Involvement of proinflammatory cytokines in angiotensin II-induced hypertension in rat

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Received June 10, 2020; Accepted July 10, 2020

DOI: 10.3892/etm.2020.9100

Abstract. Rightfully considered as essential for hydro-electrolytic homeostasis, angiotensin II (Ang II) is the main product of the renin-angiotensin system (RAS). Ang II is one of the most important factors that contribute to the regulation of systemic arterial blood pressure (ABP). This major role is based on the effects exerted by RAS: Upon the kidney (RAS involvement in the control of salt and water excretion), upon the brain (RAS involvement in the control of water intake), and upon the sympathetic nervous system. It is currently known that there is a tight bidirectional link between high ABP and chronic kidney disease (CKD). Ang II causes vasoconstriction in the renal microvasculature, predominantly in the preglomerular arterioles. High ABP affects the target organs (eyes, brain, heart, kidneys) and it is known both as a cause and as an effect of CKD. Thus, there is a positive feedback mechanism that contributes even more to the increase in ABP and the progression of CKD. Along with its main hemodynamic effects, Ang II has direct proinflammatory actions, that also affect the structure and function of the kidney and heart. This study investigated the role of RAS and Ang II in the inflammation that accompanies the hypertension experimentally induced by Ang II in rats. Our data support the hypothesis that anti-inflammatory medication might alleviate the morphological and/or functional changes of the kidneys and heart that are related to Ang II-induced hypertension.

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Key words: angiotensin II, blood pressure, proinflammatory cytokines, heart, kidneys

Introduction

The renin-angiotensin system (RAS) is a signaling cascade that governs the balance of water and electrolytes and the control of systemic arterial blood pressure (ABP), thus having a central role in the renal and cardiovascular function (1). Beyond such classical knowledge, regarding angiotensin II (Ang II), this peptide is nowa also known for its inflammatory effects, with their impact upon the structure and function of the kidneys and heart (2). Chronic RAS overactivation causes cardiovascular and renal dysfunction, which also involve the pro-inflammatory, pro-hypertrophic, and pro-fibrotic effects of Ang II (3). It is now well known that arterial hypertension affects target organs (eyes, brain, heart, kidney) (4,5), also initiating a vicious circle that promotes the sustained increase of ABP.

There has been a recent attempt to clarify the pathophysiological mechanisms that can be found at the basis of the inflammatory process associated with high ABP (6). The increasing levels of certain inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) and IL-1β, represent independent factors that predict mortality for patients with chronic kidney disease (CKD). The renal changes and the increased ABP in CKD can be related to effects of the inflammatory cytokines and chemokines (7). Inflammatory cells are present in the renal tissue in most kidney diseases, both in the immune-mediated ones and in those that occur as the effect of high ABP (8). One consequence of high ABP is heart dysfunction (9). The B-type natriuretic peptide (BNP) is released into the circulation from 'the stressed out myocardium', especially from the left ventricle. High concentrations of BNP in blood plasma can be found in patients with a heart condition, especially in those with heart failure and kidney failure (10). An important marker of the heart dysfunction is the N-terminal precursor of BNP (NT-pro-BNP).

The molecular mechanisms underlying the relationship between inflammation and hypertension are poorly understood. Within this context, the present study aims to address the role of the immune system in mediating the effects of Ang II excess upon the kidney and upon the heart. In order to achieve this, we used an appropriate experimental model and investigated the expression of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α, and also the blood plasma level of NT-pro-BNP as marker of cardiac dysfunction.

Materials and methods

Experimental model of hypertension induced by Ang II. Wistar male rats were used as the experimental animals. They were 12 weeks old and had an average weight of 250±50 g. The animals were kept in standard cages (2 rats in each cage), in a room with controlled temperature of 21±2˚C and with a light/dark cycle of 12/12 h. The rats had unrestricted access to food and water. They were allowed to accommodate for at least 4 days before the implantation of mini-pumps. Subsequently, the rats were divided into two groups: One group of 14 rats received Ang II and the other group of 14 rats (control) received vehicle (isoosmotic NaCl solution). Ang II acetate (Bachem Americas, Inc.) was administered as follows: continuously for 14 days; at a rate of 300 ng/kg/min; subcutaneously, with osmotic minipumps (Alzet, model 2001) (1 μl/h), placed in the interscapular paravertebral region (11).

