The ACE inhibitors enalapril and captopril modulate cytokine responses in Balb/c and C57Bl/6 normal mice and increase CD4^+CD103^+CD25^- negative splenic T cell numbers

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1. Introduction

Accumulating data from clinical and experimental studies have emphasized an important role of angiotensin-converting enzyme (ACE) inhibitors on non-hemodynamic, immune-mediated functions such as cytokine production [1–3]. In this regard, it has been recognized that the beneficial effects of ACE inhibitors on several cardiovascular disorders is, at least in part due to the modulation of proinflammatory cytokines. Accordingly, in chronic heart failure patients, the ACE inhibitor enalapril decreased circulating levels of interleukin (IL)-6 [1] which is induced by angiotensin II (Ang II) [4,5] and is elevated in patients with unstable angina [6]. On the other hand, enalapril-long term treated hypertensive patients produced significantly high levels of IL-10 [2]. As IL-10 deactivates macrophages, dendritic cells, indirectly prevents antigen-specific T cell activation, decreases serum cholesterol levels and exerts anti-sclerotic effects in vitro and in vivo [7–12], one could argue that, at least part of the additional beneficial effects of ACE inhibitors on renal and heart diseases are due to the increased IL-10 production. The mechanism(s) underlying immunemodulatory properties of ACE inhibitors are believed to result mostly from inhibition of Ang II formation, since in experimental models, such as apolipoprotein E deficient (apoE-KO) mice, the ACE inhibitor enalapril completely abolished Ang II-up regulated expression of the proinflammatory chemokines monocyte chemotactic protein-1 (MCP-1) and macrophage-colony stimulating factor (M-CSF) [13]. Moreover, human monocyes stimulated with tumor necrosis factor-α (TNF-α) and granulocyte macrophage-colony stimulating factor (GM-CSF) release Ang II [14], suggesting that there is a link between the rennin–angiotensin system (RAS) and cytokine production that may be independent of hemodynamic factors. So far, most of the data on non-hemodynamic effects of Ang II, as well as those of ACE inhibitors, have been obtained from human patients and experimental studies. Little attention however, has been paid to immunemodulatory roles of ACE inhibitors on systemic cytokine synthesis in non-manipulated animals. Since T lymphocytes, dendritic cells and macrophages have all the components of the RAS [15–18], we hypothesized that Ang II may act as a bridge in the vascular/angiotensin-immune system axis and ACE
inhibitors modulate both hemodynamic and immune-mediated effects of Ang II, including systemic cytokine production. To test this hypothesis, in the present work we evaluated the effects of inhibiting physiological levels of Ang II on cytokine synthesis in Balb/c and C57Bl/6 normal mice. We show that both short- and long-term treatment of mice with enalapril increased spleen cell production of IL-10 while captopril increased it only in Balb/c mice. Enalapril as well as captopril-short-term treatment enhanced IL-2 synthesis in Balb/c mice. Furthermore, CD4+CD103+ presented increased IL-10 production after enalapril treatment. Besides, the enhancement of IL-2 and IL-10 levels correlated with a slight increased CD4+CD103+CD25negative T cells numbers in spleens from enalapril-treated mice, suggesting that under physiologic conditions inhibition of Ang II formation may play an important role on the immune system by controlling cytokine expression and consequently modifying immune and regulatory functions exerted by splenic T-cell populations.

2. Materials and methods

2.1. Animals

C57Bl/6 and Balb/c mice (1–2 month old) were obtained from Oswaldo Cruz Foundation, Salvador, BA. All experiments were performed according to the Institutional Ethical Guidelines on the use of animals in research and also accordingly to Oswaldo Cruz Foundation Ethical Committee. Since C57Bl/6 and Balb/c mice are considered Th1- and Th2-prone mouse strains, we have chosen them to verify whether the genetic background of those mice would exert differential effects on the immunoregulatory properties of enalapril and captopril on cytokine synthesis.

