Insulin and amino acids independently stimulate skeletal muscle protein synthesis in neonatal pigs

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THE RELATIVE RATES OF GROWTH and protein synthesis are higher during the neonatal period than at any other stage of postnatal life (16, 20, 29, 31, 35). During the neonatal period, more rapid gains in protein mass occur in skeletal muscle than in other tissues (50). Previous studies in rats and pigs suggest that neonates utilize their dietary amino acids efficiently for growth because they are capable of a greater increase in protein synthesis in response to feeding than older animals (9, 15, 17, 18, 40, 41). The feeding-induced stimulation of protein synthesis is more dramatic in skeletal muscle than in other organs and decreases profoundly with development (7–9, 17). For example, in response to refeeding, fractional rates of skeletal muscle protein synthesis in 7-day-old pigs increase from 15 to 24%/day and in 26-day-old pigs from 4 to 6%/day (9).

Insulin is recognized as a key factor in the regulation of skeletal muscle protein synthesis in the neonate. Postprandial changes in protein synthesis are positively correlated with changes in circulating insulin concentrations in the neonatal pig (12). Studies using our novel hyperinsulinemic-euglycemic-euaminoacidemic clamp technique in neonatal pigs have shown that insulin stimulates whole body amino acid disposal in the neonate and that the insulin sensitivity and responsiveness of amino acid disposal decreases with development (48). The infusion of insulin at doses achieving fed plasma insulin levels, when amino acids and glucose are maintained at fasting levels, reproduces the feeding-induced stimulation of muscle protein synthesis (11, 49). This response of protein synthesis to insulin declines with development (11, 49) in parallel with the developmental decline in the stimulation of muscle protein synthesis by feeding (9).

The postprandial rise in amino acids also plays an important regulatory role in the stimulation of protein synthesis by feeding in the neonate. Recently, we demonstrated that the infusion of amino acids at a dose that reproduces fed-state amino acid levels increases protein synthesis in skeletal muscle of the neonate (13). This increase in muscle protein synthesis occurs in the presence of either fasting or fed insulin levels and is similar to that obtained by insulin stimulation alone. The infusion of fed levels of either insulin or amino acids, alone or in combination, increases muscle protein synthesis to within the range normally found in the fed state. However, it was not determined
whether insulin and amino acids interact at submaxi-
mal doses to stimulate skeletal muscle protein synthe-
sis in the neonate.

In the present study, we wished to determine
whether insulin and amino acids independently regu-
late skeletal muscle protein synthesis in the neonate.
Specifically, we asked whether 1) the stimulation of
muscle protein synthesis by insulin requires concur-
rent amino acid stimulation; 2) the stimulation of mus-
cle protein synthesis by amino acids requires insulin;
3) the basal fasting insulin level stimulates muscle
protein synthesis; 4) there is a dose-response effect of
amino acids on muscle protein synthesis; 5) there is
a dose-response effect of insulin on muscle protein syn-
thesis; and 6) there is an effect of amino acids on the
insulin sensitivity of muscle protein synthesis. To ad-
dress these issues, pancreatic glucose-amino acid
clamps were used in fasted neonatal pigs to block
insulin secretion while glucose and glucagon were
maintained at fasting levels. Insulin was infused to
achieve levels that simulate less than fasting (−0.5
μU/ml insulin), fasting (−2 μU/ml insulin), interme-
diate (−6 μU/ml insulin), and fed (−30 μU/ml insulin)
levels while at each insulin dose amino acids were
clamped at either the fasting or fed level. At the high-
est insulin dose, amino acids were also reduced to less
than fasting levels. The results showed that insulin
and amino acids act independently to stimulate protein
synthesis in skeletal muscle of the neonate.

METHODS

Animals. Eleven multiparous crossbred (Yorkshire ×
Landrace × Hampshire × Duroc) sows (Agriculture Head-
quarters, Texas Department of Criminal Justice, Huntsville,
TX) were housed in lactation crates in individual, environ-
mentally controlled rooms, maintained on a commercial diet
(GP-18, PMI Feeds, Richmond, IN), and provided water ad
libitum throughout the lactation period. After farrowing,
piglets were returned to the sow until studied. The study was conducted in
accordance with the guidelines for the Care and Use of Laboratory Animals.

