DIFFERENTIAL REGULATION OF CALPASTATIN PROMOTERS BY AGENTS THAT AFFECT PORK TENDERNESS

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Keywords: pork, calpastatin, cells, regulation, growth

Introduction

Variation in meat tenderness is a major factor affecting consumer choice in the UK. Despite efforts to control nutrition and breeding, and the stress associated with handling and slaughter processes, the tenderness of pork is not always of consistent quality. An important biochemical factor during conditioning is undoubtedly the calpain-specific inhibitor calpastatin. Variable quantities of calpastatin at slaughter are known to suppress calpain-mediated postmortem proteolysis to different degrees and contribute to the variation in ultimate tenderness. In support of this, high levels of calpastatin are induced by β-agonist feeding over several weeks in several livestock species and are clearly associated with increased toughness (Koohmaraie, 1996). The biochemical mechanism underlying the β-agonist-induced increase in calpastatin expression remains unproven, although cAMP responsive elements (CRE) have been identified in bovine and porcine calpastatin gene promoters that suggest β-adrenergic signalling by cAMP-mediated protein kinases and phosphorylation of CRE binding protein (CREB) (Cong *et al.*, 1998; Parr *et al.*, 2001). Adrenergic pathways are also activated in the short term by physiological stress, which is well known to affect tenderness and it has been shown that relatively short term adrenaline administration can affect calpastatin expression (Sensky *et al.*, 1996). There are at least three different promoter regions in the porcine calpastatin gene (1xa, 1xb and 1u, Parr *et al.*, 2004) that, via differential promoter usage and alternative splicing, give rise to three isoforms of calpastatin (Types I, II and III) that vary in their N terminal sequences. These promoter regions contain a number of transcription factor motifs that could affect the expression of calpastatin under different environmental conditions. Specifically, there are elements in all the promoter regions that suggest susceptibility to stimuli mediated by β-agonists, Ca^{2+} or calcineurin (Figure 1). In this study, the responsiveness of different porcine calpastatin promoter constructs to stimuli that simulate these pathways is investigated *in vitro* using a non-differentiating skeletal muscle cell line.

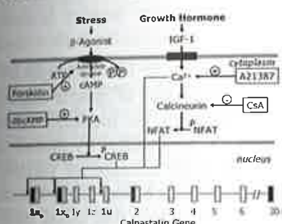

Figure 1: Potential pathways involved in the regulation of calpastatin expression and potential manipulation by different agents.

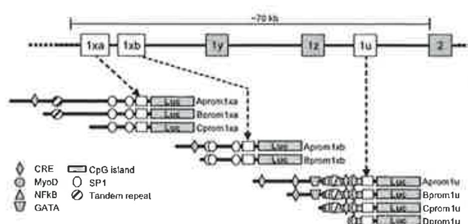

Figure 2: Promoter constructs generated to 1xa, 1xb and 1u, illustrating the sequential removal of different potential regulatory motifs.

Materials and Methods

A series of putative promoter constructs were generated by PCR in which potential transcriptional motifs were systematically removed (Parr *et al.*, 2004, Figure 2). Amplicons were ligated into the pGL3 Basic vector linked to luciferase as a reporter gene (Promega, UK) and used to transfect L6G8 myoblasts derived from rat skeletal muscle (Parr *et al.*, 2004). Cells were transfected using the GeneJuice Transfection Reagent (Novagen, UK) once they had reached 50 - 70% confluence and, immediately after transfection, cells were treated with either 2 mM dibutyryl cAMP (dbcAMP), 10 μM forskolin (adenylate cyclase activator), 1 μM A23187 (Ca^{2+} ionophore) or 10 μM cyclosporin A (CsA, calcineurin inhibitor). Firefly and *Renilla* Luciferase activities were determined using the Dual-Glo Luciferase Assay System (Promega, UK) with *Renilla* luciferase activity used to correct for the transfection efficiency. Data are expressed as the change in transfection efficiency relative to the untreated constructs and significance of treatments was determined by Student's unpaired t-test.

Results and Discussion

All promoter constructs except for Dprom1u exhibited full functionality as previously reported (Parr *et al.*, 2004). Activity of the 1xa promoter series was reduced by dbcAMP and forskolin to differing degrees depending on the degree of truncation of the construct (Table 1). A23187 had the opposite effect, increasing the activity of the 1xa promoter constructs, whilst CsA had no effect. The only treatment to affect expression of the 1xb promoter activity was A23187

which increased the activity of the Bprom1xb construct. Treatment of the 1u constructs with the different reagents generally produced the opposite effect to that seen with the 1xa promoters, with dbcAMP and forskolin having positive effects on promoter activity and A23187 suppressing activity.

Table 1: Change in percentage expression of different porcine calpastatin promoter constructs following treatment with different agents. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

	2 mM dbcAMP	10 μM forskolin	1 μM A23187	10 μM CsA
Aprom1xa	-20.2%**	-12.9%*	+8.1%	-8.7%
Bprom1xa	-12.8%	-5.4%	+11.5%*	+5.0%
Cprom1xa	-5.2%	-1.8%	+8.7%**	-1.1%
Aprom1xb	-2.7%	-5.5%	+3.7%	-4.3%
Bprom1xb	-0.6%	-4.9%	+17.7%**	+7.6%
Aprom1u	+22.8%***	+0.1%	-8.4%	+5.2%
Bprom1u	+18.2%	+19.9%*	-31.2%**	-8.2%
Cprom1u	+15.5%	+11.6%*	-42.1%**	+1.8%
Dprom1u	-6.9%	-3.1%	+2.9%	-9.0%

The results support whole animal studies indicating that all three promoters are functional and that different mRNA transcripts and protein isoforms of calpastatin are present in different muscle types and respond to β-adrenergic stimulation (Parr *et al.*, 2004). In porcine longissimus the abundance of Type I and III mRNA transcripts is comparable, while Type II is barely detectable. However, at the protein level the Type III isoform mainly derived from the 1u promoter completely dominates in porcine longissimus. The effects seen on the 1u promoter series support the known link between β-adrenergic stimulatory pathways, including β-agonist feeding and physiological stress, and calpastatin in a way that could account for the increased toughness in treated animals. The opposite effect on the 1xa promoter series is unexpected, given that CRE motifs are also found in this promoter, and this warrants further attention. Growth hormone (pST) treatment in pigs has been reported to reduce calpastatin mRNA expression in longissimus (Ji *et al.* 1998) and is believed to act by increasing IGF-1 thereby increasing Ca^{2+} concentration in muscle. A23187, which raises intracellular Ca^{2+}, had strong effects on transcriptional efficiencies with a differential effect on 1xa and 1u promoters. Ca^{2+}-dependent effects on gene expression can act by both calcineurin-dependent and independent signaling pathways, but the lack of an effect of CsA on any promoter construct suggests that calcineurin-mediated signaling can be discounted.

Conclusions

The close relationship between elevated calpastatin expression and increased toughness in livestock species indicate that calpastatin plays a critical role in determining the ultimate tenderness of meat. Reduction of muscle calpastatin pre-slaughter by selective breeding or other interventions would provide a means of reducing the incidence of toughness. This study demonstrates that the expression of calpastatin in a model system can be manipulated by factors associated with physiological stress or changes in intracellular calcium in whole animals that might account for variable rates of tenderisation. The most striking and novel result is the large differential effect of Ca^{2+} which may suggest strategies for controlling the activities of the 1xa and 1u promoters of the calpastatin gene pre-slaughter that could have a major impact on meat quality.

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