

A Circulating Substance Cross-reacting with Antiimidazoline Antibodies

Detection in Serum in Relation to Essential Hypertension

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Abstract

It has been shown in various mammal species that clonidine, a well known centrally acting hypotensive agent, acts through the activation of imidazoline receptors (IRs) in the nucleus reticularis lateralis (NRL) of the brainstem. Specific binding sites sensitive to imidazolines and insensitive to catecholamines have been detected in rat and bovine, as well as human brains. An endogenous ligand, other than catecholamines, should exist for these IRs. Such a ligand could play a role in the pathophysiology of human essential hypertension. Therefore, we developed two RIAs with polyclonal and monoclonal anticlonidine antibodies. These antibodies presented specificity spectra similar to that of the IRs: they bound imidazolines and not catecholamines at all. These RIAs were used to detect imidazoline-like immunoreactivity in the human serum. Immunoreactive substance was measured in 26 normotensive subjects' sera, and specificity of interaction between antibodies and sera was verified. None of the known endogenous substances tested so far were able to interact with the two antibodies. Immunoreactivity in 32 essential hypertensive patients' sera proved higher in ~ 30% of cases. Values of immunoreactivity positively correlated with the mean arterial pressure values. This study demonstrates the existence of an "imidazoline-like" immunoreactive substance in the human serum with high levels in some hypertensive patients. (*J. Clin. Invest.* 1993. 92:1068-1072.) Key words: imidazoline receptors • endogenous ligand • clonidine • immunoreactivity • radioimmunoassays

Introduction

Clonidine is a well known, centrally acting hypotensive substance that was described as an α_2 -adrenoceptor agonist. However, recently, animal investigations concerning the mechanism of the hypotensive effect of clonidine have led to the discovery of a new class of receptors: the imidazoline receptors (IR).¹ The demonstration of the existence of these IRs and

their involvement in the central control of blood pressure were based on the following observations:

(a) in rats, clonidine hypotensive effect originates in the nucleus reticularis lateralis region in the brainstem (1);

(b) catecholamines stereotactically injected into this region never affected arterial pressure, in contrast with imidazolines, which were hypotensive whatever their selectivity for α -adrenoceptor subtypes (2);

(c) in vivo electrochemical experiments performed in rats, confirmed that the clonidine hypotensive effect originating in the NRL region was related to imidazoline specific receptors and that sedation, the main side effect of clonidine, was due to activation of α_2 -adrenoceptors elsewhere in the brainstem, namely, the locus coeruleus (3);

(d) binding experiments with [³H]clonidine and [³H]-*para*-aminoclonidine (pac) also confirmed besides a classical α_2 -adrenergic binding of these ligands, the existence of specific imidazoline binding sites insensitive to catecholamines both in the brain and in peripheral tissues of various species, including humans, the hypotensive effect of imidazolines being related to the specific imidazoline binding (4-6);

(e) biochemical experiments (7) permitted physical separation of α -adrenoceptors and imidazoline specific binding sites leading to the conclusion that they should be considered as two different molecular entities, at least within the rabbit kidney.

As these IRs are present in the human brain (4), one could assume that a noncatecholamine endogenous ligand could exist also in humans. And if IRs are involved in the regulation of cardiovascular tone in humans as it is in animals, it could therefore also be assumed that a dysfunctioning of the "imidazoline receptor system," concerning the receptor itself, or its endogenous ligand or both, might be associated with certain forms of essential hypertension. In fact, one could suppose that the endogenous ligand of IRs may share certain structural analogies with imidazolines. To test the presence of endogenous imidazoline-like substances, we developed polyclonal and monoclonal antibodies against clonidine with *para*-aminoclonidine (pac) coupled to hemocyanine as the antigen (8, 9). As was the case with IRs, these two antibodies bound imidazoline molecules and not catecholamines at all.

We have reported elsewhere that these antibodies were able to detect immunoreactive substances in bovine brain extracts (polyclonal antibodies; reference 8) and in human brain extracts (monoclonal antibody mFE7; reference 9) containing the putative endogenous ligand for IRs.

Here we compare two radioimmunoassays developed with the polyclonal and monoclonal antiimidazolines antibodies to detect circulating immunoreactive substances in human sera from normotensive and hypertensive subjects.

