Dietary L-Arginine Supplementation during Gestation in Mice Enhances Reproductive Performance and Vegfr2 Transcription Activity in the Fetoplacental Unit1–3

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Abstract

Regarded as one of the most versatile amino acids, arginine serves as a precursor for many molecules and has been reported to improve the reproductive performance of rats and pigs. To this end, we sought to determine if dietary L-arginine alters fetoplacental vascular endothelial growth factor receptor-2 (Vegfr2) transcription activity. Eighteen wild-type FVB/N female mice were bred to homozygous FVB/N-Tg(Vegfr2-luc)-Xen male mice. Bred female mice received 1 of 2 experimental diets: one supplemented with 2.00% (wt:wt) L-arginine (+Arg) or 1 supplemented with 4.10% (wt:wt) alanine (+Ala) to serve as an isonitrogenous control for +Arg. In addition, 6 mice were fed a nonsupplemented control (Con) diet to normalize bioluminescent imaging data. All data were analyzed using ANOVA followed by Fisher’s least significant difference. Total feed intake did not differ between groups; however, mice in the +Arg group consumed more arginine (P < 0.05). Arginine supplementation increased weight gain during the latter one-third of gestation (d 12–18), total litter size, number of pups born alive, number of placental attachment sites, litter birth weight, and litter weight of pups born alive but decreased the individual birth weights (P < 0.05). During d 12–18, arginine supplementation increased (P < 0.05) the mean total Vegfr2 transcription activity and Vegfr2 transcription activity corrected for fetoplacental mass. Moreover, mice in the +Arg group had an earlier rise in Vegfr2 transcription activity. In conclusion, our results demonstrate that the beneficial effect of dietary L-arginine supplementation on mammalian reproduction is associated with enhanced Vegfr2 transcription activity in fetoplacental tissues. J. Nutr. 142: 456–460, 2012.

Introduction

Whereas arginine is considered to be a conditionally essential amino acid only for mature mammals, it is essential for young, developing mammals (1). In addition to being incorporated into many proteins and playing a role in ammonia detoxification (2), arginine is also a precursor for many molecules, including polyamines, proline, glutamate, creatine, and, most notably, NO, making arginine one of the most versatile amino acids (3). Moreover, an unusual abundance of arginine has been reported to exist in porcine allantoic fluid, suggesting a critical role for this amino acid in fetoplacental nutrition (4). Recently, it was demonstrated that arginine supplementation during gestation enhances the reproductive performance of both rats (5) and pigs (6), and expression of NOS7, the enzyme responsible for converting arginine to NO, has been shown to be increased at the site of implantation in mice (7), suggesting that NO may play an important role during embryo implantation. Although it is unclear how arginine enhances reproductive performance, it has been suggested that arginine may influence angiogenesis, a critically important process during pregnancy (6).

Angiogenesis is the process by which new vasculature develops from preexisting vascular structures. Two proteins are extremely vital for angiogenesis to occur, namely vascular endothelial growth factor (VEGF) and its cell-surface receptor, vascular endothelial growth factor receptor 2 (VEGFR2). As a proangiogenic factor, VEGF has been described as the most potent stimulator of angiogenesis and VEGFR2 is considered to be the primary receptor by which VEGF elicits its proangiogenic effects.

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3 Supplemental Tables 1 and 2 and Supplemental Figure 1 are available from the “Online Supporting Material” link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
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7 Abbreviations used: +Ala, alanine-supplemented diet; +Arg, arginine-supplemented diet; Con, control diet; GD, gestational day; NOS, NO synthase; ROI, region of interest; WT, wild type.

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effects, including the stimulation of endothelial cell growth in developing tissues (8). Expression of VEGF and VEGFR2 in fetoplacental tissues occurs early in gestation (9), and the latter one-third of gestation in mice is characterized by a tremendous amount of fetoplacental vascular growth and fetal growth (10–12), indicating the importance of VEGF, VEGFR2, and angiogenesis in the fetoplacental unit.

Previously, we described a mouse pregnancy model that allows fetoplacental Vegfr2 transcription activity to be monitored during the latter one-third of gestation in real-time and in vivo using bioluminescent imaging and the Vegfr2-luc mouse (13), which contains a transgene, comprised of a mouse Vegfr2 promoter region cloned upstream from the luciferase gene (14). In this model, when the exogenous substrate, luciferin, is administered, oxidation occurs, producing light, which is then measured using imaging equipment that is highly sensitive to low-emitting light. Using this approach, the activation of the Vegfr2 promoter sequence, referred to as Vegfr2 transcription activity, is monitored through luciferase production, which allows Vegfr2 to be studied longitudinally within the same mouse noninvasively.

