Full Paper

Tacrolimus (FK506), an Immunosuppressive Agent, Prevents Indomethacin-Induced Small Intestinal Ulceration in the Rat: Inhibition of Inducible Nitric Oxide Synthase Expression

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Received September 26, 2006; Accepted November 13, 2006

Abstract. We examined the effect of tacrolimus (FK506), an immunosuppressive drug, on indomethacin-induced small intestinal ulceration in rats. Animals were given indomethacin (10 mg/kg, s.c.), killed 24 h later, and myeloperoxidase (MPO) activity and thiobarbituric acid reactants (TBARS) were evaluated in intestinal lesions. Tacrolimus (0.3–3 mg/kg) was administered p.o. twice 0.5 h before and 6 h after indomethacin injection. The expression of inducible nitric oxide synthase (iNOS) mRNA was determined by a TaqMan real-time RT-PCR, while the activity of nuclear factor (NF)- κ B DNA-binding was analyzed by electrophoresis mobility shift assays (EMSA) 6 h after indomethacin treatment. Indomethacin provoked severe hemorrhagic lesions in the small intestine, mainly in the jejunum and ileum, accompanied with increases in MPO activity and TBARS. Oral administration of tacrolimus reduced the severity of indomethacin-induced intestinal lesions in a dose-dependent manner. The increases in MPO activity and TBARS were also significantly attenuated by tacrolimus. The expression of iNOS mRNA was markedly enhanced when examined 6 h after indomethacin administration, and this response was counteracted by tacrolimus. Indomethacin also activated NF- κ B in a tacrolimus-preventable manner. These results suggest that tacrolimus prevents indomethacin-induced small intestinal ulceration in the rat. This effect may be due to inhibition of iNOS induction through suppression of NF- κ B activation.

Keywords: tacrolimus (FK506), indomethacin, small intestinal ulceration, inducible nitric oxide synthase (iNOS), nuclear factor (NF)- κ B

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs), which have been used for the treatment with several inflammatory disorders including rheumatoid arthritis (RA), are known to cause damages in the small intestine as well as stomach as side effects. Several factors have been postulated as the pathogenic element of NSAID-induced small intestinal ulceration, including enterobacterial invasion, neutrophil activation, and nitric oxide (NO) overproduction in addition to prostaglandin (PG) deficiency (1–6). Especially, NO derived from induc-

ible NO synthase (iNOS) plays a pivotal role in the pathogenesis of NSAID-induced intestinal ulceration. Indeed, the inhibition of NO production by *N*^G-nitro-L-arginine methyl ester, a non-selective NOS inhibitor, and aminoguanidine, a selective iNOS inhibitor, as well as dexamethasone almost totally abolished the development of indomethacin-induced intestinal ulceration in rats (6–8).

Tacrolimus (FK506) is a potent immunosuppressive drug that has been widely used for organ transplantation and atopic dermatitis (9, 10). This agent reportedly prevented water-immersion stress-induced gastric lesions (11) and indomethacin-induced gastrointestinal lesions (12) in rats. However, the protective mechanism of this agent on the gastrointestinal damage remains to be elucidated. Recent clinical studies also demonstrated the

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Published in J-STAGE: January 1, 2007

doi: 10.1254/jphs.FP0061181

beneficial effect of this agent in the treatment of various autoimmune and inflammatory diseases, including RA and inflammatory bowel diseases (IBD) (13). It has further been shown that tacrolimus inhibited the induction of iNOS by suppressing the activation of nuclear factor (NF)- κ B (14, 15). The activation of NF- κ B is necessary in the induction of iNOS by cytokines and bacterial endotoxin (16). Thus it is possible that the protective effect of tacrolimus may be accounted for by the inhibition of iNOS induction through suppression of NF- κ B activation. In addition, it is clinically important that tacrolimus could be useful for not only the treatment of RA itself but also the prophylaxis of NSAID-induced intestinal toxicity in RA patients.

In the present study, we re-examined the effect of tacrolimus on indomethacin-induced small intestinal ulceration in rats and investigated the mechanisms involved in this action, especially in relation to the up-regulation of iNOS expression.

