Enhanced insulin secretion responsiveness and islet adrenergic desensitization after chronic norepinephrine suppression is discontinued in fetal sheep

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Submitted 18 September 2013; accepted in final form 13 November 2013


Small-for-gestational age or intrauterine growth-restricted (IUGR) infants are at greater risk for developing metabolic diseases such as type 2 diabetes mellitus (29, 40, 54). Impaired insulin secretion is associated with a diabetic phenotype indicating that in utero complications can permanently compromise β-cell development and function (27, 28). A fetal sheep model with placental insufficiency-induced intrauterine growth restriction shares many similarities with human IUGR fetuses, such as asymmetric growth, hypoxemia, hypoglycemia, hypoinsulinemia, and hypercatecholaminemia (epinephrine and norepinephrine [NE]) (4, 16, 18, 22, 23, 32, 41, 47). Furthermore, glucose-stimulated insulin secretion (GSIS) and β-cell mass are lower in IUGR sheep fetuses, which also replicate features in human IUGR fetuses (34–36, 41, 53). Several characteristics of the fetal IUGR environment, including hypoglycemia, hypoxemia, and hypercatecholaminemia, are proposed to cause hypoinsulinemia and pancreatic β-cell dysfunction (22).

NE inhibits insulin secretion via the α2-adrenergic receptors (ARs) in fetal sheep and other species (26, 48, 51, 56). Chronic exposure to high catecholamine concentrations often causes ARs to become desensitized by downregulation of their transcript (5, 12, 14). However, in IUGR fetal sheep islets, α2A-AR mRNA concentrations were elevated (32), and genetic variants that increase α2A-AR are associated with a higher incidence of type 2 diabetes in man (6, 17, 49). In our previous studies in IUGR sheep fetuses, we showed that elevated plasma NE concentrations chronically suppress insulin concentrations (32, 37). Strikingly, AR antagonists administered to acutely block the action of chronically elevated NE concentrations revealed an enhancement in glucose-stimulated insulin concentrations in IUGR fetuses that was equivalent to control fetuses, although β-cell mass was less in IUGR fetuses (32, 35). There was also an enhancement in fractional insulin release from isolated islets in IUGR fetuses that indicates β-cell-specific compensatory adaptations (12, 36). Therefore, chronic exposure to high NE appears to produce a β-cell compensatory response distal to the ARs and enhanced insulin stimulus-secretion coupling.

In sheep fetuses, previous experiments have demonstrated that a 7-day infusion of NE slows growth and attenuates cardiovascular and metabolite effects (3). The objective of this study was to determine whether sustained exposure to elevated NE, independent of other intrauterine growth restriction-related deficiencies, acutely enhances secretagogue-stimulated insulin secretion after the exogenous administration of NE has been terminated for ≥3 h. In addition, we associated the compensatory enhancement in insulin secretion responsiveness in NE-infused fetuses with lower expression of AR and uncoupling protein 2 (UCP2) in pancreatic islets compared with vehicle-infused controls while also demonstrating that regulatory genes for insulin secretion or β-cell function were unaffected (2, 11, 19, 42).

Materials and Methods

Ethical approval. Twelve Columbia-Rambouillet crossbred ewes carrying singleton fetuses were purchased from Nebeker Ranch (Lancaster, CA) and managed in compliance with the Institutional Animal Care and Use Committee at the University of Arizona, which approved our study and is accredited by the American Association for Accreditation of Laboratory Animal Care. Ewes were fed Standard-Bread Alfalfa Pellets (Sacate Pellet Mills) and provided water ad libitum.
Animal preparations. Animals were randomly assigned to control 
(n = 6) or NE (n = 6) treatments. At 126 ± 1 days of gestational age 
(dGA), indwelling polyvinyl catheters were surgically placed in the 
fetus and ewe, as described previously (33). Fetal catheters for blood 
sampling were placed in the abdominal aorta via hindlimb pedal 
arteries, and infusion catheters were placed in the femoral veins via 
the saphenous veins. Maternal catheters were placed in the femoral 
artery and vein for arterial sampling and venous infusions. All 
catheters were tunneled subcutaneously to the ewe’s flank, exterior-
ized through a skin incision, and kept in a plastic mesh pouch sutured 
to the ewe’s skin. Ewes were allowed to recover for 5 days before the 
first GSIS and glucose-potentiated arginine-induced insulin secretion 
(GPAIS) studies were conducted.

