Binding of endothelin to plasma proteins and tissue receptors: effects on endothelin determination, vasoactivity, and tissue kinetics

Friedrich Brunner[a,*], Heike Stessel[a], N. Watzinger[b], Bernd-Michael Löffler[c], Lionel H. Opie[d]

[a]*Institut für Pharmakologie und Toxikologie, Universität Graz, Universitätsplatz 2, 8010 Graz, Austria*
[b]*Medizinische Universitätsklinik, Universität Graz, A-8036 Graz, Austria*
[c]*F. Hoffmann-La Roche, Ltd., Preclinical Research, CH-4002 Basel, Switzerland*
[d]*University of Cape Town Medical School, Ischaemic Heart Disease Research Unit, Cape Town, South Africa*

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Abstract **In vitro binding of (3-[^{125}I]Tyr)-endothelin-1 ([^{125}I]ET-1) and (3-[^{125}I]Tyr)-big ET-1(1–38) ([^{125}I]big ET-1) to plasma proteins of healthy humans, cardiac patients and normotensive and hypertensive rats was investigated by equilibrium dialysis. Binding of both tracers was similar in plasma from healthy humans, patients with congestive heart failure, and following myocardial infarction (~60%), and marginally higher in rat plasmas (~70%). Binding of [^{125}I]ET-1 to human plasma could be explained by binding to human serum albumin. Endogenous plasma ET-1 levels were ~9 pg/ml in healthy humans, and ~12–16 pg/ml in cardiac patients; big ET-1 concentrations were approximately two- to threefold higher. ET-1 bound to plasma protein was partly lost in column extraction. In rat isolated perfused hearts, the coronary dilator and constrictor potency of exogenous free and albumin-bound ET-1 was similar, whereas the kinetics of endogenous ET-1 was impeded by tight binding to ET receptors. The data indicate that binding of ET-1 to plasma proteins is without effect on peptide vasoactivity, but binding to tissue receptors greatly impedes its tissue kinetics.**

Key words: Plasma protein binding; Endothelin-1; Big endothelin-1; Vasoactivity; Tissue kinetics; Congestive heart failure; Myocardial infarction

1. Introduction

The endothelins (ET-1, ET-2 and ET-3) are a family of polypeptides with potent local actions [1,2]. ET-1 stimulates the release of several vasoactive hormones, including atrial natriuretic peptide, aldosterone and catecholamines [3]. The ET-1 concentration in the peripheral circulation is in the order of several pg/ml, but elevated levels have been observed in a number of different disease states, including congestive heart failure [4,5], myocardial infarction [6], and hypertension [7]. However, neither the causes nor the significance of elevated plasma ET-1 concentrations are clear. The increases may reflect pathophysiological changes or simply mark the progression of endothelial cell damage.

Because the extent of drug binding to plasma protein influences the interpretation of total drug concentrations [8], we hypothesized that the knowledge of plasma protein binding of ET-1 and big ET-1 in healthy subjects and cardiac patients may be of interest in the interpretation of the plasma levels of these peptides. Therefore, the aim of the present study was (i) to investigate the binding of ET-1 and big ET-1 to plasma of healthy humans, patients with heart failure or myocardial infarction, and of normotensive and hypertensive rats and (ii) to determine the effect of plasma binding of ET-1 on its vasoactivity. An additional aspect included possible effects of protein binding on the determination of ET-1 in native plasma.

2. Materials and methods

2.1. Materials

(3-[^{125}I]Tyr)-endothelin-1 and (3-[^{125}I]Tyr)-big ET-1(1–38) were purchased from Anawa Trading (Wangen, Switzerland). Bovine serum albumin (99% purity) and human serum albumin (from fraction V, essentially fatty acid free) were from Sigma Chemie (Vienna, Austria). SB 209670 ((1*RS*,2*RS*,3*RS*)-3-(2-carboxymethoxy-4 -methoxyphenyl)-1-(3,4-methylenedioxyphenyl)-5(prop-1-yloxy)indane-2-carboxylic acid)) was a gift from the Dagenham Research Centre (Dagenham, England). Extraction was performed on silica-C2 Ethyl Cartridges (Spe-ed, 500 mg, Inovex, Vienna, Austria). Blood (5–7 ml) from healthy human volunteers (mean age 26.3 ± 1.2 years) and cardiac patients suffering from congestive heart failure (mean age 59.5 ± 2.5 years; primary causes: idiopathic and ethylic cardiomyopathy) or myocardial infarction (mean age 79.3 ± 4.6 years; maximal delay between insult and blood collection: ~7 h) was collected in K^+-EDTA-tubes, immediately centrifuged, the plasma separated and stored at −70°C pending analyses. Plasma from normotensive Sprague–Dawley (S-D) and Wistar-derived (WAG) rats (both normotensive) and genetically hypertensive (GH) rats [9] was obtained from the thoracic cavity following removal of the heart for another purpose. All rats were bred locally.

