

Folate Cofactors Regulate Serine Metabolism in Fetal Ovine Hepatocytes

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ABSTRACT

The fetal liver is the primary site of fetal serine production. The regulation of this unique fetal hepatic serine production is unknown. We hypothesized that serine production would be responsive to folate cofactor supply or hormonal regulation. To test this hypothesis, we determined the effect of key folate cofactors and insulin and glucagon on serine and glycine metabolism in primary culture of fetal ovine hepatocytes. Hepatocytes were cultured in serum-free, low-folate media [5 nM 5-methyltetrahydrofolate (THF)] with or without 50 nM 5,10-methylene-THF (MTHF) or 5-formyl-THF (FTHF). Serine and glycine production (P) and utilization (U) were determined by stable isotope dilution with [1-¹³C]serine and [1-¹³C]glycine for 24 h. The effect of insulin (1 μM) or glucagon (1 μM) was determined in a similar manner. Under basal conditions, serine P (43.2 ± 5.1 μmol/mg DNA per 24 h) is greater than serine U (24.1 ± 3.1 μmol/mg DNA per 24 h), whereas glycine U (27.3 ± 3.0 μmol/mg DNA per 24 h) exceeds glycine P (16.7 ± 1.9 μmol/mg DNA per 24 h). MTHF results in a significant decrease in serine U (16.0 ± 2.7 μmol/mg DNA per 24 h; *p* = 0.02 versus low folate), with no change in serine P. FTHF reduces serine P (36.2 ± 4.9 μmol/mg DNA per 24 h; *p* = 0.01), but does not

alter serine U. There were no effects on glycine metabolism with 50 nM MTHF or FTHF. Serine P and U were inversely correlated whereas glycine P and U were directly correlated with the media concentration of MTHF or FTHF. Glucagon treatment increased serine U by 260 ± 65% versus low folate (*p* = 0.0004) but did not change serine P. Insulin treatment led to parallel increases in both serine P and U. Both folate cofactor availability and hormone concentrations regulate serine metabolism in the fetal liver. We speculate that serine metabolism may be a marker of fetal hepatic folate cofactor supply. (*Pediatr Res* 52: 589–594, 2002)

Abbreviations

THF, tetrahydrofolate
MTHF, methylene tetrahydrofolate
FTHF, formyl tetrahydrofolate
SHMT, serine hydroxymethyltransferase
MTHFS, methenyl tetrahydrofolate synthase
MEM, minimal essential medium
BrdU, 5-bromo-2'-deoxyuridine
GC/MS, gas chromatography–mass spectrometry

Serine occupies a unique metabolic position in the fetus. Studies in the fetal sheep demonstrated that serine is one of only three amino acids to have a net production across the fetal liver (1, 2). However, serine is poorly transported across the placenta to the fetus (3, 4). Thus, the fetus must rely on endogenous production of serine to meet the majority of its serine requirements. Serine is an important amino acid for DNA and RNA synthesis and phospholipid synthesis (5, 6). In human fetuses, serine metabolism is similar to that of fetal sheep (7, 8). Indeed, human fetuses that are small-for-gestational age have selectively low levels of serine and gly-

cine, suggesting an important role for serine in fetal growth (8–10).

Little is known about the regulation of fetal hepatic serine metabolism. Others and we have demonstrated that the majority of serine produced by the fetal liver is derived from glycine (2, 11, 12). However, the regulation of fetal hepatic serine metabolism is unclear. It is now clear that fetal hepatic serine synthesis is not dependent on the ontogeny of the key enzymes of serine metabolism (Fig. 1) (13) or substrate availability (11, 14).

Serine and folate metabolism are directly linked. Studies in neuroblastoma cells that over-express MTHFS found a significant increase in serine production (15). This increase in production appears to be directly related to an increase in the availability of MTHF and a decrease in FTHF. Only limited data are available on fetal folate metabolism. Folate is both actively and passively transported across the placenta. At

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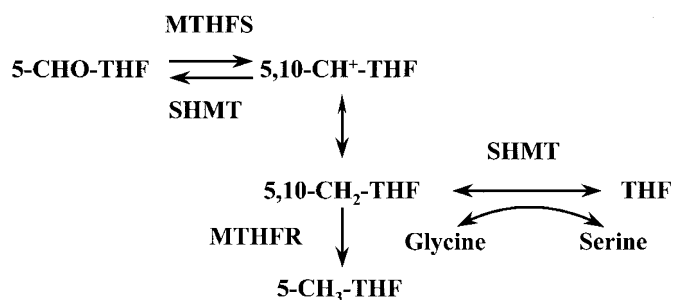


