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Allah Haafiz[1], Yuan-Xiang Pan[2], Donald Novak[1]*

Placental Amino Acid Transport System A – an Assessment of SNAT1 and SNAT2 Expression in F1 and F2 Placentas in a Rat Model of Gestational Protein Restriction*

System A łożyskowego transportu aminokwasów – ocena ekspresji SNAT1 i SNAT2 w łożyskach pokolenia F1 i F2 na szczurzym modelu ograniczenia podaży białka w ciąży

[1] Department of Pediatrics, University of Florida College of Medicine, USA
[2] Department of Food Science & Human Nutrition, University of Illinois at Urbana-Champaign, USA

Abstract

Background. Gestational protein restriction (GPR) can program a fetal phenotype prone to develop metabolic syndrome in successive generations. Although mechanisms are not well characterized, placental amino acid transport system A (SysA) activity is depressed in the setting of GPR.
Objectives. To determine mechanisms of GPR-induced SysA-adaptations in F1 and F2 placentas.
Material and Methods. Rats (F0) were pair-fed either a 19% normal protein diet (NPD) or an 8% low protein diet (LPD) through pregnancy and lactation. F1 placentas were studied for SNAT1 and SNAT2 mRNA and SNAT1 protein. Male and female offspring (F1) were bred to control animals and allowed to deliver at term at which time placentas were collected for the same studies. Transient transfection of HEK 293 cells was done using p-CMV-FLAG-SNAT2 or the control vector. After 36 hours of transfection, MeAIB transport, expression of SNAT2 mRNA and proteins were assessed.
Results. In F1 placentas, steady-state mRNA content of SNAT1 (140 ± 13 vs. 99 ± 11 arbitrary mRNA units; $p \leq 0.01$) and SNAT2 (81 ± 6, n = 10 vs. 104 ± 9 arbitrary mRNA units; $p \leq 0.001$) were higher in LPD than NPD group. An opposite but non-significant trend in mRNA expression of both isoforms was evident in F2 placentas. Despite up-regulation of mRNA in F1 placentas, SNAT1 immunoblot bands were comparable from placental-apical-membranes (0.62 ± 0.13 vs. 0.63 ± 0.13 arbitrary units; p = 0.9), basal-membranes (0.9 ± 0.14 vs. 1 ± 0.06 arbitrary units; p = 0.6) and placental-homogenates (0.5 ± 0.16 vs. 0.7 ± 0.1 arbitrary units; p = 0.3) between LPD and NPD group. Similar results were seen in F2 placental SNAT1 protein expressions. SNAT2- mRNA over-expression by transient transfection with pLPCX-FLAG-SNAT2 construct vs. control vector in HEK 293 cells resulted in up-regulation of both SNAT2 protein and Na+ dependent MeAIB transport (1243 ± 137 vs. 390 ± 27 pmole $^{-1}$mg $^{-1}$ min; $p \leq 0.0008$).
Conclusions. 1. Although GPR-induced SysA repression is associated with up-regulation of SNAT1 and SNAT2 mRNA in F1 placentas, the protein content is unchanged suggesting post-transcriptional regulation of SysA expression and function. Up-regulation of SNAT2 protein and transport activity following SNAT2-mRNA-over-expression noted in our tissue culture studies support this conclusion. 2. If maternal nutrition is optimized, GPR-induced SysA F1 placental abnormalities are not replicated in F2 placentas (**Adv Clin Exp Med 2010, 19, 5, 563–572**).

Key words: nutrient gene interaction, fetal programming, fetal origins of adult disease, metabolic syndrome, amino acid transport system A.

Streszczenie

Wprowadzenie. Ograniczenie podaży białka w ciąży (GPR) może zaprogramować u płodu fenotyp skłonności do wystąpienia zespołu metabolicznego w kolejnych pokoleniach. Chociaż mechanizmy tego zjawiska nie są dobrze poznane, wiadomo, że w warunkach GPR aktywność łożyskowego systemu A transportu aminokwasów (SysA) jest ograniczona.

