Original Research *Feature article*

Synthesis of glycine from 4-hydroxyproline in tissues of neonatal pigs with intrauterine growth restriction

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Impact statement

Intrauterine growth restriction (IUGR) adversely affects 10–15% of all human pregnancies worldwide and 15–20% of newborn piglets in the swine industry. Currently, effective nutritional support for IUGR neonates is lacking due, in part, to limited knowledge about their tissue-specific metabolism of nutrients, particularly amino acids. We recently identified a severe deficiency of glycine in IUGR piglets (an excellent animal model for studying human nutrition) as compared with age-matched littermates with normal birth weights. Results of this study indicated relatively low rates of conversion of 4-hydroxyproline into glycine (a major metabolic pathway for de no synthesis of glycine) and low activities of required enzymes in the liver, kidneys, small intestine, and skeletal muscle of 0- to 21-day-old IUGR piglets. These results have broad and important implications for improving the growth, development, and survival of not only IUGR piglets but also other mammalian neonates with IUGR, including humans.

Abstract

This study tested the hypothesis that the synthesis of glycine from 4-hydroxyproline (an abundant amino acid in milk and neonatal blood) was impaired in tissues of piglets with intrauterine growth restriction (IUGR), thereby contributing to a severe glycine deficiency in these compromised neonates. At 0, 7, 14, and 21 days of age, IUGR piglets were euthanized, and tissues (liver, small intestine, kidney, pancreas, stomach, skeletal muscle, and heart) were obtained for metabolic studies, as well as the determination of enzymatic activities, cell-specific localization, and expression of mRNAs for glycine-synthetic enzymes. The results indicated relatively low enzymatic activities for 4-hydroxyproline oxidase (OH-POX), proline oxidase, serine hydroxymethyltransferase, threonine dehydrogenase (TDH), alanine: glyoxylate transaminase, and 4-hydroxy-2-oxoglutarate aldolase in the kidneys and liver from 0- to 21-day-old IUGR pigs, in the pancreas of 7- to 21-day-old IUGR pigs, and in the small intestine and skeletal muscle (except TDH) of 21-day-old IUGR pigs. Accordingly, the rates of conversion of 4-hydroxyproline into glycine were relatively low in tissues of IUGR piglets. The expression of mRNAs for glycine-synthetic enzymes followed the patterns of enzymatic activities and was also low. Immunohistochemical analyses revealed the relatively low abundance of OH-POX protein in the liver, kidney, and small intestine of IUGR piglets, and the lack of OH-POX zonation in their livers. These novel results provide a metabolic basis to explain why the endogenous synthesis of glycine is insufficient for optimum growth of IUGR piglets and have important implications for improving the nutrition and health of other mammalian neonates including humans with IUGR.

Keywords: Amino acids, glycine, growth, metabolism, nutrition, neonates

Experimental Biology and Medicine **2023; 248: 1446–1458. DOI: 10.1177/15353702231199080**

Introduction

Intrauterine growth restriction (IUGR), defined as the impaired growth and development of the mammalian embryo/fetus or its organs during pregnancy, is a significant problem in human medicine[1] and animal production.[2] In practice, IUGR is often diagnosed as birth weight below the 10th percentile of the birth-weight-for-gestational-age reference curve in clinics[3] or less than a two standard-deviation from the mean birth weight for gestational age in animal studies.[4] IUGR adversely affects 10–15% of all human pregnancies worldwide.[5,6] Among livestock species, the pigs exhibit the most severe naturally occurring IUGR, with birth weights of 15–20% of newborns being less than 1.1 kg as compared with the mean value of 1.4 kg for piglets with normal birth weights (NBWs).[7,8] Currently, effective nutritional support for IUGR neonates is lacking due, in part, to limited knowledge of tissue-specific metabolism of nutrients, particularly amino acids.

There is evidence that glycine is a major limiting factor for the growth of both human infants[11] and piglets[12] fed bovine milk protein-based diets. Milk only meets 20% of the requirements of suckling piglets for glycine; therefore, the neonates must synthesize 80% of the needed glycine.[13] We recently discovered that 4-hydroxyproline, an abundant amino acid in the milk of sows and the plasma of piglets, is metabolized

to glycine in the tissues of NBW piglets, including liver, kidney, and skeletal muscle.[14] In addition, we have constructed a pathway diagram showing the sequence of the required reactions and enzymes.[14] However, little is currently known about this metabolic pathway in IUGR neonates. The current study was conducted with IUGR piglets (an animal model widely used in studies of human nutrition[15–17]) to address this important issue.

Materials and methods

This study was approved by the Texas A&M University Institutional Animal Care and Use Committee.

Chemicals

High-performance liquid chromatography (HPLC)-grade methanol and water were obtained from Fisher Scientific (Houston, TX). Amino acid standards (including glycine and *trans*-4-hydroxy-L-proline (4-hydroxyproline)) and other chemicals were purchased from Sigma-Aldrich (St Louis, MO).

Animals

Piglets were the offspring of Yorkshire × Landrace sows and Duroc boars. All pigs were maintained at the Texas A&M University Swine Center. The average litter size of sows was 12.4 piglets at birth. Throughout the gestation and lactation periods, sows had free access to water and corn-soybean meal based diets[18] that met National Research Council (NRC) requirements.[19] Six IUGR piglets were selected randomly (one from each litter) and euthanized on either postnatal day 0, 7, 14, or 21, when their body weights were 0.78 ± 0.07, 1.50 ± 0.18, 2.23 ± 0.29, and 3.44 ± 0.26 kg (means ± standard error of the mean [SEM], respectively). These IUGR piglets were littermates of the NBW piglets used in a previous study.[14] The newborn (0-day-old) piglets received no colostrum or water before tissue isolation within 1 h after birth.

Collection of tissues from piglets

At either 0, 7, 14, or 21 days of age, a blood sample (1 mL) was withdrawn from the jugular vein of pigs into heparinized tubes at 1 h after suckling. Thereafter, IUGR piglets were immediately anesthetized with an intramuscular injection of Telazol (10 mg/kg body weight) and then euthanized by administration of saturated KCl into the heart.[20,21] After the abdomen was opened, the heart, liver, lung, stomach (with luminal contents being removed), pancreas, jejunum (proximal half of the remaining small intestine below the duodenum, with luminal contents being removed), kidneys, gallbladder, and gastrocnemius (skeletal) muscle were quickly isolated and weighted. Tissues were cut into small portions, and samples fixed in freshly prepared 4% (wt/vol) paraformaldehyde in PBS (pH 7.2) for 24 h, followed by storage in 70% ethanol for 24 h.[22] The fixed tissues were dehydrated through a graded series of alcohol to xylene and embedded in Paraplast-Plus (Oxford Labware, St. Louis, MO) for immunohistochemical analyses.

Additional slices of fresh tissues were placed in ice-cold Krebs–Henseleit bicarbonate (KHB) buffer and used for incubation (see below). The remaining tissues were frozen in liquid nitrogen and stored at −80°C.

Production of glycine by tissues incubated with 4-hydroxyproline

Slices (~100 mg) of freshly isolated tissues from IUGR piglets were incubated at 37°C for 2 h in 2 mL of oxygenated (95% O_2/5% CO_2) KHB buffer containing 5 mM D-glucose, 20 mM HEPES (pH 7.4), and either 0, 0.1, 0.25, 0.5, 2, or 5 mM 4-hydroxyproline. This method allowed for our previous determination of conversion of 4-hydroxyproline into glycine in tissues of NBW piglets.[14] Tissue homogenates were not used to determine the formation of glycine from 4-hydroxyproline, because results of our preliminary studies indicated that its rates per g tissue were only 30–40% of those obtained for tissue slices.

The 2 h incubation of tissue was terminated by the addition of 200 µL of 1.5 M $HClO_4$. The tissue plus the incubation medium was homogenized for 2 min on ice, and the solution was then neutralized with 100 µL of 2 M K_2CO_3. After centrifugation at 600 × *g* for 5 min at 4°C, the supernatant fluid was analyzed by HPLC for amino acids.[23] In parallel experiments, concentrations of amino acids in tissues without incubation were also determined.[25] Results of our preliminary studies indicated that the rates of production of glycine from 4-hydroxyproline (e.g. 5 mM) by tissue slices from liver, pancreas, kidney, small intestine, and skeletal muscle from 21-day-old IUGR piglets were linear during the 2-h period of incubation.