Experimental design and treatment protocol. Fetal GSIS and 
GPAIS studies were performed at 132 ± 1 dGA to establish pretreat-
ment insulin secretion responsiveness. Chronic infusions of NE and 
vehicle (described below) were initiated following the studies (treat-
ment day 0). On day 7, chronic infusions were terminated for ≥3 h 
prerequisite acute insulin secretion responsiveness being evaluated. GSIS 
studies were also completed on each NE-infused fetus on day 3 
(mid-NE treatment) to evaluate the effectiveness of NE suppression 
during treatment.

In the chronic NE treatment group, NE bitartrate (Bedford Labo-
ratories) diluted with 0.3% acetic acid and saline (0.9% NaCl) was 
infused continuously in the fetal vein. During the 7-day infusion 
period, the dose of NE concentration was started at 1 μg/min for the 
first 24 h, increased to 2 μg/min for days 2–4, and finally increased to 
4 μg/min for days 5–7, as described previously (3). Control fetuses 
received an infusion of saline with 0.3% acetic acid. After the final 
GSIS study at 139 ± 1 dGA, both ewes and fetuses were euthanized 
with an overdose of pentobarbital sodium (86 mg/kg, Euthasol; Virbac 
Animal Health, Fort Worth, TX). Perirenal adipose, liver, heart, lung, 
and kidneys were dissected and weighed.

GSIS and GPAIS studies. Square-wave hyperglycemic clamps were 
performed as described previously (21). Three baseline blood samples 
were collected at 5-min intervals before the hyperglycemic clamp was 
initiated. Following the last baseline sample, at time designated 0 min, 
a glucose bolus (110 mg/kg estimated fetal weight) was given to the 
fetuses and followed immediately by a continuous infusion of 33% 
dextrose solution (Abbott Laboratories, Abbott Park, IL) that was 
warmed to maintain fetal glucose concentration at ∼2.4 mM. This is 
the recommended value to achieve maximum glucose-stimulated insulin 
concentrations in singleton sheep fetuses (21). During the hypergly-
cremic clamp, three fetal arterial plasma samples were collected every 
5 min starting from 35 min. Following the hyperglycemic clamp, an 
arginine bolus (261 mg of arginine in 0.4 M sodium acetate) was 
administered, and plasma samples were collected at 5, 10, 20, and 30 
min after the arginine bolus infusion. Maternal blood was transfused 
continuously into the fetus (5 ml/h; −60 min to completion) to 
compensate for blood collections.

Blood collection and analysis. Fetal blood was collected in syringes 
filled with EDTA (Sigma-Aldrich, St. Louis, MO) and centrifuged 
(16,000 g) for 2 min at 4°C to separate plasma. Plasma glucose 
concentrations were measured immediately with an YSI model 2700 
SELECT Biochemistry Analyzer (Yellow Springs Instruments, Yel-
low Springs, OH). Plasma insulin and NE concentrations were mea-
sured with an ovine insulin ELISA (Alpco Diagnostics, Windham, 
NH) and Noradrenaline ELISA (Labor Diagnostika Nord), respec-
tively, as described previously (20). Additional fetal blood samples 
were collected in heparin-lined syringes (Elkins-Sinn, Cherry Hill, 
NJ) for blood gas and oxygen saturation measurements with an ABL 
725 (Radiometer, Copenhagen, Denmark).

Pancreatic islet isolation. Islets were isolated from the fetal pan-
creas after a Liberase BlendZyme III (0.175 mg/ml; Roche, Indianapolis, 
IN) digestion and purified with a Histopaque density gradient 
(Sigma-Aldrich), as described previously (36, 50). After isolation, 
islets were washed in Krebs-Ringer buffer containing 0.5% BSA and 
then cultured overnight at 37°C in 95% O2-5% CO2 in RPMI 1640 
medium supplemented with 2.8 mM glucose (Sigma-Aldrich), 2% fetal 
bovine serum, and penicillin-streptomycin-neomycin (50 U, 50 μg, 
and 100 μg, respectively, Sigma-Aldrich). Islets were hand-picked 
into a 1.5-ml tube and centrifuged at 800 g for 5 min. After removing 
the supernatant, the pellets were stored at −80°C until RNA was 
extracted.

