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ORIGINAL ARTICLE

# PD123319, angiotensin II type II receptor antagonist, inhibits oxidative stress and inflammation in 2, 4-dinitrobenzene sulfonic acid-induced colitis in rat and ameliorates colonic contractility

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## Abstract

Angiotensin II, the main effector of renin angiotensin system, plays an important role in the inflammatory process and most of its effects are mediated through the AT1 receptor activation. However, the knowledge about the AT2 receptor involvement in this process is still evolving. We previously found that in an experimental model of colitis, AT2 receptor activation can contribute to the impairment of the muscle contractility in vitro in the course of inflammation. Here, we investigated the potential alleviating effects of the in vivo treatment of PD123319 (1-[[4-(Dimethylamino)-3-methylphenyl]methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid ditrifluoroacetate), AT2 receptor antagonist, in 2,4-dinitrobenzene sulfonic acid (DNBS)-induced rat model of colitis. The effects of i.p PD123319 (0.3, 3 and 10 mg/kg) administration to rats subjected to intra-rectal DNBS instillation were investigated. The study revealed that the colon injury and the inflammatory signs were ameliorated by PD123319 when visualized by the histopathological examination. The colon shortening, myeloperoxidase activity, and colonic expression of IL-1β, IL-6 and iNOS were downregulated in a dose-dependent manner in DNBS-induced colitis rats treated with PD123319 and the anti-oxidant defense machinery was also improved. The mechanism of these beneficial effects was found in the ability of PD123319 to inhibit NF-κB activation induced by DNBS. The colonic contractility in inflamed tissues was also improved by PD123319 treatment. In conclusion, our data have demonstrated previously that undescribed proinflammatory effects for the AT2 receptors in DNBS-induced colitis in rats in which they are mediated likely by NF-κB activation and reactive oxygen species generation. Moreover, when the inflammatory process is mitigated by the AT2 receptor antagonist treatment, the smooth muscle is able to recover its functionality.


**Keywords** Inflammation · Angiotensin · AT2 receptor · Inflammatory bowel disease

## Introduction

Renin–angiotensin system (RAS) is a prominent regulatory system for fluid and electrolyte homeostasis, but its impact on gastrointestinal (GI) physiology has not been explored enough. Recent data indicate that RAS is well expressed and active in the GI tract and it can also play a major role in several gut processes, including absorption, secretion, and motility (Ewert et al. 2006; Fishlock and Gunn 1970; Mastropaolo et al. 2013, 2015).

Moreover, data are accumulating suggesting an involvement of RAS in diseases such as GI cancer, mesenteric ischemia, motility disorders, and particularly inflammatory diseases (Shi et al. 2016). The RAS is a multi-component cascade with renin as the rate-limiting enzyme that cleaves angiotensinogen to angiotensin (Ang) I, which is further cleaved by angiotensin-converting enzyme (ACE) to Ang II. Ang II is the main effector of the RAS, it acts by binding to the angiotensin receptors (AT1 and AT2), which are found in many cell types including intestinal epithelial cells and mucosal immune cells (Fandriks 2011). AT1 receptors mediate all classical actions of Ang II in the target cells. AT2 receptors, indeed, in many instances counteract AT1

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receptor-mediated actions. The tissue or paracrine RAS is a major activator of oxidative stress and inflammatory processes in several tissues. In patients with active inflammation, an increase in the colonic mucosal Ang I and II concentrations has been reported (Jaszewski et al. 1990). Shi et al. (2016), using a transgenic mouse model overproducing active renin, demonstrated that the renin–Ang II cascade promotes colitis. Angiotensin II appears to drive colonic mucosal inflammation by promoting intestinal epithelial cell apoptosis and mucosal Th17 responses. A role for AT1 receptors in the inflammatory processes has been recognized since knockout mice for AT1 receptors developed less severe experimental colitis than wild-type mice (Kim and Iwao 2000; Inokuchi et al. 2005; Katada et al. 2008; Mizushima et al. 2010). Moreover, AT1 receptor blockers have been demonstrated to be efficacious in the attenuation of colonic injury in some experimental colitis. Indeed, our knowledge on the role of AT2 receptors is still incomplete and controversial.

AT2 signaling can result in either beneficial or adverse effects on proliferation, inflammation, and fibrosis in cardiovascular disease (Kim and Iwao 2000; Matavelli et al. 2011; Verdonk et al. 2012). Moreover, the stimulation of the angiotensin AT2 receptor by the Compound 21, in an experimental model of acute lung injury, induced anti-inflammatory effects (Menk et al. 2018). Recently, we have reported that in the DNBS rat model of colitis the effects of Ang II in the modulation of colonic contractility in vitro was altered with a recruitment of the AT2 receptors which, via enteric nerves and production of NO, counteract AT1 receptor excitatory effects (Zizzo et al. 2017). Transcripts encoding for the AT1 (both AT1A and AT1B receptor subtypes) and the AT2 receptors have been demonstrated in both normal and inflamed colonic tissues. Interestingly, the AT2 receptor antagonist improved the colonic contractility in inflamed tissues. Therefore, we suggested that AT2 receptor activation might represent one of the multiple mechanisms leading to the impairment of the muscle contractility in the course of inflammation. This finding has encouraged us to investigate whether the AT2 signaling could be also involved in the colon injury in the experimental colitis. In the current study, we aim to evaluate the effects of the in vivo AT2 signaling block, using PD123319, AT2 receptor antagonists, on the signs of the inflammatory disease in DNBS rat model of colitis.

## Materials and methods

### Animals

Thirty Wistar male rats (weighing 250–350 g), were purchased from ENVIGO Srl (San Pietro al Natisone UD, Italy) and were employed throughout the study. The animals were housed in temperature-controlled rooms on a 12-h light cycle at 22–24 °C and 50–60% humidity. They were fed with standard laboratory chow and tap water ad libitum. The animals were allowed to acclimatize to the housing conditions for 1 week prior to experimentation. Procedures involving the animals and their care were conducted in the conformity of the Italian "D.Lgs 26/2014" and the European directives (2010/63/EU). The animal care and handling were conducted in accordance to the provisions of the European Community Council Directive 210/63/UE, recognized and adopted by the Italian Government. The experiments had been approved by the Ethical Committee for Animal Experimentation of the University of Palermo and by the Italian Ministry of Health (Authorization N 370/2017-PR released May 3, 2017, Rome, Italy). No other methods to perform the described experiments (3Rs) were found.