Analysis of enzymatic activities of tissues

Frozen tissue (0.5 g) was homogenized in 2 mL of freshly prepared buffer consisting of 300 mM sucrose, 5 mM HEPES (pH 7.4), 1 mM EDTA, 3 mM dithiothreitol, 0.5% (vol/vol) Triton X-100, and 0.1% (wt/vol) protease inhibitor (aprotinin, chymostatin, pepstatin A, and phenylmethylsulfonyl fluoride; 5 µg/mL each) for 2 min on ice.[24] The whole homogenization mixture was transferred to a tube and centrifuged at 600 × *g* for 10 min at 4°C. The supernatant fluid was subjected to three cycles of freezing in liquid nitrogen and thawing in a 37°C water bath before use for assays of enzymatic activities of POX[24] and OH-POX, as well as 4-hydroxy-2-oxoglutarate aldolase (HOA), alanine: glyoxylate transaminase (AGT), serine hydroxymethyltransferase (SHMT), and threonine dehydrogenase (TDH).[25–27] The experimental details for these procedures have been previously published.[14] Two different amounts of a tissue extract were used for the assay of each enzyme. Maximum activities of all enzymes were measured under optimum conditions.[14,26,27] The limit of detection for the activity of each enzyme was 5 nmol/g tissue/min. Results of our preliminary studies with tissues from IUGR piglets indicated that the changes in the activities of all enzymes were linear with the time (Supplemental Table 1) and the amount of protein used in the present study.

RNA isolation and quantitative real-time polymerase chain reaction (RT-PCR) assays

Total RNA was isolated from piglet tissues using Trizol (15596026; Invitrogen) according to Jobgen *et al*.[28] The quantity and quality of the total RNA were determined using spectrometry (wavelength 230 nm). The expression of mRNAs for OH-POX (*PRODH2*), proline oxidase (*PRODH*), HOA (*HOGA*), AGT (*AGXT2*), SHMT (*SHMT2*), and TDH (*TDH*) in tissues was determined using quantitative real-time polymerase chain reaction (RT-PCR) assays.[14] Primers for the genes for glycine synthesis and swine 18S ribosomal RNA (the reference gene) were designed using Primer Express Software Version 1.5 (Applied Biosystems, Waltham, MA), and quantitative RT-PCR was performed using the ABI prism 7900HT system (Applied Biosystems) with the Power SYBR Green PCR Master Mix (4309155; Applied Biosystems), as we published previously.[14] The relative expression of mRNAs was calculated using the comparative ΔΔCq method.[12]

Immunohistochemical analyses

Immunohistochemical localization of OH-POX and POX proteins in tissue sections (~5 μm) from IUGR piglets was performed to determine their cell-specific and temporal expression, as we described previously.[29] The immunohistochemical analyses were conducted using human anti-OH-POX immunoglobulin G (IgG) (0.5 mg protein/mL; developed and kindly provided by Dr. James M. Phang, National Cancer Institute, Frederick, MD) and rabbit anti-POX polyclonal IgG (HPA020361; Sigma-Aldrich, St Louis, MO) at dilutions of 1:500 and 1:200, respectively. Antigen retrieval was performed by boiling the samples in 10 mM sodium citrate buffer (pH 6.0). Purified non-relevant rabbit IgG was used as a negative control to replace the primary antibody at the same final concentration. Immunoreactive proteins were visualized in sections using the VECTASTAIN ABC Kit (PK-6101 for rabbit IgG; Vector Laboratories, Newark, CA) and 3,3′-diaminobenzidine tetrahydrochloride (D5637; Sigma-Aldrich) as the color substrate. In our preliminary study, we used the western blotting technique[29] to validate the specific binding of the OH-POX antibody to a single protein in pig tissues (e.g. liver and kidney; Supplemental Figure 1).

Statistical analysis

Results are expressed as means ± SEM. Statistical analyses of data were performed by one-way analysis of variance using the General Linear Models procedures.[30] Differences among treatment means were determined using the Student–Newman–Keuls multiple comparison method.[30] A probability value of ≤ 0.05 indicated statistical significance.

Results

Activities of enzymes involved in glycine synthesis per g tissue

Data on developmental changes in the activities of glycine-synthetic enzymes (OH-POX, HOA, and AGT) in tissues from IUGR piglets, expressed per g tissue, are summarized in Table 1. The enzymatic activities of OH-POX in the liver and kidney did not differ ($P > 0.05$) between 0- and 7-day-old IUGR piglets but increased ($P < 0.05$) by day 14 of age. Thereafter, the activity of this enzyme continued to increase ($P < 0.05$) in the liver until day 21 of age, but did not change ($P > 0.05$) in the kidney between 14 and 21 days of age. In both the pancreas and the small intestine, OH-POX enzymatic activity was not detected in newborn IUGR piglets until postnatal day 7 after which time values increased ($P < 0.05$) to postnatal day 21. In skeletal muscle, OH-POX enzymatic activity was not detected in 0- to 14-day-old IUGR piglets, but was detected in 21-day-old IUGR piglets. Neither the stomach nor the heart of 0- to 21-day-old IUGR piglets had detectable OH-POX enzymatic activity.

HOA enzymatic activity decreased ($P < 0.05$) and increased ($P < 0.05$) in the kidney and liver of IUGR pigs, respectively, during the first week of postnatal growth (Table 1). In these two tissues, HOA enzymatic activity did not differ ($P > 0.05$) between 7 and 14 days of age, but declined ($P < 0.05$) by day 21 of age. HOA enzymatic activity was not detected in the skeletal muscle of 0- to 7-day-old IUGR piglets, but was detected in the skeletal muscle of 14- and 21-day-old IUGR piglets. In the stomach, the activity of this enzyme was not detected at 0 to 14 days of age, but was present by day 21 of age. The heart of 0- to 21-day-old IUGR piglets did not have detectable HOA enzymatic activity.

AGT enzymatic activity in the kidney did not differ ($P > 0.05$) between 0- and 7-day-old IUGR piglets, but increased ($P < 0.05$) in 14- and 21-day-old IUGR piglets (Table 1). In the liver, the enzymatic activity of AGT increased ($P < 0.05$) gradually between 0 and 21 days of age. In the pancreas, AGT activity increased ($P < 0.05$) during the first week of postnatal life, did not differ ($P > 0.05$) between 7 and 14 days of age, and increased thereafter ($P < 0.05$) by day 21 of age. In the small intestine, AGT activity increased gradually ($P < 0.05$) between 0 and 14 days of age, but did not change ($P > 0.05$) at 21 days of age. Both the skeletal muscle and the stomach contained AGT activity in all age groups of IUGR piglets, and there were no developmental changes during the postnatal period studied. By contrast, the activity of this enzyme was absent from the heart of 0- to 21-day-old IUGR piglets,

The patterns of postnatal changes in POX enzymatic activities in the pancreas and small intestine of IUGR piglets were generally similar to those for OH-POX. However, POX activity was not detected in the pancreas, but was detected in the small intestine, of newborn IUGR piglets and the stomach and heart of 21-day-old IUGR piglets (Table 1). By contrast, POX enzymatic activities in both the kidney and the liver decreased ($P < 0.05$) during the first week of postnatal life, did not differ ($P > 0.05$) between 7 and 14 days of age, and then decreased ($P < 0.05$) by day 21 of age.

SHMT and TDH enzymatic activities were restricted primarily to the liver in IUGR piglets (Table 1) in which the activities of both enzymes increased ($P < 0.05$) gradually between 0 and 21 days of age. In the pancreas, SHMT enzymatic activity was absent in newborn IUGR piglets, but

Table 1. Activities of glycine-synthetic enzymes and related enzymes in tissues of IUGR piglets expressed per g tissue.

