Selective Inhibition of Phosphodiesterase-4 Ameliorates Chronic Colitis and Prevents Intestinal Fibrosis

S. Videla, J. Vilaseca, C. Medina, M. Mourelle, F. Guarner, A. Salas, and J.-R. Malagelada
Digestive System Research Unit, Hospital Vall d’Hebron, Barcelona, Spain (S.V., J.V., C.M., M.M., F.G., J.-R.M.); and Department of Pathology, Hospital Mutua de Terrassa, Barcelona, Spain (A.S.)
Received June 10, 2005; accepted October 26, 2005

ABSTRACT
The phosphodiesterase-4 (PDE4) inhibitors may be an important target in the treatment of several inflammatory conditions. The anti-inflammatory effect of PDE4 inhibitors bears similarities with that of steroids, without interfering with the hypothalamic-pituitary-adrenal axis. We compared the effect of rolipram, a selective PDE4 inhibitor, with steroids on the clinical course of experimental colitis induced by 2,4,6-trinitrobenzenesulfonic acid (TNBS). Three groups of rats (n = 20) received TNBS. One group received methylprednisolone from day 7, another group received rolipram from the same day, and control group received no further treatment. On days 14 and 21 after TNBS instillation, sets of 10 rats underwent colonic dialysis to measure eicosanoid release. Colonic lesions were blindly scored, and proteins included 11 families of PDE enzymes (PDE1–PDE11), which are identified by substrate specificity, kinetic characteristics, and amino acid sequence (Francis et al., 2001; Banner and Trevethick, 2004). Among these enzymes, the PDE4 enzymes selectively hydrolyze cAMP and have a low affinity to cGMP (Beavo and Brunton, 2002). It is currently known that increased intracellular cAMP levels can inhibit the proinflammatory and tissue-destructive properties of several inflammatory cells by inhibiting cytokine synthesis. In fact, the PDE4 inhibitors have the ability to increase intracellular cAMP and suppress many cytokines, including TNF-α production in mononuclear cells (Sinha et al., 1995; Spina, 2004), a cytokine that plays a pivotal role in the immunomodulatory response in Crohn’s disease (Breese et al., 1994; Banner and Trevethick, 2004). In addition, the PDE4 inhibitors have been successfully used in the treatment of several inflammatory pulmonary disorders, such as asthma, chronic obstructive pulmonary disease (Donohue, 2004), and inflammatory bowel disease (Banner and Trevethick, 2004). Our aim was to compare the effect of rolipram, a selective PDE4 inhibitor, versus steroids on the development of chronic inflammatory lesions in an experimental rat model of colitis induced by intracolonic administration of 2,4,6-trinitrobenzenesulfonic acid.
trobenzenesulfonic acid (TNBS). This experimental model resembles human Crohn's disease, with transmural inflammation and serosal involvement. Chronic changes are observed from 1 to 3 weeks after TNBS enema administration (Vilaseca et al., 1990). Chronic lesions induced by TNBS are segmental and well circumscribed, consisting of mucosal ulcerations with granulation tissue at the base and mixed transmural infiltration by neutrophils, lymphocytes, and macrophages. Small granulomas are often observed in the submucosa and serosa. In severe TNBS colitis, transmural fibrosis causes stricture of the lumen, whereas uninvolved areas do not show mucosal inflammation.

Materials and Methods

Animals

Male Sprague-Dawley rats weighing 225 to 250 g (CERJ, Le Genest, France) were used. The animals were maintained in a restricted access room with controlled temperature (23°C) and 12-h light/12-h dark cycle. Standard rodent chow pellets (Biocenter, Barcelona, Spain) and tap water were provided ad libitum. All of the experiments were approved by the Animal Care and Use Committee of the Hospital Vall d’Hebron and the Autonomous University of Barcelona.

Experimental Colitis

The experimental procedure was as follows. Chow pellets were withdrawn, and rats were given drinking water containing 20% sucrose and electrolytes for 36 h prior to TNBS administration. Thereafter, rats were lightly anesthetized with ether. A rubber cannula (8 cm long, external diameter 2 mm) was inserted rectally into the colon and used to instill 1 ml of a solution containing 60 mg of TNBS (Sigma-Aldrich, St. Louis, MO) in 20% ethanol (Merck Biosciences, Darmstadt, Germany) into the colonic lumen. Chow pellets and tap water were then resumed. Body weight was routinely measured every 2nd day.

