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AN ANGIOTENSIN II RECEPTOR ANTAGONIST REDUCES INFLAMMATORY PARAMETERS IN TWO MODELS OF COLITIS

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Abstract

Little is known about the effects of the proinflammatory hormone Angiotensin II (Ang II) in inflammatory bowel disease. The aim of this study was to evaluate the effect of Valsartan (Diovan), an Ang II receptor antagonist, in two models of colitis.

METHODS—Colitis was induced in Sprague-Dawley rats by administration of trinitrobenzene sulfonic acid (TNBS; 30mg in 50% ETOH ic) or 5% Dextran Sulphate Sodium (DSS) in drinking water ad libitum for 5 days. Valsartan was administered orally in drinking water (160mg/L) during thirty days prior to the induction of the colitis, and for 5 days after. All animals were evaluated for weight change, diarrhea, myeloperoxidase activity, macroscopic and microscopic damage. Cytokine levels in the colon were measured by ELISA, real time RT-PCR and immunohistochemistry.

RESULTS—In the TNBS model, Valsartan reduced the macroscopic damage score, significantly decreased the microscopic damage ($p < 0.01$), and accelerated weight gain after colitis. In the DSS colitis model, Valsartan treated animals had less diarrhea and microscopic damage. Valsartan reduced the protein levels of TGF β ($p < 0.05$), and IL-18 in the TNBS model, and led to over-expression of IL-10 mRNA in the DSS model.

CONCLUSION—These data demonstrate a possible anti-inflammatory effect for Valsartan in colitis.

Keywords

TNBS; DSS; angiotensin; rat

INTRODUCTION

Inflammatory bowel disease (IBD), comprising both ulcerative colitis and Crohn's disease, is a chronic condition that affects many people [1]. Ulcerative colitis is mainly limited to the colon whereas Crohn's disease can involve any segment of the gastrointestinal tract, however, in both, the etiology remains unclear [2]. Both conditions result in inflammation, ulceration, edema, bleeding and diarrhea. Although many treatments are currently available, these are only

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Preliminary data from this study were previously communicated at the Experimental Biology Meetings (San Diego 2005, Washington 2007) and the World Congress of Gastroenterology (Montreal 2005).

The experiments reported herein were performed in accordance with the principles described in the "Guide for the Care and Use of Laboratory Animals", Publication No. DHHS (NIH) 86-23

effective for ameliorating the signs and symptoms of the disease. There is, as yet, no cure for this condition. The most commonly used therapies include aminosalicylates, antibiotics, corticosteroids and immunosuppressants, all of which have disadvantages. Antibiotics appear to be effective in ameliorating disease symptoms in a subpopulation of patients, however the indiscriminate use of antibiotics could change the environmental conditions of the commensal microflora and also trigger resistance [3]. Moreover, the use of immunosuppressants and anti-inflammatory drugs, such as corticosteroids, has many undesirable side effects. It is therefore important to find better treatment options with fewer side effects.

Angiotensin II (AngII) is a proinflammatory hormone that has been shown to be involved in many pathological conditions [4]. Tissue and plasma levels are increased during stress leading to a release of reactive oxygen species from vascular smooth muscle, with resulting cellular damage and inflammation [5]. Ang II participates in several key events of the inflammatory response: it increases vascular permeability (via release of prostaglandins and vascular endothelial cell growth factor/vascular permeability factor), and it participates in the recruitment of infiltrating inflammatory cells into tissues by their direct activation, or by regulation of the expression of adhesion molecules and chemokines by resident cells [6]. An angiotensin converting enzyme (ACE) blocker reduced peritubular and medullar interstitial fibrosis in the kidney of experimental animals [7]. Several studies have also demonstrated the beneficial effects of blocking the angiotensin II receptor in other organs such as the liver [8] and kidney [9], however little is known about its possible therapeutic benefits in gastrointestinal inflammation, and specifically in colitis.