To this end, the goal of this study was to determine, using a novel bioluminescent mouse pregnancy model, if the reported beneficial effect that dietary l-arginine supplementation has on mammalian reproduction is associated with enhanced fetoplacental Vegfr2 transcription activity.

**Methods**

**Mice and diets.** Care and use of mice utilized in this study were conducted in accordance with and under the approval of the Institutional Animal Care and Use Committee of Mississippi State University. Homozygous Vegfr2-luc male mice were purchased from Caliper Life Sciences and WT FVB/N female mice were obtained from an in-house colony derived from two vendors (The Jackson Laboratory and Charles River Laboratories International). Male mice were caged individually and female mice in groups of 4–5/cage until they were paired for breeding, after which bred female mice were housed individually. All mice were housed in an environmentally controlled room set at 22°C with a 12-h-light/–dark cycle and consumed feed (Purina Test Diet) and water ad libitum. The bioluminescent pregnancy model utilized in this study was based upon previous work described by Greene et al. (13).

To monitor only fetoplacental Vegfr2 transcription activity, 18 WT female mice were bred to the homozygous Vegfr2-luc male mice, restricting the Vegfr2-luciferase transgene to the fetoplacental tissues. Conversely, breeding a Vegfr2-luc female mouse would not allow fetoplacental luciferase expression to be distinguished from maternal expression originating from tissues such as the uterus (15) and vascularized corpus lutea (16), which express VEGF in abundance during gestation. After pairing WT female mice and Vegfr2-luc male mice overnight, confirmation of breeding was determined by the presence of a vaginal plug, and this day was designated as GD 1.

After observing a vaginal plug (GD 1), bred female mice were then housed individually and randomly assigned to one of three custom purified diets (n = 6/diet; Purina Lab Test Diets) (Supplemental Table 1). Bred female mice received one of two experimental diets: one supplemented with 2.00% (wt:wt) l-arginine (+Arg) or one supplemented with 4.10% (wt:wt) alanine (+Ala) to serve as an isonitrogenous control for +Arg. In addition, six mice were fed a nonsupplemented control (Con) diet to normalize bioluminescent imaging data. All three diets were formulated to be phytoestrogen and alfalfa free, because phytoestrogens can potentially influence Vegfr2 expression (17) and alfalfa can produce autofluorescence (18) that can interfere with bioluminescence imaging. Body weights were recorded daily from GD 1 to 18, and total feed intake during gestation was also recorded. Mice were imaged from GD 12 to 18 to detect luciferase activity as an indicator of Vegfr2 transcription activity. Additionally, the number of pups and litter weights were recorded at birth. After birth, all dams received the standard Con diet. Three weeks postpartum, dams were killed via cervical dislocation and the uterus was excised. The intact uterus was fixed in formalin for determining the number of placental attachment sites. Additionally, 3 wk postpartum, all pups were killed via cervical dislocation and the heart, spleen, liver, and brain were excised and weighed.

**Bioluminescence imaging.** Pregnant female mice were imaged from GD 12 to 18 based upon previous results demonstrating that light emissions were not detectable prior to this time of gestation (13). Briefly, mice were anesthetized with isoflurane (1.5–3.0%) and s.c. injected in the dorsal neck region with luciferin (150 mg/kg; Caliper Life Sciences) suspended in Dulbecco’s PBS (15 g/L). Additionally, the abdominal region of the mice was shaven to reduce the effects of hair on light scattering and/or absorption. Twenty minutes after luciferin administration, mice were imaged for 5 min ventral side up using the IVIS 100 Imaging System (Caliper Life Sciences) while maintained under isoflurane (1.50–3.00%) anesthesia based upon previous work that demonstrated this timeline to be optimal for this particular model (13).