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Cel pracy. Ocena mechanizmów przystosowania się SysA pod wpływem GPR w łożyskach pokoleń F1 i F2.
Materiał i metody. U szczurów (F0) podczas ciąży i laktacji zastosowano dietę z 19% odpowiednią zawartością białka (NPD) i z 8% małą zawartością białka (LPD). W łożyskach F1 badano zawartość białek SNAT1 i SNAT2 mRNA i SNAT1. Potomstwo płci męskiej i żeńskiej (F1) krzyżowano ze zwierzętami z grupy kontrolnej. Młode rodziły się o czasie, wtedy pobierano łożyska do badań. Wykonano przejściową transfekcję komórek HEK 293 za pomocą p-CMV-FLAG-SNAT2 lub wektora kontrolnego. Po 36 godzinach transfekcji oceniono transport MeAIB, ekspresję SNAT2 mRNA i białka.
Wyniki. W łożyskach F1, zawartość SNAT1 w stanie równowagi mRNA (140 ± 13 *vs* 99 ± 11 umownych jednostek mRNA, $p \leq 0{,}01$) i SNAT2 (81 ± 6, n = 10 *vs* 104 ± 9 umownych jednostek mRNA, $p \leq 0{,}001$) była większa w grupie LPD niż w grupie NPD. Odwrotna, ale nieistotna tendencja w ekspresji mRNA obu izoform była widoczna w łożyskach F2. Mimo zwiększonej aktywacji mRNA w łożyskach F1, prążki SNAT1 błon szczytowych łożyska (0,62 ± 0,13 *vs* 0,63 ± 0,13 umownych jednostek, p = 0,9), błon podstawnych (0,9 ± 0,14 ± 0,06 *vs* 1 umownych jednostek, p = 0,6) i homogenat łożyska (0,5 ± 0,16 *vs* 0,7 ± 0,1 umownych jednostek p = 0,3) były porównywalne między LPD a grupy NPD. Podobne wyniki obserwowano w ekspresji białka SNAT1 w łożyskach F2. Nadekspresja SNAT2 w mRNA przez przejściową transfekcję pLPCX-FLAG-SNAT2 *vs* wektor kontrolny w komórkach HEK 293 powodowała zwiększoną regulację zarówno białka SNAT2, jak i Na^+ zależnego transportu MeAIB (1243 ± 137 *vs* 390 ± 27 pmol jednostki; $p \leq 0{,}0008$).
Wnioski. Chociaż ograniczenie SysA wywołane przez GPR jest związane z nadmierną aktywacją SNAT1 i SNAT2 w mRNA w łożyskach F1, zawartość białka nie zmienia się, co sugeruje potranskrypcyjną regulację ekspresji i czynności SysA. Nadmierna aktywacja białka SNAT1 i transport zachodzący pod wpływem nadekspresji SNAT2-mRNA wykryte w badanych tkankach potwierdza ten wniosek. Jeśli stan odżywienia matki jest optymalny, to zaburzenia SysA wywołane przez GPR w łożyskach F1 nie są replikowane w łożyskach F2 (**Adv Clin Exp Med 2010, 19, 5, 563–572**).

Słowa kluczowe: interakcja gen żywienie, programowanie płodu, płodowe pochodzenie chorób dorosłych, zespół metaboliczny, system A transportu aminokwasów.

Compromised intrauterine growth is well recognized for its relationship with subsequent insulin resistance, a prime biochemical aberration linked to adulthood metabolic syndrome (MetS) [1–4]. MetS and the related cardiovascular complications are emerging global health hazards affecting 30–35% of Americans [5, 6] and comparable European populations [7]. Although intra-uterine growth-retardation (IUGR) can result from distinct etiologies, maternal malnutrition continues to be the most important preventable cause of IUGR, especially in developing countries. Underlying genomic changes linking IUGR and subsequent evolution to insulin resistance remain elusive. To elucidate these mechanisms, rodents are commonly utilized to model IUGR, often through gestational protein restriction (GPR). Indeed low-protein diet (LPD) during rodent-pregnancy induces IUGR, proportional to the degree of protein deprivation [8]. As such the link between maternal-GPR and evolution of typical biochemical and phenotypic displacements in subsequent generations is well established [9, 10]. In addition, more recently, using similar rodent model, we have documented that MetS-prone phenotype can be transmitted by paternal-GPR as well [11], expanding the scope of possible fetal metabolic changes (a.k.a "fetal programming") destined to emerge as MetS in successive generations.

Whereas these developments firmly establish the link between GPR and fetal metabolic programming, fundamental questions remain: what is the scope of placental-fetal adaptations in the setting of GPR; how do these adaptations impact fetal metabolic programming; can we utilize these adaptations to assign a relative risk of developing MetS-prone-phenotype in a given infant with IUGR; and if so, what are the most critical time-frames and interventions necessary to interrupt the metabolic and phenotypic evolution. Concerted efforts encompassing both basic and translational studies are required to answer these complicated and intertwined questions. The critical initial steps to answer these questions are tied to elucidating placental-fetal adaptive changes in the setting of GPR. Using a rat model of GPR, we have documented that placental amino acid transport System A (SysA) expression and function is regulated in response to GPR [12, 13]. Furthermore, most recently, we have documented the evolution of MetS-prone phenotype in F2 animals descending from both males and females subjected to GPR [11]. The most remarkable placental adaptation noted in this study was the significant down-regulation of SysA activity in first generation placentas, which was briskly restored when maternal diet was optimized in second generation pregnancies. The aim of the work presented here was to understand the mechanisms of placental-SysA repression in the setting of GPR. Using the same model and protocol involving two generations, we have investigated the placental sodium-coupled neutral amino acid transporter gene member 1 (SNAT1) protein and mRNA expression of SNAT1 and SNAT2, the two ubiquitous SysA-isoforms. Quite interestingly, as reported in the

result section, contrary to SysA function, transcriptional activity of both SNAT1 and SNAT2 were upregulated in first generation placentas, indicating a post-transcriptional control of SysA activity in the setting of GPR. Using tissue culture system, we then addressed the most logical next question: in biological systems, without feedback inhibitions, how efficiently is the transcriptional over-expression of SysA genes translated into enhanced SysA-activity? We addressed this question by transient transfection of highly transfectable HEK 293 cells with a FLAG-containing vector by inserting SNAT2-full-coding-sequence in the construct.

