Fetal hyperinsulinemia increases farnesylation of p21 Ras in fetal tissues

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MATERIALS AND METHODS

Materials. Bovine serum albumin and other biochemicals were from Sigma (St. Louis, MO). The anti-p21 Ras rat monoclonal IgG, Y13–259, and Protein G-PLUS/protein A-agarose immunoprecipitation reagents were from Oncogene Science (Uniondale, NY). Tritiated farnesyl pyrophosphate was from Du Pont-NEN. Bacterially expressed Ras protein with a Cys-Val-Lys-Ser COOH terminus was a kind gift from Dr. Charles Omer (Merck, West Point, PA). All supplies and reagents for SDS-PAGE were from Bio-Rad (Hercules, CA). The enhanced chemiluminescence kit was a product of Amersham.

Animal care and surgical procedure. Studies were performed in late-gestation Columbia–Rambouillet time-dated pregnant ewes obtained from a commercial breeder (Nebeker Ranch, Santa Monica, CA), as previously described (20). In brief, after hysterotomy, fetal catheters for blood sampling were inserted into the abdominal aorta via hindlimb arteries. Catheters for infusions were inserted into the femoral veins via hindlimb veins. Maternal catheters were placed via a single groin incision into the femoral artery for sampling and into the femoral vein for infusions. All catheters were tunneled subcutaneously through a maternal skin incision and maintained within a plastic pouch secured to the ewe’s flank. Catheters were flushed every other day with heparinized saline [150 U heparin/ml of 0.9% (wt/vol) NaCl in water]. All animals recovered from surgery and were standing, eating, and drinking by 6–8 h after surgery. The ewes were maintained in a temperature-controlled environment (18 ± 2°C) and were allowed ad libitum access to alfalfa pellets, water, and a mineral block. Ewes were kept in a standard cart next to similarly housed sheep. All studies were approved by the University of Colorado Health Sciences Center (UCHSC) Animal Care and Use Committee. Studies were performed at the UCHSC Perinatal Research Facility, which is accredited by National Institutes of Health, the US Department of Agriculture, and the American Association for the Accreditation of Laboratory Animal Care.

Experimental design. The experimental study was conducted after a postoperative recovery period of ≥5 days in six groups of animals. In group 1, fetuses received saline infusion; in group 2, fetuses received insulin infusion to achieve hyperinsulinemia. Insulin (pure pork insulin, Eli Lilly, Indianapolis, IN) was prepared by diluting insulin in 0.9% (wt/vol) sodium chloride in water and was given as a continuous infusion of insulin (1.0 mU·min⁻¹·kg estimated fetal wt⁻¹) to produce fetal hyperinsulinemia. Blood (0.03 ml) was sampled every 10–15 min for the remainder of the study for fetal arterial plasma glucose concentration, and a fetal intravenous infusion of D25W (25% wt/vol dextrose in water) was adjusted to maintain the hyperinsulinemic euglycemic clamp. Group 3 received fetal infusions of glucose to produce an approximately twofold increase in fetal glucose concentrations, and group 4 received similar fetal infusions of glucose and somatostatin to block fetal insulin secretion. Group 5 received maternal infusions of glucose to produce a twofold increase in fetal glucose concentrations, and group 6 received similar maternal infusions of glucose plus fetal infusions of somatostatin to block fetal insulin secretion.

All infusions were carried out between 0800 and 1200 and lasted 4 h. The stability of the steady state was judged by the lack of oscillations of >5% up or down of the mean concentration values over the sampling period.

Fetal blood samples were analyzed for hematocrit, blood oxygen content, oxygen saturation, and concentrations of plasma glucose, lactate, and insulin (20).

Maternal blood equal to the total volume of blood removed from the fetus during steady-state draws was transfused into the fetus at a relatively constant rate, with one-half of the volume given during the 20 min before the steady-state draws and the other one-half at the end of the steady-state draw (for the first steady state only, because the animal was euthanized immediately after the final blood samples were obtained). Isovolumetric transfusions also were given approximately every 30 min during the glucose clamp between the two sampling periods. Approximately 12% of fetal blood volume was replaced during the study.

