Plasma cardiotrophin-1 is elevated in human hypertension and stimulated by ventricular stretch

Chris J. Pemberton*, Sara D. Raudsepp, Tim G. Yandle, Vicky A. Cameron, A. Mark Richards

Christchurch Cardioendocrine Research Group, Department of Medicine, Christchurch School of Medicine and Health Sciences, University of Otago, 2 Riccarton Avenue, P.O. Box 4345, Christchurch, New Zealand

Received 7 December 2004; received in revised form 26 April 2005; accepted 17 May 2005

Available online 23 June 2005

Time for primary review 27 days

Abstract

Objective: Cardiotrophin-1 (CT-1) is an interleukin-6-related cytokine with known hypertrophic and protective actions upon cardiac myocytes. We provide here the first report of cardiac tissue and plasma levels of CT-1 in human and experimental hypertension, demonstrate cardiac CT-1 secretion stimulated by ventricular stretch, and characterise molecular forms of CT-1 in tissue and plasma.

Methods: CT-1 levels in human and rat plasma and in rat cardiac tissue extracts were determined by specific radioimmunoassay (RIA). Cardiac CT-1 secretion during ventricular stretch was studied in isolated, perfused hearts. Molecular forms of CT-1 were identified using RIA coupled with high performance liquid chromatography (HPLC). Results are given as mean ± SEM.

Results: Plasma levels of CT-1 in patients with untreated hypertension (UTH, 606 ± 18 pmol/L, n = 24) were significantly higher than those in age- and BMI-matched normotensive volunteers (NT, 546 ± 12 pmol/L, n = 31, P < 0.01 vs. UTH). CT-1 levels in matched patients with treated hypertension (THT, 618 ± 10 pmol/L, n = 35) were similar to those in UTH patients, but higher than in NT controls (P < 0.01). Plasma CT-1 demonstrated a weak but significant correlation with systolic blood pressure in all patients (r = 0.241, P < 0.05, n = 90). In contrast, CT-1 levels in male, 40-week-old, NT-WKY rats (1295 ± 98 pmol/L) were significantly higher than those in matched UTH-SHR (937 ± 31 pmol/L, P < 0.01). In both WKY and SHR rats, atrial tissue concentrations of CT-1 were 8-fold higher than ventricular levels. Left ventricular tissue CT-1 protein concentrations were significantly higher in 40-week-old SHR compared with age-matched WKY (SHR 12.6 ± 0.5 fmol/g vs. WKY 9.5 ± 0.8 fmol/g, P < 0.01). Ventricular stretch of Langendorff perfused, isolated WKY/SHR hearts resulted in significant, acute release of CT-1 and BNP. HPLC coupled with specific RIA revealed CT-1 in human/rat plasma, isolated rat heart perfusate, and rat heart tissue extracts to consist of complex, high molecular weight forms.

Conclusions: This is the first report to show increased levels of plasma CT-1 in hypertensive disease. CT-1 is a unique cardiac cytokine whose release is stimulated by ventricular stretch. The atrium contains the highest levels of the protein. The stored and circulating molecular form of CT-1 is complex, which may modulate its in vivo role in cardiovascular disease.