Material and Methods

Animal Studies

Study Design

All studies were approved by the Institutional Animal Care Committee at the University of Florida. Timed-pregnant Sprague-Dawley (Harlan) rats were obtained on day 5 of gestation and weight matched into low protein (8% protein diet, n = 22) and control (19% protein diet, n = 22) groups. Animals were pair-fed throughout pregnancy as we have described [14]. Maternal animals and pups (F1) were continued on their respective diets until day 21, at which time standard rat chow was given *ad libitum*. On approximately day 70 of life, both control (N = 9) and experimental F1 females (N = 10) were mated with "outside" Sprague-Dawley males; whereas, control (N = 12) and experimental males (N = 10) were mated with "outside" primiparous females of approximately the same age. These animals were offered normal diet and allowed to deliver at term when placentas were collected for the downstream analysis. All animals were housed with *ad libitum* access to water and 12 hour light-dark cycles.

Diets

Diets were purchased from Purina Inc (Indianapolis, IN). First generational experimental animals (LPD) received an 8% protein diet that consisted of 71.4% carbohydrate, 8% protein, 10% fat, 4.3% fiber. The control animals (NPD) received an isocaloric 19% protein diet that contained 60.6% carbohydrates, 19.3% protein, 10% fat and 4.3% fiber.

Sample preparation

Preparation of placental apical and basal predominant membrane vesicle/protein and enzyme determinations: Apical and basal enriched membrane preparations were prepared as previously described [15]. Similarly, activities of alkaline phosphatase, a marker for the apical membrane (AM), and [^{3}H] dihydroalprenolol binding, a marker for the basal membrane (BM), were determined by methods used previously [15]. Membrane protein content was determined by Lowry's method utilizing bovine serum albumin as standard.

RNA isolation from placenta: Total cellular RNA was isolated from single placentas using the Qiagen RNeasy Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Briefly, 20–30 mg of placenta was homogenized under liquid nitrogen and 600 µL of the manufacturer's RLT buffer (containing 10 µL/mL of β-mercapthoethanol) was added to the homogenate. The mixture was loaded onto a QIAshredder spin column and centrifuged for 2 minutes at 13,000 × *g* for 2 min at room temperature. The lysate was then mixed with an equal volume of 70% ethanol and passed through an RNeasy column by centrifugation at 10,000 × *g* for 1 minute. The column was washed once with 700 µL of the manufacturer's RW1 buffer and twice with 500 µL of Qiagen's RPE buffer, each wash consisted of a 1 min centrifugation at 10,000 × *g*. The column was centrifuged again for 1 min at 13,000 × *g* to remove any residual wash buffer. For elution of the RNA, 50 µL of RNase-free water was added to the column and incubated at room temperature for 1 minute before centrifuging the column for 1 min at 10,000 × *g* to collect the eluted RNA.

Immunoblotting

Proteins were isolated from placental membrane preparations as noted before. Protein aliquots (50 µg/lane) were separated on 10% SDS-PAGE using standard techniques and electrotransferred to a 0.45-µm nitrocellulose membrane. Immuno-blotting was performed by using anti-SNAT1 polyclonal (primary) antibody as described by Varoqui [16]. Immunoreactive bands were detected with goat anti-mouse antibody conjugated to horseradish peroxidase (HRP) and detected by enhanced chemiluminescence. Bands were analyzed by densitometry using Quantity One (Bio-Rad) software.

Measurement of Steady-state mRNA Expression

qRT-PCR: To measure the relative amount of the SNAT1 and SNAT2 mRNAs, separate quantitative real-time RT-PCR analysis were performed with a DNA Engine Opticon 2 system (MJ Research, Reno, NV) using the detection of SYBR Green I (Applied Biosystems Inc.). Each RNA sample was measured at least in duplicate. One

hundred ng of total RNA was used in each reaction. The reactions were first incubated at 50°C for 30 min followed by 95°C for 15 min and then amplification of 35 cycles of each at 95°C for 15 s, 60°C for 60 s for SNAT2 and 62.5°C for 60 s for SNAT1. The primers utilized were SNAT1: (forward 5'-3' TCAGCCTGGTACGTCGATGG, reverse 5'-3' CCAGGTTCTTCAAGAGACACAG), SNAT2: (forward 5'-3' AGAGCAATTCCAGTATTAGC, and reverse 5'-3' TTAATCTGAGCAATGCGATTGTG). After PCR, melting curves were acquired by stepwise increase of the temperature from 55°C to 95°C to ensure that a single product was amplified in the reaction. For an endogenous control, separate reactions were done for rat β-actin and asparagine synthetase mRNA. There was no difference in the expression of these genes between control and experimental placentas.

