JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2008, 59, 4, 673–689
www.jpp.krakow.pl

M.A. ENGEL[1], C.A. KELLERMANN[1], T. RAU[2], G. BURNAT[1],
E.G. HAHN[1], P.C. KONTUREK[1]

ULCERATIVE COLITIS IN AKR MICE IS ATTENUATED BY INTRAPERITONEALLY ADMINISTERED ANANDAMIDE

[1]First Department of Medicine, University of Erlangen-Nuremberg, Germany
[2]Institute of Pathology, University of Erlangen-Nuremberg, Germany

Anti-inflammatory and anti-nociceptive properties of endocannabinoids and synthetic cannabinoid compounds were described previously. We studied effects of the endogenous cannabinoid anandamide (N-arachidonylethanolamine) in experimental colitis induced by TNBS (2,4,6-trinitrobenzene sulfonic acid) in AKR mice. A scoring system was used to describe clinical and macroscopic changes. Intraperitoneally administered anandamide significantly reduced experimental colitis, quantified by macroscopical and histological scoring systems as well as pro-inflammatory cytokine mRNA expression. We conclude that systemically administered anandamide attenuates TNBS colitis in mice, and that systemically active cannabinoid compounds might have therapeutic potential for the treatment of IBD.

Key words: *cannabinoids, anandamide, colitis, TNBS, IBD*

INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic inflammatory disorders of the gastrointestinal tract characterized by infiltration of neutrophils as well as mast cells and eosinophilic granulocytes into the colonic wall, accompanied by epithelial cell necrosis and ulceration (1, 2). The distribution pattern of Crohn disease (CD) and ulcerative colitis (UC) are profoundly different. Whereas Crohn disease may produce segmental inflammation throughout the gut “from the lips to the anus”, ulcerative colitis starts in the rectum and may

continuously affect the whole colon. The etiology of IBD is still not known precisely. It is commonly accepted, that dysregulation of inflammatory mechanisms together with genetic susceptibility and environmental factors play a key pathogenetic role in IBD (3, 4). Today's therapeutic approaches include the use of immunosuppressive and anti-inflammatory drugs such as glucocorticosteroids, salicylates, azathioprine, cyclophosphamide or mycophenolate mofetil and modern immunomodulators (biologicals) like TNF alpha blockers. However, benefit for patients is still unsatisfactory, particularly in the longterm follow up (5). Maintenance of remission has a high rate of adverse effects (6) with potentially fatal complications; consequently there is a compelling need for new pharmacological approaches in the treatment of IBD.

Several experimental animal models of IBD have been developed. Among these, the intrarectal administration of 2,4,6- trinitrobenzene sulfonic acid (TNBS) has been extensively used to study the mechanisms of colonic inflammation and to test anti-inflammatory drugs (7). Similarly, as observed in inflammatory bowel diseases in humans, this experimental model involves the immune and neuroendocrine systems and leads to long-lasting ulcerative damages of the colonic mucosa (8). Colitis is induced by transrectal administration of the covalently reactive reagent TNBS, which is believed to induce a T-cell-mediated response against hapten-modified autologous proteins/luminal antigens (9).

The endogenous cannabinoid system plays a role in the control of various functions in the GI tract, including gastroprotection, intestinal motility, and secretion (10).

Δ^9-THC and a variety of natural and synthetic cannabinoids have been shown to possess anti-nociceptive and anti-inflammatory activity (10-13). Cannabis preparations have been used in folk medicine for the treatment of a wide variety of disorders, including those affecting the gastrointestinal tract (14) and there are anecdotal reports suggesting that marijuana may be effective in alleviating symptoms of Crohn disease (15). Anandamide (N-arachidonylethanolamine) and 2-AG (2-arachidonylglycerol) are the most important endocannabinoids. The endocannabinoid system consists of endocannabinoids, cannabinoid receptors, carrier proteins in the cell membrane and microsomal enzymes of degradation. At least two receptor subtypes (CB1, CB2) mediate the endocannabinoid effects and belong to the family of G-protein coupled 7-transmembrane receptors. Those receptor subtypes are furthermore activated by plant derived cannabinoid substances such as THC (tetrahydrocannabinol) of which the Δ^9-enantiomere is the main psychoactive component of the plant cannabis sativa (16).

In the present study we have studied the modulatory effects of intraperitoneally administered anandamide in the model of TNBS colitis.

MATERIALS AND METHODS

Induction of colitis and dosing regimens

AKR mice were housed under standard conditions and supplied with drinking water and food ad libitum. All animal procedures complied with the guidelines for care and use of laboratory animals of the government of the state of Bavaria, Germany and the study was approved by the local Ethics Committee of University of Erlangen-Nueremberg, Erlangen, Germany. After 24 hours of starving, colitis was induced in AKR mice (Charles River Wiga GmbH, Sulzfeld, Germany) by giving a single enema with TNBS (2,4,6-trinitrobenzene sulfonic acid) 5 mg (Sigma-Aldrich laboratories, Seelze, Germany) *via* a polyethylene catheter (outer diameter 2 mm) 4 cm from the anus. In pilot experiments, this dose of TNBS was found to induce reproducible colitis in AKR mice (data not shown). Animals were lightly anesthetized with ketamine (40 mg/kg) immediately before the procedure. TNBS was dissolved in 50% ethanol solution and the total volume of the enema was 150µl. Ethanol is required to break the mucosal barrier, whereas TNBS is believed to haptenize colonic autologous or microbiotic proteins rendering them immunogenic to the host immune system (9). Mice were maintained in a head-down position for 5 minutes to prevent solution leakage. Treatment group (n=6) received once daily intraperitoneal application of anandamide 5mg/kg (N-arachidonylethanolamine, Tocris Bioscience, Missouri, USA). Injection was performed 30 minutes before and 24-, respectively 48 hours after TNBS infusion. TNBS control group received TNBS enema without anandamide treatment (n=6), vehicle control group received only 50% ethanol solution 150 µl (n=3). For 3 days rats were monitored for colitis and then sacrificed, their colons excised and collected for further analysis. This time point was chosen because maximal acute TNBS-induced inflammation has been reported in mice after 3 days (17). At 1, 3 and 6 cm colonic segments were dissected (slice thickness 1-3 mm) and were placed in 10% neutral buffered formalin for subsequent histological analysis.