2.2. Equilibrium dialysis

All experiments were performed with an equilibrium dialysis system (Dianorm) at 37°C using teflon cells of 0.2 ml half cell volume as previously described [10]. Equilibrium time was 7 h (ET-1) and 17 h (big ET-1), respectively. The buffer cell was filled with 0.2 ml [^{125}I]ET-1 or [^{125}I]big ET-1 as tracer (10,000–12,000 cpm) dissolved in dialysis buffer of the following composition (mM): $NaH_2PO_4 \times 1$ H_2O 15, $Na_2HPO_4 \times 2$ H_2O 85, NaCl 50, Triton X-100 0.1%; pH 7.4). The second half cell was filled with the respective protein solution (plasma, bovine or human serum albumin). Following freezing and thawing, the pH of rat plasmas varied between 6.0 and 8.1 which was without effect on protein binding. Non-specific binding to the dialysis membrane (Thomapor, Reichelt Chemietechnik, Heidelberg, Germany); mol. weight cutoff: ~15,000 Da) amounted to 6% and was neglected in the calculations. The radioactivity in both cells was determined by counting 100 μl of each cell in a Gamma-counter (Packard Canberra, Vienna). Binding percentages were calculated according to the formula: % bound = $[(B-A)/B] \times 100$ with B being the molar concentration of the tracer in the protein compartment, and A in the protein-free compartment [10]. When filled with plasma, the volume of cell B increased up to 20% (ET-1) and 30% (big ET-1) due to osmosis which was taken into account in the calculations.

2.3. Determination of ET-1, big ET-1, and serum albumin in plasma

Thawed plasma was centrifuged, diluted 1:10 with RIA buffer and passed over C2-columns activated with ethanol (2 ml) and water (5 ml).

*Corresponding author. Fax: (43) (316) 323-5414.

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After washing ET-1 was eluted with 70% acetonitrile (2 ml), the eluate freeze-dried, and the residue taken up in 400 μl RIA buffer. Immunoreactive ET-1 and big ET-1 were determined by specific RIAs as previously described [11,12]. The serum albumin content of human plasmas was determined in a local clinical laboratory using conventional techniques.

2.4. Functional experiments

The effect of plasma protein binding on ET-1-induced vasodilation and vasoconstriction was studied in rat isolated hearts perfused at constant flow as described previously [13]. Concentration response curves of ET-1 (1–300 pg/ml) were established in the absence and presence of bovine serum albumin (40 g/l) using separate hearts. ET-1 solutions were incubated with bovine serum albumin for 10 min at 37°C prior to infusion into the heart.

2.5. Effect of ET-1 tissue binding on ET-1 release

The effect of binding of ET-1 to tissue ET receptors on ET-1 tissue kinetics was assessed by blocking ET receptors with the non-selective ET receptor antagonist, SB 209670 [14]. The blocker was infused with the perfusion buffer, and release of endogenous ET-1 into the coronary circulation and the interstitial fluid measured as described previously [13].

2.6. Statistics

All binding experiments were carried out in quadruplicate the determinations of ET-1 and big ET-1 plasma levels in triplicate, and the determinations of plasma albumin in duplicate. In all cases, the mean was determined and counted as 1 determination (n). Statistical differences were analyzed by unpaired two-tailed Student's *t* test. A *P*-value < 0.05 was considered significant.