RNA extraction. RNA was extracted from isolated pancreatic islets 
with the AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Valencia, CA) 
according to the manufacturer’s instructions. Quality and concentra-
tion of the RNA were determined by measuring absorbance at 260 and 
280 nm with the NanoDrop ND-1000 Spectrophotometer (Nanodrop, 
Wilmington, DE). RNA integrity was evaluated with an Experion 
Automated Electrophoresis System (Bio-Rad Laboratories, Hercules, 
CA). PCR amplification was performed on RNA (~1 μg) that was 
reverse transcribed into cDNA with Superscript III reverse transcript-
pase (Invitrogen Life Technologies, Carlsbad, CA).

Primer design and cDNA cloning. Synthetic oligonucleotide primers 
were designed against sequences for ovine α1A, α2A, α2B, α3, α7-ARs, 
β1, 2, 3-ARs (12), insulin (GenBank accession no. U00659), pancreatic 
duodenal homeobox 1 (PDX-1; JF728302), glucose transporter 2 
(GLUT2; AJ1318925), Gαs (JX101267, Gαo (JX101268), Gαs (JX102069), Gαo (JX101270, Gαq (JX101271), Gαi (forward, 5'-GAG CAC 
GAC CTC CCT CGG TGC T-3'; reverse, 5'-AGG CTC CTG CAC AGT GGT GCT-3'), Gβ3 (forward, 5'-GCG GCC GGC AGA CAT 
CCA GGA TGT GTC-3'; reverse, 5'-CTG TCC AGC GAT TGC 
TGG TGC-3'), Gβ1 (forward, 5'-GCG GCC AGC AGA CAA CCA 
TAT-3'; reverse, 5'-CTG CCG GCA CAT CCC TGC TCG-3'), Gβ2 (forward, 5'-ACA AGC GTT GAC CAT GCA TCA G-3'), Kir6.2 (forward, 5'-ACA AGC GTT GAC CAT GCA TCA G-3'), and L-type voltage-dependent calcium channel α1D 
subunit (ACNA1D; forward, 5'-GCC GGC AAC ACC CGG AGA 
TAC A-3'; reverse, 5'-AGG CCG CTC AAA GCA CCA GAT 
GCT GGT AAG G-3'). All DNA products were cloned and sequenced as described previously (12, 13).

Quantitative real-time PCR. The relative expression levels of 
mRNA transcripts were determined by quantitative PCR (qPCR) 
using SYBR Green (Qiagen) in an iQ5 Real-Time PCR Detection 
System (Bio-Rad). After an initial denaturation for 15 min, all reac-
tions went through 40 cycles at 96°C (30 s), annealing temperature (30 
and 72°C (10 s), at which point the fluorescent measurement was 
collected. Optimal annealing temperatures for different primer sets 
were determined using a temperature gradient (54–62°C) and exam-
ined on an agarose gel for specificity. melt-curve analysis, starting at 
60°C with an increase of 0.2°C every 6 s to 96°C, was performed at 
the end of the amplification to confirm product homogeneity.

PCR efficiency was determined with gene-specific plasmid DNA, 
for which threshold cycles (Ct) were linear over six orders of 
magnitude. Samples were run in triplicate. The results were normal-
ized to the reference gene ribosomal protein S15, the average ΔCt 
was analyzed by the comparative ΔCt method (Ct reference gene) 
and fold change was determined by the 2−ΔΔCt 
method (46).

Statistical analysis. Data for body and organ weights and qPCR 
(ΔCt) were analyzed by one-way ANOVA, using the general linear 
model procedure of SAS (SAS 9.3; SAS Institute, Cary, NC). Insulin 
concentrations during basal and hyperglycemic steady-state periods 
were compared between treatments and studies. GSIS responsiveness 
was taken to be the difference in mean insulin concentration between 
the hyperglycemic and basal states and was compared between 
treatments and studies. GPAIS was analyzed as the area under the 
curve relative to basal insulin concentrations for each sheep fetus 
(Priism 5.01; GraphPad Software, La Jolla, CA). Biochemical, hema-
tological, and hormone measurements for daily, GSIS, and GPAIS 
studies were analyzed by ANOVA, using the MIXED procedure with
fetus as a random effect (SAS 9.3). The midtreatment GSIS in NE fetuses was compared with pretreatment values with a nonparametric Kruskal-Wallis test (proc npar1way; SAS 9.3). All values are expressed as means ± SE. *P* values ≤0.05 were considered significant.

