Muscle Targeted Growth Promotants
Deana Hancock

[1]All right. It's my pleasure to present or introduce our third speaker this afternoon. After this speaker, we'll have a short break, and so, please stick around, and the break will be held in the back – up above this room in the back. They'll have some beverages and things up there. It's my pleasure to introduce Dr. Deana Hancock. She was born in Missouri and was raised on a dairy farm, and so, she has an agricultural background to start with. She received her bachelors and master degrees from the University of Missouri through the Department of Animal Sciences, and then, she went on to Texas Tech and received her Ph.D. in ruminant nutrition with an emphasis in growth and development. She went on to a post-doc with Lilly and then became a faculty member at Purdue University, and subsequent to that, she was hired by Elanco, and she is currently Project Manager of Development and Registration with Elanco, and she is going to be talking about kind of the next phase of how we might use some growth promotants in modifying animal performance and the impacts on meat quality. Thank you John and I'd like to thank both John and the Committee for the invitation. Its' a pleasure to be here today, and I'd also like to thank my co-authors here. Dave Anderson was a mentor for me when I first started working in the mode of action of beta agonists a number of years ago now, and then, Diane Moody was a post-doc in my lab, and today, we are going to switch gears now just a little bit from the sequencing and genome effects and switch more to a specific pharmaceutical approach to muscle growth and, specifically, the mode of action of beta agonists.

[2]So, first of all, what is a beta agonist? It binds to the beta receptor, and then, it activates, through the beta receptor, on many different cell types in the body.

[3]And here, we've got a schematic of the beta receptor, and on the right hand side, the shaded areas in green there are conserved across the human beta one, two, and three, so across the subtypes, and we see about a thirty-one percent conversation, and that's primarily in the seven transmembrane domain area, and then, on the bottom, on the left, you'll see the circled amino acids are conserved then within a receptor subtype, in this case the beta one, across humans, pigs, and sheep, and there's about an eighty percent conservation there.

[4]And one thing that is unique about the beta three – there are three subtypes; one, two, and three. The three lacks, in domain four, phosphorylation sites. So, it's thought that the beta three does not down regulate like the beta one and beta two receptors do. So, that's a bit unique about it.

[5]So, how do these beta agonists work then?

[6]They bind to the receptor at the cell membrane

[7]and stimulate the G stimulatory protein

[8]and activate adenylate cylase to increase cyclic AMP. So, this is the cyclic AMP second messenger system that these are working through,.

[9]And then, they activate PKA or Protein Kinase A and then elicit a series of events from that point on,

[10]largely enzyme phosphorylation mediated. And then, the metabolic effects that we see in the fat cells – specifically, we'll see a decrease in fat synthesis and an increase in fat breakdown, and in the muscle cell, either protein synthesis has increased or no change and protein breakdown is either not changed or decreased, and the bulk of the presentation that I'm going to spend time on today is on the mode of action of how we see this increase in muscle accretion; so the muscle cell aspects.

[11]Skipped Slide.

[12]And as we start, I wanted to first talk just a little bit about the endogenous beta agonists. And that would be – one would be Norepinephrine, and that is a Phenethanolamine or the natural Catecholamine, and it's released at the central and sypathetic nervous system endings, and it circulates in the plasma at relatively high concentrations.

[13]And then, we have Epinephrine also in the same class, and it is synthesized in the adrenal medulla, and it circulates in lower concentrations than Norepi, and it is synthesized from Norepinephrine.

[14]So, then, as we look at this molecular structure of the molecule, what specifically is this when we say Phenethanolamine, and the base structure that you'll see there in green is similar across these molecules.

