Comparison of Different Blockages on the Renin Angiotensin System in Murine Schistosomal Fibrosis

Parreira N.A.¹, Ramalho F.S.¹, Augusto M.J.¹, Rodrigues V.² and Ramalho L.N.¹,*

¹Department of Pathology Faculty of Medicine of Ribeirão Preto-FMRP-USP, Brazil
²Department of Cell and Molecular Biology and Pathogenic Bioagents of Faculty of Medicine of Ribeirão Preto-FMRP-USP, Brazil

Abstract: The inhibition of the renin-angiotensin system (RAS) display anti-fibrogenic effects in liver cirrhosis, however RAS influence on schistosomal fibrosis has not been totally clarified. Thus, our objective was to compare the activities of different antihypertensive drugs on the inhibition of the RAS in murine schistosomal fibrosis. BALB/c mice (n=40); weighing 20g were subjected to inoculation of 50 cercariae and subdivided in three groups: the first group (n=15), animals were treated with losartan (10 mgkg⁻¹ body weight); the second group (n=15) was treated with lisinopril (10 mgkg⁻¹ body weight); while the third group (n=10) was treated with proportional volume of 0.9% NaCl, daily for 12 weeks. For this purpose, histological analysis, RT-PCR of genes related to hepatic fibrosis, as well as TGFβ1 and p42/44 MAPK protein quantification were evaluated. There was observed a decrease in the collagen deposits in animals treated with losartan (p<0.01) and lisinopril (p<0.01) once compared to the controls. This improvement was accompanied by a reduction of α-SMA labeled cells (p<0.001 and p<0.001) and procollagen α1(I) gene expression in both treated groups (p<0.05 and p<0.05). However, only losartan induced diminution of TGFβ1 and TIMP1 gene expression (p<0.01 and p<0.01), and TGFβ1 protein amount. Diversely, p42/44 MAPK protein was augmented in lisinopril group. Our findings demonstrated the effect of lisinopril and losartan on the decrease of hepatic fibrosis in murine schistosomiasis. The main mechanism of this process involves anti-fibrogenic activity via inhibition of TGFβ1 and TIMP1 by losartan treatment. Nevertheless, other possible mechanisms associated to lisinopril treatment may be implicated.

Keywords: Liver, fibrosis, schistosomiasis, lisinopril, losartan, hepatic stellate cell.

1. INTRODUCTION

Estimated to four to six million people are infected with Schistosoma mansoni in Brazil, with 70% of cases concentrated in the states of Minas Gerais and Bahia [1]. The most severe form is associated with hepatosplenic hepatic fibrosis, whose base is represented by pathologic lesions described by Symmers (1903) fibrosis as "pipe stem", which basically consists of diffuse portal fibrosis without distortion of the hepatic parenchyma or interposition by regenerative nodules [2].

The main cause of the formation of liver fibrosis is due to the activation of hepatic stellate cells (HSC). Hepatic stellate cells are the major producers of extracellular matrix proteins in the tissue repair reaction in response to chronic hepatic injury [3]. Previous studies of experimental schistosomiasis revealed that activated HSC participate in the formation of periportal-nes granulomas in portal spaces [4-7]. Additionally, activated HSC were also identified in the periportal fibrous tissue of human schistosomiasis [8,9]. The HSC activation is considered a serious consequence of infection by S. mansoni in which involves the remodeling of extracellular matrix and excessive collagen deposition through activation of HSC in the portal tract [10].

Several factors are associated to the activation of HSC [11]. Among them, angiotensin II (Ang II), a major component of the Renin-Angiotensin System (RAS), promotes the activation of HSC cells to "myofibroblast-like", inducing cell contraction and increased intrasinusoidal pressure [12]. Ang II also stimulates an augment of αSMA expression by the activated HSC [13]. Bataller et al. (2000) demonstrated that Ang II-induced activation, contraction and proliferation of cultured human HSC via interaction with AT₁ receptor, these effects are blocked by the use of losartan, an antagonist of the Ang II [14].

There was also observed that losartan determined a significant inhibition of the activation and proliferation of HSC in rats, suggesting potentially be useful in controlling the genesis of reducing liver fibrosis and portal hypertension [15]. Losartan was also identified as therapeutic to Schistosoma mansoni disease, mainly in acute phases, when associated to treatment with praziquantel [16,17]. A further antihypertensive class of used by Ohishi et al. (2001) demonstrated the anti-fibrogenic effect of lisinopril, an angiotensin converting enzyme (ACE) inhibitor, on chronic CCL₄-induced hepatic fibrosis in rats [18]. Curiously,
schistosomal granuloma macrophages can produce ACE during this development, increasing the inflammatory response [19]. However, the use of captopril, an ACE inhibitor, not affects the granulomatous response [20]. Despite of the inhibition of RAS determines fibrosis diminution in diverse studies of hepatic cirrhosis, the RAS influence on schistosomal hepatic fibrosis has not been totally clarified, especially without treatment combined with praziquantel. The aim of this study was to compare the activities of different antihypertensive drugs on the inhibition of the RAS in murine schistosomal fibrosis.