**RESULTS**

**Body weights and organ weights.** Body weights were not different between control ewes (56.0 ± 4.5 kg) and ewes with NE-infused fetuses (49.8 ± 3.3 kg) or between control (3.8 ± 0.4 kg) and NE-infused fetuses (3.3 ± 0.2 kg). No treatment effects were found for fetal organ weights (Table 1).

**Daily fetal plasma biochemical values.** Fetal plasma glucose, insulin, and NE concentrations were not different between groups prior to treatment (Fig. 1). During the 7-day infusion, average NE concentrations were ninefold higher in NE-infused fetuses than control fetuses (9.343 ± 1.047 vs. 1.059 ± 142 pg/ml, *P* < 0.01; Fig. 1A). Insulin concentrations were 61% lower in NE fetuses compared with control fetuses (0.12 ± 0.01 vs. 0.31 ± 0.03 µg/l, *P* < 0.01; Fig. 1C). NE-infused fetuses had greater plasma glucose concentrations (1.54 ± 0.04 vs. 0.99 ± 0.03 mM, *P* < 0.01; Fig. 1B) and partial pressure of oxygen (PO2; 31.29 ± 0.65 vs. 22.98 ± 0.56 mmHg, *P* < 0.01; Fig. 1D) compared with controls. Hematocrit and blood pH were not different between treatments throughout the 7-day infusion period.

**GSIS studies.** Among the pretreatment GSIS studies, fetal plasma glucose concentrations of NE fetuses were not different from controls at baseline but were lower (*P < 0.05*) during the hyperglycemic clamp (Table 2). In the posttreatment GSIS studies, plasma glucose concentrations at baseline were 19% greater in NE fetuses than controls. However, posttreatment GSIS hyperglycemic glucose concentrations of NE-infused fetuses were not different from controls or from the pretreatment hyperglycemic values. During all GSIS studies, the mean glucose infusion rates needed to maintain the hyperglycemic steady state were similar between treatment groups within the study period.

In the pretreatment GSIS studies, fetal plasma insulin concentrations at baseline and hyperglycemic steady-state periods were not different between control fetuses and NE fetuses (Fig. 2). Insulin concentrations during the NE infusion were decreased at both the baseline and hyperglycemic periods (0.09 ± 0.01 µg/l, baseline; 0.40 ± 0.16 µg/l, hyperglycemic; *P* < 0.01) compared with NE pretreatment values (0.44 ± 0.05 µg/l, baseline; 0.99 ± 0.10 µg/l, hyperglycemic). Three hours after the 7-day NE infusion was terminated, NE fetuses had fivefold greater (*P < 0.01*) insulin concentrations at baseline and fourfold greater (*P < 0.01*) insulin concentrations during the hyperglycemic clamp compared with their own pretreatment GSIS studies. Posttreatment baseline and hyperglycemic insulin concentrations in NE fetuses were greater than control insulin concentrations. Among controls, insulin concentrations during the GSIS studies (both at baseline and during hyperglycemic steady state) were not different between pre- and posttreatment periods (Fig. 2).

**Hematocrit, PO2, and pH at baseline and hyperglycemic steady states were similar between NE and control fetuses prior to the chronic infusion (Table 2).** During the posttreatment GSIS, the hematocrit was 12% lower (*P < 0.01*) at baseline and hyperglycemic states in NE fetuses compared with controls. PO2 was greater (*P < 0.01*) during the baseline and hyperglycemic clamp in NE fetuses compared with controls.

During the pretreatment GSIS studies, fetal plasma NE concentrations were similar between treatment groups at baseline, but control fetuses had higher (*P < 0.05*) plasma NE concentrations at hyperglycemic state compared with NE fetuses. During the posttreatment GSIS studies, plasma NE concentrations of NE fetuses were 3.5-fold greater (*P < 0.01*) than control fetuses at baseline and hyperglycemic steady states (Table 2).

