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Rapid communication

Characterization of a long-acting recombinant human serum albumin-atrial natriuretic factor (ANF) expressed in *Pichia pastoris*

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ABSTRACT

The cardiac hormone atrial natriuretic factor (ANF) combines pharmacological properties of drugs used to treat essential hypertension (EH), congestive heart failure (CHF) and acute myocardial infarction (AMI). Treatment of CHF or AMI patients with an intravenous (*iv*) infusion of the circulating form of ANF ($ANF_{99–126}$) produces significant clinical improvement. The short half-life (5 min) and peptide nature of ANF impose logistic restrictions for chronic administration. To increase its half-life, we fused ANF and human serum albumin (HSA) mini-genes by recombination in *Pichia pastoris*. The activity of three configurations of the fusion protein was tested *in vitro* and *in vivo*. The fusion protein that comprised of C-terminus HSA connected to N-terminus ANF via a hexaglycine linker showed the best outcome; it increased cGMP production *in vitro*. *In vivo* an *iv* bolus of HSA-ANF into mice increased significantly plasma cGMP levels and lowered blood pressure (BP) for up to 6 h hence successfully extended ANF half-life in plasma while retaining its biological activity. HSA-ANF represents the basis for development in the chronic therapeutic use of ANF.

1. Introduction

The cardiac polypeptide hormone ANF participates in the modulation of renal function, vascular tone, sympathetic activity and the renin-angiotensin-aldosterone system thus affecting extracellular fluid volume, blood pressure, renal sodium excretion and cardiac pre- and afterload. ANF has a demonstrated compensatory role in EH, CHF, and following an AMI [1] suggesting it might have therapeutic applications in these clinical entities. Indeed, Carperitide, a recombinant form of the circulating human ANF peptide ($ANF_{99–126}$), has been used clinically for the treatment of acute AMI and CHF. Two multicenter trials: the PROTECT [2] and the COMPASS [3] studies confirmed the expected clinical benefits of an ANF infusion in acute heart failure.

Due to its polypeptide nature, $ANF_{99–126}$ has a short half-life in the circulation (5 min) and therefore, necessitates prolonged *iv* infusions to obtain therapeutic effects. These characteristics impose logistic restrictions for chronic clinical applications.

In order to enhance the half-life of peptides of clinical interest such as insulin, glucagon-like peptide-1, brain natriuretic peptide, granulocyte colony-stimulating factor and interferon α, the peptide of interest has been fused to human serum albumin (HSA), which has a circulating half-life in humans of about 20 days. These and other fusion proteins, now in pre-clinical or clinical development, show similar biological effects to the unfused peptide but a slower clearance rate from the circulation owing to the HSA moiety (reviewed in [4]).

Here, we report on the development and the biochemical and biological characterization of an HSA-ANF fusion protein that possesses a considerably longer half-life than native $ANF_{99–126}$ as judged by its ability to promote a significant increase in cGMP production and lower blood pressure in mice.

2. Materials and methods

2.1. Construction of recombinant ANF-HSA-expressing plasmids and cell lines

The HSA cDNA was generated by polymerase chain reaction (PCR), using the pC3-HFUS [5] construct as the template, sense oligonucleotide primer 5′-GATCCGGTAC CACAAGAGTG AGGTTGCTCA GCG-3′, antisense primer 5′-GCATGCGGCC GCTAATGGTG ATGGTGATGG TGATGTAAAG CCTAAGGCAG CTGACTTG-3′, and high fidelity *Pfu* polymerase. The resulting 1794 bp amplification product was restricted at the unique

Abbreviations: ANF, atrial natriuretic factor; EH, essential hypertension; CHF, congestive heart failure; AMI, acute myocardial infarction; BP, blood pressure; HSA, human serum albumin; cGMP, cyclic guanosine mono-phosphatase; *iv*, intravenous; HSA-ANF, human serum albumin fused to atrial natriuretic factor; IBMX, 3-isobutyl-1-methylxanthine; RP-HPLC, reverse phase- high pressure liquid chromatography; *P. pastoris*, *Pichia pastoris*.

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KpnI and NotI sites that were introduced by PCR, and ligated between the corresponding sites of pUC19 (Invitrogen, Burlington, ON, Canada) to form pUC-HSAH$_6$. Codons corresponding to residues 99–126 of preproANF, ANF$_{99-126}$, were obtained by gene synthesis, using four synthetic oligonucleotides: ANF-A, 5′-TCGAGAAAG AAGCCTGCGG AGATCCAGCT GCTTCGGGGG CAGGAT-3′; ANF-B, 5′-GGACAGGATT GGAGCCAGA GCGGACTGGG CTGTAACAGC TTCCGGTAC-3′; ANF-C, 5′-GTCCATCCG CCCCCAAGC AGCTGGATCT CCGCAGGCTT CTTTTC-3′; and ANF-D, 5′-CATGTACC GGAGCTGTT ACAGCCCAGT CCGCTCTGGG CTCCAATCCT-3′. ANF-B and ANF-C were phosphorylated using T4 polynucleotide kinase. Next, ANF-A and phosphoANF-C, and phosphoANF-B and ANF-D, respectively, were separately annealed. The resulting 96 bp XhoI–KpnI-compatible DNA fragment was gel-purified and inserted between the corresponding sites of pCDNA3.1, forming pC3-ANF. Following confirmatory DNA sequencing to ensure fidelity and, where appropriate, the avoidance of subclones containing PCR errors, the 96 bp XhoI–KpnI fragment of pC3-ANF, the 1774 bp fragment of pUC-HSAH$_6$, and the 4541 bp fragment of pPICZ9ssamp were joined in a three-part ligation to form pPZ9-ANF-HSA. This plasmid encoded prepro-α-ANF-HSA, comprising: the 80 residue yeast prepro-α factor cleavable secretory sequence; ANF$_{99-126}$; HSA$_{3-584}$; and six His residues. Following transformation of *P. pastoris* strain X-33 to Zeocin resistance, candidate expresser colonies were isolated and checked for ANF-HSA secretion using small-scale cultures. On induction of the construct's alcohol oxidase promoter with methanol, the ANF-HSA product was the single most abundant secreted product of these cells. The highest expressers were expanded as the production and back-up cell lines. Using similar methodology a prepro-α-H6HSA-G6-ANF$_{99-126}$ construct was also used.