Figure 1. Metabolic pathway of serine and folate metabolism. *MTHFR*, methylene-THF reductase; *5,10-CH⁺-THF*, 5,10-methenyl-THF; *5,10-CH₂-THF*, MTHF; *5-CH₃-THF*, 5-methyl-THF; *5-CHO-THF*, FTHF.

midgestation, only a small amount (0.5%) of maternal folate is found in the fetus, primarily in the fetal liver (16). During the third trimester, there is a more significant transfer of folate to the fetus, primarily to the liver. We have previously demonstrated that the ontogeny of the key enzymes of folate metabolism is consistent with a role in the regulation of fetal hepatic serine metabolism (17). In addition, we have shown that fetal ovine hepatocytes are an excellent model in which to study fetal hepatic serine metabolism (11, 12, 18, 19).

Based on these observations, we hypothesized that the relative supply of MTHF and FTHF to fetal ovine hepatocytes would alter serine metabolism. Specifically, we hypothesized that MTHF would increase net serine biosynthesis in fetal ovine hepatocytes. To test this hypothesis, in the first part of this study, we determined the effects of MTHF and FTHF on serine and glycine metabolism in fetal ovine hepatocytes.

Shortly after birth, there is a change from net hepatic serine production to net hepatic serine utilization (20). Coincident with this change, there is an increase in the activities of the enzymes for the use of serine for gluconeogenesis (21, 22). Inasmuch as induction of gluconeogenesis is linked to hormonal changes that occur at birth (23), it is possible that the change in serine metabolism at birth is in part related to changes in glucagon and insulin that occur after birth. Thus, the second goal of this study was to determine the effect of insulin and glucagon on serine and glycine metabolism in fetal ovine hepatocytes.

MATERIALS AND METHODS

Materials. Media used for these studies was standard MEM α compounded without any added glucose, folic acid, pyridoxine, or arginine, supplemented with 0.4 mM ornithine, prepared by Cell and Molecular Technologies, Inc (Lavallette, NJ, U.S.A.). MTHF, FTHF (6S or 6R + S), and 5-methyl-THF were from Schircks Laboratories (Jona, Switzerland). Stock solutions for culture were prepared as $\times 100$ stock solutions in 1% ascorbic acid, sterilized by filtration, and stored at -20°C until added to cultures. $1\text{-}[^{13}\text{C}]$ serine was from Cambridge Isotope Laboratories (Woburn, MA, U.S.A.), and $1\text{-}[^{13}\text{C}]$ glycine was from Isotec, Inc. (Miami, OH, U.S.A.). Stock solutions were prepared in sterile deionized water, filter sterilized, and added to media just before use. Pyridoxine and collagenase H were from Boehringer Mann-

heim Corp. (Indianapolis, IN, U.S.A.). Glucagon was from Eli Lilly and Co. (Indianapolis, IN, U.S.A.), and purified pork insulin was from Novo Nordisk A/S (Bagsvaed, Denmark). All other chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and were of the highest grade available.

Hepatocyte preparation. Fetal ovine hepatocytes were isolated by perfusion and collagenase digestion of the left lobe of the liver using techniques previously described except that collagenase H was used (11, 18). In brief, the portal vein of the left lobe was catheterized with a 6F catheter and perfused with calcium-free HEPES buffer (10 mM HEPES, 138 mM NaCl, 3 mM KCl, 0.7 mM Na₂HPO₄, pH 7.6). After 10 min, 0.025% collagenase H and 2.2 mM CaCl₂ were added to the perfusate. After collagenase digestion, hepatocytes were filtered through mesh, isolated by centrifugation, and washed three times with media. Hepatocyte viability as determined by trypan blue exclusion was $>95\%$ for all cultures. After isolation, hepatocytes were plated in standard MEM (GIBCO, Grand Island, NY, U.S.A.) supplemented with 10 nM dexamethasone, 10 IU/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, 50 $\mu\text{g}/\text{mL}$ kanamycin, and 10% FCS. After 4 h for adherence, the media was changed to MEM α supplemented with 5 nM 5-methyl-THF, 0.4 mM ornithine, 1.1 mM glucose, 2 mM lactate, 0.2% albumin, 10 nM dexamethasone, 10 IU/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, and 50 $\mu\text{g}/\text{mL}$ kanamycin (low-folate media) without FCS and with folate cofactors from 50 nM (physiologic concentration) to 2000 nM (supraphysiologic). Before any manipulations, hepatocytes were cultured for 24 h in low-folate media to further folate deplete the cells. Thereafter, media was changed every 24 h until harvest.