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**Table 1. Organ and tissue weights in control and NE-infused fetal sheep**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Weight, g</th>
<th>Control (n = 6)</th>
<th>NE (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>52.5 ± 1.7</td>
<td>50.9 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>25.0 ± 2.2</td>
<td>24.4 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>Kidneys</td>
<td>19.9 ± 1.5</td>
<td>18.4 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>98.8 ± 11.6</td>
<td>88.3 ± 5.1</td>
<td></td>
</tr>
<tr>
<td>Lungs</td>
<td>123.8 ± 8.9</td>
<td>109.8 ± 5.9</td>
<td></td>
</tr>
<tr>
<td>Perirenal adipose</td>
<td>13.8 ± 1.5</td>
<td>15.2 ± 0.8</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. NE, norepinephrine.
Post-NE infusion

GSIS, glucose-stimulated insulin secretion; NS, not significant. Differences were determined between treatment groups for pre- and posttreatment GSIS studies.

Fetal plasma insulin concentrations followed the arginine bolus reached maximum concentrations after 5 min in all fetuses. The pretreatment GPAIS area under the curve measurement was not different between controls and NE fetuses. During the posttreatment GPAIS studies, insulin concentrations of NE-infused fetuses were greater ($P < 0.05$), and the area under the curve was 3.6-fold greater ($P < 0.01$) compared with NE pretreatment and control values (Fig. 3).

**GPAIS studies.** Fetal plasma insulin concentrations following the arginine bolus reached maximum concentrations after 5 min in all fetuses. The pretreatment GPAIS area under the curve measurement was not different between controls and NE fetuses. During the posttreatment GPAIS studies, insulin concentrations of NE-infused fetuses were greater ($P < 0.05$), and the area under the curve was 3.6-fold greater ($P < 0.01$) compared with NE pretreatment and control values (Fig. 3).

**Islet gene expression.** To interpret compensatory enhancement of GSIS in NE-infused fetuses, we evaluated factors regulating aspects of insulin synthesis and secretion. Pancreatic islets from NE fetuses had lower ($P < 0.05$) mRNA concentrations for α₁D-AR, α₂A-AR, α₂C-AR, β₁-AR, G protein subunit α₂-β, and UCP2 (Fig. 4). Insulin, PDX-1, Glut2, Kir6.2, CACNA1D, other ARs, and Gox1, Gox3, Gox6, Gox7, Gox8, and Gβ₁-3 mRNA concentrations were not different in pancreatic islets (data not shown).

**DISCUSSION**

In the present study, we show that chronic infusion of NE suppressed fetal insulin concentrations throughout the 7-day treatment and was accompanied by hyperglycemia. Circulating NE concentrations resembled those observed in placental insufficiency-induced IUGR sheep fetuses, which have 69% lower insulin concentrations (36). After the NE infusion was terminated, we observed a compensatory augmentation of GSIS and GPAIS in NE fetuses, although plasma NE concentrations remained elevated compared with controls. The α₂A-
As expected, there was no difference in GSIS responsiveness between the two groups before the treatment (Fig. 2). In control fetuses, GSIS was also not different following the 7-day vehicle infusion (Fig. 2). Comparison between the two GSIS studies in control fetuses demonstrates that there is no significant developmental enhancement or maturation in β-cell function at this gestational stage (33), in contrast to that found for younger fetuses (1). During the NE treatment, insulin concentrations at basal and hyperglycemic steady states were inhibited. After removing the chronic NE infusion, basal, hyperglycemic, and GPAIS insulin concentrations were approximately fourfold higher than pretreatment measurement in NE fetuses or posttreatment measurements in control fetuses (Fig. 2). Therefore, these findings support the hypothesis that NE induces a compensatory hyperinsulin secretion state in β-cell insulin secretion responsiveness.

Enhanced insulin secretion responsiveness was observed despite higher than normal plasma NE concentrations 3 h after the chronic NE infusion was terminated. According to previous studies, the whole body clearance rate of NE is 178 ml·kg⁻¹·min⁻¹ in near-term sheep fetuses and is not dependent on plasma catecholamine concentration (43). Since the average blood volume in fetal sheep is 120 ml/kg at 131 dGA (7), theoretically, the half-life of circulating plasma NE is <2 min. However, longer than expected clearance rates were shown in a previous long-term NE infusion study (38), indicating that chronic NE exposure decreased NE clearance, as found previously (9). In the current study, our primary objective was to look at the immediate insulin secretion responsiveness following a chronic NE infusion. Despite NE’s inhibitory effect on insulin secretion, NE fetuses with relatively greater plasma NE concentrations still had enhanced insulin secretion responsiveness compared with control fetuses.