2.2. Treatment

Mice were treated with enalapril, captopril or were left on normal drinking water. Enalapril and captopril (Neo Quimica, Brazil) were dissolved in drinking water at a concentration of 0.02 and 0.2 mg/mL, respectively, and replaced every 24 h. The daily dosage was 5 mg/kg body weight for enalapril and 30 mg/kg for captopril, assuming a daily fluid intake of 5 mL. These dosages are within the usual therapeutic ranges used in mice [3,19–21] and were chosen to achieve a similar level of systolic blood pressure. We chose the ACE inhibitors enalapril and captopril to verify whether the difference in their active moieties would exert differential effects on cytokine production in healthy mice, since it has been reported that those two ACE inhibitors exert differential effects in experimental lupus disease [19].

2.3. Spleen cell culture

Spleen cells (5 × 10⁶ per mL) from each individual mouse were cultured for 24–48 h in complete medium (RPMI 1640 supplemented with 10% FCS, 2 mmol/L L-glutamine, 4 mmol/L non-essential amino acids, 5 × 10⁻⁵ M 2-mercaptoethanol, sodium pyruvate, and 10 mM Hepes). Cells were cultured in duplicate in flat-bottomed microwells and maintained in a humidified atmosphere with 5% CO₂ at 37 °C. The cultures were done either in complete medium alone or were stimulated with immobilized anti-CD3 mAb (2C 11) plus soluble anti-CD28 mAb (PV-1). Supernatants were collected and kept at −80 °C for further studies.

2.4. Measurements of cytokines and intracellular staining

Cytokine levels in culture supernatants were determined by ELISA, using monoclonal antibody pairs and recombinant cytokines purchased from PharMingen, as previously described [3]. For intracytoplasmic staining, cells were first stained with FITC-conjugated (FL-1) or Cy5-conjugated (FL-3) primary mAbs, as indicated, for 20 min at 4 °C and then fixed in 2% paraformaldehyde for 30 min. The cells were then washed and incubated in staining buffer containing 0.1% saponin for 30 min. Continuously exposed to saponin, the cells were then stained with PE-conjugated anti-murine IL-10 (Invitrogen Corporation) for 30 min at 4 °C (FL-2). After washing with staining buffer, the cells were washed again with staining buffer without saponin to allow membrane closure. A minimum of 30,000 events were collected by reading on FL-1, FL-2 or FL-3, for flow cytometric analysis. Results were analyzed using FLOWJO software (Tree Star Inc., Ashland, OR).

2.5. Staining of spleen cells for flow cytometry

Staining was performed as previously described [22], followed by analysis on a FACScan analyzer (Becton and Dickinson). In all studies, two acquisitions were performed for every sample: one for total cells, in order to determine the absolute frequencies and other deliberately gating positive events for further analysis. A total of 10,000 events were recorded per sample.

2.6. Statistical analysis

Data are reported as the means ± SE. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Mann–Whitney test and probability values of P < 0.05 were considered significant.

3. Results

3.1. Effect of enalapril and captopril on spleen cell production of IL-10

As enalapril-long-term therapy of hypertensive patients significantly increases IL-10 levels [2], we reasoned that it would be valuable to verify if enalapril would enhance IL-10 synthesis in normal mice after a long-term treatment with that ACE inhibitor. To do that, we treated different strains of mice such as C57Bl/6 and Balb/c mice with enalapril for one month. Control animals were left without treatment or were treated with captopril. The data expressed in Fig. 1A show that enalapril-long-term treatment of C57Bl/6 mice tended to increase IL-10 production; even it did not reach statistic significance when compared to untreated mice. However, enalapril-long term treated Balb/c mice produced significantly higher amounts of IL-10 than untreated mice (Fig. 1B, P = 0.033). Contrarily to what was verified for enalapril, captopril-long-term treatment of C57Bl/6 and Balb/c mice did not exert any effect on IL-10 production mice (Fig. 1A and B). It should be noted that IL-10 was not detected in supernatants from unstimulated spleen cells from enalapril- and captopril-treated mice (data not shown).