Pancreatic glucose-amino acid clamps. Pigs (n = 8–12/
treatment group; total of 90) were placed, unanesthetized, in
a sling restraint system following a 12-h fast. During a
30-min basal period before the clamp procedure was initi-
atcd, blood samples were taken and immediately analyzed
for blood glucose (YSI 2300 STAT Plus, Yellow Springs
Instrument, Yellow Springs, OH) to establish the average basal
concentration of blood glucose (19). Plasma total branched-
chain amino acid (BCAA) concentrations were determined by
rapid enzymatic kinetic assay (5) to establish the average
basal concentration of BCAA to be used in the subsequent
clamp procedure. Pancreatic glucose-amino acid clamps were
performed using techniques similar to those previously
described (45). The clamp was initiated with a primed (20 μg/
kg), continuous (100 μg·kg⁻¹·h⁻¹) somatostatin (Bachem,
Torrance, CA) infusion. After a 10-min infusion of somatosta-
tin, a continuous infusion of replacement glucagon (150
ng·kg⁻¹·h⁻¹; Eli Lilly, Indianapolis, IN) was initiated and
continued to the end of the clamp period. Insulin was infused
at 0, 10, 22, and 110 ng·kg⁻¹·min⁻¹ to achieve plasma
insulin concentrations of −0.2, 6, and 30 μU/ml to simulate
less than fasting, fasting, intermediate, and fed insulin lev-
els, respectively (9). At each dose of insulin, amino acids were
clamped at either the fasting (−500 nmol BCAA/ml) or fed
(−1,000 nmol BCAA/ml) level by adjusting the infusion rate
of a balanced amino acid mixture (see below) to maintain the
plasma BCAA concentration within 10% of the desired level
(48). At the highest insulin dose only, amino acids also were
allowed to fall below fasting levels (−250 nmol BCAA/ml) by
omitting the amino acid clamp. The amino acid mixture (13)
(48). At the highest insulin dose only, amino acids also were
content contained (mmol) arginine (20.1), histidine (12.9), isoleucine
(28.6), leucine (34.3), lysine (27.4), methionine (10.1), phenyl-
alanine (12.1), threonine (21.0), tryptophan (4.4), valine
(34.1), alanine (27.3; 38% provided as alanyl-glutamine), aspartate (12.0), cysteine (6.2), glutamate (25.8), glutamine
(17.1; 100% provided as alanyl-glutamine), glycine (54.3; 4%
provided as glycyl-tyrosine), proline (34.8), serine (23.8), taur-
ine (2.0), and tyrosine (7.2; 83% provided as glycyl-tyrosine).
Blood samples were also taken at intervals for later determi-
nation of circulating insulin, glucagon, and individual essen-
tial and nonessential amino acid concentrations.

Tissue protein synthesis in vivo. The fractional rate of
protein synthesis was measured with a flooding dose of L-[3-
H]phenylalanine (14, 26) injected 90 min after the initiation
of the clamp procedure. Pigs were killed at 2 h, and samples
of longissimus dorsi muscle were collected and rapidly frozen.

The specific radioactivity values of the protein hydrolysate,
homogenate supernatant, and blood supernatant were deter-
mined as previously described (16). Previous studies have
demonstrated that, after a flooding dose of tritiated phenyl-
alane is administered, the specific radioactivity of tissue
free phenylalanine is in equilibrium with the aminoacyl-
RNA specific radioactivity, and therefore the tissue free
phenylalanine is a valid measure of the tissue precursor pool
specific radioactivity (14).

Plasma hormones and substrates. The concentrations of
individual amino acids from frozen plasma samples obtained
at 0 and 90 min of the insulin infusions were measured with
an HPLC method (PICO-TAG reverse-phase column; Waters,
Milford, MA) as previously described (18). Plasma radioim-
munoreactive insulin concentrations were measured using a
porcine insulin radioimmunoassay kit (Linco, St. Louis, MO)
that used porcine insulin antibody and human insulin stan-
dards. Plasma radioimmunoreactive glucagon concentrations
were measured using a porcine glucagon radioimmunoassay
kit (Linco) that used porcine glucagon antibody and human
glucagon standards.

Calculations and statistics. The fractional rate of protein
synthesis (Kₚ; percentage of protein mass synthesized in a
day) was calculated as

\[ K_p \times \text{day}^{-1} = \frac{(S_f - S_i) \times (1,440/t)}{S_i} \times 100 \]

where S₀ (in dpm/nmol) is the specific radioactivity of the
protein-bound phenylalanine, S₀ (in dpm/nmol) is the specific
radioactivity of the tissue free phenylalanine at the time of
tissue collection and the linear regression of the blood specific
radioactivity of the animal at 5, 15, and 30 min against time,
t is the time of labeling in minutes, and 1,440 is the minutes-
to-day conversion.