3. Results

ET-1 binding data, ET-1 plasma concentrations, and albumin plasma concentrations for humans and rats are summarized in Table 1. Following addition of [^{125}I]ET-1 (final concentration ~5 pM) to human or rat plasma, ~60% were bound in human plasma, and ~70% in rat plasma. There was no difference between healthy human males and females, nor between patients and controls. The binding of ET-1 to rat plasma was independent of rat strain and blood pressure. Compared to healthy controls, plasma ET-1 levels were 1.7 times higher in patients with heart failure and 1.3 times higher in patients with myocardial infarction ($P < 0.05$). In rats, plasma levels were similar in all groups studied. Plasma albumin concentrations were 20% lower in both patient groups than healthy individuals ($P < 0.05$) and the albumin content of rat plasma was even lower, as reported previously [15]. When [^{125}I]ET-1 was dialyzed against human serum albumin (2–50 g/l), binding increased from 11.2 ± 5.9 to 68.0 ± 2.5% of total (Fig. 1).

Table 1
Plasma binding of [^{125}I]ET-1, plasma ET-1 concentration and plasma albumin concentration in humans and rats

	[^{125}I]ET-1 bound (% of total)	plasma ET-1 (pg/ml)	Plasma albumin (g/dl)	*n*
Human				
male	58.5 ± 1.3	9.2 ± 0.2	5.2 ± 0.1	5
female	58.0 ± 1.5	8.8 ± 0.2	4.9 ± 0.1	5
CHF	55.6 ± 0.8	15.7 ± 1.8*	4.1 ± 0.1*	14
MI	62.0 ± 2.4	12.1 ± 0.7*	4.1 ± 0.2*	7
Rats				
S-D	69.4 ± 0.9	9.0 ± 0.1	2.9 ± 0.1	11
WAG	70.6 ± 0.9	9.4 ± 0.1	n.d.	5
GH	71.7 ± 1.2	9.2 ± 0.1	n.d.	5

Binding to plasmas was determined by spiking with [^{125}I]ET-1 (~10,000 cpm) and levels of plasma ET-1 were determined by RIA as described in section 2. Probands were either healthy (males, females) or suffered from congestive heart failure (CHF) or myocardial infarction (MI). Rats were either normotensive (Sprague–Dawley, S-D; Wistar-derived, WAG) or genetically hypertensive (GH). n.d., not determined. Data are means ± S.E.M. of *n* plasmas assayed in each group. *$P < 0.05$ vs healthy controls (males).

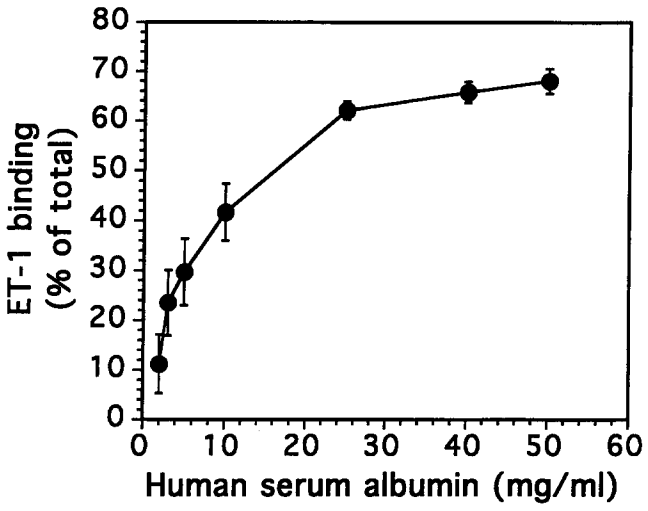

Fig. 1. Concentration-dependence of ET-1 binding to human serum albumin. Solutions of purified albumin (2–50 mg/ml) were dialyzed against phosphate buffer spiked with [^{125}I]ET-1. Data are mean values ± S.E.M. of 3 determinations in quadruplicate.

Binding of [^{125}I]big ET-1 (~5 pM) to human and rat plasma and big ET-1 plasma concentrations are shown in Table 2. Again, binding percentages were similar for all groups. Binding of [^{125}I]big ET-1 to plasma from WAG and GH rats was slightly higher than binding to plasma from S-D rats ($P < 0.05$). Big ET-1 plasma concentrations were similar in healthy human males and females (mean: 24.9 ± 0.7 pg/ml), and increased 1.8-fold in heart failure and 1.4-fold in infarct patients ($P < 0.05$). Big ET-1 levels in rats were clearly dependent on rat strain and blood pressure: in both normotensive WAG and hypertensive rats, big ET-1 levels were only one half of those in S-D rats ($P < 0.05$; Table 2).

