Maternal citrulline supplementation enhances placental function and fetal growth in a rat model of IUGR: involvement of insulin-like growth factor 2 and angiogenic factors

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Abstract

Objective: To determine the effects of maternal citrulline supplementation on fetal growth and placental efficiency in a rat model of intrauterine growth restriction (IUGR) induced by maternal protein restriction.

Methods: Pregnant Sprague–Dawley rats were randomly assigned to three groups: NP (receiving a control 20% protein diet), LP (a 4% protein diet), or LP-CIT (an LP diet along with L-citrulline, 2 g/kg/d in drinking water). On the 15th and 21st day of gestation (GD15 and GD21, respectively), dams underwent a C-section, by which fetuses and placentas were extracted. The expression of genes involved in placental growth and angiogenesis was studied by quantitative RT-PCR.

Results: Maternal citrulline supplementation increased fetal weight at GD21, and fetal weight/placental weight ratio, an index of placental efficiency, from mid gestation (p<0.001). The expression of Igf2-P0, a placenta-specific variant of insulin-like growth factor 2 (Igf2) gene, and Vegf and Flt-1, involved in angiogenic pathways, was enhanced in the LP-CIT group (versus NP, p<0.001, p<0.01, and p<0.05 for Igf2-P0, Vegf, and Flt-1, respectively).

Conclusions: In a model of IUGR induced by protein deprivation, citrulline enhances fetal growth, placental efficiency, and the expression of genes involved in angiogenesis. The relevance of such effect in human pregnancies complicated by IUGR warrants further study.

Keywords

Amino acids, angiogenesis, growth factor, placenta, transporter

Introduction

Intrauterine growth restriction (IUGR) is a major cause of perinatal mortality and morbidity [1]. Additionally, growing evidence links this condition to the long-term risk of cardiovascular and metabolic disease in adulthood [2]. IUGR most commonly arises from maternal undernutrition in developing countries, and from impaired placentation in developed countries. However, regardless of the mechanism involved, fetal growth restriction results from an imbalance between the placental capacity to supply oxygen and/or nutrients and fetal demand [3]. Clinical trials aimed at increasing placental blood flow through NO-donor administration or angiogenic factor gene therapy (EVERREST Project – NCT02097667) are currently ongoing. Yet manipulation of maternal diet also warrants investigation as an alternative, noninvasive approach. Arginine is the only endogenous precursor of NO, which is thought to improve placental vascular regulation, and may thus to be helpful in human pregnancies complicated by preeclampsia or IUGR [4]. Unfortunately, clinical trials of arginine supplementation have produced disappointing results in IUGR [5]. Increased arginine degradation by liver arginase or placental arginase and/or increased concentration of asymmetric dimethylarginine (ADMA), a competing analog of L-arginine, are observed in human IUGR [6,7], and may account for such lack of efficacy. Citrulline, a nonprotein amino acid that is virtually absent from regular diet, is a precursor of arginine, and is an attractive alternative nutrient as it escapes liver metabolism, has high bioavailability, and is quantitatively converted to arginine in vivo [8]. In a recent study, we showed that this amino acid improves fetal growth in an animal model of IUGR induced by maternal dietary protein restriction [9]. The mechanisms, however, remain to be identified particularly regarding placental changes since increased NO production, hence a possible NO-regulated angiogenesis as well, was found to be involved. The objectives of the current study therefore were to determine whether citrulline started early in gestation enhances the angiogenic pathways in placenta, and
hence prevents fetal growth restriction. To our knowledge, the current study is first to address the time-dependent effects of citrulline on placental function over the course of gestation.

**Methods**

**Materials**

Citrulline was purchased from Inresa (Bartenheim, France), and the drinking solution was prepared three times/week using deionized water, and then stored at 4 °C until use.

**Experimental design**

All procedures were carried out in accordance with current institutional guidelines on animal experimentation in France with the approval of the animal Ethics Committee of Pays de La Loire (Protocol number CEEA.2010.8).