Experimental Design

Transmural colitis was induced in three groups of 20 rats each. One group comprised 20 rats that received 5 mg/kg/day methylprednisolone (Urbason; sanofi-aventis, Bridgewater, NJ) i.m. from day 7 after enema instillation, and another group (n = 20) received no further treatment. On days 14 and 21 after induction of colitis, 10 rats/group were sacrificed based on determination of hydroxyproline content as described previously (Mourelle et al., 1996). In brief, the frozen colonic specimen was ground and approximately 300 mg of powder was extracted into 2 ml of a solution of 120 mM NaCl and 30 mM KHCO3 (pH 7.9). One hour later, the fluid was withdrawn and stored at −20°C until assayed. Eicosanoid concentrations in the dialysates were measured by specific radioimmunoassay. Titrated standards were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Antisera were purchased from Advanced Magnetics (Cambridge, MA).

Myeloperoxidase Activity. For the assay of MPO activity, the colonic specimen was homogenized in 2 ml of phosphate-buffered saline using a Tissue Tearor (model 985-370; Biospec, Racine, WI) and centrifuged (Schierwagen et al., 1990). The pellets were then homogenized in an equivalent volume of phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (Sigma-Aldrich) and 5 mM EDTA, sonicated three times for 30 s each time (Labasonic 2000; Braun), and centrifuged. Supernatants were used for determination of tissue MPO activity by a kinetic method. One unit of enzyme activity is defined as the amount of MPO that degrades 1 nmol of peroxide/min at 25°C.

TNF-α and TGF-β1 Determination. For assay of TGF-β1 and TNF-α in tissue, colonic samples were homogenized with the protease inhibitor phenyl-methyl-sulfonylfluoride (2 mM). Afterward, samples were centrifuged and the supernatants at various dilutions were used for measurement of cytokine concentration in the tissue. Assay of TGF-β1 concentration was performed by the enzyme-linked immunosorbent assay method (Genzyme, Cambridge, MA). Samples and standards were acidified using HCl for 1 h and then neutralized to pH 7.0 to 7.4 before assay (Mourelle et al., 1998). Other aliquots of the supernatants were used for TNF-α assay using a commercial enzyme-linked immunosorbent assay method for rat TNF-α (BioSource International, Camarillo, CA) (Videla et al., 1998). Results are expressed as nanograms of cytokine per square centimeter of colonic tissue.

Tissue Collagen Content. Tissue collagen content was estimated based on determination of hydroxyproline content as described previously (Mourelle et al., 1996). In brief, the frozen colonic specimen was ground and approximately 300 mg of powder was placed in a 10-ml flask containing 2 ml of 6 N HCl for hydrolysis at 105°C for 16 h. After evaporation to dryness at 50–80°C, samples were resuspended in 3 ml of sodium acetate-citric acid buffer (pH 6.0). Excess charcoal was added, and the suspension was filtered. A 2-ml aliquot was placed in a Pyrex test tube and mixed with 1 ml of chloramine T solution prepared immediately before use as described previously (Rojkind and González, 1974). Samples were kept for 20 min at room temperature, and the reaction was stopped by the addition of 0.5 ml of 2 mol/liter sodium thiosulfate and 1 ml of 1 N NaOH; the solution was saturated with 2 g of NaCl. Toluene was added, and the oxidation product of proline was extracted. Two milliliters of the aqueous layer was placed in boiling water for 30 min to convert the oxidation product of hydroxyproline to a pyrrole. The samples were cooled at room temperature, and the pyrrole obtained was extracted with toluene. Aliquots were mixed with Ehrlich's reagent, and absorbance was read at 560 nm. Results are expressed as milligrams of hydroxyproline per square centimeter of colon.

Assessment of Colonic Damage. The macroscopic lesions were scored separately by two independent observers who were unaware of the treatment applied, and the results were averaged (Videla et al., 1998; Medina et al., 2001). The macroscopic score was obtained by assessment of colonic strictures, adhesions to surrounding tissues, mucosal ulcerations, and wall thickening according to the criteria shown in Table 1. Samples were processed for the histological studies by routine techniques before embedding in paraffin. Sections were obtained from areas showing macroscopic damage, stained with hematoxylin and eosin, and coded for blind examination by two pathologists. Both pathologists examined and scored all sections according to the presence of ulcerations, degree of inflammation, depth of the lesions, and fibrosis (Table 1).
in body weight were found between the three experimental groups as above.

