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The atriopeptidase inhibitor (±)candoxatrilat reduces the clearance of atrial natriuretic factor in both intact and nephrectomized rats: evidence for an extra-renal site of action

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Atrial natriuretic factor (ANF*) is a 28 amino acid peptide with natriuretic, diuretic and vasodilator properties [1]. On exogenous administration it is rapidly cleared from the circulation resulting in a short *in vivo* half-life [2]. The peptide is degraded by the zinc-dependent neutral endopeptidase (EC 3.4.24.11, atriopeptidase) *in vitro* [3, 4], and several groups have recently reported that inhibitors of this enzyme (atriopeptidase inhibitors, API) potentiate the renal and vasodilator effects of ANF in animal models [5, 7].

(±)Candoxatrilat (*cis* - 4 - {[2 - carboxy - 3 - (2 - methoxyethoxy)propyl]- 1- cyclopentanecarbonylamine}- 1- cyclohexane carboxylic acid, UK-69,578) is a potent, selective inhibitor of atriopeptidase (K_i 36 nM) which evokes a doubling of plasma ANF levels, and increases sodium excretion and urine flow following acute administration to rodents [8]. A similar maximal increase in plasma ANF levels associated with a rise in sodium excretion (with no effect on potassium excretion) and fall in plasma renin activity is observed following acute administration of the compound to man [9]. This profile resembles that elicited by low-level ANF infusions when plasma ANF levels are increased by a similar degree [10].

The precise mechanism of action of (±)candoxatrilat *in vivo* is currently the subject of further investigation and preliminary studies have demonstrated that the natriuretic and diuretic responses to (±)candoxatrilat are blocked by ANF antiserum [11]. In the present study we sought to determine the effects of (±)candoxatrilat on the clearance of ANF in anaesthetized rats, and to define the kinetics of the clearance processes. Furthermore, since the kidney is a major site of ANF clearance [12] and atriopeptidase is found in abundance in this tissue [13], we examined whether nephrectomy altered the profile of activity of (±)candoxatrilat.

* Abbreviations: ANF, atrial natriuretic factor; API, atriopeptidase inhibitor; *AUC*, area under the plasma concentration curve; CL_p, plasma clearance; ^{125}I-ANF, (3-[^{125}I]iodotyrosyl^{28}) rat ANF; V_d, volume of distribution.

Materials and Methods

Disappearance of ^{125}I-ANF in anaesthetized rats. Male Sprague–Dawley rats (375 g) were anaesthetized with Inactin (120 mg/kg i.p.) and catheters placed in the trachea, carotid artery and femoral vein. After a 60 min stabilization period 2.5 μCi (12 ng/kg) of ^{125}I-ANF (Amersham International, U.K.) was injected i.v. in 50 μL saline, and arterial blood samples (0.3 mL) taken at 15, 30, 60, 90, 120, 180, 240 and 300 sec into syringes containing EDTA (1 mg/mL) and aprotinin (1000 kI.U./mL). Samples were centrifuged immediately to obtain plasma, which was mixed with 3 volumes of 4% (v/v) acetic acid and stored on ice prior to extraction. One hour after the initial injection of ^{125}I-ANF, animals were dosed intravenously with saline vehicle or (±)candoxatrilat (3 mg/kg) followed 10 min later by a second injection of 2.5 μCi ^{125}I-ANF, and a further eight blood samples obtained and processed as described above. Aliquots (300 μL) of plasma in acetic acid were loaded onto Sep-Pak C18 columns (Waters, U.K.), and the columns were washed with 10 mL distilled water followed by 10 mL 4% (v/v) acetic acid. Columns were eluted with 2 mL 86% (v/v) ethanol containing 4% (v/v) acetic acid, and radioactivity in the eluate determined using a gamma counter.

Recovery of 1 μCi (1.6 ng) of ^{125}I-ANF added to a 1 mL plasma was 71 ± 1.8% (N = 6). Figures given in the text are uncorrected for recovery.