Materials and Methods

Animals

Male Sprague-Dawley rats (220–240 g; Nippon Charles River, Shizuoka) were used. The experiments were performed using 6 rats per group under unanesthetized conditions, unless otherwise specified. All experimental procedures employed in the present study were approved by the Experimental Animal Research Committee of the Kyoto Pharmaceutical University.

Induction of small intestinal lesions by indomethacin

Small intestinal ulceration was induced by oral administration of indomethacin according to our previous study (7). Briefly, non-fasting animals were given indomethacin, s.c. at a dose of 10 mg/kg. Twenty-four hours later, they were killed under deep ether anesthesia, and the stomach to the ileum were removed and treated with 2% formalin for fixation of the tissue. Then, they were opened along the mesenteric attachment and examined for lesions under a dissecting microscope with square grids ($\times 10$). The area (mm^2) of macroscopically visible lesions was measured, summed per small intestine, and used as a lesion score. To highlight hemorrhagic lesions, 1% Evans blue solution was administered i.v. in a volume of 0.5 ml/animals 0.5 h before killing. The person measuring the lesions did not know the treatments given to the animals. Tacrolimus (0.3–3 mg/kg, p.o.) was given once either 0.5 h before or 6 h after the administration of indomethacin or given twice, once at 0.5 h before and once at 6 h after indomethacin treatment.

Determination of myeloperoxidase (MPO) activity and lipid peroxidation

MPO activity was measured by the modified method of Bradley et al. (17). In brief, 24 h after indomethacin treatment, the animals were killed under deep ether anesthesia, and the small intestines were removed. After rinsing the intestine with cold saline, the whole layer of the intestine was weighed and homogenized in 50 mM phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide (HTAB, pH 6.0; Sigma, St. Louis, MO, USA). The homogenized samples were subjected to freeze-and-thaw three times and centrifuged at 2,000 rpm for 10 min at 4°C. The MPO activity was determined by adding 100 μ l of the supernatant to 1.9 ml of 10 mM phosphate buffer (pH 6.0) and 1 ml of 1.5 M *o*-dianisidine hydrochloride (Sigma) containing 0.0005% w/v hydrogen peroxide. The changes in absorbance at 450 nm of each sample were recorded by a Hitachi spectrophotometer (U-2000; Hitachi, Tokyo). Sample protein content was estimated via spectrophotometric assay (Peace protein assay kit; Pierce, Rockford, IL, USA), and MPO activity was obtained from the slope of the reaction curve, according to the following equation: Specific activity ($\mu\text{mol H}_2\text{O}_2/\text{min} \cdot \text{mg protein}$) = (OD/min)/(OD/min H_2O_2) $\cdot$ mg protein.

Lipid peroxidation in the small intestine was determined as thiobarbituric acid (TBA) reactants following indomethacin treatment according to the modified method of Ohkawa et al. (18). The whole layer of the intestine was homogenized in 1 ml KCl per 100 mg wet tissue. The homogenate was supplemented with the mixture of TBA reactants and boiled at 100°C for 1 h. Then 5 ml of a mixture of *n*-butanol and pyridine was added to each reaction mixture, shaken vigorously for 1 min, and centrifuged for 10 min at 4,000 rpm. Absorbance was measured at 532 nm in a Hitachi spectrophotometer, and sample protein content was estimated via spectrophotometric assay as described above. The results were expressed as nmol per mg protein.

