Effect of the angiotensin II receptor blocker olmesartan on the development of murine acute myocarditis caused by coxsackievirus B3

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ABSTRACT

Ang II (Angiotensin II) has been shown to play a pivotal role in the pathophysiology of various organs, especially the cardiovascular system. The effects of ARBs (Ang II receptor blockers) in the treatment of hypertension, congestive heart failure and myocardial fibrosis have been analysed extensively in human trials, as well as animal models, and the focus of interest is now directed to its pleiotropic effects, especially on inflammatory disorders. To investigate the effects of a new ARB, olmesartan, on immune-mediated myocardial injury, the protective effects of olmesartan on the development of murine acute myocarditis caused by CVB3 (coxsackievirus B3) were analysed. Olmesartan and a non-specific vasodilator hydralazine lowered systolic blood pressure of mice on day 7 after virus inoculation to a similar extent. Olmesartan significantly decreased myocardial inflammation compared with controls, whereas hydralazine significantly increased this. Olmesartan significantly decreased the expression of IFN-γ (interferon-γ), FasL (Fas ligand), iNOS (inducible nitric oxide synthase) and PFP (pore-forming protein) in myocardial tissue, indicating that olmesartan suppressed the activation of infiltrating killer lymphocytes. Olmesartan also decreased the expression of CVB3 genomes in myocardial tissue as well as serum levels of 8-OHdG (8-hydroxy-2′-deoxyguanosine), a biomarker of oxidative-stress-induced DNA damage. The findings suggest that olmesartan prevents myocardial damage and may improve the prognosis of patients with acute myocarditis; however, further investigations are needed before clinical use.

INTRODUCTION

Ang II (angiotensin II) has been shown to play a pivotal role in the pathophysiology of various organs, especially the cardiovascular system. The effects of ARBs (Ang II receptor blockers) in the treatment of hypertension, congestive heart failure and myocardial fibrosis have been well analysed in human trials, as well as animal models, and the focus of interest is now directed to its pleiotropic effects, especially on inflammatory disorders. Previous studies have reported that Ang II, acting through the AT1 (Ang II type 1) receptor, plays an important role in the immunopathology involved in renal injury [1,2], chronic allograft rejection and graft coronary artery disease [3,4], as well as acute inflammation, such as viral myocarditis [5,6]. ARBs have also been reported to suppress arteriosclerotic lesions in animal models and patients with coronary artery disease or hypertension by modulating inflammatory responses [7–11]. Histological evidence of active inflammation was reported to exist in substantial portion of patients with idiopathic heart failure, such as dilated cardiomyopathy [12]. It is known that dilated cardiomyopathy or myocardial dysfunction with unknown aetiology not only develops as a consequence

Key words: angiotensin II, coxsackievirus B3, inflammation, infection, lymphocyte, myocarditis, oxidative stress.

Abbreviations: Ang II, angiotensin II; ARB, Ang II receptor blocker; AT1, Ang II type 1; CVB3, coxsackievirus B3; FasL, Fas ligand; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HR, heart rate; ICAM-1, intercellular cell-adhesion molecule-1; IFN-γ, interferon-γ; iNOS, inducible nitric oxide synthase; LFA-1, lymphocyte function-associated antigen-1; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; SBP, systolic blood pressure; PFP, pore-forming protein.

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of acute myocarditis, but can be induced by latent cardiac myocyte injury through immunological mechanisms, as well as various external stresses, including metabolic, mechanical and oxidative stress, which may play an important role in the progression of aging. In the present study, to investigate the effects of ARBs on the immune-mediated myocardial injury, the protective effects of a new ARB olmesartan on the development of murine acute myocarditis caused by CVB3 (coxsackie virus B3) was investigated.

METHODS

Animals
Five-week-old male C3H/He mice were purchased from Shizuoka Laboratory Animal Centre (Shizuoka, Japan). Full anaesthetic procedures were used and all precautions were taken to ensure that the animals did not suffer unduly during and after the experimental procedure. This work was undertaken as required by the Institutional and National Animal Care Committees.

Virus
The preparation of CVB3 (Nancy strain) was as described previously [13]. Five-week-old male C3H/He mice were inoculated intraperitoneally with \( 1 \times 10^6 \) plaque-forming units of CVB3 in 0.2 ml of PBS.

In vivo treatment of mice with olmesartan or hydralazine
Five-week-old C3H/He mice were divided into three groups (groups A, B or C), with eight mice in each group. Mice in groups B or C received olmesartan (Sankyo) or hydralazine respectively, in 0.2 ml of 0.1 % NaHCO₃ (15 mg/kg of body weight daily, intraperitoneally) from the day of virus inoculation (day 0) until day 6. Mice in group A received 0.2 ml of vehicle (0.1 % NaHCO₃) as controls in the same way.

