLRP3 inflammasome and activates caspase-1 [20]. In this study, the increase in active oxygen led to the activation of NLRP3 and induced an increase in caspase-1 in the graviditas control mice. In the graviditas gp91phox^{-/-} mice, an increase in active oxygen, NLRP3 and caspase-1 was not seen, and a decrement in the newborn weight was observed. In the graviditas IGF^{-/-} mice, although the increase in active oxygen, NLRP3 and caspase-1 was seen, a decrement in the newborn weight occurred, which was likely directly caused by the reduction of IGF-1. Furthermore, active oxygen produced from gp91phox NADPH oxidase was considered to be the

first rate-determining step, according to the finding that the newborn weight remained similar to the control mice when gp91phox^{-/-} mice were treated with an activator of ROS.

Although an increase in the plasma IL-1β level was observed in the graviditas control mice, an increase in IL-1β was not seen in the graviditas gp91phox^{-/-} mice. Caspase-1 activates proIL-1β and produces IL-1β [21], which subsequently induces the production of IGF-1 [22]. Through this mechanism, tyrosine kinase JAK-2 and transcription factor STAT-5, downstream of JAK-2, is activated by IL-1β stimulation. Additionally, it has been previously reported that STAT-5 continuously produces IGF-1 [23]. In this study, a decrement in the newborn weight in control mice was observed following IL-1β receptor inhibitor treatment, and the newborn weight of control mice was similar to the newborn weight of gp91phox^{-/-} mice. In addition, the plasma IGF-1 decreased in the control mice treated with IL-1β receptor inhibitor. Taken together, these findings suggest that an increased newborn weight is dependent on increased IL-1β and IGF-1.

Although IL-1β is cytokine which induces inflammation, IL-1β was expressed at a low level in this study and did not induce inflammation. Honsho et al. [23] has reported that low-level efficiently induces IGF-1. Therefore, we speculate that the level of IGF-1 was also efficiently induced by low-level IL-1β in this study.

Taken from the above-mentioned findings, the decrement in the newborn weight in gp91phox^{-/-} mice is likely due to a series of events from the inflammation system stemming from the lack of active oxygen production by gp91phox NADPH oxidase, which, in turn, results in low levels of IGF-1 (Figure 6). Although gp91phox NADPH oxidase has been shown to be involved in the control of ovulation [24] and a retention of graviditas [11] by producing active oxygen, our findings suggest that gp91phox NADPH oxidase also participates in fetal growth.

Conclusion

In this experiment, we showed that the newborn weight is decreased in gp91phox^{-/-} and IGF-1^{-/-} mice compared with control mice. As a mechanism of the weight decrease of these newborns, ROS produced from gp91phox NADPH oxidase activates NLRP3. The increase in IL-1β takes place because the activation of NLRP3 induces caspase-1. It is believed that fetal growth is mediated through IL-1β induction of IGF-1. Our study shows the possibility that active oxygen produced from gp91phox NADPH oxidase may play an important role during fetal growth.

Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

Authors' Contributions

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

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