Stability of MTHF. FTHF is known to be stable in culture. To determine the stability of MTHF in culture, ovine hepatocytes were cultured as above except that the media was Dulbecco's MEM/F12 without phenol red (Specialty Media, Phillipsburg, NJ, U.S.A.). The media was supplemented with 500 nM MTHF. Cells were cultured in a T75 flask with 40 mL of media. Aliquots of 10 mL of media were removed at 0, 0.5, 2, and 24 h. The media was acidified with 1 N HCl to a pH of approximately 2.0, and the acidified media was immediately added to a tube containing 60 mg of sodium borodeuteride to stabilize the MTHF. This results in reduction of the MTHF to 5-methyl-[CH₂D]-THF. The reaction was terminated with stepwise addition of 4 mL of 1 N HCl (to pH 3.0) after 30 min. The samples were then desalted using a C18 resin (100 mg) in a column. The column was washed with 4 vol of 0.01 N HCl, and eluted three times with 0.5 mL of methanol containing 1.0% vol/vol β -mercaptoethanol. Samples were dried overnight under vacuum, covered, and stored protected from light at room temperature until assay. The dried samples were resuspended in 200 μL of argon-treated deionized water, and 10 μL was injected and analyzed on a Finnigan LC/MS (Finnigan, Palo Alto, CA, U.S.A.) using the electrospray ionization mode. Ions were monitored on the negative pole. The predicted $[\text{M}-\text{H}]^{-1}$ for MTHF converted to 5-methyl-[CH₂D]-THF is 459 whereas the predicted $[\text{M}-\text{H}]^{-1}$ for native 5-methyl-[CH₃]-THF is 458.

Stable isotope studies. For stable isotope studies, hepatocytes were cultured for 24 h in low-folate media supplemented

with the folate cofactor of interest. The media was refreshed with the same media and either 1- $^{13}\text{C}_1$ serine or 1- $^{13}\text{C}_1$ glycine at approximately 10% enrichment for a further 24 h. Media was harvested at 0 and 24 h, cellular debris was removed by centrifugation, and the media was snap-frozen on dry ice and stored at -80°C until analysis. Serine and glycine ^{13}C media mole percent enrichment and concentration were determined at 0 and 24 h by GC/MS (11, 12). Serine and glycine interconversion, production, and utilization rates were determined by stable isotope dilution as we have previously described (11, 12).

Bromodeoxyuridine labeling of hepatocytes. To assess the effect of supplementation of hepatocytes with the folate cofactors on cellular division, we determined the incorporation of BrdU by immunohistochemistry. Cells were cultured as above in low-folate media with or without 500 nM supplemental folate cofactors and 80 μM BrdU for 24 h. The cells were washed three times with PBS and fixed in methanol/0.3% hydrogen peroxide. After treatment with 2 N HCl and washing with PBS, BrdU-labeled cells were detected by immunohistochemical detection using Becton-Dickinson (San Jose, CA, U.S.A.) mouse anti-BrdU antibody and detection by 3,3'-diaminobezidine using the universal peroxidase kit from Vector Laboratories, Inc. (Burlingame, CA, U.S.A.). Cells were then counterstained with hematoxylin, and 100 cells per well were counted in triplicate per condition by a single investigator (M.R.N.) blinded to the treatments.

Assays. Cellular DNA was determined by fluorimetry after solubilization in 1% SDS (24). Protein was determined by the method of Lowry *et al.* (25) as modified by Hartree (26). Cytosolic extracts were prepared from hepatocytes as previously described (14). SHMT activity was determined by the method of Geller and Kotb (27). MTHFS was determined by the method of Girgis *et al.* (15).

Statistics. Data are presented as mean \pm SD or SEM. Comparisons among groups were made by paired *t* test with significance taken at $p = 0.05$.

RESULTS

Stability of MTHF. MTHF was readily detectable by direct probe GC/MS in culture media after 0.5 and 2 h on fetal ovine hepatocytes (Fig. 2) and was present at low levels of detection even after 24 h of culture.