In addition to its vasoconstrictor activities Ang II can promote tissue inflammation, enhancing neutrophil infiltration, raising the possibility of its contribution to intestinal ulceration [10]. Moreover, Ang II is known to regulate motility in the intestine, as well as ion and water absorption via receptors in the mucosa and muscle [11]. Colonic mucosal levels of both angiotensin I and II are greater in patients with Crohn's disease, and appear to correlate with the degree of inflammation [12]. This led us to hypothesize that the inhibition of Ang II could attenuate the inflammation and subsequent damage found in acute colitis. Recently it was demonstrated that an ACE inhibitor, Captopril, could reduce the levels of Ang II and the colonic fibrosis found in an animal model of colitis [13]. Moreover, losartan, an Ang II type I receptor blocker was found to have beneficial effects in colitic mice confirming the involvement of this receptor [14].

In this study we used valsartan (Diovan), a nonpeptide angiotensin II receptor antagonist acting at the AT1 receptor subtype [15] as a possible anti-inflammatory drug to further investigate its therapeutic potential in two different animal models of acute colitis. At present, various animal models exist for the study of IBD, but none can be said to exhibit all the features of this condition. The trinitrobenzene sulfonic acid (TNBS) model is an animal model that has been used extensively. This hapten produces an inflammatory response that persists for several weeks and shows many similarities to the human condition [16,17]. Another commonly used model is the dextran sulfate sodium (DSS) model that, when given orally, produces inflammation in the colon of rodents [18].

We used both the TNBS and DSS animal models, two commonly used models of IBD, to ensure that any observed changes were not model specific.

METHODS

Colitis was induced in male Sprague-Dawley rats (250–350g; Southern Veterinary Service, Ponce School of Medicine, PR). All animals were maintained in restricted-access rooms with controlled temperature (23°C) and a 12 hour light-dark cycle. Standard laboratory chow and

drinking water were provided *ad libitum*. The Animal Care and Use Committee at Ponce School of Medicine approved all experimental procedures involving animals.

The animals were randomly assigned to one of six treatment groups: water alone (normal animals) (n=4), valsartan alone (n=4), TNBS colitis control (n=6), TNBS colitis + valsartan (n=6), DSS colitis control (n=6), DSS colitis + valsartan (n=7). All rats were weighed for the duration of the study, and were housed individually so as to monitor fluid consumption. Blood pressure (systolic, diastolic and mean) and heart rate were measured in all groups before and after the induction of colitis using a tail cuff non-invasive blood pressure (NIBP-8) monitor (Columbus Instruments, Columbus, OH using AcqKnowledge software, Biopac systems Inc, Goleta, CA).

Induction of colitis

TNBS colitis was induced using a modified version of the method of Morris *et al.*, [17]. Briefly, after lightly anesthetizing with ether, a rubber catheter was inserted rectally into the colon so that the tip was approximately 8 cm proximal to the anus. TNBS (60 mg/ml) was dissolved in 50% ethanol (vol/vol) and instilled into the lumen of the colon through the rubber catheter (total volume of 0.5ml).

DSS colitis was induced by administration of 5% DSS in drinking water *ad libitum* for 5 days [18].

Valsartan treatment

Valsartan was administered orally in distilled drinking water (160mg/L) for thirty days prior to the induction of colitis, and during the five days after until the time of sacrifice. This dose is similar to that used in previous studies with this drug in rats [19,20]. Control and normal groups received distilled drinking water only. To ensure drug consumption the volume was measured daily.

Collection of samples

All rats were euthanized with 100mg pentobarbital i.p. five days after the induction of colitis. Before sacrifice, a blinded observer identified all animals with a different code to avoid bias. Following laparotomy, the whole colon was removed and examined for macroscopic damage using a previously well-defined scoring system [16]. Four criteria were examined: the presence of adhesions (0, 1 or 2; for none, minor or major respectively), diarrhea (0 or 1; absent or present respectively), thickness (in mm), and ulceration (0 for no damage, with increasing scores depending on extent of ulceration). Sections of distal colon were taken for microscopic analysis and measurement of myeloperoxidase activity. Tissue samples were stored for RNA and protein isolation.

