Editorial Expression of Concern and Corrections

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EDITORIAL EXPRESSION OF CONCERN. PNAS is publishing an Editorial Expression of Concern regarding the following three articles:

(*i*) **PHYSIOLOGY.** "NCX-1000, a NO-releasing derivative of ursodeoxycholic acid, selectively delivers NO to the liver and protects against development of portal hypertension," by Stefano Fiorucci, Elisabetta Antonelli, Olivia Morelli, Andrea Mencarelli, Alessandro Casini, Tommaso Mello, Barbara Palazzetti, Dominique Tallet, Piero del Soldato, and Antonio Morelli, which appeared in issue 15, July 17, 2001, of *Proc Natl Acad Sci USA* (98:8897–8902; first published July 10, 2001; 10.1073/pnas.151136298);

(*ii*) **PHARMACOLOGY.** "NCX-1015, a nitric-oxide derivative of prednisolone, enhances regulatory T cells in the lamina propria and protects against 2,4,6-trinitrobenzene sulfonic acid-induced colitis in mice," by Stefano Fiorucci, Elisabetta Antonelli, Eleonora Distrutti, Piero Del Soldato, Roderick J. Flower, Mark J. Paul Clark, Antonio Morelli, Mauro Perretti, and Louis J. Ignarro, which appeared in issue 24, November 26, 2002, of *Proc Natl Acad Sci USA* (99:15770–15775; first published November 11, 2002; 10.1073/pnas.232583599); and

(*iii*) **MEDICAL SCIENCES.** "A β-oxidation-resistant lipoxin A_4 analog treats hapten-induced colitis by attenuating inflammation and immune dysfunction," by Stefano Fiorucci, John L. Wallace, Andrea Mencarelli, Eleonora Distrutti, Giovanni Rizzo, Silvana Farneti, Antonio Morelli, Jih-Lie Tseng, Babu Suramanyam, William J. Guilford, and John F. Parkinson, which appeared in issue 44, November 2, 2004, of *Proc Natl Acad Sci USA* (101:15736–15741; first published October 25, 2004; 10.1073/pnas.0404722101).

The editors wish to note that a reader has raised questions about the apparent duplication in the use of certain figures in the foregoing articles. We have been informed by the University of Perugia, Italy, of an ongoing review conducted by an inquiry committee at the university. We are awaiting the findings of the committee to determine the appropriate next steps.

Randy Schekman, *Editor-in-Chief*

www.pnas.org/cgi/doi/10.1073/pnas.0803890105

PLANT BIOLOGY. For the article "Salt tolerance of *Arabidopsis thaliana* requires maturation of *N*-glycosylated proteins in the Golgi apparatus," by Jae Sook Kang, Julia Frank, Chang Ho Kang, Hiroyuki Kajiura, Meenu Vikram, Akihiro Ueda, Sewon Kim, Jeong Dong Bahk, Barbara Triplett, Kazuhito Fujiyama, Sang Yeol Lee, Antje von Schaewen, and Hisashi Koiwa, which appeared in issue 15, April 15, 2008, of *Proc Natl Acad Sci USA* (105:5933–5938; first published April 11, 2008; 10.1073/pnas.0800237105), the authors note that the affiliation for Kazuhito Fujiyama should have appeared as Osaka University. The corrected author line, the affiliation line, and a related footnote appear below.

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NEUROSCIENCE. For the article "Organization of the core structure of the postsynaptic density," by Xiaobing Chen, Christine Winters, Rita Azzam, Xiang Li, James A. Galbraith, Richard D. Leapman, and Thomas S. Reese, which appeared in issue 11, March 18, 2008, of *Proc Natl Acad Sci USA* (105:4453–4458; first published March 7, 2008; 10.1073/pnas.0800897105), the authors note that on page 4455, right column, in "AMPAR-Type Structures," paragraph 2, line 10, the phrase "from replica labeling (20 ± 4 nm) (**28**)" should instead read: "from replica labeling (20 ± 4 nm) (**48**)." The authors also wish to add the following reference citation:

48. Tanaka J, *et al.* (2005) Number and density of AMPA receptors in single synapses in immature cerebellum. *J Neurosci* 25:799–807.

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NCX-1015, a nitric-oxide derivative of prednisolone, enhances regulatory T cells in the lamina propria and protects against 2,4,6-trinitrobenzene sulfonic acid-induced colitis in mice

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Contributed by Louis J. Ignarro, September 26, 2002

NCX-1015 is a nitric oxide (NO)-releasing derivative of prednisolone. In this study we show NCX-1015 protects mice against the S. A. development and induces healing of T helper cell type 1-mediated experimental colitis induced by intrarectal administration of 2,4,6-trinitrobenzene sulfonic acid (TNBS). The beneficial effect of NCX-1015 was reflected in increased survival rates, improvement of macroscopic and histologic scores, a decrease in the mucosal content of T helper cell type 1 cytokines (protein and mRNA), and diminished myeloperoxidase activity in the colon. In contrast to its NO derivative, only very high doses of prednisolone were effective in reproducing these beneficial effects. NCX-1015 was 10- to 20-fold more potent than the parent compound in inhibiting IFN-γ secretion by lamina propria mononuclear cells. Protection against developing colitis correlated with inhibition of nuclear translocation of p65/Rel A in these cells. *In vivo* treatment with NCX-1015 potently stimulated IL-10 production, suggesting that the NO steroid induces a regulatory subset of T cells that negatively modulates intestinal inflammation.