Tissue Culture Studies

Generation of FLAG-SNAT2 Vectors

pCMV-Tag2c (Stratagene) was used to generate a pCMV-FLAG-SNAT2 construct. A 2.5 kb cDNA fragment of rat SNAT2 containing the entire coding region and 900 bp of 3'-untranslated region was generated by XhoI restriction enzyme digest from a 2.7 kb cDNA fragment cloned in plasmid pcDNA3.1. The 2.5 kb fragment was then sub-cloned into the XhoI site in the multiple cloning region of the pCMV-Tag2c vector. The FLAG tag was oriented to be upstream to and in frame with the SNAT2 translation start codon. To eliminate a stop codon (TGA) that existed at 22 nt upstream of the SNAT2 ATG start codon, site-directed mutagenesis was conducted to mutate T to G to make GGA (Gly). The FLAG-orientation and proper alignment with SNAT2 was confirmed by DNA sequencing. Similar steps were performed to generate pLPCX-FLAG-SNAT2 construct. Preliminary studies confirmed comparable transfection efficiencies of both SNAT2 containing vectors and were therefore used interchangeably for transcriptional and transport studies. In each case, concordant FLAG-containing empty vectors were used as controls.

Transient Transfection with FLAG-SNAT2 Constructs

Typically HEK 293 cells (1.5×10^6) were platted on 60 mm dishes and grown to 50% confluence. Transfections were then carried out in triplicate either using p-CMV-FLAG-SNAT2 or the control vector using Fugene 6 (Roche, Indianapolis, IN) according to the manufacturers guidelines. The cells were grown for 36 hours at 37°C in a humidified atmosphere of 5% CO_2/95% air before downstream protein or mRNA analysis (*vide infra*).

RNA Isolation

Total cellular RNA was isolated from cells using the Qiagen RNeasy Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. For 60 mm dishes medium was aspirated and 600 μL of the manufacturer's RLT buffer containing 10 μL/mL of β-mercapthoethanol was added to each dish. The rest of the steps were identical to description in RNA isolation noted in previous section.

Protein Extraction and Immunoblotting

At completion of 36 hours of transfection the media was discarded and cells resuspended in 1 mL of lysis buffer and proteins isolated. Proteins were separated on a 7.5% SDS/PAGE and electrotransferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were incubated with 3% blocking solution (3% (w/v non-fat dry milk, 50 mM Tris-base, 0.138 M NaCl, 2.7 mM Kcl, pH 8) at room temperature for 30 minutes with 50–60 rpm agitation. Immunoblotting was performed using anti-FLAG M2 monoclonal antibody (Sigma) at 1:1000 dilutions with TBS and 3% nonfat dry milk at room temperature for 30 minutes. The blots were washed 1 × 5 min in TBS on a shaker and then incubated with peroxidase-conjugated goat anti-mouse secondary antibody (Life technologies, Gaithersburg, MD) at a 1:20000 dilution for 30 min at room temperature. The blots were then washed for 5 × 4 min in Tris-buffered saline/Tween (50 mM Tris-base, 0.138 M NaCl, 2.7 mM Kcl, pH 8 plus 0.05% tween 20).The bound secondary antibody was detected by using an enhanced chemiluminescence kit (Amersham Biosciences) and exposed to Biomax MR film (Kodak).

Transport Studies

Wells were coated with acid soluble calf skin collagen (Sigma ac-3511) at 10 μg in 0.5 ml water/ /well and left on room temperature under ultraviolet light overnight. Next day the fluid was aspirated and the wells were air dried for 4 hours under ultraviolet light. 393 T cells were seeded at 0.05 million cells/well. At 24 hours when the cells were 60–70% confluent, transfections were performed in 6 wells with 0.2 ug DNA/well containing either pCMV-Tag2c-SNAT2 or pCMV-Tag2c control vector using Fugene 6 transfection reagent. Cells in six wells were grown with out transfection as a negative control.

Transport Assay: Cells were incubated at 37°C twice for 15 min each in sodium-free Krebs-Ring-

ers phosphate buffer (choline-KRP). To initiate transport, [^{3}H]-MeAIB in 250 µl of either NaKRP (sodium-containing Krebs-Ringers phosphate buffer) or choline-KRP (37°C) was added simultaneously to each of the wells for 1 min. The transport measurement was terminated by discarding the radioactivity and rapidly washing the cells five times with 2 ml of ice-cold choline-KRP. The Na^+-dependent transport is taken as the difference between uptake in NaKRP and choline-KRP. After air drying, cellular protein was precipitated with 10% TCA, and the supernatant radioactivity analyzed by liquid scintillation counting. Proteins were solubilized in 0.2 N NaOH/0.2% SDS and analyzed for total cellular protein. The data are expressed as pmol·mg^1 protein·min^1 and presented as the averages of at least four assays.

Statistical Analyses

For both animal and tissue culture studies, two-tailed paired Student's t-test was used to determine the statistical significance. A P value ≤ 0.05 was considered significant. Results are given as the means ± standard error of the mean (SEM). The placental mRNA from first generation as well as the densitometric analysis of immunoblot bands from both generations were compared between LPD and the NPD group. F2 placental mRNA content was compared according to F1 parental lineage such that the offspring of F1 female animals were compared to the offspring of random control females. Similarly, offspring of F1 male animals were compared to offspring of random control males. Likewise, using two-tailed Student's t-test, mRNA, and Na+ dependent MeAIB transport were compared after transient transfection of HEK 293 cells with SNAT2-containg vs. respective base (empty vector).

