# Induction of Glutamate Dehydrogenase in the Ovine Fetal Liver by Dexamethasone Infusion during Late Gestation[1]

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**Glucocorticoids near term are known to upregulate many important enzyme systems prior to birth. Glutamate dehydrogenase (GDH) is a mitochondrial enzyme that catalyzes both the reversible conversion of ammonium nitrogen into organic nitrogen (glutamate production) and the oxidative deamination of glutamate resulting in 2-oxoglutarate. The activity of this enzyme is considered to be of major importance in the development of catabolic conditions leading to gluconeogenesis prior to birth. Ovine hepatic GDH mRNA expression and activity were determined in near-term (130 days of gestation, term 147 ± 4 days) control and acutely dexamethasone-treated (0.07 $mg^{-1}$ $hr^{-1}$ for 26 hr) fetuses. Dexamethasone infusion had no effect on placental or fetal liver weights. Dexamethasone infusion for 26 hr significantly increased hepatic GDH mRNA expression. This increased GDH mRNA expression was accompanied by an increase in hepatic mitochondrial GDH activity, from 30.0 ± 7.4 to 58.2 ± 8.1 U GDH/U CS (citrate synthase), and there was a significant correlation between GDH mRNA expression and GDH activity. The generated ovine GDH sequence displayed significant similarity with published human, rat, and murine GDH sequence. These data are consistent with the *in vivo* studies that have shown a redirection of glutamine carbon away from net hepatic glutamate release and into the citric acid cycle through the forward reaction catalyzed by GDH, i.e., glutamate to oxoglutarate.** Exp Biol Med 228:100–105, 2003

**Key words:** glutamate dehydrogenase; fetal liver; dexamethasone; GDH activity

The mitochondrial enzyme glutamate dehydrogenase (GDH; EC 2.1.14) catalyzes both the reversible conversion of ammonium nitrogen into organic nitrogen (glutamate production) and the oxidative deamination of glutamate, resulting in 2-oxoglutarate. The equilibrium of the enzyme favors the reverse reaction, the formation of glutamate under standard conditions (1, 2). This reverse reaction is the only pathway by which ammonia can become bound to the α-carbon of a α-carboxylic acid.

GDH activity has been documented for different physiological states, and in the myometrium, placenta, and fetal liver, brain, kidney, heart, and pancreas (3–11). The present study was stimulated by a number of studies directed at glutamine and glutamate exchange between the fetal liver and placenta (12, 13). These studies have shown that during ovine fetal life, glutamine, from maternal plasma, is transported across the placenta into the fetal circulation, from which it is taken up by the fetal liver in large quantities (14–16). The glutamine is used in part for net fetal glutamate release from the liver. The hepatic glutamate released maintains the fetal glutamate concentration and is the key determinant of glutamate delivery to the placenta. The placenta virtually clears all the glutamate from the fetal circulation, with an extraction coefficient of 75%–90% (12), and oxidizes it through the actions of placental GDH (12, 17). Recent studies have shown that a fetal dexamethasone infusion, sufficient to induce parturition, near term also causes a marked reduction in the net release of glutamate from the fetal liver. Using L-[1-$^{13}$C]glutamine, it was shown that the glutamine carbon flux was redirected into the citric acid cycle, suggesting increased placental GDH activity under these conditions (13). The present study was designed to determine whether there was an increased expression of hepatic GDH mRNA and corresponding GDH activity after such a fetal dexamethasone infusion in the near-term ovine fetus.