The effect of plasma protein binding on ET-1 extraction during plasma work-up is summarized in Table 3. Human plasma spiked with [^{125}I]ET-1 was dialyzed as above, and the contents of dialyis cells A (non-bound fraction) and B (plasma protein-bound plus free fraction) were chromatographed separately. In agreement with our previous determinations [13], ~90% of radioactive peptide loaded onto columns was recovered. However, whereas unbound (free) label was in toto contained in the elution phase, with no radioactivity appearing in the wash phase prior to elution, part of [^{125}I]ET-1 (~15%) was lost with the wash phase, when post-dialysis plasma containing albumin-bound ET-1 was chromatographed. Therefore, to determine the recovery of plasma-borne ET-1 in column chromatography correctly, radioactive tracer must be added to a plasma sample and not to buffer as done frequently.

To determine the effect of plasma protein binding of ET-1 on its vasodilator and vasoconstrictor potency, ET-1 was infused into rat hearts in the absence and presence of albumin. In the absence of albumin, ET-1 concentrations between ~1 and ~20 pg/ml were vasodilatory, whereas higher concentrations

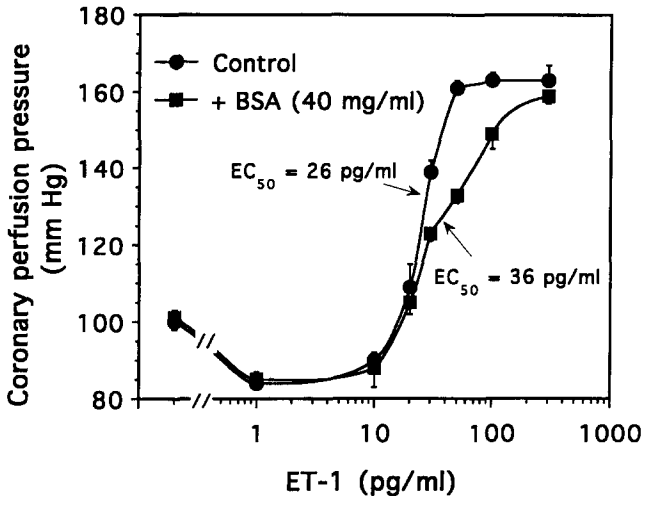

Fig. 2. Effect of bovine serum albumin (BSA) on vasodilator and vasoconstrictor effects of ET-1 in isolated perfused hearts. Initial coronary perfusion pressure was raised to 100 mmHg by perfusing at a higher than normal rate. BSA was added to ET-1 solution 10 min prior to experiment. Data are mean values ± S.E.M. of 3 determinations each.

constricted the coronary vessels resulting in an increase in coronary perfusion pressure up to ~160 mmHg (Fig. 2). However, following incubation of the peptide with serum albumin, presumably resulting in binding of ET-1 to the protein, the vasodilatory component was unaffected, but the vasoconstrictor component was blunted, resulting in a slight increase in the EC_{50} value. Serum albumin alone (vehicle) had no effect on either component (data not shown).

Finally, the effect of tissue binding of ET-1 on ET-1 release from isolated perfused hearts was investigated (Fig. 3). Under control conditions, only small amounts of ET-1 were released into the coronary effluent over 255 min of perfusion. However, when the mixed ET_A-ET_B receptor antagonist SB 209670 (5 μM) was infused with the perfusion buffer, presumably resulting in time-dependent blockade of endothelial and myocardial ET receptors, ET-1 release was steadily augmented between 2.3-fold (30 min of perfusion with antagonist) and 8-fold (3.5 h of perfusion with antagonist). Over the same time course, ET-1 secretion into interstitial fluid was always much lower than that into coronary effluent and increased only 2–3-fold (data not shown).

