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ATRIAL NATRIURETIC FACTOR IN TWO KIDNEY - TWO CLIP RENOVASCULAR HYPERTENSION IN THE RAT

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Abstract Hig levels of circulating atrial natriuretic factor (ANF) have been reported in several physiopathologic conditions like hypertension, heart and renal failure, pregnancy and high sodium intake. Nevertheless, neither relationships with water-sodium space regulation nor the role of an ANF vascular relaxant effect have been yet defined. The aim of present experiments was to characterize the contribution of circulating ANF and its vascular relaxing effects in the two kidney-two clip (2K2C) experimental model of renovascular hypertension. Complementary, plasma metabolites nitrite/nitrate of nitric oxide (NO) was examined because of mediation for both (NO an ANF) through cGMP. The results showed (two-four weeks after surgery): indirect sistolic blood pressure (mmHg), 186 ± 4 in HT and 122 ± 1 in SH ($p < 0.001$); a significant increase of plasma ANF (fmol/ml) in HT ($n = 7$, 1221 ± 253) vs. SH ($n = 9$, 476 ± 82; $p < 0.02$). Nitrate/nitrite plasma concentrations (µmol/l) were mpt different between SH and. The relaxant effect of ANF (10^{-9}, 10^{-8} and 10^{-7} M) on phenylephrine ($3{,}5 \times 10^{-6}$ M) contracted rings from HT rats was smaller than SH rats (10^{-8} M, $p < 0.05$). Contractions to phorbol 12, 13-dibutyrate (seven weeks after surgery) were significantly higher in rings from HT rats ($p < 0.001$). We conclude: 1) in addition to decreased granularity in atrial myocardiocytes, high circulating values of ANF here described suggest an increased turnover of the peptide in 2K2C hypertensive rats; 2) lower significant vascular relaxant effects in HT rats would indicate down regulation of ANF receptors in this model; the latter would derive from high plasma ANF concentration and, tentatively, because of greater activity of protein kinase C in the vascular wall; 39 similar values of plasma nitrite/nitrate in SH and HT rats would indicate a comparable NO circulating availability in both groups.

Resumen ***Factor natriurético atrial en la hipertensión dos riñones-dos clips en la rata.*** Niveles circulantes elevados del factor natriurétrico atrial (ANF) han sido referidos en varias condiciones fisiopatológicas tales como la hipertensión arterial, la insuficiencia cardíaca y renal, el embarazo y la ingesta de sodio elevada. Sin embargo, aún no está claramente establecida su participación en la regulación del espacio sodio-agua ni su importancia como relajante vascular. El objetivo del presente trabajo ha sido caracterizar la contribución del ANF circulante y sus efectos sobre el músculo liso vascular en el modelo experimental de hipertensión renovascular dos riñones dos clip (2R2C). Complementariamente, se examinó la concentración plasmática de metabolitos del óxido nítrico (NO, nitrito/nitrato), dado que para ambas sustancias (NO y ANF) los efectos son mediados por GMPc. Los resultados mostraron (dos-cuatro semanas después de la cirugía): presión arterial sistólica (indirecta, mmHg), 186 ± 4 en HT y 122 ± 1 en SH ($p < 0.001$); significando aumento del ANF plasmático (fmol/ml) en las ratas HT n = 7), 1221 ± 253 con respecto a las SH ($n = 9$), 476 ± 82 ($p < 0.02$); las concentraciones de nitrito/nitrato en plasma (µm/l) no fueron diferentes entre HT y SH. El efecto relajante del ANF (10^{-9}, 10^{-8} y 10^{-7} M) fue menor en los anillos de aorta de ratas HT ($p < 0.001$). En conclusión: 1) las altas concentraciones de ANF circulantes, acompañadas por una degranulación de los miocitos atriales, sugieren un recambio aumentado del mismo en HT 2R2C; 2) el menor efecto relajante en anillos de aorta de ratas HT pre-contraídos con Phe, indicaría una desensibilización de los receptores para ANF en este modelo, atribuible a las altas concentraciones de ANF circulante y, tentativamente, a una mayor actividad de proteína kinasa C en la pared vascular; 3) la similar concentración plasmática de nitrito/nitrato en SH e HT indicaría una disponibilidad de NO circulante comparable en ambos grupos

Key words: atrial natriuretic factor, hypertension, renovascular hypertension, vascular reactivity