Scoring Systems

Different scoring systems were used to describe clinical and macroscopical changes before tissue samples were collected. All scoring systems were used previously with slight modifications and are accepted scoring systems in colitis models (8, 18). Among the macroscopical parameters analyzed were length of the colon starting from 0.5 cm above the anus to the top of the caecum, colon weight and the consistency of any stools found within the colon. These parameters summed in the Macroscopical Score (MS) with a maximum of 11 points (*Table 1*). A semiquantitative score was used to evaluate severity of histological changes in the inflamed colon (Histopathological Colitis Score, HCS). The HCS featured the parameters inflammation extent, crypt architecture, hyperemia/edema, and infiltration with inflammatory cells, with a maximum of 12 points (*Table 2*). The scoring system used to describe the changes for each of these parameters is detailed in *Table 1*. The two scores MS and HCS were added together to provide a Major Colitis Score (MCS) with a maximum of 23 points (*Table 3*). By modifying known scoring systems we established the Major Colitis Score (MCS), which might be a useful tool for further studies in the field of experimental colitis.

Histology evaluations

Cross-sectioned segments from the colon of each animal were removed, rinsed in saline and then fixed in 10% neutral-buffered formalin. They were embedded in paraffin, sectioned and stained with haematoxylin/eosin. The sections were examined by light microscopy (Olympus Model BX-50 microscope, Olympus Instruments, Melville, NY, USA) and were scored by an investigator, blinded

Table 1. Macroscopical Score (MS)

Stool score
0= normal (well formed faecal pellets)
1= loosely shaped, moist pellets
2= amorphous, moist, sticky pellets
3= diarrhea
+ 1 occult blood in stool

Colon weight score (weight gain)
0= < 10%
1= 10 – 50%
2= >50 – 100%
3= >100 – 150%
4= > 150%

Colon length score (shortening)
0= < 5%
1= 5 – 14%
2= 15 – 24%
3= 25 – 35%
4= > 35%

The macroscopical characterization of the severity of TNBS colitis is measured through these criteria with a total maximum of 12 points.

Table 2. Histopathological Colitis Score (HCS)

Feature	Description	Score
Inflammation extent	none	0
	mucosa	1
	mucosa	2
Damage in crypt architecture	none	0
	regeneration	1
	destruction	2
Hyperemia / Edema	without	0
	mild	1
	moderate	2
	severe	3
Infiltration with inflammatory cells	without	0
	mild	1
	moderate	2
	severe	3

(ulceration and/or crypt abscess respectively +1)
The Histopathological Colitis Score is derived from the features listed above with a maximum of 11 points. These features describe inflammation criteria to determine colitis severity.

Table 3. Major Colitis Score MCS (=MS+HCS)
The Major Colitis Score (MCS) is derived from the macroscopical and microscopical (histological) features listed above with a total maximum of 23 points. Severity is graded according to the following Table:

Major colitis score MCS
0= no signs of colitis
≤ 7 (mild colitis)
≤ 14 (moderate colitis)
>14 (severe colitis)

Tables 1-3. To quantify inflammatory changes we used different score systems that have been described before with slight modifications. The Macroscopical Score (MS) is a summation of different parameters: stool score, colon weight score and colon length score (*Table 1*). The Histopathological Colitis Score (HCS) includes inflammation extent, damage in crypt architecture, hyperemia/edema and the extent of infiltration with inflammatory cells (*Table 2*). The MS and HCS together add up to the MCS (Major Colitis Score) which is graduated in a mild, moderate and severe colitis (*Table 3*).

to the experimental groups tested. Scoring method was described before (*Table 2*). Photomicrographs were taken with a Leitz Laborlux S microscope (Wild Leitz, Wetzlar, Germany) using a 4x objective and a SPOT RT digital camera (Diagnostic Instruments, Sterling Heights, MI, USA).

Reverse transcription polymerase chain reaction (RT-PCR)

The expression of TNF-α and IL-1β was analyzed by means of RT-PCR. Mucosal specimens were scraped off on ice using slide glass and immediately snap frozen in liquid nitrogen and stored at -80°C until analysis. Total RNA was extracted from mucosal samples using a guanidium isothiocyanate phenol chloroform single step extraction kit from Stratagene (Heidelberg, Germany) based on the method described by Chomczynski and Sacchi (19). Following precipitation, RNA was resuspended in RNAse-free TE buffer and its concentration was estimated by absorbance at 260 nm wavelength. Furthermore, the quality of each RNA sample was determined by running the agarose-formaldehyde electrophoresis. RNA samples were stored at -80°C until analysis.

Single stranded cDNA was generated from 5 µg of total cellular RNA using Moloney murine leukaemia virus reverse transcriptase (MMLV-RT) (Stratagene, Heidelberg, Germany) and oligo-(dT)-primers (Stratagene, Heidelberg, Germany). Briefly, 5 µg of total RNA was uncoiled by heating (65°C for 5 min) and then reverse transcribed into complementary DNA (cDNA) in a 50 µl reaction mixture that contained 50 U MMLV-RT, 0.3 µg oligo-(dT)-primer, 1 µl RNAse Block Ribonuclease Inhibitor (40 U/µl), 2 µl of a 100 mM/l mixture of deoxyadenosine triphosphate (dATP), deoxythymydine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP) and deoxycytidine triphosphate (dCTP), 10 mM/l Tris-HCl (pH=8.3), 50 mM KCl, 5 mM $MgCl_2$. The resultant cDNA (2 µl) was amplified in a 50 µl reaction volume containing 2 U Taq polymerase, dNTP (200 µM each) (Pharmacia, Germany), 1.5 mM $MgCl_2$, 5 µl 10x polymerase chain reaction buffer (50 mM KCl, 10 mM Tris-HCL, pH=8.3) and specific primers for β-actin, TNF-α and IL-1β used at final concentration of 1mM (all reagents from Takara, Shiga, Japan). The mixture was overlaid with 25 µl of mineral oil to prevent evaporation. The polymerase chain reaction mixture was amplified in a DNA thermal cycler (Perkin-Elmer-Cetus, Norwalk, CT) and the incubation and thermal cycling conditions were as followed: denaturation at 94°C for 1 min, annealing at 60°C for

45 sec and extension 72°C for 2 min. The number of cycles was 30 for β-actin, TNF-α and 29 for IL-1β. The nucleotide sequences of the primers were as follows: β-actin sense: AGACCTCTATGCCAACACAGTG, antisense: TCCTGCTTGCTGATCCACATC, TNF-α sense: AGCAAGCAGCCAACCAGG, antisense: GCCACAAGCAGGAATGAGAAG, IL-1β sense: CTCGCAGCAGCACATCAAC, antisense TGTTCATCTCGGAGCCTGTAG. The primer sequences for β-actin, TNF-α and IL-1β were based on the sequences of the published cDNAs. Polymerase chain reactions products were detected by electrophoresis on a 1.5% agarose gel containing ethidium bromide. Location of predicted products was confirmed by using a 100-bp ladder (Takara, Shiga, Japan) as a standard size marker. The gel was then photographed under UV transillumination. The intensity of PCR products was measured using video image analysis system (Kodak Digital Science) as described earlier (20). The signal for investigated mRNA was standardized against that of the β-actin mRNA from each sample and the results were expressed as analyzed mRNA/β-actin mRNA ratio as described earlier (21).