Analysis of variance (general linear modeling) was used to
assess the effect of insulin, amino acids, and their interac-

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tion. If there was an interaction between insulin and amino acids, Student's t-test was used to test for differences between groups. To determine the effectiveness of the clamp procedure, individual amino acid, glucose, and insulin concentrations in each treatment group were compared with their basal concentrations by use of t-tests. Probability values of <0.05 were considered statistically significant for all comparisons except those for plasma amino acid concentrations. Because there was an increased probability that one of the 21 amino acid comparisons in each of the nine treatment groups would be significantly different among groups by random chance, a more conservative statistical approach for amino acid comparison was used; therefore, probability values of <0.01 were considered statistically different. Data are presented as means ± SE. The insulin sensitivity of muscle protein synthesis was calculated by nonlinear regression analysis.

RESULTS

Infusions, hormones, and substrates. Fasted 7-day-old pigs were infused with somatostatin (to block insulin secretion), glucagon (at replacement levels), and glucose (as needed to maintain fasting levels). Insulin was infused at four doses to achieve levels that simulated 1) less than fasting, 2) fasting, 3) intermediate between fasting and fed, and 4) fed conditions. At each dose of insulin, amino acids were clamped at either the fasting or fed level; at the highest insulin dose, amino acids were also allowed to fall to less than fasting levels. Table 1 shows the circulating insulin, glucagon, and glucose concentrations during the infusion compared with baseline (0 time) values, when amino acids were clamped at fasting, fed, and less than fasting levels. Targeted plasma insulin levels, i.e., 0, 2, 6, and 30 μU/ml, were largely achieved in all treatment groups. Hyperaminoacidemia or hypoaminoacidemia did not alter plasma insulin or glucagon concentrations. Circulating glucose concentrations were maintained at basal fasting levels during the infusion of somatostatin, glucagon, insulin, and/or amino acids. Replacement circulating glucagon levels were achieved during the somatostatin clamp in most groups.

Circulating essential and nonessential amino acid concentrations achieved during the infusions are compared with baseline (0 time) values in Figs. 1 and 2, respectively. The circulating concentrations of both essential and nonessential amino acids were maintained at the fasting level during the euaminoacidemic clamps. The exceptions were reductions in serine, taurine, or tyrosine and an increase in citrulline in one or more groups. Hyperaminoacidemia increased the circulating concentrations of essential amino acids by ~90% and those of nonessential amino acids by ~50% (P < 0.001). In the presence of hyperinsulinemia, without amino acid infusion, essential and nonessential amino acids fell to ~50% of fasting levels (P < 0.001).

Figure 3 shows the net whole body glucose disposal and amino acid disposal rates as indicated by the glucose and amino acid infusion rates during the 2-h infusion period. Amino acids were not infused in the ~0 and 2 μU/ml plasma insulin level groups, because plasma amino acid concentrations did not fall below 10% of the basal level. Amino acids were also not infused in the hyperinsulinemic-euglycemic-hypoaminoacidemic group, thereby allowing plasma amino acid concentrations to fall to ~50% of the basal level. Insulin, but not amino acids, increased glucose infusion rates. Both insulin and amino acids increased amino acid infusion rates.

Skeletal muscle protein synthesis. To determine whether insulin and amino acids stimulate skeletal muscle protein synthesis independently in the neonate, we asked six specific questions. We asked whether 1) the stimulation of muscle protein synthesis by insulin requires concurrent amino acid stimulation; 2) the stimulation of muscle protein synthesis by amino acids requires insulin; 3) the basal fasting insulin level stimulates muscle protein synthesis; 4) there is a dose-response effect of amino acids on muscle protein synthesis; 5) there is a dose-response effect of insulin on muscle protein synthesis; and 6) there is an effect of amino acids on the insulin sensitivity of muscle protein synthesis.

Table 1. Plasma insulin, glucagon, and glucose concentrations in response to insulin and amino acid infusion during pancreatic glucose-amino acid clamps in 7-day-old pigs