Tissue	Age of piglets (days)			
	0	7	14	21
Hydroxyproline oxidase (OH-POX)				
Kidney	2.61 ± 0.09[b]	2.52 ± 0.11[b]	3.17 ± 0.17[a]	3.02 ± 0.14[a]
Liver	0.93 ± 0.05[c]	1.16 ± 0.18[c]	3.29 ± 0.21[b]	4.53 ± 0.16[a]
Pancreas	ND	1.33 ± 0.12[c]	4.01 ± 0.15[b]	7.81 ± 0.49[a]
Skeletal muscle	ND	ND	ND	0.74 ± 0.10
Small Intestine	ND	0.86 ± 0.05[c]	1.77 ± 0.09[b]	4.06 ± 0.20[a]
Stomach	ND	ND	ND	ND
Heart	ND	ND	ND	ND
Proline oxidase (POX)				
Kidney	4.18 ± 0.13[a]	2.71 ± 0.08[b]	2.62 ± 0.11[b]	1.76 ± 0.09[c]
Liver	3.85 ± 0.10[a]	1.96 ± 0.08[b]	1.92 ± 0.07[b]	1.41 ± 0.06[c]
Pancreas	ND	1.80 ± 0.09[c]	4.03 ± 0.11[b]	5.79 ± 0.20[a]
Skeletal muscle	ND	0.71 ± 0.02[c]	0.86 ± 0.03[b]	1.03 ± 0.04[a]
Small Intestine	0.81 ± 0.03[d]	1.74 ± 0.07[c]	2.81 ± 0.14[b]	4.85 ± 0.27[a]
Stomach	ND	ND	ND	0.65 ± 0.05
Heart	ND	ND	ND	0.73 ± 0.06
4-Hydroxy-2-oxoglutarate aldolase (HOA)				
Kidney	0.84 ± 0.03[a]	0.62 ± 0.02[b]	0.59 ± 0.03[b]	0.38 ± 0.02[c]
Liver	1.18 ± 0.09[b]	1.56 ± 0.07[a]	1.61 ± 0.08[a]	1.58 ± 0.07[a]
Pancreas	ND	1.40 ± 0.06	1.34 ± 0.09	1.49 ± 0.11
Skeletal muscle	ND	ND	0.06 ± 0.01	0.07 ± 0.02
Small Intestine	ND	0.42 ± 0.02[b]	0.44 ± 0.02[b]	0.58 ± 0.03[a]
Stomach	ND	ND	ND	0.38 ± 0.02
Heart	ND	ND	ND	ND
Alanine: glyoxylate transaminase (AGT)				
Kidney	1.01 ± 0.07[b]	1.05 ± 0.07[b]	1.44 ± 0.09[a]	1.47 ± 0.10[a]
Liver	1.73 ± 0.06[d]	2.41 ± 0.07[c]	3.08 ± 0.08[b]	4.02 ± 0.09[a]
Pancreas	0.92 ± 0.04[c]	2.02 ± 0.09[b]	2.06 ± 0.06[b]	2.63 ± 0.08[a]
Skeletal muscle	0.14 ± 0.01	0.15 ± 0.01	0.17 ± 0.02	0.16 ± 0.02
Small Intestine	0.42 ± 0.02[c]	0.73 ± 0.03[b]	0.91 ± 0.04[a]	0.93 ± 0.05[a]
Stomach	0.07 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.09 ± 0.01
Heart	ND	ND	ND	ND
Serine hydroxymethyltransferase (SHMT)				
Kidney	0.90 ± 0.03[b]	1.21 ± 0.14[a]	1.29 ± 0.11[a]	1.27 ± 0.05[a]
Liver	1.46 ± 0.18[d]	2.99 ± 0.20[c]	4.03 ± 0.19[b]	6.63 ± 0.22[a]
Pancreas	ND	2.25 ± 0.14[c]	4.14 ± 0.27[b]	4.82 ± 0.06[a]
Skeletal muscle	ND	ND	0.49 ± 0.02	0.58 ± 0.06
Small Intestine	ND	ND	0.47 ± 0.08	0.45 ± 0.06
Stomach	ND	ND	ND	1.01 ± 0.14
Heart	ND	ND	ND	ND
Threonine dehydrogenase (TDH)				
Kidney	ND	ND	0.07 ± 0.02[b]	0.15 ± 0.03[a]
Liver	0.53 ± 0.04[d]	0.98 ± 0.03[c]	1.43 ± 0.10[b]	1.85 ± 0.09[a]
Pancreas	ND	2.12 ± 0.09[c]	4.06 ± 0.18[b]	5.34 ± 0.20[a]
Skeletal muscle	ND	ND	ND	ND
Small Intestine	ND	ND	ND	ND
Stomach	ND	ND	ND	ND
Heart	ND	ND	ND	ND

IUGR: intrauterine growth restriction; ND: not detected.
Values, expressed as μmole/g tissue/min, are means ± SEM, n = 6.
[a–d]Within a row, means not sharing the same superscript letter differ ($P < 0.05$).

increased ($P < 0.05$) gradually between days 7 and 21 of age. In the kidney, SHMT enzymatic activity increased ($P < 0.05$) during the first week of postnatal life, but did not change ($P > 0.05$) between 7 and 21 days of age, whereas TDH enzymatic activity was not detected in 0- and 7-day-old IUGR piglets but was present at low levels in 14- and 21-day-old IUGR piglets. The skeletal muscle and small intestine of 14- and 21-day-old IUGR piglets, as well as the stomach of 21-day-old IUGR piglets contained SHMT enzymatic activity.

Activities of enzymes involved in glycine synthesis per whole tissue

The total activity of each glycine-synthetic enzyme per whole tissue in IUGR piglets (Table 2) was calculated as enzyme activity per g tissue (Table 1) × the total weight of tissue (Supplemental Table 2) to estimate the contribution of the 4-hydroxyproline pathway in a tissue to glycine provision in the whole body. For 0- to 14- and 21-day-old IUGR piglets, the liver and skeletal muscle contained the most total OH-POX activity, respectively, followed by kidney and pancreas in descending order. The total activities of OH-POX, POX, HOA, AGT, and SHMT in the kidney and liver, POX in the small intestine, TDH in the liver, as well as AGT in the pancreas, skeletal muscle, small intestine, and stomach increased gradually ($P < 0.05$) between 0 and 21 days of age. The total activities of OH-POX, POX, HOA, SHMT, and TDH in the pancreas, OH-POX and HOA in the small intestine, as well as POX in skeletal muscle increased gradually ($P < 0.05$) between 7 and 21 days of age.

Expression of mRNA for enzymes in tissues

The expression of mRNAs for OH-POX (*PRODH2*), POX (*PRODH*), HOA (*HOGA*), AGT (*AGXT2*), SHMT (*SHMT2*), and TDH (*TDH*) in the kidney, liver, pancreas, skeletal muscle, small intestine, stomach, and heart of 0- to 21-day-old IUGR piglets is summarized in Table 3. Differences in expression of these genes between days of postnatal life were generally similar to their relative enzymatic activities as summarized in Table 2. Among all the genes examined, the expression of *PRODH2* mRNA in the pancreas exhibited the greatest increase (14.1-fold) during the 3-week postnatal period, followed by *PRODH* in the pancreas and *SHMT2* in the liver in descending order. Except for *PRODH*, there were no significant changes in the abundances of mRNAs for other genes in the heart between 0 and 21 days of age. *TDH* mRNA was not detected in the skeletal muscle, small intestine, stomach, or heart of 0- to 21-day-old IUGR piglets.

Localization of OH-POX protein in tissues

Cell-specific localization of OH-POX protein in the liver, kidney, and small intestine of 0- to 21-day-old IUGR piglets is shown in Figures 1 to 3, respectively. Periportal hepatocytes are near the portal triad, perivenous hepatocytes are around the hepatic central vein, and the convoluted tubules of the kidney are the cortical portions of renal tubules; thus, these cell types can easily be identified histologically without any special staining. OH-POX protein was detected at a relatively low level in the liver of all IUGR piglets, and did not exhibit an age-dependent change in distribution among the different zones of the liver. The abundance of OH-POX protein in the liver was similar between 0 and 7 days of age and appeared to increase gradually as the age increased from 7 to 14 days. In the kidney of IUGR piglets, OH-POX protein was localized primarily to the proximal renal tubule and also appeared in distal convoluted tubules. As shown in Figure 1, OH-POX protein was expressed weakly in the kidney of newborn IUGR piglets, but became more abundant in the kidney of 14- to 21-day-old IUGR piglets. In the small intestine (jejunum) of 7-day-old IUGR pigs, OH-POX protein was detected weakly in enterocytes, but not the intestinal glands (crypts); however, at days 14 and 21 of age, OH-POX protein was expressed in both enterocytes and intestinal glands (crypts).