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1. Abbreviations used in this paper: CDS, clonidine-displacing substance; IR, imidazoline receptor; ng pac eq, nanogram *para*-aminoclonidine equivalent; NRL, nucleus reticularis lateralis; pac, *para*-aminoclonidine.

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Methods

Antibody preparation. Preparation of polyclonal antibodies has been described elsewhere (8). Briefly, *para*-aminoclonidine linked to hemocyanin with glutaraldehyde was injected into rabbits. The resulting specific antisera were characterized: the polyclonal antibodies recognized imidazolines closely related to clonidine and no known endogenous substances tested.

Monoclonal antibody (mFE7) production and characterization has been described elsewhere (9). Despite a lower affinity for free pac and clonidine than the polyclonal antibodies, the specificity was similar: mFE7 recognized iminoimidazolines and no catecholamines or other known endogenous substances tested.

Subjects and patients. Essential hypertensive patients and normotensive subjects were investigated in this study.

Serum (15–20 ml) from 26 normotensive subjects (19 males and 7 females; mean value of age \pm SD = 40.7 \pm 17.4 yr, range: 20–79 yr) were obtained from the local blood donor bank (Centre de Transfusion Sanguine, Strasbourg France) and from volunteers of the laboratory. Blood pressure parameters were: systolic arterial pressure = 116.9 \pm 13.0 mmHg, diastolic arterial pressure = 74.2 \pm 5.8 mmHg, and mean arterial pressure = 88.5 \pm 6.8 mmHg. The series of sera from hypertensive patients was provided by the Hypertension Unit of Broussais Hospital, Paris. It included samples (3–10 ml) from 32 patients with normal renal function (24 males and 8 females; mean value of age \pm SD = 45.6 \pm 12.0, range 18–71 yr). No patients received any anti-hypertensive drug, and 23 out of 32 received no other medication before study. The remaining nine were treated with anxiolytic drugs. They all had normal plasma creatinine, potassium, aldosterone, renin activity, and 24-h urinary normetanephrin and metanephrin; they could therefore be classified as essential hypertensive patients. Urinary normetanephrin and metanephrin were measured according to the method described by Trouvin and Billaud-Mesguich (10) to avoid inclusion of patients with pheochromocytoma in our study. Blood pressure parameters (mean \pm SD) were as follows: systolic arterial pressure; 156.3 \pm 17.8 mmHg; diastolic arterial pressure, 98.8 \pm 10.6 mmHg; and mean arterial pressure, 118.0 \pm 11.4 mmHg. They were therefore designated as moderate hypertensive patients.

Sample preparation. All the samples tested in this study were prepared without any blood-clotting inhibitors and after fasting (10 h). All the sera were stored frozen at -20°C until used (4–8 wk). Subsequently, sera were thawed and ultrafiltered through membranes (YM1-MW cutoff, 1,000 D-level; Amicon Div., W. R. Grace, Inc., Beverly, MA) at 4°C . Membranes were washed with 10 ml ultrapure water, and resulting dialysates lyophilized. The dry powder obtained was methanol extracted three times with decreasing volumes of methanol (4,2,1 ml; RCS 10-60; Jouan, Paris). The methanol soluble fractions were evaporated to dryness in a speedvac, reconstituted in 5 ml of ultrapure water, and stored at -20°C until analysis by radioimmunoassays described below.

Serum extracts were first tested with the monoclonal RIA. Thawed serum extracts were kept at 4°C for 3 d to allow three separate analysis. Sera extracts were frozen again and tested with the polyclonal RIA after storing 1 mo at -20°C .

Radioimmunoassays. The radioimmunoassays developed either with polyclonal or with monoclonal anticonidine antibodies were similar, except for antibody dilution and for the quantities of cold pac used to obtain the standard curves (see below).

Assay medium constituted of 100 μl antibody (for polyclonal antibody, final dilution: 1/400; for monoclonal antibody, final dilution: 1/2,000) in Tris-HCl buffer, 50 mM, pH 7.2, 100 μl Tris buffer zero standard (B_0) or known standard amount of cold pac or unknown samples (B) and 20 μl [^3H]pac (10,000–15,000 dpm, specific activity: 50 Ci/mM; New England Nuclear, Boston, MA). Dilutions of antibodies were chosen so that 50% radioligand was bound in B_0 . Incubation time was 2 h at room temperature. Separation of bound from free ligand has been performed as previously described (8, 9).