2. MATERIALS AND METHOD

The research protocol was approved by the Ethics Committee on Animal Experiments (CETEA) FMRP-USP, Protocol 037/2007. BALB/c mice (n=40) underwent 50 cercariae of subcutaneous inoculation and then were divided into three groups: group 1 (n=15) were treated daily with losartan (Merck, Rahway, NJ, USA), 10 mg.kg⁻¹ body weight intraperitoneally (i.p.) for 12 weeks, group 2 (n=15) were treated daily with lisinopril (Merck, Rahway, NJ, USA), 10 mg.kg⁻¹ body weight, i.p. for 12 weeks, while group 3 (n=10) received an equal volume of 0.9% NaCl, i.p. for 12 weeks. Following, animals were sacrificed under anesthesia with intramuscular ketamine (80 mg.Kg⁻¹ body weight) and xylazine (10 mg.kg⁻¹ body weight). Fragments of liver tissue were fixed in 10% buffered formalin for 48 hours and embedded in paraffin. Small liver samples were also collected in RNA stabilizing solution (RNA Later Stabilization Reagent, QIAGEN, CA, USA), were rinsed with phosphate-buffered saline and xylazine (10 mg.kg⁻¹ body weight). Fragments of liver tissue were fixed in 10% buffered formalin for 48 hours and embedded in paraffin. Small liver samples were also collected in RNA stabilizing solution (RNA Later Stabilization Reagent, QIAGEN, CA, USA), frozen in liquid nitrogen and stored at -80°C.

2.1. Histological Studies

Liver samples were fixed in 10% buffered formalin for 24 hours, embedded in paraffin and subjected to routine histological processing. The slides were prepared with 5 µm sections and stained with Sirius Red. The collagen deposition was measured as the percentage of Sirius Red staining (red) in ten random fields (microscopic magnification of 40×), with the help of the software Image J (Image J, 1.33u, NIH, USA).

The liver preparations were also submitted to immunohistochemical analysis. Briefly, 4-µm-thick sections mounted on poly-l-lysine-coated slides were deparaffinized, rehydrated, immersed in 10 mmol/L citrate buffer, pH 6.0, and submitted to heat-induced epitope retrieval using a vapor lock for 45 min. The slides were rinsed with phosphate-buffered saline (PBS) and immersed in 3% hydrogen peroxide for 20 min to block endogenous peroxidase. Non-specific protein binding was blocked with normal serum (Vectastain Elite ABC Kit, Universal, Vector Laboratories Inc., Burlingame, CA, USA) for 30 min. The sections were then incubated with monoclonal primary antibody specific for alpha smooth muscle actin (α-SM actin, Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:100 dilution), for 2 h at room temperature (25°C) in a humid chamber. Following washes in PBS, biotinylated pan-specific universal secondary antibody (Vectastain Elite ABC Kit, Universal, Vector Laboratories Inc.) was applied for 30 min. Next, the slides were incubated with the avidin–biotin–peroxidase complex (Vectastain Elite ABC Kit, Universal, Vector Laboratories Inc.) for 5 min. The slides were counterstained by Harris haematoxylin, dehydrated and mounted with Permount (Biomeda, Foster City, CA, USA). As negative controls, all specimens were incubated with an isotope-matched control antibody under identical conditions. The αSMA-positive cells were quantified randomly, at least 30 representative high-power fields (40×), by two of the authors (NAP and LZR), and the results are expressed as number of αSMA-positive cells by microscopic field.

2.2. Gene Expression by Real Time PCR

Liver samples stored at -80° C were used for the quantification of gene expression of Procollagen α1(I), TGFβ1 and TIMP1. The tissue samples, homogenized with the aid of a Polytron homogenizer (PT 2100, Kinematica Polytron, Newark, NJ, USA) were subjected to extraction of total RNA using refrigerated centrifuge (Mikro 220R Hettich Zentrifugen, Germany) and specific kit (RNAqueous® – 4PCR DNA-free™ RNA Isolation for RT-PCR). The complementary DNA was obtained by reverse transcription from using retro-transcription kit (Ominiscript® RT Kit, Qiagen). The simultaneous quantification in gene amplification was performed by employing StepOnePlus™ (Applied Biosystems, CA) using primers specific for the genes of Procollagen α1(I), TGFβ1, TIMP1 and 18S (Assays-on-Demand Gene Expression Products, Applied Biosystems, Foster City, CA) and Taq Polymerase (TaqMan Universal PCR Master Mix, No AmpErase UNG - 2X Applied Biosystems). There was employed for the analysis of ΔΔCT method from the difference between samples using the gene 18S reference.