Disappearance of immunoreactive ANF in anaesthetized rats. Under pentobarbitone anaesthesia (60 mg/kg i.p.) cannulae were placed in the trachea and jugular vein of male Sprague–Dawley rats (350 g). Both kidneys were exposed via a mid-line incision, and bilateral nephrectomy or sham operation carried out. After a 10 min stabilization period, (±)candoxatrilat (3 mg/kg) or saline vehicle was injected intravenously, followed 10 min later by a 200 ng (571 ng/kg) bolus of ANF 5-28 (Atriopeptin III, Cambridge Research Biochemicals, U.K.). All injections were made in a total volume of 100 μL. Animals were killed 30, 60, 90, 120, 180 or 600 sec after ANF 5-28 injection and 10 mL blood removed via cardiac puncture into syringes

containing EDTA (1 mg/mL) and protease inhibitors (Aprotinin, 1000 kI.U./mL; soybean trypsin inhibitor, 5 μg/mL; pepstatin A, 1 μg/mL). Samples were centrifuged immediately to obtain plasma, which was loaded onto Sep-Pak C18 columns (Waters, U.K.) and columns washed with 3 mL 50 mM Tris/acetate buffer pH 7.4. Following elution with 3 mL 86% (v/v) ethanol containing 4% (v/v) acetic acid, samples were evaporated under nitrogen, and the ANF concentration determined by radioimmunoassay (RIA) as described previously [8]. The within RIA coefficient of variation (*CV*) was 12.5% ($N = 4$) and the between assay *CV* 12.8% (N = 30). Recoveries of two different quantities of synthetic ANF (100 pg and 1 ng) added to 1 mL plasma were 46.6 ± 4.2% and 48.9 ± 3.7% (N = 10) respectively. Figures given in the text are uncorrected for recovery.

Pharmacokinetic analysis. Analysis of the log plasma concentration versus time data was performed by linear regression analysis using standard pharmacokinetic equations [14] based on a two-compartment system. For all analyses the correlation coefficient was greater than 0.95 (in most cases 0.98–0.99). Plasma clearance (Cl_p) was calculated by dose/*AUC* 0–∞. The apparent volume of distribution (V_d) was calculated from the equation $V_d = Cl/\beta$ where β is the rate constant of the terminal elimination phase.

Results and Discussion

Removal of ANF from the circulation is thought to be mediated by three mechanisms; metabolism by atriopeptidase, by binding to ANF-C receptors and by glomerular filtration. Receptor-mediated ANF clearance has been studied *in vivo* using ^{125}I-ANF [15], and we have employed a similar protocol to evaluate the effects of the atriopeptidase inhibitor (±)candoxatrilat. Disappearance of ^{125}I-ANF was clearly biphasic (Fig. 1) with an initial rapid half-life ($t_{\frac{1}{2}\alpha}$) of 16 sec, and a slower terminal phase ($t_{\frac{1}{2}\beta}$) of 87 sec. These data are similar to those reported previously [15, 16]. (±)Candoxatrilat (3 mg/kg i.v.) had no effect on the $t_{\frac{1}{2}\alpha}$ of ^{125}I-ANF (Fig. 1), but significantly prolonged the $t_{\frac{1}{2}\beta}$ to 139 sec ($p < 0.0001$). The V_d for ^{125}I-ANF was unaffected by treatment with (±)candoxatrilat whereas the *AUC* approximately doubled as Cl_p was substantially reduced (Table 1). This profile contrasts markedly with that reported for the specific ANF-C receptor ligand C-ANF 4-23, which upon infusion (1 μg/kg/min) to anaesthetized rats elicited no change in the elimination half-life of ^{125}I-ANF due to a concomitant reduction in both Cl_p and V_d[15]. Since concentrations of (±)candoxatrilat as high as 2 mM fail to displace ^{125}I-ANF binding to rabbit lung* (a preparation containing both type-B and type-C receptors), the effects of (±)candoxatrilat on ^{125}I-ANF clearance are unlikely to be a consequence of ANF-C receptor blockade, but are consistent with specific inhibition of atriopeptidase. As ^{125}I-ANF binding was only measured in one tissue, however, we cannot at this time exclude the possibility that the compound may affect clearance receptor subtypes not present in the lung.

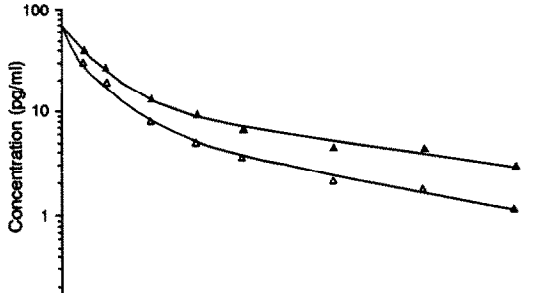

Fig. 1. Effect of (±)candoxatrilat on the plasma disappearance of ^{125}I-ANF in the anaesthetised rat. Data are from a representative experiment in one animal. During the control period (Δ), disappearance of ^{125}I-ANF was biphasic, with a $t_{\frac{1}{2}\alpha}$ of 15 sec (90% confidence limits 9–57 sec) and $t_{\frac{1}{2}\beta}$ of 102 sec (80–132 sec). Following 3 mg/kg i.v. (±)candoxatrilat (▲) the $t_{\frac{1}{2}\alpha}$ was 15 sec (11–24 sec) and elimination $t_{\frac{1}{2}\beta}$ was 140 sec (102–222 sec).