Keywords: Cytokines; Hypertension; Secretion; Ventricular stretch; HPLC

1. Introduction

Cardiotrophin-1 (CT-1), a member of the interleukin-6 (IL-6) family of cytokines, was originally identified as a factor that could induce hypertrophy of cardiac myocytes in vitro [1]. CT-1 mRNA is widely expressed in various adult human tissues including heart, liver, skeletal muscle, ovary, colon, prostate and testis [2], and in plasma CT-1 is reportedly increased in patients with congestive heart failure (CHF) [3,4]. Cardiac CT-1 appears to be predominantly derived from cardiac fibroblasts [8], and binds with the gp130/leukemia inhibitory factor (LIF) receptor heterodimer and a 80 kDa tertiary component to induce
ventricular hypertrophy in vitro [9,25]. Consistent with its likely cardiovascular role, increased ventricular mRNA expression of CT-1 has been detected in vivo in genetically hypertensive rats [10], in rats after experimental MI [11], in dogs with overt pacing induced CHF [12] and in vitro in isolated ventricular myocytes subjected to mechanical stretch [13]. CT-1 also has cardioprotective actions during ischaemia-reperfusion [14], and protects cardiac myocytes from apoptosis via a mitogen activated protein (MAP) kinase intracellular pathway [15]. Remarkably, given the promise that CT-1 holds as a potential target of therapeutic advances, few reports have documented circulating levels of the cytokine in cardiovascular disease (CVD) [3–7], presumably because of a lack of precise assay systems for its measurement. Furthermore, the relationship of ventricular load to CT-1 protein release into the circulation and a clear characterisation of its circulating form(s) have not been established. Accordingly, we have developed a sensitive radioimmunoassay (RIA) for human and rat CT-1 and used it to: 1) determine plasma concentrations and cardiac stored levels of CT-1 in normotensive WKY and hypertensive SHR rat strains; 2) document circulating plasma levels of CT-1 in clinical hypertension; 3) provide the first evidence that ventricular stretch can modulate cardiac release of CT-1 in Langendorff perfused isolated rat hearts and 4) characterised the molecular forms of immunoreactive human and rat CT-1 in plasma and cardiac tissue using high performance liquid chromatography (HPLC).

2. Methods

All animal experiments conform with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health, (NIH publication No. 85-23, revised 1996) and were approved by the Animal Ethics Committee of the University of Otago, New Zealand. All human protocols were approved by the Ethics Committee (Canterbury) of the Ministry of Health, New Zealand. All patients gave informed consent before recruitment into the study and the investigation conforms with the principles outlined in the Declaration of Helsinki.

2.1. Chemicals

Recombinant human CT-1 (rhCT-1) was obtained from Peprotech, USA. Recombinant human Leukaemia Inhibitory Factor (LIF), Interleukin-6 (IL-6), Tumour Necrosis Factor-α (TNFα) and IL-11 were purchased from Sigma (Missouri, USA). Synthetic Brain Natriuretic Peptide (BNP), Atrial Natriuretic Peptide (ANP), Endothelin-1 (ET-1) and Angiotensin II (AII) were purchased from the Peptide Institute (Osaka, Japan). A synthetic peptide sequence common to both human and rat CT-1 (Fig. 1) was synthesised by Chiron Mimotopes (Melbourne, Australia). This sequence corresponded to CT-1(106-120) with a Cys⁰ N-terminus extension and was greater than 95% pure as assessed by ESI mass spectrometry.

2.2. Studies on SHR/WKY plasma and cardiac tissue CT-1

Male SHR (n = 6) and WKY (n = 6) rats obtained from the University of Otago Hercus Taieri Research Unit were culled at age 40 weeks. Plasma was collected into chilled Na3-EDTA tubes and centrifuged to prepare plasma. The heart was removed, weighed, cut into atrial and ventricular sections, frozen in liquid nitrogen and stored at -80 °C before extraction.

2.3. Langendorff isolated perfused rat heart studies

A total of twelve male WKY and SHR rats, aged 12 weeks (6 of each strain, 270–300 g) underwent a modified isolated heart procedure, similar to that described previously [16,17]. Insertion of a liquid-filled balloon into the left ventricle allowed measurement of contractile parameters (Powerlab, AD Instruments). Hearts were paced at 350 beats per minute (Digitimer DG2A Pulse Generator, Digitimer, UK) and allowed to equilibrate for 30 min. After a 10 minute control period with the end-diastolic pressure (EDP) set at 5–6 mmHg, the left-ventricle was stretched for 20 min to achieve an EDP between 25–30 mmHg, a pressure known to increase ventricular BNP peptide secretion [16]. Hemodynamic variables and perfusate samples (for CT-1 and BNP RIA) were collected at −10, −5, 0, 2, 4, 6, 8, 10, 15 and 20 min.