Pregnant Sprague–Dawley rats were purchased from Janvier Laboratories (Le Genest Saint Isle, France). Delivered to our animal facilities on the 1st gestational day (GD1), they were individually housed under controlled conditions (22°C, 12:12-h dark–light cycle) and had free access to an adequate protein diet (NP, 20% protein) or an isoenenergetic, low protein diet (4% protein). Diets were customized and provided by Arie Block BV (Woerden, The Netherlands). Pellet intake was recorded daily. On GD2, dams of the low protein group were randomized to be supplemented or not by citrulline-enriched water. Drink intake was monitored daily so as to ensure citrulline was administered orally at a dose of 2 g/kg/d. They all had free access to additional water besides.

On gestational day 15 or 21 (GD15 or GD21, respectively), dams (3–4 rats/diet/gestational day) were anesthetized with iso-flurane and then underwent a cesarean section. Fetuses and placentas were weighed individually. Placentas were harvested, rapidly rinsed in cold 0.9% NaCl solution, then snap-frozen in liquid nitrogen and stored at −80°C until further analysis, or fixed in 4% paraformaldehyde for 48 h before being embedded in paraffin.

**Real-time quantitative PCR**

Total mRNA was purified from 30 mg-placental samples (whole thickness section) (n = 9–12/diet/gestational day) using the RNA isolation kit (NucleoSpin RNA, Macherey-Nagel, Germany) following the manufacturer’s instruction. The RNA was quantified using the Nanovue spectrophotometer (GE Health Care, France), and its integrity was confirmed by agarose gel electrophoresis. Complementary DNA (cDNA) was synthesized from 2 μg of mRNA using random hexamers (Promega) and the M-MLV reverse transcriptase (Promega).

The relative expression of mRNA transcripts was measured by quantitative real-time PCR (qRT-PCR). Two reference genes were used as internal control, Gapdh, and Ywhaz. They were chosen and validated using the Gennorm® Software (https://genorm.cmgg.be/).

A dissociation melt curve analysis was performed to demonstrate ampiclon homogeneity. The reaction mix for qRT-PCR consisted of 7 μl of 1 SYBR Green (Biorad®), 1.5 μl of each primers (2.5 mM), and 5 μl of 1/40 diluted cDNA. A negative control was included for each primer pair. mRNA expression was calculated using the 2−ΔΔCt method [10]. Control group values were used as calibrator.

**Placental morphologic analysis**

Paraffin-embedded placentas were cut into 6 μm-thickness sections. Placentas were stained with hematoxylin and eosin for general morphology. The central third part was identified by the presence of maternal artery channel as described elsewhere [11]. The thickness of each individual zone, namely labyrinthine and junctional spongiosplastablast zones (LZ and SZ, respectively), was measured by tracing perpendicularly to the fetal side with the aid of the NanoZoomer Digital Pathology System (Hamamatsu, Japan).

**Statistical methods**

Data are expressed as means ± SEM. Kruskal–Wallis test followed by Mann–Whitney U test were used for between groups comparison. Statistical analysis was performed using GraphPad Prism 5.0® (San Diego, CA), and results were considered significant if p values was <0.05.

**Results**

As expected, at 15 days of gestation, no effect of maternal dietary protein restriction on fetal weight was detected. Yet the low protein diet (LP) resulted in IUGR with a 9.1% decrease in fetal weight near term (at day 21), in comparison with NP (p<0.01). Citrulline administration increased fetal weight by 4%, compared with LP at day 21 (p<0.01) (Figure 1A, see Table 1 in Supplemental online material for more details).

