Angiotensin II receptor antagonism reverts the selective cardiac BNP upregulation and secretion observed in myocarditis

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METHODS

Animals and experimental protocol. The experimental protocol was approved by the University of Ottawa Animal Care Committee in accordance with the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals. The experiments were conducted using 8-wk-old Lewis rats obtained from Charles River (Wilmington, MA). Purified porcine myosin (Sigma) in phosphate buffer at a concentration of 10 mg/ml was emulsified with an equal amount of complete Freund’s adjuvant supplemented with 10 mg/ml of mycobacterium tuberculosis H37RA (Difco). On day 1, the rats were injected in two subcutaneous sites with the total of 0.2 ml of emulsion. The control rats received the emulsion without myosin. The animals were separated into three groups: control, EAM, and EAM + olmesartan (n = 8 in each group). Olmesartan (1 mg·kg⁻¹·day⁻¹) was administered orally every 2 days in accordance to body weight (BW) throughout the experimental period, was given to rats of the EAM + olmesartan group. The olmesartan dosage chosen does not affect content and BNP secretion in rat neonatal cardiocyte cultures but did not produce similar changes in ANF gene expression or secretion (13). It was further determined that proinflammatory cytokines, such as IL-1β and TNF-α, specifically stimulated BNP mRNA and BNP secretion in rat neonatal cardiocyte culture.

These results may underlie recent reports that have demonstrated that BNP plasma levels may increase during sepsis seemingly unrelated to hemodynamic parameters (1, 21), although no simultaneous determinations of plasma ANF levels were determined in these studies.

In the present study, experimental autoimmune myocarditis (EAM) in rats was used to determine whether the coordinate regulation of ANF and BNP observed during acute cardiac allograft rejection in humans was specific to this entity or was generally associated with myocardial inflammation. EAM is induced by T-cell activation following the immunization of rats with myosin heavy chain and histologically resembles giant cell myocarditis. Although the inflammatory response lessens after 3 wk, the animals show dilatation of the heart and congestive heart failure, thus mimicking human cardiomyopathy (9, 19). Since angiotensin II receptor type 1 antagonists have been reported to inhibit proinflammatory cytokine production in vitro and in vivo, we also tested the hypothesis that olmesartan, an angiotensin II receptor antagonist reported to reduce the severity of EAM at a low dose (1 mg·kg⁻¹·day⁻¹) without affecting blood pressure (16, 17), would revert the disregulation of ANF and BNP production observed in myocarditis, henceforth providing a mechanistic insight into this phenomenon.

THE NATRIURETIC PEPTIDES atrial natriuretic factor (ANF; and atrial natriuretic peptide) and brain natriuretic peptide (BNP) are cardiac hormones that increase in blood in pathophysiological states such as arterial hypertension and chronic congestive heart failure (17, 26). However, we previously found that during an acute cardiac allograft rejection episode in humans, plasma BNP may be elevated above prejection levels independent of hemodynamic parameters, whereas plasma ANF levels did not change in unison with BNP. Successful treatment of the acute rejection episode reversed the increased circulating BNP, suggesting that cytokines released from activated T lymphocytes may stimulate BNP secretion from cardiocytes (15).

In support of this view, we found that the conditioned medium derived from a mixed lymphocyte reaction, an in vitro model of transplantation immunity, stimulates BNP mRNA
blood pressure (27). Three weeks after the initiation of the experiments, BW and systolic blood pressure by the tail-cuff method were determined and the rats were euthanized by decapitation. Trunk blood was collected in chilled tubes containing EDTA and immediately centrifuged at 4°C. After centrifugation, the plasma was stored at -80°C. After blood collection, the heart was excised and dissected in cold saline into the right and left atrium and the right and left ventricle with their respective septa as part of the left chambers. After the hearts were weighed, the tissues were wrapped in aluminum foil and snap frozen in liquid nitrogen for a determination of natriuretic peptide content and mRNA levels. The hearts from two randomly chosen animals in each group were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned for histopathological examination after staining with hematoxylin-eosin. The pathologist was blinded to treatment group. Myocarditis was graded as follows: mild = <2 foci of inflammation/low-power field, moderate = 2–5 foci/low-power field, and severe = >5 foci/low-power field.

