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Recombinant canine single chain insulin analogues: Insulin receptor binding capacity and ability to stimulate glucose uptake

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ABSTRACT

Virtually all diabetic dogs require exogenous insulin therapy to control their hyperglycaemia. In the UK, the only licensed insulin product currently available is a purified porcine insulin preparation. Recombinant insulin is somewhat problematic in terms of its manufacture, since the gene product (preproinsulin) undergoes substantial post-translational modification in pancreatic β cells before it becomes biologically active. The aim of the present study was to develop recombinant canine single chain insulin (SCI) analogues that could be produced in a prokaryotic expression system and which would require minimal processing. Three recombinant SCI constructs were developed in a prokaryotic expression vector, by replacing the insulin C-peptide sequence with one encoding a synthetic peptide (GGGPGKR), or with one of two insulin-like growth factor (IGF)-2 C-peptide coding sequences (human: SRVSRRSR; canine: SRVTRRSSR). Recombinant proteins were expressed in the periplasmic fraction of *Escherichia coli* and assessed for their ability to bind to the insulin and IGF-1 receptors, and to stimulate glucose uptake in 3T3-L1 adipocytes.

All three recombinant SCI analogues demonstrated preferential binding to the insulin receptor compared to the IGF-1 receptor, with increased binding compared to recombinant canine proinsulin. The recombinant SCI analogues stimulated glucose uptake in 3T3-L1 adipocytes compared to negligible uptake using recombinant canine proinsulin, with the canine insulin/cIGF-2 chimaeric SCI analogue demonstrating the greatest effect. Thus, biologically-active recombinant canine SCI analogues can be produced relatively easily in bacteria, which could potentially be used for treatment of diabetic dogs.

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Introduction

Diabetes mellitus in dogs is characterised by hyperglycaemia caused by an absolute or relative deficiency in the pancreatic β cell hormone insulin (Catchpole et al., 2008). Virtually all diabetic dogs require administration of exogenous insulin to control their blood glucose concentration. Whereas recombinant insulin is used to treat human diabetic patients, in the UK currently only one licensed insulin product is available for treatment of diabetes mellitus in companion animals, consisting of purified porcine insulin (Caninsulin, MSD Animal Health)[1]. Recombinant human insulin has been used for many years in North America for treatment of canine diabetes mellitus, where until recently there was no FDA-approved insulin for dogs and cats (Rucinsky et al., 2010).

Production of purified beef and pork insulin has been scaled down, with the introduction of recombinant techniques for production of human insulin. Since the supply of bovine and porcine insulin for veterinary use generally relies on human market availability, insulin for diabetic dogs is likely to become increasingly limited. In recent years, bovine insulin products (formerly Insuvet Soluble, Lente and Protamine Zinc Insulin, Zoetis) have been withdrawn from the veterinary market. Thus, there is an anticipated need for development of recombinant canine insulin preparations.

Biologically active insulin is synthesised in pancreatic β cells by extensive post-translational modification of preproinsulin. After folding and disulphide bond formation between insulin A and B chains, cleavage of the connecting C-peptide is required for biological activity (Fig. 1A). Proinsulin demonstrates a somewhat modest 1–2% affinity for binding to the insulin receptor (INSR) compared to insulin and it is thought that there are two main reasons for this (Peavy et al., 1985); insulin C-peptide does not seem to allow enough molecular flexibility to facilitate interaction with INSR binding sites and it interferes with important receptor-binding residues, such as

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[1] See: http://www.vmd.defra.gov.uk/productinformationdatabase/SPC_Documents/SPC_124274.doc (accessed 24 September 2014).