4. Discussion

The main goal of this study was to assess the effects of ET-1 plasma binding on its vasodilator and vasoconstrictor activity and to obtain information on the effect of plasma binding of ET-1 on the determination of ET-1 plasma concentration. Another aim was to determine whether plasma protein binding of ET-1 and big ET-1 was different in cardiac patients and hypertensive rats compared to controls and whether such differences may explain increased peptide levels found in these conditions.

Following standard techniques [16], in vitro binding of ET-1 and big ET-1 in human plasma of healthy control subjects and cardiac patients was of only moderate extent, which indicates that no major effects on plasma-derived kinetic parameters, e.g. plasma clearance of exogenous or endogenous peptide are to be expected [17]. In rat plasma, binding of both ET-1 and big ET-1 was higher than in human plasma, a species difference observed previously with a variety of drugs and endogenous substances. In human, and presumably also rat plasma, binding to the albumin molecule probably accounted for most binding in plasma. Serum albumin is involved in the binding and transport of many endogenous and exogenous substances and is an important factor in the pharmacokinetics of a variety of drugs [18]. The higher binding percentage of ET-1 to rat plasma at a significantly lower plasma albumin concentration compared to human plasma (Table 1) may indicate that one or more other binding proteins of lesser importance are involved, or that the binding affinity to rat albumin is higher. This also applies for the ET-1 binding data obtained with the plasmas of cardiac patients (Table 1) and the big ET-1 binding data obtained with plasmas from congestive heart failure patients (Table 2). The nature of the binding interaction was not studied, but is presumably similar to the interaction of serum albumin with other polypeptide hormones.

The present study has confirmed previous reports which showed that plasma ET-1 and big ET-1 levels are increased in humans with failing hearts or following myocardial infarction. Due to the lack of comparable analytical procedures among laboratories and definition of a range of normal values, the significance of increased ET-1 and big ET-1 levels in these disease states is obscure. The present results show that plasma protein binding of both peptides is not increased in cardiac patients compared to controls and, therefore, the higher total plasma concentrations in the patients cannot be explained with a lower free fraction (total concentration equals free concentration/free fraction) [8]. Rather, the higher total peptide concentrations are a true reflection of increased synthesis and/or decreased removal in cardiac patients.

Of importance is the observation that binding of ET-1 to albumin had no effect on its potency as a vasodilator. Clearly, albumin-bound ET-1 is capable of activating ET receptors, probably because the binding affinity to the albumin molecule is much lower (presumably in the micromolar range) than the affinity to ET receptors (nanomolar range or lower) [19]. An insufficient time of contact (10 min) between the low (vasodilatory) concentrations of ET-1 and albumin which would lead to incomplete binding to the protein is unlikely to account for this,

Table 2
Plasma binding of big [^{125}I]ET-1 and plasma big ET-1 concentration in humans and rats

	[^{125}I]big ET-1 bound (% of total)	plasma big ET-1 (pg/ml)	*n*
Human			
male	65.0 ± 1.1	23.7 ± 0.8	5
female	n.d.	26.1 ± 0.7	5
CHF	58.4 ± 2.2	44.0 ± 7.2*	5
MI	59.1 ± 1.4*	33.8 ± 4.6*	6
Rats			
S-D	71.1 ± 0.9	45.9 ± 9.0	11
WAG	76.2 ± 1.1*	19.4 ± 0.5*	5
GH	77.6 ± 0.9*	21.3 ± 0.9*	5

Binding to plasma was determined by spiking with big [^{125}I]ET-1 (~10,000 cpm). For other details, see legend to Table 1. *$P < 0.05$ vs healthy controls (males) and S-D rats, respectively.

because the binding reaction is generally complete after several seconds. The reasons for the slight, albeit significant non-parallel displacement of the vasoconstrictor portion of the ET-1 concentration response curve are not clear.

