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Novel natriuretic peptides from the venom of the inland taipan (*Oxyuranus microlepidotus*): isolation, chemical and biological characterisation☆

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Abstract

Three natriuretic-like peptides (TNP-a, TNP-b, and TNP-c) were isolated from the venom of *Oxyuranus microlepidotus* (inland taipan) and were also present in the venoms of *Oxyuranus scutellatus canni* (New Guinea taipan) and *Oxyuranus scutellatus scutellatus* (coastal taipan). They were isolated by HPLC, characterised by mass spectrometry and Edman analysis, and consist of 35–39 amino acid residues. These molecules differ from ANP/BNP through replacement of invariant residues within the 17-membered ring structure and by inclusion of proline residues in the C-terminal tail. TNP-c was equipotent to ANP in specific GC-A assays or aortic ring assays whereas TNP-a and TNP-b were either inactive (GC-A over-expressing cells and endothelium-denuded aortic rings) or weakly active (endothelium-intact aortic rings). TNP-a and TNP-b were also unable to competitively inhibit the binding of TNP-c in endothelium-denuded aortae (GC-A) or endothelium-intact aortae (NPR-C). Thus, these naturally occurring isoforms provide a new platform for further investigation of structure–function relationships of natriuretic peptides.
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Venoms are proving to be a remarkable source of novel peptides that have potential applications in agriculture and human health. Moreover, many peptides are invaluable tools to study biochemical and physiological processes. Toxins that target ion channels and receptors have been isolated from spiders, marine snails, snakes, scorpions, and a range of other animals [1,2]. The Australian inland taipan (*Oxyuranus microlepidotus*) is regarded as the most venomous snake in the world [3]. Despite this reputation, little is known about the composition of its venom. Whole venom from *O. microlepidotus* has previously been shown to display potent neurotoxicity in neuromuscular preparations [4,5], produce relaxation in rat pre-contracted aortae [4] and a fall in blood pressure in anaesthetised rats when given intravenously [4]. Further studies on the venom found this activity to be unaffected by a nitric oxide scavenger, cyclo-oxygenase inhibition, phospholipase A inhibition or by a range of K^+ channel antagonists [6]. This suggests that the venom has a direct effect on the underlying smooth muscle.

In this work, we describe for the first time the isolation, chemical characterisation, and biological activities

☆ *Abbreviations:* LC–MS, liquid chromatography–mass spectrometry; TFA, trifluoroacetic acid; HPLC, high performance liquid chromatography.

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of three small novel peptides originating from the venom of *O. microlepidotus*.

Materials and methods

Venom collection and fractionation. Venom was collected from specimens of *O. microleptidotus* (inland taipan) from Goydnor's Lagoon, South Australia, Australia; *Oxyuranus scutellatus canni* (New Guinea taipan) from Merauke, West Papua, and *Oxyuranus scutellatus scutellatus* (coastal taipan) from Cairns, Queensland, Australia, and pooled to minimise the effects of individual variations. Whole venom was diluted to a concentration of 10 mg/mL with MilliQ water, centrifuged at 1500*g* for 2 h in Centricon 50 centrifuge filters (Millipore, 50 kDa cut-off), and the filtrate was centrifuged again at 3000 *g* using Centricon 10 tubes (Millipore, 10 kDa cut-off). The filtrates from this step were then freeze-dried and stored until needed for LC–MS analysis and RP-HPLC analysis.

LC–MS analysis. Whole venom was dissolved in 0.1% TFA to a concentration of 1 mg/mL and subjected to LC–MS analysis. On-line LC–MS (PE-SCIEX API III triple quadrupole mass spectrometer equipped with an ionspray atmospheric pressure ionisation source) was performed on a Vydac C18 analytical column (2.1 × 35 mm, 5 μm particle size, 300 Å) with Solvent A (0.05% TFA, H_2O) and Solvent B (90% acetonitrile in 0.045% TFA) at a turbospray flow rate of 130 μL/min with a variable gradient (0–20% B for 2 min and then 20–45% for 12 min followed by 45–80% over 1 min). Samples (10 μL) were injected manually into the LC–MS system and analysed in positive ion mode. Full scan data were acquired at an orifice potential of 80 V over the mass range 400–2100 Da with a step size of 0.2 amu. Data processing was performed with the aid of the software package Biomultiview (PE-SCIEX, Canada).

