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Calcineurin inhibitors, cyclosporin A and tacrolimus inhibit expression of inducible nitric oxide synthase in colon epithelial and macrophage cell lines

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Abstract

Nitric oxide (NO) production is increased in inflammatory bowel disease and selective inducible nitric oxide synthase (iNOS) inhibitors have proved to be anti-inflammatory in experimentally induced colitis. The aim of the present study was to test if drugs used in the treatment of inflammatory bowel disease effect on NO production in colon epithelial and macrophage cell lines. We tested the effects of cyclosporin A, tacrolimus (FK-506), methotrexate, sulfasalazine, 5-aminosalicylic acid and two novel TNF-α antagonists etanercept and infliximab on endotoxin-induced NO production in human T84 colon epithelial cells and in murine J774 macrophages. Cyclosporin A and FK-506 inhibited iNOS expression, and subsequent NO production, in a dose-dependent manner at therapeutically achievable drug concentrations in both cell lines. The effect was most pronounced when cyclosporin A was given 1 h prior to 4 h after endotoxin, and declined thereafter, indicating that cyclosporin A does not inhibit iNOS activity. Neither cyclosporin A nor FK-506 altered the activation of nuclear factor-κB (NF-κB) that is a critical transcription factor for iNOS. Sulfasalazine inhibited NO production slightly only when given at high (100 μM) drug concentrations. Methotrexate, 5-aminosalicylic acid and TNF-α antagonists infliximab and etanercept were practically ineffective. Two inhibitors of phosphatase calcineurin, cyclosporin A and FK-506, inhibited iNOS expression and NO production in human T84 colon epithelial cells and in murine J774 macrophages by an NF-κB independent manner. These findings are implicated in the anti-inflammatory action of these compounds.

Keywords: Nitric oxide (NO); iNOS (inducible nitric oxide (NO) synthase); Cyclosporin A; Tacrolimus; Sulfasalazine; Colitis

1. Introduction

Nitric oxide (NO) is produced in increased amounts and acts as a pro-inflammatory and cytotoxic mediator in various inflammatory diseases (Moilanen et al., 1999). NO is synthesised from L-arginine by nitric oxide synthase (NOS) enzymes. Two types of NOS enzymes have been identified: constitutively expressed Ca^{2+}-dependent nitric oxide synthase (cNOS) and inducible Ca^{2+}-independent nitric oxide synthase (iNOS) (Knowles and Moncada, 1994; Alderton et al., 2001). iNOS is induced by inflammatory cytokines and bacterial products in various cell types (Moilanen et al., 1999). When iNOS is induced, it produces high amounts of NO for prolonged periods which is related to immunoregulatory, antimicrobial and cytotoxic actions of NO.

Nitric oxide production is increased in inflammatory bowel disease where NO is mainly produced by macrophages, neutrophils and epithelial cells. In experimentally induced intestinal inflammation, NO seems to have a biphasic effect. In the early stages of inflammation, low levels of NO produced by cNOS are protective but later on, when NO is produced in high amounts by iNOS, it is associated with mucosal lesions, ulcerations, intraluminal bleeding, bowel dilatation and dysfunction, and has pro-inflammatory and destructive effects (Laszlo et al., 1994; Guslandi, 1998; Miller and Sandoval, 1999). NO production and iNOS activity have been shown to be increased in active ulcerative colitis (Kimura et al., 1997, 1998; Boughton-

