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Amino Acids and Insulin as Regulators of Muscle Protein Synthesis in Neonatal Pigs

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Neonatal Growth Characteristics

- Growth rate is higher during the neonatal period than at any other stage of postnatal life.
- In the neonate, the most rapidly growing protein compartment in the body is in skeletal muscle.
- Neonates are efficient at utilizing dietary amino acids for protein deposition.

Merits of the Neonatal Pig as an Animal Model

- Body size
- Range of experimental approaches
  - repeated sampling
  - tracer kinetics
  - feeding modality
- Large litters allow multiple treatments
- Anatomy & metabolism
- Ethical considerations are not limiting

Highly Efficient Use of Dietary Protein for Muscle Growth in the Neonate Is Due to an Elevated Response of Muscle Protein Synthesis to Feeding

- Feeding stimulates protein synthesis profoundly in very young pigs. But as they get older, the response to feeding is much smaller.
- The stimulation of protein synthesis by feeding was much greater the younger the animal.

Flooding Dose Method for Measuring Fractional Protein Synthesis Rate

- Rapid injection of large dose of tracee amino acid, along with tracer amino acid
  - 150 mM Phe (5-10x body's free pool)
  - $^{2}H_{5}$-Phe, ^{13}C-Phe, or ^{3}H-Phe
- "Flood" the free amino acid pool to minimize the differences between the extracellular (blood) and intracellular free amino acid in their isotopic enrichment (specific radioactivity)
- After injection of flooding dose, the isotopic enrichment of the protein-bound amino acid increases linearly
- Determine isotopic enrichment of Phe in protein and intracellular free pool

Flooding Dose Method for Measuring Fractional Protein Synthesis Rate – (Continued)

- Measure rate of incorporation of a labeled amino acid into tissue protein, corrected for the amount of the label in the free amino acid pool
- Ks in %/day = (S_b / S_a) x 1440/t
  - S_b = isotopic enrichment of protein-bound Phe
  - S_a = isotopic enrichment of precursor pool Phe
  - t = time of labeling in min
- Can determine acute changes in tissue protein synthesis over 5 to 30 min
- Useful for metabolic non-steady state conditions such as feeding studies or short-term hormone infusion

What factors regulate the enhanced stimulation of muscle protein synthesis by feeding in the neonate?

- **Potential regulatory factors:** *Amino acids, Glucose, Insulin*

Pancreatic-Substrate Clamp Technique

- Infuse somatostatin
  - Block insulin secretion
- Infuse insulin
  - Raise plasma insulin to desired level
- Infuse glucagon
  - Replacement levels
- Clamp glucose
  - Monitor blood glucose level every 5 min
  - Adjust dextrose infusion rate to maintain desired levels
- Clamp amino acids
  - Monitor blood levels of representative essential amino acid every 5 min
  - Adjust infusion rate of balanced amino acid mixture to maintain desired levels
- Infuse isotopic amino acid tracer
  - Measure protein synthesis

Insulin and Amino Acids Stimulate Protein Synthesis in Skeletal Muscle of the Neonate

- Raising insulin to the fed level, increased muscle protein synthesis.
- Raising amino acids to the fed level, increased muscle protein synthesis.
- Raising both insulin and amino acids together increased muscle protein synthesis, but no further than with insulin and amino acids alone.
- The increase in muscle protein synthesis with insulin and amino acids decreased with age.

Dose Response Effect of Insulin on Muscle Protein Synthesis and Stimulation by Amino Acids at Each Insulin Dose

- Insulin increased muscle protein synthesis in a dose response manner. At each dose of insulin, amino acids increased protein synthesis.

Dose Response Effect of Amino Acids on Muscle Protein Synthesis

- There is a nice dose response effect of AA on muscle protein synthesis.

Conclusions

- In skeletal muscle of the neonatal pig, the feeding-induced stimulation of protein synthesis is due to the rise in both insulin and amino acids after a meal.
- The postprandial increase in protein synthesis in liver and other visceral tissues is primarily dependent on amino acid supply.