<table>
<thead>
<tr>
<th>Hormone/Substrate</th>
<th>Amino Acid Group</th>
<th>Baseline</th>
<th>Insulin Group, μU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Insulin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>1.5 ± 0.2</td>
<td>0.6 ± 0.1a</td>
<td>2.4 ± 0.2a</td>
</tr>
<tr>
<td>Fed</td>
<td>1.7 ± 0.2</td>
<td>0.7 ± 0.2a</td>
<td>2.7 ± 0.3a</td>
</tr>
<tr>
<td>&lt;Fasting</td>
<td>1.8 ± 0.2</td>
<td>100 ± 10</td>
<td>78 ± 11</td>
</tr>
<tr>
<td>Glucagon</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>105 ± 7</td>
<td>100 ± 10</td>
<td>78 ± 11</td>
</tr>
<tr>
<td>Fed</td>
<td>101 ± 8</td>
<td>100 ± 15</td>
<td>96 ± 13</td>
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<tr>
<td>&lt;Fasting</td>
<td>102 ± 18</td>
<td>100 ± 10</td>
<td>78 ± 11</td>
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<tr>
<td>Glucose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>81 ± 3</td>
<td>84 ± 6</td>
<td>74 ± 3a</td>
</tr>
<tr>
<td>Fed</td>
<td>83 ± 2</td>
<td>85 ± 3</td>
<td>77 ± 5</td>
</tr>
<tr>
<td>&lt;Fasting</td>
<td>76 ± 1</td>
<td>100 ± 10</td>
<td>78 ± 11</td>
</tr>
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</table>

Values are means ± SE; n = 8–12 per group. Plasma concentrations of insulin are in μU/ml, glucagon in pg/ml, and glucose in mg/dl. aFasting, less than fasting. bFasted 7-day-old pigs were infused with somatostatin (to block insulin secretion), glucagon (at replacement levels), and glucose (to clamp glucose at fasting levels). Insulin was infused at 4 doses to achieve levels that simulated 1) less than fasting (~0 μU/ml), 2) fasting (~2 μU/ml), 3) intermediate between fasting and fed (~4 μU/ml), and 4) fed (~30 μU/ml) conditions. At each insulin dose, amino acids were clamped at within the fasting or fed level by use of a balanced amino acid mixture; at the highest insulin dose, amino acids also were allowed to fall to less than fasting levels. a Significantly different from baseline values (P < 0.05).
The data from pigs treated with four different doses of insulin and two to three doses of amino acids were thus analyzed in subsets, so that each question could be addressed specifically. Because muscles composed of primarily fast-twitch, glycolytic fibers predominate in the musculature of the neonatal pig and are the most responsive to anabolic agents (11, 13), we studied the longissimus dorsi muscle, which contains primarily fast-twitch muscle fibers.

Previous studies have shown that insulin infusion in the neonatal pig stimulates protein synthesis in skeletal muscle when amino acids are clamped at the fasting level (11, 48). To determine whether insulin-stimulated muscle protein synthesis requires maintenance of fasting amino acid levels, insulin was increased to the fed level (30 μU/ml) while amino acids were either clamped at the fasting level (500 nmol BCAA/ml) or allowed to fall below the fasting level (250 nmol BCAA/ml). Results were compared with those for the basal condition of fasting insulin (2 μU/ml) and amino acid (500 nmol BCAA/ml) levels (Fig. 4). Insulin increased the rate of muscle protein synthesis in the absence of amino acid infusion (*P < 0.001), although not to the rate of muscle protein synthesis when amino acids were clamped at the fasting level. Values are means ± SE; n = 8–12 per treatment group. *Statistically different from baseline values (*P < 0.01).

Fig. 1. Plasma concentrations of essential amino acids at baseline (0 time) and in the presence of 0, 2, 6, and 30 μU/ml plasma insulin levels while amino acids were clamped at 500 (A), 1,000 (B), and 250 (C) nmol branched-chain amino acids (BCAA/ml) by use of a balanced amino acid mixture in 7-day-old pigs. In all groups, glucose was clamped at the fasting level. Values are means ± SE; n = 8–12 per treatment group. *Statistically different from baseline values (*P < 0.01).

Fig. 2. Plasma concentrations of nonessential amino acids at baseline (0 time) and in the presence of 0, 2, 6, and 30 μU/ml plasma insulin levels while amino acids were clamped at 500 (A), 1,000 (B), and 250 (C) nmol BCAA/ml by use of a balanced amino acid mixture in 7-day-old pigs. In all groups, glucose was clamped at the fasting level. Values are means ± SE; n = 8–12 per treatment group. *Statistically different from baseline values (*P < 0.01).
acids were clamped at the fasting level (P < 0.001). This suggests that the stimulation of muscle protein synthesis by insulin does not require concurrent amino acid stimulation in the neonate.