Microscopic analysis

Segments of colon were fixed in 10% formalin and, after routine processing; sections were stained with hematoxylin and eosin to determine the extent of inflammatory infiltrate and the appearance of the underlying muscle layers. Histological assessment of damage was performed using previously published criteria [16]. Briefly, we evaluated loss of mucosal architecture (0→3; absent → severe), cell infiltration (0→3; absent → severe), muscle thickness (0→3; absent → severe), crypt abscess formation (0 or 1; absent or present), and goblet cell depletion (0 or 1; absent or present). All slides were analyzed by a blinded observer.

Measurement of neutrophil infiltration

Tissue myeloperoxidase (MPO) activity was determined as an index of granulocyte infiltration. MPO is an enzyme found within the azurophilic granules of neutrophils and other cells of myeloid origin. It has been demonstrated previously that these levels reflect the state of inflammation in the mucosa of the intestine [21,22]. Tissue samples were weighed and stored at -20°C until assayed within a week using a well-established assay [23]. The absorbance was measured at 460 nm at 30 second intervals. The readings were done in triplicate and the absorbance results were averaged. Average absorbance was converted to units per mg of tissue.

Immunoassays

Total protein was extracted from the colon tissues after homogenization using Trizol Reagent, following the manufacturer's specifications (Gibco BRL, Gaithersburg, MD). To determine TNF-alpha, TGF-beta, IL-10 and IL-18 levels in tissue homogenates, aliquots were assayed by sandwich ELISA using commercial kits (R&D Systems, Minneapolis, MN, or Biosource, Camarillo, CA), following the manufacturers protocol. The detection limits for TNF-alpha, TGF-beta, IL-10 and IL-18 were 5 pg/ml, 4.2 pg/ml, 10 pg/ml and 4 pg/ml respectively. Intra- and inter-assay variations for TNF-alpha and IL-10 were both less than 10%, while those for TGF-beta and IL-18 were less than 7%.

Real-Time RT-PCR

To validate data obtained with the ELISA, real-time RT-PCR was performed on selected genes, using total RNA from experimental colitis (DSS or TNBS) treated with Valsartan ($n = 4$), and control colitis with water ($n = 4$). The genes selected were TNF-alpha, TGF-beta, IL-10 and IL-18. Primers were synthesized by a commercial vendor (IDT DNA Technology, Inc., Coralville, Iowa; Table 1). Total RNA was isolated from tissues using the Trizol LS reagent. To remove contaminating DNA, samples were treated with DNase I (DNA-free, Ambion, Austin, Texas). RT was performed on the PTC-200 thermal cycler (MJ Research, Waltham, Mass) using the iScript cDNA synthesis kit (Bio-Rad, Hercules, California) following the manufacturer's protocol. After cDNA synthesis, PCR reactions were performed in triplicate with specific oligoprimers using the iQ SYBR Green Super Mix kit according to the manufacturer's recommendations (Bio-Rad, Hercules, California). The PCR amplification profile was as follows: 94°C for 4 minutes followed by 50 cycles of denaturation at $94^{\circ}\text{C}/30$ seconds, gene-specific annealing temperature/30 seconds, and extension at $72^{\circ}\text{C}/40$ seconds. Annealing temperatures per primer set were determined empirically. A melting curve was generated after each run to verify the specificity of the primers, shown by the presence of a single band and no primer-dimer artifacts. Real-time analysis of PCR amplification was performed with an iCycler iQ Optical System software, version 3.0a (Bio-Rad, Hercules, California). Relative expression levels were calculated for each sample after normalization against the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), using the delta-delta Ct method for comparing relative fold-expression differences [24,25].