Results

Placental SysA mRNA Expression

As noted in Figure 1A, in F1 placentas, expressed in arbitrary mRNA units, the steady-state

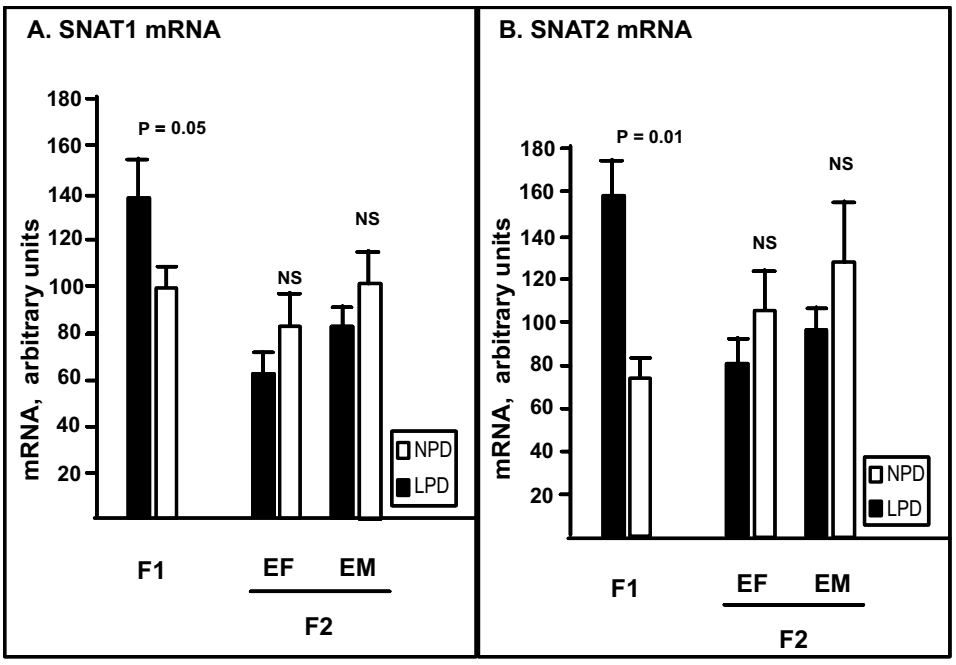

Fig. 1. Placental SysA isoform specific mRNA content determined by real time RT-PCR. Panel A depicts steady-state mRNA content of SNAT1 and panel B for SNAT2 isoform (y-axis). Respective gestations are shown on x-axis as labeled. In F1 placentas, mRNA content of both isoforms were significantly higher from LPD group (solid black bars, N = 22) than the NPD group (empty bars, N = 22). As detailed in Method's section, a reversed but non-significant trend is noted in F2 generation-placentas from both maternal and paternal-GPR descent. EM – experimental male; EF – experimental female

Ryc. 1. Zawartość swoistej izoformy mRNA w łożysku oceniona w czasie rzeczywistym za pomocą RT-PCR: Panel A przedstawia zawartość SNAT1 mRNA w stanie równowagi, a panel B izoformę SNAT2 (oś y). Odpowiednie ciąże są zaznaczone na osi x zgodnie z etykietami. W łożyskach F1 zawartość obu izoform w mRNA była istotnie większa w grupie LPD (czarne słupki, N = 22) niż w grupie NPD (puste słupki, N = 22). Jak przedstawiono w rozdziale Metody, odwrotną, ale nieistotną tendencję odnotowano w łożyskach z pokolenia F2 pochodzących od matki i ojca z GPR. EM – doświadczalny samiec; EF – doświadczalna samica