3.2. Increased IL-10 production by splenic CD4+CD103+ T cells in enalapril-treated C57Bl/6 and Balb/c mice

Since in most of experiments IL-10 levels measured by ELISA in supernatants presented a huge variability, we next evaluated whether IL-10 would be increased in the intracellular compartment of splenocytes from C57Bl/6 and Balb/c mice treated with enalapril or captopril for 7 days. In Fig. 2A, all splenic IL-10-producing cells were gated and it is shown that both C57Bl/6 and Balb/c mice treated with enalapril produced increased amounts of IL-10 when compared with controls. When all CD4 T cells were evaluated for IL-10 production we have found that the same pattern is
obtained, where splenic CD4 T cells from both C57Bl/6 and Balb/c enalapril-treated mice produced increased amounts of IL-10 when compared to controls (Fig. 2B). Also, in both strains of mice, CD4+CD103+ T cells are shown to produce increased IL-10 levels after enalapril treatment (Fig. 2C). As seen in Fig. 2D, CD4+CD103+ T lymphocytes are the main population of CD4+ IL-10 producer cells. However, after captopril treatment, IL-10 is increased only in Balb/c mice when compared to controls, in all evaluations that were performed (Figs. 2A–D). These results suggest that the genetic background of the mouse strain may influence immunoregulatory effects of those ACE inhibitors since captopril was unable to stimulate IL-10 in C57Bl/6 mice as did enalapril.

When the CD8+ T cell subpopulation from enalapril- or captopril-treated mice were analyzed it was verified that only captopril-treated Balb/c splenic cells produced higher levels of IL-10 than the controls (data not shown).

3.3. Effect of enalapril and captopril treatment on spleen cell production of IL-2

Since Ang II is considered a Th1 target peptide [23,24] and inhibition of ACE by enalapril and captopril increased IL-10 production, we next evaluated whether enalapril would decrease IL-2 since IL-10 and IL-2 are type 2 and type 1 cytokines, respectively. Balb/c mice were treated with enalapril for 7 days and control animals were left without treatment. We found that the spleen cell production of IL-2 was only significantly higher in enalapril-treated Balb/c mice compared to untreated animals (Fig. 3B, P = 0.049). To verify whether the enalapril-enhanced IL-2 synthesis was due to the genetic background of the Balb/c mouse strain, we did another set of experiments where Balb/c and C57Bl/6 mice were treated with enalapril or captopril for 7 days. Control mice were left on normal drinking water. The levels of IL-2 were evaluated by intracellular
stain of splenocytes from each individual mouse. Neither enalapril nor captopril exerts any effect on IL-2 production in C57Bl/6 mice (data not shown). However, enalapril as well as captopril significantly increased IL-2 levels in Balb/c mice (data not shown). Considering that: (a) enalapril- and captopril-short-term treatment enhanced IL-2 production in normal Balb/c mice; and (b) in general, hypertensive patients take ACE inhibitors for lifetime, we reasoned that it would be important to verify whether the effect of enalapril and captopril treatment on IL-2 would be higher after a long-term therapy. To address this issue, we treated Balb/c and C57Bl/6 mice with enalapril or captopril for one month. Control mice were left without treatment. As shown in Fig. 3C and D neither enalapril- nor captopril-long-term therapy enhanced IL-2 synthesis in both strains of mice comparing to untreated mice.

3.4. Enhanced IL-2 production correlates with increased CD4+CD103+CD25negative splenic T cells in enalapril-treated BALB/c mice

It has been extensively shown that increase in IL-2 levels is associated with expansion of regulatory T (Treg) cells [25] and we show herein that enalapril and captopril up regulated IL-2 synthesis in normal mice. Thus, we next verified whether enalapril-enhanced IL-2 levels would result in expansion of Treg cells in spleen cells from enalapril-short term treated Balb/c mice. To address this issue, we stained splenocytes from enalapril-treated Balb/c mice with anti-CD4, CD103, and CD25 antibodies and analyzed them by FACS. When spleen cells were analyzed for the expression of CD4+CD103+CD25positive (after gating on CD4+CD25+), the number of Treg cells does not seem to be higher in enalapril-treated mice than in untreated controls (Fig. 4A and B). Even though CD4+CD25positive T cell numbers were not significantly increased (Fig. 4B), CD4+CD25negative T cells seem to be slightly higher in enalapril-treated mice when compared to untreated controls (Fig. 4C). Therefore, we next analyzed CD4+CD103+ spleen T cells on gated CD4+CD25negative. As shown in Fig. 4D (after gating on R2), mild increased numbers of CD4+CD103+CD25negative T cells were found to be significant in enalapril-treated mice comparing with controls. Based on this data, we hypothesize that enalapril treatment may induce an accumulation of supposed regulatory T cells such as CD4+CD103+CD25negative which might be the source of IL-2 in spleen from enalapril-short term treated Balb/c mice.