At the end of the study, euthanasia solution was injected into the mother (12 ml intravenously) and fetus (2 ml intra-cardiac) (Sleepaway, pentobarbital sodium in 10% alcohol, Fort Dodge Laboratories, Fort Dodge, IA). At autopsy, the fetus was removed, dried, and weighed. Tissue samples obtained from liver, fat, and skeletal muscle were immediately frozen in liquid nitrogen until determinations of the FTase activity and the amounts of farnesylated p21 Ras 5–7 days later.

In vitro FTase assay. FTase activity was assayed in vitro using a modified method of Moores et al. (14). Homogenates of tissue samples were lysed in 500 μl of buffer [in mM: 150 NaCl, 5 MgCl₂, 1 phenylmethylsulfonyl fluoride, 1 dithiothreitol (DTT), 1 sodium vanadate, 1 sodium phosphate and 1% Triton X-100, 0.05% SDS, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 50 mM HEPES, pH 7.5]. Crude lysates were sonicated and centrifuged at 10,000 g. Total protein was determined by bicinchoninic acid assay (Pierce) and diluted to 0.5 mg·ml⁻¹·sample⁻¹. The in vitro filtration assay was initiated by adding a 5-μl aliquot of diluted extract to 45 μl of reaction assay solution [5 mM MgCl₂, 5 mM DTT, 100 nM ras protein, 100 nM tritiated farnesyl pyrophosphate (15 Ci/mmol), 50 mM HEPES, pH 7.5] and incubated at 37°C for 60 min. The assay was stopped with 1 ml of ice-cold 1 M HCl in ethanol, and the samples were placed on ice for 15 min. Reaction solutions were transferred to borosilicate glass tubes (12 × 75 mm), and 2 ml of ice-cold ethanol were added to each tube. Solutions were filtered through Whatman GF/C glass-fiber filters. Each filter was air dried, placed in a scintillation vial with 10 ml of scintillation fluid, and quantified by scintillation spectrometry.

Separation of farnesylated and unfarnesylated p21 Ras. Equal volumes of tissue lysate and 2% Triton X-114 were combined in a borosilicate glass tube, vortexed, and incubated at 37°C for 3 min. Solutions were kept at room temperature until phases had separated. Equal samples from each phase were placed in separate 1.5-ml Eppendorf tubes, and p21 Ras proteins were immunoprecipitated using the monoclonal antibody Y13–259. Relative amounts of p21 Ras were determined by Western blotting followed by densitometry, as previously described (3, 8).

Statistical analysis. All statistics were analyzed by Student’s t-test, with a P value of <0.05 considered significant, and by a one-way analysis of variance (ANOVA), using SAS/STAT software 6.12, with resulting P values adjusted by Dunnett’s procedure for comparing the mean of each group with a common control (group 1). Results are expressed as means ± SE.

RESULTS

Experimental groups. Fetal and maternal weight, gestational age, and plasma concentrations of glucose and insulin are summarized in Table 1. Group 1 received fetal infusions of saline and served as controls. Hyperinsulinemia in group 2 was achieved by infusion
of insulin directly into the fetal circulation (hyperinsulinemic euglycemic clamp). Fetal infusion of glucose alone (group 3) resulted in fetal hyperglycemia and hyperinsulinemia. Addition of somatostatin (group 4) blocked hyperinsulinemia in the hyperglycemic fetuses. Ewes in group 5 received an infusion of glucose, whereas their fetuses received no infusion. Fetuses in this group became both hyperglycemic and hyperinsulinemic (Table 1). An infusion of somatostatin into the fetuses whose mothers were infused with glucose (group 6) blocked fetal hyperinsulinemia, but fetuses remained hyperglycemic. There were no differences in fetal oxygen content, hemoglobin, and plasma lactate concentrations among the experimental groups (data not shown).