The ratio of fetal mass to placental mass (FPR) is commonly used as an index of placental efficiency [12]. At day 15, a significant increase in FPR was found in both restricted groups, and the rise was further enhanced in the group receiving citrulline, compared with the unsupplemented group (Figure 1B). Nonetheless, this effect was sustained throughout gestation only in LP-CIT group (LP-CIT versus LP, p<0.001) (Figure 1C). The surface area for maternal-fetal exchange was estimated by the thickness of LZ, as described in “Methods” section. It was significantly reduced in the LP group and simultaneously enhanced in the LP-CIT group by the end of gestation (Figure 1D).

The expression of Igf2 gene was measured on GD15 and GD21. Maternal protein restriction did not alter the expression of total Igf2 on GD15, but enhanced its expression on GD21 (LP versus NP, p<0.01) (Figure 2A and B). Noteworthy, there was a trend toward enhanced transcription of Igf2 on GD15 (LP-CIT versus NP, p = 0.0502) and the rise achieved significance at the end of gestation with citrulline administration (LP-CIT versus LP, p<0.01) (Figure 2B). We studied the P0 transcript of Igf2, a variant that is exclusively expressed in the LZ of murine placenta [13]. This transcript was overexpressed early from mid gestation through the end of gestation with citrulline (p<0.001), whereas the rise in expression was only significant by the end of gestation in LP group (Figure 2C and D). The genes Igf1r (Insulin-like growth factor 1 receptor) and Igf2r (Insulin-like growth factor 2...
receptor) were significantly more expressed in LP-CIT than in LP group on GD15 (Figure 2E and F).

Because angiogenesis contributes to the development of placental vascular system in LZ, we studied the expression of the gene encoding Vascular Endothelial Growth Factor (VEGF) and its receptor Flt-1 (FMS-related tyrosine kinase 1) on GD15. The transcription of both genes was significantly enhanced in the group receiving citrulline (Figure 3A and B).
We designed the primers in order to separately estimate the mRNA level of two distinct variants: Flt-1 and soluble VEGFR-1 (sFlt-1). The relative expression of sFlt-1 was reduced in LP-CIT, in comparison with NP (p < 0.05) (Figure 3C).

The activation of either growth factor or angiogenesis pathway might involve the trophoblast apoptotic state. The \textit{Bcl2} (B-cell CLL/lymphoma 2) and \textit{Bax} (Bcl2-associated X protein) genes encode antiapoptotic and proapoptotic regulators, respectively, and their transcription level was studied on GD15. \textit{Bcl2}/\textit{Bax} ratio transcription level was found to be enhanced in LP-CIT (Figure 3D).

The \textit{Igf2}-P0 transcript was proven to be a mediator of placental adaptive responses in case of undernutrition through modifications in System A placental amino acid transporters (SNAT1 and SNAT4) [14] encoded by the genes \textit{Slc38a1} and \textit{Slc38a4}, respectively. These two genes were overexpressed in the LP-CIT group at GD15 (Figure 3E and F); however, such effect was no longer detected on GD21 (Figure 4A and B in Supplemental online material).

**Discussion**

**Main findings**

In recent studies, we demonstrated that maternal dietary citrulline supplementation enhanced fetal growth in an animal model of IUGR [9]. The current study not only confirms the benefit of citrulline supplementation on fetal growth but also demonstrates that citrulline supplementation impacts the expression of placental genes playing a key role in placentation, angiogenesis, and maternal–fetal nutrient exchange.

**Impact of citrulline supplementation on placental \textit{Igf2} expression**

In the current study, the upregulation of placental IGF2 was observed in dams receiving citrulline supplementation. In rodents, \textit{Igf2}-P0 is expressed exclusively in trophoblast cells of LZ where maternal–fetal exchange takes place [13]. In our study, P0 transcript was upregulated early (on GD15) and maintained throughout gestation in dams receiving citrulline. Such increase may account for the increased LZ surface area found on GD21 in LP-CIT.