The presence of secretory like granules linked to striated cardiac muscle cells was observed by electron microscopy in guinea pig atrial as far as in 1956[1]. Many years later, in 1981, de Bold et al[2] demonstrated that intravenous administration of rat atrial homogenates enriched in such granules resulted in a fast and short but impressive diuresis, natriuresis and consistent decrease in blood presure. Later on, the peptidic nature of the natriuretic factor localed in the granules was established[3] and the fact was confirmed by immunohistochemical stud-

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ies[4]. In addition to the atrial natriuretic factor (ANF) two other natriuretic peptides, B and C types (BNP and CNP), with a high degree of sequence homology to ANF, each derived from a separate gene and with some similar biological effects, were identified during the past deca-de[5, 6].

We have previously reported that ANF specific granules as observed by electron microscopy, decrease in 2K2C rats with subacute (two weeks) and chronic (six weeks) hypertension[7]. It was suggested that vanishing of granularity in hypertensive rats 72 hs after clipping and further on, would indicate the release of the natriuretic peptide as a cooperative system which favour sodium-water equilibrium in renovascular hypertension. At least two opposite conditions could account for the results: 1) a decrease in synthesis and 2) an increased delivery of the peptide. In order to distinguish between the two possibilities, plasma ANF concentration analysis were performed in present experiments after two-three weeks of clipping both renal arteries. We speculated that, if plasma concentration were elevated, the decreased atrial granularity previously described could express the increased release of the peptide. Moreover, since renal clearance of ANF by kidneys with artery stenosis has been reported to be normal[8, 9], it would suggest an enhanced turnover of the peptide during the subacute period of 2K2C renovascular hypertension.

Concerning the hypotensive action of ANF, some authors[10] suggested that it could specially derive from a decrease in cardiac output. However, a vascular relaxant effect of ANF through cGMP has been clearly defined on aorta and renal rabbit strips[11, 12]. In this regard, the endothelium-derived relaxing factor, which is nitric oxide (NO) or a nitroso compound, also yields vasodilation by cGMP in both conductance and resistance vessels[13-16]. Consequently, in present experiments ANF and NO metabolites (nitrite/nitrate) circulating levels and vascular "in vitro" relaxation to ANF were additionally determined to characterize their relationships in the experimental 2K2C type of renovascular hypertension; as far as we know, this information has not been reported before. This model allows to particularly analize primary Goldblatt ischemic mechanisms since it excludes the involvement of an untouched contralateral kidney (as in 2K1C rats) or the simultaneous reduction in kidney mass (as in 1K1C rats), which is accompanied by a significant increase in sodium space[17].

Material and Methods

Male Wistar rats (250-270 g) were used. Rats were maintained on commercial standard food (Asociación Cooperativa Argentina) and tap water "ad libitum". Room temperature was maintained at 22 ± 1°C and the air was adequately recycled. Hypertension was elicited by applying a solid silver clip (0.29 mm lumen) to each renal artery[18] under ether anesthesia (HT rats); in control Sham rats (SH) all surgical procedures were performed except to apply the clips. The day before sacrificing the animals, indirect systolic blood pressure (BP) was determined by means of a photoelectric tail-cuff connected to an amplifier (II TC model 47, Woodland Hills, California, USA) in series with an oscilloscope (type 532, TEKTRONIC inc., Portland, Oregon, USA).

ANF radioimmunoassay

Two-three weeks after surgery, the animals were anesthetized by intraperitoneal injection of 3.5% Chloral Hydrate (0.8 ml/100 g). Blood samples for ANF analysis were obtained from the jugular vein and immediately placed in ice-chilled plastic tubes with EDTA and then centrifuged at 2,000g at 4°C for 30 min. Plasma samples were kept at -70°C until ANF assay[19]. Briefly, samples were acidified by adding 100 µl/ml of 1 M HCl and passed three times through Sep-Pak C-18 cartridges previously activated with 5 ml of acetonitrile containing 0.1% trifluoroacetic acid (TFA) followed by 5 ml of 0.1% TFA. The cartridges with the adsorbed peptide were washed with 20 ml of 0.1% TFA and then eluted with 3 ml of 80% acetonitrile containing 0.1% TFA. Samples were dried and then stored at -20°C until assayed. Lyophilized dried samples were reconstituted in 1 ml phosphate buffer (pH 7.4) containing 0.1% bovine serum albumin, 0.01% sodium azide, 0.05 M NaCl, and 0.1% Triton and supernatants were assayed for ANF by radioimmunoassay. Anti-rat ANF (99-126) antibody was purchased from Peninsula Lab. Inc. (Belmont, CA) and labeled human ANF (99-126) from New England Nuclear (Boston, MA). ANF concentration was expressed as fmol/ml of plasma.