Figures 1 and 2 show the tissular MPO and TNF-α activity on days 14 and 21 after induction of colitis. Tissular specimens from TNBS rats dosed with methylprednisolone and rolipram had significantly lower MPO activity and TNF-α content compared with control tissues from TNBS rats on day 14. However, there was no statistical difference on day 21 among the three different groups. Furthermore, tissular TGF-β1 level was significantly reduced by rolipram treatment measured on days 14 and 21 after induction of colitis, whereas methylprednisolone had no effect (Fig. 3).

Table 2 shows intracolonic release of inflammatory mediators on day 21 after induction of colitis. As shown in the graph, the release of prostaglandin E2, thromboxane B2, and leukotriene B4 was significantly lower in both treatment groups compared with control group.

The effect of methylprednisolone and rolipram on morphological lesion scores in TNBS-induced colitis is shown in Fig. 4. On day 14, both treatment groups showed significantly lower scores than control group. However, on day 21 after induction of colitis, a statistically lower score versus control group was only observed in colons from TNBS rats treated with rolipram. In fact, rolipram significantly lowered scores on strictures and wall thickness. Likewise, histological scores were significantly lower in rats that received rolipram treatment. In addition, on day 21, rolipram reduced the colonic collagen content on TNBS-induced colitis, whereas methylprednisolone had no effect (Fig. 5).

**Discussion**

Crohn’s disease is a chronic relapsing condition that can affect all parts of the gastrointestinal tract, where chronic inflammation and fibrosis of the bowel are prominent features. However, to date, there is no satisfactory treatment for inflammatory lesions and intestinal fibrosis of this disease. PDE4 belongs to an important family of proteins that regulate the intracellular levels of cyclic nucleotide second messengers. It has been shown that targeting PDE4 with selective inhibitors may offer novel therapeutic strategies in the treatment of various inflammatory conditions (Genain et al., 1995; Sommer et al., 1997; Donohue, 2004), including inflammatory bowel disease (Banner and Trevethick, 2004). In this study, we have tested the therapeutic effect of a selective PDE4 inhibitor rolipram in a chronic rat model of colitis.
induced by TNBS. We found that this compound reduced the release of inflammatory mediators, macroscopic and histological damage scores, and colonic collagen content in this experimental rat model of colitis.

PDE4 are the predominant cAMP-hydrolyzing PDE in most inflammatory cells, and in general, intracellular elevations in cAMP are associated with broad anti-inflammatory effects by suppressing the synthesis of many cytokines (Spina, 2004). For example, it has been shown that selective PDE4 inhibitors can inhibit interferon-γ production from T cells from intestinal lamina propria (Prehn et al., 2001). However, more interestingly, these compounds, including rolipram, strongly inhibit TNF-α production in monocytes and macrophages in vitro (Schade and Schudt, 1993; Seldon et al., 1995). Furthermore, the synthesis of the anti-inflammatory cytokine interleukin-10 can be enhanced by rolipram (Eigler et al., 1998) and exogenous interleukin-10 administration acts synergistically with rolipram in decreasing TNF-α production (Siegmund et al., 1997).

It is well known that TNF-α plays a pivotal role in mucosal immunoinflammatory responses in inflammatory bowel disease, and TNF-α antibodies have been successfully tested in steroid-refractory and Crohn’s disease patients with fistula (Rutgeerts et al., 1999). This cytokine is involved in the activation of neutrophils (Shalaby et al., 1985), up-regulation of adhesion molecules (Gamble et al., 1985), and induction of nitric-oxide synthase (Thiemermann et al., 1993) and matrix metalloproteinases (Pender et al., 1998) that contribute to tissue damage by degrading connective tissue extracellular matrix. We have previously shown that TNF-α is enhanced in TNBS-induced colitis, and neutralization of this cytokine with monoclonal antibodies therapy could be an important tool in the treatment of chronic intestinal inflammation (Videla et al., 1998). In the present study, we found that rolipram significantly reduced the TNF-α production on colonic tissues from TNBS rats compared with control animals, with a significantly reduction of tissular MPO activity that represents the amount of neutrophils trapped into the intestinal tissue on day 14 after induction of colitis. However, we found no significant difference on day 21 among the three experimental groups because of both tissular TNF-α, and MPO activity returned to baseline levels, as previously observed (Videla et al., 1998). Our findings are in accordance with a previous study in an experimental model of colitis induced by dextran sulfate sodium, where administration of this selective PDE4 inhibitor significantly reduced TNF-α synthesis (Hartmann et al., 2000) and MPO activity (Diaz-Granados et al., 2000) in the colon of colitic mice. In addition, mesopram, another PDE4 inhibitor, has been successfully tested for established dextran sulfate sodium-induced colitis and also prevented colitis induction in a dose-dependent manner (Loher et al., 2003). Furthermore, several studies

**Fig. 2.** TNF-α levels in colonic homogenates in the group of rats described in Fig. 1 (*, p < 0.05 versus control).

**Fig. 3.** TGF-β1 levels in colonic tissue from the same group of rats described on Figs. 1 and 2 (*, p < 0.05 versus control).

