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INHIBITION OF (−)[^{125}I]-IODOCYANOPINDOLOL BINDING TO RAT LUNG BETA ADRENOCEPTORS BY UREMIC PLASMA ULTRAFILTRATES

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Abstract—Numerous data have suggested that beta-adrenoceptor-mediated responses were decreased in uremia and that parathormone could be implicated in this phenomenon. In a previous paper we have shown that the beta$_2$ receptor density of mononuclear cells of uremic patients is significantly increased despite a significant increase in plasma epinephrine, suggesting that an endogenous substance could interfere and disregulate the beta$_2$ receptor density. In order to further evaluate this phenomenon we have firstly studied the influence of one non uremic and five uremic plasma ultrafiltrates on the binding of (−)-[^{125}I]iodocyanopindolol using rat lung beta adrenoceptors. The results show that uremic plasma ultrafiltrates induce a decrease in the B_{max} value without any variation on the K_d value.

In a second step we have assessed the ability of human synthetic 1–34 and 53–84 parathormone to interact directly with beta-adrenoceptors. No variation in the (−)-[^{125}I]iodocyanopindolol binding parameters was observed.

These results suggest that an uremic endogenous substance might interfere on the beta adrenergic receptors and that the alteration in the beta-adrenergic response in uremia is probably not due to a direct action of parathormone on the beta-adrenoceptors.

In both uremic patients and animals with experimental uremia, abnormalities in sympathetic nervous system function have been reported [1, 2]. Uremia is characterized by a decrease in beta adrenoceptors mediated responsiveness [3–5]. *In vitro* studies have demonstrated that the depressant effect of propranolol on myocardial contractile force was significantly inhibited in the presence of ultrafiltrates of uremic patients with severe hyperparathyroidism [4, 5]. This leads to the hypothesis that an endogenous substance produced in uremia might interfere with the beta-adrenergic response.

In previous data [6], it has been shown that the uremic state is associated with elevated epinephrine plasma levels concomitant with an unexpected increase of the beta$_2$-adrenergic receptor density on mononuclear cells. This phenomenon compared with what is mostly observed in normal subjects during beta-blocking treatment with respect to catecholamines [7, 8] and beta-adrenoceptors [9, 10] has suggested the role of an endogenous beta-blocking substance in uremia.

Previous studies tend to suggest that parathyroid hormone (PTH) might play a role in the beta-adrenergic response, either indirectly by increasing calcium transmembrane influx [11] or directly via an inhibitory effect on the beta-adrenoceptors [12, 13].

The use of radiolabeled antagonists of high specificity and activity has allowed the development of radioligand binding assays for detailed study of receptor function [14]. This study was performed using (−)-[^{125}I]iodocyanopindolol [(−)^{125}ICYP], characterized by its specificity for beta receptors, its high affinity constant and lack of either beta$_1$ or beta$_2$ subtype selectivity [15, 16].

In order to establish the presence or absence of an endogenous, beta-interfering substance in the uremic patients we have first compared the (−)^{125}ICYP binding parameters on beta-adrenoceptors of rat lung membranes well known to possess high density in beta$_2$-adrenoceptors [17], when incubated with uremic plasma ultrafiltrate to those determined in the same conditions with a control subject ultrafiltrate. As reference, these binding parameters have been previously determined when rat lung membranes were incubated in a physiological buffer.

In a second study, the effect of PTH on the (−)^{125}ICYP binding capacity has been assessed by incorporating different concentrations of synthetic human PTH into the physiological medium.

PATIENTS AND METHODS

Five normotensive (diastolic blood pressure ≤90 mm Hg) patients, chronically uremic maintained by intermittent haemodialysis for varying lengths of time (Table 1) were investigated. None of the uremic patients had cardiovascular disease, as detected by physical examination and echocardiography. They were dialysed 3 × 6 hr weekly. Although none of them had advanced secondary hyperparathyroidism all had high plasma PTH levels (Table 1). The patients were receiving only phosphate binding agents and vitamin D.

As control, a healthy volunteer was included in the study. All the subjects granted their informed consent.