2.4. Extraction of CT-1 immunoreactivity from human/rat plasma, isolated rat heart perfusate and rat cardiac tissue

Extracts of human and rat plasma and isolated heart perfusate were prepared for the measurement of CT-1 immunoreactivity, using solid-phase SepPak C₁₈ cartridges as previously described [17]. Rat cardiac tissue extracts were prepared as previously described [18].
2.5. Anti CT-1 antibody generation

Synthetic Cys\(^6\)-CT(106-120) was coupled to maleimide treated/ECMS derivatised bovine serum albumin (BSA) as previously described \[30\], and injected intradermally into two New Zealand White rabbits over 5–6 sites. Rabbits were bled 11–13 days after injection and the procedure repeated 4–6 weekly until adequate antiserum titre obtained. One rabbit (A44) produced adequate antiserum that was used to measure CT-1 in both rat and human species.

2.6. Preparation of \(^{125}\)I-radiolabeled recombinant human CT-1

Full length recombinant hCT-1 (2.5 \(\mu\)g) was iodinated using 0.5 mCi Na\(^{125}\)I in the presence of 5 \(\mu\)g chloramine T in 5 \(\mu\)l of 0.5 M phosphate buffer, pH 7.3 for 20–25 s. The reaction was stopped by addition of 50 \(\mu\)g cysteine HCl in a further 5 \(\mu\)l of phosphate buffer. The resulting iodinate was loaded onto a 10 cm RP300 Brownlee HPLC column (Applied Biosystems, San Jose, CA) and eluted with a gradient of 0% to 100% acetonitrile in 0.1% trifluoroacetic acid (TFA) over 30 min at a flow rate of 1 ml/min.

### Table 1

Haemodynamic and demographic data for healthy volunteer and hypertensive human patients groups

<table>
<thead>
<tr>
<th></th>
<th>Normotensives ((n = 31))</th>
<th>Untreated hypertensives ((n = 24))</th>
<th>Treated hypertensives ((n = 35))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>61.3 ± 1.8</td>
<td>65.1 ± 1.3</td>
<td>65.7 ± 1.8</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>26/5</td>
<td>18/6</td>
<td>32/3</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>116.9 ± 1.7</td>
<td>153.9 ± 2.5*</td>
<td>140.7 ± 2.2*</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>75.1 ± 1.3</td>
<td>95.1 ± 1.9*</td>
<td>80.9 ± 1.2*</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>24.4 ± 0.4</td>
<td>26.0 ± 0.9</td>
<td>26.5 ± 0.4*</td>
</tr>
<tr>
<td>Medications (%)</td>
<td>–</td>
<td>–</td>
<td>ACE inhibitor (31%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>b-blocker (31%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ca(^{2+}) blocker (17%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Diuretic (39%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AT(_1)R blocker (6%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(\alpha)-blocker (10%)</td>
</tr>
</tbody>
</table>

All data given as mean ± s.e.m where appropriate. \(* = P < 0.05\) versus corresponding normotensive value.

Fig. 2. Representative standard curve and serial dilutions of human plasma and rat heart extracts in the CT-1 radioimmunoassay exhibited dilution of immunoreactivity parallel to the standard curve. Numbers above white triangles indicate dilutions of human plasma, whereas numbers/bars over the filled squares indicate rat heart dilutions.

Fig. 3. A: Plasma concentrations of CT-1 (pmol/L) in normotensive humans (NT), age and BMI matched patients with untreated hypertension (UTH) and in age matched treated hypertensives (THT). Plasma CT-1 in both UTH and THT patients were higher than NT levels (\(** = P < 0.01\)). B: regression analysis of plasma CT-1 versus systolic blood pressure in all human patients revealed a weak but significant association \((P = 0.023, r = 0.241)\). C: CT-1 plasma concentrations in matched, 40 week old, male WKY and SHR rats \((** = P < 0.01\)).
2.7. Radioimmunoassay (RIA) measurement of immunoreactive CT-1 and rat BNP