### DNBS-colitis induction and treatment protocol

Rats were randomly assigned in six groups (five animals each): (1) sham group; (2) colitis group; (3) sham group + PD123319 (10 mg/kg/day i.p); (4) colitis group + PD123319 (0.3 mg/kg/day i.p); (5) colitis group + PD123319 (3 mg/kg/day i.p); (6) colitis group + PD123319 (10 mg/kg/day i.p). Colitis was induced by intracolonic [i.c.] instillation of DNBS, as already described (Zizzo et al. 2017, 2019). Briefly, rats were fasted overnight and then under light anesthesia with 1% isoflurane (Merial Italia Spa, Assago,MI,Italy),a solution of 15 mg of 2,4-dinitrobenzene sulfonic acid (DNBS; Sigma-Aldrich Inc., St Louis, MO, USA) in 50% ethanol, total volume of 0.25 mL, was injected into the colon through an 8-cm plastic catheter (PE90).

Rats were then maintained in an inclined position for 5 min and allowed to recover with food and water supplied. Sham animals received 0.25 mL of vehicle alone (50% ethanol). Previous experiments demonstrated that 15 mg DNBS induced reproducible colonic inflammation manifested by body weight decrease as well as macroscopic damage and biochemical alterations characteristic of the disease (Zizzo et al. 2019).

PD123319, AT2 receptor antagonist (Sigma-Aldrich Inc., St Louis, MO, USA), was intraperitoneally [i.p.] administered once a day for 6 days starting 30 min before the induction of colitis (day 0). PD123319 was dissolved in 0.5 mL water solution, prepared just before the injection, at a dose range from 0.3 to 10 mg/kg. At day 6, rats were sacrificed and the abdomen was immediately opened and the appearance of colon was then examined. Distal colon was removed, opened longitudinally, and gently cleaned of fecal content using saline and was processed for assessment by the macroscopic, histological scores and biochemical markers. No

mortality was observed in any group throughout the study. The severity of colitis was evaluated by independent observers blinded to the identity of the treatments, in accordance with the criteria previously reported by Zizzo et al. (2017, 2019) and efforts were made to minimize the suffering of animals.

## Assessment of colitis damage

### Body weight change and stool consistency

During the experimental period, the animals were observed daily. Each day, the body weight and the stool consistency were calculated. The body weight change was calculated as the percent difference between the body weight before the experimental manipulation (100%) and the daily body weight. For the analysis of stool consistency, three grade scores were used (0, normal; 2, loose; and 4, diarrhea).

### Macroscopic scores

After the sacrifice, the distal colon was collected and for each specimen wet weight (mg) and its length (cm) were measured. Then the weight/length ratio was calculated as indicator of colonic edema. The colon damage was scored according to the criteria described by Appleyard and Wallace (1995), as outlined in Table 1. The cumulative score of the different parameters represents a numerical index determining the severity of the macroscopic damage (Macroscopic Damage Score).

### Analysis of microscopic inflammatory damage

Full-thickness colon biopsy specimens were fixed in 4% formaldehyde for 24 h (Sigma-Aldrich, Inc., St. Louis, USA), embedded in paraffin, and cross sections of 5 μm were stained with hematoxylin and eosin (Bio-Optica Milano SpA, Italy). Histological sections were examined using a conventional microscope (Olympus BX50, Olympus Optical Co., Japan). The colon microscopic damage was scored on a 0–10 scale as described by Hunter et al. (2005), as reported in Table 2.

### Myeloperoxidase assay

Myeloperoxidase (MPO) activity, a marker for neutrophil inflammation, was estimated according to the method of Moreels et al. (2001). Samples were blotted dry, weighted, and placed in a potassium phosphate buffer pH 6.0 containing 0.5% hexadecyltrimethylammonium bromide (5 g tissue per 100 mL buffer). The samples were then placed on ice, homogenized for 30 s and subjected to two sonication and freeze-thawing cycles. The suspension was centrifuged

**Table 2** Microscopic scoring system adapted from Hunter et al. (2005)

| Histological finding | Normal | Minimal | Mild | Maximal |
|---|---|---|---|---|
| Inflammatory infiltrate | 0 | 1 | 2 | 3 |
| Number of layers infiltrated | 0 | 1 | 2 | 3 |
| Mucosal tissue damage | 0 | 1 | 2 | 3 |
| Mucosal oedema | | | | |
| Absent = 0 | | | | |
| Present = 1 | | | | |

**Table 1** Criteria for macroscopic scoring system adapted from Appleyard and Wallace (1995)

| Feature | Score |
|---|---|
| Ulceration | |
| Normal aspect mucosal | 0 |
| Localized hyperemia with no ulcers | 1 |
| Ulceration without hyperemia/bowel wall thickening | 2 |
| Ulceration with hyperemia/bowel wall thickening at one site, | 3 |
| Two or more sites of ulceration with hyperemia/bowel wall thickening | 4 |
| Major damage (necrosis) extended > 1 cm along the length of colon | 5 |
| When an area of damage extended > 2 cm along the length of colon; the score was increased by one for each additional cm involved | 6–10 |
| Adhesion | |
| No adhesions | 0 |
| Minor adhesions (colon can be separated easily from the other tissue) | 1 |
| Major adhesions | 2 |
| Thickness | |
| Maximal bowel wall thickness (×) in mm was added to above score | X |

at 15,000*g* for 15 min at 4 °C. Aliquots (0.1 mL) of the supernatant were added to 2.9 mL of o-dianisidine solution (16.7 mg of o-dianisidine in 1 mL methanol, 98 mL of 0.05 M potassium phosphate buffer pH 6.0, and 1 mL of a 0.5% $H_2O_2$ solution as a substrate for MPO enzyme). The absorbance rate was monitored at 460 nm for 60 s using a spectrophotometer (Beckman Coulter Inc, CA, USA). One unit of MPO activity was defined as the amount of the enzyme converting 1 µmol $H_2O_2$ to $H_2O$ in 1 min at room temperature and was expressed as units per gram tissue (U g tissue$^{-1}$).

### ELISA assay for inflammatory cytokines

10 mg of colon tissue was homogenized in 10 mL of lysis buffer (supplied by ELISA kits), after centrifugation at 14,000 rpm at 4 °C for 30 min. 100 µL of the supernatant was used to test interleukin-1β (Thermo Fisher Scientific, Rockford, IL, USA) and interleukin-6 (Cloud-Clone Corp., Houston, TX, USA) expression, according to the manufacturer's instructions. Briefly, 100 µL of the samples was incubated in duplicate at 37 °C for 1 h in supplied multiwell plates, followed by the addition of prepared "detection reagent A" (100 µL) and incubation at 37 °C for 1 h. Then the solution was eliminated and the multiwell plates were washed three times with washing buffer. 100 µL of the prepared "detection reagent B" was added to the samples and then incubated at 37 °C for 30 min. After washing three times in washing buffer, 90 µL of "substrate solution" was added and incubated at 37 °C for 15 min. The reaction was stopped with 50 µL of "stop solution" and read at 450 nm immediately with the GloMax® Discover Microplate Reader (Promega, Milano, Italy). As a reference for quantification, a standard curve was made.