A set of standard unlabeled pac samples was freshly prepared in the following concentration range: stock solution of 10 mg/ml pac in Tris buffer was diluted to obtain 0.1, 0.25, 0.75, 1, 2.5, 5, 7.5, and 10 ng pac in the assay for polyclonal antibodies, and 1, 5, 10, 20, 40, 60, 80, 100, 200, and 300 ng pac in the assay for the monoclonal antibody. These standards were carried out through the RIA in triplicate. The radioactivity counted in the supernatants was used to calculate bound (B) [^3H]pac for each point of the standard curves. These values were divided by the count of the zero standard (B_0) and the logit transformation plotted vs. the natural logarithm, \log_e , of the amount of cold pac to construct the calibration curves. Results of unknown samples were expressed in arbitrary units as ng pac equivalent (ng pac eq) as determined by extrapolation of calibration curves and related to the initial volume of serum. Each determination was performed in triplicate, and each unknown sample was tested at least at three dilutions in three independent experiments. Nonspecific binding was measured in the presence of 100 μg unlabeled pac in the assay.

Saturation experiments of monoclonal antibody mFE7 were carried on in the absence and presence of serum extracts. Isotopic dilutions were used because of the intermediate affinity of this antibody for free pac. Increasing amounts of cold pac (10 to 80 ng/100 μl) were incubated with 100 μl serum extract, mFE7 antibody (100 μl ; final dilution: 1/1,000) and 25 μl of [^3H]pac (1 nM) were then added. The procedure described above was then used to measure bound radioactivity and the binding values obtained by computer analysis of data by the program EBDA (Elsevier BIOSOFT Advanced Technology, Cambridge, UK [11]).

Results

Sensitivity of radioimmunoassays. Typical calibration curves, generated according to the proposed protocol, are shown in Fig. 1.

The RIA procedures appeared to be most suitable for the detection of pac levels in the range from 0.25 to 10 ng/100 μl for polyclonal antibodies and from 5 to 100 ng/100 μl for the monoclonal antibody. Thus, the polyclonal RIA appeared about ten times more sensitive than the monoclonal RIA, due to the higher affinity of polyclonal antibodies for free pac ($K_d = 13$ nM) compared to monoclonal antibodies ($K_d = 180$ nM).

Immunoreactivity in the human samples. In the serum extracts of normotensive subjects, immunoreactivity was detectable at low level (Fig. 2, lines 1 and 3) with the two radioimmunoassays. Mean values (mean \pm SD) of immunoreactivity were 7.6 \pm 12.1 ng pac eq/ml ($n = 26$) with monoclonal antibodies and 11.9 \pm 19.2 ng pac eq/ml ($n = 26$) with polyclonal antibodies.

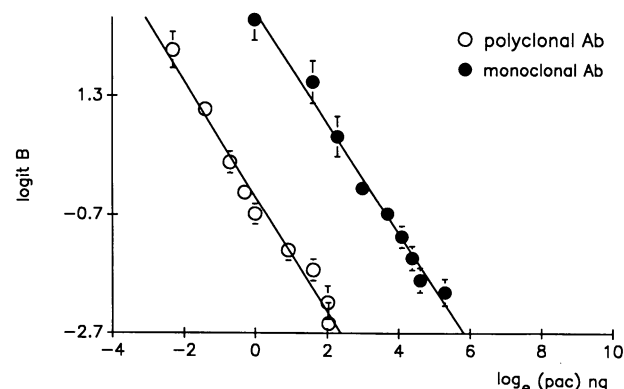


Figure 1. Typical calibration curves expressed on a log-logit scale of polyclonal and monoclonal RIA. Each point is the mean \pm SEM of nine independent experiments effected in triplicates.