Antiserum A44 was used in the CT-1 RIA at a final dilution of 1:3000. The cross reactivity of this antiserum with recombinant human Leukemia Inhibitory Factor (LIF), Interleukin-6 (IL-6), Tumor Necrosis Factor-α (TNFα) and IL-11 was less than 0.6% in each case. Cross reactivity with synthetic Brain Natriuretic Peptide (BNP), Atrial Natriuretic Peptide (ANP), Endothelin-1 (ET-1) and Angiotensin II (AII) was less than 0.03% in all cases. All sample extracts, radioactive trace, standard and antiserum solutions were diluted in RIA buffer [19]. The assay incubate consisted of 100 μL of extracted sample, or standard (0–3200 pmol/L of full length recombinant human CT-1) combined with 100 μL of antiserum A44 and 100 μL of full length recombinant 125I-human CT-1 trace (4000–6000 cpm). The tubes were incubated for 24 h at 4 °C and then free and bound CT-1 were separated by solid phase second antibody method (donkey anti-rabbit Sac-Cel, Immunodiagnostic Systems, Boldon, UK). Sac-Cel (1 ml) diluted in 5% dextran solution (final Sac-Cel concentration 5%) was added to each tube, the solution vortexed and incubated at room temperature for 30 min. The tubes were then centrifuged at 2800 × g for 10 min at 20 °C and decanted, with the resulting pellet counted in a Gammamaster (LKB, Uppsala, Sweden). Rat BNP concentrations were determined using a modified commercial RIA (Phoenix Pharmaceuticals, CA).

2.8. High Performance Liquid Chromatography (HPLC) of CT-1

Plasma, isolated heart perfusate and cardiac extracts were dried under air, reconstituted in 20% acetonitrile/0.1%TFA/0.15 M NaCl/0.01% Triton X-100 buffer (with and without 0.1 M dithiothreitol (DTT) or 20% β-mercaptoethanol) and first subjected to size exclusion (SE) HPLC on a Superdex G75 Superose column (Pharmacia Biotech, Sweden), using

![Fig. 5. Isolated heart perfusate levels of CT-1 (panel A) and BNP (panel B) during ventricular stretch in SHR and WKY heart preparations. Panel A; relative to the 10 minute control period, perfusate CT-1 concentrations increased acutely in WKY and SHR hearts (WKY max+ 83%, P<0.01; SHR max+ 36%, P<0.05) in response to ventricular stretch, but were not maintained for the entire stretch period. The peak height difference between WKY and SHR heart CT-1 secretion (47%) was not significant. Panel B; relative to the control period, ventricular stretch resulted in acute increases in perfusate BNP in WKY (max+ 28%) and SHR (max+ 18%) hearts (*, P<0.05; **, P<0.01).](image)

![Fig. 4. CT-1 concentrations in cardiac tissue extracts from 40 week old WKY and SHR strains. Atrial tissue CT-1 (fmol/g) tended to be higher in age matched 40 week old SHR compared with WKY controls (P=0.412, NS). Ventricular CT-1 levels in SHR were significantly higher than WKY (SHR 12.6±0.5 fmol/g vs. WKY 9.5±0.8 fmol/g, **P<0.01). Note that atrial levels of CT-1 were ~8-fold greater than ventricular in both WKY and SHR animals (P<0.01 for both WKY and SHR).](image)
an isocratic gradient of the above buffer at a flow rate of 0.25 ml/min. Fractions were collected at 2 min intervals and subjected to CT-1 RIA to establish immunoreactive molecular size. Immunoreactive CT-1 fractions from SE-HPLC were then pooled, lyophilised, reconstituted in 20% acetonitrile/0.1%TFA and eluted from a 5 µ reverse phase (RP)C18 Jupiter column (Phenomenex, Torrance, CA) with a gradient of 20%–80% acetonitrile over 60 min at a flow rate of 1 ml/min. Fractions were collected, dried, reconstituted and re-subjected to CT-1 RIA. RP-HPLC was calibrated with CT-1 (Peprotech, USA) whereas SE-HPLC was calibrated with CT-1 and markers ranging between \( M_r = 66,500 \) and \( M_r = 6,000 \) (Sigma-Aldrich).

2.9. Healthy and hypertensive human blood sampling

Blood samples for measurement of human plasma CT-1 were drawn from 31 normotensive volunteers (NT) randomly selected from the community, who were not taking any blood-pressure medications and had no evidence of cardiovascular or endocrine illness. Blood samples were also drawn from age-and BMI-matched patients with known, treated hypertension (THT, \( n = 35 \)). A separate cohort with untreated hypertension were recruited (UTH, \( n = 24 \)), who had BP > 140/90 on two independent measurements. Haemodynamic and demographic data for these patient groups are given in Table 1. Blood samples were drawn from a forearm vein into chilled \( Na_3\)-EDTA vacutainers, centrifuged and stored at \(-80^\circ C\) until assay.