Inflammatory bowel disease (IBD) is a family of chronic, relapsing, and tissue-destructive diseases characterized by dysfunction of mucosal T cells, abnormal cytokine production, and cellular inflammation that ultimately leads to damage of the intestinal mucosa (1). Clinically, IBD is subdivided into Crohn's disease (CD) and ulcerative colitis. Although the etiology of IBD remains unknown, there is circumstantial evidence to link IBD to a failure of the mucosal immune system to attenuate the immune response to endogenous antigens (2). Support for this view has come from animal models of colitis, which consistently exhibit an imbalance of regulatory cytokines, most notably an excessive production of IFN-γ, a typical T helper cell (Th)-1 cytokine (1, 2). Indeed an increased release of this cytokine has been described in CD patients and several models of murine colitis including the hapten model of colonic inflammation induced by intrarectal delivery of 2,4,6-trinitrobenzene sulfonic acid (TNBS; refs. 3–6). In this model intestinal inflammation develops as a result of the covalent binding of the haptenizing agent to autologous host proteins with subsequent stimulation of a delayed-type hypersensitivity to TNBS-modified self antigens. Although the relationship of this model to human disease is imperfect (1–4), the hapten-induced colitides display CD-like features, notably transmural mononuclear inflammation and predominant Th-1 activity of the mucosal leukocytes. Inflammation and cytokine production in TNBS-treated mice, as well as in CD patients, is associated with activation of transcription factors such as nuclear factor (NF)-κB (7–11). NF-κB/Rel is a family of transcription factors that participate in the activation of immune-regulatory genes including cytokine, cell-surface receptor, and acute-phase genes (7). The prominent pathogenetic role of NF-κB/Rel in TNBS colitis is clear from the observation that antisense oligonucleotides to p65/Rel A abrogate colitis (10).

Glucocorticoids are potent antiinflammatory drugs and exert their antiinflammatory action in this model through inhibition of lymphocyte proliferation and synthesis of proinflammatory cytokines as well as by down-regulating specific adhesion molecules resulting in redistribution of lymphocyte traffic (11). The broad effects of glucocorticoids are generally mediated through binding of glucocorticoids to cytoplasmic receptors (GRs). Although activation of gene expression by glucocorticoids generally requires binding of a GR dimer to a specific DNA site, some effects exerted by glucocorticoids are mediated instead by protein–protein interactions between the GR and transcription factors such as NF-κB/Rel (12–16). Glucocorticoids are commonly used to treat IBD patients (11); however, the clinical effects are often transitory, and disease recurs on tapering the drug, whereas high doses are accompanied by serious side effects and dependence (17). Therefore, modified forms of steroids active at lower therapeutic doses would fulfill an urgent clinical need.

NO-releasing steroids are a recently described class of antiinflammatory compounds obtained by coupling an NO-releasing moiety with a glucocorticoid (18, 19). One of these compounds, NCX-1015, an NO-releasing derivative of prednisolone, was demonstrated to be more effective than prednisolone in reducing inflammation, inhibiting cytokine and chemokine generation, and up-regulating the expression of the steroid-sensitive cell-surface marker CD163 in human peripheral blood mononuclear cells (18). Moreover, NCX-1015 was found to be more potent than prednisolone in reducing disease activity in a rat model of arthritis (19). In the present study, we assessed the effect of NCX-1015 in the TNBS mouse model of colitis and evaluated its efficacy in comparison with prednisolone as an inhibitor of nuclear translocation of NF-κB and generation by lamina propria mononuclear cells (LPMCs) of Th-1 cytokines *in vivo* and *in vitro*.

Materials and Methods

Study Protocols. BALB/c and male Swiss Albino mice (6–8 weeks old) were purchased from Charles River Breeding Laboratories (Milan) and Bantin & Kingman (Hull, U.K.). Acute colitis was induced according to a published method (3, 6). Briefly, mice

Abbreviations: IBD, inflammatory bowel disease; CD, Crohn's disease; Th, T helper cell; TNBS, 2,4,6-trinitrobenzene sulfonic acid; NF, nuclear factor; GR, glucocorticoid bound to cytoplasmic receptor; LPMC, lamina propria mononuclear cell; MPO, myeloperoxidase; H&E, hematoxylin/eosin.