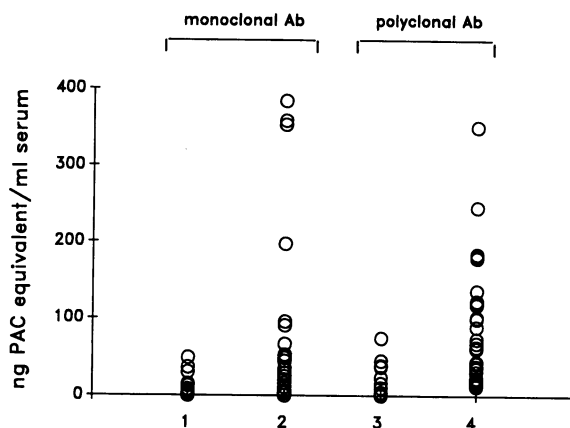


Figure 2. Imidazoline-like immunoreactivity measured in serum extracts from normotensive (lines 1 and 3) and hypertensive (lines 2 and 4) subjects with monoclonal RIA (lines 1 and 2), and polyclonal RIA (lines 3 and 4). Each point represents a different individual. The results are expressed as ng pac eq/ml serum, calculated by extrapolation of standard curves as described in Methods. Results are the mean of at least three determinations at two dilutions of serum extracts in triplicate.

ies. The ranges of values were respectively 0 to 48.7 ng pac eq/ml and 0 to 74.3 ng pac eq/ml.

However, significant differences with these values were noted in the hypertensive group. As can be seen in Fig. 2, lines 2 and 4, some higher immunoreactivity values were found in this group. The mean value (mean \pm SD) with monoclonal RIA was 58.5 \pm 106.4 ng pac eq/ml serum ($n = 32$, values range: 0–384). The polyclonal RIA assays gave a mean value of 77.9 \pm 77.6 ng pac eq/ml ($n = 32$, values range: 0–350). Considering a basal arbitrary upper limit of 50 ng pac eq/ml for monoclonal RIA and of 100 ng pac eq/ml for polyclonal RIA observed in the normotensive group, 25 and 28.1% (with monoclonal and polyclonal RIAs, respectively) of the hypertensive patients studied here showed high immunoreactivity values. Interestingly, none of these patients was treated with anxiolytic drugs.

Monoclonal mFE7 and rabbit polyclonal anti-*para*-aminoclonidine antibodies gave similar qualitative and quantitative results, as the values obtained with both correlated very well ($r = 0.78$, $P < 0.01$, $n = 58$).

Specificity of the interaction between immunoreactive substance and the antibodies. Parameters of pac standard curves and serum sample dilution curves were compared. Dilution of the extracted samples yielded curves with slopes identical to the pac standard curve (Fig. 3). We can thus assume that homologous interactions occur between antibodies and pac, and antibodies and immunoreactive substances. In addition, analysis with monoclonal RIA of immunoreactivity present in human serum extracts supplemented with pac at concentrations from 10 to 80 ng/assay demonstrated additivity of reactivity of pac and imidazoline-like immunoreactivity. Scatchard analysis of saturation data in the absence and the presence of serum extracts led to the following results. The K_d value decreased in the presence of serum extract: 168 \pm 44 nM vs. 599 \pm 190 nM (mean \pm SEM, $n = 5$, $P < 0.05$) and the B_{max} value remained unchanged with 3.98 \pm 0.74 vs. 3.72 \pm 0.93 nmol/mg protein. Serum extracts behave as competitive inhibitors of pac binding on the antibody.

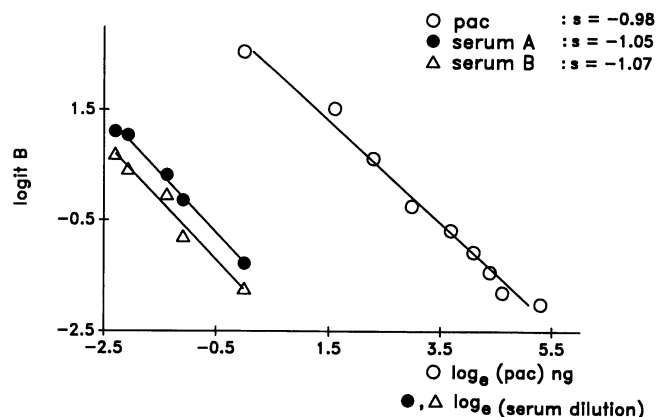


Figure 3. RIA response curves (log-logit scale) based on serial dilutions of pac (\circ) and of serum extracts (\bullet , Δ). Representative experiments of two different extracts, each point tested in triplicate. Curve equations were: $y = -1.07x - 1.26$ for serum A; $y = -1.05x - 1.81$ for serum B; $y = -0.98x + 2.9$ for pac.