**Image analysis.** All images were analyzed using Living Image 2.50 software (Caliper Life Sciences). Measurements were made by drawing ROI on the bioluminescent images. A 4.25- × 5.5-cm primary ROI was drawn (Supplemental Fig. 1), covering the abdominal region of the mouse being measured while a smaller background ROI (1.50 cm × 1.90 cm) was positioned on the ventral neck region to correct for autofluorescence (19). The light emissions from background ROI were subtracted from the light emissions from the primary ROI. The bioluminescent data are presented as total light emissions (photons per second) relative to the Con group and as light emissions corrected for fetoplacental mass (p × μs−1·g−1) relative to the Con group, which was obtained by dividing the total light emissions for each day by the amount of weight gain relative to the initial body weight recorded on GD 1. In addition, daily bioluminescent data separated by treatment group are expressed as light emissions corrected for fetoplacental mass (p × μs−1·g−1).

**Statistical analysis.** Data were analyzed using ANOVA with repeated measures when appropriate. For repeated measures, least square means were calculated and separated using Tukey’s honestly significant difference when the P value from the ANOVA was <0.05. Least square means were considered to be significantly different at a value of P ≤ 0.05 and tended to differ at a value of P ≤ 0.10. Data are presented as least square means ± SEM. One mouse from the +Ala group was removed from the statistical analysis due to data points being identified as extreme outliers through a box-plot IQR analysis.

**Results**

**Reproductive performance and neonatal tissue weights.** Total feed intake did not differ among mice (P > 0.10), indicating that total feed consumption did not contribute to any observed differences (Table 1). However, mice receiving the +Arg diet consumed more arginine (1530 ± 45.3 mg) compared to +Ala group (332 ± 49.6 mg; P < 0.0001) (Table 1). Weight gain during GD 12 to 18 was 17% greater (P < 0.01) for mice in the +Arg group compared to those in the +Ala group (Table 1). Additionally, total litter size was 102% greater (P < 0.01) for mice in the +Arg group and the number of pups born alive to +Arg mice was 95% greater (P < 0.01) than that observed in the +Ala group (Table 1). Compared to the +Ala group, arginine supplementation during gestation increased (P < 0.05) the number of placental attachment sites by 66% (Table 1). Likewise, the litter birth weight was 83% greater (P < 0.01) for mice in the +Arg group compared to mice consuming the +Ala diet, and the litter weight of pups born alive was 78% greater (P < 0.01) when mice received arginine supplementation (Table 1). Conversely, the individual birth weight of pups born...
TABLE 1  Reproductive performance of mice fed either the +Ala or +Arg diet during gestation

<table>
<thead>
<tr>
<th>Dietary treatment</th>
<th>+ Ala</th>
<th>+ Arg</th>
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<tbody>
<tr>
<td>Feed intake</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total feed intake, g</td>
<td>59.3 ± 2.16</td>
<td>59.9 ± 1.97</td>
</tr>
</tbody>
</table>
| Total l-arginine intake, mg | 332 ± 49.6 | 1530 ± 45.3 *
| Reproductive performance |        |
| Weight gain (GD 12–18), g | 5.95 ± 0.260 | 7.01 ± 0.230 *
| Total pups born per litter, n | 4.20 ± 0.730 | 6.50 ± 0.670 *
| Total pups born alive per litter, n | 4.20 ± 0.750 | 6.17 ± 0.690 *
| Placental attachment sites, n | 5.80 ± 1.100 | 9.67 ± 1.00 **
| Litter birth weight of all pups born, g | 5.40 ± 0.870 | 9.88 ± 0.790 *
| Litter birth weight of all pups born alive, g | 5.40 ± 0.920 | 9.62 ± 0.840 *
| Individual birth weight of pups born alive, g | 1.32 ± 0.0500 | 1.18 ± 0.0400 **

1 Values are least square mean ± SEM, n = 5 (+Ala) or 6 (+Arg). Asterisks indicate different from +Ala: *P < 0.01; **P < 0.05. +Ala, L-alanine supplemented diet; +Arg, L-arginine supplemented diet.

alive was 11% less (P < 0.05) in pups born to +Arg dams compared to pups from dams consuming the +Ala diet (Table 1). When evaluating neonatal organ weights as a percentage of body weight 3 wk postpartum, no differences were found between dietary treatments for heart and spleen tissue weights. However, liver and brain tissue weights did differ. Interestingly, the liver weight of pups exposed to the +Arg diet during gestation was 5% less (P < 0.05) compared to pups exposed to the +Ala diet (Supplemental Table 2). In contrast, the brain weight of pups exposed to the +Arg diet during gestation was 18% greater (P < 0.0001) compared to pups exposed to the +Ala diet (Supplemental Table 2).