After a 6 hr fast, without smoking and xanthined beverage for 12 hr and bed rest of 1 hr, 100 ml of plasma ultrafiltrate was obtained *in vivo* from the

Table 1. Clinical characteristics of the control subject and the five uremic patients

Subjects	Age (yr)	SBP (mmHg)	DBP (mmHg)	Duration of treatment (months)	PTH plasmatic (mUI/ml)
Control	31	120	80	0	2.1
U_1	45	125	70	70	9.4
U_2	24	110	61	38	13
U_3	54	132	66	114	11.5
U_4	72	140	90	6	7.5
U_5	71	140	90	6	5.8

SBP: systolic blood pressure; DBP: diasolic blood pressure.

uremic patients and the normal subject during the first 10 min of haemodialysis, using a Biospal 2400 kidney equipped with its highly permeable polyacrylonitrile membrane (AN 69). Thus a pure plasma ultrafiltrate was collected from the outflow of the dialysis compartment. The uremic patients were dialysed not less than 48 hr after the previous dialysis treatment. The ultrafiltrates were stored at −80° until used. Epinephrine (E), norepinephrine (NE), PTH, sodium, potassium, calcium, phosphorus, chloride, creatinine and urea levels were determined in the ultrafiltrates. Before starting the haemodialysis session, 10 ml of blood were collected in order to determine plasma PTH levels.

Determination of ultrafiltrate catecholamines. Catecholamines were measured according to Brown and Jenner [18] by double isotopic enzymatic assay with a sensitivity of 1.5 pg/ml for both E and NE. The coefficients of variation were 3.9% (80 ± 2.6) and 3.1% (420 ± 13) for E and NE respectively (intra assay) and 4.5% and 4.2% (inter assay). However, the coefficient of variation for low values of E was slightly greater, i.e. 10% (25.1 ± 2.5).

Plasma and ultrafiltrate PTH determinations. PTH was determined by radioimmuno-assay with an anticarboxyl terminal antibody according to Franchimont *et al.* [19] with a sensitivity of 1 mUI/ml. The coefficients of variation were 20.2% (17.5 ± 3.5) and 23.6% (1.9 ± 0.4) for high and low values respectively (intra-assay) and 21.9% (10.6 ± 2.2) and 9.4% (3 ± 0.2) (inter-assay).

Biochemical ultrafiltrate assays. Sodium, potassium and chlore were assayed using flame photometry. The phosphor determination was based on the measurement of the unreduced phosphomolybdate complexes using the Beckman phosphorus reagent kit. Urea and creatinin were quantitated with a Beckman analyser II. Calcium was titrated using the methymol-blue method with the calcium kit Bio-Merieux.

Preparation of the rat lung membranes. Male Wistar rats (150 g body weight) were anesthetized with 5 mg/100 g weight pentobarbital and killed by decapitation. Lungs were rapidly excised and cleaned in ice-cold isotonic buffer. The lungs were homogenized at 4° with an Ultra-Turrax® (IKA-WERK) (3 × 5 sec) in TSE medium (buffer A) (Tris–HCl 20 mM, sucrose 0.25 mM and EDTA 1.0 mM, at pH 7.4). Homogenates were centrifuged according to the method of Paris [20]. The crude membrane pellet was diluted in buffer B (NaCl 140 mM, KCl 5.4 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 0.8 mM and Tris–HCl 50 mM, pH 7.4).

The protein concentration was measured by the Lowry method [21]. Membranes were then frozen at −80° until used.

Binding experiments and data analysis. Direct beta adrenergic receptor binding assays were performed in the buffer B, as reference and in plasma ultrafiltrate of both uremic patients and the normal subject. The ultrafiltrates and buffer B were adjusted to pH 7.4 at 4°. In all assays the membranes were at a final concentration of 0.05 mg of protein per ml. Nineteen different concentrations of $(-)^{125}$ICYP ranging from 10^{-12} to 6 10^{-10} M with or without 10^{-6} M (±) propranolol were used in a final volume of 0.5 ml.

In a second step, in order to determine the efficacy of three different uremic plasma ultrafiltrates in inhibiting $(-)^{125}$ICYP specific binding, samples were incubated with increasing concentrations of ultrafiltrates (uremic ultrafiltrates were diluted with the control ultrafiltrate until a dilution of 1/256).