Nitrite-nitrate in plasma

Nitrite/nitrate plasma levels were measured in SH and HT rats after four weeks of surgery by the fluorometric assay described by Misko et al.[20] Briefly, plasma samples were filtered through 5 000 cutoff microcentrifuge filters (Sigma Chemical Co St. Louis, MO) for 45 min at 7 500g. Nitrate was converted to nitrite by the action of 20 mU Nitrate Reductase from Aspergillus species (Boehringer Mannheim Biochemical, Mannheim, Germany) in presence of 40 µM NADPH and 20 mM TRIS pH 7.6. The reaction was stopped after 5 minutes at 20°C, by dilution with equal volume of destilled water followed by the addition of 2, 3-diaminonaphthalene (DAN, 0.05 mg/ml in 0.62 M HCl) for determination of nitrite. After a 10 min incubation at 20°C, the reaction was stopped with NaOH 2.8 N. DAN reacted with nitrite to form 1-(H)-naphthotriazole, a fluorescent product. Fluorescense was measured in a JASCO FP-770 fluorometer. Nitrite/nitrate plasma concentration was expressed as µmol/l.

Contractility of aorta rings

To study vascular contractility, the same animals in which blood samples for ANF were obtained were decapitated and the abdominal aorta was removed and placed in cold Krebs-bicarbonate solution: (mM) NaCl, 120; KCl, 4.8; KH_2PO_4, 1.2; $MgSO_4.7H_2O$, 1.3; $CaCl_2$, 1.6; $NaHCO_3$, 25; Dextrose, 10; $CaNa_2.EDTA$, 0.03. The excess of adventitia was excised and rings of the arteries were cut (3 mm wide) to be suspended in tissue baths with Krebs solution conveniently gassed by 95% O_2 and 5% CO_2. Tension development was registered by isometric force transducers (GRASS FT03) connected to an amplifier in series with a PC with a special computer program for registration of vascular smooth muscle contraction. After one hour of equilibration at 2 g of basal tension (readjusted every fifteen min), contractions were induced in abdominal aorta rings of SH and HT by 3.5 x 10^{-6} M phenylephrine (Phe, SIGMA) for

three min; then, relaxations to three different doses of ANF (10^{-9}, 10^{-8} and 10^{-7} M) were tested on the same rings and results were expressed as percent relaxation of Phe contraction. In another group of rats (seven weeks after clipping), 10^{-5} M phorbol 12, 13-dibutyrate (PDBu, SIGMA) was used to stimulate protein kinase C in order to induce contraction as an indirect index of protein kinase C activity. Results were expressed as mg of tension development.

Statistical analysis

Results were expressed as means ± SEM. The unpaired Student's t-test were used and differences at a level of $p < 0.05$ were considered significant.

Results

Blood pressure

Values (mean ± SEM) in HT rats were significantly higher than in SH rats ($p < 0\ 001$): 186 ± 4 vs 122 ± 1 mmHg.

Kidney mass

Striking similarities were observed in kidney mass (expressed in g) of HT and SH rats in all groups. The fact indicates that fairly comparable mass of functional kidney tissue was present in SH and clipped rats. Group of rats in which ANF and vascular contractility were determined: left kidney: HT (n = 7) 1.36 ± 0.04 vs SH (n = 9) 1.35 ± 0.04; right kidney: HT (n = 7) 1.41 ± 0.05 vs SH (n = 9) 1.40 ± 0.04. Group of rats in which nitrite/nitrate were determined, left kidney: HT (n = 8) 1.31 ± 0.06 vs SH (n = 9) 1.25 ± 0.04; right kidney: HT (n = 8) 1.33 ± 0.04 vs SH (n = 9) 1.28 ± 0.03.

ANF and nitrite/nitrate in plasma

Plasma ANF value (fmol/ml)) (Fig. 1, upper panel) were higher in HT (n = 7, 1 221 ± 253) than in SH rats (n = 9, 476 ± 82, $p < 0.02$). On the other hand, nitrite/nitrate plasma concentration (µmol/l) did not differ (Fig. 1, lower panel) between HT and SH rats: HT (n = 8) 25.9 ± 2.0 vs SH (n = 9) 22.4 ± 3.3, NS.

