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The impact of growth promoters on muscle growth and the potential consequences for meat quality

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ABSTRACT

To meet the demands of increased global meat consumption, animal production systems will have to become more efficient, or at least maintain the current efficiency utilizing feed ingredients that are not also used for human consumption. Use of growth promoters is a potential option for increasing production animal feed efficiency and increased muscle growth. The objective of this manuscript is to describe the mechanisms by which the growth promoters, beta-adrenergic agonists and growth hormone, mediate their effects, with specific consideration of the aspects which have implications for meat quality.

1. Introduction

The predicted increase in world population to 9 billion by 2050 is expected to be associated with an increase in the urban population, with an estimated 70% being in an urban environment compared to 49% today and, hopefully, the inevitable increase in incomes (FAO, 2009). To meet this demand it is estimated that the food production will have to increase, particularly for commodities associated with higher incomes, such as meat, with the predicted demand more than doubling by 2050 (FAO, 2009).

To date, significant advances in animal genetics and animal nutrition have been made to meet the increasing demand. To achieve the maximum growth potential, high quality feed ingredients are required. Feed ingredients account for a large proportion of the overall costs of animal production, particularly in non-ruminant species (Patience, Rossoni-Serao, & Gutierrez, 2015). Continuing to rely on the same ingredients, in competition with human nutrition and biofuels, mean prices will inevitably increase. Therefore the cost of meat and animal products will also increase. It has been estimated that for many agricultural commodities the rate of production has already reached a peak (Seppelt, Manceur, Liu, Fenichel, & Klotz, 2014). Hence, if we are to continue to meet the demand for animal products, we cannot simply feed more animals the same feed ingredients, as that would require more crops, land and water (Foresight, 2011; Godfray et al., 2010). Therefore the aim of current research is to improve the efficiency with which animals utilize their feeds, giving more product for the same amount of feed or the same amount of product for less feed.

Through selective breeding and improved diet formulations over the last 20–30 years, feed efficiency of pigs (Patience et al., 2015) and chickens (Siegel, 2014) has improved, with Feed Conversion Ratio (FCR) values of 2.0 or less currently achievable (i.e. >50% efficiency). The UN suggests productivity is likely to be enhanced in the future through better animal disease control, improved irrigation and water management practises, and better fertilizers (FAO, 2009). In terms of animal production the increase in productivity could also be increased through the continued utilization of genetic selection through breeding programmes. Also it is probably inevitable that molecular biology technologies will be accepted and GMO organisms will be utilized for animal production. However throughout the world there is an increasing utilization of growth promoters. A goal of all these technologies is to increase the efficiency of feed utilization that ideally results in an increase in lean carcass weight. A predominant group of growth promoters are those which are based on endocrine factors such as anabolic steroids, growth hormone (GH, also called somatotropin, ST) and beta-adrenergic agonists (BA). These agents have metabolic modifying characteristics that result in enhanced growth. In addition there are a variety of other types of growth promoters, such as antibiotics, whose predominant affect is thought to be the increased efficiency of utilization of nutrients from the gut. Although the use of growth promoting agents is banned in the European Union (EU), they are legally used in many other countries.

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Increases in lean tissue deposition (increased muscle mass), decreases in fat deposition, along with repartitioning nutrients away from fat to muscle growth, are some of the predominant objectives of animal production systems focused on generating meat. Therefore these are key features of animal growth that growth promoters are designed to influence. The actions of hormonal based growth promoters' are through mechanisms that influence processes at a cellular level. In addition to affecting protein turnover to increase protein accretion, they also have effects on energy metabolism. This manuscript will examine the effects of hormonal based growth promoters on carcass parameters that influence meat quality, particularly growth hormone (GH) and beta-adrenergic agonists (BA), and will predominantly focus on their effects in pigs.