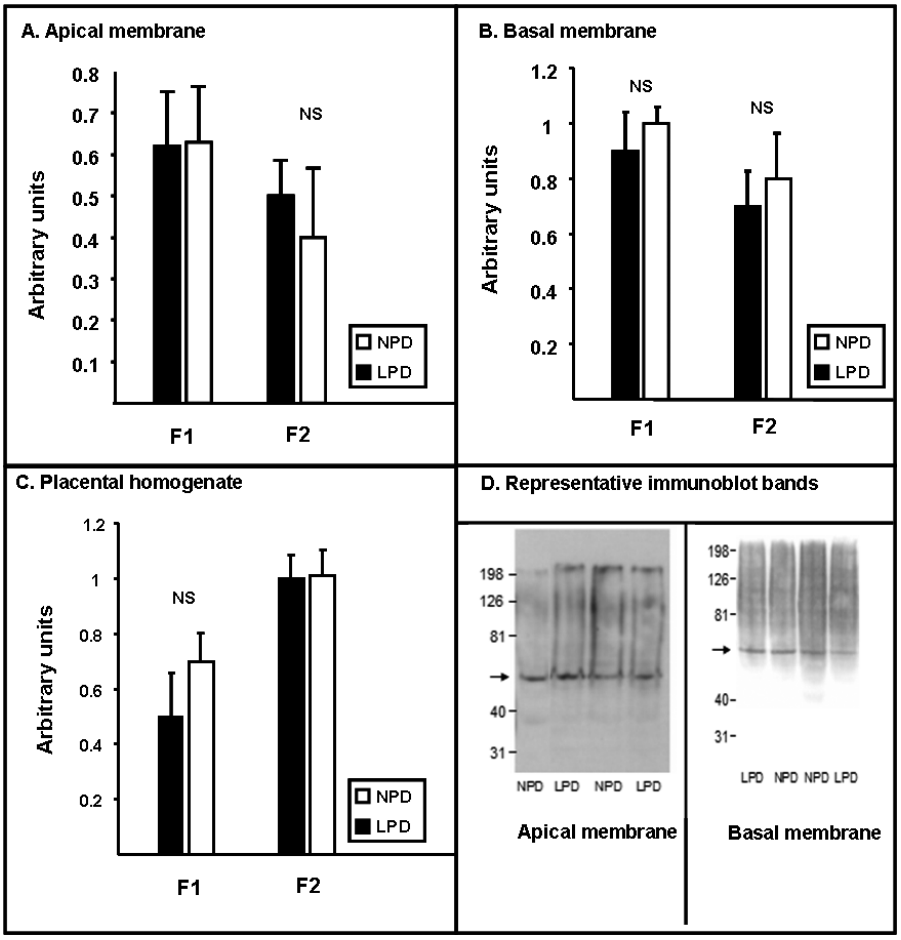

Fig. 2. Placental SNAT1 protein expression. Shown are the quantitative assessment of immunoblot bands determined by densitometry analysis from apical membranes (A), basal membranes (B), and placental homogenates (C). In panel D, a representative immunoblot from F1 apical membrane (left panel) and F2 basal membrane (right panel) are shown. As noted SNAT1 expression was comparable between LPD (solid black bars) and NPD group (empty bars) in both generation placental-membranes and placental-homogenates

Ryc. 2. SNAT1 w łożysku. Ilościowa ocena prążków immunoblot ustalona na podstawie analizy densytometrycznej błon szczytowych (A), błony podstawnej (B), homogenatu łożyska (C). Na panelu D przedstawiono typowe prążki błony szczytowej F1 (panel lewy) i błony podstawnej F2 (prawy panel). Ekspresja SNAT1 była porównywalna między grupą LPD (czarne słupki) a grupą NPD (puste słupki) w błonach i homogenatach z łożyska w obu pokoleniach

mRNA content of SNAT1 was higher in LPD than NPD group (140 ± 13 vs. 99 ± 11; $p \leq 0.01$, N = 22). A non-significant decrease in mRNA expression of SNAT1 is seen in second generation placentas of animals from LPD decent (both genders, Figure 1A). For example, steady-state mRNA contents of SNAT1 in F2 generation placentas from maternal GPR-background (EF) was relatively lower in LPD than NPD group (61 ± 12, n = 10 vs. 82 ± 11; p = 0.2, n = 9). Similar but more pronounced depression of SNAT1 mRNA content (nearly reaching statistical significance) is noted in F2 placentas of paternal (EM) descent (81 ± 6, n = 10 vs.104 ± 12; p = 0.052, n = 9). As noted in Figure 1B, the results for SNAT2 mRNA for both-generation-placentas closely resemble the results seen for SNAT1. For example, in F1 placentas SNT2 mRNA was higher in LPD than NPD group (81 ± 6, n = 10 vs. 104 ± 9; $p \leq 0.001$, n = 12). Similarly, SNAT2 mRNA content in F2 placentas from EF origin (83 ± 10, n = 10 vs.102 ± 16; p = 0.31, n = 9) as well as from paternal (EM) descent (99 ± 8, n = 10 vs. 130 ± 11; p = 0.20, n = 9) were depressed as compared to the control group, although the difference was not statistically different.

Placental SysA Protein Expression

Although SNAT1 mRNA was up-regulated in F1 placentas, densitometric analysis of immunoblot bands (depicted in Fig. 2) revealed no significant difference in the expression of SNAT1-protein on AM (0.62 ± 0.13 vs. 0.63 ± 0.13 arbitrary units; p = 0.9, n = 6) between LPD and NPD membrane preparations. Similar results were seen