Purification and sequence determination. Venom samples were fractionated by reversed-phase HPLC using a Vydac C18 column (20 × 250 mm, 10 μm, 300 Å) on a Waters 600 HPLC system. The following gradient conditions were used: 0–20% in 5 min (4% gradient), 20–60% over 40 min (1% gradient), and then 60–80% in 5 min (4% gradient). Fractions were collected manually by monitoring the absorbance at 214 nm. Sequence determination was performed on isolated fractions containing the masses 3651, 3661, and 4112 Da. The purified peptides were reduced and maleiidylated prior to sequence determination using Edman degradation chemistry on an Applied Biosystems 477A Protein Sequencer.

Aortic ring experiments. Male Sprague–Dawley rats were killed and the descending thoracic aorta was rapidly removed and flushed with physiological solution (see below). After removal of adhering fat and connective tissue, 5 mm rings were mounted under 10*g* resting tension, between two stainless steel hooks, in organ baths (37 °C) containing carbogenated (95% O_2 and 5% CO_2) physiological salt solution of the following composition (mM): NaCl, 118.4; $NaHCO_3$, 25; glucose, 11; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; and $CaCl_2$, 2.5. The lower hook was attached to a tissue holder and the upper hook to a force displacement transducer (Grass FTO3). Where indicated, endothelial cells were removed by gentle rubbing of the intimal surface with a wire. To confirm the presence or absence of endothelial cells, tissues were pre-contracted with a sub-maximal concentration of phenylephrine (0.3 μM) and the response to acetylcholine (ACh, 10 μM) was observed. ACh-induced relaxation greater than 80% of maximum indicated the presence of endothelium; whereas ACh-induced relaxation less than 10% indicated the absence of endothelium. Ring preparations under resting tension were conditioned by two contraction/relaxation cycles with 40 mM KCl. Preparations were then pre-contracted with 40 mM KCl and venom peptides were tested at different doses. Responses were expressed as a percentage relaxation of the KCl response.

Determination of cell cyclic GMP elevations. The human embryonic kidney 293 cell line was used for the stable expression of rat GC-A (GC-A/293). Cells expressing rat GC-A (GC-A/293) in the 293 cell line were as previously described [7] and determination of cGMP elevations was undertaken as previously described [8]. Responses were expressed as a percentage of the effect produced by ANP.

Results and discussion

A preliminary LC–MS screening of the whole venom permitted us to detect molecular weight components from 2 to 20 kDa (data not shown). The fractionated venom also revealed the clear presence of minor components in the 3–4 kDa range (Fig. 1). Three components, with molecular weights of 3651, 3661, and 4112 Da, were purified by preparative RP-HPLC. Edman degradation sequencing provided the full sequences which revealed a high degree of homology between the three components and the circulating isoforms of ANP and BNP (Fig. 2). The three snake venom natriuretic-like peptides were designated TNP-a, TNP-b, and TNP-c, respectively, according to the source (taipan venom) and chronological order of isolation. Swiss-Prot Accession Nos. are: TNP-a P83224 (*O. microlepidotus*), P8322 (*O. s. canni*), and P83225 (*O. s. scutellatus*); TNP-b P83227