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(fasted for 2 days) were anesthetized with halothane and O_2, and a 3.5-F catheter was inserted carefully into the colon such that the tip was 4 cm proximal to the anus. To induce colitis, 1.0–2.5 mg of TNBS in 50% ethanol (to break the intestinal epithelial barrier) was administered slowly into the lumen via the catheter fitted onto a 1-ml syringe. Control mice received 50% ethanol alone by using the same technique described above. The total injection volume was 100 μl.

Protection Against TNBS-Induced Lethality. In the first study we examined the effect of NCX-1015 and prednisolone on lethality caused by TNBS. In this protocol (16), mice (12 per group) were injected intrarectally with 50% ethanol or 1, 1.5, 2, or 2.5 mg per mouse of TNBS. TNBS-treated mice then were treated s.c. with 5 mg/kg/day prednisolone or NCX-1015 for 7 days. Surviving mice were killed 8 days after TNBS administration, colonic inflammation was scored, and myeloperoxidase (MPO) activity was measured.

Prevention of TNBS-Induced Colitis. After instillation of 1.5 mg per mouse of TNBS, animals were allocated randomly into one of three treatment groups [placebo, NCX-1015 (5 or 0.5 mg/kg/day s.c.), or prednisolone (10 or 5 mg/kg/day s.c.)] and followed for 7 days. Mice were monitored for the appearance of diarrhea, loss of body weight, and overall mortality. At the end of the experiment, surviving mice were killed, blood samples were collected by cardiac puncture, and a 7-cm segment of the colon was excised for macroscopic and microscopic damage evaluation. Colonic IL-2, IL-10, IFN-γ, and tumor necrosis factor-α (ELISA and RT-PCR) and MPO activity were assessed according to published methods (6). In duplicate experiments, the colons of surviving mice were excised for LPMC preparation.

Treatment of Established Colitis. In this protocol, mice were treated therapeutically with NCX-1015 (0.5 and 5 mg/kg/day) or prednisolone (5 and 10 mg/kg/day) from day 21 to 35 after TNBS administration. Animals then were killed 35 days post-TNBS injection.

Histological Grading of Colitis. For histological examination, a sample of colonic tissue located precisely 2 cm above the anal canal was obtained, fixed in 10% buffered formalin phosphate, embedded in paraffin, sectioned, and stained with hematoxylin/eosin (H&E). The degree of inflammation on microscopic cross sections was graded semiquantitatively from 0 to 4 (0, no signs of inflammation; 1, very low level of inflammation; 2, low level of leukocyte infiltration; 3, high level of leukocyte infiltration, high vascular density, and thickening of the colon wall; and 4, transmural infiltrations, loss of goblet cells, high vascular density, and thickening of the colon wall) (3). Slides were examined with a BX60 microscope (Olympus, Tokyo), and images were acquired by a SPOT-2 electronic camera (Diagnostic Instruments, Burroughs, MI) and digitized with specific software (Delta Sistemi, Rome). Grading was done in a blinded fashion.

Isolation and Culture of LPMCs. LPMCs were isolated from freshly obtained colonic specimens according to the method of Van der Heijden and Stok (20) as described (6). LPMCs from mice subjected to different treatment regimens were suspended in complete medium (RPMI medium 1640/10% heat-inactivated FCS/3 mM L-glutamine/10 mM Hepes buffer/10 μg/ml gentamycin/100 units/ml penicillin/100 units/ml streptomycin) and cultured at a concentration of 10^6 cells per ml. To measure cytokine production, 10^6 LPMCs were aliquoted into uncoated culture wells for 48 h (to measure production by unstimulated cells) or onto wells containing immobilized murine anti-CD3ε antibody (clone 145-2C11, PharMingen) and 1 μg/ml soluble anti-CD28 (clone 37.51, PharMingen) to measure production by stimulated cells. The culture supernatants then were harvested and assayed for IL-10 and IFN-γ concentration by using commercially available ELISA assays (Endogen, Cambridge, MA).

p65/Rel A Assay. Nuclear extracts from LPMCs were prepared by using TransFactor extraction kit (BD Bioscience/CLONTECH) according to manufacturer instructions. After centrifugation at $20,000 \times g$ for 5 min at 4°C, supernatants (nuclear extracts) were assayed for p65 content. An equal amount of nuclear lysate was added to incubation wells precoated with the DNA-binding consensus sequence. The presence of translocated p65 subunit was then assessed by using Mercury TransFactor kit (BD Biosciences/CLONTECH) according to manufacturer instructions. Plates were read at 655 nm, and results were expressed as OD.

Intravital Microscopy of the Mouse Mesentery Microcirculation. Male Swiss Albino mice were used. Control animals were injected i.p. with 100 μl of vehicle (peanut oil), and experimental animals were treated with 0.5 mg/kg NCX-1015 or 5 mg/kg prednisolone. All animals were injected i.p. with 10 ng of IL-1β 1 h after steroid administration. The mouse mesenteric preparation was executed as described (21).

Data Analysis. All values in the figures and text are expressed as mean ± SE of n mice per group. Comparisons of more than two groups were made with a one-way ANOVA (22). For intravital microscopy, statistical differences were calculated on original values by using one-way ANOVA followed by Bonferroni test for intergroup comparisons.