Glycine synthesis from 4-hydroxyproline in tissues

Except for the liver and skeletal muscle of 0- to 7-day-old IUGR piglets as well as the kidney of newborn piglets, concentrations of glycine in extracts of tissues from IUGR piglets after a 2-h period of incubation were greater ($P < 0.05$) than those for tissues without incubation due to net protein degradation (Table 4). Except for the pancreas from newborn IUGR piglets and for skeletal muscle from 0- to 14-day-old IUGR piglets, increasing 4-hydroxyproline concentration in incubation medium from 0 to 5 mM increased ($P < 0.05$) concentrations of glycine in the extracts of tissues from IUGR piglets after a 2 h period of incubation by 22–135%. In both the pancreas from newborn IUGR piglets and the skeletal muscle from 0- to 14-day-old IUGR piglets, the addition of 0.1 to 5 mM 4-hydroxyproline to incubation medium had no effect ($P > 0.05$) on concentrations of glycine in the extracts of tissues from IUGR piglets after the 2 h incubation.

Discussion

Glycine has critical physiological functions in nutrition, metabolism, and health. For example, beyond serving as a building block for proteins (including collagen), glycine participates in one-carbon metabolism essential for DNA synthesis and cell growth, and is also required for the synthesis of heme, purines, creatine, glutathione, and bile salts.[13,31,32] About 80% of dietary glycine is used for protein accretion in neonatal pigs,[13] as glycine accounts for 11.5% of total amino acids in tissue proteins of the whole body.[33] Thus, a deficiency of glycine results in growth restriction and oxidative stress in young pigs,[12] indicating that glycine is a nutritionally essential amino acid in neonates. In this regard, it is noteworthy that we recently discovered that NBW piglets are capable of synthesizing glycine from 4-hydroxyproline in a tissue- and age-specific manner.[14] This study shows that the same metabolic pathway occurs, but is less active, in the liver, kidney, pancreas, skeletal muscle, and small intestine of IUGR piglets (Tables 1–4).

Glycine is the most abundant amino acid in the plasma of sow-reared piglets with NBW (~1–1.2 mM).[13,34] We have recently reported that concentrations of glycine in the plasma of 0- to 21-day-old IUGR piglets (~0.5–0.6 mM) is only 50% of that for age-matched NBW piglets; thus, this severe deficiency of glycine limits the growth of IUGR piglets.[35] At present, little is known about the mechanisms responsible for glycine deficiency in young mammals (including piglets) with IUGR. To address this issue, we examined key enzymes in the metabolic pathways for de novo synthesis of glycine in IUGR piglets (Table 1). Serine hydroxymethyl transferase and threonine dehydrogenase are key enzymes for the synthesis of glycine from serine and threonine, respectively, in animals.[26,36] Hydroxyproline oxidase also plays an important role in the conversion of 4-hydroxyproline into glycine in

Table 2. Activities of glycine-synthetic enzymes and related enzymes per whole tissue in IUGR piglets.

Tissue	Age of piglets (days)			
	0	7	14	21
Hydroxyproline oxidase (OH-POX)				
Kidney	14.4 ± 0.51^{d}	26.1 ± 1.1^{c}	49.7 ± 2.7^{b}	75.5 ± 3.5^{a}
Liver	19.0 ± 0.96^{d}	47.0 ± 7.3^{c}	224 ± 14^{b}	465 ± 16^{a}
Pancreas	ND	2.68 ± 0.24^{c}	12.1 ± 0.45^{b}	35.7 ± 2.2^{a}
Skeletal muscle	ND	ND	ND	1019 ± 15
Small Intestine	ND	45.5 ± 2.5^{c}	145 ± 7.4^{b}	512 ± 25^{a}
Stomach	ND	ND	ND	ND
Heart	ND	ND	ND	ND
Proline oxidase (POX)				
Kidney	23.1 ± 0.71^{c}	28.1 ± 0.87^{b}	41.0 ± 2.1^{a}	44.0 ± 2.4^{a}
Liver	78.5 ± 2.0^{c}	79.5 ± 3.2^{c}	131 ± 5.0^{b}	145 ± 5.7^{a}
Pancreas	ND	3.63 ± 0.18^{c}	12.2 ± 0.35^{b}	26.4 ± 0.90^{a}
Skeletal muscle	ND	426 ± 14^{c}	768 ± 22^{b}	1418 ± 52^{a}
Small Intestine	16.9 ± 0.56^{d}	92.1 ± 3.7^{c}	230 ± 11^{b}	612 ± 34^{a}
Stomach	ND	ND	ND	10.1 ± 0.73
Heart	ND	ND	ND	14.0 ± 1.1
4-Hydroxy-2-oxoglutarate aldolase (HOA)				
Kidney	4.65 ± 0.18^{c}	6.42 ± 0.21^{b}	9.24 ± 0.45^{a}	9.50 ± 0.55^{a}
Liver	24.1 ± 1.8^{d}	63.3 ± 3.0^{c}	110 ± 5.5^{b}	162 ± 6.8^{a}
Pancreas	ND	2.82 ± 0.12^{c}	4.05 ± 0.27^{b}	6.80 ± 0.48^{a}
Skeletal muscle	ND	ND	53.5 ± 4.5^{b}	96.3 ± 6.9^{a}
Small Intestine	ND	22.3 ± 1.1^{c}	36.0 ± 1.6^{b}	73.1 ± 3.2^{a}
Stomach	ND	ND	ND	5.93 ± 0.27
Heart	ND	ND	ND	ND
Alanine: glyoxylate transaminase (AGT)				
Kidney	5.59 ± 0.38^{d}	10.9 ± 0.69^{c}	22.6 ± 1.4^{b}	36.8 ± 2.6^{a}
Liver	35.3 ± 1.3^{d}	97.7 ± 2.7^{c}	210 ± 5.8^{b}	413 ± 9.4^{a}
Pancreas	0.97 ± 0.05^{d}	4.07 ± 0.18^{c}	6.22 ± 0.19^{b}	12.0 ± 0.36^{a}
Skeletal muscle	43.8 ± 3.8^{d}	89.9 ± 6.6^{c}	152 ± 14^{b}	220 ± 25^{a}
Small Intestine	8.75 ± 0.42^{d}	38.6 ± 1.7^{c}	74.4 ± 3.0^{b}	117 ± 6.7^{a}
Stomach	0.24 ± 0.02^{d}	0.54 ± 0.03^{c}	0.82 ± 0.05^{b}	1.40 ± 0.09^{a}
Heart	ND	ND	ND	ND
Serine hydroxymethyltransferase (SHMT)				
Kidney	4.98 ± 0.15^{d}	12.5 ± 1.5^{c}	20.2 ± 1.7^{b}	31.8 ± 1.3^{a}
Liver	29.8 ± 3.7^{d}	121 ± 7.7^{c}	274 ± 13^{b}	681 ± 23^{a}
Pancreas	ND	4.53 ± 0.28^{c}	12.5 ± 0.82^{b}	22.0 ± 0.29^{a}
Skeletal muscle	ND	ND	437 ± 17^{b}	798 ± 77^{a}
Small Intestine	ND	ND	38.4 ± 4.8^{b}	56.7 ± 6.2^{a}
Stomach	ND	ND	ND	15.8 ± 2.2
Heart	ND	ND	ND	ND
Threonine dehydrogenase (TDH)				
Kidney	ND	ND	1.10 ± 0.28^{b}	3.75 ± 0.65^{a}
Liver	10.8 ± 0.73^{d}	39.7 ± 1.1^{c}	97.4 ± 7.2^{b}	190 ± 9.5^{a}
Pancreas	ND	4.27 ± 0.18^{c}	12.3 ± 0.57^{b}	24.4 ± 0.91^{a}
Skeletal muscle	ND	ND	ND	ND
Small Intestine	ND	ND	ND	ND
Stomach	ND	ND	ND	ND
Heart	ND	ND	ND	ND

IUGR: intrauterine growth restriction; ND: not detected.
Values, expressed as μmole/whole tissue/min, are means ± SEM, n = 6.
$^{a–d}$Within a row, means not sharing the same superscript letter differ ($P < 0.05$).

animal tissues.[14] Only by analyzing the expression of these enzymes and the formation of glycine, can we understand the capacities of key tissues for endogenous glycine synthesis via different pathways in IUGR piglets.

As indicated in our recently published work,[14,37] 4-hydroxyproline is a major substrate for glycine synthesis via the OH-POX pathway in 0- to 21-day-old pigs with NBW. This explains, in part, why glycine remains abundant in the plasma of neonatal pigs although this nutrient is relatively low in porcine milk.[9,10] Results of this study indicated that the liver and skeletal muscle were the major sites for the metabolism of 4-hydroxyproline to form glycine in 0- to

Table 3. Expression of mRNAs for glycine-synthetic enzymes and related enzymes in tissues of IUGR piglets.