Table 1. BW, systolic blood pressure, and relative heart chamber wet weights of rats with EAM

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BW, g</th>
<th>Systolic Blood Pressure, mmHg</th>
<th>LA Weight-to-BW Ratio</th>
<th>RA Weight-to-BW Ratio</th>
<th>LV Weight-to-BW Ratio</th>
<th>RV Weight-to-BW Ratio</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>314.8±6.6</td>
<td>116.4±4.2</td>
<td>9.04±0.39</td>
<td>10.66±0.76</td>
<td>165.5±3.3</td>
<td>34.0±1.9</td>
</tr>
<tr>
<td>EAM</td>
<td>247.8±1.5§</td>
<td>110.1±4.2</td>
<td>10.07±0.27*</td>
<td>15.78±0.93§</td>
<td>234.1±19.0§</td>
<td>50.3±4.5§</td>
</tr>
<tr>
<td>EAM + olmesartan</td>
<td>255.0±5.1§</td>
<td>108.9±2.2</td>
<td>7.96±0.78†</td>
<td>13.47±1.14*</td>
<td>169.0±4.1‡</td>
<td>43.2±3.6†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8 animals for each group. BW, body weight; EAM, experimental autoimmune myocarditis; LA, left atrium; RA, right atrium; LV, left ventricle; RV, right ventricle. *P < 0.05 vs. control group; †P < 0.05 vs. EAM group; ‡P < 0.01 vs. EAM group; §P < 0.01 vs. control group.

Immunoreactive ANF and BNP determination in plasma and tissue. Plasma samples were acidified by adding 100 μl/ml of 1 M HCl and passed through Sep-Pak C18 cartridges (Millpore) that were prewetted with 5 ml of 80% acetonitrile (ACN) in 0.1% trifluoroacetic acid (TFA) and 10 ml of 0.1% TFA. The cartridges with the absorbed peptides were washed with 20 ml of 0.1% TFA and eluted with 3 ml of 60% ACN in 0.1% TFA. Tissue samples were homogenized in 10 vol of an extracting mixture consisting of 0.1 N HCl, 1.0 mol/l acetic acid, and 1% NaCl and centrifuged at 10,000 g for 30 min at 4°C. The supernatants were then extracted with the use of Sep-Pak C18 cartridges as described above for plasma, except that the elution was with 80% ACN in 0.1% TFA. The eluates from tissue or plasma were freeze-dried and processed for RIA. Immunoreactive (ir) ANF and irBNP were determined by RIA as previously described with anti-rat ANF99-126 and anti-rat BNP64-95 sera, respectively (17). The ANF and BNP antisera showed <0.01% cross-reactivity with BNP and ANF peptides, respectively.

Fig. 1. Histopathology of experimental autoimmune myocarditis (EAM). A and B: low-power views of normal and of EAM left ventricular (LV) apexes, respectively. D and E: corresponding higher magnifications. The latter demonstrates the presence of giant multinucleated cell present within the inflammatory reaction. C, F (ventricle), and I (atrium): abatement of myocarditis following olmesartan (Olm) treatment. G and H: normal and EAM atrial muscle, respectively. Original magnifications: A–C, ×100; and D–I, ×400 (hematoxylin-eosin stain).
**Plasma cytokine array.** Plasma cytokine concentration was evaluated using RayBio Rat Cytokine Antibody Array (RayBiotech). One milliliter of the plasma was applied to the array and was processed according to the manufacturer’s instructions. Membrane signals were quantitated by lucigenin-enhanced chemiluminescence using a Kodak 1D chemiluminescence imaging system. The analysis of image files was done using the RayBio Analysis Tool program. The results were expressed as relative densities compared with those of positive controls (biotin-labeled antibody) after the blank subtraction (BSA) included in each membrane. Cytokine signals were below that of the control signal.