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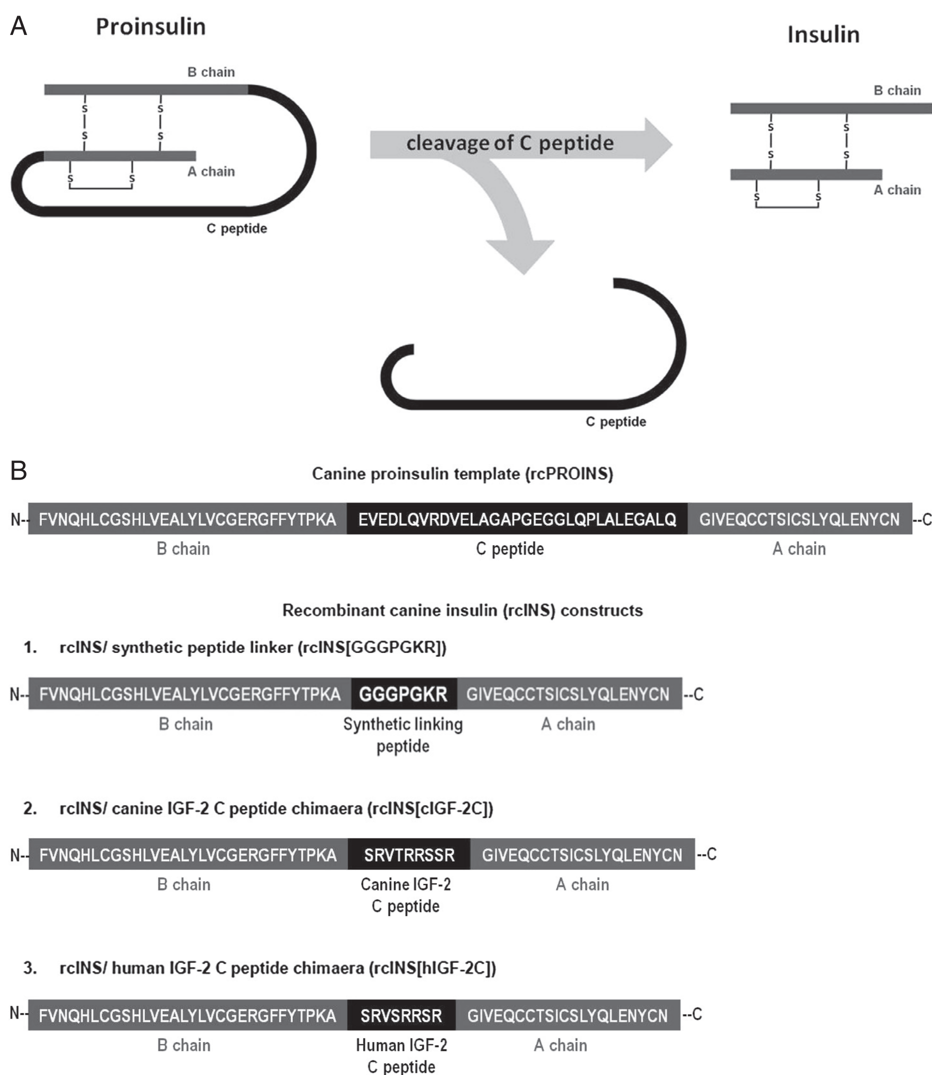

Fig. 1. (A) Post-translational modification of proinsulin to yield the biologically-active insulin and C-peptide. (B) Details of single chain insulin analogues adapted from recombinant canine proinsulin (rcPROINS) for the present study. Three different recombinant canine insulin analogues (rcINS) were generated, where the insulin C-peptide was substituted with different linking peptide sequences.

glycine at position A1. This presents a challenge for commercial production of recombinant insulin, since most methods are based on use of prokaryotic expression systems, with bacteria and yeast lacking the necessary cellular machinery and enzymes required for correct folding and processing of proinsulin to insulin.

The first recombinant insulin to become commercially available (Humulin, Eli Lilly) was based on a process whereby insulin A and B chains were produced separately in bacteria, then combined to form the biologically active molecule (Riggs and Itakura, 1979). However this process is somewhat inefficient and, subsequently, different techniques have been employed for commercial production of recombinant human insulin, which usually involves synthesis of a precursor molecule that is subjected to chemical and/or enzymatic modification (Christensen et al., 1991).

An alternative approach to synthesis of recombinant insulin is to produce single chain insulin (SCI) analogues, which do not require post-translational modification to exert their biological activity (Kristensen et al., 1995). In SCI analogues, the proinsulin C-peptide is substituted with alternative linking peptide sequences that allow folding and disulphide bond formation between A and B chains, but which do not require cleavage and interfere with binding to the INSR much less than the native C-peptide. One such construct, developed for gene therapy of diabetes mellitus, involved substituting the insulin C-peptide with a synthetic peptide linker (Lee et al., 2000).

There are other members of the insulin superfamily, with the most important being the insulin-like growth factors, IGF-1 and IGF-2 (Chan and Steiner, 2000). Unlike insulin, IGF-1 and IGF-2 do not require cleavage of their C-peptide to bind to their cognate receptors. IGF-2 has been implicated in the syndrome of non-islet cell tumour hypoglycaemia, which involves production of an IGF-2 related peptide by tumour cells that acts on insulin receptors to cause hypoglycaemia (Boari et al., 1995; LeRoith, 2004; Zini et al., 2007). Thus, insulin and IGF-2 both have glucose-lowering properties that might be exploited for developing a novel therapeutic agent for canine diabetes mellitus.

The aim of the present study was to develop canine SCI analogues, whereby the insulin C-peptide was substituted with either a synthetic peptide linker or the human or canine IGF-2 C-peptide sequences, to express these recombinant SCI analogues in bacteria and to assess their ability to bind to the INSR and stimulate glucose uptake in cultured cells. The hypothesis was that such SCI analogues would be biologically active, without any requirement for post-translational modification.

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