Effect of folate cofactors on serine metabolism. The effect of supplementation with 50 nM of the various folate cofactors on serine metabolism is shown in Tables 1 and 2. Under standard low-folate conditions, serine production exceeded utilization, resulting in a net serine production. Supplementation with 50 nM MTHF results in a significant reduction in serine utilization compared with low folate. The result is an increase in net serine production compared with low folate. In contrast, 50 nM 6S-FTHF results in a reduction in serine production compared with low folate. There was no effect on serine and glycine interconversion percentages (Table 2).

Effect of folate cofactors on glycine metabolism. The effect of supplementation with 50 nM of the various folate cofactors on glycine metabolism is shown in Tables 1 and 2. Under

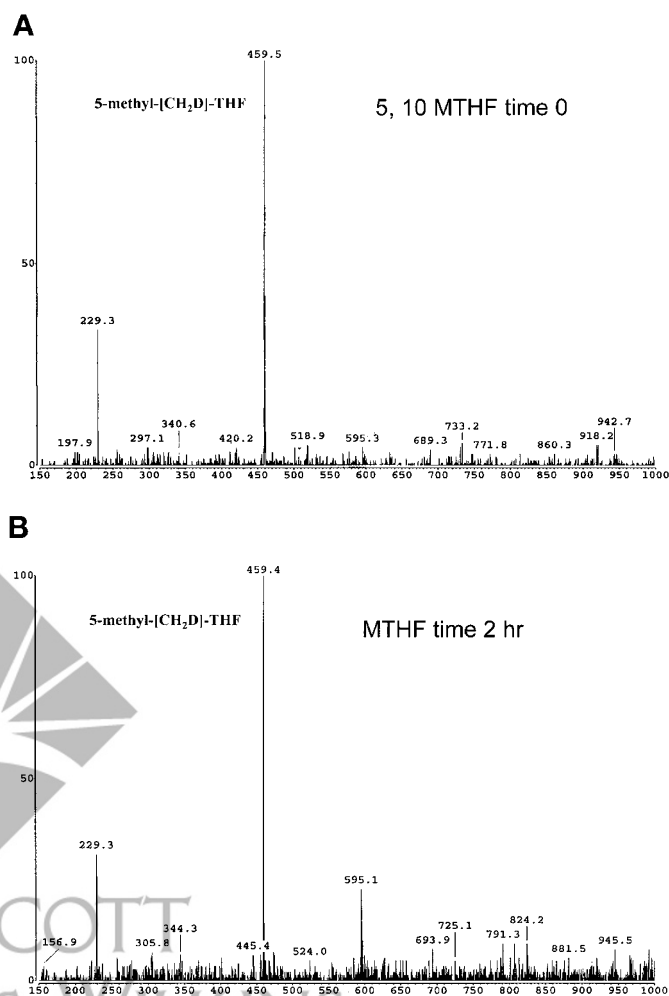


Figure 2. Persistence of MTHF in culture. Direct probe MS analysis of media supplemented with 500 nM MTHF before (time 0 h, *A*) and after application for 2 h (time 2 h, *B*) on fetal ovine hepatocytes. Media preparation and extraction is outlined in the text. Note the strong peak at M/Z 459 representing the 5-methyl- $[\text{CH}_2\text{D}]$ -THF derivative of MTHF.

low-folate conditions, glycine utilization exceeded production with net glycine utilization. There were no changes in glycine metabolism with 50 nM MTHF or FTHF. However, there was a significant difference between glycine production and utilization between MTHF- and FTHF-supplemented cultures (Table 1).

Concentration effect of folate cofactors on serine and glycine metabolism. Increasing the media concentration of MTHF (50 to 2000 nM) did not further affect the changes in serine metabolism (Fig. 3). However, increasing FTHF concentrations (50–2000 nM) did cause a further decrease in both serine production and utilization (Fig. 3). Increasing MTHF concentrations to 500 nM did result in further increases in glycine production and utilization that declined at 2000 nM (Fig. 4). In contrast, there was no effect of increasing concentrations of FTHF (up to 2000 nM) on glycine production or utilization (Fig. 4).