Insulin and Amino Acid Signaling Pathways Leading to Translation Initiation

- When insulin binds to its receptor, it induces the autophosphorylation of the receptor on its tyrosine residues, followed by the activation of its tyrosine kinase activity.
- The activated receptor binds to IRS-I, leading to phosphorylation of IRS-1 on tyrosine residues and its activation.
- IRS-I in turn activates PI3 kinase.
- Activation of PKB inhibits TSC1 and 2 which are inhibitors of mTOR and likely do so by inhibiting Rheb.
- MTOR is activated by insulin and by amino acid stimulation.
- MTOR activates S6K1, a translation initiation factors which leads to the activation of ribosomal protein S6 which has been thought to enhance translation of specific mRNAs, including those that are involved in the translational machinery.
- mTOR also phosphorylates 4EBP1. 4E-BP1 is a repressor protein that binds to eIF4E and blocking eIF4E binding to eIF4G.
- Phosphorylation of 4E-BP1 by activated mTOR releases eIF4E, which can then bind to eIF4G which binds to mRNA and increase translation initiation.

Feeding-Induced Activation of IR, IRS-1, PI 3-Kinase, and PKB Decreases With Development in Skeletal Muscle

- Feeding increased the phosphorylation of the insulin receptor in 7 day old pigs and this response to feeding was much lower in 26 day old pigs.
- This developmental change in the feeding-induced activation of the insulin receptor was transduced down the signaling pathway to IRS-1, PI 3-kinase, and protein kinase B.
- There was a profound effect of feeding on the activity of the insulin signaling proteins and that response decreased with development.

Feeding-Induced Activation of S6K1 and 4E-BP1, Dissociation of 4E-BP1•eIF4E, and Association of eIF4E•eIF4G Decrease with Age in Muscle

- Feeding increased the phosphorylation of **S6K1** and this response decreased with development.
- The phosphorylation of 4E-BP1, that repressor protein that binds to eIF4E, was also increased in response to feeding and the response decreased with development.
- The increase in the phosphorylation of 4E-BP1 in response to feeding caused the dissociation of 4E-BP1 from eIF4E, and thus a decrease in the amount of the inactive 4E-BP1.eIF4E complex.
- This resulted in the association of eIF4E with eIF4G in an active complex which binds to mRNA and the 43S preinitiation complex.
- All of these responses decreased with development.

Conclusion

The high rate of muscle protein synthesis in neonates in response to feeding is due to the enhanced activation of positive regulators of the insulin and nutrient signaling pathways.

Negative Regulators of Insulin and Amino Acid Signaling Pathways

- Studies using largely in vitro and cell culture systems have identified new signaling components that are negative regulators of the insulin and amino acid signaling pathways leading to translation initiation.
- But little is known about the regulation of these proteins in the whole animals under physiologically relevant conditions.
- We elected to conduct a more detailed study of the role of development in the activation and protein abundance of these signaling components.

PTP1B Is a Protein Tyrosine Phosphatase that Dephosphorylates the Insulin Receptor and IRS-I, and Decreases Insulin Signaling

- Protein tyrosine phosphatase 1B is a protein tyrosine phosphatase that dephosphorylates the insulin receptor and IRS-1 and decreases insulin signaling.
- It does so by associating with the internalized insulin receptor and a protein called Grb2.

Activity of PTP1B, which Dephosphorylates the Insulin Receptor and IRS-I, Increases with Development in Muscle

- We found that PTP1B activity in muscle was lower in 7 than in 26 day-old pigs.
- We also examined PTP1B association with insulin receptor, which is required for it to be active and this also increased with development.
- This data is consistent with our hypothesis that the activity of this negative regulator would be low in neonatal muscle where insulin signaling is so active.

PTEN Is a Lipid Phosphatase that Antagonizes the Action of PI3 Kinase

- PTEN (phosphatase and tensin homologue on chromosome 10) is a lipid phosphatase that converts phosphatidylinositol 3,4,5trisphosphate back to phosphatidylinositol 4,5bisphosphate and therefore antagonizes the effects of PI 3-kinase, which activates PKB.