2.3. Western Blotting

The levels of TGFβ1 and p42/44 were quantified using Western blot method. Cell extracts were obtained...
from frozen liver samples using the detergent-based lysis buffer, containing protease and phosphatase inhibitors, then were centrifuged at 14000 rpm for 10 min at 4°C. Samples were subjected to 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked one hour in 5% bovine serum albumin in Tris-buffer saline with Tween 20, room temperature. The membranes were incubated with primary antibodies, overnight 4°C, to TGFβ1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:200 dilution), p42/44 MAPK-monoclonal antibody primary - ERK (Cell Signaling Technology, Danvers, MA, USA, 1:1000 dilution) and beta-actin (Sigma, St Louis, MI, USA, 1:200 dilution) as a control. Next, the membranes were washed with buffer and incubated with secondary antibody conjugated to peroxidase. Blots were visualized using ECL Prime kit (Amersham, USA). Resulting blots were scanned with ImageQuant LAS 4000 (GE Healthcare, USA). Relative densities of the bands were analyzed optical densities of specific proteins were quantified with the ImageQuant TL software (GE Healthcare, USA).

2.4. Statistical Analysis

In view the nature of the data for statistical comparisons was applied nonparametric Kruskal-Wallis and Mann-Whitney U. Probability values less than 0.05 are considered significant. Statistical analysis was performed with the aid of a computer equipped with the GraphPad Prism 5.0 (GraphPad Software Inc., CA, USA).

3. RESULTS

Figure 1A-C shows the collagen amount stained by Sirius red in the control, lisinopril and losartan groups. The collagen deposits are accumulated mainly around schistosomal granulomas. A similar pattern was observed in the HSC labeled by αSMA immunohistochemistry (Figure 1D-F). Both lisinopril and losartan (13.6 ± 3.2 and 14.2 ± 4.3) reduced the collagen extent in comparison to the control animals (21.4 ± 5.2) (P<0.01) (Figure 1G). Similarly, the number of αSMA marked cells was diminished by lisinopril and losartan treatment (3.2 ± 1.0 and 5.0 ± 0.8) in contrast to the control group (8.8 ± 2.0) (P<0.001) (Figure 1H). Lisinopril and losartan administration also decreased the Procollagen α1(I) gene expression (92.6 ± 89.9 and 130.7 ± 89.6) when compared to the control group (310.7 ± 79.6) (P<0.05) (Figure 1I). However, the TIMP1 and TGFβ1 gene expression could be reduced from control group (291.5 ± 34.8 and 39.0 ± 14.6) only by losartan treatment (14.1 ± 4.9 and 14.3 ± 6.7) (P<0.001 and P<0.001, respectively) (Figure 1J-K). The TIMP1 and TGFβ1 gene expression were similar between lisinopril (298.4 ± 83.1 and 23.7 ± 14.7) and control groups (291.5 ± 34.8 and 39.0 ± 14.6) (P>0.05 and P>0.05, respectively) (Figure 1J-K). Losartan treatment, additionally decrease TGFβ1 protein amount, without any difference among lisinopril and control groups. On the other hand, lisinopril augmented the p42/44 MAPK quantity in relation to the losartan and control animals (Figure 1L).

4. DISCUSSION

The findings observed by the present study show evidences of anti-fibrogenic activity of antihypertensive agents, lisinopril and losartan, on the murine schistosomal fibrosis.

The main pharmacological inhibitors of RAS are the antagonists of Ang II receptors and the ACE inhibitors. Firstly, losartan, an Ang II receptor antagonist, was demonstrated as an anti-fibrogenic agent in different models of hepatic cirrhosis [15,21]. Losartan was amply reported to blockage the HSC activation via TGFβ1 stimulation by Ang II [22-24]. Moreover, losartan induce a diminution of collagen production by HSC that were activated by Ang II [25]. Attia et al. 2013 using telmisartan showed that there was a reduction in the diameter of the granulomas and fibrotic areas [17]. This study was in accordance with the findings observed by El-Lakkany et al. 2011, in which losartan resulted in regression of hepatic granulomatous lesions by Schistosoma mansoni, with reduction of the dia-meter of the granuloma [26]. Losartan can also restrict the TIMP1 activity, and consequently permits the interstitial collagenases to degrade extracellular matrix, as well as collagen reabsorption [27]. Furthermore, observations in vitro demonstrate that HSC present AT1 receptors [14] and the AT1 receptor blockage may alleviate liver fibrosis [28]. In accordance, our results presented losartan as an important anti-fibrogenic agent in hepatic schistosomal fibrosis, which reduced collagen deposits, αSMA positive-cells population and procollagen α1(I) gene expression. We also observed, through gene expression and protein quantification, a diminution of TGFβ1 production in the animals treated with losartan. Additionally, losartan could decrease TIMP1 gene expression.
Figure 1: Representative photomicrographs of Control, Lisinopril, and Losartan groups. Liver sections were stained by Sirius red coloration (magnification, ×100) (A–C), or α-SMA immunohistochemistry (D–F). Effect of Lisinopril and Losartan treatment on Sirius red staining percentage (G); on α-SMA positive cell number (H); on Procollagen α1 gene expression (I); on TIMP1 gene expression (J); on TGFβ1 gene expression (K) and on TGFβ1 and p42/44 MAPK protein amount (L). *P<0.05 Control × Lisinopril; **P<0.05 Control × Losartan.