To determine whether the stimulation of muscle protein synthesis by amino acids requires insulin, insulin was reduced to nearly zero by somatostatin infusion, and amino acids were either maintained at the fasting level (500 nmol BCAA/ml) or raised to the fed level (1,000 nmol BCAA/ml; Fig. 5). Raising amino acids from the fasting to the fed level, when insulin was reduced to nearly zero, increased muscle protein synthesis (P < 0.02). This suggests that the stimulation of muscle protein synthesis by amino acids does not require insulin in the neonate.

To determine whether basal fasting insulin levels stimulate muscle protein synthesis, insulin levels were either reduced to nearly zero or were maintained at the fasting level (2 µU/ml) while amino acids were maintained at the fasting level (500 nmol BCAA/ml) or raised to the fed level (1,000 nmol BCAA/ml; Fig. 6). An increase in fractional rates of protein synthesis from 10.2 ± 0.8 to 13.9 ± 0.6%/day (P < 0.002) suggests that basal fasting insulin levels stimulate protein synthesis in neonatal muscle.

To determine whether there is a dose-response effect of amino acids on muscle protein synthesis, amino acids were allowed to fall below fasting (250 nmol BCAA/ml), remain at fasting (500 nmol BCAA/ml), or raised to fed levels (1,000 nmol BCAA/ml). In each group, insulin was infused at the fed level (30 µU/ml), because insulin infusion is required to reduce circulating amino acids below fasting levels by promoting amino acid disposal (48). Protein synthesis rates

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**Fig. 3.** Whole body net glucose disposal rates (A) and amino acid disposal rates (B) in the presence of −0, 2, 6, and 30 µU/ml plasma insulin levels while amino acids were clamped at 500, 1,000, and 250 nmol BCAA/ml by use of a balanced amino acid mixture. In all groups, glucose was clamped at fasting levels. Values are means ± SE; n = 8–12 per treatment group. Amino acid disposal rate was the average rate of infusion of leucine from a balanced amino acid mixture necessary to maintain circulating BCAA near either the basal fasting level (500 nmol/ml) or twofold the fasting level (1,000 nmol/ml). Amino acids were not infused in the −0 and 2 µU/ml insulin/fasting amino acid treatment groups and were allowed to fall to below fasting level in the −30 µU/ml insulin/less than fasting amino acid level (250 nmol BCAA/ml) group. Glucose disposal rate was the average rate of infusion of dextrose necessary to maintain circulating glucose near the fasting level. Hyperinsulinemia increased glucose and amino acid disposal rates. *Significantly different from previous insulin level within the same amino acid (AA) level (P < 0.05). †Significant effect of amino acids within the same insulin group (P < 0.05).

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**Fig. 4.** Fractional protein synthesis rates (Ks) in longissimus dorsi muscle of 7-day-old pigs in the presence of hyperinsulinemia (~30 µU/ml) and either euaminoacidemia or hypoaminoacidemia (500 or 250 nmol BCAA/ml) compared with basal fasting condition (~2 µU insulin/ml and 500 nmol BCAA/ml). Values are means ± SE; n = 8–12 per treatment group. Insulin increased protein synthesis rates during hypoaminoacidemia (P < 0.001). Protein synthesis rates were further increased during euaminoacidemia (P < 0.001). *Significantly different from basal fasting condition (P < 0.05). †Significantly different from hyperinsulinemic-euaminoacidemic group (P < 0.05).

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**Fig. 5.** Fractional protein synthesis rates in longissimus dorsi muscle of 7-day-old pigs in the presence of hypoinsulinemia (~0 µU/ml) with either euaminoacidemia (500 nmol BCAA/ml) or hyperaminoacidemia (1,000 nmol BCAA/ml). Values are means ± SE; n = 8–12 per treatment group. Hyperaminoacidemia in the presence of hypoinsulinemia increased muscle protein synthesis (P < 0.02). †Significantly different from hypoinsulinemic-euaminoacidemic group (P < 0.05).
increased progressively as circulating amino acids were increased to fasting ($P < 0.001$) and then fed ($P = 0.06$) levels. Thus amino acids increase muscle protein synthesis in a dose-response manner.

Figure 8 compares the dose-response effect of insulin on muscle protein synthesis in the presence of euaminoacidemia (500 nmol BCAA/ml) or hyperaminoacidemia (1,000 nmol BCAA/ml) amino acid levels. There was a progressive increase in protein synthesis rates as the level of insulin was increased ($P < 0.005$). Amino acids increased muscle protein synthesis ($P < 0.05$) at each dose of insulin except the highest dose (30 μU/ml), where there was a tendency for amino acids to stimulate muscle protein synthesis ($P < 0.10$). Thus there was a dose-response effect of insulin on muscle protein synthesis in the presence of fasting or fed amino acid levels. The effects of insulin and amino acids were additive until maximal rates of protein synthesis were achieved.