2. Growth hormone

Growth hormone is a peptide hormone produced by the anterior pituitary and is involved in the processes of development and growth that includes skeletal muscle, bone and adipose tissue (Beermann, 1994). Increased plasma GH has the effect of redirecting nutrients away from adipose tissue and toward muscle and bone (Etherton & Bauman, 1998). The effect of GH is to stimulate the production of insulin like growth factor (IGF-1) from the liver. These observations led to the hypothesis that GH effects, particularly on growth, were mediated by IGF-1 originating from the liver (Daughaday, 2000). However GH can stimulate IGF-1 expression in other tissues, particularly bone but also skeletal muscle (Brameld et al., 1996; Velloso, 2008: Wang, Bikle, & Chang, 2013). In the circulation IGF-1 is associated with insulin like growth factor binding proteins (IGFBP) which prolong its half-life (Boisclair, Rhoads, Ueki, Wang, & Ooi, 2001). The effect of IGF-1 can be mediated through dimers of the IGF-1 receptor as well as IGF-1 receptor/insulin receptor hybrids (Denley, Cosgrove, Booker, Wallace, & Forbes, 2005) and subsequently activates multiple signalling pathways. In muscle the predominant pathways are the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK/ERK pathway, which tends to be associated with proliferative growth, whilst the protein kinase B -mechanistic target of rapamycin - ribosomal protein S6 kinase (Akt–mTOR–S6K) pathway is predominantly responsible for protein synthesis but also affects protein degradation (Clemmons, 2009).

Growth hormone is approved for use in 14 countries, including Australia, for administration to pigs to improve growth characteristics (Dunshea, Cox, Borg, Sillence, & Harris, 2002). As the GH is a peptide it has to be administered by regular injection. Its action on pig growth has been well characterized, reducing feed intake whilst simultaneously increasing lean and reducing fat deposition (Etherton et al., 1987). Unsurprisingly, due to the signalling pathway activated by GH-IGF-1 axis, increased protein accretion in GH treated pigs is stimulated by protein synthesis rather than a decrease in protein degradation. However there are inconsistent reports in the literature with some reporting similar increases in protein synthesis and breakdown but overall net increased nitrogen balance (Tomas et al., 1992), whilst others have reported that protein synthesis is increased but protein degradation appears not to be affected (Bush et al., 2003) and some have described even a decrease in degradation (Vann et al., 2000). The effect of exogenous GH on adipose tissue is to reduce fat synthesis which results in reductions in back fat (Krick et al., 1992). Dunshea, D'Souza, Pethic, Harper, and Warner (2005) carried out a comprehensive review on the effects of GH on pig meat quality. Using a meta-analysis approach on published data, they concluded that GH decreased intramuscular fat by 12% and increased shear force by 9%. The effects on shear force are unlikely to be due to a large decrease in the proteolytic activity. Therefore the increased shear force is likely to be due to changes in temperature transfer to the meat, either during chilling the carcass, thereby affecting rigour development or effects of heat transfer during cooking which influences denaturing of proteins. Overall Dunshea et al. (2005) concluded that GH causes a small increase in shear force and the sensory perception of meat from treated pigs is tough, but it was unlikely that that this could be detected by consumers.

3. Beta-adrenergic agonists

The BAs are analogues of the endogenous catecholamines, adrenaline and noradrenaline. When administered to livestock species they have positive effects on growth and nutrient repartitioning. Beta-2 adrenergic receptor specific agonists, such as clenbuterol and cimaterol, have the greatest growth effect; however ractopamine, which binds to both beta-1 and -2 adrenergic receptors (Mills, Kissel, Bidwell, & Smith, 2003), also has similar growth effects. The BA generally have positive effects on weight gain, FCR, and act as strong repartitioning agents, increasing muscle growth whilst decreasing adipose tissue deposition (Meersman, 1998).

The BAs mediate their growth effects through the BA receptor. The subtypes of beta adrenergic receptors (BAR) vary depending on the tissue. For example, in pigs skeletal muscle has more BAR2 than BAR1 whilst in adipose tissue it is the reverse (Liang & Mills, 2002). BAR3 are thought to be present on porcine adipocytes (McNeel & Mersmann, 1995). The BAR activates the adenylate cyclase pathway which subsequently produces cyclic adenosine monophosphate (cAMP), which then activates protein kinase A. This kinase can alter enzyme activity through phosphorylation. For example the phosphorylation dependent cascade results in phosphorylase being activated and glycogen being degraded to glucose-1-phosphate. In addition, protein kinase A can activate the transcription factor, cAMP response element binding protein (CREB), which then regulates the transcription of genes that have a functional cAMP responsive element within their regulatory regions (Altarejos & Montminy, 2011). Therefore these agents have an immediate effect on enzyme activity, but can also alter transcription of a number of genes.