### Analysis of reactive oxygen species (ROS) generation

To assess ROS generation by fluorimetric analysis, 10 mg of tissue was homogenized with 1 mL of phosphate-buffered saline (PBS), pH 7.2 and 10 µL of protease inhibitor (Amersham Life Science, Munich, Germany) was added, and centrifuged at 14,000 rpm, for 30 min at 4 °C. After centrifuging, 100 µl of the supernatant was incubated with 1 mM dichlorofluorescein diacetate (DCFH-DA) for 10 min at room temperature in the dark. The conversion of non-fluorescent DCFH-DA to the highly fluorescent compound 2′, 7′ dichlorofluorescein (DCF) by esterase activity can be used to monitor the presence of peroxides due to the oxidative burst in the tissue. Therefore, the emitted fluorescence is directly proportional to the concentration of hydrogen peroxidase inside the sample. The sample was analyzed by fluorimeter (Microplate reader WallacVictor 2—1420 Multilabel Counter; PerkinElmer, Inc.). The excitation filter was set at 485 nm and the emission filter was set at 530 nm.

### mRNA analysis by real-time PCR

Quantitative real-time PCR (qRT-PCR) was used to quantify messenger RNA encoding for neuronal NOS (nNOS, NOS-1), endothelial NOS (eNOS, NOS-3), and inducible NOS (iNOS, NOS-2) levels in the rat colon. The expression of nNOS, eNOS, and iNOS was normalized to that of beta actin, a housekeeping gene that is not subjected to regulation. Total RNA was extracted from whole thickness colonic preparation devoid of mucosa layer from control and DNBS rats using RNAeasy lipid tissue mini kit (Qiagen, Valencia, CA). 2 ng of total RNA was used to synthesize the first strand cDNA using RT FirstStrand kit (Qiagen, Valencia, CA). Synthesized cDNAs were amplifed using SYBR Premix Ex Taq II (TaKaRa, Bio Inc Foster City, CA) and StepOne Real-Time instrument (Applied Biosystems, Foster City, CA). Gene expression was performed in triplicate, using specific primers and amplification conditions. The oligonucleotide primers for rat nNOS, eNOS, and iNOS and for rat beta actin were as follows: nNOS forward: 5′-CGT CCGTGACTACTGTGACAA-3′, reverse: TGTCCAAAT CCATCTTCTTGG, iNOS forward: 5′-AGAAGGGGACGA ACTCAGC-3′, reverse: 5′-TCGAACATCGAACGTCTC AC-3′, eNOS forward: 5′-TGACCCTCACCGATACAACA -3′, reverse: 5′-CGGGTGTCTAGATCCATGC-3′, beta actin forward: 5′-CTAAGGCCAACCGTGAAAAG-3′, reverse: 5′-GCCTGGATGGCTACGTACA-3′.

Temperature cycling consisted of an initial denaturation step for 10 min, followed by 45 cycles of amplification. Each cycle consisted of denaturation for 15 s at 95 °C, annealing for 30 s at 60 °C, and extension for 30 s at 72 °C. Samples were incubated for an additional 10-min period at 72 °C for terminal elongation after the completion of the final cycle. On the basis of the Ct value (threshold cycle—the number of reaction cycles after which fluorescence exceeds the defined threshold) of the examined gene and of the internal control gene, the relative expression level of RNA was calculated according to the $2^{-\Delta\Delta Ct}$ approximation method algorithm.

### Nitric oxide (NO) concentration assay

10 mg of rat colon tissue was homogenized with 1 mL of PBS, pH 7.2, and centrifuged at 14,000 rpm, for 30 min at 4 °C. After centrifuging, an aliquot of 100 µL of the supernatant was incubated with equal volumes of Griess reagent (1% sulphanilamide in 5% phosphoric acid and 0.1% *N*-(1-naphthyl)-ethylenediamine), the absorbance was immediately red at 450 nm (GloMax® Discover System, Promega), at 485 nm (excitation), and at 520 nm (emission)

in a fluorescence microplate reader. NO concentration was evaluated using a standard curve.

### Total protein extraction and western blotting

Total proteins were prepared by homogenizing 10 mg of rat colon tissue in 1 mL solubilizing buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 2 mM phenylmethylsulfonyl fluoride PMSF), 1 mM DTT, 0.1% SDS, protease inhibitor (Amersham Biosciences, Milan, Italy) and phosphatase inhibitor (cocktail II and III; Sigma-Aldrich, Milan, Italy). Total proteins in the lysates were quantified by Bradford method (Bio-Rad). Protein samples (30 µg) were submitted to 10% SDS-PAGE and were transferred onto nitrocellulose filters. The Western blot was incubated with Phospho-NF-κB p65 (Ser536) Antibody (1:1000; Cell Signaling, Boston, USA) and β-actin (1:1000; Sigma-Aldrich Inc., St Louis, MO, USA) antibodies. Band intensities were analyzed with ImageJ and expression was adjusted to β-actin expression. The protein levels were expressed as intensity relative to control.

### Evaluation of the mechanical activity of colonic longitudinal muscle strips in vitro

Smooth muscle strips (10 mm in length) from the rat distal colon were cut in longitudinal direction. In DNBS-treated animals, tissues were taken from the region of colon immediately adjacent to the gross macroscopic damage area. The strips were then suspended into the four channels of a vertical organ bath, each containing 10 mL of oxygenated (95% O2 and 5% $CO_2$) Krebs solution and maintained at a constant temperature of 37 °C. The distal end of the strips was tied to an organ holder and the proximal end was connected, by a silk thread, to the isometric force transducer (FORT 25 Ugo Basile Biological Research Apparatus, Comerio VA, Italy). Mechanical activity was digitized by an A/D converter, displayed and recorded on a personal computer, using the PowerLab/400 system (Ugo Basile, Biological Research Apparatus, Comerio VA, Italy). Preparations were subjected to an initial tension of 500 mg and were then left to equilibrate for at least 30 min, until stable spontaneous mechanical activity was observed. Preparations from different groups of rats were challenged with 10 µM carbachol (CCh) or with high KCl (60 mM) and with 1 µM isoproterenol (Iso).

