

# Biological basis of the ractopamine response

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**ABSTRACT:** The potential for beta-adrenergic receptor ( $\beta$ AR) agonists to modify growth rate and body composition has been investigated for over 20 yr. Ractopamine was developed by Elanco Animal Health and is the first  $\beta$ AR ligand to be cleared for use in pigs in the United States, which occurred in 2000. Ractopamine is structurally similar to the natural catecholamines epinephrine and norepinephrine and binds with high affinity to  $\beta$ AR in pig adipose and muscle tissue. The family of  $\beta$ AR, however, belongs to a much larger family of structurally related G-protein coupled receptors (GPCR) and there is little or no information available as to whether ractopamine binds and signals through additional GPCR. The question of whether  $\beta$ AR mediate the growth response in pigs and other animals is not fully settled, but primary attention has been given to understanding how  $\beta$ AR might mediate increased growth and protein accretion, and whether effects are direct on adipose and muscle tissue or mediated by secondary factors. Ractopamine is a racemic mixture of four isomers resulting from two asymmetric carbons. The RR isomer (levorotatory at both carbons) has the

highest affinity for the pig  $\beta_1$ AR and  $\beta_2$ AR and  $K_d$  values are essentially equivalent for both subtypes ( $\sim 25$  nM). Other isomers have from 3- to 600-fold lower affinity. The RR isomer appears to mediate the growth response in rats and likely is the active isomer in pigs. Therefore, ractopamine can be considered nonselective in binding to either the  $\beta_1$ AR or  $\beta_2$ AR in pigs. It is not known, however, whether the  $\beta_3$ AR or additional subtypes may mediate a ractopamine response. Direct activation of  $\beta$ AR in adipocytes promotes triglyceride hydrolysis and decreases fatty acid and triglyceride synthesis, thus leading to less lipid accumulation. Fat accretion in pigs given ractopamine is not consistently reduced, which may result from  $\beta$ AR down-regulation. Ractopamine consistently increases muscle protein accretion in pigs. Responses are maximal within the 1st wk and decline toward zero over 4 to 6 wk. Curiously,  $\beta$ AR down-regulation is not significant in skeletal muscle. The mechanism responsible for increased protein accretion is not clear, but cumulative evidence points to a direct effect, possibly involving both protein synthesis and degradation.

Key Words: Adipose Tissue, Beta-Adrenergic Agonists, Livestock, Muscle

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## Introduction

Early reports of the effects of beta-adrenergic receptor ( $\beta$ AR) ligands on growth in rodents (Emery et al., 1984) and lambs (Baker et al., 1984) appeared nearly 20 yr ago. Stock and Rothwell were investigating the anti-obesity effects of sympathomimetic drugs in rodents. These authors observed that whereas a nonselective ligand reduced weight gain and body fat, a  $\beta_2$ AR-selective ligand (clenbuterol) increased food intake, weight gain, and protein accretion (Emery et al., 1984).

Clenbuterol also increased protein accretion in lambs (Baker et al., 1984). Increased growth was not observed with a  $\beta_1$ -selective ligand in rodents (Emery et al., 1984; Convey et al., 1987). It cannot be expected, however, that the  $\beta_2$ AR mediates the growth response in other species because the distribution of  $\beta$ AR subtypes may well differ. Increased protein accretion and leanness have been observed in all livestock species, with cattle and sheep generally responding to a greater extent than pigs, and pigs greater than chickens (Mersmann, 1998; Moody et al., 2000). That qualitative changes are similar across species suggests a common mechanism of action, although differences between species may indicate a more complex model. Insight into the mechanism of  $\beta$ AR-mediated growth is obtained by comparing the responses of  $\beta$ AR ligands and somatotropin, which exhibits a similar net increase in protein accretion (Nossaman et al., 1991). Ractopamine and other phenethylamines typically increase carcass dressing percentage in pigs, whereas somatotropin decreases this parame-

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ter. This difference reflects the selective effect of phenethanolamines on skeletal muscle vs the general growth enhancement and greater effect on organ growth by somatotropin. The combination of ractopamine and somatotropin are additive on growth (Jones et al., 1989). It might be inferred, therefore, that ractopamine does not share a common mediator or signaling pathway with somatotropin, and that  $\beta$ AR activate targets unique to muscle.