Effect of folate cofactors on SHMT and MTHFS. One mechanism by which folate cofactors might affect the changes in serine and glycine biosynthesis would be through modulation of the specific activity of SHMT or MTHFS. The activity

Table 1. Effect of folate cofactor supplementation on serine and glycine metabolism*

	Low folate (n)	MTHF (n)	6S-FTHF (n)
Serine production μmol/24 h/mg DNA (n)	43.2 ± 5.1 (20)	39.4 ± 4.3 (21) <i>p</i> = 0.06	36.2 ± 4.9 (15) <i>p</i> = 0.01/0.29
Serine utilization μmol/24 h/mg DNA (n)	24.1 ± 3.1 (20)	16.0 ± 2.7 (18) <i>p</i> = 0.02	15.7 ± 3.4 (13) <i>p</i> = 0.20/0.92
Net serine production μmol/24 h/mg DNA (n)	16.2 ± 2.7 (16)	20.4 ± 3.7 (14) <i>p</i> = 0.07	16.3 ± 3.7 (10) <i>p</i> = 0.006/0.27
Glycine production μmol/24 h/mg DNA (n)	16.7 ± 1.9 (21)	21.6 ± 2.3 (21) <i>p</i> = 0.10	14.3 ± 2.1 (15) <i>p</i> = 0.13/0.001
Glycine utilization μmol/24 h/mg DNA (n)	27.3 ± 3.0 (20)	35.0 ± 3.9 (21) <i>p</i> = 0.11	21.8 ± 3.5 (12) <i>p</i> = 0.48/0.003
Net glycine production μmol/24 h/mg DNA (n)	-9.3 ± 2.1 (17)	-11.1 ± 2.8 (18) <i>p</i> = 0.16	-5.5 ± 1.4 (12) <i>p</i> = 0.35/0.20

* After preconditioning in low-folate media for 24 h, hepatocytes were cultured for 24 h with 50 nM of the indicated factor. Serine and glycine production and utilization were determined by stable isotope dilution. Values are mean ± SEM. Comparisons were by paired *t* test between cultures performed in parallel (thus not necessarily all values compared). The *p* values for MTHF are for comparison with low-folate media; *p* values for 6S-FTHF are for comparison with low-folate/MTHF.

Table 2. Effect of folate cofactors on serine and glycine interconversion*

Media	% Ser C to Gly	% Gly P from Ser	% Gly C to Ser	% Ser P from Gly
Low folate	53 ± 31 (9)	53 ± 16 (8)	48 ± 35 (17)	41 ± 30 (15)
MTHF	65 ± 34 (10)	51 ± 22 (8)	54 ± 38 (18)	43 ± 31 (18)
6S-FTHF	54 ± 37 (6)	65 ± 22 (5)	77 ± 20 (11)	60 ± 27 (13)

* After preconditioning in low-folate media for 24 h, hepatocytes were cultured for 24 h with no supplemental folate cofactor or 50 nM of MTHF or 6S-FTHF. Serine and glycine interconversion was determined by stable isotope methodology as outlined in the text. Values are mean ± SD for (n) samples.

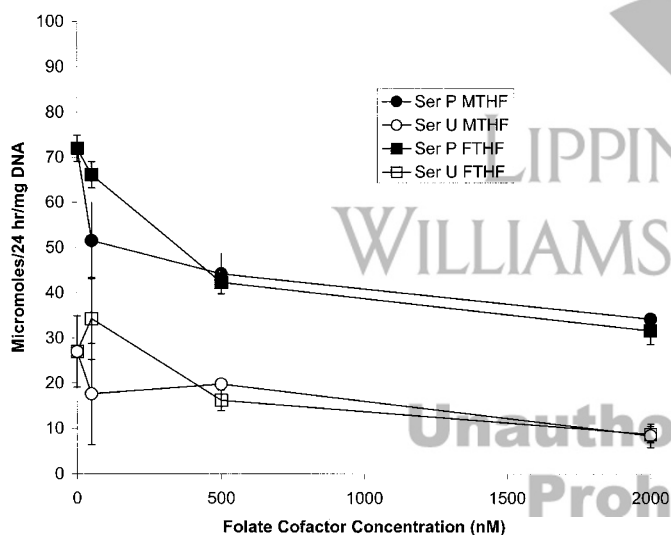


Figure 3. Concentration effect of folate cofactors on serine metabolism. Fetal ovine hepatocytes were cultured in the presence of increasing doses of MTHF or FTHF. Serine (Ser) production (P) and utilization (U) were determined by GC/MS and stable isotope tracing as outlined in the text. Values are mean ± SD for three determinations.

of SHMT and MTHFS were not affected by culture in low-folate media compared with standard media (1 μM folate) or low-folate media supplemented with 50 nM MTHF or 50 nM 6 R+S FTHF (Table 3).