Figure 9 shows a nonlinear regression model of muscle protein synthesis in the presence of euaminoacidemia (500 nmol BCAA/ml) or hyperaminoacidemia (1,000 nmol BCAA/ml) amino acid levels. The half-maximum response of protein synthesis by amino acids (AA) concentration. The half-maximum response of protein synthesis to insulin was estimated at 2.1 μU/ml in the presence of euaminoacidemia and at 1.9 μU/ml in the presence of hyperaminoacidemia. Values are means ± SE; $n = 8–12$ per treatment group.

**Fig. 6.** Fractional protein synthesis rates in longissimus dorsi muscle of 7-day-old pigs are compared during hypoinsulinemia (~0 μU/ml) and euinsulinemia (~2 μU/ml) in the presence of euaminoacidemia (500 nmol BCAA/ml). Values are means ± SE; $n = 8–12$ per treatment group. Raising insulin from 0 to 2 μU/ml increased protein synthesis rates ($P < 0.002$). *Significantly different from hypoinsulinemic-euaminoacidemic group ($P < 0.05$).

**Fig. 7.** Fractional protein synthesis rates in longissimus dorsi muscle of 7-day-old pigs in the presence of hyperinsulinemia (~30 μU/ml) are compared during hypoinsulinemia (250 nmol BCAA/ml), euaminoacidemia (500 nmol BCAA/ml), or hyperaminoacidemia (1,000 nmol BCAA/ml). Values are means ± SE; $n = 8–12$ per treatment group. Raising amino acids increased protein synthesis rates progressively ($P < 0.05$). †Significantly different from hyperinsulinemic-hypoaminoacidemic group ($P < 0.001$).

**Fig. 8.** Fractional protein synthesis rates in longissimus dorsi muscle of 7-day-old pigs at ~0, 2, 6, and 30 μU/ml plasma insulin levels during euaminoacidemia (500 nmol BCAA/ml) and hyperaminoacidemia (1,000 nmol BCAA/ml). Protein synthesis rates increased progressively as the insulin dose was increased ($P < 0.005$). Hyperaminoacidemia increased protein synthesis at the ~0, 2, and 6 μU/ml insulin doses ($P < 0.05$), with a nonsignificant increase at the ~30 μU/ml insulin dose ($P < 0.10$). Values are means ± SE; $n = 8–12$ per treatment group. The effects of insulin and amino acids were additive until maximal rates of protein synthesis were achieved at the fed insulin and amino acid levels. †Significantly different from euaminoacidemic group within same insulin group ($P < 0.05$).

**Fig. 9.** Nonlinear regression analysis of muscle protein synthesis vs. plasma insulin in the presence of euaminoacidemia and hyperaminoacidemia. A curvilinear relationship between protein synthesis and plasma insulin is demonstrated that is influenced by amino acid (AA) concentration. The half-maximum response of protein synthesis to insulin was estimated at 2.1 μU/ml in the presence of euaminoacidemia and at 1.9 μU/ml in the presence of hyperaminoacidemia. Values are means ± SE; $n = 8–12$ per treatment group.
did not change with amino acid supplementation. The half-maximum response of protein synthesis to insulin was estimated at 2.1 μU/ml when amino acids were at the fasting level and 1.9 μU/ml when amino acids were at the fed level. This suggests that amino acids do not change the sensitivity of muscle protein synthesis to insulin in the neonate.

**DISCUSSION**

Previous studies have shown that both insulin and amino acids play important roles in the feeding-induced stimulation of protein synthesis in skeletal muscle of the neonate (10, 11, 13, 49). Reproduction of postprandial protein synthesis rates in skeletal muscle in the neonate can be achieved by the infusion of insulin to the fed level when amino acids and glucose are clamped at basal fasting levels (49). Furthermore, the infusion of amino acids to fed levels, while glucose and insulin are at fasting levels, can also reproduce the feeding-induced stimulation of muscle protein synthesis in the neonate (13). However, due to the achievement of maximal rates of muscle protein synthesis rates by the infusion of insulin and/or amino acids to achieve fed levels in previous studies, it was not discerned whether there are additive effects of insulin and amino acids at submaximal levels. The results of the present study showed that both insulin and amino acids stimulated muscle protein synthesis in a dose-response manner and that the effects of insulin and amino acids were additive until maximal rates were achieved. Furthermore, the results suggest that insulin and amino acids act independently to stimulate the postprandial rise in skeletal muscle protein synthesis in neonates.