[15]And what changes, as we look at some different beta agonists here, are the groups on the left-hand side as well as all the way out on the right-hand side. Those can change, and that's what makes the different molecules have slightly different specificity, and what we've got circled in the oval here are the similarities or that Phenethanol main nucleus across all of these different compounds, but there are some selectivity differences. Ractopamine, for instance, the molecule that Elanco has, is classified as a beta one selective antagonist versus the others that you'll see here are beta two selective antagonists, and this is from a prototypical classical assay where they are tested, and the relative specificity is identified, and Ractopamine, for instance, binds at both beta one and beta two. It just preferentially selects for the beta one receptor in these prototypical assays, and then, you'll see one instance on Zilpaterol there, even though it's a ring structure, it still has that Phenethanolamine structure in it, and as far as approved molecules for use, Ractopamine is approved in the United States and a number of outside of the United States countries. Zilpaterol is approved in Mexico and South Africa in beef cattle.

[16]So, a brief summary here of the typical growth and carcass effects that we see; we'll see about a four day reduction in days to market to the similar end weight with less feed, and the yield on the animals is increased, whether you look at the dressing or the trim cuts. So, total yield of lean meat is increased, and this is while they maintain the pork quality attributes, and this comes from our original submission package with Ractopamine.

[17]Some things to think about though when we start to look at different Phenethanolamines and responses that we see and some similarities and some differences and some factors that contribute to some of these responses are things like dietary protein. Typically, in the monogastrics anyway, we see an enhanced response when we supplement either with dietary protein or amino acids, and we've seen that across several species, and duration of treatment and on down to age and weight kind of could be lumped together I think. Basically, as the animals are older and near finishing, we've got an increase in fat accretion. Protein accretion is slowing down. We tend to see a bigger response there, and again, that is across multiple species and multiple compounds that we see that type of response. Then, with dosage, we also see a differential affect on growth and leanness. Typically, it takes a higher dose to get the carcass leanness response maximized relative to the growth response, and then lastly, genetics, we have seen effective responses across different genetic lines, and this now needs to be expanded to beef. We've had several studies in cattle with optiflex across different biological types and see the typical Ractopamine response.

[18]And initially, these compounds were named repartitioning agents, and one of the first papers was with Ricks, et al., in 1984 here at the Reciprocal Meats Conference and then some additional work from Dale Bauman and Dunshea, but basically, they have taken from the nutrient pool and slowed down lipolysis and lipid accretion, and fat deposition then is reduced by six percent. And muscle accretion is increased,

[19]and with Ractopamine in particular, we see an increase in protein synthesis with very similar rates of protein degradation, and not all molecules are having

this same affect on protein synthesis and degradation, and we'll expand on that more as we go through the talk, but protein accretion increased by thirty percent.

[20]So, as I indicated, I want to spend most of the time then today talking about this muscle growth and what's driving these responses at the muscle level. And before I move into that though, a couple of comments about direct and indirect, and we are going to study that a little bit at the current state on muscle, but it was originally thought too that the indirect effects from endocrine effects might be part of the response, and through a number of studies now, which we'll review a few those, we don't really think that that's the predominant factor that's driving this response, and we do know that with several of these molecules we do get an increased blood flow. So, nutrients are increased to the tissue bed.

[24]So, to start out with the muscle protein metabolism schematic here, I just want to use this introductory slide to comment about the two pathways to get this protein accretion. We've got the synthetic side, and then, we've got the degradation side. So, if we increase synthesis or decrease degradation or shift those two in proportion, we can have an accretion effect and through various different parts of this pathway.

[25]And again, this question of, at the muscle level though, is it direct or indirect effects that we are seeing to drive this response, and I think there is a lot of evidence today now that would start to suggest that we are looking at some direct effects at the muscle level, and that's what I want to spend the bulk of the time today walking through that – why we feel that that's the case.

[26]But some early work here out of Don Byrem's lab while he was at Cornell with the closed arterial infusion model, and this is in steers on the hind limb. They infused Cimaterol on one and a controlled solution on the other, and what they saw was an increased fractional rate of protein accretion across a number of durations here. So, in that closed system, it looked like it was a direct effect on protein accretion, and then, other evidence to support that effect is if you have an animal with inherited dystrophy, you will have a beta agonist effect in that model, or if you denervate and induce muscle wasting or recently there has been some… induced wasting showing that the beta agonist will reverse that, and in endotoxemia or food deprivation and diabetic models, you'll see a beta agonist effect and then also in models where they have hypophysectomized, castrated, or adrenalectomized. So, if you remove those tissues, you will still see a beta agonist effect. So, all of this leads up to suggest that there is a direct affect on the skeletal muscle.