Hyperglycemia was observed throughout the NE treatment and represents a confounding factor that might also negatively influence β-cell function. The 55% increase in fetal plasma glucose concentrations is most likely due to reduced glucose oxidation in peripheral tissues of the NE fetus, which is further supported by the chronic hyperoxemia (3, 39). Previous studies in fetal sheep have found that chronic hyperglycemia for 7–10 days results in impaired β-cell function and decreased insulin secretion (10). Additionally, 2 wk of pulsatile hyperglycemia also lowers GSIS in fetal sheep and increases the rate of accumulation for reactive oxygen species in isolated pancreatic islets at 11.1 mM glucose compared with controls (20).

Together, these findings indicate that fetal sheep islets exposed to hyperglycemia exhibit glucotoxicity and β-cell dysfunction (25). Interestingly, the islets from NE fetuses, also exposed to chronic hyperglycemia, were not affected in a similar fashion, as insulin secretion was enhanced after the 7 days of exposure. This shows that NE may protect islets from the ensuing hyperglycemia and subsequent glucotoxicity, possibly by inducing metabolic quiescence (30, 31).

Chronic exposure of high catecholamine concentrations has been shown to persistently reduce both mRNA and protein expression of ARs (5, 12, 14) and related G proteins (52), which is also supported in the current study (Fig. 4). NE inhibits insulin secretion principally through α₂-ARs, but not α₁-ARs or β-ARs (31). Thus, this lower expression of α₂A-AR and α₂C-AR might lead to decreased adrenergic inhibition of insulin secretion (51, 55). The decreased expression of Gαi-2 is also postulated to lower NE inhibitory effects on insulin secretion (52). Therefore, desensitization of both α₂-ARs and Gαi-2 contributes to a higher insulin concentration as well as higher insulin responsiveness after the 7-day NE infusion is removed, whereas plasma NE concentrations remain greater than controls.

As the exclusive proton leak regulator in the mitochondria of the β-cell, UCP2 negatively affects β-cell responsiveness by decreasing ATP production (15, 57). Chronic hyperglycemia stimulates UCP2 expression in rat and human islet cultures (8, 44). In glucokinase knockout mice, whether incubated with or without chronic hyperglycemia, decreased rates of glucose utilization lower UCP2 expression and activity in pancreatic islets (15). Thus, expression and function of UCP2 are associated with glucose metabolism in islets, which is suppressed by α₂-AR stimulation (31).

In conclusion, a 7-day infusion of NE in fetal sheep chronically suppresses plasma insulin concentrations and lowers α₂-ARs and Gαi-2 mRNA in islets. After NE infusion is terminated, NE-infused fetuses have enhanced insulin secretion responsiveness to both glucose and arginine stimulation, although NE concentrations are greater than controls. These findings demonstrate that 7-day NE suppression during late gestation contributes AR desensitization and a compensatory enhancement of β-cell function. In addition, lower expression of UCP2 in pancreatic islets may facilitate enhanced insulin stimulus-secretion coupling. Therefore, these data begin to explain how endocrine factors such as catecholamines may facilitate prenatal adaptations for thriftness in utero (24) but
contribute to postnatal catchup growth, early onset obesity, and other metabolic diseases.

ACKNOWLEDGMENTS

We thank Miranda J. Anderson and Mandie M. Dunham for technical assistance.

GRANTS

Funding for this work was from the National Institute of Diabetes and Digestive and Kidney Diseases (R01-DK-084482; S. W. Limesand, principal investigator). X. Chen was supported by Grant no. 20710916, Southwest University, China. D. T. Yates and A. R. Macko were supported by T32-HL-7249, and D. T. Yates was supported by a US Department of Agriculture-National Institute of Food and Agriculture Fellowship (no. 2012-67012-19855). A. S. Green was supported from F32-DK-088514. The content of this article is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

DISCLOSURES

The authors have no conflicts of interest, financial or otherwise, to declare.

AUTHOR CONTRIBUTIONS


REFERENCES