## Material and Methods

**Animal Preparation.** This study was approved by the University of Colorado Health Sciences Center Animal

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Care and Use Committee. Twelve time-mated 2- to 3-year-old Columbia-Rambouillet ewes pregnant with a single fetus were used. Surgery was performed to insert catheters for dexamethasone infusion at approximately 120 days of gestation (term 147 ± 4 days). Preoperatively, the ewe was given 500 mg of ampicillin and 500 mg of gentamycin intramuscularly. Surgery was performed under a combination of general pentobarbital (65 mg/ml) and spinal anesthesia (2 ml of 1% pontocaine) after a 48-hr fast with free access to water. During surgery, general anesthesia was maintained with intermittent i.v. infusion of pentobarbital via the jugular catheter. The fetus was anesthetized with pentobarbital via the maternal circulation. The uterus was exposed via a midline laparotomy incision. A 7- to 9-cm uterine incision was then made, a fetal forelimb was exteriorized, and an incision was made on the medial site of the knee joint. The brachial vein was located and a polyvinyl catheter (1.4 mm OD) was inserted. An amniotic catheter was also attached to the fetus by attachment to the brachial vein catheter for the injection of antibiotics into the amniotic cavity. The catheters were flushed every other day with heparinized saline (30 U/ml). At least 5 days were allowed for full recovery as assessed by normal $O_2$ content and glucose concentration in the fetal circulation before dexamethasone infusion was commenced.

**Experimental Design.** On the day of study, at approximately 130 days of gestation, a bolus of 0.2 mg of dexamethasone was administered to the fetus via the fetal brachial vein followed by a continuous infusion (0.07 $mg^{-1}$ $hr^{-1}$) for 26 hr. This infusion rate and duration was based upon previous studies that concluded that this regime was sufficient to induce metabolic changes that were consistent with the onset of parturition (18). A group of five ewes comprised the control group. These animals had undergone fetal surgery for use in other studies, but problems with catheter patency made them unsuitable for their planned studies and thus available as controls in this study. After the experimental period, the fetus was delivered by cesarean section with i.v. pentobarbital anesthesia (25 mg/kg). The fetal liver was collected and weighed. The right lobe was removed and the left lobe was sectioned into slices after trimming the peripheral tissues. Approximately 10 g were placed in mitochondrial isolation medium (IM) buffer (220 m*M* mannitol, 70 m*M* sucrose, 2 m*M* Hepes, and 0.5 mg/ml bovine serum albumin [BSA], pH 7.4; 4°C) for mitochondrial fraction isolation, which proceeded immediately. Mitochondria, for GDH activity determination, were obtained as previously described (19). Mitochondrial pellets were resuspended in IM buffer and were stored at −80°C. GDH activity was measured within 3 weeks of mitochondrial pellet preparation. The remaining liver slices were frozen in liquid nitrogen and were stored at −80°C until later total cellular RNA extraction.

**Enzymatic Analysis.** The catalytic activity of GDH was determined at room temperature by measuring the decrease in absorbance due to the oxidation of NADH (20). Briefly, mitochondrial samples were thawed on ice, diluted 1:1 with IM, sonicated, and centrifuged at 100,000*g* for 1 hr at 4°C. The supernate was further diluted 1:20 with IM prior to assay. Ten to 50 μl of diluted sample was added to a reaction mixture (64.5 m*M* TEA, 3.22 m*M* EDTA, 129 m*M* ammonium acetate, pH 8.0, 17.6 m*M* NADH, 96 m*M* ADP, and 160 kU/l LDH). Absorbance was read at 339 nm until all endogenous pyruvate had been consumed. Once a steady state had been achieved, 100 μl of 2-oxoglutarate (7 m*M*) was added and changes in absorbance (OD) were recorded every 30 sec for up to 5 min. Sample volumes that produced a linear decrease in absorbance no greater than 0.05/min over the 5-min oxidation period were used. NADH oxidation was linear with time and enzyme concentration under conditions reported in the original method (20). Samples were also assayed for citrate synthase (CS) activity as previously described (21). GDH activity was than expressed as units of GDH activity/units of CS activity, where a unit was defined as the change in OD per minute at 25°C.

**GDH Reverse Transcription-Polymerase Chain Reaction (RT-PCR).** An ovine GDH cDNA probe was generated through RT-PCR. PCR primers were chosen based on published sequences of the human and rat GDH (X07769 and X14223, respectively): upper primer 5′–GCGAGGACGACCCCAACTT-3′ and lower primer, 5′–TCAATGCCAGGACCAATAA-3′. The RT reaction was carried out using 1 μg of ovine liver total cellular RNA (tcRNA) using SuperScriptII (Gibco-BRL, Gaithersburg, MD). Thermal cycling using Taq DNA polymerase included 35 cycles at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for an additional 1 min. The PCR reaction was held at 72°C for a final extension for 10 min before being cooled to room temperature. An aliquot of the product was fractionated on a 1.5% agarose gel and was stained with ethidium bromide. Liver tcRNA samples yielded a single product of approximately 480 base pairs. After PCR, the product was cloned into pCR 2.1 (Invitrogen, Carlsbad, CA) and was then transformed using a INVαF' One Shot kit (Invitrogen).