Lastly, direct binding assays were performed in a PTH buffering solution. Synthetic human 1–34 and 53–84 PTH (Sigma, St. Louis, MO) was added to the medium B in order to achieve a concentration of 0.05, 0.1, 1, 10 and 50 ng/ml. All the analyses were performed at 4° and equilibrium was achieved after 18 hr. This low temperature was chosen in order to prevent proteolysis, when the incubating medium was the plasma ultrafiltrate or PTH buffering solution. When equilibrium was reached samples were rapidly filtered under vacuum through Whatman GF/F glass fiber filters. Each filter was washed twice with 10 ml of buffer B. Radioactivity was determined in a scintillation spectrometer (Packard Tricarb 460 CD). For direct specific study, binding was defined as total bound radioactivity minus the radioactivity not displaced by 10^{-6} M propranolol. Each value was the mean of three measurements. The two parameters B_{max} (the binding capacity) and K_d (the dissociation constant) were estimated using a method described previously [22]. It consisted of fitting simultaneously total and non-specific binding data. Non-linear regression, based upon a uniformly weighted sum of squares, was performed using a Gauss Newton iterative minimization method.

RESULTS

The biochemical parameters obtained with plasma ultrafiltrates from the uremic patients are compared with those of the control subject and the buffer medium in Table 2. There is no evident difference in the composition of the electrolytes, except the absence of phosphorus, urea and creatine in the buffer medium. Epinephrine concentrations were variable, mostly higher in uremic subjects compared to the control. Norepinephrine plasma ultrafiltrate levels in the uremic patients and the control subject were in the same range. As expected, PTH, creatinine and urea were higher in the uremic patients compared to the control subject. Plasma PTH levels are also

Table 2. Biochemical characteristics of the buffer, the plasma ultrafiltrate of the control subject and uremic plasma ultrafiltrate

	Na^+ (mM)	K^+ (mM)	Ca^{2+} (mM)	Ph (mM)	Cl^- (mM)	E (ng/ml)	NE (ng/ml)	PTH (mUI/ml)	Urea (mM)	Creat (μM)
Buffer	140	5.4	1.80	0	149	0	0	0	0	0
Control	136	3.5	1.65	1.25	103	0.054	0.395	<1	7.50	70
U_1	147	5.6	1.60	1.65	95	0.078	0.223	5	14.20	340
U_2	147	5.6	1.60	2.15	116	0.129	0.506	9	24.70	1100
U_3	143	5.1	1.55	1.25	113	0.089	0.846	3.7	19.40	650
U_4	144	4.3	1.45	2.20	111	0.052	0.301	3.7	18.70	630
U_5	153	3.9	2	0.90	104	0.178	0.520	1	49.40	850

Various ions were measured: phosphorus (ph) epinephrine (E) norepinephrine (NE), the carboxyl terminal fraction of PTH, urea and creatinin.

higher in the uremic patients (Table 2). The (−)^{125}ICYP binding parameters, K_d and B_{max}, determined in the buffer medium, and in the normal subject ultrafiltrate are similar (Table 3). Surprisingly, as shown in Table 3, the beta adrenoceptors characteristics are very different when the rat lung membranes are incubated with uremic plasma ultrafiltrates.

All seven curves were treated simultaneously, either with a common B_{max} value and individual K_d values or with a common K_d value and individual B_{max} values in order to determine if the alteration of one parameter was predominant to the other or if both parameters were altered. The model was selected according to a goodness of fit criterion based on the correlation coefficient r between observed and computed binding values. All data were analyzed with a uniform weighting where all weights were set equal to one. Best fits were observed for the model where all curves were assumed to have the same K_d value ($1.72\ 10^{-11}$ M) and different B_{max} values (Fig. 1, Table 4). Compared to the control, a 60% to 90% decrease in receptor density was observed after incubation in uremic ultrafiltrates.

Figure 2 shows that increasing ultrafiltrate concentrations inhibited the specific binding of (−)^{125}ICYP to rat lung beta adrenoceptors and confirms the precedent results. Inhibition of the binding of (−)^{125}ICYP appeared to follow a simple inhibition with a pseudo Hill coefficient (nH) of approximately 1 (Fig. 2).

No correlation was found between the decrease of the beta adrenergic receptors density and the urea, creatinine or PTH ultrafiltrate levels.