Results

NCX-1015 Protects Mice from Development of TNBS Colitis. First we characterized the development of colitis in animals subjected to intrarectal administration of TNBS. Whereas control (ethanol-treated) mice had no macroscopic lesions, TNBS administration resulted in a dose-dependent inflammation. As reported earlier

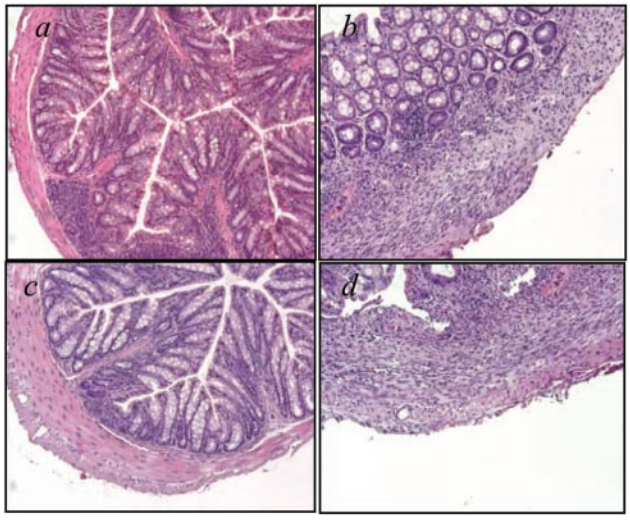

Fig. 1. Early administration of NCX-1015 protects against TNBS colitis development. (*a*) Photomicrograph of H&E-stained paraffin section of distal colon (magnification, ×200) from control (ethanol-treated) mice. (*b*) Photomicrograph of H&E-stained paraffin section of distal colon (magnification, ×200) from a TNBS-treated mouse on day 7 showing severe transmural colitis with bowel-wall thickening, inflammatory infiltrates, and marked increase of lymphoid follicles size-associated with adherence to surrounding tissues. (*c*) Effect of daily administration of 5 mg/kg NCX-1015. Note the marked reduction of colitis. (H&E; magnification, ×200.) (*d*) Effect of 5 mg/kg/day prednisolone on histological colitis in TNBS-treated mice. (H&E; magnification, ×200.)