**Sequencing Data.** Sequencing of the DNA was conducted on several clones using an ABI 377 automated DNA sequencer with dye rhodamine terminator chemistry using both the M13 Reverse and M13 forward primers. Nucleotide sequence verification was performed using basic local alignment search tool (BLAST; National Center for Biotechnology Information, Bethesda, MD). The amplified product sequenced corresponded to nucleotides 192–672 of human GDH (X07769) and was submitted to GenBank (accession number AY102935).

**RNA Preparation and Northern Hybridization.** Total cellular RNA was prepared using TRI-REAGENT (Molecular Resource Center, Cincinnati, OH) as previously described (22). Total cellular RNA (20 μg) was denatured and electrophoresed in a 1% agarose gel, glyoxal/DMSO in 0.01 *M* $PO_4$ buffer (pH 7.4) and was transferred to an $N^+$ Hybond membrane (Amersham, Pis-

cataway, NJ) using a pressure blotter PosiBlot (Stratagene, La Jolla, CA) under alkaline conditions (10 m*M* NaOH). After transfer, membranes were UV crosslinked and rinsed in 6× SSC (0.72 *M* NaCl, 40 m*M* $Na_2HPO_4.7H_2O$, and 4 m*M* EDTA) before the integrity of RNA and transfer efficacy was confirmed by methylene blue staining (23). The membranes were then rinsed in diethyl pyrocarbonate-treated water until the membrane was clear of background stain. These procedures were conducted at room temperature. Stained membranes were photographed, and samples not displaying distinct and clear 28S and 18S rRNA were discarded.

Transferred membranes were prehybridized (6× SSC, 50% [v/v] formamide, 10% [w/v] sulfate Dextran, 7% [w/v] sodium dodecyl sulfate [SDS], 5 μg/ml denatured salmon sperm DNA, and 100 5 μg/ml yeast tRNA (Gibco-BRL) for 2–4 hr at 42°C. Membranes were then hybridized with a 481 base pair *EcoRI-EcoRI* $^{32}$P-labeled ovine-specific GDH cDNA fragment at a concentration of 1–2 × $10^6$ cpm/ml. After a 16-hr hybridization period at 42°C, membranes were washed twice with 2× SSC/0.1% SDS for 20 min at room temperature and then twice with 0.1× SSC/0.1% SDS for 15 min each at 65°C. The molecular size of ovine GDH-specific band was estimated from a 0.4- to 9.5-kb RNA ladder (Gibco-BRL). Each gel was run with a sample of postnatal lamb and maternal liver, which served as a positive control for GDH expression. Membranes were exposed to a phosphorus plate for 12 hr and were scanned by a Storm system (Molecular Dynamics, Sunnyvale, CA). Resulting images were analyzed through ImageQuant (Molecular Dynamics) generating data in the form of mRNA expression volumes. To correct for loading and transfer differences after GDH quantification, the membranes were stripped (0.5% boiling SDS allowed to cool to room temperature) and reprobed with an 18s rRNA probe. 18s rRNA levels were measured by hybridization with an oligonucleotide probe (5′-ACGGTATCTGATCCGTCTTCGAACC-3′) labeled with $^{32}$P-dCTP using terminal deoxytransferase (Boehringer Mannheim Biochemicals, Indianapolis, IN). After this, the membranes were subjected to the same washing procedures and image analysis as described.

**Statistical Analysis.** Gestational age and fetal and placental weights and values from the enzymatic analysis are reported as means ± SEM. Results from the phosphorus imaging scanning, a ratio of ovine GDH mRNA expression compared wih 18s rRNA expression, are reported as means ± SEM. Differences among groups were analyzed by Student's *t* test with significance set at $P < 0.05$.