**RNA extraction and RT-PCR.** RNA from tissue samples (n = 4, each chamber, each group) was extracted using TRIzol (GIBCO) following manufacturer’s instructions. RT was performed with SuperScript II Reverse Transcriptase (Invitrogen) using oligo(dT)12–18 (Invitrogen) according to the manufacturer’s instruction. Real-time PCR was performed using a LightCycler FastStart DNA Master SYBR Green I (Roche) according to the manufacturer’s instruction. The primers sequences and the thermal profiles of each primer are shown in supplemental Tables 1 and 2, respectively (note: all supplemental material can be found with the online version of this article). Results were normalized to glucose-6-phosphate dehydrogenase mRNA as an internal control and are thus shown as relative mRNA levels. Molecular sizes of specific amplicons following PCR were validated by electrophoresis together with a 123 bp DNA ladder (Invitrogen) in a 1% agarose gel and ethidium bromide staining.

**Statistical analysis.** All the results are expressed as means ± SE. ANOVA was performed to determine statistical differences among multiple groups using Systat. When significance was obtained by ANOVA, Bonferroni/Dunn test post hoc analysis was used to determine pairwise differences. A level of P < 0.05 was considered significant.

**RESULTS**

**Anatomical parameters and blood pressure.** The BW of EAM rats and EAM rats treated with olmesartan was significantly decreased compared with that of the control rats. Blood pressure did not differ among the groups. Relative heart chamber weights in EAM rats were significantly increased. Olmesartan administration significantly decreased compared with that of the control rats. Olmesartan administration to the EAM rats significantly reversed plasma BNP to control levels.

**Histopathology.** Full sections of myocardium from the different heart chambers were examined. Representative pictures are shown in Fig. 1. When compared with those of normal hearts (Fig. 1, A, D, and G), the sections from EAM animals were remarkable for severe granulomatous myocarditis in the ventricular (Fig. 1, B and E) and the atrial myocardium (Fig. 1H). The ventricular myocardium had patchy areas of active myocarditis (Fig. 1B). The granulomatous myocarditis inflammatory cells were mixed in nature, including lymphocytes, macrophages, plasma cells, and Langhans giant cells (Fig. 1, E and H). No necrotizing granulomas were seen. The cardiomyocytes were replaced and destroyed by the inflammatory cells. No significant fibrosis was seen.

Olmesartan administration significantly reduced the severity of the pathological changes in both the atrial and the ventricular myocardium (Fig. 1, C, F, and I). The right atrium and lower interatrial septum had residual mild healing myocarditis. This was mixed in cellular composition with macrophages, plasma cells, lymphocytes, and prominent numbers of neutrophils. No scarring was noted. Giant cells were absent.

**Plasma and cardiac ANF and BNP concentrations.** Plasma irANF levels showed no difference among the different experimental groups (Fig. 2). On the other hand, plasma BNP levels were significantly increased in EAM rats compared with the control rats. Olmesartan administration to the EAM rats significantly reversed plasma BNP to control levels.

Atrial irANF and irBNP content in EAM rats were significantly decreased compared with that of the control rats except that BNP content in the left atria, although lower, did not reach a statistically significant difference (Fig. 3). Olmesartan administration to the EAM rats significantly increased irANF but not irBNP content in the atria. Ventricular irANF and irBNP content in EAM rats were significantly increased compared with that of the control rats. Olmesartan administration to the EAM rats significantly reversed ventricular irANF and irBNP content to control levels.

**Relative ANF- and BNP-specific cardiac mRNA levels.** Left atrial ANF and BNP mRNAs tended to be lower compared with those of the control rats. Olmesartan administration to the EAM rats did not affect levels of expression (Fig. 4). In contrast, EAM rats showed strong ANF and BNP gene expression in the right atria that was normalized by the administration of olmesartan. Left ventricular (LV) and right ventricular ANF and BNP mRNAs in EAM rats were significantly increased, paralleling closely the changes seen in ANF and BNP content. Olmesartan administration significantly reversed changes in ventricular gene expression.

**Plasma cytokine levels.** Plasma lipopolysaccharide-induced CXC chemokine (LIX), leptin, monocyte chemotactic protein-1 (MCP-1), and tissue inhibitor of metalloproteinase-1 (TIMP-1) levels in EAM rats were significantly increased (P = 0.0393, P = 0.0367, P = 0.0447, and P = 0.0152, respectively) compared with those of the control rats (Fig. 5).

**Cytokine gene expression in the heart.** LIX, MCP-1, and TIMP-1 relative mRNA levels were significantly increased in EAM hearts compared with the control rat hearts with the
exception of LIX levels in the left atria and TIMP-1 levels in the left atria and left ventricles. Olmesartan treatment decreased specific mRNA levels for these cytokines (Fig. 6).