Lisinopril, as well as others ACE inhibitors, was equally reported as a significant agent to reduce fibrosis in experimental cirrhosis [18,29], however these agents were not yet investigated in hepatic
schistosomal fibrosis. Similarly to Ang II receptor antagonists, ACE inhibitors also have been pointed to induce both TGFβ1 control and TIMP1 diminution as one of the principal events in their anti-fibrogenic role [30,31]. The present study demonstrated an expressive anti-fibrogenic activity by lisinopril, which resulted in collagen deposits, αSMA positive-cells population and procollagen a1(1) gene expression reduction. Though, there was not observed any effect of lisinopril in TGFβ1 gene expression or protein amount, as well as in TIMP1 gene expression.

About ACE inhibitors, we believe that other pathways act mainly bradykinin. More than to blockage the conversion of Ang I to Ang II, the ACE inhibitors can also reduce the degradation of endogenous bradykinin to inactive peptides [32]. Furthermore, bradykinin is produced during the inflammatory response to Schistosoma mansoni, and may contribute to granuloma development [33]. In spite of infused bradykinin can display anti-fibrogenic effects in CCl4 rats through the inhibition of TGFβ1 and TIMP1 pathway [34], bradykinin was also reported inducing an increase of αSMA expression by mesenchymal stem cells via TGFβ1 activation [35]. Moreover, the endogenous bradykinin may exhibit other scenery, mainly during schistosomal fibrosis, which involves TGFβ1 augment during this inflammatory response [36]. Thus, in relation to our study, the anti-fibrogenic effects observed in the lisinopril group may be associated not to the diminution of Ang I to Ang II conversion, using another pathway diverse to TGFβ1 and TIMP1 levels reduction, but to endogenous bradykinin increase. In fact, bradykinin enhance may be related to an increase of P42/44 MAPK expression, a pro-apoptotic factor to HSC [37]. In agreement, we observed an augment of P42/44 MAPK protein in the animals treated with lisinopril. Surely treatment with lisinopril requires more detailed studies on the channels of action about the schistosomal fibrosis.

In conclusion, both lisinopril as losartan showed a reduction of liver fibrosis in the murine schistosomiasis. The main mechanism of this process involved the anti-fibrogenic activity through inhibition of TGFβ1 and TIMP1. Thus the blockage of the production and interaction of Ang II with the AT1 receptor consists as a potential treatment of schistosomal fibrosis.

RESEARCH SUPPORT

Research supported by FAPESP (nº2008/01296-2), CNPq (nº 302547/2012-6) and FAEPa.

REFERENCES

[17] El-Lakkany NM, El-Maadawy W, Ain-Shoka A, Badawy A, Hammam O, Ebeid F. Potential antibiotic effects of AT1 receptor antagonist, losartan, and/or praziquantel on acute
http://dx.doi.org/10.1111/j.1440-1681.2011.05575.x

http://dx.doi.org/10.1016/S1386-6346(01)00102-4


http://dx.doi.org/10.1016/0014-4894(91)90015-O

http://dx.doi.org/10.1016/j.bpg.2011.02.005

http://dx.doi.org/10.1111/j.1478-3231.2005.01192.x

http://dx.doi.org/10.1016/j.cca.2005.06.014

http://dx.doi.org/10.1016/j.bpg.2011.02.005

http://dx.doi.org/10.1002/hep.20628

http://dx.doi.org/10.1186/1756-3305-6-199

http://dx.doi.org/10.1186/1756-3305-6-199

http://dx.doi.org/10.1074/jbc.M310284200

http://dx.doi.org/10.1016/j.biopharm.2007.10.020

http://dx.doi.org/10.1007/s10620-007-9941-v

http://dx.doi.org/10.1111/j.1440-1746.2006.04663.x

http://dx.doi.org/10.1016/0003-0888(98)0003-0


http://dx.doi.org/10.1053/j.gastro.2007.09.023

http://dx.doi.org/10.1016/j.cellsig.2008.06.021

http://dx.doi.org/10.1016/j.exppara.2007.06.002

http://dx.doi.org/10.1096/fj.04-1847fje