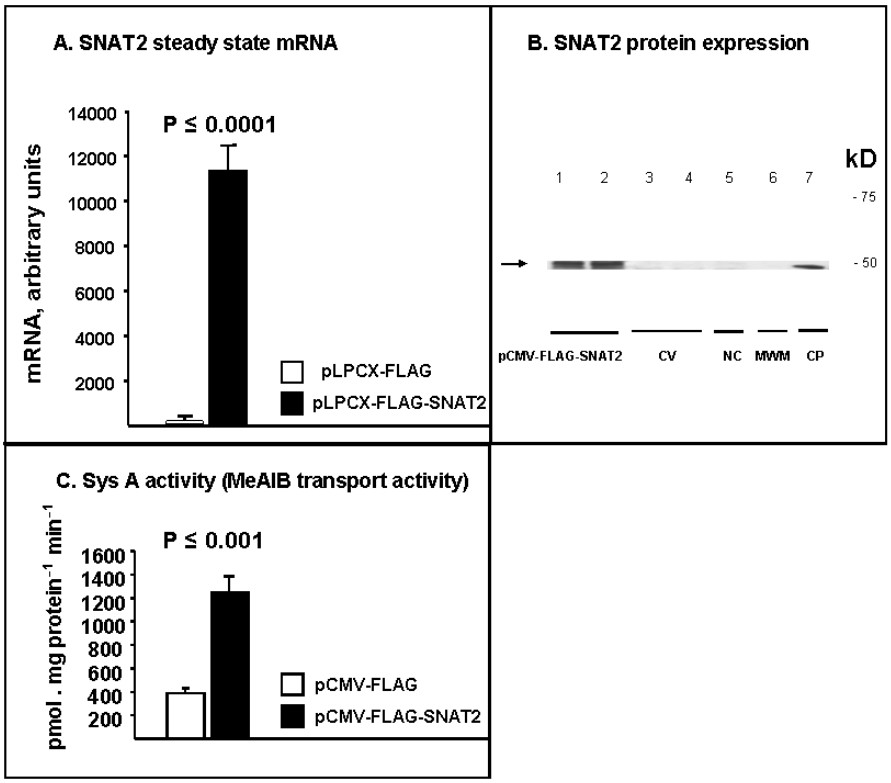

Fig. 3. SysA functional up-regulation by SNAT2-over-expression by transient transfection. Separate experiments were performed for mRNA by q-PCR, western analysis and transport studies. Typically HEK 293 cells grown to 50% confluence when transfection was carried out using either pLPCX-FLAG-SNAT2 or p-CMV-FLAG-SNAT2 (as shown). After 36 hours total RNA and proteins were isolated or transport studies performed. Panel A depicts significant induction of SNAT2 steady-state mRNA with transfection of the vector construct containing SNAT2 whole length coding sequence (solid black bar) vs. base FLAG vector without SNAT2 component. Panel B shows over-expression of SNAT2 protein depicted by anti-FLAG antibodies. Panel C highlights significant higher MeAIB (specific SysA substrate) transport in cells treated with vector construct containing SNAT2 whole length coding sequence (solid black bar) vs. base FLAG vector without SNAT2 component

Ryc. 3. Czynnościowa nadmierna aktywacja SysA przez nadekspresję SNAT2 przez przejściową transfekcję. Przeprowadzono oddzielne badania dla mRNA za pomocą q-PCR, techniki western blot i badań transportu. Zazwyczaj komórki HEK 293 mnożyły się o 50%, gdy przeprowadzono transfekcję za pomocą pLPCX-FLAG-SNAT2 lub p-CMV-FLAG-SNAT2. Po 36 godzinach wyizolowano całkowite RNA i białka lub przeprowadzono badanie transportu. Panel A przedstawia istotne wywołanie SNAT2 w mRNA w stanie równowagi za pomocą transfekcji wektora zawierającego całą długość kodowanej sekwencji SNAT2 (czarne słupki) w porównaniu z bazą wektora FLAG bez komponentu SNAT2. Panel B pokazuje nadekspresję białka SNAT2 za pomocą przeciwciał anty-FLAG. Panel C pokazuje istotnie większy transport MeAIB (typowy substrat SysA) w komórkach pod wpływem wektora zawierającego całą długość kodowanej sekwencji SNAT2 (czarne słupki) w porównaniu z bazą wektora FLAG bez komponentu SNAT2

for BM-preparations (0.9 ± 0.14 vs. 1 ± 0.06 arbitrary units; p = 0.6, n = 4) and placental homogenate preparations (0.5 ± 0.16 vs. 0.7 ± 0.1 arbitrary units; p = 0.3, n = 4). Furthermore, analysis of immunoblot bands from F2 placentas were also comparable for SNAT1-expression between LPD and NPD placental-AM-preparations (0.5 ± 0.08, n = 6 vs. 0.4 ± 0.16 arbitrary units; p = 0.6, n = 3); BM-preparations (0.7 ± 0.12, n = 4 vs. 0.8 ± 0.16 arbitrary units; p = 0.8, n = 3); and placental-homogenate-preparations (1 ± 0.08 vs. 1.1 ± 0.09 arbitrary units; p = 0.5, n = 8). SNAT2 immunoblotting was not performed because of a lack of suitable antibody when these studies were performed.

SNAT2-over-expression by Transient Transfection

As shown in Fig. 3, transient transfection of HEK 293 cells with pLPCX-FLAG-SNAT2 construct resulted in more than 100-fold induction of SNAT2 mRNA over control (11227 ± 1126 vs. 81 ± 11; p ≤ 0.0001, n = 4). A commensurate SNAT2 protein over-expression is noted by strong bands detected by anti-FLAG antibodies at the site of detection of control protein (CP, Fig. 3C). Similarly, in line with SNAT2 mRNA and protein over-expression, Na+ dependent MeAIB (specific SysA substrate) transport was significantly enhanced by

transfection with SNAT2-containing vs. control vector 1243 ±137 vs. 390 ± 27 pmole $^{-1}$mg $^{-1}$ min; $p \leq 0.0008$, $n = 4$). Collectively, these results suggest that in biological systems, without any feedback modulation, transcriptional over-expression of SNAT-isoforms increase protein and functional activity of SysA.