**Effectiveness of pancreatic glucose-amino acid clamp.** To isolate the independent effects of insulin and amino acids on skeletal muscle protein synthesis in the neonate, we used our previously reported pancreatic glucose-amino acid clamp (45). We chose this method because it is preferable to the diabetic animal model, which is fraught with confounding metabolic effects including aberrations in plasma glucose and ketone body concentration (15, 32, 33, 38). Using the pancreatic glucose-amino acid clamp, we largely achieved the targeted insulin and amino acid levels, which were within the narrow physiological range. We also reduced insulin and amino acids below fasting levels, which in the case of insulin was at the detectable limit of the assay and in the case of amino acids required insulin stimulation to promote amino acid uptake. The infusion procedure also achieved the maintenance of glucose and glucagon at fasting levels for the duration of the experiment. The choice of omitting an amino acid clamp in one insulin-stimulated treatment group not only served to test the independent effects of insulin on muscle protein synthesis but also demonstrated the profound effects of physiological levels of insulin on whole body amino acid disposal in the neonate by reducing circulating amino acid levels by one-half. Thus the amino acid clamp is particularly useful in rapidly growing animals and has been recently used in the fetal lamb model to examine the effects of hormonal stimulation (42, 44). Use of a balanced amino acid mixture to clamp amino acids at fasting levels also largely prevented the reduction in nonessential amino acids that can occur with the use of current commercial formulas (11, 13, 44, 48, 49).

**Effect of insulin and amino acids on muscle protein synthesis.** Although we previously demonstrated that muscle protein synthesis rates can be raised to fed rates by the infusion of insulin when amino acids are clamped at fasting levels (49), a potential stimulatory effect of the amino acids infused to maintain fasting amino acid levels could not be ruled out. In the present study, we showed that infusing insulin to simulate fed insulin levels stimulated muscle protein synthesis even when amino acids were allowed to fall to one-half of basal fasting amino acid levels by the omission of an amino acid clamp. Although the rates of muscle protein synthesis observed were submaximal, suggesting that insulin’s stimulatory effect on muscle protein synthesis is blunted if basal amino acids levels are not maintained, the results more importantly indicate that insulin’s stimulatory effect on muscle protein synthesis in the neonate does not require concurrent amino acid stimulation. This finding further defines insulin’s role as a regulator of the postprandial stimulation of muscle protein synthesis in the young growing animal, as previously described in hindlimb of the young lamb (47), and in just-weaned but growing rats (24). However, studies in more mature animals (3, 4, 36, 39) and humans (21, 23, 27, 30, 34, 37, 43) found insulin to have little, if any, effect on muscle protein synthesis in the absence of amino acid infusion, a finding which contrasts markedly with those studies conducted during early growth and development. For example, a recent in vitro study suggests that the postprandial rise in muscle protein synthesis requires both insulin and amino acids in young adult rats (3). This suggests that the response of muscle protein synthesis to insulin wanes and is lost with development.

In a recent study (13), a stimulatory effect of amino acids on protein synthesis, in the presence of fasting insulin levels, was demonstrated in skeletal muscle of young postnatal pigs. Furthermore, studies in young, adult, and elderly populations have also shown that amino acids, either alone or concurrent with insulin infusion, have a stimulatory effect on muscle protein synthesis and are a primary physiological regulator of protein synthesis in skeletal muscle (6, 28, 46). In this study, we wished to determine whether or not insulin plays a permissive role in amino acid-stimulated muscle protein synthesis in neonates. Our data showed that increasing circulating amino acids to fed levels in the near absence of insulin increased muscle protein synthesis. However, the increase in protein synthesis was less than that which occurred in the presence of fasting insulin levels due to a reduction in the baseline rate of protein synthesis. In fact, the rates of muscle protein synthesis achieved by amino acid stimulation in the near absence of insulin was similar to that

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achieved by insulin stimulation when amino acids were below fasting levels. These results suggest that the stimulation of skeletal muscle protein synthesis by amino acids does not require insulin in the neonate. The finding contrasts with the recent study of Anthony et al. (1), in which somatostatin infusion blocked the stimulation of skeletal protein synthesis by leucine in mature rats. This suggests that, although the stimulation of muscle protein synthesis by insulin and amino acids in the neonate is independent, with maturation insulin is required to play a permissive role in amino acid-stimulated muscle protein synthesis. Although leucine increased muscle protein synthesis in alloxan-induced diabetic rats (2), in a severely diabetic model (partial pancreatectomy), the protein synthesis-stimulatory effect of resistance exercise was blocked by insulin deficiency (22).