The biphasic elimination profile of exogenous ANF and the effects of an API ((±)candoxatrilat) on this profile can both be defined on an individual animal basis using ^{125}I-ANF (as illustrated in Fig. 1), because the technique permits multiple sampling in a single animal. In order to rule out the possibility of radiolabelled fragments being present in the extracted plasma, which could interfere with the interpretation of these results, we undertook a series of confirmatory experiments using a specific RIA to monitor the disappearance of a 200 ng i.v. bolus of ANF 5-28 from plasma. A relatively high dose of ANF 5-28 was chosen, in order to follow the disappearance of ANF through several half-lives. Since the RIA allowed only one ANF sample per animal, individual clearance curves could not be constructed, hence pharmacokinetic data from these experiments (Table 2) are derived from a single ANF disappearance curve with multiple measurements ($N = 5$–6 animals) per time point.

ANF 5-28 was rapidly cleared from the plasma of anaesthetized rats, appearing to follow a biphasic elimination profile. Although the initial rapid phase could not be accurately quantified, the terminal $t_{\frac{1}{2}\beta}$ was determined to be 51 sec (90% confidence limits 46–57 sec, Table 2A). (±)Candoxatrilat (3 mg/kg i.v.) prolonged the terminal $t_{\frac{1}{2}\beta}$ of ANF 5-28 (to 73 sec) and decreased Cl_p, whilst V_d was unchanged. The absolute values of $t_{\frac{1}{2}\beta}$ and Cl_p obtained using ANF 5-28 (Table 1A) are similar to those for ^{125}I-ANF (Table 1), whilst the *AUC* values are considerably higher because of the large dose of ANF administered. The reason for the difference in V_d obtained in the two studies is not readily apparent, although saturation of the receptor-mediated clearance mechanism by high doses of ANF may be involved and this possibility is currently the subject of further investigation. Since endogenous ANF levels are elevated in certain disease states, saturation of ANF-C receptors may be of significance clinically under these circumstances. We noted that the effects of (±)candoxatrilat were entirely consistent between the two studies and conclude that the inhibitor reduces the clearance of both ANF 5-28 and ^{125}I-ANF and prolongs the elimination half-life.

The role of the kidney in ANF clearance has been highlighted by several groups [12, 17, 18], in particular binding to vascular ANF-C receptors [9], filtration at the glomerulus, and inactivation by atriopeptidase [4] may all play a role in the removal of ANF from the renal circulation. As we have already demonstrated the effects of (±)candoxatrilat are not mediated via ANF-C receptor mediated clearance, however, further investigation was required to establish that these effects occurred independent of glomerular filtration. Thus, we examined the effects of the API on the clearance of ANF 5-28 (200 μg i.v.) in nephrectomized rats (Table 2B) since this allowed us to monitor the activity of (±)candoxatrilat in the absence of glomerular filtration. Compared to normal (sham-operated) rats, the terminal elimination $t_{\frac{1}{2}\beta}$ for ANF 5-28 in nephrectomized animals was doubled (119 sec), AUC was increased, Cl_p reduced and V_d unchanged. Similar effects of nephrectomy on the pharmacokinetics of ANF have been described pre-

* Greengrass PM, unpublished observations.

Table 1. Effects of (±)candoxatrilat on the i.v. pharmacokinetics of ^{125}I-ANF (12 ng/kg)

Treatment	$t_{i\alpha}$ (sec)	$t_{i\beta}$ (sec)	*AUC* (ng sec/mL)	V_d (mL/kg)	Cl_p (mL/min/kg)
(A) Vehicle					
Control	16 ± 0.6	87 ± 4	2.60 ± 0.34	582 ± 80	289 ± 48
Vehicle	17 ± 1	95 ± 5	2.82 ± 0.28	570 ± 74	248 ± 30
(B) (±)Candoxatrilat					
Control	14 ± 0.6	86 ± 5	2.74 ± 0.12	500 ± 57	237 ± 16
(±)Candoxatrilat	16 ± 0.8	139 ± 10*	4.70 ± 0.83*	535 ± 67	167 ± 25*

Data was derived by analysis of the disappearance curves of ^{125}I-ANF in anaesthetized rats receiving (A) vehicle (0.9% saline) and (B) (±)candoxatrilat (3 mg/kg i.v.), as described in Materials and Methods, and represent the mean ± SEM of eight (vehicle) or nine [(±)candoxatrilat] animals. Statistical analysis was by paired Student's *t*-test, based on a normal distribution of the data.