The first BA to be licenced for use as a feed additive was ractopamine for use in pigs in 1999. A significant advantage of these agents when compared to GH is that they can be administered in feed. The predominant effect of BA is to increase lean deposition whilst also decreasing fat deposition (Meersman, 1998). The BAs have strong effects on muscle hypertrophy and appear to be differentially effective across farm species, with ruminant species (cattle and sheep) responding the strongest, being particularly effective in older animals (Meersman, 1998). The early studies on the effects of BA in livestock indicated that these agents had a strong effect of decreasing protein degradation without increasing protein synthesis (Bohorov, Buttery, Correia, & Soar, 1987). However subsequent studies in pigs treated with ractopamine have indicated that protein synthesis, particularly of myofibrillar proteins, is stimulated (Adeola, Ball, & Young, 1992). The effects on fat deposition are not as clear as for GH, particularly in pigs, however it appears that ractopamine reduces backfat thickness in pigs, but this is not as dramatic as the effect of BA in ruminant species (Dunshea et al., 2005). After reviewing a large number of studies, Dunshea et al. (2005) concluded that the use of BA in ruminants increased the shear force, with cimaterol increasing shear force by 60%. Using a meta-analysis approach on published data they also described how, in pigs, the BAs ractopamine and salbuterol had no effect on IMF, whilst cimaterol caused a decrease. All these BA had the effect of increasing shear force in pigs, but again cimaterol had the greatest negative effect, but the effects were not as great as in ruminants. Unlike GH, the effect of BA on tenderness appears to be mediated through their strong inhibitory effects on protein degradation (see below), rather than effects on IMF.

4. Fibre type and meat quality

Skeletal muscle is made up of muscle fibres which have differing contractile and metabolic characteristics. Muscle fibres can be histochemically classified according to their actomyosin ATPase activity into three types, types I, IIA and IB, which have a contractile and

associated metabolic characteristics of slow twitch oxidative, fast twitch oxidative and fast twitch glycolytic, respectively (Brooke & Kaiser, 1970). When considered at a molecular level the differences in fibre type are, in part, determined by the myosin heavy chains (MyHC) they express, as this protein is a determinant of muscle contractile speed and thereby the associated metabolism required to support this. There are four MyHC isoforms (types I, IIa, IIx and IIb) which has led to a revision in the classification of fibre types as types I, IIA, IIX and IIB with a rank order of contraction speed of I < IIA < IIX < IIB (Schiaffino & Reggiani, 1996). The MyHCIIb isoform is expressed in rodents, but absent from most large animals such as cattle and sheep, although pigs do express this isoform (Lefaucheur, 2010). To support the contraction of these fibres there are associated metabolic characteristics. At one extreme, the slow twitch fibres that express high levels of MyHCI protein have a dependence on oxidative (aerobic) metabolism, as they can maintain a low power output. These fibres have a good blood supply and tend to have a low glycogen and high triglyceride content and fatigue slowly, so are used for sustained contraction. In contrast, the fast twitch type II fibres have a dependence on glycolytic (anaerobic) metabolism. These fibres have a relatively low blood supply and have a high glycogen content and low triglyceride content and fatigue relatively quickly so are used for rapid, intense contraction, which cannot be sustained for long periods. The type IIB fibres have the highest glycolytic metabolism capacity. The types IIA and IIX fibres have metabolic characteristics that, although still enable a high degree of glycolytic metabolism, increasingly have the capacity to carry out oxidative metabolism, hence they are classified as oxidative-glycolytic, particularly the type IIA fibres which have the greatest aerobic capacity (Schiaffino & Reggiani, 1996). For glycolytic fibres (types IIA, IIX and IIB) there is not a simple relationship between the expression of the fast MyHC protein isoforms (IIa, IIx and IIb) and the fibre type classification based on contractile and metabolic characteristics. For example in Large White pig longissimus muscle, fibres classified as type IIB included MyHC IIx expressing fibres, as well as hybrid fibres expressing both MyHC IIx and IIb (Lefaucheur, 2010). These hybrid fibres are a particular characteristic of the fast glycolytic fibres, which means using MyHC expression data to determine fibre type (as normally defined by contractile and metabolic characteristics), should be done with caution.