2.2. Recombinant ANF purification and characterization

The recombinant ANF-HSA was purified from media conditioned by *P. pastoris* cells permanently transformed with pPZ9-ANF-HSA and induced with methanol as per previously published protocols using nickel-chelate affinity chromatography [6]. Briefly, the media was neutralized to pH: 8, precipitates removed, and the clarified media concentrated by ultrafiltration. The concentrated media was passed over Ni-NTA-agarose, and specifically bound proteins eluted with an imidazole gradient of 10–200 mM. Appropriately enriched fractions were identified by SDS-polyacrylamide gel electrophoresis, pooled, concentrated to > 1 mg/ml total protein concentration, aliquoted and frozen. The fusion protein was further purified by RP-HPLC using a semi-preparative Vydac C_{18} column eluted with a linear gradient of 15–40% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 3 mL/min [7,8]. The samples of interest were pooled and freeze dried. N-terminal amino acid sequencing and mass spectrometry (MS) was used to determine integrity and primary structure.

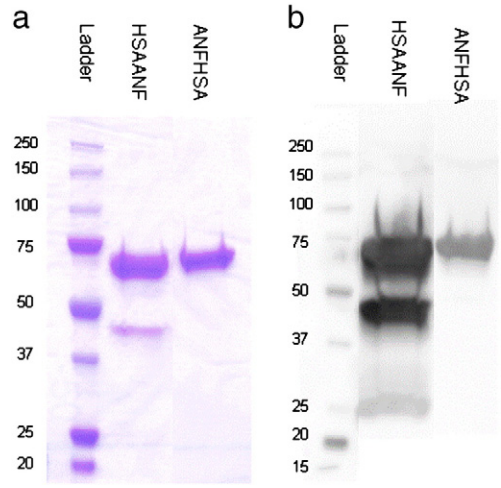

Fig. 1. a) This panel shows a Coomassie-stained SDS gel from a clone secreting either HSA-ANF or ANF-HSA sampled at 96 h. The fusion proteins (2.5 μg) were recognized by Western blotting with an anti-histidine antibody in panel b). Molecular weight markers, (Ladder). Both chimeric proteins, ANF-HSA and HSA-ANF co-migrate with the ~75 KD marker.

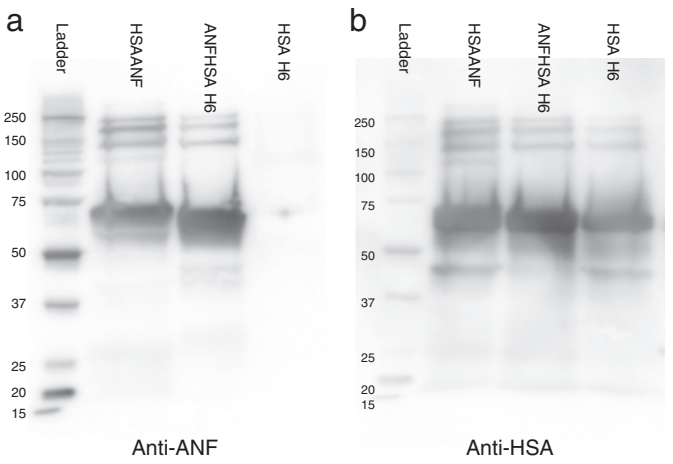

Fig. 2. a) Western blotting using 2.5 μg of protein probed with anti-ANF and b) anti-HSA antibodies.

2.3. In vivo biological activity

All animal experimentation was carried out in accordance with the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals. The protocol was approved by the University of Ottawa Animal Care Committee. Animals, CD1 mice, received an *iv* bolus via tail vein of HSA-ANF (430.8; 324.8 and 212.8 mg/kg),

MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGY SDLEGDFDV

AVLPFSNSTNNGLLFINTTIASIAAKEEGVSLEKR''SLRRSSCFGGRMDRI

GAQSGLGCNSFRY''HKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPFEDH

VKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMAD

CCAKQEPERNECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYL

YEIARRHPYFYAPELLFFAKRYKAAFTECCQAADKAACLLPKLDELRDEG

KASSAKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAEVSKLVTDLT

KVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHC

IAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHP

DYSVVLLLRLAKTYKTTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIK

QNCELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCK

HPEAKRMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFS

ALEVDETYVPKEFNAETFTFHADICTLSEKERQIKKQTALVELVKHKPKA

TKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQAALGLHHHH

HH

Fig. 3. Alignment of the chimeric protein. The alignment starts with the cleavable yeast pre-pro factor sequence (80 aa) then ANF (between double asterisks) followed by the His tag.