Immunohistochemistry

Immunohistochemical analysis of formalin-fixed and paraffin-embedded colonic tissue was carried out using the Streptavidin-biotin-peroxidase method [26]. After antigen retrieval, using the citrate buffer epitope retrieval method, sections were incubated at room temperature for one hour with a primary antibody followed by Multi Link for 20 minutes (HK268, BioGenex, CA, USA). The specimens were immunostained with the Streptavidin-Peroxidase (HK3200804, BioGenex) reagent for 20 minutes, and the reaction product was developed with diaminobenzidine chromogen (DAB HK124, BioGenex). The specimens were counterstained with hematoxylin. Negative controls were made using phosphate-buffered saline (PBS) instead of the primary antibodies. The primary antibodies used were anti-rat TNF-alpha antibody

(R&D Systems) using 0.1µg/ml dilution, TNF-R1 and TNF-R2 (Santa Cruz, CA, USA). In addition, colonic tissue was immunoassayed for the following antibodies: IL-10 goat polyclonal antibody (Santa Cruz), TGF-beta1 rabbit polyclonal antibody (Santa Cruz), and anti-rat IL-18 (dilution 15µg/ml; R&D). The working dilution of TGFbeta1, IL-10, TNF-R1 and TNF-R2 antibodies was 1/200.

Materials

TNBS was obtained from Fluka Chemika (Buchs, Switzerland), DSS was obtained from ICN Biomedical Inc. (Aurora, Ohio) and Sodium Pentobarbital was obtained from Veterinary Laboratories, Inc. (Lenaxa, Kansas). Valsartan (Diovan®) was obtained from Novartis. Histological supplies were obtained from Fisher Scientific (Pittsburg, P.A.). All other chemicals were obtained from the Sigma Chemical Company (St. Louis, MO).

Statistical analysis

Values are presented as mean $\pm$ SEM where “n” represents one tissue from one animal used for a single replicate of an experiment. Differences between the means of each group were analyzed using an ANOVA followed by a Tukey-Kramer Multiple comparisons test. For the weight change analysis, an ANOVA with Dunnett-multiple comparisons test was used to compare all time points to day zero, and all treatment groups to water alone at each time point. Statistical analyses were performed using GraphPad Instat V3.0 (GraphPad Software, San Diego CA). In all cases, $p < 0.05$ was considered to represent a significant difference.

RESULTS

Valsartan treatment did not change blood pressure or fluid consumption

Valsartan treatment had no significant effects on the blood pressure (systolic, diastolic and mean) or heart rate in normal animals without colitis; or before or after the induction of colitis in either model. There was also no difference in the rate of fluid consumption between the water and Valsartan treated groups in either model before the induction of colitis. The average rate of fluid consumption for all groups was 1.78 ml/hr. The valsartan treated animals thus received the equivalent of 22.04 mg of Valsartan/kg of body weight per day.

Valsartan treatment reduced weight loss following induction of TNBS colitis

All animals were weighed throughout the course of the study. There was no significant difference in the weight of the animals between the different treatment groups at the start of the study, before the administration of Valsartan (average body weight 304.50 ± 5.78 g, $n=6-7$ rats per group). Moreover, there was no significant difference in the weight of the animals after 30 days of Valsartan and prior to the induction of colitis (average body weight 428.52 ± 7.43 g, $n=6-7$ rats per group).

Following the induction of TNBS-colitis, both the Valsartan treated animals and the untreated controls began to lose weight, and weighed significantly less than the water controls from day 2 until the time of sacrifice ($p < 0.01$; Figure 1). The untreated TNBS-colitis animals weighed significantly less at all time points after the induction of colitis reaching a maximum weight loss of 11.44% of their original body weight four days after the administration of TNBS ($p < 0.01$; Figure 1). In contrast, the weight of the Valsartan treated animals was not significantly different from their original weight until day four, at which time the animals reached a maximum weight loss of 8.17% (Figure 1). These animals also regained weight faster following the induction of colitis, demonstrating that the weight loss occurred as a consequence of the TNBS induced colitis and was ameliorated by Valsartan administration. There was no significant difference in the weight of DSS/colitis animals compared to their original weight,

whether treated with Valsartan or not, until day 5 by which time they weighed significantly more than on day 0. There was no significant decrease in the weight of the non-colitis animals treated with either water alone or Valsartan alone.

Valsartan decreased damage scores in TNBS colitis and incidence of diarrhea in DSS colitis

Both TNBS and DSS caused colonic ulceration and inflammation as evidenced by a significant increase in the macroscopic damage score (Figure 2), occurrence of diarrhea (Figure 3), and microscopic damage score (Figure 4b).