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Smith et al., 1993; Lundberg et al., 1994). NOS inhibitors with relative selectivity towards iNOS have been reported to have anti-inflammatory effects in endotoxin-induced intestinal barrier dysfunction in rats (Unno et al., 1997), in trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats (Zingarelli et al., 1998) and in spontaneous colonic inflammation occurring in HLA-B27 transgenic rats (Aiko et al., 1998). In recent studies, a highly selective iNOS inhibitor 1400 W has proved to have a significant anti-inflammatory action in severe TNBS-induced colitis (Kankuri et al., 2001; Menchen et al., 2001) and in endotoxin-provoked injury in rat gastroduodenal microvasculature (Kiss et al., 2001). These results suggest that inhibition of iNOS in gut inflammation is of therapeutic value. The aim of the present study was to investigate the effects of drugs used in the treatment of inflammatory bowel disease on L-arginine NO-pathway in two different cell lines, in human intestinal T84 epithelial cells and in murine J774 macrophages. The latter cell line was used because iNOS pathway is well characterised in that cell line. We used also human intestinal T84 epithelial cells, which are appropriate for the pathogenesis of inflammatory bowel disease, and which have recently been shown to express iNOS and produce NO when exposed to bacterial endotoxin (Lahde et al., 2000). In these conditions, we investigated the effects of two calcineurin inhibitors cyclosporin A and tacrolimus (FK-506), two TNF-α antagonists infliximab and etanercept and three other drugs for inflammatory bowel disease sulfasalazine, methotrexate and 5-aminosalicylic acid, on cellular iNOS expression and NO production in response to inflammatory stimuli.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle medium and its supplements were from Gibco BRL (Paisley, Scotland, UK). Cyclosporin A was supplied by Calbiochem (La Jolla, CA). Infliximab was from Centocor (Leiden, The Netherlands), etanercept was from Wyeth Lederle (Espoo, Finland) and methotrexate from Orion (Espoo, Finland). All other reagents were from Sigma (St. Louis, MO).

2.2. Cell culture

Human T84 colon epithelial cells and murine J774 macrophages were obtained from American Type Culture Collection (Rockville, MD). Cells were cultured at 37 °C (in 5% carbon dioxide) in Dulbecco's Modified Eagle's Medium (DMEM) with glutamax-I containing 10% (5% in T84 cells) heat-inactivated foetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (250 ng/ml). Cells were harvested with trypsin-EDTA. Cells were seeded in 24-well plates for nitrite measurements, in 6-well plates for Western blot analysis and in 10-cm dishes for electrophoretic mobility shift assay. Confluent cells were exposed to fresh culture medium containing the compounds of interest.

2.3. XTT-test

Cell viability was tested using Cell Proliferation Kit II (Boehringer Mannheim, Indianapolis, IN). Cells were incubated with the tested compounds for 20 h before addition of sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) (final concentration 0.3 mg/ml) and *N*-methyl dibenzopyrazine methyl sulfate (1.25 mM). Then, cells were further incubated for 4 h and the amount of formazan accumulated in growth medium was assessed spectrophotometrically. Triton-X treated cells were used as a positive control.

2.4. Nitrite determinations

Measurement of nitrite accumulation into the culture medium was used to determine NO production. At indicated time points, the culture medium was collected and nitrite was measured by Griess reaction (Green et al., 1982).

2.5. Western blot analysis

After desired time of incubation, cell pellets were lysed in ice-cold extraction buffer (10 mM Tris-base, 5 mM EDTA, 50 mM NaCl, 1% Triton-X, 0.5 mM phenylmethyl sulfonyl fluoride, 2 mM sodiumorthovanadate, 10 μg/ml leupeptin, 25 μg/ml aprotinin, 1.25 mM NaF, 1 mM sodiumpyrophosphate and 10 mM *n*-octyl-β-D-glucopyranoside). After extraction by incubation on ice for 15 min, samples were centrifuged and the resulting supernatant was diluted 1:4 and boiled for 5 min in sample buffer (6.25 mM Tris–HCl, 10% glycerol, 2% SDS and 0.025% 2-mercaptoethanol) and stored at −20 °C until analysed. Coomassie blue method was used to measure the protein content of the samples (Bradford, 1976). Protein samples (20 μg) were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 8% polyacrylamide gel and transferred to nitrocellulose membrane. iNOS protein was detected and identified by Western blotting using rabbit polyclonal antibody (M-19 for murine J774 macrophages and N-20 for human T84 epithelial cells) obtained from Santa Cruz Biotechnology, Santa Cruz, CA.

2.6. Preparation of nuclear extracts

Cells were seeded on 10-cm dishes and were grown to confluency. Cells were incubated with the compounds of interest for 30 min. After incubation, cells were washed with ice-cold phosphate buffered saline (PBS) and solubilised in hypotonic buffer A (10 mM HEPES–KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiotreitol, 0.2 mM phenylmethyl sulfonyl fluoride, 10 μg/ml leupeptin, 25 μg/ml

aprotinin, 0.1 mM EGTA, 1 mM Na_2VO_4, 1 mM NaF) and incubated for 10 min on ice. Thereafter, cells were vortexed for 30 s and the nuclei were separated by centrifugation at 4 °C, 15 000 rpm and 10 s. Nuclei were suspended in buffer C (20 mM HEPES–KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiotreitol, 0.2 mM phenylmethyl sulfonyl fluoride, 10 μg/ml leupeptin, 25 μg/ml aprotinin, 0.1 mM EGTA, 1 mM Na_3VO_4, 1 mM NaF) and incubated on ice for 20 min. Nuclei were vortexed 30 s and nuclear extracts were obtained by centrifugation at 4 °C and 15 000 rpm for 2 min. Coomassie blue method was used to measure the protein content of the samples (Bradford, 1976).