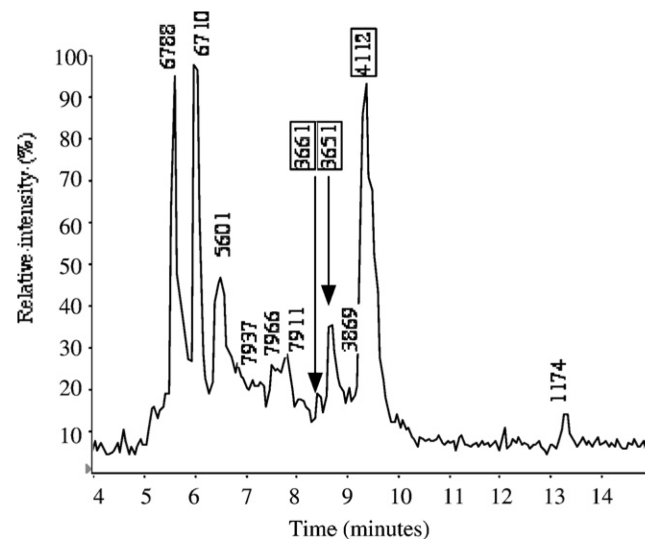

Fig. 1. LC–MS profile of <10 kDa centrifuge fraction of *O. microlepidotus* (inland taipan) venom. Mass in Daltons is given above each major peak. On-line LC–MS of venoms was performed on a Vydac C18 analytical column (2.1 × 35 mm, 5 μm particle size, 300 Å) with Solvent A (0.05% TFA, H_2O) and Solvent B (90% acetonitrile in 0.045% TFA) at a turbospray flow rate of 130 μL/min. The solvent delivery and gradient formation were achieved using an Applied Biosystems 140 B solvent delivery system. The variable gradient was 0–20% in the first 2 min and then 20–45% over the next 12 min followed by 45–80% over the next minute. Electrospray mass spectra were acquired on a PE-SCIEX triple quadrupole mass spectrometer that was equipped with an ionspray atmospheric pressure ionisation source. Samples (10 μL) were injected manually into the LC–MS system and analysed in positive ion mode. Full scan data were acquired at an orifice potential of 80 V over the mass range 400–2100 Da with a step size of 0.2 amu.

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ANP human            SLRRSSCFGGRMDRIGAQSGLGCNSFRY
ANP rat              SLRRSSCFGGRIDRIGAQSGLGCNSFRY
BNP human         SPKMVQGSGCFGRKMDRISSSSGLGCKVLRRH
BNP rat   IQERLRNSKMAHSSSCFGQKIDRIGAVSRLGCDGLRLF
DNP                  EVKYDPCFGHKIDRINHVSNLGCPSLRDPRPNAPSTSA
TNP-b              SDPKIGDGCFGLPLDHIGSVSGLGCNRPVQNRPKK
TNP-a              SDSKIGDGCFGLPLDHIGSVSGLGCNRPVQNRPKK
TNP-c              SDSKIGNGCFGFPLDRIGSVSGLGCNRIMQNPPKKFSGE
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Fig. 2. Comparison of isolated peptides with the invariant residues of the natriuretic peptide family and previously isolated venom natriuretic peptides.

(*O. microlepidotus*), P83229 (*O. s. canni*), and P83228 (*O. s. scutellatus*); and TNP-c P83230 (*O. microlepidotus* and P83231 (*O. s. canni*).

The human natriuretic peptide family [atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), and C-type natriuretic peptide (CNP)] display hypotensive and vasodilator activity, and exert their effects primarily through the membrane-bound guanylyl cyclases [9]. Natriuretic peptides have been isolated from a variety of animal venoms [10–12]. In addition, a novel peptide, *Dendroaspis* natriuretic peptide (DNP), has been isolated from the venom of the green mamba (*Dendroaspis angusticeps*) and is functionally similar to ANP in stimulating the production of guanylate cyclase via GC-A [13]. DNP has been shown to produce relaxation in canine arterial smooth muscle through both GC-A (endothelium-denuded aortic rings) and the NPR-C receptor (endothelium-intact aortic rings) [14].