In the TNBS model of colitis, Valsartan pretreatment reduced the overall macroscopic damage by nearly 25% (Figure 2). This reduction was observed in each of the individual scoring criteria: adhesions, colon thickness, and ulcer score, however there was no effect on the incidence of diarrhea. In contrast, in the DSS-colitis model, treatment with Valsartan decreased the incidence of diarrhea (a major clinical symptom in patients with IBD), by 50% (Figure 3). No differences in the other parameters were noted in this model.

There was a significant increase in the total microscopic damage score in both models of colitis (Figure 4). Animals receiving valsartan alone had no differences in any of the individual parameters analyzed compared to those receiving only water (Table 2). The total microscopic damage observed in the TNBS-model of colitis was significantly reduced by administration of Valsartan ($p<0.01$; Figure 4). A significant reduction in the extent of cellular infiltration was observed ($p<0.01$), together with a decrease in all of the other parameters apart from the muscle thickening (Table 2). Valsartan treatment in the DSS model did not significantly reduce the total microscopic score but tended to decrease the loss of mucosal architecture and the amount of cellular infiltration.

The levels of myeloperoxidase were significantly higher in those animals receiving TNBS than in those receiving water ($p<0.01$) or valsartan ($p<0.01$) alone (Table 3). The administration of Valsartan had no significant effect on the myeloperoxidase levels in the TNBS colitis animals. There were no significant differences in myeloperoxidase levels in the DSS model (Table 3).

Valsartan modulated cytokine expression in TNBS colitis

To investigate the role of the angiotensin system in the immune regulation, local cytokine levels in the colonic tissue were quantified by ELISA. As can be seen in Figure 5, Valsartan treatment significantly decreased the levels of TGF-beta, a fibrotic agent ($p<0.05$) in the TNBS-colitis but had no effect in the DSS-model. The levels of the proinflammatory cytokine, IL-18, were decreased by more than 50% with Valsartan treatment in both models of colitis, almost reaching significance in the DSS-model ($p=0.059$). The levels of the anti-inflammatory cytokine IL-10 which were undetectable in the TNBS model increased with Valsartan treatment. No significant differences were seen in the levels of TNF-alpha in either model (Table 4).

To quantify the fold expression of selected genes involved in colonic inflammation, Real Time RT-PCR of the following genes were performed: TNF-alpha, TGF-beta 1, IL-18 and IL-10. A fold difference of over 2 was considered to be over-expressed (38). In the TNBS colitis rats treated with Valsartan there was essentially no difference in the expression of any of the genes. In contrast, in the DSS colitis animals treated with valsartan, IL-10 was over-expressed with a down-regulation in the expression of TNF alpha (Figure 6)

Immunohistochemical analysis for cytokine staining showed a positive reaction of the inflamed distal colon from TNBS colitis rats for TNF-alpha, TGF-beta and IL-18 expression but very few positively stained cells for IL-10 (Figure 7). In each case the cells stained appeared to be inflammatory cells, particularly neutrophils, but also occasionally macrophages. In either case not all the neutrophils or all the macrophages were stained and there was no specific pattern

apparent. In contrast, in those animals treated with Valsartan an increase in IL-10 expression was observed, with staining of neutrophils and some lymphocytes, whereas positive staining for the other cytokines was diminished (Figure 7). Valsartan treatment in the DSS colitis model had little effect on the expression of any of the cytokines examined (data not shown).

DISCUSSION

The renin-angiotensin system, from an evolutionary point of view, is a very old system with pro-inflammatory effects on different tissues. In addition to endocrine effects, it also has paracrine and autocrine actions. Angiotensin antagonist drugs are currently used extensively to treat hypertension. Recently there has been some evidence indicating a proinflammatory effect for Ang II, and the beneficial effects of the use of an Ang II receptor antagonist, olmesartan medoxomil, in hypertensive patients with vascular microinflammation [27]. Valsartan is a specific Ang II type 1 receptor antagonist which acts by binding to the AT-1 receptor and displacing Ang II from this receptor. This drug has been used for treating mild to moderate hypertension in humans [28,29]. In our study, we selected an AT1 receptor blocker, instead of an ACE inhibitor, due to its more selective effects, thus avoiding mechanisms such as those dependent on cell chymase for angiotensin II formation. In this way we were also able to obtain a more direct anti-inflammatory effect, which is dependent on angiotensin II blockage.