2.7. Electrophoretic mobility shift assay (EMSA)

Single-stranded oligonucleotides (5′-AGTTGAGGGGACTTTCCCAGGC-3′ and 3′-TCAACTCCCCTGAAAGGGTCCG-5′; Amersham Pharmacia Biotech (Piscataway, NJ)) containing the nuclear factor-κB (NF-κB)-binding sequences were annealed and 5′ ^{32}P-endlabeled with DNA 5′-End Labeling Kit (Boehringer Mannheim). For binding reactions, 10 μg of nuclear extract was incubated in 20 μl of total reaction volume containing 0.1 mg/ml (poly)dI-dC, 1 mM dithiotreitol, 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 200 mM KCl and 10% glycerol for 20 min at room temperature. Thereafter, 0.2 ng of ^{32}P-labeled oligonucleotide was added and reaction mixture was incubated for 10 min at room temperature. Protein/DNA complexes were separated from DNA probe by electrophoresis on 4% polyacrylamide gel. Gel was dried on filter paper and autoradiographed with intensifying screen at −70 °C.

2.8. Statistics

Results are expressed as mean ± standard error of mean (S.E.M.). Statistical significance was calculated by analysis of variance supported by Dunnett adjusted significance levels. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Effects of cyclosporin A and FK-506 on NO production

Bacterial endotoxin (lipopolysaccharide, LPS) induced iNOS expression and NO production in human T84 epithelial cells and in murine J774 macrophages. Cyclosporin A inhibited NO production (measured as nitrite accumulation into the culture medium) in a dose-dependent manner in both cell lines. Exposure to increasing concentration of cyclosporin A resulted in a 34% and 10% (1 μM), 59% and 30% (3 μM) and 80% and 52% (10 μM) inhibition of NO production during a 24-h incubation in T84 and J774 cells, respectively (Fig. 1A). FK-506 (tacrolimus) is another inhibitor of Ca^{2+}/calmodulin-dependent calcineurin. Mimicking the action of cyclosporin A, FK-506 had also a dose-dependent inhibitory action on endotoxin-induced NO production (Fig. 1B). Cytotoxic action of cyclosporin A or FK-506 as a contributing factor was ruled out by XTT-test.

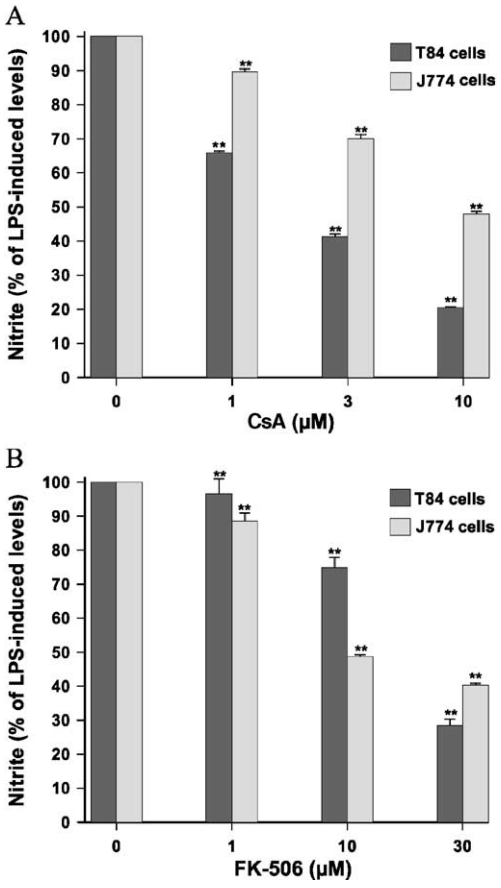

Fig. 1. (A) Effects of increasing concentrations of cyclosporin A (CsA) on endotoxin (lipopolysaccharide, LPS; 1 μg/ml)-induced NO production in T84 and in J774 cells during a 24-h incubation time. (B) Effects of increasing concentrations of FK-506 on endotoxin (1 μg/ml)-induced NO production in T84 and in J774 cells during a 24-h incubation time. NO production was determined by measuring nitrite accumulation into the culture medium by Griess reaction. Mean ± S.E.M., $n = 6$. ** indicates $P < 0.01$ as compared to cells incubated without cyclosporin A or FK-506.