Mice were killed on day 7, serum samples were taken and stored at −80 °C, and the hearts were sectioned laterally approximately midway between the apex and atria, which resulted in cross-sections of both ventricles. Half of each heart was fixed in 10 % (v/v) buffered formalin and used for histology. The other half of each heart was frozen in liquid nitrogen and used for PCR.

Histology
The cross-sections of formalin-fixed heart tissue from mice in each group were stained with haematoxylin and eosin prior to being photographed. The total area of the myocardium and the areas of inflammation (consisting of cell infiltration and necrosis) were scanned. The percentage area of the myocardium undergoing inflammation was determined by analysis performed using the public domain NIH Image program.

Preparation of RNA and cDNA synthesis
The procedures for preparation of total cytoplasmic RNA from heart tissue and cDNA synthesis were as described previously [14].

Amplification of cDNA by PCR
To examine semi-quantitatively the expression of mRNAs of cytokines and other immune mediators in the heart tissues, single-stranded cDNA was amplified in a DNA thermal cycler (PerkinElmer) with 1 unit of ExTaq DNA polymerase (TAKARA BIO) using 5′- and 3′-primers specific for IFN-γ (interferon-γ), FasL (Fas ligand), iNOS (inducible nitric oxide synthase), PFP (pore-forming protein), CVB3 and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) respectively. The primer sequences, annealing temperature and the number of cycles for IFN-γ, GAPDH, FasL, PFP and CVB3 genes were as described previously [15–18]. PCR was performed as follows: denaturation at 94 °C for 1 min, primer annealing for 1 min and primer extension at 72 °C for 2 min. Expression of these mRNAs was examined using ethidium-bromide-stained agarose gels. The relative expression levels of the PCR products were quantified by analysis performed using the public domain NIH Image program.

Measurement of serum concentration of 8-OHdG (8-hydroxy-2′-deoxyguanosine)
Serum 8-OHdG concentration was measured by a Highly Sensitive 8-OHdG Check ELISA [Japan Institute for the Control of Aging (JaICA), Nikken SEIL] which is a competitive ELISA for quantitative measurement of the oxidative DNA adduct 8-OHdG in various biological samples, such as DNA of tissue, serum, urine etc., using an anti-(8-OHdG) monoclonal antibody (according to the manufacturer’s instructions). The measurement range of this kit was 0.125–10 ng/ml.

Statistical analysis
Values are means ± S.E.M. One-way ANOVA (P value corrected by using Bonferroni/Dunn’s modulus for multiple comparison) was used to evaluate differences among the three groups. Wilcoxon’s two-rank-sum test (Mann–Whitney test) with P values corrected by the Bonferroni method was used to evaluate differences between the two groups.

RESULTS

Effects of olmesartan or hydralazine on arterial SBP (systolic blood pressure) and HR (heart rate)
As shown in Table 1, there were no significant differences in arterial SBP on day 0 between mice in groups A
Table 1  Effects of olmesartan and hydralazine on arterial SBP and HR before and after treatment

<table>
<thead>
<tr>
<th></th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
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<tbody>
<tr>
<td>SBP (mmHg)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Day 0</td>
<td>94.96 ± 1.29</td>
<td>95.58 ± 1.73</td>
<td>97.18 ± 2.47</td>
</tr>
<tr>
<td>Day 7</td>
<td>91.35 ± 2.62</td>
<td>73.70 ± 2.98**</td>
<td>75.08 ± 2.85**</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>583.8 ± 19.4</td>
<td>592.8 ± 28.6</td>
<td>603.7 ± 22.4</td>
</tr>
<tr>
<td>Day 7</td>
<td>562.5 ± 15.9</td>
<td>587.5 ± 31.8</td>
<td>634.2 ± 30.8</td>
</tr>
</tbody>
</table>

*P < 0.01 compared with group A (vehicle-treated control).

Effects of olmesartan and hydralazine on the development of myocardial inflammation

The incidence of myocarditis was 100% in all of the groups. Figure 1 shows a representative section of the heart of a mouse from the vehicle-treated control group (group A; Figure 1A), the olmesartan-treated group (group B; Figure 1B) and the hydralazine-treated group (group C; Figure 1C) respectively. Extensive cell infiltration and necrosis were seen in the control group (Figure 1A), whereas both cell infiltration and necrosis were less severe in the olmesartan-treated group (Figure 1B). However, both cell infiltration and necrosis were more severe in the hydralazine-treated group (Figure 1C). Quantification of the histological study (Figure 2) revealed that the area of myocardium undergoing inflammation was significantly decreased (P < 0.015) in the olmesartan-treated group (group B), but significantly increased (P < 0.015) in the hydralazine-treated group (group C), compared with the vehicle-treated control group (group A). Thus blockade of the AT1 receptor significantly decreased myocardial inflammation induced by CVB3.