Effect of folate cofactors on BrdU labeling. There was a significant increase in BrdU labeling in cells cultured in low-folate media supplemented with 500 nM MTHF compared with cells cultured in low-folate media or low-folate media supplemented with either 5-methyl-THF or 6S-FTHF (*p* < 0.0005; Fig. 5).

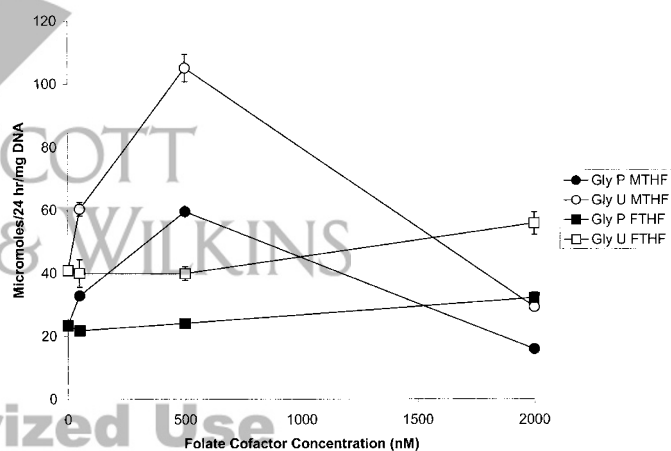


Figure 4. Concentration effect of folate cofactors on glycine metabolism. Fetal ovine hepatocytes were cultured in the presence of increasing doses of MTHF or FTHF. Glycine (Gly) production (P) and utilization (U) were determined by GC/MS and stable isotope tracing as outlined in the text. Values are mean ± SD for three determinations.

Effect of hormonal supplementation on serine and glycine metabolism. The effect of hormonal supplementation on serine and glycine metabolism is shown in Table 4. Insulin treatment (1 μM) results in a generalized increase in all variables compared with no insulin treatment. Glucagon specifically increased serine utilization with no effect on serine production.

DISCUSSION

To date, the regulation of fetal hepatic serine biosynthesis has been unclear. Others and we have demonstrated that glycine, glycolytic intermediates, and glucose are the primary precursors for the unique net fetal hepatic serine biosynthesis

Table 3. Activity of MTHFS and folate supplementation*

Media/condition	SHMT (nmol/min/mg protein)	MTHFS (nmol/min/mg protein)
Native liver	21.1 ± 9.3	0.197 ± 0.122
Standard media	12.1 ± 2.3	0.064 ± 0.026
Low-folate media	12.6 ± 3.4	0.087 ± 0.032
5,10 MTHF	15.1 ± 1.7	0.032 ± 0.017
6 R + S FTHF	11.9 ± 0.9	0.115 ± 0.067

* Values are mean ± SD of three separate determinations.

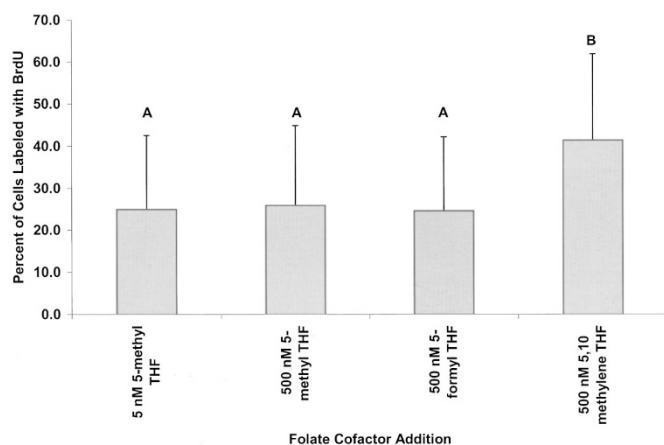


Figure 5. Effect of folate cofactor supplementation on BrdU labeling. Fetal ovine hepatocytes were cultured for 24 h in low-folate media or media supplemented with 500 nM of the indicated folate cofactor. BrdU labeling was determined as detailed in the text. Different letters indicate significant differences. B was significantly different from A by paired two-tailed *t* test at *p* < 0.0005.