Table 3. Individual characterisation of the binding parameters, K_d and B_{max}, of (−)^{125}ICYP on rat lung beta-adrenoceptors, with buffer, control and uremic plasma ultrafiltrates as incubating medium

	B_{max} (fmol/mg of protein)	K_d (10^{-11} M)
Buffer	472.13 ± 11.65	1.49 ± 0.24
Control	451.49 ± 23.03	0.74 ± 0.28
U_1	78.71 ± 11.65	10.97 ± 3.62
U_2	251.09 ± 25.81	4.86 ± 1.36
U_3	236.64 ± 32.11	3.75 ± 1.55
U_4	82.55 ± 28.13	3.90 ± 2.51
U_5	75.67 ± 20.65	7.33 ± 4.92

When synthetic human PTH was added in the medium B no variation in B_{max} or K_d were observed.

DISCUSSION

The striking difference observed between the (−)^{125}ICYP binding capacity of the beta-adrenergic receptors, when incubated in the normal subject ultrafiltrate compared with that in the uremic ultrafiltrates is an experimental fact.

Differences in the electrolytes composition and epinephrine levels are irrelevant, since the binding parameters of the (−)^{125}ICYP determined in the normal subject ultrafiltrate, did not differ from those obtained in the physiological medium B. Thus the results observed between the uremic and the normal subject ultrafiltrate are probably linked to the uremic state. These results demonstrate that plasma uremic ultrafiltrates may possess an endogenous substance able to non competitively antagonize the binding of (−)^{125}ICYP on the beta adrenergic receptors. This substance is dialysable but its effects on the (−)^{125}ICYP binding capacity are not correlated to the urea, creatinine and PTH concentrations of

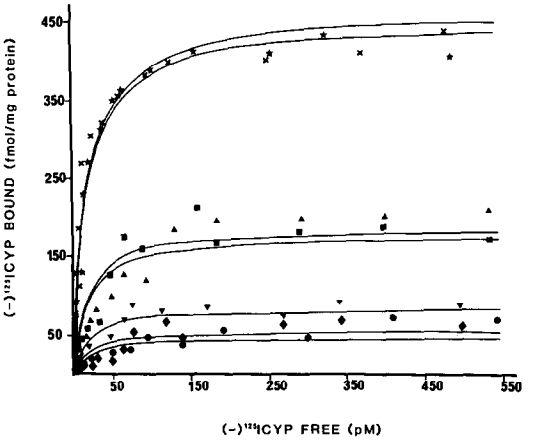

Fig. 1. Simultaneous mathematical treatment of the seven saturating curves of (−)^{125}ICYP on rat lung beta-adrenoceptors with buffer or plasma ultrafiltrates as incubating medium. All the curves have the same K_d value: $1.72\ 10^{-11}$ M and different B_{max} values: ✕, buffer B; ★, control; ●, U_1; ▲, U_2; ■, U_3; ▼, U_4; ◆, U_5 plasma ultrafiltrates.

Table 4. Simultaneous characterisation of the binding parameters, K_d and B_{max}, of $(-)^{125}$ICYP on rat lung beta-adrenoceptors as defined in Fig. 1

	B_{max} (fmol/mg of protein)	K_d (10^{-11} M)
Buffer	448.41 ± 12.18	
Control	461.06 ± 13.76	
U_1	44.80 ± 9.16	
U_2	174.84 ± 9.64	1.72 ± 0.15
U_3	174.95 ± 9.64	
U_4	83.20 ± 10.40	
U_5	53.46 ± 10.06	

uremic plasma ultrafiltrates. Furthermore uremia is characterized by a decrease in beta adrenoceptors mediated responsiveness [3–5] suggesting a decrease in beta-adrenoceptor function. Brodde and Daule [23] have shown that cyclic AMP levels in lymphocytes following isoprenaline stimulation are diminished in patients on maintenance hemodialysis. They have also observed an increase of the $beta_2$-adrenergic receptor density on the lymphocytes, but without statistical significance. This fact was associated with a rise of catecholamine plasma levels. We have shown in a previous study [6] that patients undergoing chronic haemodialysis possess a statistically significant increase in $beta_2$-adrenergic receptors, associated also with high plasma epinephrine levels. These data show that the situation observed in the uremic patients contrasts with the down regulation usually observed in normal subjects [24].