### $\beta$ -Adrenergic Receptors

The  $\beta$ AR are cell-surface receptors that belong to large class of 7-transmembrane domain proteins. A subset of these proteins is the large family of G-protein coupled receptors (**GPCR**) to which the  $\beta$ AR belongs (Strosberg, 1996). Although ractopamine and other phenethanolamines are known to bind to  $\beta$ AR in cell membranes from different species (Colbert et al., 1991; Spurlock et al., 1993), it is less certain whether this class of compounds or specific members of this class of compounds mediates their effects solely through the  $\beta$ AR. Part of the uncertainty is that over 1,000 GPCR have been identified that are bound by a wide array of ligands from small amines to lipids to proteins (Strosberg, 1996). The GPCR may have evolved from a common ancestral gene forming subtle structural changes to accommodate the wide variety of ligands. Although it is easy to demonstrate binding and signaling through the  $\beta$ AR, it is more difficult to prove exclusivity to this receptor class and the possibility of binding to other receptor classes cannot be ignored. In addition, the natural catecholamines also bind to the alpha-adrenergic receptors ( $\alpha$ -AR), which signal quite differently from the  $\beta$ AR. Ractopamine is purported to have low affinity for  $\alpha$ -AR in rodents (Colbert et al., 1991), but I am unaware of published data for the pig or any other species.

Three  $\beta$ AR subtypes have been cloned from several species, including the pig (Liang et al., 1997; Cao et al., 1998; Smith et al., 2001). The  $\beta$ AR subtypes share approximately 50% sequence homology, with the greatest homology in the transmembrane domains where the ligand binding pocket is formed. Subtype diversity provides for greater control of metabolic processes and provides the pharmacologist the means to better define metabolic targets. The  $\beta$ AR subtypes exhibit ligand selectivity such that the rank order of affinity for norepinephrine is  $\beta_1$ AR >  $\beta_2$ AR >  $\beta_3$ AR. Epinephrine binds the  $\beta_1$ AR and  $\beta_2$ AR with a similar affinity but has a greater affinity for the  $\beta_2$ AR than does norepinephrine. The  $\beta_1$ AR and  $\beta_2$ AR are co-expressed in most tissues of the body but the ratio of these  $\beta$ AR subtypes can vary, such that in the rat, the heart is predominantly  $\beta_1$ AR, whereas the lung and skeletal muscle have mostly  $\beta_2$ AR. The  $\beta_3$ AR has a limited pattern of expression and is found most abundantly in white and brown adipose tissue.

Subtypes also differ in regulation by phosphorylation and gene expression and G-protein selectivity (Strosberg, 1996). Multiple subunits of the G-proteins have been identified that bind  $\beta$ AR with varying affinity and show differential tissue expression. All of this leads to considerable diversity in the response to a given ligand. Sequence homology for any given subtype across species is typically high and on the order of 90%. Despite the high homology, one cannot assume that the pharmacological properties of a given subtype will be the same across species. Whether a given ligand exhibits high or low affinity for a  $\beta$ AR and is able to signal through G-proteins is highly dependent on amino acid sequence, which differs across species. For example, the antagonist ICI 118,551 is highly selective for the  $\beta_2$ AR in humans and rats and is used extensively to target this subtype. However, ICI 118, 551 is not  $\beta_1$ - $\beta_2$ AR selective in the pig and cannot be used to distinguish these subtypes (Mersmann et al., 1993; Liang and Mills, 2002). Similarly, BRL 37344, which is selective for the  $\beta_3$ AR in the rat, is  $\beta_2$ AR-selective in the pig (Liang and Mills, 2002). The distribution of  $\beta$ AR subtypes may also differ by species. This is most evident for the  $\beta_3$ AR, which is the predominant subtype in rodent adipose tissues and the primary regulator of lipolysis and thermogenesis. In humans and pigs, however, the  $\beta_3$ AR represents less than 10% of the  $\beta$ AR subtypes in white adipose tissue, and less than 2% of the subtypes in other major tissues (McNeel and Mersmann, 1999). The  $\beta_1$ AR is the predominant subtype in most pig tissues, comprising nearly 80% in adipose, 72% in heart, 65% in lung, 60% in skeletal muscle, and 50% in liver (McNeel and Mersmann, 1999; Liang et al., 2002).