2.10. Statistical analysis

All data is presented as mean±SEM. Analysis of single time-point plasma hormone concentrations was carried out using unpaired, two tailed students \( t\)-test. Hemodynamic and peptide RIA timecourse data from isolated heart experiments were analysed with two-way ANOVA for repeated measurements followed by Least Significant Difference (LSD) post hoc test. In all statistical analyses, a value of \( P<0.05 \) was considered statistically significant.

3. Results

3.1. Radioimmunoassay for CT-1

Using antiserum A44, the RIA for CT-1 had the following characteristics: mean zero binding (\( B_o \)) of \( 28±1\% \), detection limit of \( 28±2\) pmol/L, 50% displacement of radiolabeled trace at 424.2 pmol/L and non-specific binding of assay buffer equal to \( 4.2±0.1\% \) over 21 consecutive assays. The recovery of recombinant human CT-1 from human and rat plasma and isolated heart perfusate was low, being \( 26±3\% \) (\( n = 15 \)). The inter-assay coefficient of variation (CV) was \( 14–17\% \), whereas intra-assay CV was \( 4–6\% \) between 120 and 800 pmol/L. As shown in Fig. 2, dilutions of SepPak cartridge extracts from human and rat samples diluted in parallel with the standard curve.

3.2. Immunoreactive CT-1 in human and rat plasma and SHR and WKY cardiac tissue

Mean plasma CT-1 levels in untreated hypertensive humans (UTH, \( 606±18\) pmol/L, \( n = 24 \)) were significantly higher than those in age-and BMI-matched normotensives (NT, \( 546±12\) pmol/L, \( n = 31 \), \( P<0.01 \), Fig. 3A), CT-1 levels in treated hypertensives (THT, \( 618±10\) pmol/L, \( n = 35 \)) were similar to levels in UTH but were significantly higher than those in NT patients (\( P<0.01 \)). Plasma CT-1 showed a weak but significant positive correlation with systolic blood pressure in all patient groups (\( r = 0.241 \), \( P<0.05 \), \( n = 90 \), Fig. 3B). In 40 week old normotensive WKY rats, plasma CT-1 levels were \( 1295±98\) pmol/L (\( n = 6 \), Fig. 3C) which were significantly higher than those in matched, untreated hypertensive SHR animals (SHR \( 937±31\) pmol/L, \( P<0.01 \) vs.

### Table 2

<table>
<thead>
<tr>
<th>Contractile variables in WKY and SHR isolated heart preparations during ventricular stretch experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY time (min)</td>
</tr>
<tr>
<td>------------------------</td>
</tr>
<tr>
<td>(-10)</td>
</tr>
<tr>
<td>(0)</td>
</tr>
<tr>
<td>(2)</td>
</tr>
<tr>
<td>(6)</td>
</tr>
<tr>
<td>(10)</td>
</tr>
<tr>
<td>(20)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SHR time (min)</th>
<th>EDP (mmHg)</th>
<th>DP (mmHg)</th>
<th>(+dp/dr\ (mmHg/sec^{-1}))</th>
<th>(-dp/dr\ (mmHg/sec^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-10)</td>
<td>5±3</td>
<td>56±3</td>
<td>1278±173</td>
<td>904±114</td>
</tr>
<tr>
<td>(0)</td>
<td>6±3</td>
<td>55±3</td>
<td>1248±151</td>
<td>884±91</td>
</tr>
<tr>
<td>(2)</td>
<td>17±9</td>
<td>72±3</td>
<td>1478±39</td>
<td>1095±16</td>
</tr>
<tr>
<td>(6)</td>
<td>27±10</td>
<td>94±4</td>
<td>1968±161</td>
<td>1405±83</td>
</tr>
<tr>
<td>(10)</td>
<td>34±9</td>
<td>99±4</td>
<td>2117±221</td>
<td>1517±109</td>
</tr>
<tr>
<td>(20)</td>
<td>28±7</td>
<td>106±4</td>
<td>2329±376</td>
<td>1671±218</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM. There were no significant differences in variable between WKY and SHR preparations. EDP=end diastolic pressure; DP=developed pressure; \(+dp/dr\)=maximum positive rate of pressure development; \(-dp/dr\)=maximum negative rate of pressure development.
Cardiac tissue extracts from WKY and SHR rats contained immunoreactive CT-1, with concentrations in the atrium (80–100 fmol/g in both strains) ~8-fold higher than in the ventricle (Fig. 4). Ventricular CT-1 concentrations in SHR were significantly (30–40%) higher than WKY (SHR 12.6 ± 0.5 fmol/g vs. WKY 9.5 ± 0.8 fmol/g, P < 0.01, Fig. 4).