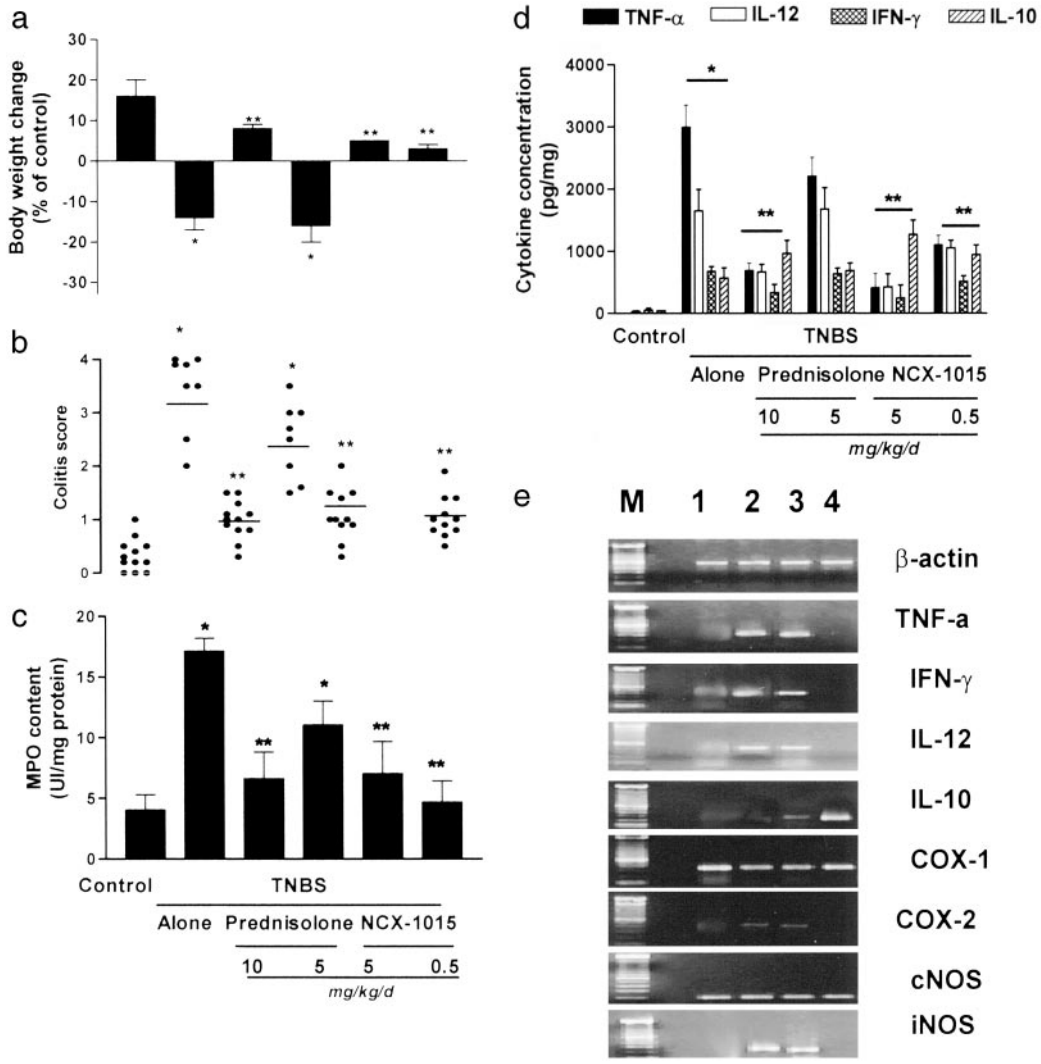

Fig. 2. (*a–c*) Early administration of NCX-1015 protects against TNBS colitis development. Colitis was induced by intrarectal administration of 1.5 mg of TNBS per mouse, and animals were killed 7 days after colitis administration. Data are mean ± SE of 10–12 mice. *, $P < 0.05$ versus control (ethanol-treated mice), and **, $P < 0.05$ versus TNBS alone. (*d*) NCX-1015 suppresses Th-1 cytokine production and stimulates IL-10 secretion. Data are mean ± SE of 10–12 mice. *, $P < 0.05$ versus control, and **, $P < 0.05$ versus TNBS alone. (*e*) RT-PCR analysis of expression inflammatory mediators in colons obtained 7 days after colitis induction. M, molecular weight markers; lane 1, control (ethanol-treated); lane 2, a colon from a mouse treated with TNBS alone; lane 3, colon of a mouse treated with TNBS plus 5 mg/kg NCX-1015; lane 4, colon of a mouse treated with TNBS plus 5 mg/kg/day prednisolone. Each RT-PCR is representative of at least three separate experiments.

(6), rectal instillation of 2.5 mg per mouse of TNBS in 50% ethanol induces colitis in >95% of BALB/c mice. Mice treated in this way manifest a severe illness characterized by diarrhea and a profound and sustained weight loss resulting in a mortality of 75%. On microscopic examination the colons of TNBS-treated mice demonstrated a transmural inflammation involving all layers of the bowel wall with a marked increase in the thickness of muscular layer and adherence to surrounding tissues compared with control sections (Fig. 1 *a* and *b*). Histologically lamina propria-infiltrating cells included neutrophils, monocytes, and lymphocytes, and immunohistochemical analysis revealed CD11b-positive cells, i.e., granulocytes and monocytes (data not shown). In addition, a superficial inflammation characterized by epithelial cell loss, patchy ulceration, pronounced depletion of mucin-producing goblet cells, and reduction in the density of the tubular glands was observed. Early administration of 5 mg/kg/day NCX-1015 (Fig. 1*c*) but not prednisolone (Fig. 1*d*) reduced overall mortality, which was 34% and 67%, respectively ($P <$ 0.05, NCX-1015 in comparison with TNBS alone).

Because these studies demonstrated that NCX-1015 protected against TNBS-induced colitis, experiments were carried out to define the relative potency of the new compound in comparison with prednisolone. In these experiments animals were injected with 1.5 mg per mouse of TNBS. The development of colitis was prevented by early administration of NCX-1015 (Fig. 2 *a–c*). The two doses of NCX-1015 tested, 0.5 and 5 mg/kg/day (equivalent to 0.33 and 3.3 mg/kg/day prednisone, respectively) effectively attenuated the severity of the wasting syndrome, ameliorated the colitis score, and reduced the colonic MPO activity. NCX-1015 administration also reduced the colonic mRNA and protein content of tumor necrosis factor-α, IL-12, and IFN-γ (Fig. 2 *d* and *e*). NCX-1015 also reduced the expression of inducible NO synthase and cyclooxygenase-2 but in contrast did not inhibit colonic expression of IL-10 mRNA or protein (Fig. 2 *d* and *e*). In fact, IL-10 expression was enhanced in mice treated with NCX-1015. These changes were observed also after prednisolone administration, although only the higher dose was effective (Fig. 2 *a–e*)

