



SPLASH! milk science update OCTOBER 2013 issue

This month's issue covers how milk supports muscle-building in elderly consumers, how kangaroo milk suggests new treatments for premature infants, the discovery of a simple property of breastmilk that protects against infant bowel disease, and the relationship between insulin and lactation. Enjoy!

Ripped in Retirement

- **It is possible to maintain and grow skeletal muscle mass in old age by careful management of diet and exercise.**
- **The most important dietary elements are essential amino acids, especially leucine, which mediates mRNA translation for muscle building.**
- **Whey and casein proteins from milk both help in building muscles, even though they are absorbed by the body at different rates.**

Many of the changes that happen with aging are hard to explain. Among them is a difficulty in maintaining and growing muscle mass. This is known as sarcopenia and has been estimated to account for 1.5% of total healthcare expenditures in Western countries. Over time, researchers have shown that a careful combination of resistance training, plus a diet containing sufficient and particular amino acids, can keep you looking buff well into your autumn years.

It all comes down to a simple relationship: if your body makes more muscle than it breaks down, you will gain muscle. Conversely, if—as often happens in old age—you break down more muscle than you synthesize, you will become less muscular.

But the simplicity of the problem stops there. On the upside, as theory has developed, it has become more optimistic. The understanding that muscle metabolism becomes fundamentally compromised with age has been replaced with the idea that muscle building is merely harder to trigger in older bodies. What's needed to counteract aging is stronger muscle-building stimuli.

This assertion is derived from data that indicates the muscle synthesis rates in people who were fed increasing amounts of protein after resistance exercise. In young people, the body temporarily hikes muscle manufacture in a dose-dependent manner up to about 20 g of protein (ingested after resistance exercise). But when young people consume more than that amount, the extra amino acids are oxidized rather than used to stimulate muscle synthesis.

Neither protein digestion nor protein absorption rates slow down in the elderly. But greater protein consumption is still required to spur muscle growth.

For instance, in the previous experimental set up, when older people are fed 35 g of protein following resistance exercise, muscle synthesis rates rise beyond the level that they reach when this same group is fed 20 g. (Recall that 20 g is the amount that prompts maximum muscle synthesis in the young.) But what counts as the maximum rate of muscle synthesis stimulated by eating protein after resistance training seems to be about the same for adults of all ages—it's just that older people need to eat more protein to make it happen. Put another way, although the body's response to muscle-building stimuli becomes blunted over time, it is not diminished.



What causes the blunting isn't clear. There are two main theories, which are not mutually exclusive. One suggests the delivery of essential amino acids to muscle cells is limited by blood flow. The second involves insulin, inflammation, and an abnormally high level of lipid in the blood—hence anti-inflammatory drugs are sometimes prescribed to thwart the blunting of the muscle-building response.

What is better understood is that only essential amino acids appear to stimulate muscle synthesis—with one having a particularly important role. Leucine jump-starts mRNA translation—protein making—for muscle growth. It does this by interacting with a ribosomal protein (S6) and other proteins called initiation factors 4E (eIF4E) and 4G (eIF4G), in the cytoplasm of muscle cells. Because branched-chain amino acids such as leucine appear in the bloodstream in direct correspondence to their level in the diet, leucine's effect in prompting protein-making in muscles can be improved by changing what you eat.

This is where a few intriguing studies come in. They deal with two categories of leucine-rich proteins that are by far the most-studied in the context of muscle synthesis: whey and casein proteins, which are both found in milk.

The important difference between whey and casein is the rate at which they are absorbed by the body. Whey is broken down quickly and its constituents exert a muscle-building effect about 3 hours after ingestion. Casein, meanwhile, forms an insoluble ball in the stomach, which slows digestion down. Its constituents therefore take 6-8 hours before getting to work in muscle tissue.

This was recently demonstrated in a neat study¹ that followed the muscle building effect of the two protein types. When participants in this study consumed a meal containing both whey and casein proteins, each with a different label, the amino acids derived from casein were incorporated into muscle over a much more sustained period than those derived from whey—proving the point about their absorption rates.

Sports coaches should take note of this: it suggests, for example, that a casein-rich supplement would be most appropriate before bedtime, while a whey-rich one might be more useful in the middle of the day when there is rarely long to wait before the next meal.