The use of somatostatin in the pancreatic glucose-amino acid clamp allowed us to reduce endogenous insulin to near zero so as to determine whether basal fasting insulin could stimulate muscle protein synthesis in the neonate. The results show that the neonatal muscle is so sensitive to insulin that even basal fasting insulin levels have a stimulatory effect on protein synthesis. This enhanced sensitivity of neonatal muscle protein synthesis to insulin may contribute to the high efficiency rates of protein deposition and the rapid growth rate seen at this age. Furthermore, this finding exposes the vulnerability of the young patient with diabetes, in whom muscle protein synthesis rates may be suppressed due to the absence of insulin.

Having previously shown that fed levels of amino acids stimulate skeletal muscle protein synthesis (13), we further wished to determine whether there was a dose-response effect of amino acids on muscle protein synthesis. The results showed a progressive increase in muscle protein synthesis rates, as circulating amino acid concentrations were increased by infusion from below-fasting to fasting levels and finally to fed levels, indicating that amino acids stimulate skeletal muscle protein synthesis in neonatal pigs in a dose-response manner.

Our previous studies (48) showed that maximal rates of skeletal muscle protein synthesis were achieved by the infusion of insulin to simulate insulin levels in the fed steady state (10 mU/ml) with no further increase in muscle protein synthesis at insulin levels that reproduce the immediate postprandial period (30 μU/ml) or with pharmacological doses (800 μU/ml). In the present study, four doses of insulin (~0, 2, 6, and 30 μU/ml) were infused to simulate below-fasting, fasting, intermediate between fasting and fed, and postprandial fed insulin levels, to define the dose-response effect of insulin on muscle protein synthesis in neonatal pigs. We demonstrated a progressive increase in muscle protein synthesis rates as the insulin level was increased to maximum physiological fed levels. Furthermore, at each dose of insulin except the highest dose, an increase in amino acids from the fasted to the fed level increased muscle protein synthesis. This suggests that the stimulation of muscle protein synthesis by insulin and amino acids is additive until maximal rates of protein synthesis are achieved.

Garlick and Grant (25) previously reported that amino acids enhanced the sensitivity of skeletal muscle protein synthesis to insulin in postabsorptive, young, weaned rats, such that maximal rates of protein synthesis were achieved at lower insulin concentrations when amino acids were concurrently infused. We observed that, because amino acids increased the baseline rate of muscle protein synthesis and tended to increase the maximum rate of muscle protein synthesis, amino acids did not affect the responsiveness of muscle protein synthesis to insulin in neonates. Furthermore, we demonstrate that the half-maximum response of protein synthesis to insulin was similar at both fasting and fed circulating amino acid levels, indicating that amino acids do not alter the sensitivity of muscle protein synthesis to insulin in the neonate. Differences in our results in neonatal pigs and those by Garlick and Grant in young, weaned rats could be attributed to stage of development at the time of study. Our findings suggest that insulin and amino acids independently stimulate muscle protein synthesis in the neonate and imply independent action of insulin and amino acids on the intracellular signaling pathways that regulate muscle protein synthesis.

Perspectives. Our previous studies showed that feeding stimulates skeletal muscle protein synthesis in neonates (9, 17) and that the magnitude of the feeding response can be reproduced by the infusion of insulin and/or amino acids (11, 13, 49). This suggests that the postprandial rise in insulin and amino acids mediates the feeding-induced stimulation of muscle protein synthesis in neonates. Using our pancreatic glucose-amino acid clamps in the present study allowed us to look in more detail at the individual roles of insulin and amino acids in this response. The results highlight the exquisite insulin sensitivity of skeletal muscle protein synthesis in the neonate, in that the basal fasting insulin level stimulated protein synthesis and insulin increased protein synthesis in the absence of amino acid infusion. In addition, the results showed that amino acids stimulated muscle protein synthesis in the absence of insulin, thereby increasing the basal rate of muscle protein synthesis, but did not enhance the sensitivity of muscle protein synthesis to insulin. Thus the results suggest that insulin and amino acids act independently to stimulate protein synthesis in skeletal muscle of the neonate. The ability of skeletal muscle protein synthesis to respond to the postprandial rise in both insulin and amino acids likely contributes to the efficient utilization of dietary amino acids for protein deposition and the rapid gain in skeletal muscle mass in the neonate. These findings highlight the importance of a protein-containing diet for skeletal muscle growth in the neonate.

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