### Solutions and drugs

Krebs solution consisted of (mM): NaCl = 119; KCl = 4.5; $MgSO_4$ = 2.5; $NaHCO_3$ = 25; $KH_2PO_4$ = 1.2; $CaCl_2$ = 2.5; and glucose = 11.1. Drugs used were: carbamylcholine chloride (CCh), Isoproterenol (Iso), and potassium chloride (KCl) from Sigma-Aldrich Inc. All drugs were dissolved in distilled water. Working solutions were then dissolved in Krebs solution. The method of delivery and the doses of the drugs used were selected on the basis of the available literature.

### Data analysis and statistical tests

All data are mean ± SEM: '*n*' indicates the number of animals. Statistical analysis was performed using *GraphPad Prism 6.0 software*, utilizing one-way ANOVA and Dunnett's multiple comparison test and considered significant if $P$ value was < 0.05.

## Results

As previously reported (Zizzo et al. 2019), in DNBS groups, we observed sustained body weight loss and diarrhea during the 6 days after the start of the induction of the colitis, while sham groups exhibited continuous body weight gain and no diarrhea (Fig. 1).

The treatment with AT2 receptor antagonist, PD123319 (0.3, 3, and 10 mg/kg i.p) dose-dependently reduced in DNBS animals, the drop in the body weight as well the occurrence of diarrhea (Fig. 1A, B). PD123319, *per se*, at the higher dose used, 10 mg/kg, had no effect on sham animals (Fig. 1A, B).

### Macroscopic scores

Rats challenged with DNBS showed intense damage in the large intestine, leading to high macroscopic scores. Distal colon appeared dilated, thickened, generally highly vascularized with ulcerations or necrotic tissue. Diffuse adhesions between colon and other organs were observed. The colonic weight/length ratio, a reliable index of oedema tissue, was significantly increased in colitis rats compared to sham groups. PD123319, at the dose of 0.3, 3, or 10 mg/kg, reduced the total macroscopic damage score, reaching the statistical significance at 3 or 10 mg/kg ($P < 0.05$) (Fig. 2A). The colonic weight/length ratio was also reduced by PD123319 in comparison to DNBS group, confirming the relief of the colitis severity. Sham animals treated with PD123399 did not show any difference in the macroscopic score and in the colonic weight/length ratio compared to sham animals receiving only the vehicle (Fig. 2B).

### Microscopic score, myeloperoxidase (MPO) assay, and inflammatory cytokine levels

The severity of the colon tissue damage was explored by hematoxylin and eosin (H&E) staining. The results showed that the microscopic evaluation of colon sections stained

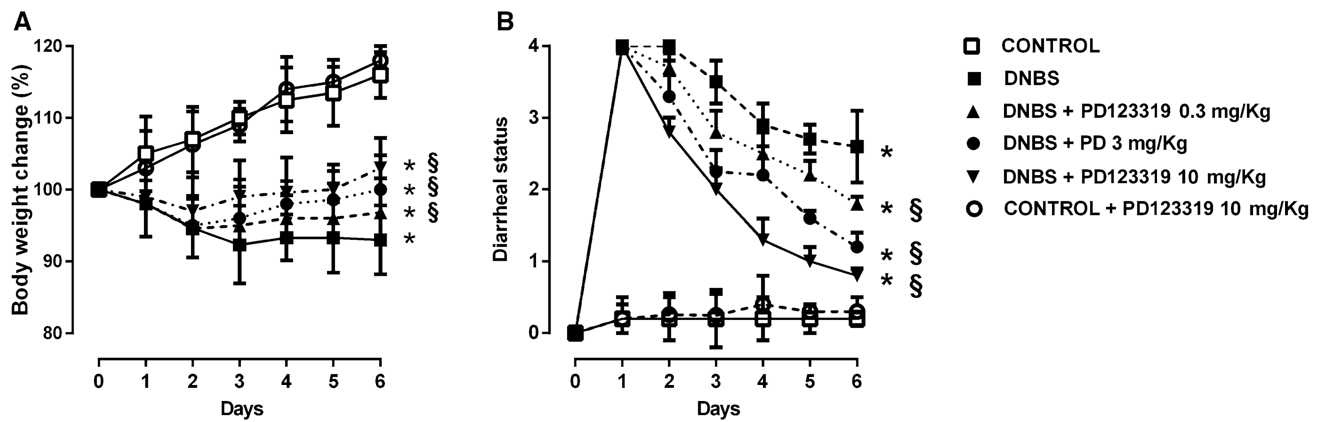


**Fig. 1** PD123319 ameliorates clinical signs, body weight loss, and incidence of diarrhea in DNBS-induced colitis group. **A** Body weight changes, calculated as percent difference relative to the original body weight, and **B** stool consistency in vehicle or in 0.3, 3, and 10 mg/kg PD123319-treated rats with or without colitis induction. Data are mean ± SEM $n=5$ for each group. *$P<0.05$ versus Sham group; $^{§}P<0.05$ versus DNBS group

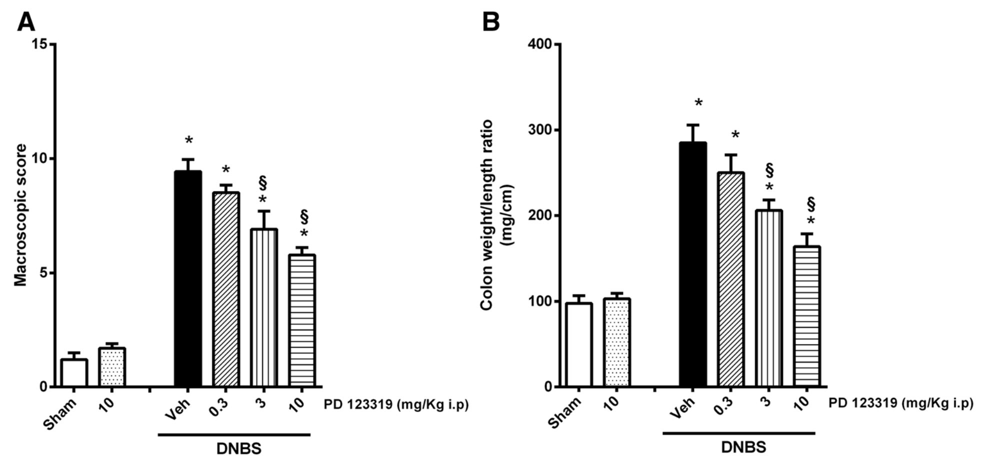


**Fig. 2** PD123319 attenuates macroscopic colonic damage in DNBS-induced colitis group. **A** Macroscopic score and **B** colon weight/length ratio in vehicle or in PD123319-treated rats with or without colitis induction. Data are mean ± SEM. $n=5$ for each group. *$P<0.05$ versus Sham group; $^{§}P<0.05$ versus DNBS group

with hematoxylin/eosin was in line with observation of the macroscopic parameters (Fig. 3A). Histological analysis of distal colon sections from sham group and sham group treated with PD123399 showed intact epithelium, absence of oedema, and normal muscle architecture (Fig. 3A). Severe microscopic damage, characterized by a significant depletion of goblet cells and crypts, increased infiltration of inflammatory cells, severe submucosa oedema, thickening of smooth muscle, and disruption of colonic architecture were observed in colon specimens from DNBS group (Fig. 3A). PD123319 at the doses of 3 mg/kg and 10 mg/kg distinctly reduced the colonic lesions by improving the microscopic score, indicating that it has protective effects against the histopathological damage of colon of DNBS animals (Fig. 3B).