Determination of mRNA expression for iNOS and tumor necrosis factor (TNF)- α

The expressions of mRNAs for iNOS and TNF- α were analyzed by RT-PCR and quantified by TaqMan real-time RT-PCR. In brief, the animals were killed under deep ether anesthesia 6 h after indomethacin treatment, and the small intestines were removed, frozen in liquid nitrogen, and stored at -80°C until use. The total RNA was extracted from the whole layer of small intestine using Separose RNA-I (Nacalai Tesque, Kyoto). First strand cDNA primed by random hexamers was reverse-transcribed with TaqMan Reverse Tran-

scription Reagents (Applied Biosystems, Foster City, CA, USA). For RT-PCR, an aliquot of the first strand cDNA sample served as a template in 35 cycles of PCR reaction with 1 min of denaturation at 95°C and 1 min of extension at 68°C using the Advantage 2 polymerase mixture (BD Biosciences Clontech, Palo Alto, CA, USA). The sequences of the sense and anti-sense PCR primers for iNOS were 5'-CGGTTCACAGTCTTGGTGAAAG-3' and 5'-CAGGTGTTCCCCAGGTAGGTAG-3', respectively, giving rise to a 780-bp PCR product, and those for TNF- α were 5'-GTCCAA CAAGGAGGAGAAGTT-3' and 5'-TCCTGGTATGAA GTGGCAAATC-3', respectively, giving rise to a 250-bp PCR product. For rat GAPDH, a house keeping gene, the sense and antisense primers were 5'-GAA CGGGAAGCTCACTGGCATGGC-3' and 5'-TGAGGT CCACCACCCTGTTGCTG-3', respectively, giving rise to a 311-bp PCR product. A portion of the PCR mixture was electrophoresed in a 1.8% agarose gel in Tris-EDTA-acetic acid buffer (40 mM Tris, 2 mM EDTA, and 20 mM acetic acid, pH 8.1), and the gel was stained with ethidium bromide and photographed (BioDoc-It system; UVP, Upland, CA, USA).

For TaqMan real-time RT-PCR, the reaction mixture was prepared by the TaqMan Universal PCR Master Mix with pre-designed and pre-labeled TaqMan PCR primer and probe sets for rat iNOS (NOS2) (Assay ID: Rn00561646_m1), rat TNF- α (Assay ID: Rn00562055_m1), and rat GAPDH endogenous control (purchased from Applied Biosystems TaqMan Gene Expression Assays Inventoried). Real-time PCR was performed using an ABI PRISM 7500 Sequence Detection System instrument and software (Applied Biosystems). The expression levels of iNOS and TNF- α mRNAs were standardized to GAPDH mRNA, and the mRNA levels of iNOS and TNF- α in the small intestinal tissue of control and tacrolimus-treated rats were expressed as the ratio to the mean value for normal small intestinal tissue.

Electrophoretic mobility-shift assay (EMSA) for determination of NF- κ activity

Nuclear protein extraction for EMSA was performed according to the method supplied with the nuclear extract kit (Active Motif, Carlsbad, CA, USA). In brief, the animals were killed under deep ether anesthesia 6 h after indomethacin treatment, and the small intestines were removed, frozen in liquid nitrogen, and stored at -80°C until use. The whole layer of the small intestine was isolated from each of 6 rats, pooled, and homogenized (Ultra-turrax, T8) in a hypotonic buffer. Tissue homogenates were lysed for 15 min on ice and then centrifuged at 850 $\times$ g for 10 min. The pellets were

suspended in hypotonic buffer, lysed for 15 min on ice, and then centrifuged at 14,000 $\times$ g for 30 s. The pellets were suspended in the complete lysis buffer containing 10 mM dithiothreitol and protease inhibitor cocktail supplied by the kit, lysed for 30 min on ice, and then centrifuged at 14,000 $\times$ g for 10 min. The supernatants (nuclear fraction) were stored at -80°C until use.

EMSA was performed according to the method supplied with EMSA Gel-Shift Kits (Panomics, Inc., Redwood City, CA, USA) with the biotin-labeled transcription factor (TF) probe, whose sequence was 5-AGTTGAGGGGACTTTCCAGGC-3. Nuclear protein extracts were incubated with the TF probe in the binding buffer including poly d (I-C) supplied by the kit at room temperature for 30 min. For the competition assay, unlabeled probe (Cold TF probe) was added to the binding buffer. The mixture was electrophoresed on a 6% polyacrylamide gel at 4°C and transferred electrophoretically to a nitrocellulose membrane. The protein/DNA complex was visualized by a chemiluminescence-imaging system using VersaDoc 5000 (BIO-RAD).