Based on the association between the metabolic characteristics of muscle and fibre type, it would be expected that IMF content would be lower in muscles that contain a high proportion of fast fibre types, particularly those containing a high proportion of type IIB fibres, for example in pigs. Recent work in pigs has indicated no clear relationship between proportions of types I and IIB fibres and IMF content (Kim et al., 2013), although the total number of fibres and density of type IIB fibres were positively related to the IMF content (Kim et al., 2013). Henckel, Oksbjerg, Erlandsen, Barton-Gade, and Bejerholm (1997) made a similar observation in pigs, that the frequency of type IIB fibres was positively correlated to IMF. However those who have reviewed this area have concluded that there does not appear to be a clear relationship between the quantity of intramuscular adipocytes and fibre type (Lefaucheur, 2010; Hocquette et al., 2010). In relation to the metabolic activity of type IIB fibres, these fast-twitch glycolytic fibres have a higher dependence on glycolytic metabolism, therefore they have higher glycogen contents. As a result, muscles that have a high proportion of these types of fibres tend to have an increased rate of postmortem pH decline, along with a lower ultimate pH, as well as decreased water holding capacity and a paler colour (Choe et al., 2008; Larzul et al., 1997; Ryu & Kim, 2005). The fast type fibres have greater cross sectional areas (CSA) than the slow fibres, with the type IIB fibres having the greatest CSA. However muscles containing fibres with a high CSA have been reported as having a lower meat quality in both cattle (Renand, Picard, Touraille, Berge, & Lepetit, 2001) and pigs (Karlsson et al., 1993; Kim et al., 2013). However the relationship between CSA per se and meat quality is not absolutely clear. Studies that have reported this relationship have often done so in animals that have variant genes or are subject to growth promoter treatments, which show a change in CSA, but this is associated with alterations in metabolic activity (Lefaucheur, 2010). For example, although callipyge lambs do have fibres with a large CSA, their muscles contain very high concentrations of calpastatin, which is thought to be a more significant factor in causing their extreme toughness (Duckett, Snowder, & Cockett, 2000).

5. Proteolytic systems and meat quality

The final tenderness of meat depends on the degree of alteration of the muscle structural and associated proteins postmortem (Hopkins & Thompson, 2002). Specific myofibrillar, myofibril cytoskeleton and costamere proteins are subjected to cleavage, with selected and restricted cleavage of the major myofibrillar proteins such as actin and myosin (Goll, Thompson, Taylor, & Christiansen, 1992; Taylor, Geesink, Thompson, Koohmaraie, & Goll, 1995a; Lametsch et al., 2003). There are several endogenous proteolytic systems present in muscle, which could participate in postmortem proteolysis, these include the cathepsin, proteasome, caspase and calpain systems (Kemp, Sensky, Bardsley, Buttery, & Parr, 2010). Generally it is considered that the cathepsin system does not play a role in meat tenderisation, as there is little association between cathepsin activity and the variation in the tenderness of meat (Whipple et al., 1990).