We used two of the most commonly employed animal models of colonic inflammation: the TNBS and DSS models. TNBS is a hapten that produces colitis via activation of Th1 cells. In this study, TNBS with ethanol produced a large inflammatory response as evidenced by increases in both macroscopic and microscopic damage. Similar damage has previously been reported in studies using TNBS to induce colitis [16,17]. Animals treated with valsartan showed a decrease in these parameters, thus our data suggests that an angiotensin II receptor antagonist can decrease the colonic inflammation in the TNBS animal model. When we examined the cytokine production we also observed a decrease in inflammatory cytokines such as TGF- β and IL-18, with an increase in the anti-inflammatory cytokine IL-10 in the valsartan treated animals. TGF- β has been shown to have effects on gastrointestinal mucosal integrity and repair in vitro, but its effects in vivo are still unclear. It has been suggested that it is required for intestinal mucosal healing with its modulation playing a role in susceptibility to injury in colitis [30]. Interestingly, TGF- β and IL-10 appear to have interrelated roles in the regulation of colitis. Fuss *et al* have suggested that the regulatory effects of IL-10 are possibly indirect, and are related to its ability to facilitate the regulatory role of TGF- β [31]. TNBS induces the activation of Th1 by CD4, and previous studies have shown that CD4⁺CD25⁺ T cells express high levels of TGF- β 1 in a surface-bound and/or a secreted form, after stimulation [32]. Our data were confirmed by a decrease in positive cell staining by immunohistochemistry for the proinflammatory cytokines in the valsartan treated animals, with an increase in IL-10 positive cells.

The DSS model of colitis is different to that of the TNBS model. DSS is cytotoxic to epithelial cells and appears to be phagocytosed by macrophages, and this action obstructs the relationship between intestinal lymphocytes and epithelial cells [33]. DSS produces a mild to moderate inflammation in the intestinal tract, characterized by bloody diarrhea. In this study we did not find any significant differences in macroscopic and microscopic damage between the control and valsartan-treated group in the DSS model. We did however find a large decrease in the incidence of diarrhea in these animals following valsartan treatment compared with their untreated colitis controls. One possible explanation for our results can be the mechanism by which DSS induces acute colitis. It seems to result from an alteration in the colonic epithelium with resulting bacterial translocation that perpetuates inflammation and does not produce alteration of B and T cell responses [34]. In this model the over expression of IL-18 protein was decreased in those animals treated with Valsartan. IL-18 has been shown to be increased

and play an important role in the primary stages of inflammation in DSS colitis, and its inhibition with a recombinant IL-18 binding protein was able to attenuate intestinal damage in both this model [35], as well as the TNBS model [36]. In the present study valsartan treatment caused an upregulation in the gene expression of IL-10, a cytokine which is known to be therapeutic in both the DSS and TNBS models of colitis. Lindsay *et al.*, have demonstrated the therapeutic efficacy of an adenoviral vector encoding IL-10 (AdvmlIL-10) on stool markers of inflammation and histological scores in mice [37,38]. However, although IL-10 polymorphisms have been found in patients with IBD, results in clinical trials have been disappointing [39].

Ang II receptors are found throughout the intestine. Binding for the Ang II type 1 receptor has been demonstrated in the muscularis mucosa, enteric nerves and epithelial cells of the colon, and specifically within the intestine of Sprague-Dawley rats [40]. Mucosal levels of Ang I and II have been found to be increased in colonic biopsies from patients with Crohn's disease, with the source most likely to be a local angiotensin cascade, or the endothelium of the microvasculature [12]. Our results in large part concur with the recent study by Inokuchi and colleagues who demonstrated amelioration of TNBS colitis in angiotensinogen gene knockout mice, and the beneficial effects of prophylactic treatment with the Ang II type I receptor blocker, losartan [14].