And when it comes to the elderly, there isn't always a clear sarcopenia-beating winner between whey and casein supplementation. One related finding² is particularly noteworthy, however. It measured the net balance of one amino acid—phenylalanine—in the legs of 15 elderly people and compared what happened when those people ate whey versus a mixture of corresponding essential amino acids. Curiously, the whey group incorporated more phenylalanine into their limb: they grew their leg muscles more than when the same amino acids were delivered in a non-whey source.

So whey, at least, must help muscle building through more ways than the amino acids it contains. The Arizona State University researchers behind the study think the key to this might lie with increased insulin secretion, a known inhibitor of muscle protein breakdown. Whatever the answer, they have uncovered another helpful detail that could improve the health of many.

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Kangaroo Tips for Human Preemies

- **Kangaroos and their cousins give birth to premature young that develop outside of the womb.**
- **Kangaroo milk protects premature young and helps them develop.**
- **Components of kangaroo milk or concepts from their lactation strategy may be mimicked to improve the survival of human preemies.**



Newborn tammar wallaby. Photo courtesy of Prof. Kevin Nicholas.

If a human mother were like a kangaroo, her “baby” would be born after only one month of gestation. Immediately after birth, her embryonic “baby” would crawl-climb up to one of her nipples and attach to one nipple, and not let go for the next 15 weeks. The “baby” nurses continually from the same nipple, drinking milk that is entirely different in composition from the milk consumed by the baby’s older brother or sister from the mother’s other nipple. The older sibling does not nurse continually. He or she bounces off to play and eat other food, and comes back to sip at the “Fountain of Mom” using the nipple not occupied by the newborn.

In the real world, human mothers are not like kangaroos. When an embryo is born, it dies. Human babies become viable at 23-24 weeks gestation, but even with the best care, only about half of them survive, and those who do survive have lifelong disabilities. The World Health Organization reports that more than 20 million infants are born with low birth weights each year, representing 15.5% of all births. More than 95% of these babies are born in developing countries. Many premature or low-birth weight babies have underdeveloped lungs that make breathing difficult, and the baby’s digestive system is not yet mature enough to effectively process

breast milk. The lesson from kangaroos is that life may be possible outside of the womb at a younger age of gestation, but that life needs to be supported by a continuous supply of milk that is specially formulated for their developmental stage.

What’s so special about kangaroo milk?

Kangaroos are large, so scientists mostly study their smaller cousin, the tammar wallaby. Painstakingly conducted cross-fostering studies in tammars—where a baby born from one mom is nursed by another another—demonstrate developmental stage differences. Trott et al. (2003) and Waite et al. (2005) examined the effect of fostering groups of 60-day-old tammar young onto a group of host mothers at a more advanced stage of lactation. The rate of growth of the foster pouch young (those suckling milk meant for older tammars) was significantly increased relative to the control pouch young (those drinking age-appropriate milk). Subsequent experiments (Kwek et al., 2009) extended these studies and showed the more “mature” milk not only increased growth of the fostered young, but also accelerated development of the stomach. This development appeared to result from direct action of the milk on the stomach, although colonization of the gut, which is also controlled by the milk, most likely contributed to this process (Kwek et al., 2009). Therefore, as a general concept, it may be assumed that the progressive changes in marsupial milk composition have evolved to regulate specific developmental processes.

Human babies born too soon have fragile lungs, intestines, and skin, amid a sea of hospital pathogens. What is in kangaroo milk that allows their fragile babies to survive pathogens outside of the womb? Some antibacterials, such as cathelicidin and WFDC-2, are present only during very early lactation or during weaning (Kwek et al., 2013). In early lactation, these antibacterials may protect the baby from pathogens in the environment, while their function during weaning may be to protect the mammary gland from infection (Kwek et al., 2013).

A major challenge for scientists is to identify similarly helpful compounds in human milk and understand how they can be used as pharmaceuticals and nutraceuticals for improved health outcomes in premature infants. Recent studies on one of those antibacterials—cathelicidin—highlighted how difficult that challenge can be. Different parts of the same protein can have different functions depending on the stage of lactation. During early lactation, the more proteins were produced from the part of the cathelicidin gene that is anti-bacterial, presumably to protect the young against infection (Wanyonyi et al., 2012). The expression of this same part of the gene increased again in late involution (weaning), presumably to protect the mammary gland from mastitis (Wanyonyi et al., 2012). In contrast, there was a three-fold increase in expression of a different part of the same gene in mid-lactation, which has the capacity to increase mammary gland growth and thus increase milk production (Wanyonyi et al., 2013). Thus, for scientists it is not even as simple as finding one magic gene. We must understand the “when,” “what,” and “how” of every component in milk.