[27]And at the muscle level itself, it has now been found that there are beta receptors at the muscle level, and beta one, two, and three are all on the skeletal muscle cell

[28]with some interesting species differences though. The pig predominantly carries the beta one receptor subtype; whereas the bovine and rat has predominantly the beta two receptor subtype. So, we need to keep that in mind as we look at some of these mechanistic studies that we'll look at there in a moment.

[29]And then, also interesting in some work with Ron Young and some internal work that we've had with beta agonists in poultry, typically, we'll see a much larger response here in the dark muscle, and I think a lot of that is partly attributed to the fact that the dark muscle has more beta receptors than the white muscle, and we've seen that in both turkeys and broilers.

[30]So, this cyclic AMP cascade that we talked about initially is that which is mediating this response or not, and I hope through the next few slides I'll be able to convince you that, at the muscle level, it probably is.

[31]Initially though, that was not the thought. The thought was that it was independent of the beta agonist pathway, and that was largely driven from a couple of early papers where they tried to block the beta agonist effect, specifically the muscle effect, with the antagonist Propranolol, and they could not block the effect.

So, they thought that it was due to something independent of that pathway.

[32]But more recently, the anabolic effects are thought to be through the beta agonist pathway and for these following reasons. Propranolol has been shown to block the muscle accretion effect, when it is administered either IP or at higher oral dosages, and I'll show you some of that data. It also, when selective beta or an adrenergic antagonist had been used, they had been able to show the blockage, and muscle cells, as I indicated, do have beta agonist receptors, and beta agonists increase cyclic AMP in muscle cell culture and in muscles in vivo, and again, they've been shown to reverse the effects with antagonists or beta blockers.

[33]Some of that data is here on the left hand side. The first coming out of Warner Bergen's lab, a negative control showing very little cyclic AMP levels, and these are in mouse C2 C12 cell cultures, but as you increase Isoproterenol in the lowest dose on the right hand of that black bar graph and increase the dosage, you'll see a linear increase in cyclic AMP response or cyclic AMP concentrations in the cell culture, and then,

[34]also some work coming out David Smith's lab showing that both in myoblast and in myotubes that Ractopamine, given in culture, will increase cyclic AMP and that if you give Propranolol in that cell culture system you can block that cyclic AMP response. So, it's an indication that there is a direct effect, and it's directly mediated through the beta agonist.

[35]Then, if we switch from the cell culture and move into the rat skeletal muscle cell slices or muscle slices, the graph on the left is a typical dose concentration response curve of cyclic AMP with the beta agonist. In this case, the first on the farthest left is Isoproterenol, which is a beta one, two, and three agonist. It hits all three receptors, and it's very potent, and if you see the… EPI and EPI responses there, they shift that curve to the right. So, they are less potent than Isoproterenol but very similar in their potency, and those would be the two endogenous…. Then, on the far right, the upper graph shows a blockage with Propranolol, a non-selective antagonist, and at higher dosages, it will shift the curve to the right. So, we are seeing a blockage there, and then, if we look more specifically and tease this out with a specific receptor antagonist and in this case at the beta two antagonist in a dose… effect, you'll see the shift of the curve to the right and then a dose-dependent fashion. So, we are seeing a cyclic AMP response that is blocked specifically by the beta two receptors or antagonists, and then, on the bottom graph is a beta one antagonist blocker, and we don't see as much of an effect there until we get into very high levels. So, it certainly looks like, in this model, which again, is a rat which is predominantly beta two receptors in their muscle, a beta two effect. Then, if we look at rat skeletal muscle and look at cell membranes on the left, Isoproterenol, again, a very nice increase and dose responsive in cyclic AMP production, and then, Clenbuterol is a beta two agonist and increases cyclic AMP as well, and then, on the right, if you take a biopsy tissue sample, we see very similar responses. We've got a Clenbuterol response there, and then, interestingly in the green, in this paper also, both the gross and the gastrocnemius weight was increased with the beta agonist and then could be blocked by the beta two antagonist but not the beta one antagonist.