The beneficial effects shown by PD123319 were confirmed biochemically. The colonic damage induced by DNBS was associated with an increased myeloperoxidase levels in comparison with sham group and sham group treated with PD123399, indicative of the massive neutrophil infiltration that had taken place in the inflamed tissue. PD123319 treatment in DNBS animals reduced the enzyme activity, confirming a lower leukocyte infiltration in the colonic tissue as observed in the histological studies. Once more, the effect was dose dependent (Fig. 3C).

Similarly, other markers of inflammation, such as the levels of the proinflammatory cytokine IL-1β and IL-6 were analyzed in the colonic samples to better characterize the anti-inflammatory effect exerted by the AT2 receptor antagonist. As expected, the expression of the proinflammatory cytokines was upregulated in DNBS rats in comparison with the sham group and sham group treated with PD123399. Treatment with PD123319 counteracted the increase in proinflammatory cytokines when compared with the DNBS group (Fig. 4A).

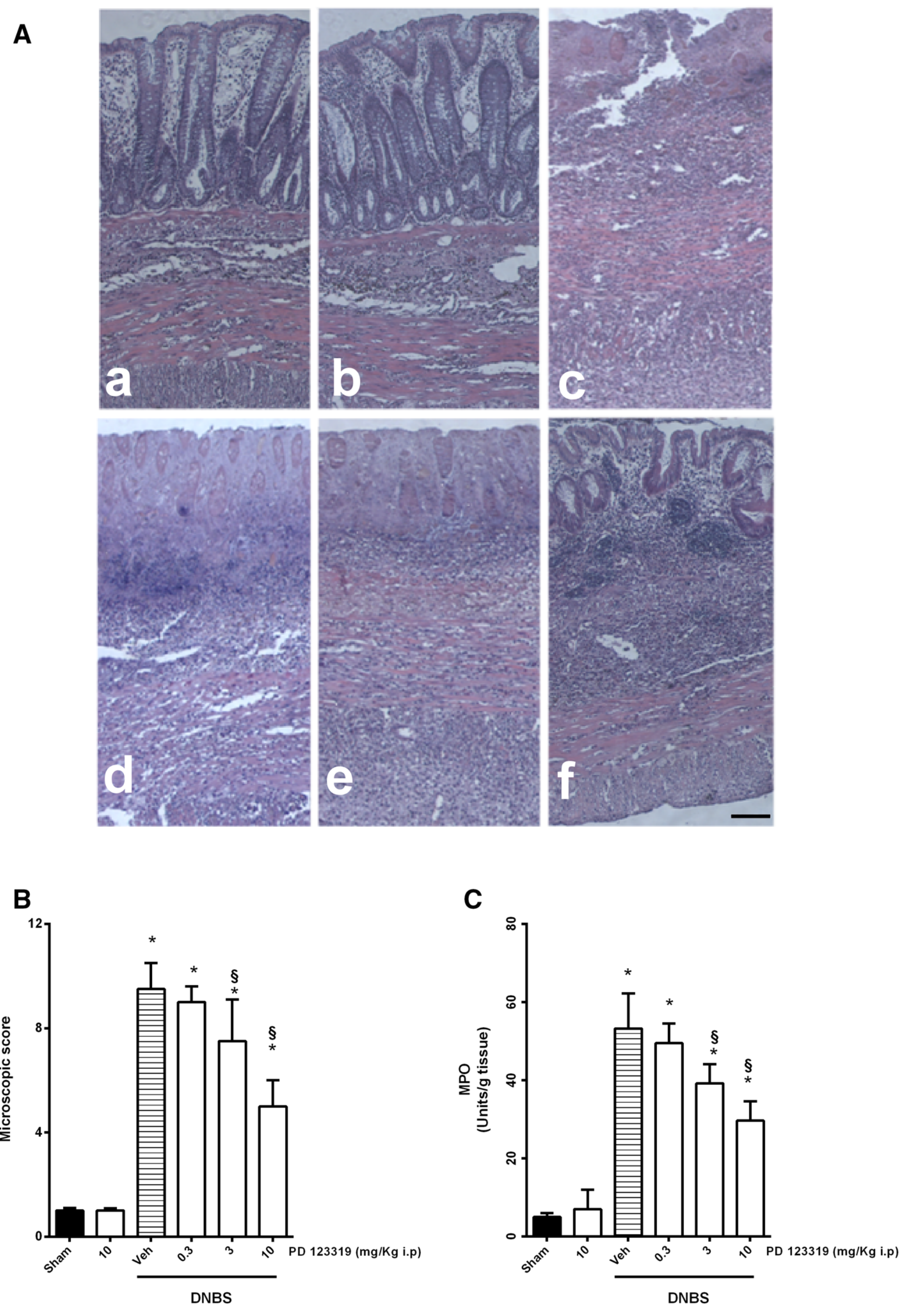


**Fig. 3** PD123319 ameliorates histological damage in DNBS-induced colitis group. **A** Representative H&E-stained colon sections from (a) Sham, (b) Sham animal treated with PD123319 (10 mg/kg i.p), (c) DNBS or (d) DNBS animal treated with PD123319 (0.3 mg/kg i.p), (e) DNBS animal treated with PD123319 (3 mg/kg i.p), (f) DNBS animal treated with PD123319 (10 mg/kg i.p). Scale bar corresponds to 100 μm and applies throughout. **B** Histological score and **C** colonic myeloperoxidase activity in vehicle or PD123319-treated rats with or without colitis induction. Data are mean ± SEM. $n=5$ for each group. *$P$ <0.05 versus Sham group; $^{\S}P$ <0.05 versus DNBS group

## NOS expression

As NO is an important component of immune and inflammatory responses, we next investigated in colonic tissue samples whether the treatment with PD123319 has any effect on the expression of the different isoforms of NOS, by investigating expression of eNOS, nNOs, and iNOS mRNA by qRT-PCR. DNBS administration induced an increase in the mRNA expression for iNOS and eNOS. The treatment with PD123319 downregulated the increase in mRNA expression of iNOS, whereas unaffected the increase in eNOS expression (Fig. 4B).