**DISCUSSION**

The elevation of plasma BNP levels found in EAM rats in the absence of changes in plasma ANF levels in these investigations is similar to that reported previously by our laboratory in episodes of acute cardiac allograft rejection in humans (15, 18). Separate quantifications of heart chambers were carried because our laboratory has shown previously that changes in ANF and BNP gene expression may occur in a chamber-specific manner (26). Unexpectedly, ANF and BNP content of both the left and right atria in EAM animals decreased compared with that of the control rats. In the right atria, the decrease in ANF and BNP concentration was accompanied by a pronounced increase in ANF and BNP message, suggesting that the rate synthesis of these peptides was not sufficient to prevent a partial depletion of a stored hormone. However, in the left atria, this relationship did not hold, suggesting that other factors, perhaps related to the pathophysiology of EAM, play an overriding role in ANF and BNP gene expression and storage. Overall, BNP gene expression increased relatively more than that of ANF, as shown in Fig. 3.

The uncoordinated increase in ANF and BNP transcripts, peptide contents, and plasma levels in EAM rats is unlikely due to hemodynamic changes induced by the disease, including heart failure. In humans, increased circulating BNP during cardiac allograft rejection does not correlate with right atrial pressure, pulmonary artery pressure, pulmonary capillary wedge pressure, or cardiac index (18). Additionally, IL-1β and TNF-α increase the secretion and expression of BNP but not of ANF in neonatal rat cardiocyte culture (13) in the absence of any mechanical stimulus.

The profile of irANF and irBNP increase in EAM rat ventricles is different from those described previously by our laboratory in aortic banding rats (17). Although 3 wk of EAM and 6 wk of aortic banding increased LV weight-to-BW ratio to a similar degree, 6 wk of aortic banding increased ventricular ANF and BNP content only twofold, and there was no difference between ANF and BNP peptide content or transcript levels. Aortic banding also increased both plasma irANF and irBNP levels to a similar degree. It appears that in myocarditis, inflammation stimulates ventricular ANF and BNP gene expression through factors that are different from hemodynamic or hormonal stimuli seen in hemodynamic overload models.

To identify factors that are unique in regulating ANF and BNP gene expression and cardiac content in myocarditis, and with the view that our previous work implicated cytokines in the selective upregulation of BNP (12), we carried out studies in plasma using cytokine arrays. There were four cytokines that showed a significant difference in plasma levels between control and EAM rats. Because of those four cytokines, three (LIX, MCP-1, and TIMP-1) are produced largely in the heart, we determine their level of cardiac expression (2, 5, 20). Significantly, the pattern of gene expression found for these cytokines was largely similar to the level of expression and content of ANF and BNP in the different heart chambers, and

![Graphs showing cardiac ANF and BNP content in control, EAM, and Olm-treated EAM rats](image-url)
so was the effect of olmesartan. A causal relationship among these observations remains to be demonstrated.

LIX is induced in cardiocytes by oxidative stress and TNF-α. In cardiac ischemia, the gene expression of LIX is elevated and the increased LIX recruits neutrophils to the sites of inflammation (2). MCP-1, a member of the C-C chemokine family, plays an important role in the migration of monocytes to the inflammation site. The gene expression of MCP-1 is elevated in various cardiovascular diseases such as acute myocardial infarction and myocarditis (5, 8). TNF-α stimulates MCP-1 production, and MCP-1 stimulates IL-1β production and gene expression in rat cardiocyte culture (3). The increased levels of chemokines such as LIX and MCP-1 and their potential to stimulate IL-1β production may explain why the conditioned medium from the mixed lymphocyte reaction selectively promotes BNP gene expression and secretion but lacks intrinsic significant concentrations of IL-1β (13). In the same experimental model as used in the present work, IL-1β protein expression was found increased by Western blot analysis. Olmesartan treatment (1 mg·kg⁻¹·day⁻¹) reversed this increase (16). The fact that IL-1β did not appear elevated in the plasma of EAM animals may be due to the fact that spillover into the general circulation is not sufficient to increase circulating levels as detected by the array. Finally, there appear to be

Fig. 4. Relative ANF and BNP mRNA levels in control, EAM, and EAM rats treated with Olm (n = 4 animals for each group). *P < 0.05 and **P < 0.01 vs. control; ***P < 0.01; †P < 0.05; ††P < 0.01 vs. EAM rats.