### Effects of fetal hyperinsulinemia on prenylation of fetal p21 Ras

Infusion of insulin directly into the fetal circulation increased the activity of FTase and the amounts of farnesylated p21 Ras in skeletal muscle, fat, and liver (Figs. 1, A-C, and 2) compared with tissues obtained from control animals, which had received saline infusions. The amounts of farnesylated p21 Ras in tissues of control animals ranged from 38 to 48% of the total.

![Fig. 1. Effect of fetal hyperinsulinemia on farnesyltransferase (FTase) activity in fetal liver (A and D), fat (B and E), and skeletal muscle (C and F). Tissue samples from the 6 experimental groups were obtained at the end of various infusions, as described in Experimental design. Results represent means ± SE of 3–4 independent determinations per group. *P < 0.01 vs. control. Glc, glucose; SST, somatostatin; MGlc, maternal infusion of glucose; FSST, fetal infusion of somatostatin; SAL, (in A, B, and C), fetal saline infusion and (in D, E, and F) maternal saline infusion.](http://ajpendo.physiology.org/)

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**Table 1. Fetal characteristics and fetal levels of glucose and insulin**

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<th>Group 1</th>
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| Fetal weight, g | 2,766 ± 181 | 3,138 ± 429 | 2,919 ± 55 | 3,054 ± 103 | 3,576 ± 136 | 3,506 ± 730 |
| Maternal weight, lb | 127 ± 9 | 126 ± 12 | 119 ± 13 | 140 ± 4 | 101 ± 2 | 139 ± 7 |
| Gestational age, days | 135 ± 1 | 136 ± 1 | 131 ± 1 | 128 ± 1 | 134 ± 2 | 134 ± 3 |
| Plasma glucose, mg/dl | 18 ± 0.4 | 17 ± 2 | 40 ± 0.9* | 36 ± 0.5* | 42 ± 4* | 37 ± 0.7* |
| Plasma insulin, ng/ml | 0.24 ± 0.05 | 3.23 ± 1.2* | 0.47 ± 0.10* | 0.25 ± 0.05 | 1.35 ± 0.55* | 0.10 ± 0.01 |

1 Average value from the 30th min of infusion to the end of the infusion. *Significantly greater than control value (group 1), analyzed by ANOVA. n, number of fetuses in each group.
cellular Ras. A 4-h infusion of insulin increased these amounts to an average of 65% ($P < 0.001$).

Infusions of glucose into the fetal circulation resulted in fetal hyperinsulinemia and increased the activity of FTase and the amounts of farnesylated p21 Ras in fetal skeletal muscle, fat, and liver to 58% of the total cellular Ras ($P < 0.01$; Figs. 1, A-C, and 3). Because infusion of glucose stimulated fetal insulin secretion and made these animals hyperinsulinemic, we performed additional experiments to distinguish the effects of hyperglycemia from those of hyperinsulinemia. In these experiments, in addition to an infusion of glucose, fetuses received an infusion of somatostatin to block endogenous hyperinsulinemia while animals remained hyperglycemic (Table 1, group 4). The addition of somatostatin completely prevented increases in the activity of FTase and the amounts of farnesylated p21 Ras in all three tissues (Figs. 1, A-C, and 3), demonstrating that these increases were caused by hyperinsulinemia and not by hyperglycemia.

**Effects of maternal hyperglycemia on prenylation of fetal and maternal p21 Ras.** To examine whether maternal hyperglycemia can influence the amounts of farnesylated p21 Ras in fetal tissues, pregnant ewes were infused with glucose, which produced fetal hyperglycemia and hyperinsulinemia, whereas the fetuses received either no infusion (group 5) or somatostatin (group 6), which inhibited fetal hyperinsulinemia. The activity of FTase and the amounts of farnesylated p21 Ras in fetal skeletal muscle, fat, and liver were significantly increased by the maternal infusion of glucose (Figs. 1, D-F, and 4). Infusion of somatostatin into the fetal circulation blocked fetal insulin secretion and prevented increases in the activity of FTase and the

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**Fig. 2.** Effect of fetal hyperinsulinemia on the amounts of farnesylated p21 Ras in fetal skeletal muscle, fat, and liver. Results are expressed as means ± SE of 7 independent measurements in tissues obtained from 4 control (CNT) and 3 hyperinsulinemic (Ins) animals. $P < 0.01$ vs. control in all tissues.