## Nitrite and ROS production

As shown in Fig. 4, the level of nitrites and ROS at 6 days after induction of colitis, was significantly increased in DNBS group compared with the sham group ($P<0.05$). Treatment with different doses of PD123319 downregulated the generation of nitrites and ROS in colitic rats (Fig. 4C, D).

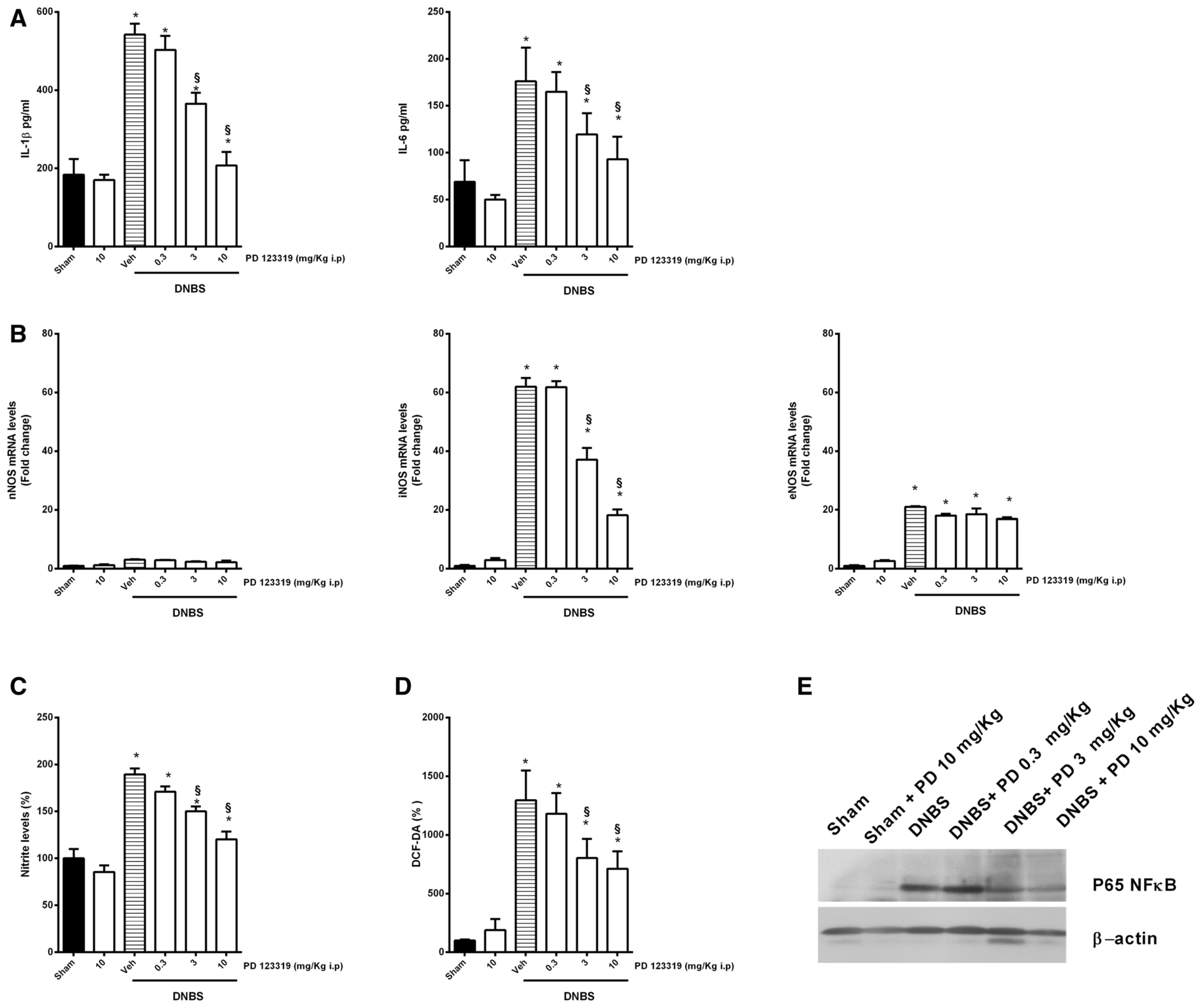


**Fig. 4** Effects of PD123319 on the proinflammatory cytokines, NOS expression, nitrite, ROS levels, and NF-κB activation. **A** IL-1β, IL-6 levels were assayed by ELISA; **B** nNOs, iNOS, and eNOS mRNA expression were evaluated by qRT-PCR; **C** nitrite levels were measured using Griess reagents (for nitrite); **D** ROS levels were detected by DCF-DA fluorescence measurement. Data are mean ± SEM. $n = 5$ for each group. *$P$ <0.05 versus Sham group; $^{\S}P$ <0.05 versus DNBS group. **E** Immunoblotting assay of the expression of NF-κB p65 fraction in vehicle or PD123319-treated rats with or without colitis; β-actin was used as marker

## Expression of p65-NF-κB

As already reported (Zizzo et al. 2019), rats treated with DNBS showed a significant increase in the colonic expression of activated NF-κB p65 subunit. PD123319 significantly decreased the expression of NF-κB p65, indicating that downregulation of this nuclear transcription factor may be implicated in PD123319 beneficial protective effects against colitis (Fig. 4E).

## Colonic smooth muscle motility

As previously shown (Zizzo et al. 2017), in in vitro studies, PD123319 treatment was able to improve colonic contractility in inflamed tissues, significantly increasing the amplitude of the spontaneous colonic contractions, as well as the pharmacologically evoked contractile and relaxant responses. In the present study, in vivo treatment with PD123319 matched the in vitro effects, being PD123319 able to induce a dose-dependent increase in the amplitude of the colonic