Endogenous constituents of serum are known to provoke nonspecific interference in antigen-antibody interaction. We therefore studied the possible influence of urea in our radioimmunoassays. This substance appeared to have no effect in our assays up to a concentration of 1 mM. No correlation between urea concentration in the serum extracts tested and the level of immunoreactivity was found, even when assuming a 100% recovery of serum urea after sample preparation steps.

Relationships between blood pressure parameters and immunoreactivity. To test the involvement of the immunoreactive substance in hypertensive pathogenesis, we examined the possible relationships between mean arterial pressure and the values of immunoreactivity obtained with the two RIAs. Individual values of the imidazoline-like immunoreactivity correlated positively with the mean arterial pressure values, $r = 0.554$, $P < 0.01$ for polyclonal RIA (Fig. 4) and $r = 0.39$, $P < 0.01$ for monoclonal RIA.

Heart rates of patients with high levels of immunoreactivity were not significantly different ($P = 0.54$) from those with low levels: 69.1 \pm 11.0 beats/min (mean \pm SD, $n = 9$) and 71.4 \pm 9.3 beats/min (mean \pm SD, $n = 23$), respectively.

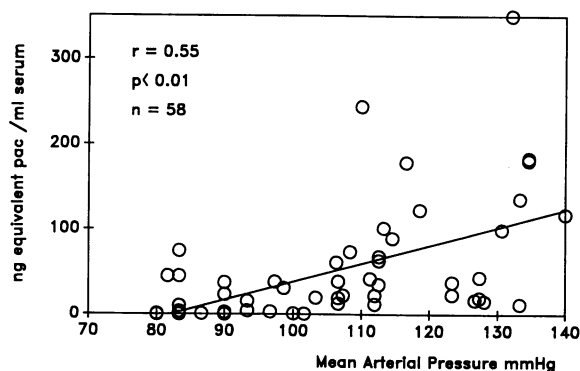


Figure 4. Relationships between imidazoline-like immunoreactivity (measured with polyclonal antibodies) and mean arterial pressure. Correlation coefficients were calculated from linear regression by the least-squares method.

Discussion

Antibodies raised against therapeutic agents have generally been used in bioavailability and pharmacokinetic studies, in addition to therapeutic drug monitoring in patients. Several RIAs with clonidine-specific polyclonal antibodies have been developed for these purposes (12–15). Assays with these antibodies were conducted as usually described for drug-specific RIAs: (a) accuracy was determined with biological fluids (serum, urine) with added known levels of drugs; (b) so-called “matrix effects” were abolished in the assays by dilutions of analytical samples in biological fluids identical to those used for calibration curves, except that they were drug free; (c) specificity of RIAs was generally assessed by determining cross-reactivities of drug metabolites; (d) pharmacokinetics in human plasma were studied after oral or intravenous administration of clonidine. In contrast, data presented in this paper only concern clonidine- or clonidine-like drug-free subjects, and thus concern clonidine-free serum samples. Even in the absence of clonidine and clonidine-like drugs, both polyclonal and monoclonal anticlonidine antibodies detected immunoreactivity in human serum.

What is the nature of the substances responsible for this immunoreactivity? Several criteria have been tested to assess the specificity of the interaction between fluid extracts and the antibodies. Linearity of results obtained with serial dilutions of samples, as well as parallelism observed between standard curves and sample curves, showed that the interactions of pac and endogenous substance with antibodies are similar. In addition, serum extracts that have been supplemented with pac gave a curve that was superimposable on the pac standard curve when corrected for endogenous immunoreactivity. Despite slightly different specificity spectra, the two different antibodies gave the same qualitative and quantitative results. As the material used in this study was of limited purity, we cannot exclude the possibility that known biological substances are recognized by the antibodies. Therefore, to determine specificity spectra of our two antibodies, we focused our attention on endogenous substances present in human serum and potentially capable of interacting with pac binding on the antibodies. As shown elsewhere (8), catecholamines (noradrenaline, adrenaline, dopamine) and their metabolites (DOPAC, DOP-PEG, dihydroxymandelic acid) as well as histamine, purine, adenine, and adenosine were unable to cross-react with polyclonal antibodies. We recently completed that list with arginine, guanidine, guanosine, carnosine, histidine, and tyrosine that all were ineffective on pac binding on the antibodies. Similarly, monoclonal antibody mFE7 specificity has been reported elsewhere (9) and showed the same results. All known endogenous substances we have so far tested with the two antibodies appeared without effect in the RIA described here.