3.3. Isolated heart studies of endogenous CT-1 secretion

In vitro baseline haemodynamic variables for these experiments were not significantly different between WKY and SHR preparations (Table 2). Ventricular stretch resulted in a prompt (within 4 min) rise in perfusate CT-1 (P < 0.01, Fig. 5A) and BNP (P < 0.05, Fig. 5B) in both WKY and SHR hearts (Table 2). In SHR hearts, the peak increase in CT-1 was lower than that observed in WKY hearts (P = NS).

3.4. Analysis of plasma and cardiac tissue CT-1 on HPLC

SE-HPLC unexpectedly revealed recombinant human CT-1 to have an apparent molecular weight of 66,000, 3× greater than the predicted monomer. Endogenous CT-1 in human plasma extracts comprised two peaks at ~M_r 66,000 (Peak I) and ~M_r 47,000 (Peak II) which are 3× and 2× larger than the expected monomer weight (~M_r 21,500) of CT-1, respectively (Fig. 6A). Incubation of recombinant and endogenous human CT-1 with 100 mM DTT failed to completely reduce the protein to a monomeric weight on SE-HPLC, with the product eluting at ~M_r 47,000 (Fig. 6B). Similar results were obtained when CT-1 was reduced with 20% β-mercaptoethanol (results not shown). When analysed further by RP-HPLC, peak II eluted earlier than peak I (Fig. 6C). Recombinant CT-1 eluted at the same position as peak I on both SE-HPLC and RP-HPLC modalities. Thus, under all HPLC conditions used here, we observed no immunoreactive human CT-1 that eluted near the predicted monomeric weight of ~M_r 21,500.

Rat cardiac tissue, plasma extracts and isolated heart perfusate extracts all contained immunoreactivity consistent with peaks I and II observed in human samples (Fig. 7).
suggesting that rat CT-1 has an in vivo structure similar to that of human CT-1.

4. Discussion

With the platform of our specific RIA for CT-1 to work from, we report the following original findings; 1) plasma levels of CT-1 in humans with treated or untreated hypertension are significantly higher than age and BMI matched normotensives; 2) plasma levels of CT-1 in 40 week-old, untreated hypertensive SHR are lower than those of age matched WKY; 3) cardiac atrial tissue levels of CT-1 in 40 week-old WKY and SHR rats are ~8-fold higher than ventricular levels; 4) 40 week-old SHR have increased ventricular tissue concentrations of CT-1 compared with age and gender matched WKY; 5) the heart is a potential source of circulating CT-1 and cardiac CT-1 secretion is stimulated by ventricular stretch/pressure; and 6) the structure of endogenous cardiac tissue and plasma CT-1 in humans and rats appears to be complex, possibly consisting of homodimers or homotrimers.