Tissue	Age of piglets (days)			
	0	7	14	21
Hydroxyproline oxidase (*PRODH2*)				
Kidney	1.00 ± 0.07^{b}	1.05 ± 0.06^{b}	1.37 ± 0.08^{a}	1.40 ± 0.09^{a}
Liver	1.00 ± 0.08^{c}	1.09 ± 0.09^{c}	1.87 ± 0.10^{b}	2.39 ± 0.13^{a}
Pancreas	1.00 ± 0.07^{d}	2.82 ± 0.14^{c}	9.31 ± 0.78^{b}	15.2 ± 0.53^{a}
Skeletal muscle	1.00 ± 0.08^{d}	1.64 ± 0.15^{c}	2.26 ± 0.10^{b}	3.86 ± 0.32^{a}
Small Intestine	1.00 ± 0.09^{d}	2.37 ± 0.18^{c}	3.40 ± 0.22^{b}	4.35 ± 0.24^{a}
Stomach	1.00 ± 0.10	1.14 ± 0.07	1.17 ± 0.12	1.09 ± 0.13
Heart	1.00 ± 0.08	1.20 ± 0.09	1.10 ± 0.07	1.15 ± 0.10
Proline oxidase (*PRODH*)				
Kidney	1.00 ± 0.02^{a}	0.68 ± 0.02^{b}	0.66 ± 0.04^{b}	0.43 ± 0.03^{c}
Liver	1.00 ± 0.04^{a}	0.53 ± 0.04^{b}	0.52 ± 0.02^{b}	0.36 ± 0.02^{c}
Pancreas	1.00 ± 0.09^{d}	2.38 ± 0.15^{c}	6.07 ± 0.46^{b}	8.76 ± 0.59^{a}
Skeletal muscle	1.00 ± 0.08^{d}	2.44 ± 0.18^{c}	3.28 ± 0.25^{b}	4.37 ± 0.29^{a}
Small Intestine	1.00 ± 0.06^{d}	2.63 ± 0.14^{c}	4.36 ± 0.31^{b}	7.54 ± 0.52^{a}
Stomach	1.00 ± 0.08^{b}	1.16 ± 0.12^{b}	1.21 ± 0.08^{b}	2.46 ± 0.21^{a}
Heart	1.00 ± 0.09^{b}	1.14 ± 0.10^{b}	1.19 ± 0.15^{b}	2.73 ± 0.25^{a}
4-Hydroxy-2-oxoglutarate aldolase (*HOGA*)				
Kidney	1.00 ± 0.04^{a}	0.74 ± 0.06^{b}	0.73 ± 0.04^{b}	0.32 ± 0.02^{c}
Liver	1.00 ± 0.06^{b}	1.59 ± 0.11^{a}	1.63 ± 0.13^{a}	1.67 ± 0.11^{a}
Pancreas	1.00 ± 0.08^{b}	2.38 ± 0.16^{a}	2.54 ± 0.23^{a}	2.60 ± 0.17^{a}
Skeletal muscle	1.00 ± 0.09^{b}	1.07 ± 0.07^{b}	2.32 ± 0.16^{a}	2.45 ± 0.19^{a}
Small Intestine	1.00 ± 0.08^{c}	2.08 ± 0.12^{b}	2.27 ± 0.19^{b}	2.91 ± 0.22^{a}
Stomach	1.00 ± 0.07^{b}	1.07 ± 0.10^{b}	1.13 ± 0.07^{b}	2.73 ± 0.25^{a}
Heart	1.00 ± 0.12	1.21 ± 0.13	1.15 ± 0.10	1.06 ± 0.16
Alanine: glyoxylate transaminase (*AGXT2*)				
Kidney	1.00 ± 0.08^{b}	1.12 ± 0.05^{b}	1.65 ± 0.09^{a}	1.68 ± 0.10^{a}
Liver	1.00 ± 0.05^{d}	1.65 ± 0.10^{c}	2.45 ± 0.15^{b}	3.50 ± 0.18^{a}
Pancreas	1.00 ± 0.06^{c}	1.91 ± 0.07^{b}	2.07 ± 0.11^{b}	2.93 ± 0.13^{a}
Skeletal muscle	1.00 ± 0.07	1.08 ± 0.09	1.18 ± 0.13	1.22 ± 0.10
Small Intestine	1.00 ± 0.09^{c}	2.04 ± 0.15^{b}	2.92 ± 0.18^{a}	3.06 ± 0.14^{a}
Stomach	1.00 ± 0.08	1.22 ± 0.13	1.17 ± 0.09	1.08 ± 0.12
Heart	1.00 ± 0.10	1.03 ± 0.11	1.06 ± 0.08	1.14 ± 0.15
Serine hydroxymethyltransferase (*SHMT2*)				
Kidney	1.00 ± 0.04^{b}	1.93 ± 0.10^{a}	2.18 ± 0.12^{a}	2.21 ± 0.17^{a}
Liver	1.00 ± 0.09^{d}	2.14 ± 0.21^{c}	3.36 ± 0.37^{b}	5.73 ± 0.40^{a}
Pancreas	1.00 ± 0.08^{d}	2.47 ± 0.19^{c}	4.50 ± 0.34^{b}	5.29 ± 0.32^{a}
Skeletal muscle	1.00 ± 0.07^{d}	1.35 ± 0.09^{c}	2.06 ± 0.10^{b}	3.19 ± 0.25^{a}
Small Intestine	1.00 ± 0.03^{d}	1.44 ± 0.08^{c}	3.37 ± 0.31^{b}	4.33 ± 0.34^{a}
Stomach	1.00 ± 0.06^{b}	1.14 ± 0.07^{b}	1.22 ± 0.10^{b}	2.90 ± 0.16^{a}
Heart	1.00 ± 0.08	1.07 ± 0.12	1.12 ± 0.08	1.17 ± 0.11
Threonine dehydrogenase (*TDH*)				
Kidney	1.00 ± 0.13^{c}	1.08 ± 0.06^{c}	2.74 ± 0.14^{b}	4.65 ± 0.15^{a}
Liver	1.00 ± 0.06^{d}	1.48 ± 0.10^{c}	2.06 ± 0.12^{b}	2.52 ± 0.07^{a}
Pancreas	1.00 ± 0.09^{d}	4.17 ± 0.32^{c}	10.4 ± 1.2^{b}	16.7 ± 1.6^{a}
Skeletal muscle	ND	ND	ND	ND
Small Intestine	ND	ND	ND	ND
Stomach	ND	ND	ND	ND
Heart	ND	ND	ND	ND

IUGR: intrauterine growth restriction; ND: not detected.
Values are means ± SEM, n = 6.
[a–d]Within a row, means not sharing the same superscript letter differ ($P < 0.05$).

14- and 21-day-old IUGR piglets, respectively (Table 2). A novel and important finding of this study is that the activities of enzymes for the conversion of 4-hydroxyproline into glycine (measured during a period of 15-, 20-, or 30-min incubation depending on tissue) are much lower in the liver, kidney, pancreas, skeletal muscle, and small intestine from IUGR piglets (Table 1) than for NBW piglets in our recent work.[14] For example, the enzymatic activities of renal OH-POX in 0- and 7-day-old IUGR piglets were only 40% and 75% of those for NBW piglets, respectively (Supplemental Figure 2). In addition, the enzymatic activities of hepatic OH-POX in 0-, 7-, 14-, and 21-day-old IUGR piglets were only 10%,