**Fig. 3.** Effect of fetal glucose infusion on the amounts of farnesylated p21 Ras in fetal tissues. Glucose was infused either alone (solid bars) or with somatostatin (checkered bars). Results are expressed as means ± SE of 7 independent measurements in tissues obtained from 4 control, 3 glucose-infused, and 3 glucose + somatostatin-infused animals. $P < 0.01$ vs. control and vs. somatostatin infusions.
amounts of farnesylated p21 Ras in fetal tissues (Figs. 1, D-F, and 4), supporting the role of fetal hyperinsulinemia to augment prenylation of p21 Ras in fetal tissues.

We also assessed the influence of fetal hyperinsulinemia on the amounts of farnesylated p21 Ras in fetal WBC. Freshly isolated WBC (buffy coat) were obtained at the end of experimental infusions, lysed, and used to determine the amounts of farnesylated p21 Ras, as described in MATERIALS AND METHODS. Infusion of glucose to mothers increased farnesylated p21 Ras in fetal WBC from 40 to 58% of total cellular Ras (P < 0.01). This increase was completely eliminated by fetal infusion of somatostatin (P < 0.001, group 6 vs. group 5; Fig. 5).

Interestingly, maternal hyperglycemia that provoked maternal hyperinsulinemia also increased the amounts of farnesylated p21 Ras in maternal skeletal muscle (Fig. 6A) and maternal WBC (Fig. 6B). These experiments confirm our previous findings in mice, rats, dogs, and humans that in vivo hyperinsulinemia increases the amounts of farnesylated p21 Ras in various tissues (3).

DISCUSSION

Contribution of hyperinsulinemia to fetal growth is a well-known physiological phenomenon with a poorly understood biochemical mechanism (reviewed in Refs. 4, 13, 15). With a lack of direct molecular evidence of the growth-promoting action of insulin in fetal tissues, effects of hyperinsulinemia on fetal growth have been assigned to the following three potential mechanisms (reviewed in Ref. 9): 1) enhancement of the uptake and utilization of nutrients by insulin-sensitive fetal tissues, 2) direct mitogenic activity, and 3) stimulation of release and/or interactions with insulin-like growth factors (IGF) and their binding proteins. All of these three have been reviewed previously and are beyond the scope of this discussion. Notwithstanding these three potential mechanisms, our current studies provide additional insight into the mechanism of action of fetal hyperinsulinemia on fetal growth.

Here, for the first time, we demonstrate that fetal hyperinsulinemia causes significant increases in the activity of FTase and the amounts of farnesylated p21 Ras in fetal tissues (Figs. 1, D-F, and 4), supporting the role of fetal hyperinsulinemia to augment prenylation of p21 Ras in fetal tissues.

We also assessed the influence of fetal hyperinsulinemia on the amounts of farnesylated p21 Ras in fetal WBC. Freshly isolated WBC (buffy coat) were obtained at the end of experimental infusions, lysed, and used to determine the amounts of farnesylated p21 Ras, as described in MATERIALS AND METHODS. Infusion of glucose to mothers increased farnesylated p21 Ras in fetal WBC from 40 to 58% of total cellular Ras (P < 0.01). This increase was completely eliminated by fetal infusion of somatostatin (P < 0.001, group 6 vs. group 5; Fig. 5).