Statistical analyses

Results are presented as means ± standard error of the mean (SEM). The number of animals (n) that is quoted throughout the manuscript refers to the number of animals. Accordingly for inter-individual statistical comparisons the Mann-Whitney U test was used (Statistica®, StatSoft, USA). Differences were considered statistically significant at $p<0.05$ and this is indicated with an asterisk in the figures.

RESULTS

Intraperitoneal application of anandamide attenuates macroscopical severity of TNBS-induced colitis

To study the involvement of the endocannabinoid anandamide in colonic inflammation, we used the TNBS model of colitis. Intrarectal administration of 150 µl of 50% ethanol did not induce detectable inflammation in the vehicle group after 3 days. The colons of these AKR mice were macroscopically and histologically normal (data not shown, n=6, $p<0.05$).

After intrarectal administration of TNBS (5 mg/150 µl per mouse), macroscopical and histopathological evaluation of AKR mice colons revealed stronger inflammation as compared to the anandamide treatment group.

Macroscopical score (MS) consisted of the three parameters stool score, change of colon weight and colon length, which were altered significantly. TNBS-control group showed higher stool score (3.17±0.31, n=6, $p<0.05$) than AKR mice treated with anandamide once daily (1.33±0.33, n=6, $p<0.05$) (*Fig. 1A*). Anandamide treatment decreased colon weight gain and increased colon length respectively in comparison to vehicle group. TNBS treated AKR mice demonstrated higher colon weight gain (colon weight score 3.5±0.22, n=6, $p<0.05$) than vehicle group, whereas anandamide treatment reduced colon weight gain (colon weight score 0.83±0.4, n=6, $p<0.05$) (*Fig. 1B*). Furthermore anandamide treatment reduced colon length reduction (colon length score 2.00±0.26, n=6, $p<0.05$) compared to TNBS-control group (colon length score

0.50±0.22, n=6, p<0.05) (*Fig. 1C*). Macroscopical Score (MS) in the anandamide group was lower than in the TNBS group (2.67±0.67 vs. 8.67±0.56, n=6, p<0.05) (*Fig. 2*).

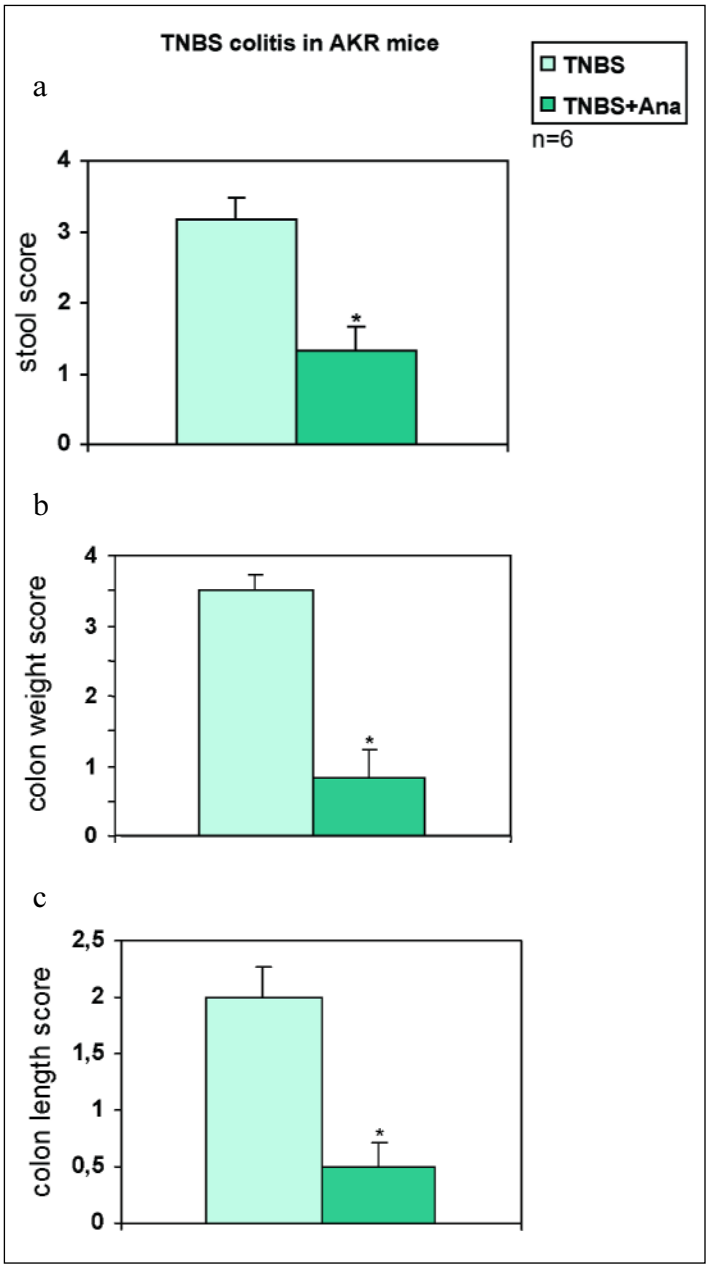

Fig. 1A-C Effect of anandamide on parameters of TNBS colitis: stool score (*1a*), colon weight score (*1b*) and colon length score (*1c*). Data are means ± SEM. Differences are statistically significant at p<0.05 indicated with *. (Mann-Whitney U test)

Intraperitoneal application of anandamide attenuates histological severity of TNBS-induced colitis

Histological analysis confirmed the macroscopic observations showing no inflammation in vehicle control group (data not shown). Histopathological Colitis Score (HCS) consisted of five criteria as described in methods. Anandamide treatment reduced inflammation extent, loss of crypt architecture, hyperemia, edema and the extent of infiltration with inflammatory cells (*Table 2*), especially neutrophil granulocytes and lymphocytes. Quantitatively the HCS was reduced in the anandamide group compared to TNBS control group (3.67±0.56 *vs*. 8.83±0.75, n=6, $p<0.05$) (*Fig. 3*). In particular, a notable disruption of the epithelial structure with necrosis and infiltration of neutrophils and lymphocytes, with acute inflammation extending into the submucosa and even muscle layer, was detected in animals with TNBS colitis without anandamide treatment. Those animals revealed a more severe transmural colitis, severe loss of epithelium, thickening of the bowel wall, inflammatory infiltrates, and stronger increase in lymphoid-follicle size (*Fig. 5A-D*).