The proteasome is a multicatalytic protease complex involved in the regulation of a number of basic cellular pathways, by their degradation of proteins in the cytosol and nucleus (Coux, Tanaka, & Goldberg, 1996). Proteolysis by the proteasome is an ubiquitin-dependent process; polyubiquitinated proteins are subsequently recognized by the proteasome, which then degrades them (Taillandier, Combaret, Samuels, Bechet, & Attaix, 2004). This process is ATP-dependent requiring a number of enzymes to ubiquitinate target proteins. In muscle there are ubiquitin system ligases which appear to be critical to the proteasomes involvement in protein degradation. These are muscle RING finger 1 (MuRF1) and muscle atrophy F-box (MAFbx) (Bodine & Baehr, 2014.). These ligases are strongly associated with muscle atrophy conditions (Glass, 2005). Although in vivo the proteasome requires ATP for target proteins to be ubiquitinated, the proteasome can carry out proteolytic activity without requiring ATP or ubiquitin (Peters, Franke, & Kleinschmidt, 1994). Indeed proteasome activity is maintained during the postmortem conditioning period, with reported substantial activity still detectable at 7 days postmortem and at pH levels of less than 6 (Lamare, Taylor, Farout, Briand, & Briand, 2002). Taylor et al. (1995b) and Robert et al. (1999) found that the proteasome was capable of causing proteolysis of myofibril proteins including nebulin, myosin, actin and tropomyosin in bovine myofibrils. However, as emphasized by Koohmaraie and Geesink (2006), the degradation pattern of myofibrillar proteins in incubations with proteasome is not the same as that seen in postmortem muscle, although this does not appear to exclude the proteasome from making a contribution to the process of postmortem proteolysis (Houbak, Ertbjerg, & Therkildsen, 2008).

The caspase system is involved in the process of programmed cell death (apoptosis). The system consists of a number of enzymes whose substrates include cytoskeletal proteins which are degraded both in apoptosis and in postmortem muscle. It has been proposed that the protease family of caspases could be active postmortem and contribute to tenderization (Sentandreu, Coulis, & Ouali, 2002; Ouali et al., 2006; Kemp et al., 2010; Kemp & Parr, 2012). Of particular interest are the effector caspases, such as caspases 3 and 7, which are activated by upstream initiator caspases, such as caspase 9, and once activated target and cleave specific substrates, resulting in cell disassembly (Fuentes-Prior & Salvesen, 2004). There have been descriptions of caspase activity as well as cleavage of indicative caspase substrates which are associated with meat tenderization, including potential effects on the calpain inhibitor, calpastatin (Kemp & Parr, 2012). However others have reported no changes in caspase activity postmortem, suggesting a lack of involvement in tenderization (Underwood, Means, & Du, 2008), whilst others

have indicated associations between caspase activity and myofibril protein degradation as well as postmortem proteolysis (Huang, Huang, Xu, & Zhou, 2008; Huang, Huang, Zhou, Xu, & Xue, 2011). However whether this proteolytic system plays a major role in influencing tenderness is debatable. If it does have a role in the postmortem proteolysis which influences meat tenderness, it is likely to be contributory rather than causative.

Of all the proteolytic systems the calpain system is the one which has the strongest experimental evidence demonstrating a role in postmortem proteolysis (Kemp et al., 2010; Koohmaraie & Geesink, 2006; Sentandreu et al., 2002). In skeletal muscle, the calpain system consists of three proteases, ubiquitously expressed isoforms Calpain-1, Calpain-2, and p94 (or Calpain-3) and the calpain-specific endogenous inhibitor, calpastatin (Goll, Thompson, Li, Wei, & Cong, 2003; Wendt, Thompson, & Goll, 2004). Experimental evidence suggests Calpain-1 has the most significant role in postmortem proteolysis and meat tenderisation. Much of this evidence has been based on observations on the association between Calpain-1 activity, as well as expression, to tenderness (Koohmaraie & Geesink, 2006). The important role of Calpain-1 in the tenderisation process was further strengthened by observations made in calpain-1 knockout mice, which indicate a direct effect of Calpain-1 in postmortem proteolysis and thereby the development of tender meat (Geesink, Kuchay, Chishti, & Koohmaraie, 2006). A consistent observation of the calpain system's involvement in tenderness is that high levels of calpastatin are associated with poor quality meat; the model being that high levels of calpastatin reduce the activity of calpain (predominantly Calpain-1) thereby reducing the proteolysis required for tender meat (Kemp et al., 2010).