The present study demonstrates a beneficial effect of Valsartan in both models of colitis, with some involvement of the immune system, however it is not clear which cells are the major target. As mentioned before many cells in the colon express Ang II receptors, including those on vascular endothelial cells, and the involvement of the microvascular system most likely plays an important role. Although this study did not investigate changes in microcirculation related with the colitis, we and others have previously demonstrated the importance of blood flow and microvascular changes to progression of the colitis in the TNBS animal model [41, 42]. Treatment with Losartan has been shown to attenuate leukocyte recruitment following ischemia-reperfusion injury in the intestine and suggests the contribution of the Ang II receptors to the inflammatory state [43].

A recent Australian-based analysis of the angiotensinogen-6 variant found a significant association with Crohn's disease, also supporting the therapeutic potential for either angiotensin-converting enzyme inhibitors or angiotensin II receptor antagonists [44]. ACE inhibitors have had mixed results in animal models of colitis. Wengrower *et al.*, demonstrated that prophylactic administration of Captopril in TNBS colitis could reduce damages scores and fibrosis after 21 days, as well as the levels of Ang II and TGF-beta [13]. On the other hand treatment with the same inhibitor administered after the induction of acute colitis did not show significant benefits in the observed lesions at the dose tested [45].

In summary, our results demonstrate that specific inhibition of Angiotensin II can reduce some of the parameters associated with intestinal inflammation. This study suggests a possible anti-inflammatory effect for Valsartan in colitis via modulation of the immune system, as shown by a blockade of both TGF-beta and IL-18 over expression. However, these results appear to depend on the model of colitis studied, and require more investigation in order to evaluate the therapeutic potential for the use of these receptor antagonists in inflammatory bowel disease.

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Figure 1.

Effects of Valsartan, an Ang II inhibitor, on weight change following induction of TNBS or DSS colitis. Animals receiving Valsartan lost less weight and regained weight faster after TNBS induction compared to the untreated controls (values are mean and s.e. of 4–7 rats/group; *p<0.05, **p<0.01 compared to day 0; #p<0.05, ##p<0.01 compared to water alone at the same time point).

Figure 2. Effect of Valsartan, an Ang II inhibitor, on macroscopic damage scores in colitis. Administration of Valsartan decreased the total macroscopic damage score in the TNBS colitis rats but not the DSS colitis (values are mean and s.e. of 4–7 rats/group; * $p < 0.05$ vs Valsartan alone, ** $p < 0.01$ vs water alone).

Figure 3.

Effect of Valsartan, an Ang II inhibitor, on incidence of diarrhea in TNBS and DSS colitis. Administration of Valsartan decreased the incidence of diarrhea in DSS colitis by 50% but had no effect in the TNBS colitis model (values are expressed as absolute % of animals with diarrhea).

A B **Figure 4.**

Effects of Valsartan, an Ang II inhibitor, on microscopic damage in TNBS and DSS colitis. A) Animals receiving water alone showed normal architecture with no microscopic damage. Administration of TNBS or DSS resulted in disruption of the normal mucosal architecture, an increase in cellular infiltration and muscle thickening, and goblet cell depletion. These changes were less apparent in the Valsartan treated animals (x10). B) Valsartan significantly reduced the microscopic damage found in the TNBS colitis rats (values are mean and s.e. of 4–7 rats/group; # $p < 0.05$ vs Valsartan alone, ** $p < 0.01$ vs water alone, *** $p < 0.001$ vs water alone).

Figure 5. Effects of Valsartan, an Ang II inhibitor, on tissue levels of TGF beta in TNBS and DSS colitis. Valsartan significantly reduced the levels of TGF beta in TNBS colitis but had no effect in the DSS model (values are mean and s.e. of 5–7 rats/group; * $p < 0.05$ vs TNBS alone).

Figure 6.