The role of \textit{Igf2}-P0 was clearly established by deleting or partially inactivating its transcription in mouse placenta [14]. Deficient \textit{Igf2}-P0 transcription impairs the growth of placentas, several days before it impacts fetal growth [15], suggesting \textit{Igf2}-P0 plays a significant role under conditions of malnutrition, but this effect may operate only over a limited time window, mainly in early and mid-gestation, the critical stages of placental development. Though upregulation of nutrient transporters has been observed, \textit{Igf2}-P0 null placentas show a lesser surface area for exchange and a thicker barrier between the maternal and fetal vessels in the labyrinthine zone compared to their wild-type counterparts [16].

In our study, we found a trend toward upregulation of \textit{Igf2}-P0 transcription throughout gestation in dams fed an LP diet, but the rise was significant as early as GD15 only in dams.
receiving citrulline. The rise in \( \text{Igf2} \) was accompanied by a decrease in \( \text{Slc38a1} \) and \( \text{Slc38a4} \) expression at mid gestation; however, this diminution was effectively corrected with citrulline as demonstrated in our results. Such finding suggests early and timely administration of citrulline may interact with the adaptation of placenta to undernutrition, trigger the adaptive transport by system A, and enhance the growth of fetuses born from undernourished dams. In fact, in our restricted dams supplemented with citrulline, we observed enhanced expression of \( \text{Slc38a1} \) and \( \text{Slc38a4} \) but this was limited to GD15, which underlines the time-dependent impact of \( \text{Igf2-P0} \) transcript on the SNAT transporter system.

Alternatively, because citrulline is transported by system A, the ability of citrulline to increase SNAT activity may enhance local citrulline availability, and thereby stimulate its target, i.e. endothelial NOS/NO machinery, in arterial endothelial cells [17]. It is noteworthy that \( \text{eNOS} \) transcription was enhanced in LP-Cit without a significant change in \( \text{iNOS} \) mRNA level on GD15 in the present study (data not shown). Since \( \text{eNOS} \)-derived NO can strongly induce the \( \text{iNOS} \) expression under some circumstances [18], this finding suggests that citrulline administration may not activate \( \text{iNOS} \), thus avoiding the harmful effects of excess NO production associated with inflammatory response.

Implication of Igf2 in placental angiogenesis

The present study suggests the effect of citrulline supplementation may involve improved establishment of placental vascular network. In fact, our study suggests that the placental adaptation to low protein diet could be mediated through IGFII, and might induce the expression of genes in VEGF pathway, as \( \text{Vegf} \) and Flt-1 m-RNA transcription was substantially increased. Indeed, VEGF is known to be a trigger for early vasculogenesis and an important regulator of angiogenesis in placental vascular growth [19]. This ligand exerts its multiple biologic effects through its interaction with two receptor tyrosine kinases, Flt-1 (VEGF receptor 1, VEGFR-1) and KDR (VEGF receptor 2, VEGFR-2). Flt-1 is required for endothelial morphogenesis, whereas VEGFR-2 is involved primarily in mitogenesis [20]. We measured the expression of both Flt-1 and sFlt-1, the latter encoding a soluble variant known to be an endogenous inhibitor of VEGFR-1 [21]. There was a trend toward an increased Flt-1 to sFlt-1 ratio (\( p = 0.07 \), LP versus LP-CIT). In other models of preeclampsia/IUGR induced by hypoxia or ischemia, sFlt-1 production is known to be clearly increased [22]. As our model did not involve hypoxia, we speculate that this may reflect an adaptive response of placenta to undernutrition, designed to prioritize angiogenesis rather than placental growth. Citrulline-supplementation predominantly affected functional receptor (Flt-1) expression so citrulline may improve the efficacy in adaptive responses to the low protein diet.