The study protocol was approved by the Ethics Committee of the 'Grigore T. Popa' University of Medicine and Pharmacy (Iasi, Romania). Regarding the use of laboratory animals and the biological preparations and samples for scientific research, all the procedures applied in the present study comply with the internationally accepted rules and guidelines from: i) the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes (12); ii) the Universities Federation for Animal Welfare; iii) the International Association for the Study of Pain (IASP).

Animal sacrifice and kidney harvesting. Prior to their sacrifice, the rats were anesthetized with ketamine, administered intraperitoneally at a dose of 12.5 mg per 100 g body weight. The ketamine solution from 5 ml vials (ketamine 50 mg/ml) was first diluted in physiological saline (NaCl 0.9 g/dl) in a 1:4 ratio, to the final ketamine concentration of 12.5 mg/ml, and this solution was given intraperitoneally in a dose of 1 ml per 100 g body weight. After sacrifice, the kidneys were removed and subjected to homogenization for their further use.

Systolic ABP measurement was performed non-invasively by tail-cuff pletysmography (BIOPAC), in the beginning of the study, and then on days 3, 6, 9, 12 and 14 (13).

Assessment of IL-1β and NT-pro-BNP in the blood plasma. The concentrations of IL-1β and NT-pro-BNP were assessed by ELISA, in samples of blood plasma prepared as follows. Blood was collected from the aorta on anticoagulant (heparin), immediately before animal sacrifice. Within 30 min after blood sampling, the samples were centrifuged at room temperature, for 20 min at 2,000 x g . The obtained plasma was immediately stored at -80˚C until further processing. For ELISA Quantikine® kits were used, rat IL-1β/IL-1F2 immunoassay (R&D Systems, Inc.) and USCN Life Science, Inc. In each case, the detailed instructions from the producer were followed.

IL-1β, IL-6 and TNF-α assessment in kidney homogenate. The kidney homogenate was obtained by passing the kidney through a fine sieve, using a syringe plunger. The sieve was washed with 2 ml of Krebs serum. The operation was repeated 3 times. After manual processing, the samples were centrifuged at room temperature, for 20 min at 2,000 x g and stored at -80˚C until further use.

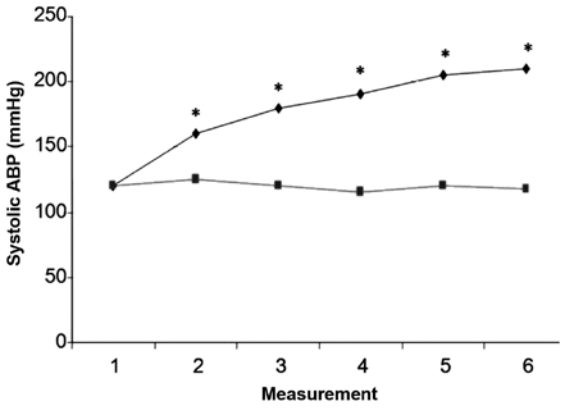

Figure 1. The systolic arterial blood pressure (ABP) is higher in male rats treated with angiotensin II (diamonds) vs. control (squares); *P<0.05.

Evaluation of gene expression for IL-6 and TNF-α. For IL-6 and TNF-α qualitative evaluation was performed, using RT-PCR, based on the reverse transcription kit Enhanced Avian HS-100 RT-PCR (Sigma-Aldrich; Merck KGaA). For TNF-α the primer sequence was AAGTTCCCAAATGGGCTC, while for IL-6 the primer sequences were TTCCCTACTTCACAAGTC and CTAGGTTTGCCGAGTAGA (14).

Statistical analysis. The statistical interpretation of data was performed using GraphPad Prism 5.0. The data were analyzed with Student's t-test or analysis of variance (ANOVA) for comparison between groups with statistical significance defined. Significance of differences between the studied groups was determined with least significant difference (LSD) test. A P-value ≤0.05 was considered statistically significant (15).