In summary, the Major Colitis Score (MCS) was reduced in the anandamide group compared to TNBS control group (6.33±0.92 vs. 16.50±0.99, n=6, $p<0.05$) (*Fig. 4*). According to the scoring system, TNBS treated animals showed severe colitis, whereas in the anandamide group colitis was significantly attenuated.

RT-PCR analysis

In control mice, mRNA for the pro-inflammatory cytokines IL-1β and TNF-α was detected as a weak signal. The exposure to TNBS and induction of colitis was associated with a significant increase in the mRNA expression for IL-1β and TNF-α in colonic tissue. Treatment with anandamide caused a significant decrease in the mRNA expression for IL-1β and TNF-α compared to the TNBS group (*Fig. 6*).

DISCUSSION

The present study demonstrates that systemically applied anandamide attenuates macroscopic and histological features of colitis and suppresses the expression of pro-inflammatory cytokines in the colonic mucosa in TNBS colitis. Besides classical cellular mechanisms of acute and chronic inflammatory responses the endocannabinoid system is implicated in human IBD and experimental models of colitis. Through acting *via* CB1 receptors, that are mainly expressed on neurons, anandamide might have an inhibitory effect on the neurogenic component of colitis. Functional CB1 receptors have been identified on enteric neurons (16, 21-24, 44). Furthermore, the gastrointestinal tract produces at least two endocannabinoids, namely anandamide and 2-AG (10, 16, 25, 26). The CB1 receptor belongs to the

class of G-protein coupled-receptors (27) and its activation reduces neurotransmitter release by inhibition of the fusion probability of synaptic vesicles with the synaptic membrane. The inhibitory G-protein α subunit inhibits the

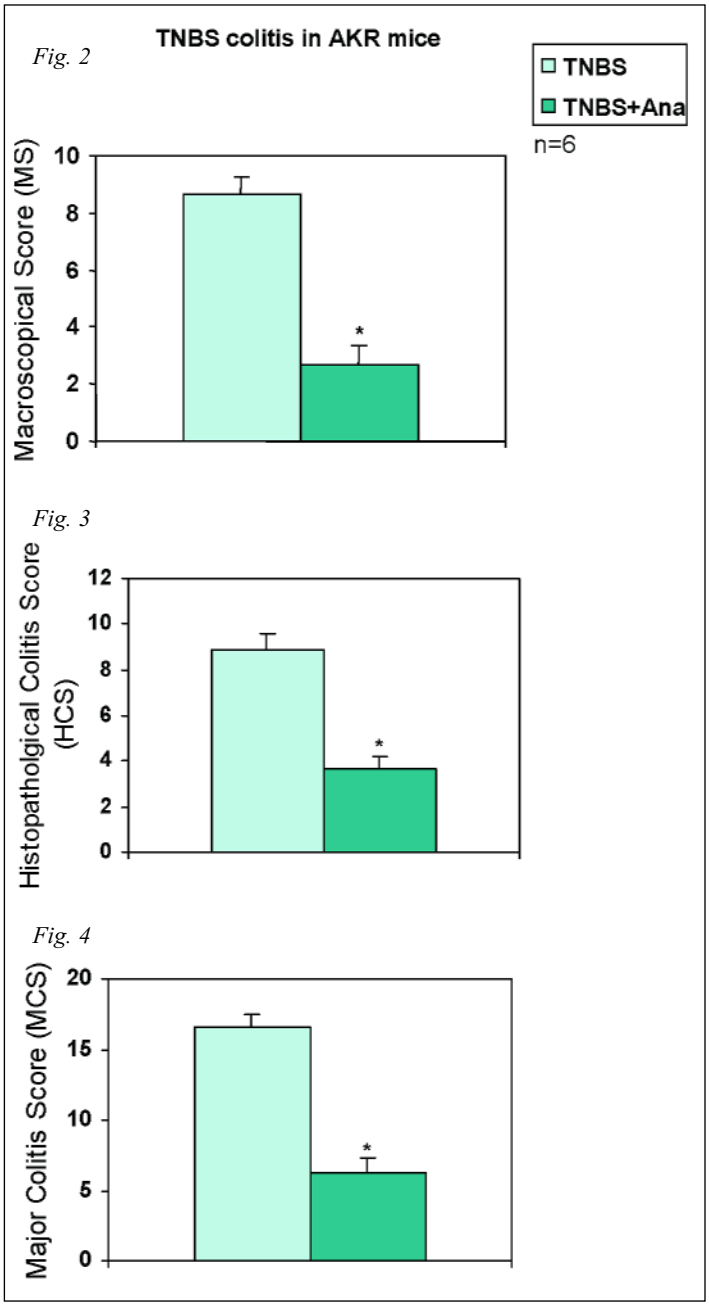

Fig. 2-4. Effect of anandamide on the macroscopical score (MS) (*Fig. 2*), histopathological colitis score (HCS) (*Fig. 3*) and the major colitis score (MCS) (*Fig. 4*).