Contractility of aorta rings

A significant lower contractile response to 3.5×10^{-6} M Phe was observed in aorta rings obtained from HT rats ($p < 0.05$; Fig. 2 upper panel) as compare with rings

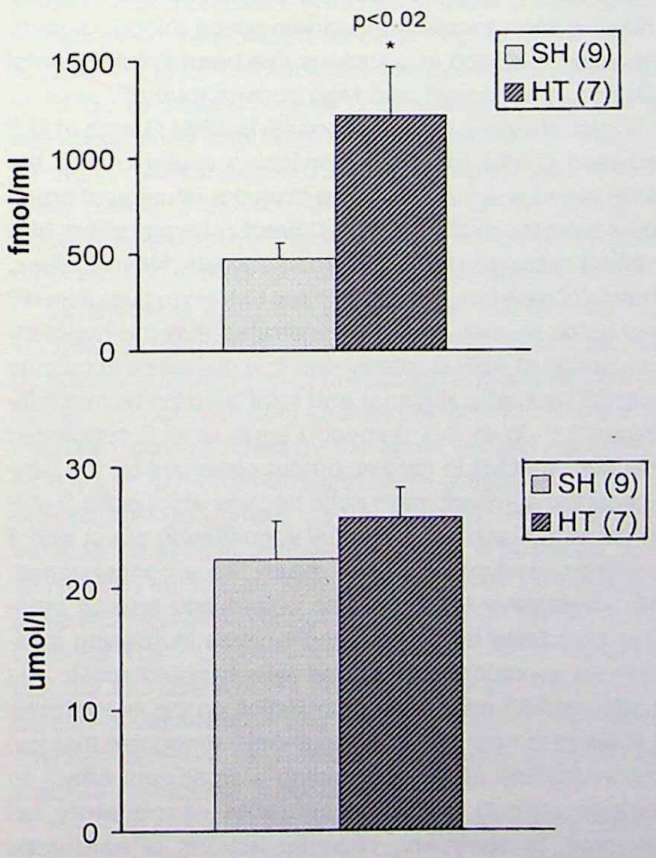

Fig. 1.– ANF (*upper panel*) and nitrite/nitrate (*lower panel*) concentration in plasma.

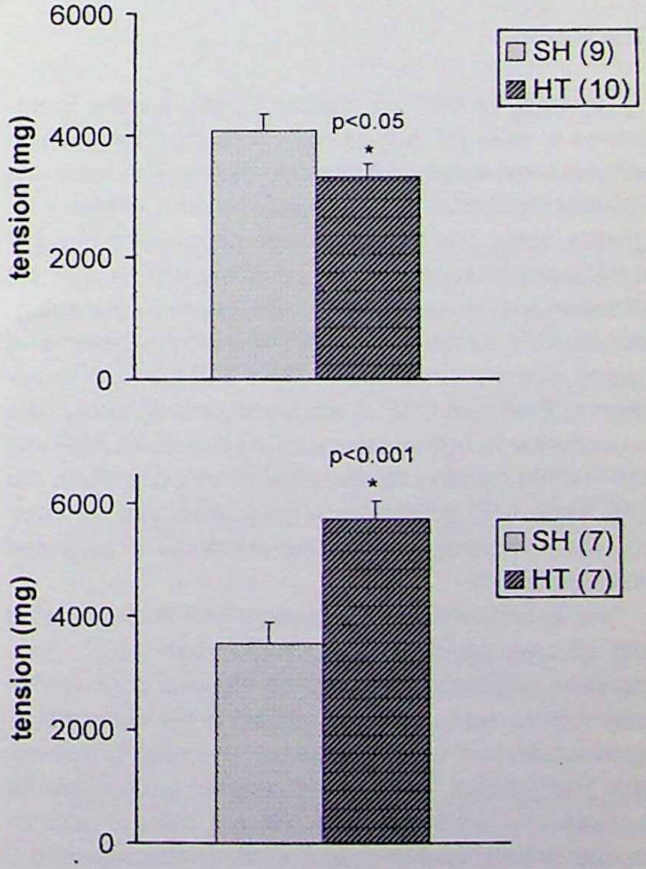

Fig. 2.– Contraction of abdominal aorta rings to phenylephrine 3.5×10^{-6} M (*upper panel*); contraction of aorta rings to phorbol 12, 13-dibutyrate 10^{-5} M (*lower panel*).