Structural similarity between the three peptides and the natriuretic peptides led us to investigate their activity. Pharmacological analysis of TNP-a, TNP-b, and TNP-c confirmed their natriuretic activity but also found significant differences, despite the highly conserved sequences (Fig. 2). Only TNP-c was equipotent to ANP and DNP, producing a near 100% relaxation in pre-contracted aortae. These effects were consistent between endothelium-intact (NPR-C mediated) and -denuded rings (GC-A mediated). TNP-a and TNP-b produced only minor (approximately 20% maximum) relaxation effects in the endothelium-denuded aortic rings but no relaxation in intact rings. The lack of activity of TNP-a and TNP-b on the GC-A receptor preparation was confirmed on cloned cell lines over-expressing this receptor (Fig. 3). In addition, neither TNP-a or TNP-b was able to competitively inhibit the binding of TNP-c to either the endothelium-intact or endothelium-denuded aortic rings (Fig. 4).

Structure–activity relationships for ANP peptides are far from clear as researchers in the past two decades have compared data from differing ANP/BNP orthologues with assays performed on differing tissue, receptor, and animal models [15–17]. Moreover, no high quality tertiary structures have been available for any ANP-like ligand [18] to help interpret the plethora of

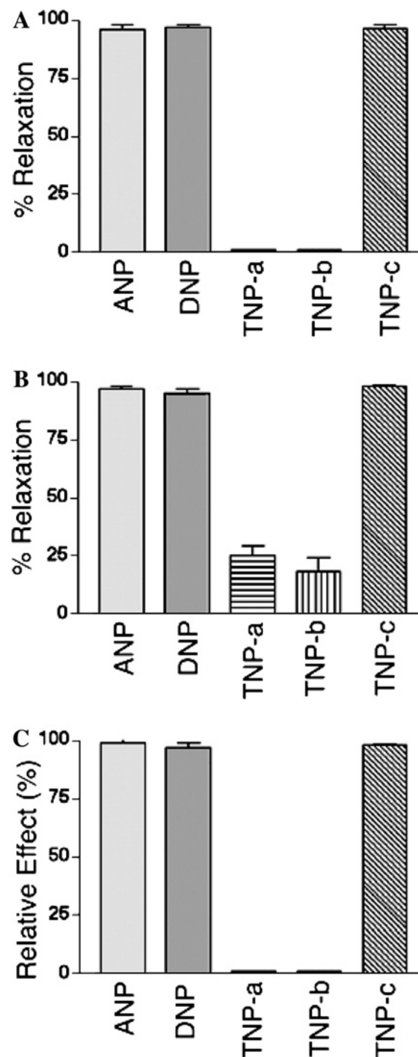

Fig. 3. Comparative effects of venom derived natriuretic peptides (0.1 μM) on rat (A) endothelium-intact aortae and (B) endothelium-denuded aortae pre-contracted with 40 mM KCl ($N = 5$). (C) 0.1 μM comparative cCMP production in 293 cells over-expressing GC-A. $N = 3$.

data. Nevertheless, there is a general consensus that for ANP–GC-A receptor interaction residues Phe 8, Met 12, and Ile 15 within the loop and Leu 21 within the C-terminal tail are critical for receptor binding [15,19–21]. The recent crystal structure [22] of NPR-C