Effect of Valsartan on cytokine gene expression assessed by real time RT-PCR. The data were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and are presented as valsartan-treated colitis relative to untreated colitis using the delta-delta Ct method for comparing relative fold-expression differences.

Figure 7.

Effect of Valsartan on cytokine expression assessed by immunohistochemistry. Left hand panel: Animals with TNBS colitis showed positively stained inflammatory cells, mostly neutrophils with some macrophages for (A) TNF-alpha, (B) TGF-beta and (C) IL-18 but little staining for (D) IL-10. Right hand panel: Valsartan treatment reduced the number of cells staining positively for (E) TNF-alpha (F) TGF-beta and (G) IL-18, and increased the number of neutrophils and lymphocytes staining positively for (H) IL-10 (x40).

Table 1
Primer Sequences Used for Real Time RT-PCR Assays

Gene Name	Primer Sequence
IL-18F	atatcgaccgaacagccaac
IL-18R	tggcacacgtttctgaaaga
TNF-alphaF	tctgtctactgaacttcgggt
TNF-alphaR	tagttggtgtctttgagatcc
TGF-betaF	atatagcaacaattcctggcg
TGF-betaR	gccgcacacagcagttctytc
IL-10F	gcaaggcagtgagcaggtgaa
IL-10R	gaataaatagaatgggaactga
GAPDHF	ggcattgtctctaatgacaa
GAPDHR	tgtgagggagatgctcagtg

Table 2

Effect of Valsartan on Microscopic damage in the colon

CRITERIA	MEAN SCORE				
	Water	Valsartan	Water/TNBS	Valsartan/TNBS	Valsartan/DSS
Loss of Mucosal Architecture	0.00 ± 0.00	0.50 ± 0.29	2.80 ± 0.20**	1.60 ± 0.40	1.28 ± 0.28
Cellular infiltration	1.00 ± 0.00	1.50 ± 0.29	3.00 ± 0.00*	1.20 ± 0.58##	1.00 ± 0.00
Muscle Thickening	0.00 ± 0.00	0.50 ± 0.29	1.60 ± 0.40	1.60 ± 0.40	1.71 ± 0.28 ^Δ
Goblet Cell Depletion	0.00 ± 0.00	0.00 ± 0.00	0.80 ± 0.20	0.40 ± 0.24	0.86 ± 0.14
Crypt Abscess Formation	0.00 ± 0.00	0.00 ± 0.00	1.00 ± 0.00	0.40 ± 0.24	0.86 ± 0.14 ^Δ
Total Damage Score	1.00 ± 0.00	2.50 ± 0.64	9.20 ± 0.37***	5.20 ± 0.97###	5.71 ± 0.61 ^Δ

Values are means ± SEM n=4-7 rats per group.

* p<0.05

** p<0.01

*** p<0.001 vs. water alone

^Δ p<0.05 vs. valsartan alone

p<0.01 vs. water/TNBS

Table 3

Effect of Valsartan on tissue myeloperoxide levels in acute colitis

	No colitis	TNBS	DSS
<u>Water</u>	0.27 ± 0.08	14.08 ± 1.84 ^{**}	0.81 ± 0.22
<u>Valsartan</u>	0.56 ± 0.06	29.59 ± 10.82 ^{##}	0.72 ± 0.15

Values are mean and s.e. of 4–7 rats/group.

^{**}
p<0.01 compared to water alone

^{##}
p<0.01 compared to valsartan alone.

Table 4
Effect of Valsartan on tissue cytokine levels in acute colitis

	IL-18 (pg/ml)	IL-10 (pg/ml)	TNF-alpha (pg/ml)
Water/TNBS	37.95 ± 14.48	0.00 ± 0.00	0.65 ± 0.57
Valsartan/TNBS	18.71 ± 7.27	2.82 ± 2.49	0.95 ± 0.55
Water/DSS	41.45 ± 12.63	16.58 ± 9.33	0.46 ± 0.46
Valsartan/DSS	20.53 ± 3.92	15.09 ± 11.73	1.11 ± 0.11

Values are mean and s.e. of 4–7 rats/group.