Abundance of PTEN, a Negative Regulator of PI 3-K Action, Increases with Age. Phosphorylation of PTEN, which Decreases PTEN Activity and Increases Insulin Signaling, Decreases with Age.

- PTEN abundance is higher in 26 day old pigs compared to 7 day old pigs. So there is less of the negative regulator in early life.
- PTEN phosphorylation is higher in 7 day old pigs compared to 26 day old pigs, which would result in a higher insulin signaling in the younger animals.

Protein Phosphatase 2A Negatively Regulates Insulin Signaling by Inhibiting PKB and S6K1

- PP2A is a phosphatase that negatively regulates insulin signaling by inhibiting the phosphorylation of PKB and S6K1.
- Phosphorylation of PP2A on Tyr 307 inhibits PP2A activity.

Abundance of PP2A, a Negative Regulator of Signaling to PKB and S6K1, Decreases with Age. Phosphorylation of PP2A, which Inhibits PP2A Activity and Increases Insulin Signaling, Decreases with Age.

- PP2A abundance is higher in 7 day old pigs compared to 26 day old pigs. This is not what you would expect for a negative regulator of insulin signaling.
- However, the phosphorylation of PP2A at tyrosine 307 is high in 7 day old pigs and non detectable in 26 day old pigs.
- Because phosphorylation of PP2A on tyrosine 307 suppress its activity, this suggests that there may be a lot of PP2A in newborn muscle but it is not active.

AMP-Activated Protein Kinase Is an Energy Sensor that Down-Regulates mTOR

- AMP kinase is activated by an increase in AMP levels within the cell.
- Cellular or metabolic stress like heat shock, hypoxia, glucose deprivation, or muscle contraction, that raises AMP levels or lowers ATP levels in cells, causes AMPK activation.
- AMP kinase phosphorylates TSC2 and thereby decrease the activation of mTOR.

Abundance and Phosphorylation of AMPKα, a Negative Regulator of mTOR Signaling, Do Not Change with Age or Feeding

- There was no effect of development or feeding on the abundance or phosphorytion of AMPK.
- So AMPK does not appear to be a regulator of the changes in insulin signaling in response to feeding.

Tuberous Sclerosis Complex 1/2 Inhibits mTOR Signaling

- Tuberous sclerosis complex 1 and 2 (gene products of hamartin and tuberin) are regulators of mTOR. The mechanism by which they regulate protein synthesis is not really clear. However, in vitro studies indicated that TSC2 can be inactivated by growth factor stimulation of PKB.
- There is some evidence that TSC1/2 responds to amino acid stimulation although there is also some evidence to the contrary.
- Phosphorylation of TSC2 by PKB is thought to inhibit the TSC1/2 complex and convert Rheb to its active form that activates TOR.
- AMPK has the opposite effect. AMPK activates TSC1/2 which decreases mTOR activity.

Abundance of TSC2, a Negative Regulator of Protein Synthesis, Increases with Age. Phosphorylation of TSC2, which Decreases TSC2 Activity, Increases with Feeding and Decreases with Age.

- TSC2 abundance is lower in 7 than in 26 day old pigs. This is consistent with TSC2 being a negative regulator of mTOR.
- In both age groups, feeding significantly enhance the phosphorylation of TSC2 at Thr1462 and 7 day old pigs have higher TSC2 phosphorylation. Phosphorylation at this site suppress the activation of TSC1/TSC2 complex.
- This is what you would predict for a negative regulator of insulin signaling.

Conclusion

The high rate of muscle protein synthesis in the neonate is due to the low activation of negative regulators of the insulin and amino acid signaling pathways.

Conclusion

The postprandial rise in insulin stimulates skeletal muscle protein synthesis through the activation of insulin-signaling components leading to mRNA translation in the neonate.

Conclusion

The postprandial rise in amino acids regulates mRNA translation through the activation of the mTOR-signaling pathway.

Conclusion

The high rate of muscle protein synthesis, and thus growth, in the neonate is due largely to an enhanced activation of insulin and amino acids signaling components after a meal.