**Table 2**

<table>
<thead>
<tr>
<th></th>
<th>PGE₂ (ng/ml)</th>
<th>TXB₂ (pg/ml)</th>
<th>LTB₄ (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>22 ± 3</td>
<td>8 ± 1.3</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>Methylprednisolone</td>
<td>2 ± 0.4*</td>
<td>2 ± 0.4*</td>
<td>0.2 ± 0.1*</td>
</tr>
<tr>
<td>Rolipram</td>
<td>4 ± 0.3*</td>
<td>1 ± 0.2*</td>
<td>0.1 ± 0.1*</td>
</tr>
</tbody>
</table>

* p < 0.05 versus TNBS.

**Fig. 4.** Morphological scores of colonic lesions on days 14 and 21 after induction of colitis. Macroscopic and histological scores were obtained according to the criteria stated in Table 1 (*, p < 0.05 versus control).

**Fig. 5.** Collagen content in colonic tissue from the same groups as described on Fig. 4 on day 21 after induction of colitis (*, p < 0.05 versus control).
have revealed a beneficial effect of rolipram in other inflammatory disorders. For instance, in a rat model of experimental autoimmune encephalomyelitis, where TGF-α synthesis plays an important role, rolipram decreased disease activity (Sommer et al., 1995). In an experimental model of autoimmune demyelinating disease, rolipram also protected against this condition even when administered after sensitization to central nervous system antigens (Genain et al., 1995). In another experimental model in rats, rolipram also decreased clinical activity of experimental arthritis (Nyman et al., 1997).

Interestingly, our study shows that rolipram effectively induced histopathological remission of chronic inflammatory lesions, with a marked reduction of colonic collagen content compared with methylprednisolone and control groups on day 21 after induction of colitis. This effect could be explained not only by a significantly reduction of TNF-1 levels in bronchoalveolar fluid, but they differ from the recent results in a mice renal fibrosis model, where a selective PDE4 inhibitor significantly reduced TGF-1 mRNA (Lange-Sperandio et al., 2005). TGF-1 is a cytokine that plays an important role in regulating collagen synthesis, repair, and regeneration after tissue injury and may be produced in intestinal wall by different cell populations. In humans, TGF-1 increased collagen type III in intestinal lamina propria fibroblasts isolated from Crohn’s disease patients, especially in fibroblasts derived from strictures compared with fibroblasts from noninflamed tissue (Stallmack et al., 1992). Furthermore, growing evidence supports the hypothesis that TGF-1 plays an important role in the development of several chronic fibrotic diseases by enhancing extracellular matrix deposition, such as liver cirrhosis (Castiglia et al., 1991) and pancreatic fibrosis (Van Laethem et al., 1996). A previous study in mice has also shown that subcutaneous injection of TGF-1 increases the expression of collagen synthesis (Roberts et al., 1986).

In addition, in vitro studies have found that TGF-1 induces collagen deposition by intestinal smooth muscle cells (Graham et al., 1990). Moreover, we have previously shown that increased TGF-1 levels in rat colonic tissue are associated with increased collagen deposition, whereas its neutralization with specific antibodies reduces collagen content in the colonic wall (Mourelle et al., 1998).

In conclusion, our findings show that rolipram is useful in the treatment of tissue injury and fibrosis associated with chronic colonic inflammation. Therefore, selective PDE4 inhibitors raise the possibility of a therapeutic approach to human Crohn’s disease.

References


Sinha B, Semmler J, Eisenhut T, Eigler A, and Endres S (1995) Enhanced tumor necrosis factor suppression and cyclic adenosine monophosphate accumulation by...

Address correspondence to: Dr. Carlos Medina, Department of Integrative Biology and Pharmacology, Institute of Molecular Medicine for the Prevention of Human Diseases, University of Texas-Health Science Center at Houston, 6770 Bertner Ave., Delton A. Cooley Building, c950B, Houston, TX 77030. E-mail: cmedinammar@hotmail.com