Results

Arterial hypertension induced by Ang II. The systolic ABP was similar in the two groups of rats before the treatment (~120 mmHg) and it increased gradually and significantly in rats treated with Ang II, whereby on day 14 it reached 208±2 vs. 120±5 mmHg the same day in the control group (Fig. 1). Ang II treatment finally raised systolic ABP above 206 mmHg in 4 rats (28.57%) and below 206 mmHg in the other 10 rats (71.43%).

Blood plasma concentration of NT-pro-BNP. After 14 days of continuous administration of Ang II, or of plain vehicle respectively, the blood plasma concentration of NT-pro-BNP in rats who were given Ang II was clearly higher (mean value 0.42 pg/ml) than in rats from the control group (mean value 0.16 pg/ml) (Fig. 2), and the difference was statistically significant (P=0.011).

Blood plasma concentration of IL-1β. After 14 days of continuous administration of Ang II, or of plain vehicle respectively, the blood plasma concentration of IL-1β was somewhat higher in rats who were given Ang II (mean value 40.4 pg/ml) than in rats from the control group (mean value 32.43 pg/ml) (Fig. 3); however, this difference was not statistically significant.

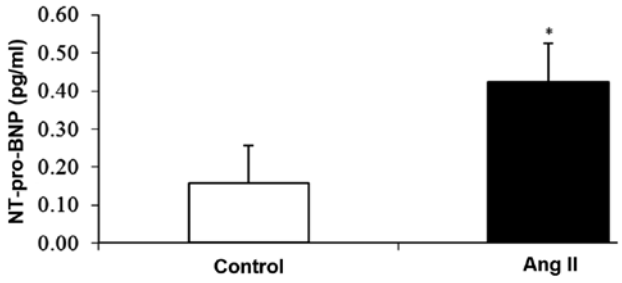

Figure 2. The blood plasma concentration of the N-terminal precursor of B-type natriuretic peptide (NT-pro-BNP) is higher in male rats treated with angiotensin II (Ang II, black column) vs. control (white column); *P<0.05.

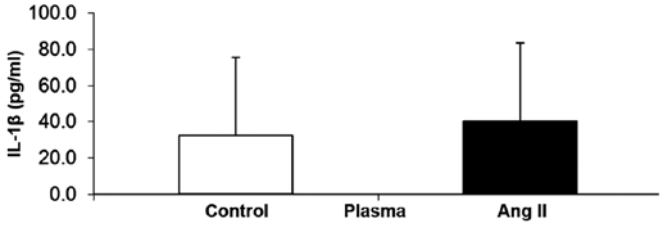

Figure 3. The blood plasma concentration of interleukin-1β (IL-1β) is similar in male rats treated with angiotensin II (Ang II, black column) vs. control (white column); P>0.05.

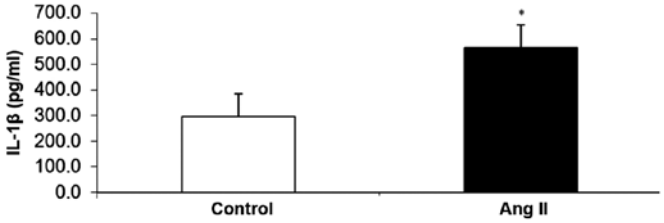

Figure 4. The concentration of interleukin-1β (IL-1β) is higher in the kidney homogenate from male rats treated with angiotensin II (Ang II, black column) vs. control (white column); *P<0.05.

IL-1β concentration in the kidney homogenate. On the contrary, at the experiment end on day 14, the IL-1β concentration in the kidney homogenate was significantly higher (P=0.0000008) in rats treated with Ang II than in the control group (mean values 564.3 vs. 297.49 pg/ml (Fig. 4).