### Ractopamine Isomers

Phenethanolamines are one class of compounds that bind to  $\alpha$ AR and  $\beta$ AR and are characterized by a substituted aromatic ring and an ethanolamine side chain with various substitutions on the aliphatic nitrogen (Smith, 1998; Ruffolo, 1991). Epinephrine, norepinephrine, and ractopamine belong to this class. Interaction of ligands with amino acids of the  $\beta$ AR involves the positively charged nitrogen, the  $\beta$ -hydroxyl group, and substituents on the aromatic ring. The  $\beta$ -hydroxyl group generates a chiral center and has two possible optically active isomers; R(-) and S(+). The  $\beta$ AR are stereoselective, showing an approximate 100-fold higher affinity for R(-) (Ruffolo, 1991). Ractopamine contains two chiral centers and a possible four stereoisomers, RR, RS, SR, and SS (Ricke et al., 1999). The RR isomer is lipolytic (Yen et al., 1983) and accounts for enhanced growth in rats (Ricke et al., 1999). Structurally, therefore, the ractopamine isomer responsible for modifying growth and composition is the one most likely to bind the  $\beta$ AR. The selectivity of pig  $\beta$ AR for ractopamine stereoisomers has been determined using the cloned  $\beta_1$ AR and  $\beta_2$ AR expressed in Chinese hamster ovary cells (**CHO**; Kissel et al., 2001). The RR

isomer has the highest affinity for each subtype and has nearly equivalent binding affinity (25 nM). The rank order of affinities for each subtype was RR > RS > SR > SS, and  $K_d$  values ranged from 25 to 16,600 nM. Except for the RR isomer, other isomers exhibited preferential binding to the  $\beta_2$ AR. Receptor activation and adenylyl cyclase stimulation indicated that the  $\beta_2$ AR was a more functional target than the  $\beta_1$ AR. None of the isomers significantly stimulated adenylyl cyclase through the  $\beta_1$ AR in CHO cell membranes, although isoproterenol was effective. In membranes from CHO cells expressing the  $\beta_2$ AR, the RR isomer was most effective and a small response was observed for SR. One of the hallmarks of pig  $\beta$ AR is the remarkable structural requirement these receptors have for adenylyl cyclase activation. The RR isomer was at best 50% as effective as isoproterenol in adenylyl cyclase activation. Ractopamine and most other  $\beta$ AR ligands evaluated for livestock growth are only partial agonists at pig  $\beta$ AR (Mills and Mersmann, 1995). Lipolysis in adipocytes is a more sensitive test of  $\beta$ AR activation than is activation of adenylyl cyclase. Because pig adipocytes contain nearly 80%  $\beta_1$ AR, we evaluated whether the RR isomer stimulated lipolysis via the  $\beta_1$ AR or  $\beta_2$ AR. Results indicated that both  $\beta$ AR subtypes were used, indicating that ractopamine could signal through the  $\beta_1$ AR and  $\beta_2$ AR. To summarize what is known about ractopamine selectivity for pig  $\beta$ AR subtypes, the RR isomer exhibits equal affinity for  $\beta_1$ AR and  $\beta_2$ AR, with other isomers showing a small preference for  $\beta_2$ AR. No ractopamine isomer was as efficacious as isoproterenol and some may be functional antagonists. The RR isomer was the most efficacious and appears to exhibit better efficacy through the  $\beta_2$ AR than through the  $\beta_1$ AR, although signaling appears to occur through both subtypes. A similar finding was noted in rats, in which ractopamine was more efficacious at the AR than at the  $\beta_1$ AR (Colbert et al., 1991).

### Adipose Tissue Metabolism

Ligands for  $\beta$ AR were first screened for their anti-obesity properties, so it is expected that adipose tissue accretion should decrease. Direct activation of  $\beta$ AR in adipose tissue and increased protein kinase A activity leads to activation and translocation of hormone-sensitive lipase with subsequent triglyceride hydrolysis. Activation of protein kinase A is also antilipogenic due to phosphorylation and inactivation of glucose transport and acetyl-CoA carboxylase and reduced expression of lipogenic genes (see Mills and Mersmann 1995; Mersmann, 1998). Pigs fed ractopamine consistently have a reduced percentage of carcass fat (Moody et al., 2000). However, the rate of fat accretion is not consistently reduced (Dunshea, 1993) and expression and activity of lipogenic genes may or may not be altered (Liu et al., 1994). The reduction in carcass fat may reflect more a dilution by increased protein accretion than a direct effect on adipose tissue. Ractopamine acutely increases