Next generation “Kangaroo care”

Around the world, skin-to-skin contact between adult and baby, known as “Kangaroo care,” is a common, cost-effective technique to keep babies warm, especially those that are born too soon. In addition to temperature regulation, Kangaroo care helps maintain the baby’s heart and respiratory rate. In premature and low-birth weight babies, Kangaroo care is associated with improved weight gain, lower stress levels, improved cognitive and motor development, and reduced pain response. In full-term babies, Kangaroo care is also known to promote successful breastfeeding. Now that we know kangaroos produce special milk to protect their immature young, perhaps we can expand our definition of Kangaroo care for preemies to include kangaroo milk-inspired therapeutics.

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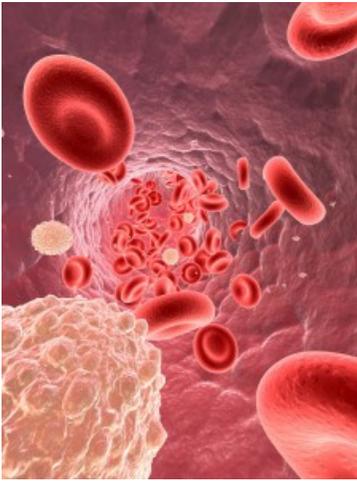
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Nipping NEC in the Bud

- **Scientists have explained in specific terms why breast milk is protective against bowel tissue death in infants, a disease called necrotizing enterocolitis (NEC).**
- **Previous experiments had shown that a molecule called receptor toll-like receptor 4 (TLR4) plays a crucial role in the development of the disease.**
- **The new work describes how TLR4 regulates blood flow to the tissues of the small intestine through a signaling pathway that involves nitric oxide.**
- **Because breast milk contains a precursor to nitric oxide, infants who drink it will have improved blood flow to their intestines—and thus a lower chance of developing NEC.**

As little as four years ago, an article appeared in the journal *Neonatology* with the title ‘Necrotizing Enterocolitis – 150 Years of Fruitless Search for the Cause’¹. This illness, called NEC for short, is on the rise. It is far too often fatal for the very young infants whom it afflicts. Recently, however, a team of researchers working in Pittsburgh made a breakthrough. They unraveled a molecular mechanism that allows NEC to develop—and in doing so, they also showed what it is about breast milk that protects babies from the disease.



NEC occurs in the intestines. Typically, as it develops, a newborn baby's gut tissue becomes inflamed because bacteria in the lumen ravage intestinal wall cells. In severe cases, patches of gut tissue die, and a hole may even appear in the intestine that allows partially digested glop to leak into the abdominal cavity.

That the vast majority of NEC cases occur in preterm infants is an undisputed medical fact. But why it develops in some premies but not in others has been much harder to pin down. When NEC was first noticed in French hospitals in the nineteenth century, it was observed to occur in clusters, with dramatic variation in the incidence between hospitals. As germ theory became widely accepted, such a pattern became suggestive of an infectious agent. These days young infants are considered at risk because the vasculature of their intestines is not fully developed, and also because their intestines are highly permeable, which makes it easier for bacteria to stray from the gut lumen.

It is also well established that premie units do a better job of preventing NEC when they feed young infants breast milk than specialist infant formula. Last year, the American Academy of Pediatrics issued a policy statement² that concluded, based on the findings of four randomized clinical trials, “feeding preterm infants human milk is associated with a significant reduction (58%) in the incidence of NEC”.

But the particulars of NEC—what exactly causes it, and what specific constituents of breast milk prevent it—have been stubbornly elusive for many decades. That is, until recently.

A group in Chicago and another in Pittsburgh made headway into this problem around six years ago, when they focused their efforts on understanding how a receptor called lipopolysaccharide receptor toll-like receptor 4, or TLR4 for short, might contribute to NEC's development. TLR4 is unusual among receptors bobbing about on the outside of human cells because it transmits a signal to the inside of the cell whenever it comes into contact with the fatty coating of a bacterium. Both research groups established that mutant mice unable to make TLR4 receptors on any of their cells are less likely to develop NEC than normal mice. This finding might sound odd, but it makes sense given a bit more theory.