Changing animal nutrition has been shown to alter calpain expression and is associated with changes in meat quality. In pigs, a moderate reduction in both protein (14.95% vs 11.08%) and digestible energy (13.81 MJ vs 12.55 MJ) intake has been shown to attenuate expression of Calpain-1 mRNA and this was associated with decreased Warner-Bratzler shear force (Tang et al., 2010). However, much earlier studies in lambs found that a more severe reduction in feed intake over 6 weeks (so average daily live weight gain was 14% of controls), did not alter calpain or calpastatin activity (Higgins, Lasslett, Bardsley, & Buttery, 1988). Changes in the calpain system activity have also been described associated with increased feed efficiency. Pigs selected for low residual feed intake (RFI), where their observed feed intake is lower than expected (based on body weight gain and back fat depth), have reduced protein degradation compared to those with high RFI (Cruzen et al., 2013). Relative to high RFI pigs, low RFI animals had lower proteasome activity, greater calpastatin activity and decreased Calpain-1:calpastatin activity ratio. These changes were associated with slowed postmortem muscle proteolysis, indicated by decreased Troponin T degradation. This study is consistent in showing that a decrease in the proteolytic process and subsequent reduction in meat tenderness is predominantly associated with increased inhibition of calpains. In addition, this study suggests that if increased feed efficiency is achieved by a decrease in protein degradation, this could have a detrimental effect on meat quality.

Our studies on a random selection of commercially slaughtered pigs have shown that a high level of calpastatin (both activity and protein levels) in the first few hours after slaughter are associated with an increased incidence of toughness at 8 days postmortem (Sensky et al., 1998; Parr et al., 1999). The observations that Calpain-1 and calpastatin are important determinants for tender meat has lead to efforts to identify genetic markers that could be used to select for postmortem tenderisation of meat. Genetic tests for variation in both the Calpain-1 (CAPN1) and calpastatin (CAST) genes, which are associated with tenderness, have been identified for cattle (Page et al., 2004; White et al., 2005; Casas et al., 2006). In pigs, similar efforts have identified genetic markers for CAST which have been suggested to be markers of pork meat quality traits (Ciobanu et al., 2004; Nonneman et al., 2011), but as yet predictive variations in CAPN1 have not been reported.

6. Effects of BA and GH on fibre type, proteolytic systems and metabolism

The objective of administration of the growth promoters is to ideally increase feed efficiency but at the same time increase lean tissue deposition (increase muscle mass) and decrease fat deposition, thereby repartitioning nutrients away from fat to muscle growth. Given that post-natal muscle growth involves hypertrophy rather than hyperplasia it might be expected that stimulated growth would result in increased muscle fibre hypertrophy. Of the growth promoters BA and GH, the former has the strongest muscle growth effects. Both BA and GH have been shown to increase muscle fibre hypertrophy (Kim & Sainz, 1992), but to a more limited extent for GH (Solomon, Campbell, & Steele, 1990). In GH treated animals there appears to be no effect on fibre type distribution and this is particularly the case for pigs (Aalhus, Best, Costello, & Schaefer, 1997; Oksbjerg et al., 1995). However for animals treated with BA there are well documented effects on fibre type distributions. Early studies in lambs indicated that the BA cimaterol (10 ppm, oral dose, for 5 weeks) caused an increase in type II fibres compared to type I (Beermann et al., 1987). In pigs treated over a four week period with the BA, ractopamine (20 ppm, oral dose), there was a sustained induction of MyHCIIb in longissimus muscle (Gunawan, Richert, Schinckel, Grant, & Gerrard, 2007). This change in gene expression takes place early in the treatment, within one day for MyHCIIb. Likewise in pigs following seven days of treatment with ractopamine (10 ppm, oral dose), in longissimus muscle there was a trend for MyHCIIb mRNA to increase and for MyHCIIa mRNA to decrease (Brown et al., 2012) and this was before a significant increase in muscle weights was observed (Ryan et al., 2012). In the same study, pigs were also treated with GH for seven days (10 mg/pig, intramuscular injection every 2 days). There was a trend for decrease in MyHCIIb gene expression in muscles with GH, but no indication of changes in muscle weights (Brown et al., 2012). Similarly in lambs, following a six day treatment with cimaterol (10 ppm, oral dose), there was a decrease in MyHCIIa isoform gene expression and an increase in MyHCIIx/b, which consisted of an induction of MyHCIIb, a MyHC not normally expressed in sheep muscles (Hemmings, Daniel, Buttery, Parr, & Brameld, 2015). This switch to fast MyHC gene expression was accompanied by an increase in selective muscle weights and CSA (unpublished observations). It was interesting to note that although MyHC gene expression was altered after six days, the quantity of MyHC protein did not change (Hemmings et al., 2015). However there was a decrease in isocitrate dehydrogenase activity which suggested that there was a change to a less oxidative metabolism, before there was a change in the contractile proteins associated with a fast-glycolytic fibre type (Hemmings et al., 2015).