Statistical analyses

Data are presented as the mean $\pm$ S.E.M. from 4–6 rats per group. Statistically analyses were performed using a two-tailed Dunnett's multiple comparison test, and values of $P < 0.05$ were regarded as significant.

Results

Effects of tacrolimus on indomethacin-induced small intestinal ulceration

Subcutaneous administration of indomethacin (10 mg/kg) caused severe hemorrhagic lesions in the small intestine, mostly in the jejunum and ileum, with a lesion score of 350.7 ± 23.0 mm² (Figs. 1 and 2). Oral administration of tacrolimus (0.3, 1, and 3 mg/kg) dose-dependently reduced the severity of these intestinal lesions when administered twice, once at 0.5 h before and once at 6 h after indomethacin injection, with a significant effect being observed even at 0.3 mg/kg; the inhibitions were 46.3%, 66.5%, and 90.9%, respectively. Likewise, this agent (3 mg/kg) given once either 0.5 h before or 6 h after the indomethacin injection also significantly decreased the severity of these lesions, the inhibition being 68.8% and 53.8%, respectively, although these effects were less potent than that obtained by the two administrations ($\times 2$) (92.2%).

Effects of tacrolimus on the increase in MPO activity and lipid peroxidation induced by indomethacin

In the normal small intestine, the MPO activity was 0.005 ± 0.003 μ mol H₂O₂/min $\cdot$ mg protein (Fig. 3A).

Fig. 1. Macroscopic observation of indomethacin-induced small intestinal lesions in rats. Animals were given indomethacin s.c. in a dose of 10 mg/kg and killed 24 h later. Tacrolimus (3 mg/kg) was given twice, once at 0.5 h before and once at 6 h after administration of indomethacin. To delineate the lesions, 1% Evans blue was administered i.v. in a volume of 0.5 ml/animal at 0.5 h before killing. A and C: control (vehicle alone); B and D: tacrolimus; C and D: magnification of A and B, respectively.

Subcutaneous administration of indomethacin markedly increased the MPO activity in the small intestine, reaching the level of $0.120 \pm 0.023 \mu\text{mol H}_2\text{O}_2/\text{min} \cdot \text{mg}$ protein 24 h later. The increased MPO activity was significantly counteracted by tacrolimus (10 mg/kg), the value being $0.008 \pm 0.003 \mu\text{mol H}_2\text{O}_2/\text{min} \cdot \text{mg}$ protein. Likewise, lipid peroxidation as determined as the amount of TBARS in the small intestine was significantly increased from $19.2 \pm 1.7 \text{ nmol/g tissue}$ to $37.7 \pm 5.1 \text{ nmol/g tissue}$ 24 h after administration of

Fig. 2. Effect of tacrolimus on indomethacin-induced small intestinal lesions in rats. Animals were given indomethacin s.c. in a dose of 10 mg/kg, killed 24 h later, and the area (mm²) of lesions was measured under a dissecting microscope ($\times 10$). In panel A, tacrolimus (0.3, 1, and 3 mg/kg) was given twice, once at 0.5 h before and once at 6 h after, while in panel B, this agent (3 mg/kg) was given once either 0.5 h before (Pre) or 6 h after (Post) and twice, once at 0.5 h before and once at 6 h after ($\times 2$) indomethacin treatment. Data are presented as the mean $\pm$ S.E.M. from 6 (A) or 4 rats (B). *Statistically significant difference from the control at $P < 0.05$.

indomethacin, which is about two times greater than that in the normal small intestine (Fig. 3B). The increase in TBARS in response to indomethacin treatment was significantly reduced by tacrolimus (10 mg/kg), the values being $24.6 \pm 1.6 \text{ nmol/g tissue}$.