Gene expression of the kidney inflammatory markers IL-6 and TNF-α. The expression of IL-6 and TNF-α was examined from a qualitative point of view by RT-PCR, in order to evaluate the role of Ang II as a proinflammatory molecule in kidney disease. The expression of IL-6 and TNF-α in the kidney was increased in rats subjected to chronic Ang II (14 days) in comparison to the control group (Fig. 5). The bands from ~950 bp are due to genomic DNA contamination of the RNA samples. We note that it is possible to obtain a diminution of the signal for DNA only when the cDNA exists as a matrix.

Discussion

Cardiac muscle expansion, due to an increased interior volume of the heart chambers and/or to an increased myocardial transmural pressure (as occuring in heart failure, myocardial infarction, or cardiomyopathy), causes the release of the natriuretic peptides of cardiac origin: ANP (atrial natriuretic peptide) and BNP (natriuretic peptide of the brain; a misnomer). Mostly by promoting sodium and water excretion, ANP is permanently involved in the homeostasy of the extracellular fluid volume (of volemia actually) and its blood plasma concentration is closely related to the left atrial pressure. BNP is released when myocardial stress occurs and its concentration is directly related to left ventricular pressure and volume (16).

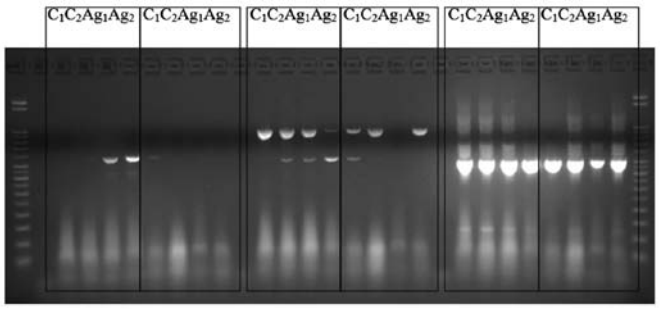

Figure 5. Gene expression for interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) in rat kidney homogenate, in rats treated with angiotensin II (Ag_1 and Ag_2) vs. control (C_1 and C_2). Results of agarose gel electrophoresis, after amplification with the primers for: IL-6 (left), TNF-α (middle), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; right). In each of these three groups of eight wells, the first extraction corresponds to the first four wells (left subgroup of four) and the second extraction corresponds to the rest (right subgroup of four).

A significant increase in systolic ABP was found in rats treated with Ang II for 14 days (Fig. 1) and this high ABP was accompanied by an increase in the blood plasma concentration of NT-pro-BNP (Fig. 2). NT-pro-BNP, an established biomarker of systolic and/or diastolic heart failure, is released by the stressed myocardium. Such a myocardial stress, occuring in the rats treated with Ang II, is due to the increased ABP, but is also a direct consequence of the proinflammatory effect of Ang II upon the ventricular myocardium.

High ABP may lead to left ventricular hypertrophy and ultimately to heart failure. Hildebrandt *et al* (16) demonstrated in humans an association between increased NT-pro-BNP in blood plasma and the high ABP associated with left ventricular hypertrophy. On the other hand, Jeppesen *et al* (17) found that in humans an increase in blood plasma NT-pro-BNP is accompanied by a decrease in the risk of developing hypertension, because BNP determines the increased urinary sodium elimination, vasodilatation, and decreased ABP (17,18). Recent data show that BNP has an impact on the cardiac remodeling process that occurs in hypertension, due to its anti-hypertrophic, anti-proliferative and anti-inflammatory effects (19). In patients with CKD, left ventricular hypertrophy occurs, with increased left ventricular volume and pressure. Subsequent changes in myocardial structure, calcification, fibrosis and collagen accumulation, all accompany the ensuing systolic and diastolic cardiac dysfunction (20). Moreover, the concentration of NT-pro-BNP in blood plasma increases in parallel with the decline in renal function in patients subjected to dialysis (21). However, the cardiac dysfunction is mild in rats treated with Ang II for 14 days.

IL-1β, is a well known pro-inflammatory cytokine, released as a consequence of oxidative stress, and is involved in the fight against infections in autoimmune and metabolic diseases. There is an increased concentration of proinflammatory cytokines in blood plasma of patients with cardiac dysfunction (22). IL-1β is involved in the pathophysiology of heart failure by promoting the remodelling of the left ventricle. Our study reveals an increase of IL-1β in Ang II treated rats vs. control, in the kidney homogenate (Fig. 4), but not in blood plasma (Fig. 3).