## Results

Tissue was collected at approximately 130 days of gestation after fetal treatment. A 26-hr dexamethasone infusion had no significant effects on fetal or fetal liver weights (Table I).

**Table I.** Placenta and Fetal Parameters in Control, (5) and Dexamethasone, (7) Infused Fetus Near Term (130 Days of Gestation). Values are Mean ± SEM

| | Control | Dexamethasone |
|---|---|---|
| Gestational age (days) | 130 ± 2 | 130 ± 2 |
| Placental weight (g) | 306.5 ($n = 2$) | 370.8 ± 25.8 ($n = 4$) |
| Fetal weight (g) | 2937.8 ± 322.2 | 3247.7 ± 207.3 |
| Fetal liver weight (g) | 116.8 ± 11.0 | 103.1 ± 5.0 |

The nucleotide sequence of ovine cDNA for GDH is shown in Figure 1. The cDNA sequence for ovine GDH (GenBank Accession number AY102935) exhibits 95.2, 91.7, and 90.9% homology with human (X07769, corresponding to bp 192–672), rat (X14223), and murine (X51081) sequences, respectively. In northern analysis, a single transcript of approximately 2.8 kb was displayed, which is in agreement with other published GDH message data (24).

Dexamethasone infusion for 26 hr resulted in significantly elevated GDH mRNA expression ($P < 0.02$; Fig. 2). This increased GDH mRNA expression was accompanied by an increase in mitochondrial GDH activity from 30.0 ± 7.4 to 58.2 ± 8.1 units of GDH activity/units of CS activity ($P < 0.03$; Fig. 3). Moreover, there was a significant correlation between GDH mRNA expression and GDH activity ($P < 0.001$, $r^2 = 0.52$; Fig. 4).

## Discussion

These results demonstrate that under the influence of a fetal dexamethasone infusion near term, sufficient to induce parturition, the expression and activity of GDH in the fetal liver is increased. GDH is exclusively located in the mitochondrial matrix and catalyzes the reaction:

$$\text{glutamate} + NAD^+ + H_2O \leftrightarrows \alpha\text{-ketoglutarate} + NH_4 + NADH + H^+$$

The equilibrium coefficient of this reaction is $1\sim10 \times 10^{-14}$, which strongly favors glutamate production or what is termed the reverse reaction (2). Together with recently published data demonstrating that fetal dexamethasone infusion decreases fetal glutamate output (13), the results reported here demonstrate that the activity of GDH is increased and altered to favor the forward reaction, that is, the production of α-ketoglutarate and associated reductive units. This dexamethasone-induced change in GDH activity could, in the normal course of events, be ascribed to the increased need for citric acid cycle intermediates for glycogen synthesis. In the fetal sheep, glucocorticoid infusions have been demonstrated to increase fetal liver glycogen concentrations (25), presumably through glucogenic enzymes such as GDH and cytosolic phosphoenolpyruvate carboxykinase, changes that occur naturally as parturition approaches.

The observation of increased hepatic GDH mRNA expression after fetal infusion of dexamethasone is the first