The net deposition of protein often results in an increase in protein synthesis, however alterations in protein degradation can also take place. Growth hormone stimulated increases in IGF-1 potentially mediates its effects through MEK/ERK and Akt–mTOR–S6K pathways. Although IGF-1 stimulated protein synthesis can be influenced by both pathways, the effect of IGF-1 on protein degradation is mediated by the latter. The Akt–mTOR–S6K pathway also controls protein degradation in skeletal muscle by AKT phosphorylating the forkhead box protein (FOXO) transcription factors, thereby reducing the gene expression of the ubquitin ligases MAFbx and MuRF1 (Glass, 2005). These two ubiquitin ligases are important in muscle protein turnover, as they target myofibrillar proteins for degradation through the ubiquitin-proteasome system (Bodine & Baehr, 2014).

For BA, early reports on the mechanism of its action described how clenbuterol treatment for five weeks (10 ppm, oral dose) significantly reduced muscle protein degradation in lambs (Bohorov et al., 1987), although subsequent studies have shown BA also stimulate protein synthesis, for example in pigs given ractopamine for four weeks (20 ppm, oral dose) (Adeola et al., 1992). The characteristic effect of orally administered BA such as clenbuterol or cimaterol on protein degradation is an

increase in the expression and activity of calpastatin (Higgins et al., 1988; Parr, Bardsley, Gilmour, & Buttery, 1992). This observation has been confirmed by others, particularly in ruminants (Kretchmar, Hathaway, Epley, & Dayton, 1990; Wheeler & Koohmaraie, 1992), but also in pigs (Parr, Sensky, Bardsley, & Buttery, 2001). In a more recent study, Douillard et al. (2012) described how over expression of exogenous calpastatin in mouse tibialis anterior attenuated the muscle hypertrophy and the shift to a fast muscle fibre type, stimulated by clenbuterol treatment for 21 days (subcutaneous injection, 1 mg/kg body weight/day). This was in spite of AKT and associated factors being phosphorylated, an event which is normally associated with increased muscle synthesis. The authors suggested that inhibition of clenbuterol stimulated hypertrophy by calpastatin over-expression did not involve inhibition of protein synthesis. They also suggested that the exogenous calpastatin expression inhibited calpain's role in remodelling the muscle myofibrillar proteins. Care must be taken in accepting this interpretation, as Douillard et al. (2012) described how in control mice treated with only clenbuterol for 21 days (subcutaneous injection, 1 mg/kg body weight/day) there was decreased calpastatin expression. This contradicts previous studies in rodents which have shown BA increases calpastatin in mice treated with formoterol for 28 days (intraperitoneal injection, 100 μg/kg body weight/day) (Koopman et al., 2010) and rats treated with clenbuterol for 3 days (intraperitoneal injection, 3 mg/kg body weight/day) (Goncalves et al., 2012). Overall the majority of studies demonstrate treatment with BA increases muscle calpastatin. The effect of BA on normal muscle is to enhance protein synthesis and reduce calcium-dependent proteolysis, which appears to be mediated through an increase in calpastatin. However in mice with denervated muscle, which induces atrophy, treatment with clenbuterol for 3 days (intraperitoneal injection, 3 mg/kg body weight/day) stimulated protein synthesis and inhibited the proteolysis mediated by both the proteasome and lysosome, reduced expression of the ubiquitin ligases, MAFbx and MuRF1, as well as cathepsin L, without a significant effect on calcium dependent proteolysis (Goncalves et al., 2012). This suggests that the calpain system mediated proteolysis is associated more with normal muscle protein turnover, rather than that associated with atrophy.