[36]And in another paper here, I want to pull out and show the route of the beta blockers. I think that probably had some affect on some of those early conclusions too, but if you block with the beta blocker before you challenge with the Clenbuterol here, you see saline and the beta blocker are very similar on their cyclic AMP production and then an elevation with Clenbuterol. So, time of administration relative to the challenge is important.

[37]And then, the dosage of the beta blocker, if you look at rat body weight gain, gastrocnemius weight, or the protein content in that muscle with the Clenbuterol or the beta two agonist, you will see an increase in all three of those, and at the low dose of Propranolol, we are not seeing a blockade of that effect, but when you increase the dose five fold, there is a nice blockade, and it is consistent across all three parameters.

[38]And then, back to this receptor selectivity question again, this is the beta two blocker; the one one eight five five one, and again, it's the same type of pattern in body weight, muscle weight, and muscle protein content. When we give the specific beta blocker at the same dose as Propranolol, we see a blockade, again, suggesting a beta two mediated event here with Clenbuterol in the rat skeletal muscle.

[39]Now, this is a different paper taking kind of a different tangent on this, but what they did was they chemically blocked rat skeletal muscle and showed a deprecation in Norepinephrine in two different muscle cell types, when they did that, and they also showed a fifteen to twenty-five percent increase in proteolysis, when they blocked the Norepinephrine response.

[40]And they showed that this is basically time, muscle, and calpain specific, and on the bottom… is days after the blockade of Norepinephrine being one, two, or four, and in the middle, you'll see the white bars are the soleus muscle, and there is a significant increase in the calcium-dependent proteolysis very acutely, and then, no response in a more chronic or four-day administration, and this is in the soleus, and it is not absorbed in the EDL. So, that is part of the reason for their conclusions there.

[41]And then also of interest, if you look - if you add Isoproterenol to this system, then what they've observed is a decrease in protein degradation of thirteen to twenty-seven percent in the two different muscles.

[42]So, removal increases proteolysis and the addition of a beta agonist decreases proteolysis.

[43]So, now what I want to do is kind of start to tease out different parts of the pathway and specifically look at fractional synthesis rate, and this is with Ractopamine in pig tissue or in pigs, and they used a six-hour infusion with 14C-Tyrosine and with a twenty-one or thirty-five day administration of Ractopamine at twenty parts per million, and from Warner Bergen's lab, showing an increase in the fractional protein synthesis rate.

[44]And then, another lab using a different label, 14C-Lysine in this case, showed very, very similar responses showing Ractopamine increasing the fractional protein synthetic rate in those tissues or in the animals.

[45]So then, how did this occur, and I want to start walking through the cascade here at the transcription level.

[46]What we know with the beta agonist is that the myocin mRNA abundance has increased in cattle fed Ractopamine and Clenbuterol, and the alpha actin mRNA has been increased with pigs fed Ractopamine, and in sheep, alpha actin has also been shown to increase with a beta two agonist, and all three of these bullets have basically been interpreted as increased rate of transcription or stability of the message. It's really just a concentration at that point and time, and as far as the exact underlying mechanism there, it is one of those two effects.

[47]On the genes for muscle or for the skeletal muscle proteins and calpastatin are what we call the CRE or cyclic AMP response element,

[48]and so, there is some work that's been done with human and rodent muscles to show that there are cyclic AMP response elements on genes, and basically what they've done here are the promoter deletion assays to interrogate what regions on the gene express that activity, and this is with a promoter expression assay, in this case the CAT assay, and so, if you delete these areas on the second line down there, you'll see that we've lost part of the Norepinephrine response for the cyclic AMP response. So, it's in that part of the gene region that we know there's an element that is responsive specifically to this cyclic AMP response, and up in Quadrant A there, what I wanted to show was that basically they've shown that this

is a beta agonist response with the Propranolol blockage there with the reduced line.