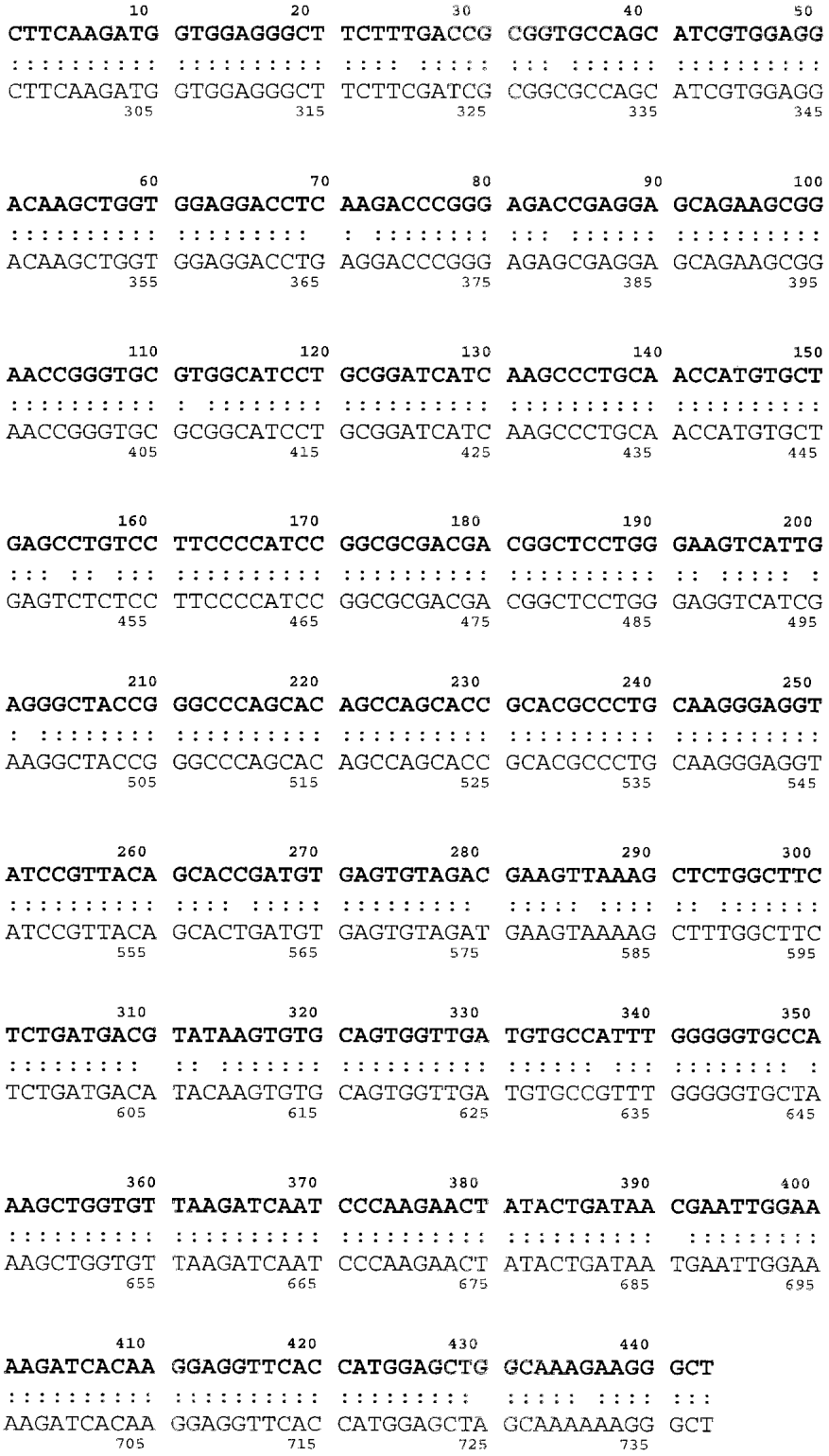


**Figure 1.** The sequence for sheep cDNA for glutamate dehydrogenase (GenBank accession number AY102935) aligned with the sequence for human GDH (M20867).

report of *in utero* alterations in liver enzymatic activity in the fetal sheep relative to glutamine/glutamate metabolism. Glucocorticoid-induced increases in hepatic GDH activity reported in this study are similar to those found previously in adult (4) and fetal (3, 9, 24) rats, and the measured GDH activity nearly doubled in this study after the 26-hr dexamethasone administration. In the rat, a relatively low activity of hepatic GDH has been described with activity increasing just prior to birth (3, 24), where an increase in glucocorticoid concentration would be expected. Moreover, maximal GDH mRNA expression levels are generally reached 2 days prior to birth, declining to prebirth levels,