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Ras proteins are the key elements in the mitogenic signaling pathways of practically all growth factors and growth-promoting agents (1, 11). They exist either in an inactive GDP-bound form or in an active GTP-bound conformation. Guanine nucleotide exchange factors promote dissociation of GDP from the inactive Ras-GDP complex, allowing Ras proteins to bind GTP (2, 12). This activation of Ras proteins by GTP loading required posttranslational modification of Ras proteins (farnesylation) and their anchoring at the plasma membrane (17, 18).

The process of farnesylation of Ras is catalyzed by a ubiquitous enzyme, farnesyltransferase (reviewed in Ref. 21), the activity of which is augmented by insulin (3, 5, 8). Insulin promotes phosphorylation of the FTase α-subunit and increases FTase enzymatic activity both in vivo and in vitro (3, 5, 8). We have previously shown that endogenous or experimental hyperinsulinemia increases FTase activity in adult mice, rats, dogs, and humans (3). These increases are accompanied by increases in the amounts of farnesylated p21 Ras in skeletal muscle, fat, liver, and WBC. The effect of insulin on prenylation of p21 Ras does not result automatically in enhanced activation of this signaling intermediate. Farnesylated p21 Ras is translocated to the plasma membrane, where it can exist in either a GDP- or GTP-associated form. However, the presence of greater amounts of farnesylated p21 Ras at the plasma membrane subsequently results in increased activation of p21 Ras in response to other growth factors (10). The present study is in concert with these previous observations and demonstrates increased amounts of farnesylated p21 Ras in hyperinsulinemic ewes and their fetuses. Circulating monocytes possess insulin receptors and respond to hyperinsulinemia with increased amounts of farnesylated p21 Ras in a manner similar to other insulin target tissues (3). Thus easily obtainable WBC can serve as surrogates of the effect of hyperinsulinemia on prenylation in vivo.

Two important observations were made in the course of this study. First, we convincingly demonstrated that fetal hyperinsulinemia causes significant increases in the activity of FTase and the amounts of farnesylated p21 Ras in fetal tissues. Inhibition of endogenous insulin secretion with somatostatin abrogated the increases in the activity of FTase and the amounts of farnesylated p21 Ras, supporting the causative role of fetal hyperinsulinemia; hyperglycemia alone did not affect the amounts of farnesylated p21 Ras in fetal tissues. Second, we demonstrated that maternal hyperglycemia increased the activity of FTase and the amounts of farnesylated p21 Ras in fetal tissues via augmented fetal insulin secretion. In these experiments, fetal infusion of somatostatin prevented increases in activity of FTase and the amounts of farnesylated p21 Ras in fetal tissues, defining insulin as the causative factor in regulating farnesylation of p21 Ras.

Numerous studies from different laboratories have indicated that the Ras pathway does not contribute to the mechanism of the metabolic effects of insulin. In contrast, this pathway is considered to be an extremely important signaling element of the mitogenic action of insulin and other growth factors (1, 2, 12, 21). Our extensive, previously published in vitro data indicate that greater availability of farnesylated p21 Ras augments mitogenic cellular responsiveness to IGF-I, epidermal growth factor, platelet-derived growth factor, and other growth-promoting factors (6, 10). This “priming” effect of insulin is specific to this hormone and is not mimicked by IGF-I or other growth factors (6). Moreover, the effect of insulin on farnesylation of p21 Ras requires the presence of intact insulin receptors and is not mediated via the IGF-I receptor (6).

In conclusion, we previously postulated that hyperinsulinemia increases mitogenic cellular responses by providing greater availability of farnesylated p21 Ras (3, 5–8, 10). We have now shown that, in the case of maternal hyperglycemia during pregnancy, the resulting fetal hyperinsulinemia increases the amounts of
farnesylated p21 Ras in fetal tissues. These increases create a “fertile” milieu for the mitogenic action of other growth factors such as IGF-I. Thus elevated levels of fetal insulin (which is a minor mitogen in its own right) prime the responsiveness of fetal tissues to other growth factors, resulting in fetal macrosomia.

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REFERENCES