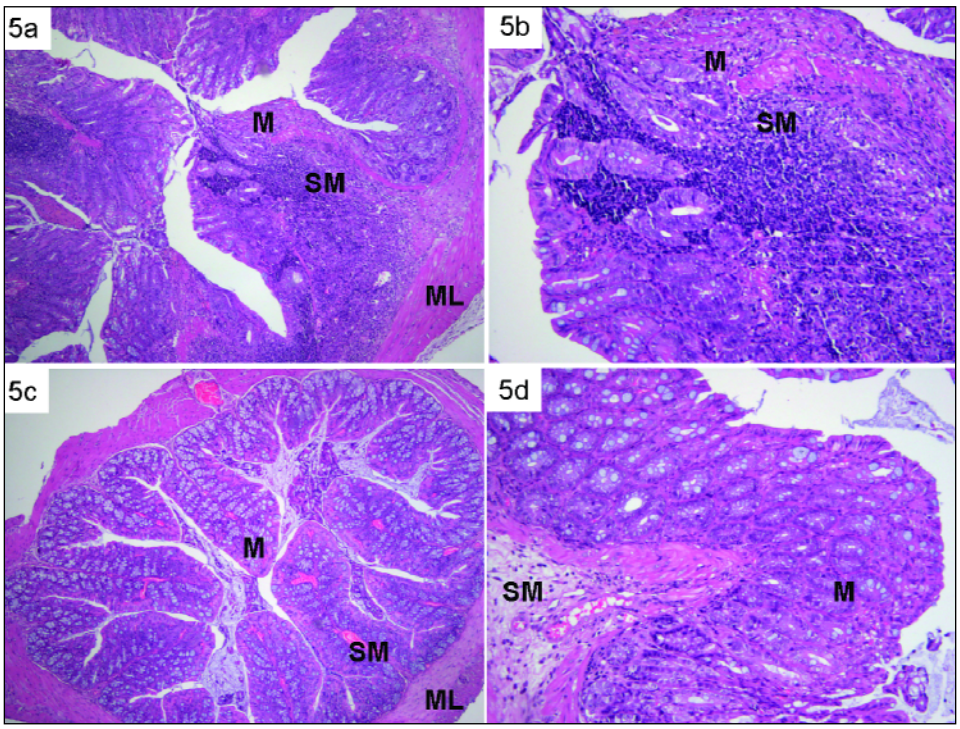

Fig. 5. Upper panel (*5A, B*): TNBS colitis showed disruption of the epithelial structure, necrosis and infiltration of neutrophils and lymphocytes with acute inflammation extending into the submucosa and thickening of the bowel wall. Inflammation severity was increased in the middle and distal parts of the mouse colon.
Lower panel (*5C, D*): Effect of anandamide treatment on TNBS colitis: significant attenuation of colonic inflammation without evidence for necrosis and reduced infiltration by inflammatory cells. M, mucosa; SM, submucosa; ML, muscle layer.

adenylyl cyclase reducing cAMP production and by that proteinkinase A (PKA) activity. Consequently, numerous phosphorylation processes are inhibited, that result in a reduction of the conductance of L-, PQ- and T-type calcium channels (28-31). Additionally, the G-protein's α- and βγ-subunit directly exert inhibitory effects on these calcium channels. The reduction of intracellular calcium levels reduces the degree of calcium-calmodulin-complex formation and consequently attenuates the activity of calcium-calmodulin-dependent kinase II (CamK II), which finally reduces phosphorylation of numerous synaptic proteins (*e.g.* synaptophysin) and inhibits vesicular exocytosis (32).

Massa *et al*. (2004) showed that the endogenous cannabinoid system is involved in the modulation of the acute phase of experimental colitis induced by DNBS (2,4-dinitrobenzen sulfonic acid) (a similar model of transrectal colitis induction) (33). This finding was supported by increased levels of transcripts

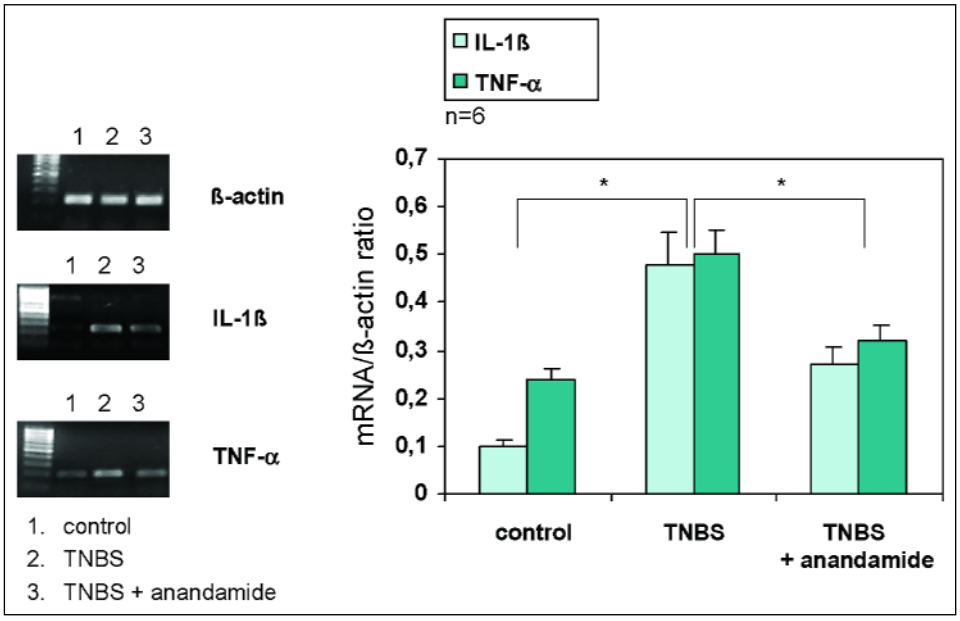

Fig. 6. mRNA expression for proinflammatory cytokines IL-1β and TNF-α in mouse colon of control mice, after TNBS colitis induction and after TNBS colitis treated with anandamide . *In vivo* rectal application of TNBS resulted in colonic inflammation with a strong increase in IL-1β and TNF-α mRNA expression. Systemical treatment with anandamide in animals with TNBS colitis showed attenuation of the experimental colitis with correlative decrease in IL-1β and TNF-α mRNA expression. Asterisks (*) indicate significance ($p<0.05$).

coding for CB1 receptors in wild-type mice after induction of inflammation. The number of CB1-expressing cells was found to be significantly increased after inflammation, without any simultaneous significant increase of the total number of neurons. Thus, it appeared that neurons that express undetectable or very low levels of CB1 receptor in basal conditions, started to express high levels of this receptor to enhance endocannabinoid signalling during inflammatory responses in the colon (33). Based on this observation it is possible that the exogenous application of the CB1 agonist anandamide in our experiment has anti-inflammatory properties in the activated status of the endocannabinoid system during inflammation.

A strong elevation of anandamide, but not 2-AG, levels was found in the colon of DNBS-treated mice, in the colon submucosa of TNBS-treated rats, and in biopsies of patients with ulcerative colitis (4). The anti-inflammatory effect of the activated endocannabinoid system is supported further by studies using VDM-11 (an anandamide reuptake inhibitor) that significantly elevated anandamide levels in the colon of DNBS-treated mice and concomitantly attenuated inflammation

(4). Consistent with these findings we could attenuate inflammation in mice with TNBS colitis by (exogenously) elevating anandamide levels systemically.