In the present study, the enhanced expression of \( \text{Igf2} \) and its receptors, especially \( \text{Igf2r} \), may be linked to the simultaneous increased transcription of VEGF and Flt-1, especially in the absence of a hypoxic trigger. Indeed, Herr et al. showed that IGFII regulates placental growth by promoting endothelial cell migration and uterine vessel formation under physiological conditions, \( \text{in vitro} \) and \( \text{in vivo} \) [23]. Interestingly, the expression of \( \text{Igf1r} \) and \( \text{Igf2r} \) was enhanced on GD15 only in LP-CIT. This finding is in accordance with our initial hypothesis whereby citrulline administration may enhance the ability of placenta to survive under adverse nutritional conditions, and this ability is mediated through IGFII, its placental variant, and their receptors.

A crosstalk between VEGF and Bcl2 has been demonstrated by several \( \text{in vitro} \) studies. The addition of VEGF to serum-starved human umbilical vein endothelial (HUVE) cells enhanced the expression of Bcl2 after 36 h [24]. Interestingly, HUVE cells grown in standard 10% fetal calf serum did not respond to VEGF stimulation. Early dietary protein restriction in pregnant rats was found to blunt the vasodilatory effect of VEGF on uterine artery [25]. In such setting, the significant rise in Flt-1 transcription we observed in LP-Cit (versus LP), in combination with the enhanced induction of Bcl2 and increased LZ thickness, suggests that citrulline improved the efficacy of VEGF pathway. The underlying mechanisms warrant further investigation.

Finally, the current findings raise two intriguing questions. First, is the effect observed on placental gene expression specific for citrulline? Even though no placebo was tested in the current study, the effect of citrulline is unlikely to be due to nitrogen supply \( \text{per se} \), since supplementation of pregnant rats with an isonitrogenous mix of nonessential amino acids failed to improve fetal growth [9]. Second, is the effect of citrulline relevant to human pregnancies affected by IUGR? The experimental model used likely is relevant for the IUGR observed in the developing world, where >30 million infants are born with IUGR yearly as a consequence of maternal undernutrition [1]. In contrast, alterations in placental blood flow and preeclampsia are the leading causes of IUGR in Western societies. However, literature evidence suggests that both settings share similarities. Protein restriction or maternal undernutrition reduce placental blood flow in pregnant ewes and rodents [26], and impair vasodilation in uterine arteries in pregnant rats [25]. Similarly, impaired placental transport of amino acids has been found to precede growth alterations in a rat model of IUGR induced by low protein diet [27], implying decreased amino acid transport may be a cause, rather than a consequence of IUGR in that model. As mentioned above, alterations in placental amino acid transport have been documented in human IUGR of vascular origin [28]. Such evidence collectively argues for the potential relevance of the experimental model used in the current study for human IUGR. In addition, the recent trial of citrulline supplementation by Powers et al. in obese pregnant women did not mention any adverse outcomes [29]. The latter trial was also the sole clinical trial to date published regarding citrulline supplementation in pregnancy, and showed a significant decrease of uterine pulse index, which reflected improved placentation.

Conclusion

In summary, the current report confirms maternal oral supplementation with citrulline enhances fetal growth and placentation in a rat model of IUGR induced by a low protein diet. Our findings furthermore support the hypothesis...
that citrulline ameliorates placental adaptation by enhancing the expression of Igf2, and the expression of pivotal genes for placental angiogenesis and survival. The putative benefit of dietary citrulline supplementation in human pregnancies with IUGR would clearly warrant investigation.

Acknowledgements

We thank Guillaume Poupeau and Vincent Dochez for their help with surgical procedures, Myriam Robard and her technicians for their staining procedure of placentas. N.-T.T., A.B., N.W. and D.D. designed the research; N.-T.T., A.B., V.A., E.M. and I.G. conducted the experiments; N.-T.T., V.A. and D.D. wrote the article; N.W. and D.D. had primary responsibility for final content. All authors read and approved the final manuscript.

Declaration of interest

None of the authors had any conflict of interest. N.-T.T., V.A. and D.D. had primary responsibility for final content. All authors read and approved the final manuscript.

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Supplementary material available online