In patients with CKD, left ventricular hypertrophy and contractile myocardial dysfunction are independent predictive factors of mortality. Blood plasma IL-1β is increased in CKD patients (23), by a dual mechanism: due to the increased uric acid concentration in blood serum, which leads to increased oxidative stress, and also due to the release of IL-1β from the myocardium in left ventricular dysfunction (24). Studies in rats with heart failure have demonstrated an increase in proinflammatory cytokines (IL-1β, IL-6 and TNF-α) not only in plasma but also in peripheral tissues, associated with an increase in the concentration of all components of RAS. In the experimental model of hypertension induced by chronic Ang II administration, Navar *et al* (25) have identified new mechanisms, involving a role for increased expression of intrarenal angiotensinogen, which in turn is modulated by the increased expression of TNF-α and IL-6 due to the chronic Ang II treatment.

It is known that renal Ang II exerts proinflammatory effects by stimulating renal infiltration with T lymphocytes and macrophages (26). These cells, which belong to the immune system, secrete inflammatory cytokines such as IL-6 and TNF-α (27). We observed increased gene expression for IL-6 and TNF-α in the kidney homogenate from Ang II-treated rats vs. control (Fig. 5). In chronic Ang II treatment, the elevation of intrarenal Ang II also involves IL-6, by its effect of increasing the local gene expression for angiotensinogen; this contributes to the increased ABP and to the renal impairment (28).

The inflammatory effects observed in the present study cannot be discussed in terms of their true cause, e.g. the results themselves do not allow us to really discern whether hypertension-related inflammation is due to hypertension itself, to Ang II, or to both. However, we prefer the third explanation, whereby the direct pro-inflammatory effects of Ang II are in fact part of an aggravating positive feedback loop (6-8,11), at least in this model of hypertension induced by Ang II. Moreover, this should occur in any circumstance of chronic high ABP, depending upon the degree of RAS involvement in each situation. Obviously, the two causative and/or mediating mechanisms could be decoupled in separate experimental models. For example in any hypertension model where ABP still increases despite the treatment with an antagonist of Ang II receptors. This would allow examination of the components of the pro-inflammatory environment which are induced by hypertension but are independent of Ang II. Such studies are available in the wide field devoted to the use of Ang II antagonists for the treatment of systemic arterial hypertension.

In conclusion, chronic Ang II increases ABP and this affects the target organs. Increased NT-pro-BNP in blood plasma reveals dysfunction of the heart left ventricle. The mechanism of renal impairment, in the hypertension induced by chronic Ang II, includes immune cell infiltration of the kidney, with the secretion of pro-inflammatory cytokines: IL-1β, IL-6 and TNF-α. Our findings suggest that the anti-inflammatory medication, which inhibits the immune system, might be useful to alleviate the kidney dysfunction in hypertension.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

All authors have substantially contributed to each of the following aspects of the article: Conception and design of the study (mainly MAM, DMT, DEB, DNS and ILS); execution of the experiment (mainly MAM and DMT); analysis and interpretation of the data (mainly MAM, DMT, DNS and DCB); drafting the manuscript (mainly MAM, DMT, ILS and DEB); revising the manuscript critically for important intellectual content (mainly DNS, ILS, DCB and DEB). All authors have read and approved the final version of the manuscript. Thus, each author has participated sufficiently in the study and takes public responsibility for appropriate portions of the content. The authors agree to be accountable for all aspects of the study in ensuring that questions related to the accuracy or integrity of any part of the study are appropriately investigated and resolved.

Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of the 'Grigore T. Popa' University of Medicine and Pharmacy (Iasi, Romania) and fulfils all the requirements of the guide issued by the International Society of Pain Study (IASP) and the European Council Committee (86/609/EEC) regarding the use of laboratory animals and biological preparations. The internationally accepted rules on animal studies were respected.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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