Of great significance is our observation that the rate of apparence of ET-1 in the coronary effluent was considerably increased following infusion of SB 209670, a non-peptide ET receptor antagonist, compared to control rates in the absence of blocker. One of us has previously shown that the coronary endothelium of isolated perfused rat hearts releases ET-1 both into the coronary effluent and the cardiac interstitial fluid, but the rate of release into the coronary lumen was much higher than that towards the interstitium [13]. The present data show that ET-1 is probably actually released preferentially to the abluminal side of endothelial cells, followed by tight binding to and time-dependent saturation of ET receptors of cardiomyocytes and/or endothelial cells. In the presence of the antagonist SB 209670, however, the binding capacity of tissue receptors was greatly reduced, resulting in diffusion of the peptide back into the coronary lumen. Because occupation of the receptors by antagonist is time-dependent, the rate of appearance of ET-1 in the coronary lumen increased steadily (Fig. 3). Thus, our previous observations of increased plasma levels of endogenous ET-1 in rats following administration of the antagonist Ro 46-2005 [20] or bosentan [21] are most likely due to displacement of ET-1 from tissue receptors. The present data also support the model of stoichiometric binding of ET-1 to receptors according to which most of tissue ET-1 is bound to receptors with consequent low levels of free peptide both in tissues and plasma [22].

Finally, the present study shows that binding of ET-1 to serum albumin also affects the determination of ET-1 in human plasma. We found that ~15% of ET-1 added to the plasma compartment was contained in the breakthrough phase and the following wash phase, whereas for the dialysis buffer compartment which contained only unbound peptide, no ET-1 was measured in these phases. Therefore, plasma protein binding impedes adsorption of ET-1 onto the column prior to elution, resulting in reduced recovery. In part, this may also be reflected in the lower plasma big ET-1 concentrations in Wistar-derived and genetically hypertensive rats compared to Sprague–Dawley rats (Table 2) due to higher binding in the former two groups. The loss is probably still greater when using more hydrophobic columns (C18) for which extraction efficiency is frequently only 60–70% for free peptide [23]. Therefore, standardized extraction and assay procedures need to be established for a meaningful interpretation of ET-1 and big ET-1 plasma levels.

Table 3
Effect of plasma protein binding on column chromatography of ET-1

	Dialysis cell A		Dialysis cell B	
	cpm	%	cpm	%
cpm added onto column	8076 ± 201	100	20657 ± 329	100
Recovery in wash phase	36 ± 36	0.4 ± 0.4	3120 ± 475	15.1 ± 2.3
Recovery in eluate	7268 ± 258	90.0 ± 2.9	15586 ± 844	75.4 ± 3.7
Total recovery	7303 ± 259	90.4 ± 3.1	18723 ± 787	90.6 ± 3.3

Plasma of healthy probands was spiked individually with ~10,000 cpm [^{125}I]ET-1 and dialyzed in quadruplicate. Part of the contents (100 μl) of dialysis cell A (buffer containing unbound peptide) and B (plasma containing unbound and bound peptide) of each replicate (total volume: 400 μl) were added separately onto preconditioned C2-columns, followed by washing of columns and elution of peptide. Data are means ± S.E.M. of 8 plasmas assayed.

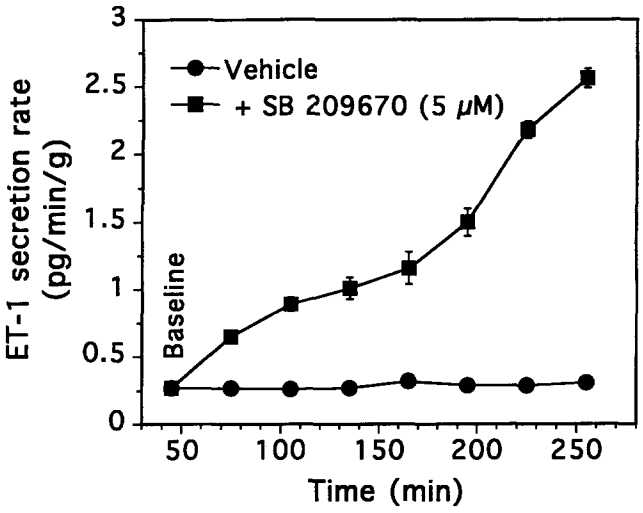

Fig. 3. Effect of blockade of ET receptors on secretion of endogenous ET-1 into coronary effluents of isolated perfused hearts. Following establishement of baseline conditions (45 min), hearts were perfused with vehicle or SB 209670 and ET-1 secretion rate determined by RIA. Mean values ± S.E.M. of 3 determinations each.

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