In the present study specific parameters for quantification of acute colitis such as colon shrinkage and colon weight gain were used. Colon weight gain and shrinkage commonly occur in TNBS colitis. The former is indicative of glandular hypersecretion, muscular hypertrophy and edematous swelling of the colonic wall, while colon shrinkage is commonly observed in chemically induced colitis and is indicative of neuromuscular-induced contraction of the colon smooth muscle (34, 35). Contractility of smooth muscle is essentially increased in the early stages and decreased in the later stages of inflammation (36, 45). Massa *et al*. (2004) showed increased spontaneous activity in intracellular recordings from circular smooth muscles in mouse colon of CB1 knockouts in comparison to wildtype mice after induction of DNBS colitis, emphasizing a unique role of CB1 receptors in tonic smooth muscle contraction in the gut (33). Our results indicate that anandamide reduced smooth muscle contraction in the inflammatory state of the colon compared to TNBS controls, which has its quantitative correlate in colon length shortening. In the pathologic state of chemically induced colitis in mice, exogenous application of anandamide could attenuate smooth muscle contraction, which might, in a clinical context, have its correlation in the attenuation of acute abdominal pain.

Accumulating data indicates other targets of cannabinoids than CB1 receptors. Pharmacological stimulation of cannabinoid receptors with the potent agonist HU210 induced a reduction of DNBS colitis (33). HU210 is able to stimulate both cannabinoid receptors CB1 and CB2 (27, 37). It was demonstrated, that anandamide acts not only through CB1 receptors, but also through other targets including CB2-receptors, that are mainly expressed on immune cells (38). By that, anandamide could exert immunomodulating effects that contributed to the attenuation of TNBS-colitis.

Another target of anandamide's inhibitory action is the transient receptor potential vanilloid 1 (TRPV1) (39). TRPV1 immunoreactivity has been shown to be present in intrinsic and extrinsic neurons innervating the GI tract (18). In the human colon, vanilloid receptor TRPV1 is overexpressed both in afferent nerve terminals and in epithelial cells during inflammation (7). The vanilloid receptor type 1 (TRPV1) is a nonselective cation channel with six transmembrane domains and is a member of the transient receptor potential (TRP) channel family. This receptor type is activated by capsaicin (the pungent ingredient of chilli pepper), temperature and protons (40). TRPV1 deficient mice showed increased susceptibility to DNBS induced colitis (8). Genetic deletion of both the cannabinoid receptor type 1 (CB1) and TRPV1 receptors led to decreased protection against DNBS-induced colitis (33). However, capsazepine as a TRPV1 antagonist attenuated disease severity in dextran sulphate sodium (DSS)-induced colitis in mice, which might be due to the inhibitory properties of capsazepine on calcium channels (18).

It has also been demonstrated that cholera toxin–induced accumulation of intestinal fluid in mice is modulated by activation of the endogenous cannabinoid system acting through CB1, but not through TRPV1 nor CB2 receptors (41). Moreover, CB1 receptors were also shown to modulate gastrointestinal motility during croton oil–induced inflammation in mice (24). The fact that high doses of exogenously locally applied anandamide may also induce intestinal inflammation (42) indicates that modulation of inflammation by cannabinoids is dose-dependent. One could speculate that the balanced endogenous synthesis and release of anandamide, which is different under physiological and pathological states and its controlled action at CB1, CB2 and TRPV1 receptors, might mediate protection against colon inflammation.

For the protective effects of anandamide on TNBS colitis vasodilatatory effects of anandamide might be responsible. Anandamide is metabolized by FAAH, resulting in arachidonic acid formation, which is a substrate of COX-2. By that it leads to the formation of a 'PGE2 like' product, which binds to the EP4 receptor in vascular smooth muscle cells and causes vasorelaxation (46). Herradon *et al*. (2007) furthermore concluded that anandamide may act on an endothelial non-CB1/non-CB2 cannabinoid receptor promoting NO synthesis with subsequent vasorelaxation (46). The role of NO in the pathophysiology of inflammatory bowel disease is currently under debate (47, 48). High concentrations of nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) is associated with ulcerative colitis (47) and the level of iNOS-derived NO correlates with disease activity in ulcerative colitis (49). Expression of iNOS is up-regulated in Crohn disease as well as in experimental colitis models (48). However, data on the role of the NO pathway in inflammation remains controversial since numerous animal studies have described an improvement of experimental colitis with iNOS inhibition (50, 51), but other reports showed ineffectiveness (52, 53) or detrimental effects of those inhibitors (54-55). Interestingly, only iNOS blockade showed protective effects in a model of DSS colitis, since the non-selective NOS inhibitor L-NAME had no effect on colonic inflammation and even aggravated inflammation given in the first three days of DSS treatment (56). Accordingly, DSS-induced colitis was significantly attenuated in mice genetically deficient in iNOS (57). In contrast, Aoi *et al*. showed that NO, produced by mainly iNOS and partly by eNOS, contributed to the healing of DSS-induced colonic lesions through upregulation of vascular endothelial growth factor (VEGF) (58).

Finally, in our set of experiments, we cannot exclude a central component of the anti-inflammatory effects of anandamide during TNBS-induced colitis since we used a systemic application approach. Previously, the cannabinoid receptor agonists were shown to suppress inflammation-induced gastrointestinal hypermotility when administered intracerebroventricularly (25, 26, 43). These results indicate that cannabinoids may also act in the central nervous system to exert their anti-inflammatory activities.

Taken together, the present study showed anti-inflammatory effects of anandamide on experimental colitis. The activation of the endocannabinoid system may be useful to control colonic inflammation in patients with IBD.

Acknowledgements: This study was supported by a grant from *Johannes und Frieda Marohn-Stiftung* of the University Erlangen-Nuremberg. Thank you to the technical assistance at the Institute of Pathology of the University of Erlangen for preparing the histological slices.

Abbreviations: 2-AG, 2-arachidonylglycerol; anandamide, N-arachidonylethanolamine; CamK, calcium-calmodulin-dependent kinase; cAMP, cyclic adenosine monophosphate; CB, cannabinoid; CD, Crohn Disease; COX, Cyclooxygenase; DNBS, 2,4-dinitrobenzene sulfonic acid; DSS, dextran sulphate sodium; HCS, Histopathological Score; IBD, Inflammatory Bowel Disease; MS, Macroscopical Score; MCS, Major Colitis Score; NO, nitric oxide; PGE, Prostglandine E; PKA, protein kinase A; TNBS, (2,4,6-) trinitrobenzene sulfonic acid; TRPV, transient receptor potential vanilloid; UC, Ulcerative Colitis; VEGF, vascular endothelial growth factor.

Conflicts of interest statement: None declared.