Our recent studies have sought to examine the effects of GH or BA treatment on muscle proteolytic systems. In pigs treated with either ractopamine (20 ppm, oral dose) or GH (10 mg/pig, intramuscular injection every 2 days) for 27 days there was a significant effect of this BA on muscle weights and this was associated with a switch to increased expression of the MyHC isoforms associated with fast fibre types, whereas there was no effect of GH (Brameld et al., 2015). In terms of effects on meat quality, there was an indication of an increase in shear force in BA treated animals ($P = 0.107$). There was a trend ($P < 0.1$) for increased calpastatin protein (detected by western blot) in BA treated animals, but there was no effect on caspase 3/7 activity (when assessing activity these two caspases are indistinguishable) (Mareko, Ryan, Brown, Brameld, & Parr, 2013a). When proteasome activity was assessed there was a change in the activity of its subunits; these have trypsin-like, caspase-like and chymotrypsin-like activity. When these activities were assessed individually, the caspase-like activity was significantly reduced in both BA and GH treated pigs, and the chymotrypsin-like activity was increased in BA treated animals only (Mareko, Ryan, Brown, Brameld, & Parr, 2013b). Overall these data would suggest that the effect of the BA ractopamine on muscle proteolysis is not as great as some of the other BAs used in earlier studies, particularly cimaterol and clenbuterol.

In addition to examining changes in proteolytic activity in pigs treated with the GH (10 mg/pig, intramuscular injection every 2 days) and ractopamine (20 ppm, oral dose) for 27 days, we also examined the effect of these growth promoters on gene expression in the Longissimus muscle at days 1, 3, 7, 13 and 27 of treatment. The changes in the transcriptome were measured using a pig transcriptome microarray. Although our previous studies had indicated stimulatory effects of BAs on the gene expression of the calpain system in pigs, particularly increases in calpastatin gene expression (Parr et al., 2001), we found no consistent significant effects on gene expression of any major proteolytic system in muscle across the time course. However as might have been expected, the greatest gene expression responses were seen with ractopamine treatment. There was an increase in the expression of genes encoding glycolysis enzymes but a decreased expression of genes associated with oxidative respiration. However the most predominant effect was a co-ordinate increase in serine synthesis pathway gene expression, with increased expression of phosphoglycerate dehydrogenase (PHGDH), phosphoserine-aminotransferase (PSAT) and phosphoserine phosphatase (PSPH). This was confirmed at the protein level, as PHGDH was significantly increased with BA (Brameld et al., 2015). As had been observed in the past, BA had a stronger muscle hypertrophic effect than GH. The effects of ractopamine on proteolytic systems do not appear to be as potent as previous described for the standard beta-2 agonists, such as cimaterol and clenbuterol. However, ractopamine does appear to be mediating its effect on muscle in pigs through biosynthetic pathways.

7. Conclusion

The consumption of meat as a protein source is predicted to increase dramatically in the next few decades. This requirement along with the increasing pressure of feed availability will demand that animal production systems are efficient. To achieve this it is very likely that the use of growth promoters will increase, as well as the possible use of genetically modified organisms. The current licenced growth promoters, which have direct effects on muscle growth as well as fat deposition, were discovered over 30 years ago. Although their effects on meat quality tend to be negative, they do not appear, at least in pigs, to have such drastic effects which cause a significant number of consumers to perceive poor meat quality. However it is likely there will be an increased incentive to develop new types of growth promoters. Fast glycolytic fibres appear to be the most receptive to growth stimuli whilst slow aerobic fibres appear to be relatively resistant to anabolic stimuli. One of the major limitations of muscle growth is that fibre number is essentially fixed at birth for most species, which means post-natal anabolic agents mediate their effects via hypertrophy. One of the consequences of hypertrophy is oxygen becomes less available, therefore glycolytic dependent metabolism becomes more favourable. Care has to be taken to ensure new types of growth promoters do not target mechanisms which result in increased CSA of fast glycolytic fibres and significant decreases in protein degradation (proteolytic systems), as there is then likely to be a significant decline in meat quality.

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