Discussion

In this work, we sought to determine the molecular mechanisms of down-regulation of placental SysA activity in the setting of GPR. We document the following interesting observations: 1) GPR-induced SysA repression is associated with up-regulation of SNAT1 and SNAT2 mRNA in F1 placentas. 2) Despite the transcriptional up-regulation, the protein contents of SNAT1 in apical and basal membranes were not over-expressed. 3) Transient transfection of highly transfectable HEK-293 cells with SNAT2-containing vector resulted in more than 100-fold-up-regulation of SNAT2 mRNA; SNAT2 protein over-expression, and a commensurate enhancement in SysA transport activity. 4) When next-generation maternal nutritional protein intake is optimized placental-SysA-activity is normalized.

The rat is commonly utilized to study maternal-fetal effects of GPR and is well known to produce abnormal fetal growth. Although the precise patterns of fetal involvement depend upon the timing, severity and the duration of GPR, the most commonly affected systems include cardiovascular, renal and endocrine systems [17]. Placental amino acid transport and its regulation, particularly in the setting of GPR is an important determinant of the fetal nutritional environment. In this regard, SysA bears special significance, because, it transports a wide range of amino acids (including alanine, asparagine, cysteine, glutamine, glycine, methionine, and serine) and its function is extensively regulated through hormonal inputs [18, 19]. Over the years, we and others have consistently documented that in the pregnant rat dams, dietary protein restriction reduces the transfer of ^{14}C-labeled MeAIB, a specific substrate of SysA, from the maternal blood into the fetus. As such a consensus has emerged that placental SysA activity is down-regulated in the setting of GPR [11]. The work presented here is the first step addressing the mechanisms of this repression. We report that contrary to SysA repression, SNAT1 protein contents of placental homogenates as well as both apical and basal membranes are comparable between LPD and NPD group (Fig. 2). Conversely, the transcriptional activities of placental SNAT1 and SNAT2, two of the most important SysA isoforms are up-regulated (Fig. 1). Further work will be needed to decipher the molecular basis of this dichotomy. Possible mechanisms include regulation of post-transcriptional mRNA processing, nuclear export, stability and translation of mature mRNAs, and altered quantity or interaction of cytoplasmic mRNA-binding proteins. It is important to realize that although a feedback post-transcriptional-control of SysA proteins is logical and is supported by our work involving tissue culture studies (Fig. 3), an explanation purely based on feed-back-inhibition of transcription appears too simplistic: because, the exchange of amino acids from the mother to the fetus involves not only placental uptake and excretion by transport systems (such as SysA), but also an extensive amino acid metabolism within the placenta. Similarly, in growth-restricted pregnancies such as those due to GPR, substantial alterations in the maternal neuroendocrine milieu are known to occur which can modulate maternal-fetal transfer of nutrients [20]. When viewed from this (limited) perspective, our finding of increased SNAT1 and SNAT2 mRNA may indicate an attempt by the syncytiotrophoblast to enhance the uptake of SysA substrates in the face of maternal substrate deprivation, a phenomenon well known cell culture models [21]. Again, given the complexity of maternal-placental-fetal interactions, extrapolations from culture models are not adequate to explain these observations.

Although the transcriptional control of SysA (primarily SNAT2) has been characterized recently [22], the lack of reliable anti-SNAT2 antibodies remains a formidable challenge to investigate translational or post-translational steps regulating the expression of SysA genes. Nonetheless, based upon data related to amino acid control of protein synthesis one can speculate that similar translational control exists for SysA proteins as well. For example, work done by Kimball at al. strongly supports a key role for amino acids as signaling molecules in the regulation of protein synthesis at translational level [23–26]. One signaling pathway that is becoming better defined involves the protein kinase mTOR (mammalian target of rapamycin) [27, 28]. Interestingly, the activity of mTOR must be maintained in order for amino acids to mediate changes in the phosphorylation status of translational proteins such as 4E-BP1 and S6K1. This may be relevant to the GPR model used in this study as mTOR appears to be a "junction point" between signals generated by amino acids and those generated by growth factors such as insulin [29]. Both types of signaling are required for optimal mRNA translation. Understanding the biochemical links between SysA and the mTOR

pathways will facilitate the understanding of the mechanism(s) by which the supply of SysA substrates (small neutral amino acids) affect the actions of growth/trophic factors at the translational level under conditions such as gestational protein restriction. Our ongoing work is focused at investigation of the maternal and fetal hormonal factors involved in SysA post-transcriptional regulation in GPR-pregnancies. Important questions emerging from this work include: 1) what are the cellular targets of post-transcriptional regulation of SysA proteins in the setting of GPR; what are the maternal and fetal adaptations linked to post-transcriptional regulation of SysA proteins; and can the placental, fetal and maternal adaptations predict the latter evolution of MetS-prone phenotype. These questions are the focus of our ongoing investigations.

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Address for correspondence:

Donald Novak
Box 100296
Department of Pediatrics
University of Florida College of Medicine
Gainesville, FL 32610-0296
Tel.: 352 392 64 10
E-mail: novakda@peds.ufl.edu

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