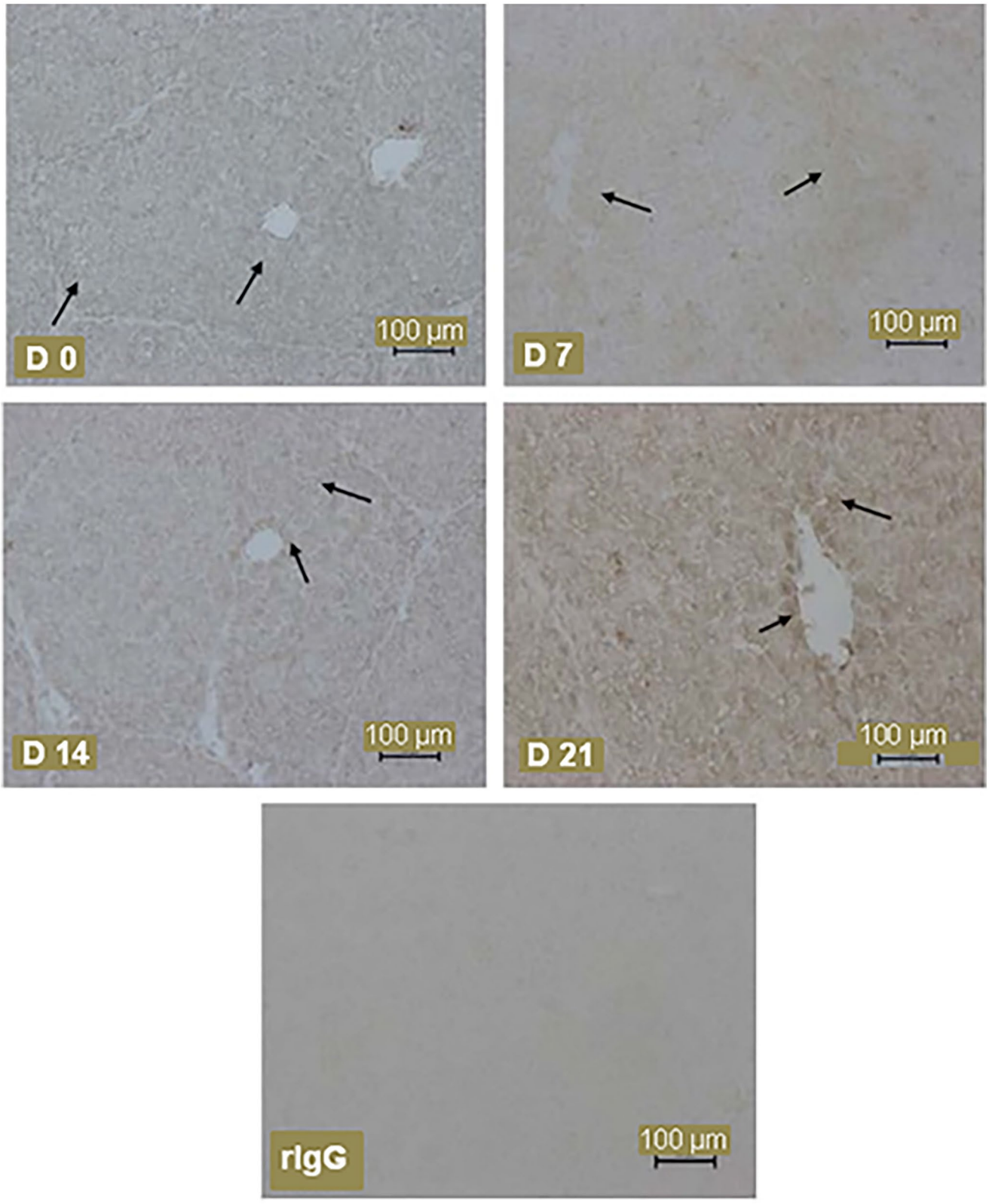

Figure 1. The localization of 4-hydroxyproline oxidase in the liver of 0-, 7-, 14-, and 21-day-old piglets with intrauterine growth restriction. 4-Hydroxyproline oxidase protein was detected at a relatively low level. No age-dependent changes in the distribution of this protein were detected among the different zones of the liver. The abundance of OH-POX protein in the liver appeared to be greater in 21-day-old pigs than in 0-, 7-, and 14-day-old pigs. Arrows indicate the expression of 4-hydroxyproline oxidase in periportal and perivenous hepatocytes. D, day; rIgG, rabbit immunoglobulin G, served as the negative control.

14%, 60%, and 76% of those for NBW piglets, respectively. The enzymatic activities of pancreatic OH-POX in 0-, 7-, 14-, and 21-day-old IUGR piglets were only 0%, 34%, 45%, and 51% of those for NBW piglets, respectively (Supplemental Figure 2). Furthermore, the enzymatic activities of jejunal OH-POX in 7-, 14-, and 21-day-old IUGR piglets were

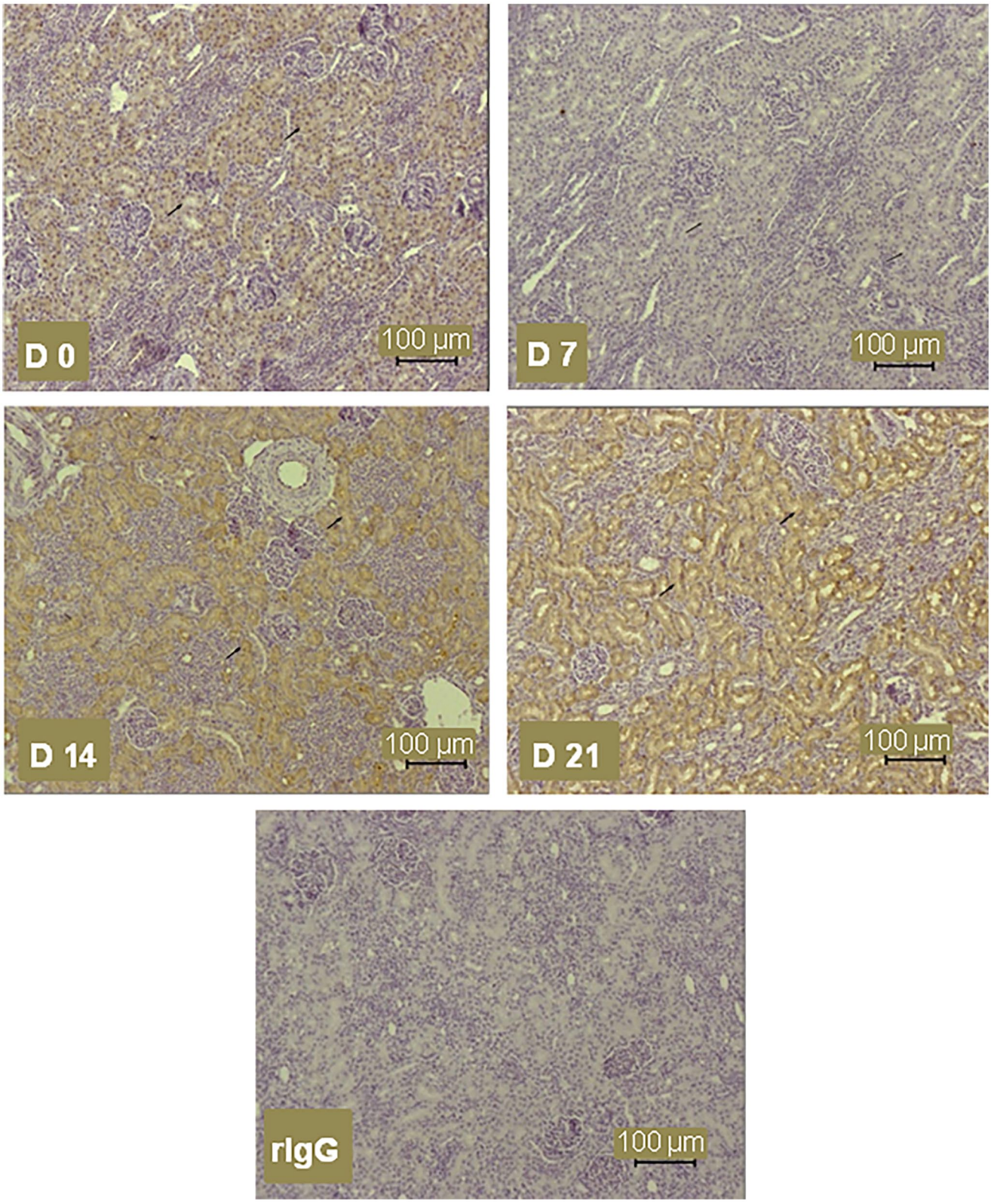

Figure 2. The localization of 4-hydroxyproline oxidase protein in the kidneys of 0-, 7-, 14-, and 21-day-old piglets with intrauterine growth restriction. Arrows indicate the expression of 4-hydroxyproline oxidase in a subset of the convoluted tubules. D, day; rIgG, rabbit immunoglobulin G, served as the negative control.

only 43%, 78%, and 65% of those for NBW piglets, respectively (Supplemental Figure 2). Of particular importance is the finding that OH-POX activity in the skeletal muscle of IUGR piglets was not detected in the first 2 weeks of postnatal life (Table 1), as compared with the onset of OH-POX activity in the skeletal muscle of 14-day-old NBW pigs.[14] At 21 days of age, OH-POX enzymatic activity in the skeletal muscle of IUGR piglets was only 33% of that for NBW piglets (Supplemental Figure 2). This may be considered as an inborn error of glycine synthesis in IUGR pigs.