[49]And then, in rat cardiac alpha myocin heavy chain, again, the same type of thing has been done here. And you'll see on the bottom right hand side the cyclic AMP responsiveness has been reduced in that region; so, from minus seventy-four to minus thirty-nine in that gene is where there's an element that is responsive to cyclic AMP,

[50]and then, this group has just further interrogated that fine mapping there, if you will, and determined that it's really in that minus sixty-one to thirty-nine area, and you'll see the reduction down to one point two relative to the five and six fold increases, and then, on the right hand side of the CAT assay there, you can see the loss of reporter gene.

[51]And bovine calpastatin also has the cyclic AMP response elements, and it's basically similar types of experiments that were done here just showing what region in the gene has the cyclic AMP response element.

[52]And the B Panel here, what I wanted to show is that they basically have mutated a four nucleotide sequence there and can abolish that effect. So, they know the specific responsive element there.

[53]On translation, I've not been able to find a whole lot of information with the beta agonist and certainly not in livestock species or rodent or human literature to really understand and determine what's going on at the level of translation. Most of it has been at transcriptional regulation.

[54]Proteolysis though, there has been quite a bit of work done with the beta agonist to understand mechanism of action that is occurring there, and it has largely been focused with the calpain/calpastatin system. Other systems have been studied, but this is where it looks like a lot of the effects are.

[55]And this is with the beta two agonist in steers and fractional degradation was reduced twenty-seven percent after three weeks, and this is out of Koohmaraie's lab.

[56]And then, this is with Cimaterol on alpha actin calpastatin mRNA expression, and again, this is in that closed arterial system that Don Byrem has, and we really didn't see a whole lot of activity on either alpha actin or the micro… or skeletal muscle calpain, but where the saw the bulk of the activity was an increase in messenger RNA for calpastatin, and then also, in the right hand corner there, in that study, they saw an increase in activity of – or increased activity of fifty-one percent.

[57]And then, to tease this out a little more, and again, this is some work from the bovine calpastatin mRNA expression with Cimaterol administration. With the three subtypes of Cimaterol, it's really the three point eight kilobase that was increased in these studies.

[58]And then, again, with the beta two agonist in both sheep and bovine, calpastatin mRNA and activity has been shown to be increased by a number of labs.

[59]And then, there has been some work with Epinephrine and Clenbuterol on calpastatin in pigs, and Epinephrine infusion for seven days increased calpastatin activity in porcine skeletal muscle. It increased the one thirty-five kDa calpastatin, and the infusion for seven days didn't change calpastatin mRNA in this case in pig skeletal muscle, and a single oral dose of Clenbuterol increased calpastatin mRNA protein in calpastatin activities sixteen hours post-feeding.

[60]And then, this is some work that a graduate student did with Dave and I looking at the effects of Ractopamine in pigs on calpain and calpastatin activity, and basically, we had an experimental design of ten percent proteins; so, lower than the NRC requirement, and eighteen percent crude proteins, which would have been more

than adequate for the pigs on Ractopamine, and we did not see any changes in uM calpain or calpastatin, but we did see actually an increase in the mM calpain activity, and other work with Bergen's lab has shown no affect on the calpains with Ractopamine in pigs.

[61]One thing we did see though was we looked at the skeletal muscle calpain expression levels, and we did see, regardless of protein level, a reduction in the skeletal muscle calpain expression levels.

[62]And one other comment about calpastatin and its phosphorylation; there are phosphorylation sites on bovine calpastatin, and this does occur by the Protein Kinase A cascade of events. So, one of the things that I think we need to probably look at is a little more research on that area.