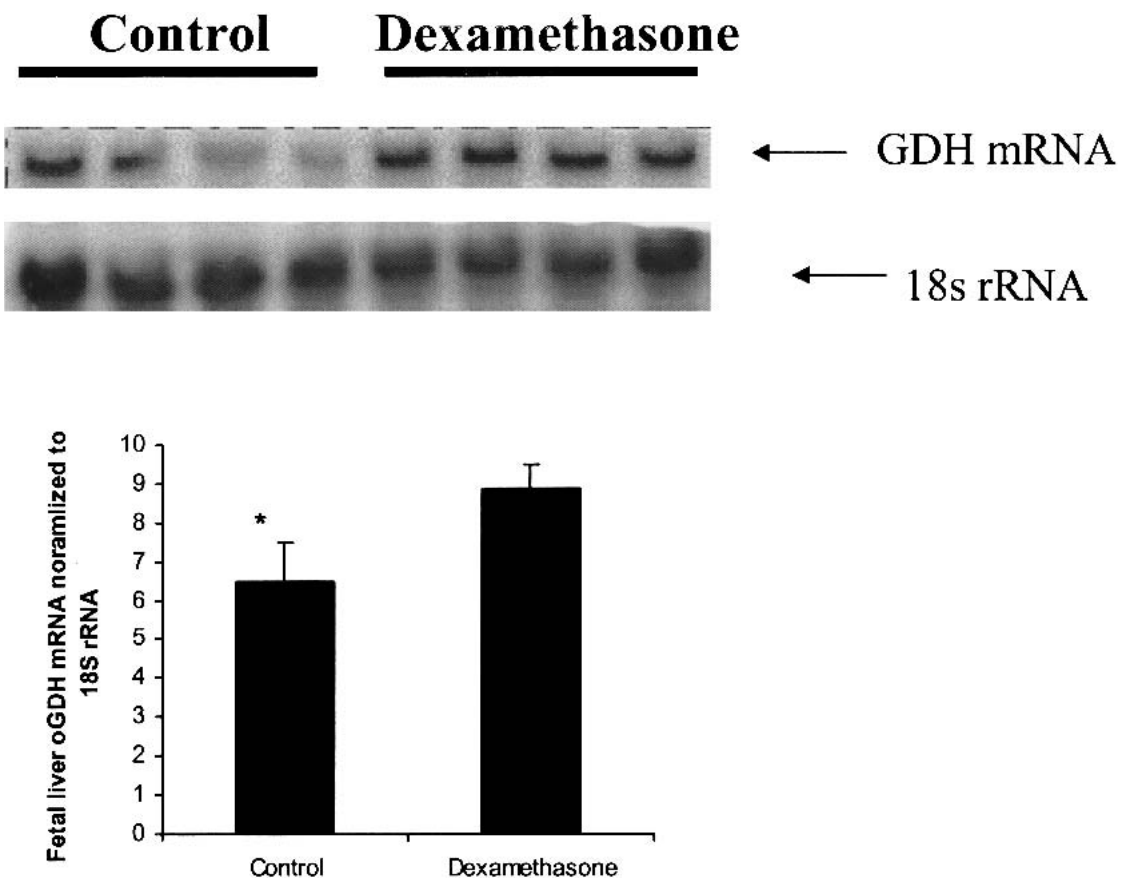


**Figure 2.** Northern blot analysis of oGDH mRNA fetal hepatic levels of control and dexamethasone-infused fetuses after 26 hr of dexamethasone infusion. A single transcript was determined at approximately 2.8 kb. Quantification of the oGDH mRNA levels normalized to the signal from 18s mRNA is shown below. Values are means ± SEM.

and then rising again to peak levels at 3–4 postnatal weeks (24). Of additional interest is the observation that in the rat placenta, as gestation advances, GDH activity actually decreases (9), suggesting a change in the placental metabolism of glutamate and placental production of ammonia. This change may occur as the placental role in fetoplacental amino acid metabolism decreases and the fetus becomes the major regulator of fetoplacental amino acid metabolism. During the prenatal period, GDH mRNA expression is regulated at both the translational level as well as at the pretranslational level, whereas after birth, GDH expression is thought to be regulated predominantly at the pretranslational level (24). Studies in adult rats have shown that $T_3$ treatment and cortisol increase the level of GDH, suggesting hormonal regulation of this gene (5). The induction of GDH activity by thyroid hormones is partly inhibited by blocking *de novo* protein synthesis, suggesting that $T_3$ is involved in altering gene transcription as well as specific enzyme characteristics (5). During fetal life, regulation of GDH through thyroid hormone interactions have not yet been reported, though the branched chain amino acids, leucine, isoleucine, and valine are also reported to enhance GDH activity during fetal life (26).