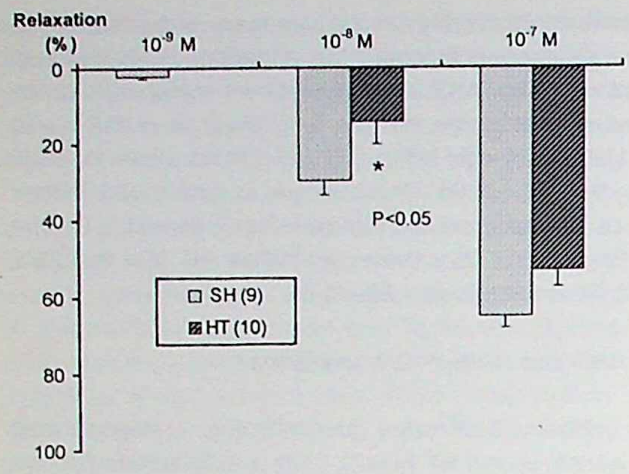

Fig. 3.– Percent relaxation to atrial natriuretic factor in abdominal aorta rings precontracted by 3.5 x 10^{-6} M phenylephrine.

from SH rats. On the contrary, PDBu induced higher contractions (Fig. 2, lower panel) on aorta rings from HT rats vs rings from SH rats ($p < 0.001$). Relaxation responses to ANF on Phe precontracted aorta rings were smaller in rings from HT rats as compared with SH rats, but results were significant only for one of the three concentrations used (Fig. 3).

Discussion

The study of ANF contribution in renovascular hypertension is relevant in view of its recognized interaction with the renin-angiotensin system (RAS). The latter is a powerful vasoconstrictor (specially on renal vessels), increases water and sodium reabsorption and stimulates aldosterone release. On the contrary, ANF is a direct endogenous antagonist to the renin-angiotensin-aldosterone system[21, 22] and may induce peripheral vasodilation. Furthermore, the RAS and the ANF (in addition to BNP and CNP of the same peptide family) are both present in central neurons. The actions of ANF and BNP in this location appear also to be opposite to the RAS since ANF inhibits thirst associated with dehydration and hemorrhage[23] and suppresses vasopressin and ACTH release[24].

The information in the literature has clearly related ANF circulating levels to water-sodium balance[25-28]. Nevertheless, distinction between the stimulus originated in total sodium space or particularized to the interstitial or intravascular fluid compartment has been poorly defined. With this respect, in the 1K1C experimental model or renovascular hypertension, in which a characteristic increase in total exchangeable sodium was reported[17], particularly high ANF circulating values were observed[28]. Furthermore, elevated plasma ANF were also found in humans without edema with both unilateral and bilateral renal artery stenosis or with essential hypertension[29]. On the contrary, in patients with cirrhosis and edema (in the advanced period, when blood volume is contracted in spite of high total extracelular fluid), circulating ANF was reported to be within normal ranges[30].

Up to date, little information is available about ANF in experimental unilateral renal artery stenosis[31, 32] and no data was found in the literature concerning the experimental 2K2C model. In these latter 2K2C hypertensive rats we have previously observed the increase in ^{22}Na space four weeks after clipping when hypertension was moderate (BP < 170 mmHg), but no difference with controls was found in severe hypertension (BP > 170 mmHg)[33]. In present experiments the mean BP of hypertensive rats reached 186 ± 4 mmHg after three weeks; thus this group of severe 2K2C hypertensive rats should be devoided of water-sodium expansion. Consequently, high ANF plasma levels in present experiments might not be necessary ascribed to an increased total water-sodium space. This assertion is in agreement with the high circulating ANF levels described in 2K1C rats[34] in which hypertension is not either accompanied by water-sodium retention as long as the contralateral untouched kidney remains undamaged. Furthermore, our results would support that the primary specific stimulus to ANF release is cardiac muscle stretch[35] which might derive from high BP and/or the primary tendency of increasing *intravascular* fluid volume space (blood volume), the latter observed in situations like heart failure[36], renal failure[37], pregnancy[38] and high sodium intake[30].