Figure 1  Histological analysis of the effects of olmesartan and hydralazine on the development of myocarditis

Cross-sections of a heart of a mouse (day 7) in the vehicle-treated control group (A), the olmesartan-treated group (B) and the hydralazine-treated group (C) stained with haematoxylin and eosin. Scale bar, 100 μm.
Effects of olmesartan treatment on the expression of pro-inflammatory cytokine transcripts and CVB3 genomes in ventricular tissue

To investigate the effects of AT1 receptor inhibition on the activation of infiltrating cells, the expression of IFN-γ, which is mainly expressed by infiltrating cells and can be a good marker of activation [19], and FasL, which is expressed on infiltrating cells and has been shown to play important roles in myocardial injury [15], were examined. Furthermore, the expression of iNOS, which is induced by inflammation and may have a cytotoxic effect, and a cytolytic factor PFP, known to be expressed by killer lymphocytes such as natural killer cells and cytotoxic T-lymphocytes [13], as well as CVB3 genomes, were examined. cDNAs were amplified from ventricular tissue of mice from the vehicle-treated control group (group A) and olmesartan-treated group (group B) by semi-quantitative PCR. Expression of GAPDH transcripts as an internal standard showed that almost equivalent amounts of RNA were prepared from each mouse. The relative expression of these immune mediators, corrected for the expression of GAPDH, are summarized in Figure 3(B). Olmesartan treatment significantly decreased the expression of IFN-γ, FasL, iNOS and PFP, suggesting that blockade of the AT1 receptor inhibited the activation of infiltrating cells. Olmesartan treatment also decreased the expression of CVB3 genomes in ventricular tissue, although the difference between the two groups was not significant statistically.
**Effects of hydralazine treatment on the expression of pro-inflammatory cytokine transcripts and CVB3 genomes in ventricular tissue**

Because hydralazine treatment significantly increased myocardial inflammation induced by CVB3, the effects of hydralazine treatment on the expression of the transcripts of these immune mediators, as well as CVB3 genomes, were examined. Figure 4(A) shows the PCR products obtained by ethidium-bromide-stained agarose gel electrophoresis. Expression of GAPDH transcripts as an internal standard showed that almost equivalent amounts of RNA were prepared from each mouse. The relative expression levels of these immune mediators, corrected for the expression of GAPDH, are summarized in Figure 4(B). Hydralazine treatment significantly increased the expression of IFN-γ, FasL, iNOS and PFP, suggesting that hydralazine facilitated the activation of infiltrating cells. Hydralazine treatment also significantly increased the expression of CVB3 genomes in ventricular tissue, indicating aggravation of viral myocarditis.

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Effects of olmesartan treatment on the serum levels of 8-OHdG

It has been reported that increased oxidative stress exacerbates viral infection by potentiating virulence [20]. Therefore to investigate the effects of AT1 receptor inhibition on oxidative stress, we measured serum levels of 8-OHdG in mice from the vehicle-treated control group (group A) and the olmesartan-treated group (group B). The serum concentration of 8-OHdG in the olmesartan-treated group was decreased compared with control group (0.176 ± 0.051 compared with 0.210 ± 0.044 ng/ml respectively); however, the difference was not significant statistically.

DISCUSSION

In the present study, a new ARB, olmesartan, has been shown to markedly reduce myocardial injury involved in murine acute myocarditis caused by CVB3. This was associated with a significant decrease in the expression of IFN-γ, FasL, iNOS and PFP in myocardial tissue, indicating that olmesartan suppressed the activation of infiltrating killer lymphocytes. Olmesartan also decreased (but not significantly) CVB3 genomes in myocardial tissue, as well as serum levels of 8-OHdG, a marker of oxidative stress. However, hydralazine significantly increased myocardial injury with a significant increase in the expression of IFN-γ, FasL, iNOS and PFP and CVB3 genomes.

The effect of olmesartan or hydralazine treatment on myocardial injury without viral infection was also examined. No significant injury and only minimal expression of IFN-γ, FasL, iNOS and PFP transcripts were observed in either group, as in normal myocardial tissues (results not shown).