Table 4. Effect of insulin and glucagon on serine and glycine metabolism (percent of low-folate values)*

	Insulin 1 μM	Glucagon 1 μM
Serine production	254 ± 116 (<i>p</i> = 0.009)	121 ± 45 (<i>p</i> = 0.15)
Serine utilization	403 ± 206 (<i>p</i> = 0.006)	260 ± 65 (<i>p</i> = 0.0004)
Glycine production	206 ± 65 (<i>p</i> = 0.03)	142 ± 48 (<i>p</i> = 0.04)
Glycine utilization	200 ± 92 (<i>p</i> = 0.02)	170 ± 62 (<i>p</i> = 0.02)

* After preconditioning in LF media for 24 h, hepatocytes were cultured for 24 h with or without 1 μM of the indicated hormone. Glycine and serine production and utilization were determined by stable isotope dilution. Values are mean ± SD. Comparisons with low folate were by paired *t* test.

(11, 28). The primary metabolic pathway involved is SHMT and the glycine cleavage system (12).

In this study, we have demonstrated that, in fetal ovine hepatocytes, provision of excess MTHF leads to a direct reduction in serine utilization and an increase in net serine production. Inasmuch as MTHF is the primary folate cofactor for serine biosynthesis from glycine via SHMT, this would be consistent with continued flux of glycine to serine via SHMT with a concomitant decrease in the flux of serine to glycine. However, the reduction in serine utilization is not solely isolated to the flux through SHMT, as the overall percentage of serine carbon used for glycine synthesis did not change (Table 2). As serine to glycine conversion via SHMT is a major pathway for serine utilization (11, 12), the net result of MTHF supplementation is a decrease in glycine production from serine. Previous work by Girgis *et al.* (15) has shown that increasing MTHF by overexpression of MTHFS led to an

increase in serine accumulation. This work confirms their finding and extends the observation to demonstrate that extracellular MTHF can also lead to an increase in serine, primarily owing to a decrease in serine utilization.

Supplementation with 50 nM FTHF results in a decrease in serine production (Table 1). In neuroblastoma cells, FTHF excess did reduce serine levels (15). Although polyglutamated FTHF is a known slow tight binder of SHMT (29), the mechanism by which extracellular FTHF might influence serine production is unclear. Cellular transport of FTHF appears to be via the reduced folate carrier (30). Thus, intracellular FTHF might increase serine to glycine flux through SHMT to increase the relative abundance of MTHF. The dose response for FTHF on both serine and glycine metabolism does suggest that transport of FTHF is a factor in the regulation of serine and glycine metabolism. Another possible mechanism would be internalization and polyglutamation of FTHF with resultant inhibition of SHMT and a reduction in the flux through SHMT. Whatever the mechanism, this work does parallel the work of Girgis *et al.* (15), demonstrating a lower rate of serine production with FTHF supplementation.

Provision of these cofactors at 50 nM does not alter serine biosynthesis by altering enzyme activity (Table 3). Thus, the increase in serine production is most likely directly related to the provision of excess cofactor.

There is an additional biologic effect of supplementation with MTHF. Cellular division as determined by BrdU labeling is specifically increased with supplementation with MTHF but not with 5-methyl-THF or FTHF. Thus MTHF may regulate the availability of one-carbon precursors for DNA synthesis and cellular division. This provides another mechanism by which folate cofactors and serine metabolism may interact to affect fetal growth.

At birth, there is a shift from net fetal hepatic serine production to net utilization. As many of the changes in metabolism that occur at birth are regulated by changes in glucagon and insulin concentrations (23, 31, 32), we sought to determine the effect of these hormones on serine metabolism. Glucagon treatment dramatically increased serine utilization, converting the ovine hepatocytes to net serine utilization. Although insulin did not alter the net metabolism of serine, it did increase both serine production and utilization. These findings are consistent with a role for glucagon in regulation of the change in hepatic serine metabolism that occurs at birth. The up-regulation of utilization is most likely related to an increase in the utilization of serine for gluconeogenesis. However, the effect of glucagon on folate cofactor supply has not been investigated.

CONCLUSIONS

In this study, we have demonstrated a direct relationship between fetal serine metabolism and specific folate cofactor availability. We speculate that fetal serine metabolism may be a reflection of the fetal folate status. In that regard, it is of interest that intrauterine growth-retarded human fetuses have low serine levels (9, 10). This study has demonstrated that fetal hepatic serine metabolism is regulated in part by the availability of folate cofactors and the balance of insulin and glucagon,

and provides the clues to suggest that abnormalities of fetal serine metabolism may be related to fetal folate status and might impact fetal growth.

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