Effect of tacrolimus on the increase in the mRNA expression of iNOS and TNF- α induced by indomethacin

Subcutaneous administration of indomethacin markedly enhanced the expressions of iNOS and TNF- α mRNAs in the small intestine 6 h later; the expressions, as assessed by TaqMan real-time RT-PCR, were 78.7 ± 17.1 and 60.8 ± 19.6 times greater than the mean values

Fig. 3. Effects of tacrolimus on the changes in MPO activity (A) and TBARS (B) induced in the small intestine by indomethacin in rats. Animals were given indomethacin s.c. in a dose of 10 mg/kg and killed 24 h later. Tacrolimus (3 mg/kg) was given p.o. twice, once at 0.5 h before and once at 6 h after administration of indomethacin. Data are presented as the mean $\pm$ S.E.M. from 6 rats. Statistically significant difference at *P* < 0.05: * from the control (vehicle alone) and [#] from normal.

of normal rat small intestinal tissue, respectively (Fig. 4). These enhancements were both significantly counteracted by tacrolimus (3 mg/kg) when administered twice, once at 0.5 h before and once at 6 h after indomethacin treatment; the inhibitions were 96.7% and 93.5%, respectively.

Effect of tacrolimus on NF- κ B activation induced by indomethacin

Subcutaneous administration of indomethacin induced a marked activation of NF- κ B (Fig. 5). The activation of NF- κ B was apparently inhibited by tacrolimus (3 mg/kg) when given twice, once at 0.5 h before and once at 6 h after indomethacin treatment. When cold probe was added to the binding buffer for the competitive assay, all of the shifted bands disappeared.

Fig. 4. Effects of tacrolimus on the enhancement of iNOS and TNF- α mRNAs expression induced by indomethacin in the rat small intestine. Animals were given indomethacin s.c. in a dose of 10 mg/kg and killed 6 h later. Tacrolimus (3 mg/kg) was given p.o. 0.5 h before administration of indomethacin. A: analyses of iNOS and TNF- α mRNAs expression by RT-PCR. B and C: quantification of iNOS and TNF- α mRNAs expression by TaqMan real-time RT-PCR. Data are presented as the mean $\pm$ S.E.M. from 6 rats. Statistically significant difference at *P* < 0.05: * from the control (vehicle alone) and [#] from normal.

Discussion

The present study showed that the immunosuppressing agent tacrolimus prevented indomethacin-induced small intestinal ulceration in rats and further demonstrated that this agent also potently suppressed the enhancement of iNOS mRNA expression as well as the activation of NF- κ B in the small intestine following administration of indomethacin. It is thus assumed that tacrolimus exerts a protective effect against indomethacin-induced small intestinal ulceration through inhibition of iNOS induction.

NSAIDs are some of the most frequently used drugs

Fig. 5. Effect of tacrolimus on the changes in NF- κ B activation induced by indomethacin in the rat small intestine. Animals were given indomethacin s.c. in a dose of 10 mg/kg and killed 6 h later. Tacrolimus (3 mg/kg) was given p.o. 0.5 h before administration of indomethacin.

for the treatment of several inflammatory disorders including RA. It is well known that the administration of an NSAID such as indomethacin causes gastrointestinal damage in humans and laboratory animals as an adverse reaction (3, 5, 19, 20). Especially, gastrointestinal disorders are the most common side effect in RA patients who used an NSAID for a prolonged period. Tacrolimus (FK506), an immunosuppressive drug isolated from *Streptomyces tsukubaensis* (9, 10), has been widely used for organ transplantation and atopic dermatitis. Recent clinical studies demonstrated the beneficial effect of this immunosuppressive agent for the treatment of various autoimmune and inflammatory diseases, including RA and IBD (13). It has also been reported that tacrolimus significantly inhibited water-immersion stress-induced gastric lesions (11) and indomethacin-induced gastrointestinal damage (12) in rats. Consistent with the latter study, the present study confirmed that tacrolimus, given twice, once at 30 min before and once at 6 h after indomethacin, dose-dependently prevented the development of small intestinal lesions in response to indomethacin. In addition, we observed that tacrolimus when given once either 0.5 h before (pre) or 6 h after indomethacin (post) also significantly suppressed the severity of these lesions, yet the effects by the single administration were less potent than that obtained by the twice administration. It is assumed that only a single injection of tacrolimus at 6 h after indomethacin may be too late to effectively prevent the onset of intestinal lesions in response to indomethacin and that the effect of this agent given once 30 min before indomethacin may not be durable enough to cover the critical period for the lesion formation.