Evidence has accumulated that Ang II activates multiple intracellular signalling pathways through the AT1 receptor, including MAPKs (mitogen-activated protein kinases), PLC (phospholipase C), the JAK/STAT (signal transducer and activator of transcription) pathway and Rho kinases, leading to the activation of various transcription factors, such as NF-κB (nuclear factor κB), AP-1 and NF-AT (nuclear factor of activated T-cells) [21]. Because these transcription factors critically regulate the expression of various cytokines, cell-adhesion molecules and chemokines, which play a pivotal role in the development of inflammation, it is reasonable for olmesartan to suppress acute myocarditis, as shown in the present study. An ACE (angiotensin-converting enzyme) inhibitor has also been shown to ameliorate acute murine viral myocarditis [22], strongly supporting the critical role for Ang II in the immunopathology of myocarditis. On the other hand, it is known that hydralazine may cause lupus-like disease. Yung et al. [23] reported that T-cells treated with DNA methylation inhibitors, such as hydralazine, overexpress LFA-1 (lymphocyte function-associated antigen-1), which results in autoreactivity and autoimmunity, leading to lupus-like disease. As we reported previously [24], the LFA-1/ICAM-1 (intercellular cell-adhesion molecule-1) pathway plays a pivotal role in the development of myocardial injury in this model of viral myocarditis, and enhancement of LFA-1 expression on T-cells could be one of the mechanisms responsible for aggravating myocardial injury following hydralazine treatment. Furthermore, hydralazine is known to induce myocardial ischaemia, which may facilitate myocardial injury by viral myocarditis.

It is known that immunosuppressive therapy with corticosteroids aggravates acute viral myocarditis by enhancing virus titres. Immunomodulation therapy specifically targeting co-stimulatory molecules, such as ICAM-1 and FasL, can decrease myocardial damage without inhibiting virus clearance [15,24]. Furthermore, using a murine model of acute myocarditis, we have reported that immunomodulation therapy targeting co-stimulatory molecules, including B7-1 [25], CD40L [26] and 4-1BB [27] in addition to ICAM-1 [24] and FasL [15], can significantly attenuate myocardial inflammation. Treatment with an anti-(B7-1) monoclonal antibody was the most effective, followed by treatment with an anti-(ICAM-1) monoclonal antibody. The effect of olmesartan treatment on the development of myocardial inflammation in the present study was less than that with the anti-(B7-1) and -(ICAM-1) monoclonal antibodies, but was more than with treatment anti-(CD40L), -(4-1BB) and -(FasL) monoclonal antibodies. The present study has also shown that olmesartan can significantly decrease myocardial damage without enhancing viral replication as effectively as these specific immunomodulation therapies. Although these immunomodulation therapies may not be directly applicable to clinical use, blockade of co-stimulatory signals appears to be effective in the treatment of patients with acute myocarditis. For clinical use, development of humanized monoclonal antibodies or synthetic compounds that effectively block co-stimulatory signals will be necessary. On the other hand, ARBs including olmesartan are widely used in the treatment of hypertension, heart failure and cardiac hypertrophy. Because CVB3 is the most common pathogen in human myocarditis, the results of the present study suggest that olmesartan may play an important role in suppressing myocardial inflammation as well as vascular inflammation, such as atherosclerosis, in these patients treated with olmesartan [11]. It is known that external stresses, such as viral infection, heat shock and ischaemia/reperfusion, as well as unknown autoimmune mechanisms, can induce myocardial inflammation which, in turn, cause not only acute, but persistent, myocardial injury. Only a portion of patients with acute myocarditis go on to develop dilated cardiomyopathy, whereas, during the course of aging, most people will suffer, to a greater or
lesser extent, from some form of myocardial inflammation, such as atherosclerosis. Another ARB, candesartan, has also been reported to reduce myocardial injury in murine myocarditis caused by encephalomyocarditis virus [5]. Although this virus is not common in humans, chronic antihypertensive therapy with ARBs, including olmesartan, appear to play an important role in suppressing myocardial, as well as vascular, inflammation and protect from the progression of myocardial dysfunction and atherosclerosis.

It is known that Ang II mediates ROS (reactive oxygen species) generation through activation of NADPH oxidase [28]. Tsuda et al. [29] reported that olmesartan and oestrogen synergistically attenuate atherosclerosis at least partly via inhibition of oxidative stress. Deoxyguanosine is one of the constituents of DNA and is converted into 8-OHdG when oxidized. 8-OHdG is stable in vivo after it is excised from DNA by the repair enzyme system and is ultimately released into blood and excreted via urine. Therefore the concentration of 8-OHdG can be measured to determine total oxidative stress in vivo. In the present study, olmesartan has been shown to decrease (although not significantly) the serum concentration of 8-OHdG in mice with acute viral myocarditis, supporting the antioxidative stress effect of olmesartan in myocardial inflammation, as in vascular inflammation. Although oxidative stress is known to exacerbate viral infection, it appears that the contribution of an antioxidative stress effect of olmesartan on the suppression of myocardial inflammation was at most partial, because the anti-inflammatory effect was much more than antioxidative stress effect. In addition to the virus-induced immune-mediated inflammation, ARBs have been shown to play a protective role in another inflammatory disorder induced by an external stress, namely myocardial ischaemia/reperfusion [30,31]. This may at least partly contribute to the protective effects of ARBs in viral myocarditis. However, because results of the present study were from an experimental model, further investigation is needed to assess whether ARBs such as olmesartan really improve myocardial injury in patients with acute myocarditis.

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