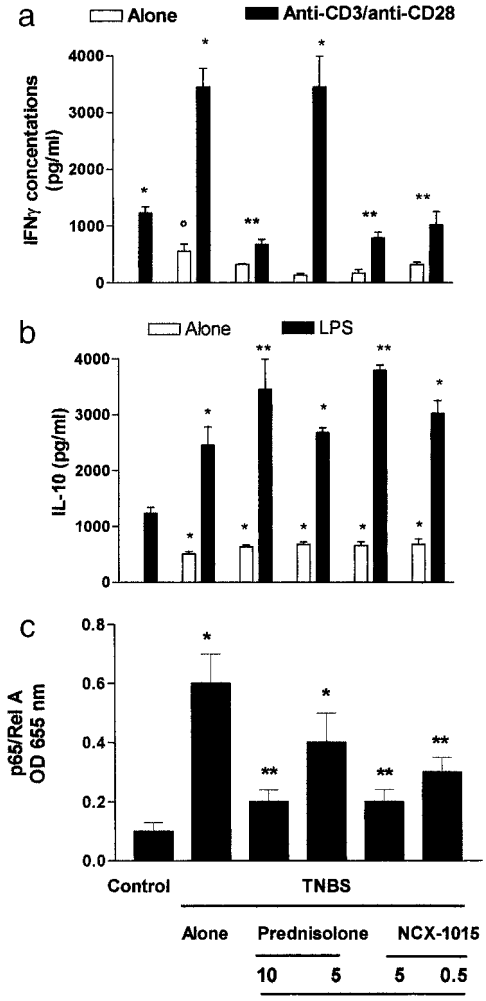

Fig. 3. Early administration of NCX-1015 abrogates IFN-γ production (*a*) while stimulating IL-10 release (*b*) by LPMCs. LPS, endotoxin. Data are mean ± SE of four experiments. Each LMPC culture was prepared by using 4–6 mice treated as described in *Materials and Methods* and killed 7 days after TNBS. *, $P < 0.05$ versus control (ethanol-treated) mice, and **, $P < 0.05$ versus TNBS alone. (*c*) Early administration of NCX-1015 abrogates NF-κB translocation in nuclear extracts from LPMCs. *, $P < 0.05$ versus control, and **, $P < 0.05$ versus mice treated with TNBS alone.

NCX-1015 Inhibits Cytokine Production and Nuclear Translocation of NF-κB in LPMCs. Next we analyzed IFN-γ production by LPMCs in NCX-1015-treated animals. As shown in Fig. 3, exposure to anti-CD3/anti-CD28 antibodies stimulated IFN-γ release from LPMCs obtained from control (ethanol-treated) mice as well as those given intrarectal TNBS. However, LPMCs prepared from mice given TNBS per rectum released significantly higher amounts of IFN-γ than LPMCs obtained from control mice. Treating colitic mice with NCX-1015 abrogated IFN-γ production. Again, only the higher dose of prednisolone (10 mg/kg/day) was effective in reducing IFN-γ production by TNBS-challenged LPMCs stimulated with anti-CD3/anti-CD28. Finally, exposure to TNBS resulted in marked increase of IL-10, a Th-regulatory cytokine. Interestingly, treatment of mice with 5 mg/kg/day NCX-1015 increased IL-10 expression *in vivo* and potentiated IL-10 release by LPMCs *ex vivo* after a challenge with anti-CD3/anti-CD28. Indeed, LPMCs prepared from TNBS-administered mice and treated with either dose of NCX-1015 generated significantly higher levels of IL-10 than cells prepared from control mice or mice injected with TNBS (Fig. 3*b*).

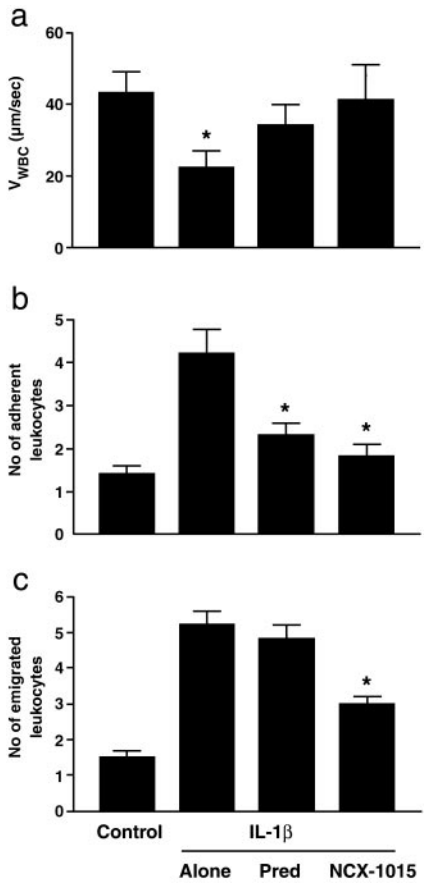

Fig. 4. NCX-1015 inhibits IL-1β-induced leukocyte rolling, adhesion, and emigration. Mice were treated i.p. with vehicle (100 μl of peanut oil), prednisolone (pred, 5 mg/kg), or NCX-1015 (0.5 mg/kg) 1 h before IL-1β administration (10 ng i.p.). Control mice received sterile saline (0.25 ml i.p.). The mesenteries were exposed 2 h later (1–3 vessels observed for each mouse) to quantify leukocyte-rolling velocity (V_{WBC}) (*a*), cell adhesion (*b*), and cell emigration (*c*). Data are mean ± SEM of $n = 4$–5 mice per group. *, $P < 0.05$ versus IL-1β-treated group.