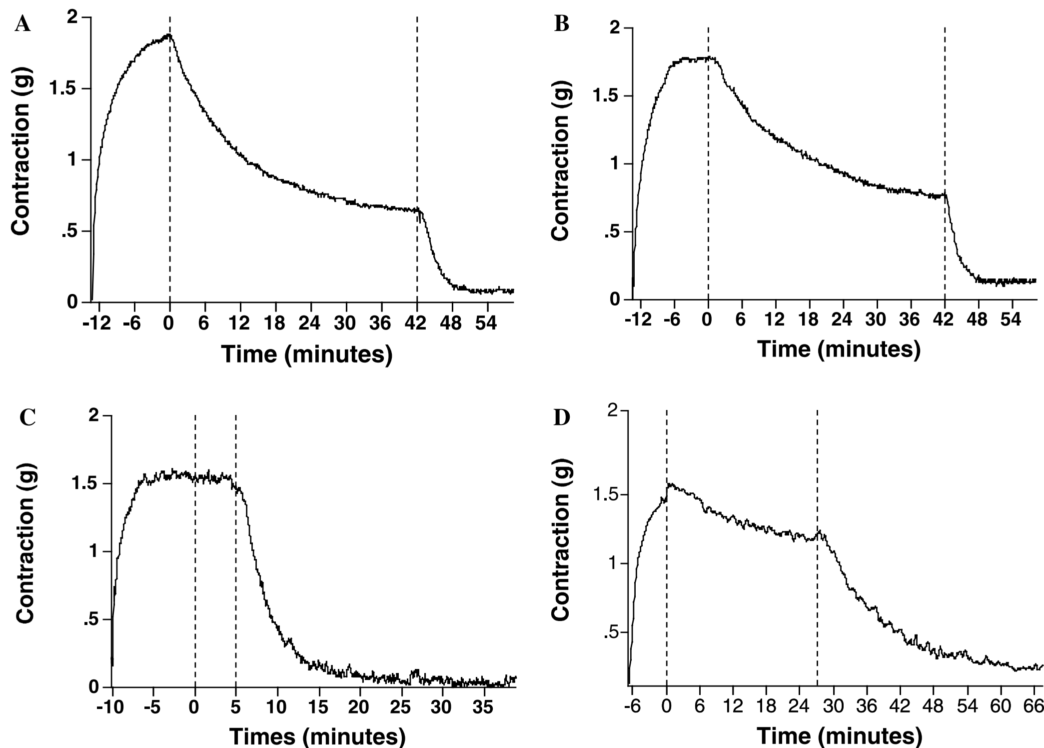

Fig. 4. TNP-c (10 nM; $t = 0$) followed by TNP-c (0.1 μM; $t = 42$) on (A) endothelium-denuded and (B) endothelium-intact aortae pre-contracted with 40 mM KCl. TNP-b (0.1 μM; $t = 0$) followed by TNP-c (0.1 μM) on (C) endothelium-denuded ($t = 5$) and (D) endothelium-intact aortae pre-contracted with 40 mM KCl ($t = 27$). $N = 3$.

complex with CNP suggests that Phe 8, Gly 9, and Arg 14 are conserved contact residues between all natriuretic peptides.

Examination of the sequences of TNP-a, TNP-b, and TNP-c showed that despite the strong homology with both ANP and BNP the venom peptides differed in several important areas. TNP-a, TNP-b, and TNP-c have the invariant basic residue at position 5 in the ring replaced by a proline residue and importantly TNP-a and TNP-b have the invariant arginine residue (i.e., R14) at position eight of the loop replaced by histidine. Further, the C-terminal tails of TNP-a, TNP-b, and TNP-c were as long or longer than the length previously shown to be the minimum for full activity [19,23]. Interestingly, the C-terminal tails shared little homology with this highly conserved region in ANP and all contained multiple proline residues within the C-terminal tail. However, it is notable that the two peptides (i.e., TNP-a and TNP-b) that are inactive in assays specific for ANP/BNP activity contain proline residues at position three in the C-terminal tail whereas TNP-c, which is equipotent to ANP, contains a conserved hydrophobic binding residue in that position. This single change may prove to be the most important of all in dictating the activity of the peptides.

In summary, we have isolated and characterised three new ANP-like peptides (TNP-a, TNP-b, and TNP-c) from the venom of the inland taipan (*O. microlepidotus*). The peptides are highly homologous within the loop region and all contain a novel C-terminal tail. The most active peptide, TNP-c, displayed equipotent activity on the GC-A receptor. Conversely, TNP-a and TNP-b are the first ANP/BNP-like natriuretic peptides to be inactive upon the GC-A based assays and were only weakly active on the NPR-C receptor. The proline residue at position three within the C-terminal tail may contribute to the potency of these molecules.

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