[63]And one last thing that I want to talk about here is the amino acid substrates and basically the fueling of the system,

[64]and again, in this model that we have of the protein-restricted diet, ten percent versus eighteen percent. On that protein restriction diet, on nitrogen retention, we didn't see a Ractopamine effect at the whole body level, when they were deficient in protein, but you do see the typical effect there when adequate protein is available. It's the same way with the average daily gain. You don't see the response unless the diet is sufficient, but what was interesting, if you look at loin eye area, even though the animals are deficient, so back to this repartitioning effect, they are laying down the protein in the loin eye area, and we use urinary creatinine as a marker of anabolic activity, and you'll see the same pattern there. It wasn't quite significant, but it was the same pattern as the loin eye measures.

[65]And then, we also looked at alpha actin expressions, and again, in those pigs, the machinery was there. It was just that they did not have the substrate to elicit all of the responses in terms of the whole body growth and nitrogen metabolism response.

[66]So, in summary, to conclude on some of the effects on protein metabolism, with Ractopamine being more beta one selective, what we've seen is primarily an increase in protein synthesis, and we have seen increases in mRNA for the myofbrillar proteins, actin, and myosin, and minimal effects on the calpain/calpastatin system, but we have shown that substrate amino acids are required to maximize the performance and lean growth.

[67]And then, the beta two agonists, Clenbuterol, Cimaterol, and the… compound, on protein metabolism, some studies have shown an increase in muscle protein synthesis. Clenbuterol has increased the abundance of mRNA for myofbrillar proteins, specifically myocin. The… compound has been shown to increase mRNA for actin, and then, all three have a consistent effect of increasing calpastatin. Thus, a large effect is mediated through the protein degradation pathways.

[68]A couple of recent technologies and tools that I think will help even better understand the mode of action of the beta agonist and how we are eliciting this muscle effect, but there are anti-sense… now that can be used as more specific beta blockers, monoclonal antibodies to the beta receptors. We now have Chinese hamster ovary cells that express the porcine and bovine beta receptors. So, there are more tools to understand mechanism at the receptor level, and then, we've also got the knockout and knockin models as well as humanized models now, and then, I should have put micro-array technology on here. I think there's a lot we can learn from that too with the more system approach versus the targeted gene approach here, and I guess one other comment that I wanted to make on the prior two talks about porcine sequence and how that might impact the industry, and I think with the pharmaceutical industry we see impacts there too, because this target that we have here, the beta receptor – well, some of the fallout of the sequencing events are going to be new targets for new growth promotants.

[69]And in conclusion and some particular research needs for the future, we've got

a lot of data now from the rodent literature that does show a direct and mediated by beta agonist effect and specifically through the cyclic AMP cascade, but we need this type of information confirmed in the livestock species, and we also need to know the receptor subtype proportions across various muscles and across species. We have some information, but there is still a lot that we could learn there in basically studying the cyclic AMP effects largely like we've seen in the rodent literature today, and then the beta blockade pathways, and that doesn't mean that there's not some additional pathways or cross-top or indirect actions that might be going on as well.

[70]And then also the rat skeletal muscle alpha actin promoter does have a cyclic AMP responsive element that, again, with the livestock genes, it would be nice to have this confirmed and also to identify what these cis and trans activating factors are on the genes that mediate the response and confirm that it is PKA mediated through this cyclic AMP cascade, and then, the deletion analysis has indicated that the calpastatin gene promoter does have at least one cyclic AMP responsive element, and again, isolation of those transacting factors and confirmation of the PKA mediated effect, and then, as I indicated in the talk, some additional work on calpastatin and the phosphorylation events, and then, there is a void on protein translation data.

[71]And in summary then, we didn't spend a lot of time on adipose tissue today, but the effects are certainly there, and it has effects on the muscle, and we've walked through some of those, and I want to leave you with the fact that a lot of these things though are very dependent on a number of things, and it is a systems approach as well, and we've got species effects, compound effects, beta receptor selectivity, dosage effects, duration, sampling relative to that dosing, and we've temporal effects on a number of fronts, and then, we've go the age and weight and nutrition, and I think we could probably add several other things onto that list.