Although LPMCs prepared from control mice killed 7 days after administration of 50% ethanol demonstrated only low levels of nuclear p65/Rel A in nuclear extracts, TNBS administration resulted in a strong translocation of p65/Rel A. This effect was abrogated by NCX-1015, whereas only the high dose of prednisolone was effective (Fig. 3*c*).

NCX-1015 Is More Potent than Prednisolone in Reducing Leukocyte–Endothelium Interaction. As shown in Fig. 4*a*, exposure to IL-1β produced an intense inflammatory response in postcapillary venules characterized by a sharp reduction in leukocyte-rolling velocity (V_{WBC}) associated with a high number of adherent and emigrated leukocytes as assessed 2 h postinjection. i.p. administration of animals with prednisolone (5 mg/kg) or NCX-1015 (0.5 mg/kg) reversed the IL-1β-induced fall in cell-rolling velocity. Cell flux was 36.7 ± 11.2 cells per min in vessels of mice treated with the cytokine, and no differences were observed in the animals treated with prednisolone (43.3 ± 11.8 cells per min) or NCX-1015 (34.8 ± 8.7 cells per min). The venular shear rate in mice injected with vehicle was calculated as 249.6 ± 15.7 per sec. This was not significantly altered after treatment with either prednisolone (224.4 ± 20.6 per sec) or NCX-1015 (290.5 ± 13.4 per sec). Treatment with 5 mg/kg prednisolone attenuated cell adhesion (although this difference did not reach statistical significance) without affecting cell emigration (Fig. 5 *a* and *b*).

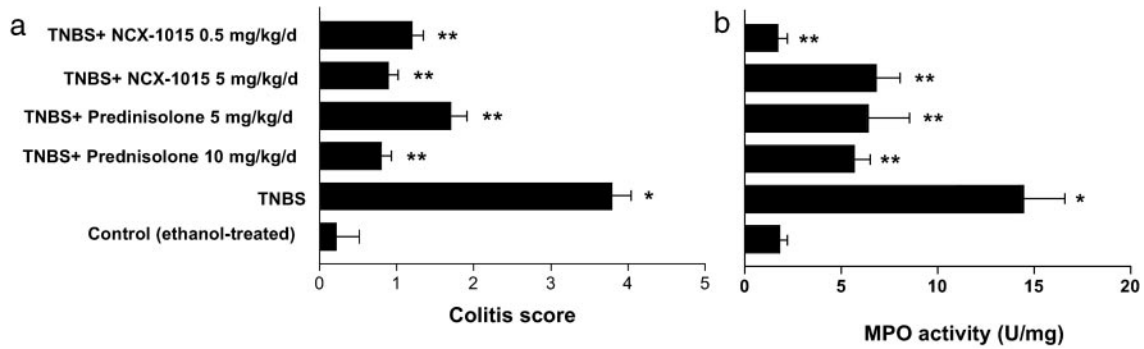

Fig. 5. Therapeutic administration of NCX-1015 heals established colitis. NCX-1015 reverses colitis score (*a*) and MPO activity (*b*) in mice with established colitis. Data are mean ± SE of 8–10 mice. *, $P < 0.05$ versus control (ethanol-treated) mice, and **, $P < 0.05$ versus TNBS alone.

In contrast, the NCX-1015 significantly reduced the extent of both cell adhesion (Fig. 4*b*) and emigration (Fig. 4*c*) by ≈50% when compared with vehicle-treated mice even when given a lower dose ($P < 0.05$).

NCX-1015 Is More Effective than Prednisolone in Healing Established TNBS Colitis. Finally, we wanted to determine whether NCX-1015 administration was effective during a later phase of the disease when colitis is fully established. As demonstrated in Fig. 5, treatment of mice with NCX-1015 markedly reduced the colitis score and MPO activity (Fig. 5) and also was effective in reducing plasma and mucosal concentrations of IL-12, IFN-γ, and tumor necrosis factor-α (data not shown).

Discussion

Intrarectal administration of TNBS in mice induces an IL-12-driven transmural inflammation that shares similarities with CD (3–7). The TNBS colitis model has been used extensively in the past to test the effect of antiinflammatory drugs. However, mesalamine (23) and the glucocorticoids budesonide (24), dexamethasone (25), and methylprednisolone (25) have proven to be only of limited efficacy in reducing the colitis score, prostaglandin E_2 production, and MPO or matrix metalloproteinase activity associated with this disease model. The poor efficacy of these treatments in TNBS-induced colitis is in line with the weak response of CD patients when compared with patients suffering from ulcerative colitis (1, 17). Confirming previous studies, we have now demonstrated that colitis induced in mice by TNBS is generally resistant to steroid administration, because only a very high dose of prednisolone (10 mg/kg/day) protected against disease development and/or induced remission of established disease. In contrast, administration of NCX-1015, an NO-releasing derivative of prednisolone, in doses of 0.5–5 mg/kg/day protected mice against death induced by TNBS and strongly inhibited colonic inflammation. The inhibition of colitis score in this model by NCX-1015 at 5 mg/kg/day (≈80%) compares favorably with that achieved by anticytokine therapies such as anti-IL-12 (3), anti-IFN-γ (4), and anti-tumor necrosis factor-α mAbs (26). A striking difference between the two steroid molecules was the superior potency of NCX-1015 in suppressing Th-1-type cytokine generation. Our *in vitro* studies demonstrated that NCX-1015 was ≈20-fold more potent than prednisolone in inhibiting IFN-γ release induced from LPMCs by anti-CD3/anti-CD8 stimulation, highlighting the importance of NO-mediated interactions in suppressing local Th-1 response in the inflamed colon.