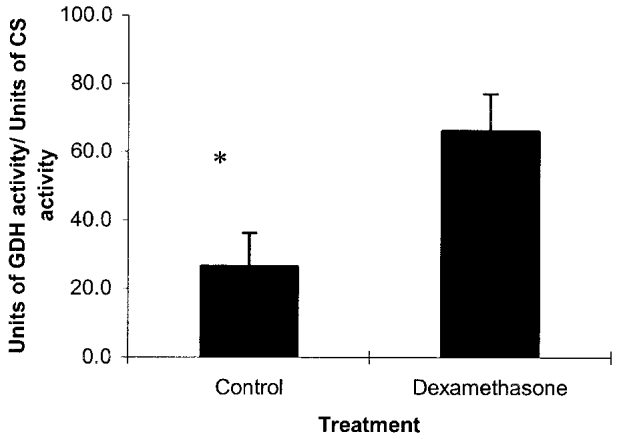


**Figure 3.** Hepatic GDH activity per citrate synthase activity for control and dexamethasone-treated fetuses after 26 hr of dexamethasone infusion. Values are means ± SEM.

In conclusion, we have demonstrated that infusion of dexamethasone induces significant increases in GDH message expression and GDH activity. These data are consistent with the *in vivo* studies, which have shown a redirection of glutamine carbon away from net hepatic glutamate release and into the citric acid cycle through the forward reaction catalyzed by GDH, i.e., glutamate to oxoglutarate.

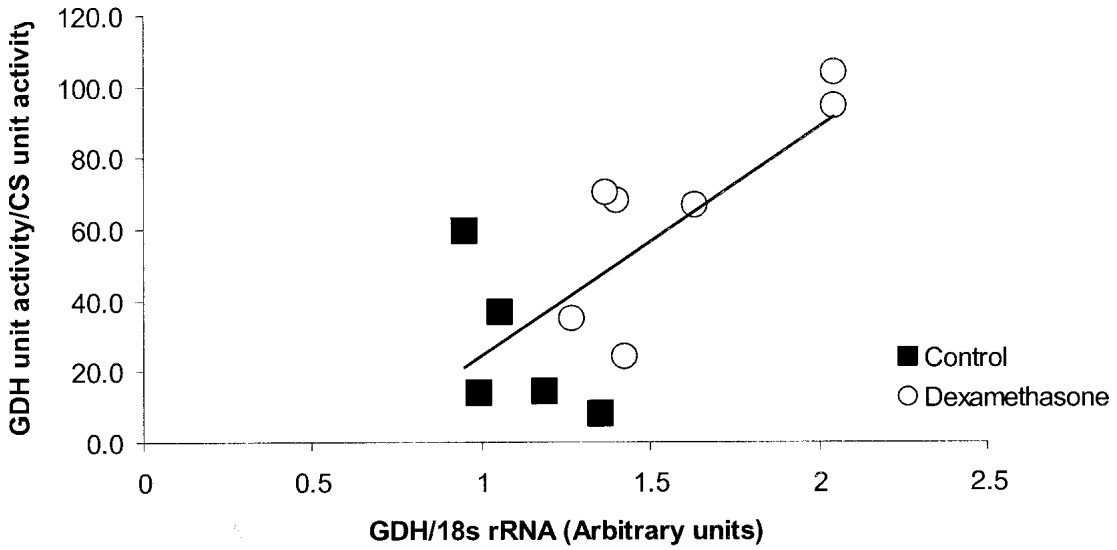


**Figure 4.** Relationship between hepatic GDH mRNA expression and activity in control and dexamethasone-treated fetuses after 26 hr of dexamethasone infusion. Y = 64.294x – 39.879, $r^2$ = 0.5223. Values are means ± SEM.

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