Our obtained plasma CT-1 levels of ~550 pmol/L in healthy humans agree with those reported by Asai et al. [7] who also reported ~550 pmol/L using a direct, unextracted RIA procedure. In contrast, healthy volunteer levels reported by Talwar et al. [4–6], Ng et al. [22] and Lopez et al. [40] are an order of magnitude lower, ranging between 25–50 pmol/L, using ELISA based measurement systems. Our RIA procedure measures CT-1 immunoreactivity in extracted plasma samples. Given our findings that recombinant human CT-1 has a poor recovery rate from C18 cartridges (~25%), this translates to a direct RIA value of ~3.5 nmol/L which is 7× that reported by Asai et al. [7] and 70× that reported by Talwar et al. [4–6]. Our RIA is directed to a different epitope on the CT-1 molecule compared with the RIA of Asai et al. [7], yet SE-HPLC demonstrates both assays identify immunoreactive CT-1 species (and also in our case, rat cardiac tissue) to elute on SEHPLC consistent with recombinant human CT-1.

We show here that plasma CT-1 levels are raised in patients with untreated hypertension and that plasma CT-1 concentrations correlate with systolic pressures. In this regard, there are two important limitations to our study; first, we did not measure ventricular mass index and thus cannot conclude whether reductions in LVH are important in maintaining plasma CT-1 levels and second, the reductions in systolic pressure observed in our treated hypertensive group may not have been enough to induce a decrease in plasma CT-1. Recently, and in agreement with our observations, Lopez et al. [40] reported that plasma CT-1 levels are elevated in patients with untreated hypertension, especially so in those with left ventricular hypertrophy (LVH). Thus, a logical target for future studies will be to determine whether reductions in LVH induced by angiotensin II receptor antagonists, angiotensin II converting enzyme inhibitors and calcium channel blocker therapies have efficacy in reducing plasma CT-1.

At the neurohumoral level, plasma levels of CT-1 related cytokines such as IL-6 are elevated in human hypertension [32,33] and have been linked with vascular inflammation and endothelial damage, possibly through an angiotensin II related mechanism [36]. The plasma profiles of CT-1 and IL-6 in hypertensive states may therefore represent a competing balance from a non-inflammatory state to one of increasing inflammation and atherosclerosis (in which plasma CT-1 levels are elevated to match those of IL-6). Such a hypothesis receives support from a single study in which treatment with CT-1 in a chronic hypoxic pulmonary hypertension model protected endothelial function of the pulmonary artery, decreased pulmonary arterial pressure and attenuated right ventricular hypertrophy [37].

At 40 weeks, hypertension is well established in SHR strains, with systolic blood pressures some 50–60 mmHg higher than WKY controls [23]. In contrast with our human studies, we found that plasma CT-1 levels in untreated hypertensive SHR aged 40 weeks were significantly lower than those of matched WKY. This is in general agreement with a previous study in which plasma CT-1 levels in 10 week old SHR tended to be lower (but not significantly) than age matched WKY [31]. Why the SHR should show an opposite plasma CT-1 profile compared with human studies is unclear, but a recent study showing that LIF and IL-6, but not CT-1, are important contributors to angiotensin II induced LVH in SHR suggests that there may be important hormonal points of difference [41]. Although ventricular CT-1 protein levels in 40 week old SHR were significantly higher than WKY controls, plasma levels of CT-1 in SHR were lower than in WKY. This is in stark contrast with the situation in humans and with cardiac hormones such as ANP/BNP, which demonstrate a positive correlation between ventricular peptide/protein content, and corresponding plasma hormone levels in cardiovascular disease states [26]. Furthermore, CT-1 does not possess a classical signal sequence like BNP or ANP [1], which could render the protein subject to a different modality of secretion, unlike the regulated and constitutive modes known for ANP/BNP, respectively.

Our novel finding of ~8-fold higher CT-1 levels in the atrium of both WKY and SHR animals compared with ventricular levels is intriguing. This pattern of cardiac protein concentration is similar to that observed for the natriuretic peptides in mammalian cardiac tissue [18,26]. Our observation that ventricular CT-1 protein is increased in 40 week old SHR animals is consistent with experimental reports describing increased ventricular CT-1 mRNA levels in 4 week old hypertensive SHR stroke prone rats [10] and in 11 week old Dahl-salt sensitive (DS) rats with ventricular hypertrophy [24] and a clinical observation of increased ventricular CT-1 protein and RNA content in failing human hearts [20]. In this latter report, increased ventricular CT-1 protein was counter-balanced by a reduction in LIF receptor...
protein (although LIF receptor mRNA increased by 91%) and this was suggested to be a compensatory response to reduce excessive activation of the hypertrophic actions of CT-1.