Although several mechanisms may account for the increased potency of NCX-1015 in protecting against colonic inflammation relevant to the parent compound, we have demonstrated that NCX-1015 potently inhibits nuclear translocation of NF-κB/Rel A *in vivo*. Analysis of p65/Rel A content in nuclear extracts of LPMCs prepared from NCX-1015-treated mice revealed that a significantly lower amount of this transcription factor was translocated into the nucleus in comparison with TNBS-treated mice. Inhibition of NF-κB by glucocorticoids may be attributed to at least two different mechanisms (27, 28): protein–protein interaction of GR and NF-κB that would prevent nuclear translocation of NF-κB to the nucleus and/or increased synthesis of IκB-α, an inhibitory protein that would sequester NF-κB in an inactive cytoplasmic form (27, 28). Because NCX-1015 was significantly more effective than prednisolone in reducing nuclear translocation of NF-κB, it seems that this effect was mediated or enhanced greatly by the NO-releasing moiety of the molecule. Indeed, NO has been demonstrated to inhibit NF-κB activation, although the mechanism is not clearly defined yet (29–34). NO not only stabilizes and/or induces the synthesis of IκB-α, but also causes the *S*-nitrosylation of cysteine in the p50 and p65/Rel A subunits leading to the formation of intersubunit disulfide bonds that prevents p50 and p65/Rel A binding to DNA. Although both mechanisms might potentiate the inhibitory effect of a glucocorticoid on NF-κB actions, our *in vitro* studies with LMPCs demonstrate that NCX-1015 does not induce IκB-α synthesis (data not shown), suggesting that inhibition of p65/Rel A binding to DNA is the main mechanism. Although we have not assessed whether *in vivo* treatment with NCX-1015 results in p50/p65 *S*-nitrosylation, antiinflammatory drugs carrying the same NO-releasing moiety of NCX-1015 potently inhibit proinflammatory targets by inducing *S*-nitrosylation of cysteine residues (35). It therefore is possible that release of NO at the site of GR–NF-κB interaction by NCX-1015 causes the *S*-nitrosylation of the p50/p65 subunit of this transcription factor, thereby blocking its actions.

An equally important finding was the demonstration that the *in vivo* administration of NCX-1015 increased IL-10 production. IL-10 exerts antiinflammatory activity and has been recognized recently as a signature cytokine for a subset of $CD4^+/CD25^+$ T lymphocytes (Tr1) that exert regulatory function on the immune response (36–40). Glucocorticoids induce the development of Tr1 cells with IL-10 acting as a positive autocrine factor even in the absence of antigen-presenting cells (36–40). Interestingly, Tr1, IL-10-secreting, cells potently inhibit NF-κB and other transcription factors that are required for Th-1 and Th-2 differentiation. In the present study we have demonstrated that NCX-1015 induces the generation of IL-10 protein and mRNA by LPMCs both *in vivo* and *in vitro*, although the mechanism(s) is unknown. Interestingly, previous studies have demonstrated that exposure to NO favors the development of Th-2-type immunity (40) and releases IL-10, suggesting that prolonged exposure to NCX-1015 *in vivo* might not only suppress Th-1 cytokine but also induce a Tr1 and IL-10 response.

In our intravital model, NCX-1015 was ≈10-fold more potent than prednisolone in reducing leukocyte adherence to endothelial cells in the mouse mesenteric circulation, an action that is likely to contribute to the antiinflammatory activity of this new glucocorticoid. Adherence of polymorphonuclear leukocytes to endothelial cells largely depends on activation/induction of adhesion molecules on both neutrophils and endothelial cells (41). NF-κB-binding sites are expressed in the promoter region of several adhesion-molecule genes, including ICAM-1, a potential target of steroid action (42), and it is probable that the increased potency of NCX-1015 in this model is due to the synergism between GR and NO as inhibitors of NF-κB-mediated events.

In conclusion, we demonstrated that the addition of an NO-releasing moiety to prednisolone results in a chemical entity with extended antiinflammatory activity in comparison with the native steroid. By inducing IL-10-secreting cells, NCX-1015 potently suppresses intestinal inflammation in a rodent model of colitis, suggesting a potential utility in the treatment of patients with IBD.

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