Fig. 5. Cytokine plasma levels in control (white bar) and EAM (black bar) rats (n = 4 animals for each group). *Lipopolysaccharide-induced CXC chemokine (LIX), P = 0.0393; leptin, P = 0.0367; monocyte chemotactic protein-1 (MCP-1), P = 0.0447; or tissue inhibitor of metalloproteinase (TIMP-1), P = 0.0152, respectively; vs. control rats.
no reports in the literature linking LIX or MCP-1 to ANF or BNP gene expression or secretions either in vivo or in vitro.

Matrix metalloproteinases are a family of proteolytic enzymes responsible for the degradation of extracellular matrix and are involved in the remodeling process that follows myocardial injury. TIMP-1 is upregulated in various cardiovascular diseases (6, 10, 24). In the present investigations, TIMP-1 mRNA levels in EAM rats increased only in the right atrium and right ventricle, and no elevation of TIMP-1 mRNA levels was seen on the left side of the heart. The reason for this is not apparent. TIMP-1 has not been previously associated with either ANF or BNP production. However, in neonatal cardiac myocyte cultures, IL-1β and TNF-α stimulated TIMP-1 gene expression (11). Additionally, plasma BNP and plasma TIMP-1 levels were found positively correlated in heart failure patients (25).

Omesartan, an angiotensin II type I receptor antagonist, has, in addition to this pharmacological action, a beneficial effect in terms of improved plasma levels of C-reactive protein, TNF-α, IL-6, and MCP-1 in hypertensive patients (4). Previous studies have shown that olmesartan administration to EAM rats decreases ventricular IL-1β expression (16), suggesting that it improves myocarditis by inhibiting both the action of angiotensin II and TNF-α (16), and that of agents associated with the inflammatory process. This is in line with observations (7) with the angiotensin I-converting enzyme (ACE) inhibitor captopril, which was shown to prevent EAM although a contribution of blood pressure lowering effects could not be discounted. Rapamycin, a potent immunosuppressant, was shown to ameliorate EAM and reduce plasma levels of BNP (14). However, rapamycin significantly increased blood pressure while lowering BNP plasma levels, making the interpretation of results difficult but suggesting that the main determinant of the increase in BNP plasma levels in EAM is not the hemodynamic parameters but the inflammatory process per se.

The dosage schedule of olmesartan used in this study (1.0 mg·kg⁻¹·day⁻¹) reversed the increase in heart weight, plasma irBNP levels, ventricular irANF and irBNP content, and rela-
tive mRNA expression in EAM rats to control levels without affecting blood pressure as measured by tail plethysmography. However, the plethysmographic blood pressure measurements cannot discern subtle changes in blood pressure, and such changes could partially or wholly explain the effects observed in BNP gene expression.

In our previous studies using a low dose of ramipril in aortic-banded rats, we were also able to revert the LV weight-to-BW ratio to control levels without affecting blood pressure (17). However, ramipril decreased plasma and ventricular irANF and irBNP content and relative mRNA expression only partially. Although the differences in experimental models are very significant, it may be worth exploring the possibility that the ability of olmesartan to revert to control levels in both anatomical parameters and BNP gene expression in EAM is due to the additional known action of olmesartan on cytokines. In rats with acute myocardial infarction, olmesartan administration improved cardiac function and reduced the infarct size better than ramipril, and it, but not ramipril, decreased macrophage infiltration and decreased IL-1β mRNA levels (22). In human peripheral blood mononuclear cells, ramipril did not suppress the genetic expression of IL-1β or TNF-α as did other ACE inhibitors (23).

In conclusion, the work presented here is the first description of a relationship between the production of cardiac BNP (an important antifibrotic agent), inflammation, and angiotensin II. It is also the first demonstration of an instance in which BNP is upregulated in an experimental setting in vivo. It is also the first demonstration of an instance in which BNP is upregulated in an experimental setting in vivo.

**REFERENCES**


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