REFERENCES

1. Podolsky DK. Inflammatory bowel disease. *N Engl J Med* 1991; 325: 928-937.
2. Podolsky DK. Inflammatory bowel disease. *N Engl J Med* 1991; 325: 1008-1016.
3. Hollander D. Inflammatory bowel diseases and brain-gut axis. *J Physiol Pharmacol* 2003; 54 Suppl 4: 183-190.
4. D'Argenio G, Valenti M, Scaglione G, Cosenza V, Sorrentini I, Di Marzo V. Up-regulation of anandamide levels as an endogenous mechanism and a pharmacological strategy to limit colon inflammation. *FASEB J* 2006; 20: 568-570.
5. Hanauer SB. Medical therapy for ulcerative colitis 2004. *Gastroenterology* 2004; 126: 1582-1592.
6. Sands BE. Therapy of inflammatory bowel disease. *Gastroenterology* 2000; 118(2 Suppl 1): S68-S82.
7. Sartor RB. Animal models of intestinal inflammation. Relevance to inflammatory bowel disease. In Inflammatory bowel disease. New York, Elsevier Science. 1991; pp. 337-353.
8. Massa F, Sibaev A, Marsicano G, Blaudzun H, Storr M, Lutz B. Vanilloid receptor (TRPV1)-deficient mice show increased susceptibility to dinitrobenzene sulfonic acid induced colitis. *J Mol Med* 2006; 84: 142-146.
9. Wirtz S, Neufert C, Weigmann B, Neurath MF. Chemically induced mouse models of intestinal inflammation. *Nat Protoc* 2007; 2: 541-546.
10. Di Carlo G, Izzo AA. Cannabinoids for gastrointestinal diseases: potential therapeutic applications. Expert Opin. Investig. *Drugs* 2003; 12: 39-49.
11. Zurier RB. Prospects for cannabinoids as anti-inflammatory agents. *J Cell. Biochem* 2003; 88: 462-466.
12. Richardson JD, Kilo S, Hargreaves KM. Cannabinoids reduce hyperalgesia and inflammation via interaction with peripheral CB1 receptors. *Pain* 1998; 75: 111-119.
13. Dembinski A, Warzecha Z, Ceranowicz P *et al*. Cannabinoids in acute gastric damage and pancreatitis. *J Physiol Pharmacol* 2006; 57 Suppl 5: 137-154.
14. Abel EL. Marijuana: the first twelve thousand years. New York, Plenum Press. 1980, pp. 289.

15. Grispoon L, and Bakalar JB. Marijuana: the forbidden medicine. New Haven, Yale University Press 1997, pp. 184.
16. Pertwee RG. Cannabinoids and the gastrointestinal tract. *Gut* 2001; 48: 859-867.
17. Keates AC, Castagliuolo I, Cruickshank WW *et al*. Interleukin 16 is up-regulated in Crohn's disease and participates in TNBS colitis in mice. *Gastroenterology*. 2000; 119: 972-82.
18. Kimball ES, Wallace NH, Schneider CR, D'Andrea MR, Hornby PJ. Vanilloid receptor 1 antagonists attenuate disease severity in dextran sulphate sodium-induced colitis in mice. *Neurogastroenterol Motil*. 2004; 16: 811-818.
19. Chomczynski P, Sacchi N. The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: twenty-something years on. *Nat Protoc* 2006; 1: 581-585.
20. Harsch IA, Brzozowski T, Bazela K, *et al*. Impaired gastric ulcer healing in diabetic rats: role of heat shock protein, growth factors, prostaglandins and proinflammatory cytokines. *Eur J Pharmacol* 2003; 481: 249-260.
21. Konturek PC, Brzozowski T, Sulekova Z, Meixner H, Hahn EG, Konturek SJ. Enhanced expression of leptin following acute gastric injury in rat. *J Physiol Pharmacol* 1999; 50: 587-595.
22. Coutts AA, Irving AJ, Mackie K, Pertwee RG, Anavi-Goffer S. Localisation of cannabinoid CB1 receptor immunoreactivity in the guinea pig and rat myenteric plexus. *J Comp Neurol* 2002; 448: 410-422.
23. Pinto L, Izzo AA, Cascio MG, *et al*. Endocannabinoids as physiological regulators of colonic propulsion in mice. *Gastroenterology* 2002; 123: 227-234.
24. Jaggar SI, Sellaturay S, Rice AS. The endogenous cannabinoid anandamide, but not the CB2 ligand palmitoylethanolamide, prevents the viscero-visceral hyper-reflexia associated with inflammation of the rat urinary bladder. *Neurosci. Lett* 1998; 253: 123-126.
25. Izzo AA, Fezza F, Capasso R, *et al*. Cannabinoid CB1-receptor mediated regulation of gastrointestinal motility in mice in a model of intestinal inflammation. *Br J Pharmacol* 2001; 134: 563-570.
26. Izzo, AA, Mascolo N, and Capasso F. The gastrointestinal pharmacology of cannabinoids. *Curr Opin Pharmacol* 2001; 1: 597-603.
27. Howlett AC, Barth F, Bonner TI, *et al*. International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacol Rev* 2002; 54: 161-202.
28. Mackie K, Hille B. Cannabinoids inhibit N-type calcium channels in neuroblastoma-glioma cells. *Proc Natl Acad Sci USA* 1992; 89: 3825-3829.
29. Twitchell W, Brown S, Mackie K. Cannabinoids inhibit N- and P/Q-type calcium channels in cultured rat hippocampal neurons. *J Neurophysiol* 1997; 78: 43-50.
30. Scroggs RS, Fox AP. Distribution of dihydropyridine and omega-conotoxin-sensitive calcium currents in acutely isolated rat and frog sensory neuron somata: diameter-dependent L channel expression in frog. *J Neurosci* 1991; 11: 1334-1346.
31. Gebremedhin D, Lange AR, Campbell WB, Hillard CJ, Harder DR. Cannabinoid CB1 receptor of cat cerebral arterial muscle functions to inhibit L-type Ca2+ channel current. *Am J Physiol* 1999; 276(6 Pt 2): H2085-H2093.
32. Hemmings HC Jr, Nairn AC, McGuinness TL, Huganir RL, Greengard P. Role of protein phosphorylation in neuronal signal transduction. *FASEB J* 1989; 3: 1583-1592.
33. Massa F, Marsicano G, Hermann H, *et al*. The endogenous cannabinoid system protects against colonic inflammation. *J Clin Invest* 2004; 113: 1202-1209.
34. Diaz-Granados HK, Lu J, McKay DM. Dextran sulfate sodium-induced colonic histopathology, but not altered epithelial ion transport, is reduced by inhibition of phosphodiesterase activity. *Am J Pathol* 2000; 156: 2169-2177.