4. Discussion

In this study, we investigated the effect of two ACE inhibitors, enalapril and captopril on systemic cytokine production in Balb/c and C57Bl/6 normal mice and showed that enalapril increased IL-10 synthesis in both mouse strains while captopril enhanced it only in Balb/c mice. These results suggest that the genetic background of the C57Bl/6 mouse strain might be underlying the effect of captopril on IL-10 production. Besides, captopril-increased IL-10 levels was only observed by the intracellular stain method suggesting that captopril is lesser potent than enalapril to enhance IL-10 synthesis in C57Bl/6 mice. The specific mechanism(s) by which enalapril seems to be more potent than captopril in enhancing IL-10 production in this strain of mice are not completely understood. However their differential effects IL-10 synthesis in C57Bl/6 mice cannot be explained only by their ability to inhibit Ang II generation. At least one could speculate that the different chemical-structure of enalapril and captopril would be the original cause of their differential immunoregulatory effect on IL-10 synthesis whether directly or via secondary pathways. Independent of the mechanism by which enalapril and captopril enhance cytokine production, it exerts an import impact on cytokine net work and consequently on cells of the immune system, including T lymphocytes. Therefore, to better understand the effect of enalapril-enhanced cytokine levels on T cells, we evaluated the expansion of Treg cells in spleen from enalapril-treated mice. Recently, it has been described that enhanced IL-2 levels are involved on generation and expansion of CD4+CD25positive Treg cells [25] and one of the main important ways to identify functional Treg is by staining spleen cells with CD4, CD25 and mostly with CD103 markers [26]. Since enalapril up regulated IL-2 synthesis, we investigated if those enhanced levels of IL-2 would target the development of extra thymic Treg. To do that, we stained spleen cells from enalapril-treated mice with antibodies to CD4, CD25 and CD103 and analyzed them by FACS. As shown in Fig. 4, enalapril increased CD4+CD103+CD25negative T cell numbers comparing to controls. According to the data, we speculate that the ACE inhibitor enalapril
targets extrathymic expansion of Treg. Although it is widely demonstrated that mostly regulatory T cell are of thymic origin some Treg can also be induced extrathymically [27,28]. In such case, elevated TGF-β levels may favor extrathymic Treg cells by inducing CD103 expression [29,30]. Whether enalapril-therapy exerts any effect on TGF-β expression has to be determined. The accumulation of supposed and potentially regulatory cells such as CD4+CD103+CD25-negative in enalapril-treated mice also raises the question whether Treg cells of this phenotype could be the result of CD25-positive T cells that reverted into CD25-negativeCD103+ Treg cells.

Macrophages, NK, dendritic and B cells also produce IL-10 in some circumstances [31,32]. In our results, CD4+CD103+ are the main T cells that produce IL-10 when compared to CD8 T cells, in this experimental system. In case of measuring a helper activity, IL-2 produced by activated CD4+ Th cells may induce NK cells to produce IFN-γ, which in turn are able to allow type-1 polarized dendritic cells. Such DC1 combine three different conditions for obtaining a type-1 immunity: (1) fully-mature status; (2) high responsiveness to CCR7 ligands; (3) high IL-12p70-producers [33]. In such a case, the higher amount of IL-10 in enalapril-treated mice may be a result of total IL-10 splenocyte-producing cells, but CD4+CD103+ are the main splenic T cells producing this cytokine after enalapril treatment when compared to CD8+ T cells, in both strains of mice. In addition, our results showed that IL-10 seems to be slightly in CD8+CD103+ after EP or CP treatment in Balb/c mice. Furthermore, captopril may stimulate IL-10 production by CD4+CD103+ only in Balb/c mice. Whether the enhancement of CD4+CD103+CD25-negative T cells that we verified in our experimental system is related to enhanced IL-2 and IL-10 levels, and whether they exert regulatory activities, it needs to be further evaluated.

Concluding, our results suggest that systemic inhibition of Ang II by ACE inhibitors play an important role on the immune system by modifying immune and regulatory functions exerted by splenic T-cell populations. Our results have important implications to our understanding of regulatory T cell function mainly indicating an additional use for enalapril in the future.

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References