A single previous report has documented a “step-up” in plasma CT-1 concentrations between the aortic root and coronary sinus in humans [7], and the ventricular gene expression of CT-1 correlates with increasing blood pressures post-MI in Dahl rats [24]. Furthermore, a study of human aortic stenosis has suggested that plasma CT-1 levels correlate with trans-valvular aortic pressure gradients, a surrogate for cardiac stretch [39]. In using the isolated heart preparation, we have removed potentially confounding factors such as multiple organ secretion and show for the first time that ventricular stretch/pressure is a stimulus for CT-1 release. The secretion profiles of CT-1 and BNP observed in our heart preparations are similar to previous reports studying stretch mediated cardiac natriuretic peptide secretion [34,35] and suggest that the abilities of 12 week old SHR and WKY to secrete CT-1 and BNP are similar. It may be that, like ANP and BNP, a portion of CT-1 in cardiac tissue is present as a readily releasable pool for secretion [26,34]. Whether endothelin-1 and angiotensin II are pivotal in mediating stretch/pressure induced CT-1 release remains to be seen, but data from in vitro isolated cardiac myocyte studies, [8,21] clearly demonstrate that passive CT-1 release is dependent upon the actions of both factors. The possibility remains that the heart is not the only secretor of CT-1, and that contributions of other organs/cell types (e.g. CSF, liver, circulating peripheral monocytes) to in vivo plasma levels of CT-1 require investigation.

In providing the first detailed HPLC/RIA analysis of CT-1 structure, we show CT-1 has a complex circulating and stored structure. Thus, both human and rat plasma, along with rat cardiac extracts and isolated heart perfusate all contained immunoreactive CT-1 proteins whose size was 2–3 times the predicted monomeric molecular weight for CT-1 (cf. 21.5 kDa). Whether or not circulating immunoreactive CT-1 contains the entire protein remains to be determined by amino acid sequencing, but structure analysis suggests the entire protein could circulate [1]. Remarkably, neither 100 mM DTT nor 20% β-mercaptoethanol treatment was able to reduce endogenous or recombinant human CT-1 to a monomer. The non-reducible nature of CT-1 on our HPLC systems is notable; adipocytokines such as Resistin/FIZZ3 have extremely complex tertiary/quartenary structures and form oligomers of multiple molecular weights which do not reduce easily using DTT or β-mercaptoethanol [28,29]. It is possible that CT-1 is heavily glycosylated; indeed, glycosylation is responsible for higher molecular weight forms of many proteins on HPLC and/or gel electrophoresis, such as adiponectin, and can have pronounced effects on biological function [27]. The majority of affinity purified CT-1 from human plasma was shown by Asai et al. [7] to elute consistent with recombinant CT-1 on SEHPLC, with a smaller portion eluting as a higher molecular weight form, but no estimate of molecular weight was given for either peak.

In conclusion, we describe a novel RIA for the measurement of CT-1. Using this RIA we report significant elevations in plasma CT-1 in untreated human hypertension which appear to be unmodified by pharmacological treatment. In contrast, untreated experimental hypertension in 40 week old SHR resulted in suppressed plasma CT-1 levels. We provide the first direct evidence that the heart secretes CT-1 and that this secretion is increased by ventricular stretch in both normotensive (WKY) and hypertensive (SHR) hearts. Consistent with previous reports describing increased ventricular CT-1 mRNA in experimental hypertension and CHF [10–12] and in human CHF [20], we show that ventricular CT-1 protein levels in 40 week old SHR animals are increased. Our HPLC observations suggest that CT-1 in humans and rats has a previously unappreciated complex biochemical structure. Further work examining the interactions of CT-1 and IL-6 in the development of hypertension and inflammation are warranted, particularly whether measurement of plasma CT-1 in hypertensive patients with LVH has merit as a predictor of future adverse cardiac events, such as has been demonstrated for IL-6 [38].

Acknowledgements

We thank Stephanie Moran for collection of blood samples. A.M.R. holds the National Heart Foundation of New Zealand Chair of Cardiovascular Studies.

References