* $P < 0.05$ compared to control.

Table 2. Effects of (±)candoxatrilat on the i.v. pharmacokinetics of ANF 5-28 (571 ng/kg)

Treatment	$t_{i\beta}$ (sec)	*AUC* (ng sec/mL)	V_d (mL/kg)	Cl_p (mL/min/kg)
(A) Sham-operated				
Vehicle	51 (46–57)*	152	274	225
(±)Candoxatrilat	73 (62–88)*	227	265	150
(B) Nephrectomized				
Vehicle	119 (94–163)*	404	243	85
(±)Candoxatrilat	468 (329–805)*	1581	243	22

Data was derived from single disappearance curves of ANF 5-28 in (A) intact (sham-operated) and (B) bilaterally nephrectomized rats, as described in Materials and Methods. Animals received either vehicle (0.9% saline) or (±)candoxatrilat (3 mg/kg i.v.) 10 min prior to injection of ANF 5-28. Curves were constructed using $N > 5$ animals per time point, and analysed over the 60–180 sec period (Group A) or the 60–300 sec period (Group B).

* 90% confidence limits of $t_{i\beta}$.

viously [20]. Following (±)candoxatrilat (3 mg/kg i.v.) the $t_{i\beta}$ of ANF 5-28 was extended further (to 468 sec), and Cl_p was reduced, whilst V_d remained unaltered (Table 2B). These data clearly demonstrate that (±)candoxatrilat is able to modulate ANF clearance independent of glomerular filtration rate, and reveal an important extra-renal site of action for the compound. Interestingly, the reduction in Cl_p following (±)candoxatrilat in nephrectomized rats appears to be of similar magnitude to that observed in sham-operated animals (i.e. 60–80 mL/min/kg) indicating that the renally-located atriopeptidase, whilst making an important contribution to ANF metabolism within the kidney tubule, plays a relatively minor role in the clearance of ANF from plasma. We conclude that, in addition to its effects on the kidney enzyme, (±)candoxatrilat attenuates ANF metabolism by inhibiting atriopeptidase in other regions of the cardiovascular system. For example, enzyme localized on the plasma membrane of the neutrophil [21], or vascular endothelium [22] may be important sites of metabolism *in vivo*, and the possibility that (±)candoxatrilat may inhibit the enzyme at these sites merits further investigation.

In summary, we have demonstrated that in anaesthetized rats, (±)candoxatrilat reduces the clearance of both ^{125}I-ANF and ANF 5-28, and prolongs the elimination half-life. These effects occur independent of glomerular filtration, since they are not attenuated by nephrectomy. Moreover, they cannot be attributed to blockade of ANF-C receptors. Our findings suggest that (±)candoxatrilat exerts its activity *in vivo* by specific inhibition of atriopeptidase at both renal and extra-renal locations.

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Fluctuation of fetal rat hepatic histidine decarboxylase activity through the glucocorticoid-ACTH system

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In 1960, Kahlson and coworkers [1] reported that large amounts of histamine are produced and released into the general circulation by the action of hepatic L-histidine decarboxylase (HDC) in fetal rat near the approach to parturition. In an attempt to determine the physiological action of histamine, Taguchi *et al.* first purified and characterized fetal rat hepatic HDC [2], and very recently Joseph *et al.* followed the characterization and expression of cDNA for this enzyme [3]. However, the mechanism regulating HDC synthesis, as well as HDC fluctuation, in fetal rat liver is unknown. We previously reported that glucocorticoids stimulate a *de novo* synthesis of HDC from mastocytoma P-815 cells [4] and glandular stomachs of rats [5]. Furthermore, Liggins *et al.* [6] reported that the fluctuation of the cortisol concentration in fetal lamb plasma is ACTH dependent in late pregnancy. These results encouraged us to determine whether the observed fluctuation of fetal rat HDC was associated with a change