The Chicago team reckoned³ that if a lack of TLR4 protects against NEC, perhaps the aberrant overproduction of TLR4 by intestinal cells would make it easier for bacteria to infect gut tissue. The group in Pittsburgh then showed this in the lab⁴.

In their most recent paper⁵, the Pittsburgh team demonstrated that TLR4 signaling also regulates the perfusion of blood to the small intestine. Blood perfusion matters in the development of NEC because cells that don't receive enough oxygen and nutrients will eventually die. The key cellular enzyme in this particular causal chain is called eNOS (endothelial nitric oxide synthase). Its concentration in a cell's cytoplasm is reduced by the chemical signals that TLR4 sends when it's in contact with a bacterium. And when the cytoplasm has less eNOS, it also has less nitric oxide (NO), because eNOS makes NO.

At this point, it's important to realise that NO is a very influential molecule: NO causes blood vessels to dilate and thus allows more blood to flow through them—which is why it's central to the biochemical mechanism of Viagra. In summary, then, the Pittsburgh researchers found that whenever a bacterium meets a TLR4 receptor, blood flow to the intestines is turned down by a very tiny smidgen. When lots of these TLR4 receptors start firing, blood flow is turned down enough to cause NEC.

To prove this, the team again turned to mutant mice that do not produce TLR4. Crucially, these particular mutant mice only lacked TLR4 on endothelial cells (cells that line the interior of blood vessels) in their intestines. That genetic change was enough to reduce the odds of the mice developing NEC, indicating to the team which cells they needed study in greater detail to figure out how TLR4 signaling contributes to NEC.

The researchers then ran through a series of experiments. They fluorescently labeled platelets to show visually that when endothelial TLR4 comes into contact with bacterial fatty coating, blood flow to that patch of the intestines shuts down. Conversely, when the same fatty coating touches endothelial cells of mutant mice lacking TLR4, blood perfusion to that patch of the intestines holds steady.

The researchers also demonstrated a distinction in the amount of eNOS made by endothelial cells. The mutants' endothelial cells, without any TLR4, carried on making lots of eNOS when the cell touched some bacterial fatty coating. These cells therefore remained well stocked with NO when a bacterium was nearby. But in the same situation, the normal mice's endothelial cells made less NO—which is why their intestinal blood vessels started to narrow. The gene involved in translating the TLR4 signal into an instruction to reduce eNOS levels is called MyD88 (myeloid differentiation primary response gene 88).

Finally, given all of the above, the Pittsburgh researchers asked why breast milk prevents NEC. The answer is somewhat surprising.

Breast milk contains tiny amounts of sodium nitrate, the same stuff—'saltpeter'—that was once exported from mines in Peru, Bolivia, and Chile in vast quantities and sent to Europe for industrial processes such as fertilizer manufacturing. (Indeed, the trade led to a war between those South American countries.) The small amount of sodium nitrate in breast milk is converted to NO in a newborn infant's gut. And this extra supply of NO keeps blood vessels in a breastfed baby's intestines dilated—even when bacteria and TLR4 tell the baby's intestinal endothelial cells to make less NO.

After so many years of research into NEC, with so few hard facts to show for the effort, these findings are exciting. Although NEC may have various causes, this work points clearly to a way in which infant formula could be improved—just add sodium nitrate!—to better protect newborns from the disease. And, by exposing some of the specific metabolic pathways involved, this research opens up whole new avenues of investigation into how NEC might be beaten. It gives hope to a lot of very little people.

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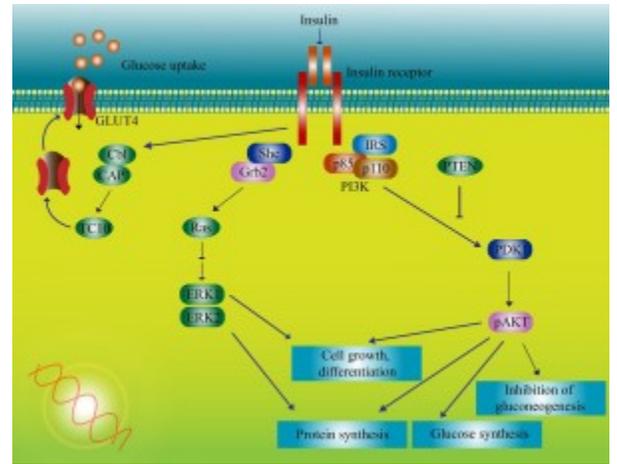
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Insulin Control = More Mammary Tissue, More Milk, Bigger Babies