35. Egger B, Bajaj-Elliott M, MacDonald TT, *et al*. Characterization of acute murine dextran sodium sulphate colitis: cytokine profile and dose dependency. *Digestion* 2000; 62: 240–248.
36. Hosseini JM, Goldhill JM, Bossone C, Pineiro-Carrero V, Shea-Donohue D. Progressive alterations in circular smooth muscle contractility in TNBS-induced colitis in rats. *Neurogastroenterol Motil* 1999; 11: 347-356.
37. Howlett AC, Bidaut-Russell M, Devane WA, Melvin LS, Johnson MR, Herkenham M. The cannabinoid receptor: biochemical, anatomical and behavioral characterization. *Trends Neurosci* 1990; 13: 420-423.
38. Eisenstein TK, Meisler JJ, Wilson Q, Gaughan JP, Adler MW. Anandamide and Delta9-tetrahydrocannbinol directly inhibit cells of the immune system via CB2-receptors. *J Neuroimmunol* 2007; 189: 17-22.
39. Di Marzo V, De Petrocellis L, Fezza F, Ligresti A, Bisogno T. Anandamide receptors. *Prostaglandins Leukot Essent Fatty Acids* 2002; 66: 377-391.
40. Ahluwalia J, Urban L, Bevan S, Nagy I. Anandamide regulates neuropeptide release from capsaicin-sensitive primary sensory neurons by activating both the cannabinoid 1 receptor and the vanilloid receptor 1 in vitro. *Eur J Neurosci* 2003; 17: 2611-2618.
41. Izzo AA, Capasso F, Costagliola A, *et al*. An endogenous cannabinoid tone attenuates colera toxin-induced fluid accumulation in mice. *Gastroenterology* 2003; 125: 765-774.
42. McVey DC, Schmid PC, Schmid HH, Vigna SR. Endocannabinoids induce ileitis in rats via the capsaicin receptor (VR1). *J Pharmacol Exp Ther* 2003; 304: 713-722.
43. Smith SR, Denhardt G, Terminelli C. The anti-inflammatory activities of cannabinoid receptor ligands in mouse peritonitis models. *Eur J Pharmacol* 2001; 432: 107-119.
44. Storr M, Sibaev A, Marsicano G, *et al*. Cannabinoid receptor type 1 modulates excitatory and inhibitory neurotransmission in mouse colon. *Am J Physiol Gastrointest Liver Physiol* 2004; 286: G110-G117.
45. De Man JG, Moreels TG, De Winter BY, *et al*. Disturbance of the prejunctional modulation of cholinergic neurotransmission during chronic granulomatous inflammation of the mouse ileum. *Br J Pharmacol* 2001; 133: 695-707.
46. Herradon E, Martín MI, Lopez-Miranda V. Characterization of the vasorelaxant mechanisms of the endocannabinoid anandamide in rat aorta. *Br J Pharmacol* 2007; 152: 699-708.
47. Cross RK, Wilson KT. Nitric oxide in inflammatory bowel disease. *Inflam Bowel Dis* 2003; 9: 179-189.
48. Grisham MB, Pavlick KP, Laroux FS, Hoffman J, Bharwani S, Wolf RE. Nitric oxide in chronic gut inflammation: controversies in inflammatory bowel disease. *J Invest Med* 2002; 50: 272-283.
49. Guihot G, Guimbaud R, Bertrand V, *et al*. Inducible nitric oxide activity in colon biopsies from inflammatory areas: correlation with inflammation intensity in patients with ulcerative colitis but not with Crohn's disease. *Amino Acids* 2000; 18: 229-237.
50. Menchen LA, Colon AL, Moro MA, *et al*. N-(3-(aminomethyl)benzyl)acetamidine, an inducible nitric oxide synthase inhibitor, decreases colonic inflammation induced by trinitrobenzene sulfonic acid in rats. *Life Sci* 2001; 69: 479-491.
51. Kankuri E, Vaali K, Knowles RG, *et al*. Supression of acute experimental colitis by a highly selective inducible nitric oxide synthase inhibitor, N-{3-(aminomethyl)benzyl}acetamidine. *J Pharmacol Exp Ther* 2001; 298: 1128-1132.
52. Vardareli E, Dundar E, Angin K, Saricam T, Inal M. Effects of intrarectal and intraperitoneal NG-nitro-L-arginine methyl ester treatment in 2,4,6-trinitrobenzene sulfonic acid induced colitis in rats. *Exp Toxicol Pathol* 2003; 55: 271-276.

53. Armstrong AM, Campbell GR, Gannon C, Kirk SJ, Gardiner KR. Oral administration of inducible nitric oxide synthase inhibitors reduces nitric oxide synthesis but has no effect on the severity of experimental colitis. *Scand J Gastroenterol* 2000; 35: 832-838.
54. Dikopoulos N, Nussler AK, Liptay S, *et al*. Inhibition of nitric oxide synthesis by aminoguanidine increases intestinal damage in the acute phase of rat TNB-colitis. *Eur J Clin Invest* 2001; 31: 234-239.
55. Yoshida Y, Iwai A, Itoh K, *et al*. Role of inducible nitric oxide synthase in dextran sulfate sodium-induced colitis. *Aliment Pharmacol Ther* 2000; 14 Suppl 1: 26-32.
56. Rumi G, Tsubouchi R, Nishio H, Kato S, Mozsik G, Takeuchi K. Dual role of endogenous nitric oxide in development of dextran sodium sulfate-induced colitis in rats. *J Physiol Pharmacol* 2004; 55: 823-836.
57. Hokari R, Kato S, Matsuzaki K, *et al*. Reduced sensitivity of inducible nitric oxide synthase-deficient mice to chronic colitis. *Free Radic Biol Med* 2001; 31: 153-163.
58. Aoi Y, Terashima S, Ogura M, Nishio H, Kato S, Takeuchi K. Roles of nitric oxide (NO) and NO synthases in healing of dextran sulfate sodium-induced rat colitis. *J Physiol Pharmacol* 2008; 59: 315-336.

Received: May 13, 2008
Accepted: November 6, 2008

Author's address: Matthias A. Engel, MD, First Department of Medicine, University of Erlangen-Nuremberg, Ulmenweg 18, D-91054 Erlangen, Germany. Phone: +49 9131 8545027; fax: +49 9131 8535025; e-mail: Matthias.Engel@uk-erlangen.de