plasma free fatty acids in vivo (Veenhuizen et al., 1987) and in vitro (Liu et al., 1989), so there is little doubt that ractopamine is an agonist in pig adipose tissue. However, the response to ractopamine may be short-lived because total  $\beta$ AR are down-regulated by nearly 50% within the first 7 d of administration (Spurlock et al., 1994). Because ractopamine isomers have only limited ability to activate pig  $\beta$ AR, down-regulation may limit the effectiveness of ractopamine and lead to the variation in carcass fat. Implementing feeding strategies to circumvent down-regulation (McElligot et al., 1989) may enhance the ability of ractopamine to limit fat accretion. McElligot et al. (1989) used intermittent feeding of clenbuterol to prevent down-regulation, and Herr et al. (2001) used a step-up dosing procedure to accommodate the loss of  $\beta$ AR. Both strategies appear to show a greater response than chronic administration of a single concentration.

### Muscle Metabolism

The initial finding that  $\beta$ AR ligands increase muscle mass was a surprise, particularly given that catecholamines are more closely linked with degradative processes and energy expenditure. Considerable effort has gone into understanding the link between  $\beta$ AR activation and increased protein accretion. Most evidence points to the  $\beta$ AR as mediating the growth response. Whereas initial reports indicated that skeletal muscle growth was not prevented by  $\beta$ AR antagonists (Reeds et al., 1988), subsequent reports showed that the  $\beta$ AR does mediate the growth response (Choo et al., 1992). For ractopamine, because the RR isomer accounts for the growth response and also is the most potent and efficacious ligand for the  $\beta$ AR, mediation by the  $\beta$ AR is indicated. The question of whether effects are direct or indirect on skeletal muscle remains unanswered, but direct effects seem likely. Unfortunately, in vitro studies have not provided the evidence for a direct link, adding to the uncertainty. Byrem et al. (1998) showed that infusion of cimaterol to the hind limb of cattle increased amino extraction across the limb, supporting the idea that direct effects could account for increased protein gain. In addition, there is little evidence for alteration of blood hormone concentrations that might contribute to increased growth (see recent reviews, see Mersmann 1998; Moody et al., 2000). Blood flow to skeletal muscle is increased at least transiently, which would increase the provision of nutrients and perhaps contribute to the growth response (Byrem et al., 1998; Mersmann, 1998).

The primary effect of  $\beta$ AR ligands is to cause fiber hypertrophy without a concomitant increase in DNA, indicating that protein synthesis, degradation, or both are affected. Both sides of the equation have been implicated as contributing to increased gain. Protein degradation is not typically measured directly, but rather as the difference from measured rates of synthesis and gain. If increased rates of synthesis are not observed

then protein degradation is assumed to be responsible (Reeds et al., 1986). Protein synthesis is increased in several species, but responses may be transient (Bergen et al., 1989; Maltin et al., 1989; Byrem et al., 1998). Increased mRNA abundance for myofibrillar proteins and calpastatin has been observed (see Moody et al., 2000), which would favor a role for both increased synthesis and reduced degradation. How might  $\beta$ AR mediate changes in protein synthesis and degradation? A direct link between cAMP and transcriptional regulation of the genes for myosin heavy chain in cardiomyocytes (Gupta et al., 1996) and bovine calpastatin (Cong et al., 1998) has been shown. It is not known whether either gene alone is rate-limiting for synthesis and degradation and would be sufficient to modify protein metabolism, but given the complexity of protein metabolism, involvement of additional regulatory signals seems likely. In addition to direct effects of cAMP,  $\beta$ AR have been shown to activate alternative signaling cascades (MAP kinase) in common with insulin (Luttrell et al., 1998). Insulin promotes protein synthesis and inhibits protein degradation and the signaling cascades have been linked to protein metabolism (Dennis et al., 1999). The potential involvement of these pathways in the  $\beta$ AR alteration of protein metabolism needs exploration.

### Implications

The recent approval by the FDA of ractopamine for use in pigs to enhance growth and feed efficiency should usher in expanded research to identify the pathways activated by beta-adrenergic receptor ( $\beta$ AR) ligands that are linked to protein metabolism and to identify strategies to take full advantage of the potential provided by this class of compounds. Receptor down-regulation may well limit the effectiveness of ractopamine, particularly in adipose tissue. Further work is needed to determine why  $\beta$ AR in skeletal muscle are not as susceptible to down-regulation as adipose tissue and whether this apparent difference is critical to the net response of ractopamine.

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