- Insulin is important for lactation, but its effects are so widespread that its specific effects on mammary gland biology are difficult to study.
- This problem was overcome by the development of laboratory mice missing the insulin receptor in milk-producing cells.
- Using these mice, a profound effect of insulin on mammary gland development during pregnancy was revealed.
- Without normal insulin effects, the neonatal mice had a severely retarded growth rate.
- Metabolic disorders caused by obesity and gestational diabetes may have similar effects in pregnant and lactating mothers.

Pregnancy hormones transform cells in the mammary gland into milk-producing cells. After birth, hormones such as insulin regulate milk production. Does insulin also influence mammary gland cells during pregnancy? A recent study by Peggy Neville and her colleagues from the University of Colorado, (Neville MC et al., 2013) demonstrated that insulin is a primary driver of cellular transformation during pregnancy.

We know insulin as a regulator of blood sugar, but it also influences cell growth and differentiation. This is especially relevant to mammary tissue during pregnancy and lactation. A role for insulin in lactation has been accepted for some time, but some questions have remained about its role during pregnancy when the mammary gland is developing—and the relative roles of insulin and insulin-like growth factors. Clarifying the role of insulin in lactation was the aim of the study by Neville and colleagues. They used a mouse model system, and in an elegant approach, bred a mouse line in which the receptor for insulin was selectively deleted in the cells that produce milk.



Insulin signaling pathway

Insulin is a ubiquitous hormone found throughout the tissues of the body and is essential to life. This has made the study of insulin a little complicated since any attempts to modulate the level of insulin have a marked impact on the entire system, and without careful regulation, will result in metabolic meltdown. Understanding its role in mammary gland development during pregnancy needed a clever approach. Like all hormones, insulin needs a receptor to perform its normal functions. In fact, all of the relevant actions and effects of insulin happen inside cells as a result of the signals that insulin receptors convey to the cellular machinery, including the engine room of the cell, structures called mitochondria, and the genes that turn “on” and “off.” Neville found a way to understand the effects of insulin by targeted deletion of the insulin receptor in the mammary gland of selected mice. In this way, the insulin continued to have its normal effect on all tissues in the body except for the mammary glands.

The first observations in these mice were clear: the baby mice (pups) were very small. The gain in weight of these pups was severely restricted, indicating that the mother was unable to produce enough milk for normal growth rates. In fact, their growth rate was only about 25% of normal, and at weaning, the mice were very small. When the mammary gland structure and contents were examined, the investigators found far fewer milk-producing cells than in normal mice, and most of the milk protein and fat normally found in these cells was lacking. Thus, there was a reduction in both the total amount of mammary tissue, and of the milk-producing capacity of the remaining tissue. Clearly, the ablation of the insulin receptor had profound effects on the mammary glands of these mice.

Knowing that a number of growth factors related to insulin—insulin-like growth factors (IGFs)—may use the same receptors as insulin, Neville and her team set out to prove that the effects were due to insulin, and not these other factors, but how to do this was the challenge. They turned to their experience with cell culture systems, especially the growth of a test tube version of the mammary gland milk-producing cell structure, variously referred to as acini or mammospheres. The development of these structures was sensitive to insulin, but insensitive to IGFs. Once formed, they were induced to secrete milk, and again, the secretion was found to be insulin-sensitive, but was not IGF-sensitive.

Finally, the team analyzed the differences in the expression profiles of 22,000 genes from milk producing cells with and without the insulin receptor. They found a definite pattern of differences in gene families that were associated with mammary gland differentiation and function. The patterns indicated that insulin is important for turning on the cell program that leads to milk production in late pregnancy.

These findings suggest significant implications for the current surge in gestational diabetes seen throughout the western world and highlight the potential impact of metabolic disturbances caused by obesity and diabetes on lactation. Diabetic, obese mothers have a difficult time producing milk, and we’re just now starting to understand why.

Neville MC, Webb P, Ramanathan P, Manninno MP, Pecorini C, Monks J, Anderson SM, Maclean PS. (2013) The insulin receptor plays an important role in secretory differentiation in the mammary gland. *Am J Physiol Endocrinol Metab* [Epub ahead of print] PubMed PMID: 23982156.

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