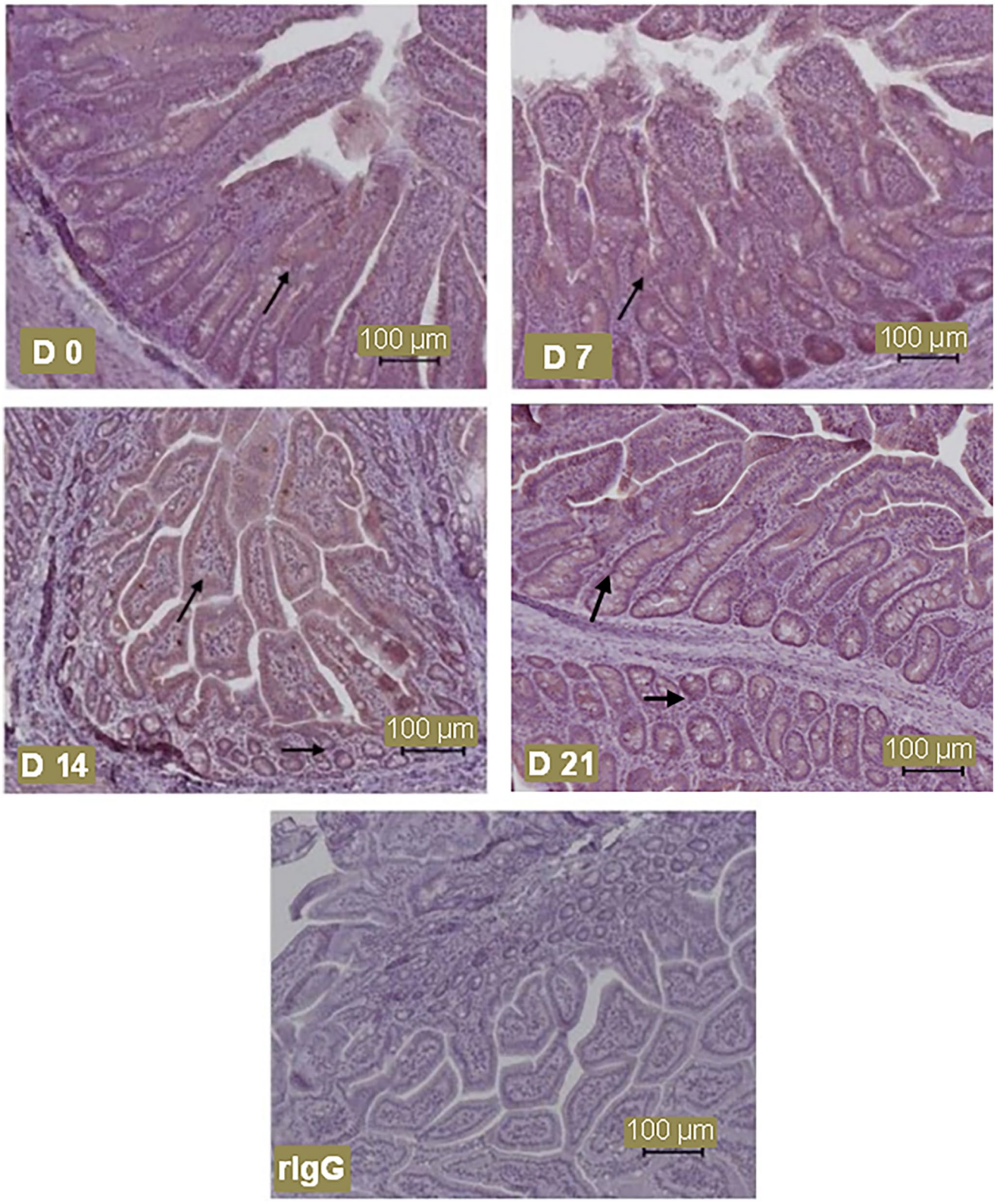

Figure 3. The localization of the 4-hydroxyproline oxidase protein in the small intestine (jejunum) of 7-, 14-, and 21-day-old piglets with intrauterine growth restriction. 4-Hydroxyproline oxidase protein was very weakly detected in the small intestine of newborn pigs. In the jejunum of 7-day-old pigs, OH-POX protein was expressed only in enterocytes, but not intestinal glands (crypts); however, at 14 and 21 days of age, OH-POX protein was expressed in both enterocytes and intestinal glands (crypts). Arrows indicate the expression of 4-hydroxyproline oxidase in enterocytes and crypts. D, day; rIgG, rabbit immunoglobulin G, served as the negative control.

It should be made clear that the activities of enzymes in tissues may not always be accurate indicators of actual metabolic fluxes in tissues.[38] This is because the measurement of enzymatic activity is performed under optimal conditions, such as saturated concentrations of substrates, which do not occur in tissues under physiological conditions. For

Table 4. Concentrations of glycine in medium plus tissue from IUGR pigs after a 2-h period of incubation.

Tissue	No incubation	Concentrations of 4-hydroxyproline in incubation medium (mM)					
		0	0.1	0.2	0.5	2	5
0-day-old pigs							
Liver	1.12 ± 0.06^{f}	$1.27 \pm 0.07^{e,f}$	1.42 ± 0.07^{e}	1.67 ± 0.06^{d}	1.92 ± 0.09^{c}	2.18 ± 0.09^{b}	2.43 ± 0.10^{a}
Pancreas	1.35 ± 0.08^{b}	3.17 ± 0.12^{a}	3.21 ± 0.10^{a}	3.25 ± 0.17^{a}	3.23 ± 0.16^{a}	3.19 ± 0.13^{a}	3.12 ± 0.11^{a}
Kidney	1.41 ± 0.08^{f}	1.64 ± 0.10^{f}	2.11 ± 0.12^{e}	2.42 ± 0.11^{d}	2.78 ± 0.09^{c}	3.14 ± 0.11^{b}	3.51 ± 0.12^{a}
Small intestine	1.15 ± 0.05^{b}	1.91 ± 0.09^{a}	1.96 ± 0.08^{a}	1.94 ± 0.07^{a}	1.96 ± 0.07^{a}	1.92 ± 0.06^{a}	1.99 ± 0.09^{a}
Skeletal muscle	1.37 ± 0.06	1.55 ± 0.08	1.60 ± 0.08	1.57 ± 0.09	1.64 ± 0.10	1.59 ± 0.07	1.61 ± 0.08
7-day-old pigs							
Liver	1.85 ± 0.06^{f}	2.06 ± 0.07^{f}	2.28 ± 0.07^{e}	2.55 ± 0.09^{d}	2.86 ± 0.07^{c}	3.18 ± 0.08^{b}	3.45 ± 0.08^{a}
Pancreas	1.98 ± 0.05^{f}	2.57 ± 0.06^{e}	2.71 ± 0.06^{e}	2.93 ± 0.07^{d}	3.18 ± 0.05^{c}	3.38 ± 0.07^{b}	3.60 ± 0.09^{a}
Kidney	2.15 ± 0.06^{g}	2.61 ± 0.07^{f}	2.88 ± 0.09^{e}	3.16 ± 0.11^{d}	3.44 ± 0.09^{c}	3.71 ± 0.09^{b}	3.97 ± 0.11^{a}
Small intestine	1.92 ± 0.05^{e}	3.70 ± 0.06^{d}	3.82 ± 0.09^{d}	$3.99 \pm 0.08^{c,d}$	$4.16 \pm 0.11^{b,c}$	$4.34 \pm 0.12^{a,b}$	4.51 ± 0.10^{a}
Skeletal muscle	1.63 ± 0.07	1.76 ± 0.08	1.81 ± 0.08	1.80 ± 0.11	1.78 ± 0.10	1.82 ± 0.08	1.84 ± 0.12
14-day-old pigs							
Liver	1.36 ± 0.04^{g}	2.89 ± 0.10^{f}	3.22 ± 0.13^{e}	3.67 ± 0.11^{d}	4.29 ± 0.14^{c}	4.95 ± 0.09^{b}	5.53 ± 0.07^{a}
Pancreas	1.74 ± 0.06^{g}	2.66 ± 0.08^{f}	3.09 ± 0.10^{e}	3.71 ± 0.09^{d}	4.47 ± 0.10^{c}	5.16 ± 0.13^{b}	5.78 ± 0.09^{a}
Kidney	2.32 ± 0.04^{g}	2.68 ± 0.06^{f}	3.02 ± 0.08^{e}	3.39 ± 0.10^{d}	3.87 ± 0.12^{c}	4.54 ± 0.15^{b}	5.08 ± 0.17^{a}
Small intestine	2.66 ± 0.08^{g}	3.90 ± 0.10^{f}	4.21 ± 0.09^{e}	4.53 ± 0.10^{d}	4.85 ± 0.10^{c}	5.17 ± 0.13^{b}	5.59 ± 0.11^{a}
Skeletal muscle	1.05 ± 0.07^{b}	1.84 ± 0.09^{a}	1.86 ± 0.11^{a}	1.92 ± 0.08^{a}	1.89 ± 0.08^{a}	1.87 ± 0.10^{a}	1.90 ± 0.10^{a}
21-day-old pigs							
Liver	1.50 ± 0.06^{g}	2.96 ± 0.10^{f}	3.49 ± 0.12^{e}	4.05 ± 0.14^{d}	4.82 ± 0.17^{c}	5.68 ± 0.16^{b}	6.31 ± 0.19^{a}
Pancreas	1.24 ± 0.04^{g}	3.02 ± 0.05^{f}	3.59 ± 0.10^{e}	4.38 ± 0.16^{d}	5.42 ± 0.27^{c}	6.37 ± 0.31^{b}	7.09 ± 0.37^{a}
Kidney	1.80 ± 0.06^{g}	2.81 ± 0.08^{f}	3.10 ± 0.09^{e}	3.40 ± 0.12^{d}	3.89 ± 0.10^{c}	4.43 ± 0.09^{b}	4.89 ± 0.12^{a}
Small intestine	1.67 ± 0.07^{g}	3.20 ± 0.08^{f}	3.91 ± 0.07^{e}	4.68 ± 0.10^{d}	5.27 ± 0.19^{c}	5.88 ± 0.25^{b}	6.63 ± 0.28^{a}
Skeletal muscle	1.62 ± 0.05^{e}	2.79 ± 0.07^{d}	2.86 ± 0.09^{d}	$2.98 \pm 0.11^{c,d}$	3.24 ± 0.12^{c}	3.55 ± 0.10^{b}	3.88 ± 0.09^{a}