The lack of statistically significancy of the $beta_2$ adrenergic density variation in Brodde's study may be due to the fact that the blood samples were taken

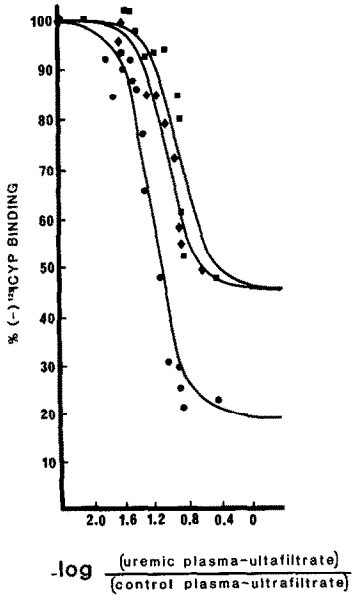

Fig. 2. Displacement curves of $(-)^{125}$ICYP binding on rat lung beta-adrenoceptors with increasing uremic ultrafiltrate concentrations. The dilution from plasma ultrafiltrate varying from 1/256 to 1: ●, U_1; ■, U_3; ◆, U_5 plasma ultrafiltrate.

10–20 hr after the last dialysis, whereas in our study [6] the blood samples were drawn 48–72 hr after the last dialysis. Therefore it is possible that the accumulation of an endogenous substance during uremia modulates the number and response of the beta adrenergic receptors and impairs the down-regulation of the beta-receptors by epinephrine. Accordingly, human subjects treated with beta-antagonists such as propranolol demonstrated mostly up regulation leading to an increase in beta-adrenoceptor density [9, 10, 24]. On the other hand beta antagonists have been demonstrated to increase epinephrine level in normal volunteers [7, 8] suggesting a comparable situation with respect to epinephrine and beta-adrenoceptor up-regulation between uremia and beta blockade in man. Therefore the role of an endogenous beta-blocking agent during chronic renal failure is suggested. According to our results, this endogenous agent seems at least acting on the $beta_2$-adrenoreceptors.

The endogenous uremic substance thought to interfere with the beta-adrenergic response has not yet been clearly identified. Previous studies, however, tend to prove that PTH might play a role either indirectly enhancing the rate of calcium entry into the cell [11] or directly via an effect on beta-adrenoceptor. In fact, *in vitro* studies have demonstrated that the depressant effect of propranolol on myocardial contractile force was significantly inhibited by ultrafiltrates of uremic patients with severe hyperparathyroidism [4–13] and that this effect was reversed after parathyroidectomy [5]. It has been shown that 1–34 and 1–84 synthetic bovine PTH induced a positive dose dependent chronotropic effect [12] and that this effect was not modified by propranolol, suggesting that PTH effect was due to calcium entry into heart cells [25]. Nevertheless, it has been described that 1–34 synthetic bovine PTH significantly inhibited the cardiodepressant effect of the propranolol and when beta-adrenoceptors were blocked with nadolol, PTH no longer inhibited the cardiodepressant effect of propranolol [13]. This leads to the conclusion that PTH probably affects cardiac muscle contraction by at least two mechanisms, one of which involves non-adrenergic transmembrane calcium flux and the second beta-adrenoceptors. In our study, no correlation was found between the PTH levels in the plasma ultrafiltrates and the $(-)^{125}$ICYP binding capacity. Furthermore the addition of 1–34 or 53–84 human synthetic PTH in the physiological medium did not alter the $(-)^{125}$ICYP binding. Thus a direct interaction between PTH and beta-adrenergic receptors does not seem to occur. Nevertheless, in uremic patients the hyperparathyroid state is associated with an altered heart-rate responsiveness to isoprenaline [3] and parathyroidectomy is followed by a significant improvement in left ventricular ejection function [26] indicating that the hyperparathyroid state during chronic renal failure obviously impaired the beta-adrenergic response. Our study and the literature data have two implications. The first concerns the fact that an uremic endogenous substance interferes probably on the beta-adrenergic receptors and may be implicated in the altered regulation and response of the beta-adrenoceptor in uremia. The second is

that PTH does not seem to act directly on the beta-adrenoceptors but contributes indirectly to alter the beta-adrenergic response in uremia.

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