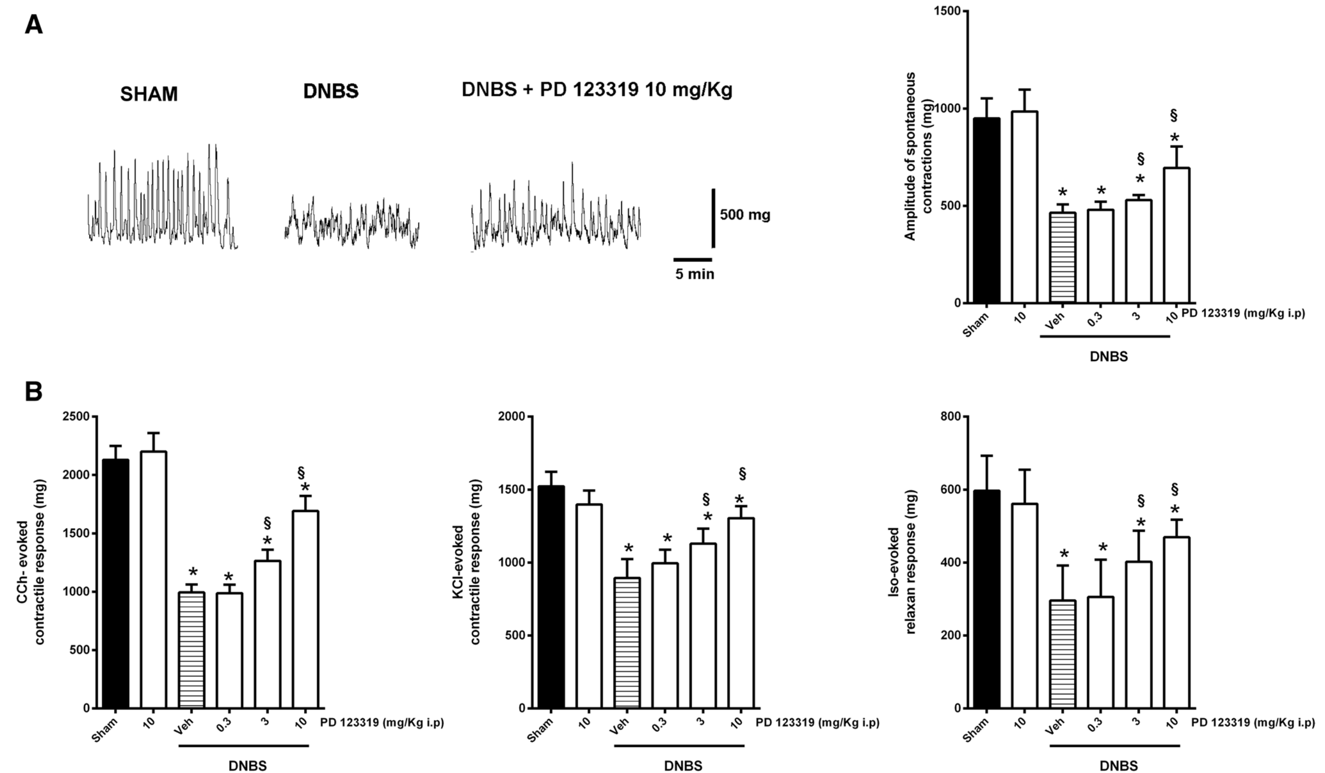


**Fig. 5** Effects of PD123319 on the in vitro contractility of the distal colon. Histograms showing **A** the amplitude of spontaneous mechanical activity, **B** the amplitude of the contractile response evoked by CCh (10 µM) and KCl (60 mM) and the amplitude of the relaxant response evoked by Iso (1 µM), in the longitudinal colonic muscle strips of rat colon from vehicle or PD123319-treated rats with or without colitis induction. Data are mean ± SEM and are expressed in absolute value ($n = 5$ for each group). The graphed value for the sham bar is the mean of the control data obtained before each treatment. *$P$ <0.05 versus Sham group; $^{§}P$ <0.05 versus DNBS group

spontaneous contractions compared to DNBS preparations (Fig. 5A) as well the contractile and relaxant responses to CCh (10 µM) or KCl (60 mM) and Iso (1 µM) (Fig. 5B).

## Discussion

The current study highlights the alleviating effects of PD123319, an Ang II AT2 receptor antagonist, treatment in DNBS-induced colitis rats, an experimental model of human IBD. These beneficial effects were associated with modulation of colonic NF-κB, inflammatory cytokines, and ROS. PD123319 improved also the colonic contractility in inflamed tissues. Therefore, this study supports our previous hypothesis that the AT2 receptor activation might represent one of the multiple mechanisms involved in the inflammation process in the intestinal tract, leading also to the impairment of the muscle contractility.

Increasing evidence suggests that the renin–angiotensin system may play a role in the inflammation processes. It is well recognized that Ang II acts as a powerful proinflammatory mediator through the stimulation of the AT1 receptors, while the AT2 receptors are believed to form part of the so-called "protective arm of RAS" (Horiuchi 2016). However, the exact functions and molecular mechanisms of the AT2 receptors remain mysterious. The AT2 receptor signaling activates phosphatases and production of ceramides, arachinodate, and nitric oxide/cGMP, and regulates apoptosis/inhibition of cell growth, vasodilatation (Matsubara 1998, de Gasparo et al. 2000). This receptor has been reported to be involved in tissue remodeling and also to promote inflammation in different experimental conditions. Adverse effect of the AT2 receptor signaling has been also demonstrated in renal and vascular tissue (Akishita et al. 2000, Ruiz-Ortega et al. 2001, 2003, 2006).

RAS has been implicated in the pathogenesis of IBD, being Ang II involved in several key steps of the inflammatory cascade that ultimately provokes intestinal injury and ulceration (Hume and Radford-Smith 2008; Mizushima et al. 2010; Arab et al. 2014). The AT1 are the main receptors expressed in the adult organism, while the AT2 receptors are only sparsely expressed in healthy adult tissue. However, in case of tissue damage, AT2 receptors are strongly re-expressed and/or upregulated. In particular, the AT2 receptor density increases in tissues under pathologic conditions in which inflammation and tissue remodeling

occur (Namsolleck et al. 2014; Karnik et al. 2015; Carey 2017).

In this study, we investigated the effects of PD123319, an Ang II AT2 receptor antagonist, on the progression of intestinal inflammation in the DNBS-induced colitis rat model. The rationale for this study had come from our previous investigation in vitro (Zizzo et al. 2017) showing that in DNBS rats there was a recruitment of the AT2 receptors which could contribute to the general depression of intestinal contractility. The AT2 receptor blockade with PD123319 improved per se colonic contractility, suggesting that during inflammation there may be a tonic activation of the AT2 receptors. It has been reported that intestinal smooth muscle function disturbances represent an important consequence of inflammation and they would contribute to the pathophysiology of cramps and abdominal pain, the most common clinical symptoms of IBD. During inflammation, the gut resident and the recruited immune cells play a vital role in the structural remodeling and in the change of smooth muscle morphology (e.g., hypertrophy) and function that can impact on contractility.

In the present study, we have shown that in vivo PD123319 treatment improves also the colonic contractility, increasing the amplitude of both spontaneous and chemically evoked contractile activity, supporting our hypothesis that inflammation in our model would recruit the AT2 receptors that contribute to the impairment of smooth muscle function.

PD123319 has been recognized as an useful tool for studying the AT2 receptor-mediated responses in our previous studies as well as in other studies (Mastropaolo et al. 2013, 2015; Zizzo et al. 2017; Karnik et al. 2015; Munoz et al. 2017).