Certain causes of false positive results due to nonspecific interactions could also be ruled out. Arndts et al. (13) attributed the erroneous blank values they obtained with a clonidine RIA to matrix effects. The dilution of plasma samples in normal human plasma was sufficient to abolish these effects. In our case, we tested immunoreactivity in serum extracts devoid of proteins and salts. Ultrapure water was used to reconstitute the samples and Tris buffer was the diluent for both standard and samples curves. In addition, dilutions of samples did not abolish the immunoreactivity.

Naturally occurring substances such as urea are known to inhibit antigen–antibody interactions. We were unable to find any correlation between urea concentration in the blood sample extracts and immunoreactivity levels. Drugs or metabolites could also interfere with RIA. For example, Farina et al. (12) demonstrated that salicylic acid decreased the sensitivity of an immunological assay of clonidine. Our first group of sera was provided by healthy normotensive donors. Despite the fact that they received no medication, a basal level of immunoreactivity was observed with both antibodies. None of the hypertensive patients received any antihypertensive treatment. Associated treatments consisted essentially of benzodiazepine anxiolytics, and no drugs had structural analogies with imidazolines. We have at the present time no evidence that immunoreactivity observed in the serum could be attributed to nonspecific interaction in the RIAs.

The chemical nature of the substance present in the serum is, for the moment, unknown, but some indications can be drawn from the method of preparation of serum extracts. The immunoreactive substance appears not to be a protein: it has a molecular mass < 1,000 D, it is methanol- and water-soluble, and its structure must be related to that of clonidine or *para*-aminoclonidine according to the strict specificity of the antibodies used in this study.

The plasma level of the imidazoline-like immunoreactivity was much higher in some patients with essential hypertension receiving no antihypertensive treatment. The immunoreactivity could reach levels as high as ten times the values observed in normotensive subjects. High levels of the immunoreactive substance were observed in ~ 30% of the hypertensive patients. One can assume that, since some borderline hypertensives were included in the group of hypertensive patients, this percentage was even underestimated.

We did not observe differences between heart rate values among the groups of patients whether they exhibited high or low immunoreactivities. These results seem to indicate that the autonomic nervous system activities in both groups of patients were similar. The correlation between mean arterial pressure and immunoreactivity is particularly interesting; an abnormal high serum level of this substance might therefore be associated with certain forms of the so-called essential hypertension. The presence of high serum level of clonidine-like substances has also been described in pregnancy-induced hypertension (16).

The question remains whether the immunoreactive substance detected with anticlonidine antibodies and the putative endogenous ligand for IRs are identical. Indirect evidences argue in favor of this hypothesis. (a) Specificities of our antibodies are limited to imidazoline-containing substances; (b) the two antibodies we used proved capable to detect immunoreactivity in bovine and human brain extracts containing the putative endogenous ligand of IRs; (c) an immunoreactive compound supposed to be a CDS (clonidine-displacing substance)-like substance was detected with polyclonal anticlonidine antibodies in rat serum extracts and proved able to contract rat gastric fundus smooth muscle (17); (d) Synetos et al. (18) reported the presence of CDS, supposed to contain the endogenous ligand of IRs, in human serum. Only the purification of both the endogenous ligand to IRs and the imidazoline-like immunoreactive circulating substance will provide an unequivocal proof of their identity.

Biological effects of CDS, related to vasomotor tone, have been described: it proved hypertensive in animal models (19)

and contracted blood vessels in a dose-dependent manner (18).

Thus, if the substance we detected with higher circulating levels in some hypertensive patients is related to CDS, then this one might contribute to maintain an elevated arterial pressure and clonidine-like drugs might therefore be antihypertensive through inhibition of the CDS effects. Again, the complete purification of these compounds will clarify this point.

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