IUGR: intrauterine growth restriction.
Values, expressed as nmol/mg of fresh tissue/2 h, are means ± SEM, n = 6.
[a–g]Within a row, means not sharing the same superscript letter differ ($P < 0.05$).

this reason, we determined the rates of glycine synthesis from 0 to 5 mM 4-hydroxyproline in piglet tissues incubated for 2 h (Table 4). Another novel and salient finding of this study is that, consistent with enzymatic activities, the rates of glycine production from 4-hydroxyproline in the kidney, liver, pancreas, skeletal muscle, and small intestine of IUGR piglets (Table 4) were generally much lower than those for age-matched NBW piglets in our recent work.[14] For example, the rates of conversion of 5 mM 4-hydroxyproline into glycine in the kidneys of 0-, 7-, 14-, and 21-day-old IUGR piglets were only 15%, 15%, 45%, and 44% of those for NBW piglets, respectively. In addition, the rates of conversion of 5 mM 4-hydroxyproline into glycine in the liver of 0-, 7-, 14-, and 21-day-old IUGR piglets were only 8%, 12%, 25%, and 34% of those for NBW piglets, respectively. Likewise, the rates of conversion of 5 mM 4-hydroxyproline into glycine in the pancreas of 7-, 14-, and 21-day-old IUGR piglets were only 18%, 25%, and 32% of those for NBW piglets, respectively. Similarly, the rates of conversion of 5 mM 4-hydroxyproline into glycine in the small intestine of 7-, 14-, and 21-day-old IUGR piglets were only 37%, 16%, and 25% of those for NBW piglets, respectively (Supplemental Figure 3). Furthermore, in contrast to NBW piglets, there was no synthesis of glycine from 4-hydroxyproline in the skeletal muscle of 0- to 14-day-old IUGR piglets, and the rate of conversion of 5 mM 4-hydroxyproline into glycine in the muscle of 21-day-old IUGR piglets was only 17% of that for NBW piglets. Taken together, our results explain why glycine is severely deficient in IUGR piglets,[35] and why glycine is likely a major limiting factor for their optimal growth and development.[7]

Besides the OH-POX-glycine pathway, the SHMT-glycine pathway was also impaired in tissues of IUGR piglets as indicated by the much lower activity of SHMT (Table 1) when compared with age-matched NBW littermates.[14] By contrast, TDH enzymatic activity in tissues was similar between IUGR (Table 1) and NBW piglets.[14] The amounts of serine and threonine available in sow's milk barely meet demands for whole-body protein synthesis in piglets.[39] It is unlikely that threonine, an amino acid that is not synthesized de novo in animal cells, is a quantitatively significant substrate for glycine production in sow-reared piglets.[37] At present, little is known about whole-body synthesis of serine or the conversion of serine into glycine in piglets.[32] Future studies are warranted to address this issue.

Immunohistochemistry is widely used in biomedical research to identify the cell- and tissue-specific expression of proteins,[29] such as OH-POX in the liver, kidney, and small intestine of IUGR pigs (Figures 1 to 3). Results of this study revealed a major difference in the metabolism of glycine in the livers of IUGR versus NBW piglets. The mammalian liver has two different types of hepatocytes (periportal and perivenous) and three distinct zones (the periportal (zone I), transitional (zone II), and perivenous (zone III)), with the periportal and perivenous hepatocytes possessing very different metabolic activities.[40–43] The cell-specific localization of enzymes in these cell types provides a basis for metabolic

zonation in the liver.[40] Interestingly, we found that, in NBW piglets, OH-POX is expressed in the periportal zone (containing ~80–85% of total hepatocytes[40]) of the liver in the first week of postnatal life to produce glycine.[14] In contrast, this zonation of the OH-POX protein was absent from the livers of IUGR piglets. A low activity of OH-POX in both the periportal zone and the perivenous zone (containing ~10–15% of total hepatocytes) likely results in a reduced rate of glycine synthesis in IUGR piglets. *In vivo* measurement of glycine flux through the liver is required to test this hypothesis and better understand the role of 4-hydroxyproline in the nutrition and health of not only piglets[37,44,45] but also other mammals including humans.[46–49]

In conclusion, results of the present study revealed relatively low enzymatic activities of OH-POX and SHMT, as well as low rates of glycine synthesis from 4-hydroxyproline in the liver, kidneys, small intestine, and skeletal muscle of 0- to 21-day-old IUGR piglets. The enzymatic activities of TDH in those tissues did not differ between IUGR and NBW piglets, and threonine is unlikely to be a significant precursor for glycine synthesis in milk-fed neonates. Furthermore, in contrast to NBW piglets, there was no zonation of OH-POX protein in the liver of IUGR piglets. These novel findings provide a biochemical basis to explain why the endogenous synthesis of glycine is insufficient for optimal growth of IUGR piglets. This new foundational knowledge has important implications for improving the growth, development, and survival of IUGR neonates (including pigs and humans) through dietary supplementation with glycine.

AUTHORS' CONTRIBUTIONS

GW designed and supervised the study. SH, WH, and GW performed the experiment. SH and GW statistically analyzed experimental data, summarized results, and wrote the manuscript. FBW and GAJ contributed to data interpretation and manuscript revisions. All authors read and approved the final manuscript.

ACKNOWLEDGEMENTS

We thank Dr. James M. Phang, National Cancer Institute (Frederick, MD 21702, USA) for kindly providing hydroxyproline oxidase antibody; Drs Gayan Nawaratna, Xiaoqiu Wang, and Barry D Long for technical assistance; and Drs John T Brosnan and Margaret E Brosnan for helpful discussion.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This research was supported by the Agriculture and Food Research Initiative Competitive Grant (No. 2014-67015-21770) from the USDA National Institute of Food and Agriculture.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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(Received May 17, 2023, Accepted August 4, 2023)