Bioluminescence assessment of fetoplacental Vegfr2 transcription activity. There were no diet × day interactions (P > 0.10) for fetoplacental Vegfr2 transcription activity, yet arginine supplementation increased (P < 0.01) the mean total Vegfr2 transcription activity in fetoplacental tissues by 56% during GD 12–18. Using bioluminescence imaging to assess total fetoplacental Vegfr2 transcription activity, a qualitative difference was also visualized between mice in the +Ala and +Arg groups (Fig. 1A). A pseudo-color scheme is applied to the images, with the red areas representing the greatest amount of Vegfr2 transcription activity and the purple-blue areas representing the least amount of Vegfr2 transcription activity. To this end, qualitative and visual differences were noted between mice consuming different diets.

To account for changes in fetoplacental mass, bioluminescent data were corrected by dividing the daily light emission values by the amount of weight gain experienced by the dam relative to GD 1 (Fig. 1,B–D). When the data were corrected for fetoplacental mass, the mean fetoplacental Vegfr2 transcription activity was 46% greater (P < 0.05) in mice in the +Arg group compared to those in the +Ala group (Fig. 1B). Moreover, when the data were separated by treatment group, the profiles for daily fetoplacental Vegfr2 transcription activity were different (Fig. 1C,D). In mice consuming the +Arg diet, fetoplacental Vegfr2 transcription activity remained unchanged (P > 0.05) from GD 13 to 15 and there were no daily increases during GD 12–18. In sharp contrast, for mice consuming the +Arg diet, fetoplacental Vegfr2 transcription did not change from GD 12 to 13 but did increase abruptly (P ≤ 0.05) on GD 14 and remained elevated throughout the remainder of gestation, revealing an earlier rise in Vegfr2 transcription occurring in tissues of the arginine-supplemented fetoplacental unit.

Discussion

Maternal nutrition during gestation is known to affect fetal growth and development through its influence on the intrauterine environment (20). Here, we report an association between maternal dietary L-arginine supplementation, enhanced reproductive performance, and fetoplacental Vegfr2 transcription activity using a novel bioluminescent mouse pregnancy model. The model utilized in the current study offers the ability to monitor fetoplacental Vegfr2 transcription activity noninvasively and longitudinally within the same mouse. Previously, we demonstrated that the data obtained using this approach parallel Vegfr2 gene expression data obtained from traditional molecular analysis (13,21). Not only does this approach reduce the total number of mice needed to monitor physiological events over time, but it also has the potential to reduce individual variation associated with end-point analysis by allowing more than one measurement to be obtained from the same mouse. Moreover, the results from the current study demonstrate that this novel bioluminescent model is capable of monitoring fetoplacental Vegfr2 transcription activity under experimental conditions during the latter one-third of mouse gestation in a longitudinal and noninvasive manner.

Arginine is a versatile amino acid, serving as a precursor for many molecules, including NO (3). NO mediates a number of reproductive processes in both males and females (22). In females, NO is produced in the endometrium (23) and is involved in embryo implantation and development (7,24,25). The importance of NO during implantation and embryo development along with the abundance of arginine found in porcine allantoic fluid reported by Wu et al. (4) suggest a crucial role for arginine in fetoplacental nutrition. Consistent with the previous findings observed in rats and pigs (5,6), dietary L-arginine supplementation enhanced the reproductive performance of mice by increasing the litter size and the number of placental attachment sites in the current study. With dietary L-arginine supplementation, though, the individual weight of the pups born alive was reduced, which is a known consequence of increased litter size in mice (26); however, the individual birth weights of the pups from +Arg dams were still within the normal range for the FVB/N mouse strain (27). Together, these results highlight the beneficial effect that dietary L-arginine supplementation has on mammalian reproduction and are in agreement with previous work performed by Zeng et al. (5), who reported an increase in litter size and number of placental attachment sites in rats supplemented with 1.3% (wt:wt) L-arginine. Interestingly, in the current study, gestational dietary L-arginine supplementation appeared to affect both the liver and brain weights of neonates; however, at this time, a definitive explanation for this phenomenon cannot be offered, suggesting the need for further investigations.