Concerning vascular responses, in 1984 García et al.[12] provided for the first time convincing evidence that the fall in blood pressure resulting from the infusion of crude atrial extracts might involve a direct relaxant effect of a purified natriuretic factor on blood vessels. Nevertheless, dilation of resistance-sized arteries has been questioned[39] and some studies have demonstrated that the hypotensive action of ANF is mainly due to a decrease in cardiac output[10]; actually, regional and total resistance might increase[10, 40]. With this respect, Lappe et al.[10] suggested that the rapid fall in cardiac output observed by the infusion of the synthetic natriuretic peptide atriopeptin II (AP II) would indicate a preferential venodilation effect with a marked reduction in venous return. As a consequence, the progressive fall in cardiac output and arterial pressure stimulates baroreflex mechanisms increasing sympathetic vasoconstrictor tone which could mask any moderate ANF peripheral vasodilation on the arterial side. It is worth to note, the same authors[10] recognize that low concentrations of AP II (avoiding a significant effect on cardiac output) cause measurable vasodilatory responses. In summary, vascular actions of natriuretic peptides generate conflicting results in relation to the use of different doses and/or whether the effect is observed on isolated blood vessels or in the entire animal. In our

2K2C experimental model, neither the diuretic-natriuretic effect, the possible depressor effect on cardiac output and/or peripheral vasodilation via ANF, nor its well known opposite action on RAS, were effective for high circulating ANF to inhibit the increase of BP. It could be speculated that any intent to decrease BP in these 2K2C rats would derive in renal ischemia and thus in renin secretion, counteracting cardiovascular depressor effects of ANF.

Our results showed that rings of abdominal aorta of 2K2C rats stimulated by 3.5×10^{-6} M Phe (a submaximal dose) contracted less than rings from SH rats (Fig. 2). Accordingly, we have previously reported that strips of the same vessel (abdominal aorta) and experimental model (2K2C), contracted less than controls to norepinephrine, indicating that vessels from this type of renovascular hypertensive animals would be less responsive to catecholamines[41]. With regard to relaxation, a lower relaxant effect to ANF on rings from hypertensive rats was observed in present experiments (significant for the medium dose used, 10^{-8} M). García et al.[27] and others[33] have reported down-regulation of ANF receptors in presence of high plasma values of ANF in 2K1C and 1K1C hypertensive rats. Thus the high plasma values observed in our 2K2C rats could be responsable, in part, for lower peripheral vasodilation mediated by down-regulation of ANF receptors. With this respect, protein kinase C has been postulated to be a regulator of ANF-B receptors[42]; to further approach this possibility, responses to PDBu (a protein kinase C stimulant) on aorta rings were examined and tension development was found to be significantly increased in rings from HT rats (Fig. 2, lower panel). This fact suggests that greater activity of protein kinase C in the vessel wall may contribute to down-regulation of ANF-B receptors in 2K2C rats.

On the other hand, it is a well known fact that vascular endothelium plays a major role in controlling vascular tone by releasing relaxing and contracting factors[43]. Among the former, NO or a closely related compound mediates its vascular relaxant effect by the same second messenger (cGMP) as ANF. Nevertheless, ANF activates membrane particulate guanylate cyclase while NO activates intracellular soluble guanylate cyclase; accordingly, ANF stimulation correlates with plasma cGMP concentration while NO stimulation correlates with cGMP content in the vascular wall[44]. Consequently, we would like to speculate that the NO effect might be particularly ascribed to local paracrine vasodilation and elevated plasma ANF would specially account for humoral modulation of peripheral vascular resistance in our 2K2C rats.

It can be concluded that high circulating values of ANF, along with the previously described decrease in granularity of atrial myocardiocytes, suggest an increased turnover of the peptide in 2K2C hypertensive rats. We further speculate that the primary stimulus for releasing ANF peptide is cardiac muscle stretch by high BP and/or the increase (or the tendency to increase) of the intravascular fluid volume; high total sodium space, in the presence of simulatneous normal or decreased blood volume and without hypertension (like in the advanced period of cirrhosis), would be denied as responsable for elevated circulating ANF. Moreover, high levels of ANF here reported might be particularly involved in humoral modulation of peripheral vascular resistance in renovascular hypertension. According to the literature, NO and CNP (which belongs to the peptide natriuretic family) are better candidates for local paracrine regulation of blood vessels[44-46].

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