Several factors have been postulated as the pathogenic elements of intestinal ulceration induced by indomethacin, including PG deficiency, bile acid, and

bacterial flora (1–5, 21). Boughton-Smith et al. (22) reported that bacterial endotoxin from *E. coli*, a predominant member of the rat gut bacterial flora, enhanced the intestinal permeability through increased expression of iNOS. Whittle et al. (6) showed that microvascular injury in the rat jejunum, which commenced 18 h following indomethacin, was associated with the expression of iNOS and was inhibited by the delayed administration of NO synthase inhibitor, suggesting a key pathogenic role of iNOS/NO in this phenomenon. Accordingly, it is considered that the release of bacterial products such as endotoxin may contribute to indomethacin-induced small intestinal ulceration through overproduction of NO due to up-regulation of iNOS expression in the small intestine. Interestingly, because we found in this study that tacrolimus potently suppressed the induction of iNOS mRNA following the administration of indomethacin, it is assumed that the intestinal protective effect of this agent is brought about by inhibition of iNOS expression.

It is known that NO interacts with superoxide radicals to produce a cytotoxic peroxynitrite, which has a deleterious influence on the gastrointestinal mucosal integrity (23–26). Thus, a detrimental role of NO in indomethacin-induced intestinal lesions may be explained by a cytotoxic effect of peroxynitrite. Indeed, TBARS and MPO activity in the small intestine were significantly increased after indomethacin treatment, and these responses were significantly inhibited by tacrolimus at the dose that prevented the development of intestinal lesions. On the other hand, Bertrand et al. (27) demonstrated the involvement of TNF- α in the pathogenic mechanism of these lesions, especially in the early event preceding the elevation of NO production and MPO activity as well as lesion formation. TNF- α is known to induce the expression of iNOS in various cells including leukocytes (28, 29). In the present study, we observed the increased expression of TNF- α mRNA in the small intestine following the administration of indomethacin and showed that this increase was totally attenuated by tacrolimus. Thus, it is possible that the protective effect of tacrolimus may be accounted for by inhibition of the induction of both iNOS and TNF- α after indomethacin treatment.

The immunosuppressive effect of tacrolimus results from interfering with the activation of T cells and the production of T cell-derived cytokines such as interleukin-2 and interferon- γ (30). The latter effect is known to be due to inhibition of calcineurin phosphatase, the enzyme involved in the activation of the nuclear factor of activated T cells that is a transcription factor required for the expression of the cytokine gene in T cells. In addition, it has been reported that tacrolimus inhibits

iNOS mRNA expression by inhibiting the activation of NF- κ B (14, 15). NF- κ B leads to the expression of various genes encoding for cytokines, growth factors, cell adhesion molecules, and inflammatory enzymes (31). The promoters of genes encoding iNOS and other cytokines, including TNF- α , contain a consensus sequence for the binding of NF- κ B, the event necessary for their induction by cytokines and bacterial endotoxin (16). Kaibori et al. (14) demonstrated that tacrolimus inhibits iNOS expression at a step of NF- κ B activation in rat hepatocytes. The present study also showed that the administration of indomethacin caused the activation of NF- κ B in the small intestine, and this activation was totally inhibited by tacrolimus. These findings strongly suggest that the intestinal protective effect of tacrolimus is attributable to inhibition of iNOS and TNF- α inductions through suppressing the activation of NF- κ B following the administration of indomethacin.

In conclusion, tacrolimus prevents indomethacin-induced small intestinal ulceration. This effect may be brought about by inhibition of iNOS induction through suppression of NF- κ B activation. It is therefore assumed that tacrolimus is useful for not only the treatment of RA itself but also the prevention of NSAID-induced intestinal toxicity in RA patients.

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