Because dietary L-arginine supplementation began on GD 1, which is well before the time of embryo implantation in mice (GD 4–5), it is tempting to speculate that the observed increased number of placental attachment sites suggests that arginine supplementation may have a positive influence on embryo implantation. Recent reports have demonstrated that arginine supplementation increases expression of inducible and endothel...
lial NOS at the implantation sites of rats (5). NO, an arginine metabolite, is produced by the endometrium (23), and the peri-implantation period in mice is characterized by elevated NOS expression at the site of implantation (7), which may suggest an important role for arginine in embryo implantation. Furthermore, inhibition of NO synthesis impairs embryo development at the 2-cell and morulae stages (24) and decreases the blastocyst development rate in cultured embryos (25), providing additional evidence of NO’s role in embryonic survival. It is also worth noting that embryos express amino acid transporters, and the activity of b<sup>0</sup>, a transport system for arginine, increases by 30-fold during the transition to the blastocyst stage (28). Taken together, the previous body of literature (5,7,24,25) and the current data warrant the need to further investigate whether arginine supplementation indeed influences embryo survival and implantation through an NO-mediated event, translating into enhanced mammalian reproductive performance.

In pigs, dietary l-arginine supplementation has also been proposed to enhance mammalian reproduction by altering angiogenesis during gestation (6). Angiogenesis is an important process during gestation, and without it, pregnancy demise may ensue (29,30). Expression of VEGF and its receptor (VEGFR2), which is upregulated in response to VEGF expression (31), is vital for proper angiogenesis to occur (8), making Vegfr2 an ideal marker for angiogenic activity. In the current study, dietary arginine supplementation positively influenced fetoplacental Vegfr2 transcription activity. Arginine might influence Vegf expression and, therefore, Vegfr2 expression by serving as a precursor for NO, which has previously been demonstrated to increase Vegf mRNA and protein in keratinocytes (32) and vascular smooth muscle cells (33,34). NO induces expression of hypoxia-inducible-factor-1 (35), a stimulator of Vegf transcription (36), providing further evidence that dietary l-arginine supplementation may alter angiogenesis by serving as NO precursor in fetoplacental tissues.

In the current study, dietary l-arginine supplementation resulted in an earlier increase in fetoplacental Vegfr2 transcription, indicating that l-arginine supplementation may create a more favorable in utero environment during earlier and enhanced vascular development of the fetoplacental unit. The Chinese Meishan pig is known to produce 3–5 more pigs per litter than U.S. and European breeds of pigs (37,38) and, interestingly, this particular breed exhibits an increase in placental Vegf and Vegfr2 gene expression earlier in gestation compared to the Yorkshire breed of pig at a time when the Chinese Meishan placenta becomes more vascularized relative to the Yorkshire placenta (39). The Vegf and Vegfr2 profiles of the Chinese Meishan pig placenta (39) are similar to the trend for Vegfr2 transcription activity in the fetoplacental unit observed in the current study whereby arginine supplementation resulted in an earlier rise in fetoplacental Vegfr2 transcription activity compared to the activity observed in the mice in the +Ala group. Interestingly, this earlier increase in fetoplacental Vegfr2 transcription activity observed in the +Arg mice occurs at a time during mouse gestation when the placenta undergoes rapid vascular development and the fetus experiences tremendous growth (10–12). This may suggest that dietary l-arginine supplementation promotes more favorable conditions for fetal survival in a uterine environment with more offspring and offers another explanation for the beneficial effects that dietary l-arginine supplementation has on mammalian reproduction.

In summary, dietary l-arginine supplementation increased the total number of viable pups born per litter, demonstrating the beneficial effect that l-arginine has on mammalian reproduction. Furthermore, we demonstrated a relationship between arginine supplementation and fetoplacental Vegfr2 transcription activity utilizing a novel bioluminescent mouse pregnancy model. Our results illustrate that the positive effects attributed to dietary arginine supplementation, in terms of litter size, are associated with more Vegfr2 transcription activity and an earlier
increase in Vegfr2 transcription activity in fetoplacental tissues, which may create a favorable environment for fetal survival during the latter one-third of gestation.

Acknowledgments

J.G., B.R., and P.R. conceived the study and participated in the design of the study; J.G., C.D., S.B., J.F., and P.R. performed all experiments; J.G. conducted the statistical analysis; and J.G., J.F., and P.R. interpreted the data and drafted the manuscript. All authors read and approved the final manuscript.

Literature Cited

9. Dumont DJ, Fong GH, Purcell TL, Given R, Chwalisz K, Garfield RE. Nitric oxide synthase transcription activity in fetoplacental tissues, which may create a favorable environment for fetal survival during the latter one-third of gestation.