PD123319 has a high affinity for the AT2 receptor (Ki ≈ 12 nM) and is approximately 10,000-fold more selective for the AT2 than the AT1 receptors (Karnik et al. 2015). Nakajima et al. (1995) reported that in rats 20 mg/kg/day i.p. of PD123319 resulted in a plasma drug level of about 248 nM in the circulation. Therefore, even at the highest dose used in the present work (10 mg/kg/d i.p), PD123319 should result in an effective AT2 receptor blockade without affecting the AT1 receptor.

Our results show that PD123319 treatment was able to mitigate, in a concentration-dependent manner, the DNBS-induced loss of body weight and the occurrence of diarrhea, to reduce the increase in colon weight/length induced by inflammation and the macroscopic score. We also observed that PD123319 had protected the histological integrity of the colon tissue, alleviating the severity of colonic injury. Moreover, in the inflamed areas, the inflammatory cell infiltration was noticeably reduced by the AT2 receptor antagonist treatment as demonstrated by the histology and by a significant reduction in the MPO levels, widely used indicator of neutrophil influx into inflamed gastrointestinal tissue (Pulli et al. 2013).

In relation to the inflammatory process, Ang II has been demonstrated to regulate several proinflammatory genes such as cytokines, chemokines, and adhesion molecules. As we have already reported (Zizzo et al. 2017,2019), and shown in the present study, in DNBS-induced experimental colitis, there is high tissue level of proinflammatory mediators such as cytokines (i.e., IL-1β and IL-6) which in turn would lead to chronic inflammation with ulceration and lesions of the colonic mucosa and leukocyte infiltration (Kandhare et al. 2012).

PD123319 counteracted the increase in proinflammatory cytokines when compared with the DNBS group, allowing us to speculate that the decrease in the levels of proinflammatory cytokine mRNAs, likely reducing the expression of the proteins, is one of the mechanisms activated by PD123319 to mitigate DNBS colitis. Consistent with our observations, Gandhi et al. (2017) demonstrated that in pancreatic ductal adenocarcinoma, the silencing of the AT2 receptors correlated with lower mRNA expressions of key proinflammatory genes (IL-1β and IL-6).

Numerous studies have shown that NF-κB, redox-sensitive transcription factor, is a key regulator of inflammation, innate immunity, and tissue integrity and that NF-κB phosphorylation and its nuclear translocation correlate with the severity of intestinal inflammation (Salminen et al. 2008; Lawrence 2009; Buhrmann et al. 2011).

NF-κB plays a critical role in the regulation of gene expression of inflammation-related molecules including adhesion molecules, chemokines, and cytokines (Liu et al. 2017). It is generally reported that AT1 receptor downstream signaling leads to the activation of NF-κB (Ruiz-Ortega et al. 2001). However, the existence of the AT2/NF-κB pathway has also been demonstrated. Ang II via AT1 and AT2 receptors and NF-κB pathway is involved in the inflammation processes at kidney level (Ruiz-Ortega et al. 2001; Klahr and Morrissey 2000; Esteban et al. 2003, 2004). Our data indicate that in PD123319-treated DNBS animals, there is also a decrease in the expression of NF-κB p65 and this may suggest that the potential role of the AT2 receptors in the intestinal inflammatory process in DNBS rats may be mediated by NF-κB activation that modulates upregulation of multiple proinflammatory genes.

Various studies based on animal models, as well as in humans, indicated that NO may be involved in gastrointestinal inflammation and that it may have a pathogenetic role in IBD (Ikeda et al. 1997; Kimura et al. 1998). NOS is responsible for NO synthesis from L-arginine in mammalian cells. There are three isoforms of NOS: nNOS, iNOS, and eNOS. Changes in NOS enzymes in inflammatory conditions have been investigated in many studies. In our model, inflammation induces an increase in the level of eNOS and

iNOS isoforms. An increase in eNOS expression has been reported in other models of experimental colitis where a protective role has been highlighted assuming the production of NO, eNOS derived, would improve blood flow, would reduce leucocyte and platelet recruitment and oxidative stress, leading to the reduction of inflammation (Sasaki et al. 2003; Vallance et al. 2004). Therefore, our data support this hypothesis. On contrary, the upregulation of iNOS has been shown to correlate with prolonged colonic inflammation, especially within epithelial cells around inflammatory loci (Kolios et al. 1998) NO produced by the iNOS may theoretically exacerbate the clinicopathological features of IBD by direct cytotoxicity, activation of neutrophils, vasodilatation, reduced smooth muscle tone, increased production of nitrosamines (to cause cancer), and interaction with superoxide to form the highly toxic peroxynitrite radical (Ribbons et al. 1995; Middleton et al. 1993; Ohshima and Bartsch 1994). Peroxynitrite (Rachmilewitz et al. 1993; Conner et al. 1996) has been suggested as the potential mediator of colitis induction. In our model, AT2 receptor antagonist treatment markedly reduced iNOS expression and nitrite levels suggesting that AT2 receptor recruitment in inflammation, increasing iNOS activity, would enhance the proinflammatory colitis response in rats.

Colonic inflammatory status is closely related to the formation of reactive compounds from activated neutrophils and phagocytes, and generating oxidative stress (Bhattacharyya et al. 2014; Perez et al. 2017). Administration of DNBS has resulted in disturbance of redox state in the colonic tissue. PD123319 treatment induced a concentration-dependent decrease in ROS, suggesting that the AT2 receptor activation could be linked to elevated oxidative stress and inflammation.

Silencing of the AT2 receptors in pancreatic ductal adenocarcinoma correlates also with lower mRNA levels of ROS inductive genes (Gandhi et al. 2017).

An open question remains the nature of cells playing a role in the beneficial effects of PD123319. The AT2 receptors have been reported to be present on immune cells, as memory T cells, although there have been limited studies on the colonic epithelial and muscular cells (Hirasawa et al. 2002; Altara et al. 2016). Further experiments are needed to investigate this issue.

In conclusion, our results suggest that AT2 receptors, likely via the NF-κB pathway, could play a key role in the inflammatory events in the course of DNBS-induced experimental colitis. The AT2 receptor antagonism by PD123319 leads to a reduction in the inflammatory response and in the oxidative stress in colon of DNBS rats. Moreover, when the inflammatory process is mitigated by the AT2 receptor antagonist treatment, the smooth muscle is able to recover its functionality. Accordingly, further studies are required to elucidate the exact mechanism(s) through which the AT2 receptor recruitment is involved in the progression of intestinal inflammatory disease.

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## Compliance with ethical standards

**Conflict of interest** The authors declare no conflict of interest.

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