The time course of the inhibitory action of cyclosporin A on NO production is shown in Fig. 2. The inhibition was most pronounced when cyclosporin A was given from 1 h prior to 4 h after endotoxin. The suppressive action declined, and in the case of T84 cells was totally reversed, if cyclosporin A was given at 8 h or later following endotoxin. These data suggest that cyclosporin A inhibits iNOS expression rather than iNOS activity (Fig. 2).

3.2. Effects of cyclosporin A and FK-506 on iNOS protein expression

In the further studies, we investigated the effect of cyclosporin A and FK-506 on iNOS expression by Western blot (Fig. 3). Cells cultured in the absence of endotoxin did not

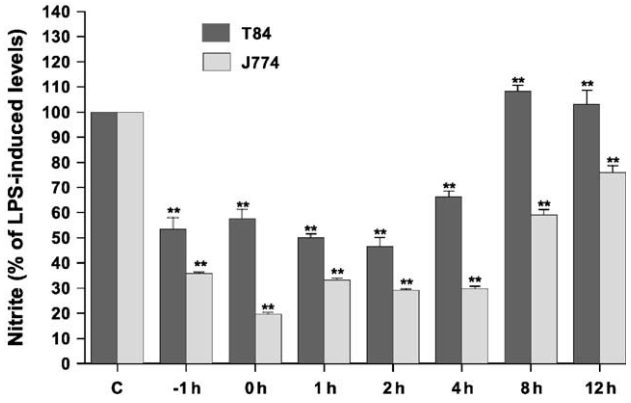

Fig. 2. Time course of the inhibitory action of cyclosporin A. T84 and J774 cells were incubated for 24 h with endotoxin (lipopolysaccharide, LPS; 1 μg/ml) and cyclosporin A (3 μM) was added at indicated time points. NO production was determined by measuring nitrite accumulation into the culture medium by Griess reaction. Mean ± S.E.M., $n=6$. ** indicates $P<0.01$ as compared to cells incubated without cyclosporin A.

contain detectable amounts of iNOS protein. iNOS expression was significantly enhanced in both cell types following exposure to endotoxin. Cyclosporin A (1–10 μM) and FK-506 (1–30 μM) suppressed this endotoxin-induced iNOS expression in a dose-dependent manner in both cell types.

3.3. Effects of cyclosporin A and FK-506 on transcription factor NF-κB activation

NF-κB is an important transcription factor of iNOS (Xie et al., 1994). Therefore, we measured the effects of cyclosporin A on NF-κB activation by electrophoretic mobility shift assay (EMSA) (Fig. 4). In the absence of endotoxin, there was a low basal activity of NF-κB that was significantly enhanced following endotoxin. Cyclosporin A or FK-506 had no effect on endotoxin-induced NF-κB activation.

3.4. Effects of methotrexate, sulfasalazine, 5-aminosalicylic acid, infliximab and etanercept on NO production

Out of the five other drugs tested, sulfasalazine (100 μM) inhibited endotoxin-induced NO production by 26%

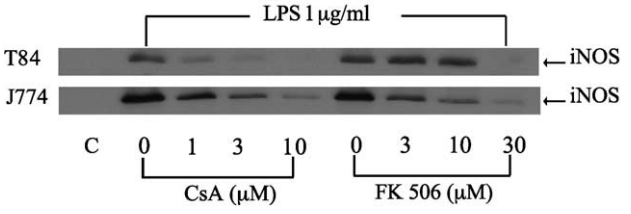

Fig. 3. The effects of cyclosporin A (CsA) and FK-506 on endotoxin (lipopolysaccharide, LPS)-induced iNOS protein expression in T84 and J774 cells as detected by Western blot analysis. Cells were incubated for 24 h with compounds of interest and iNOS was detected by immunoblot with specific antibody against iNOS. The figure shows a representative of four separate experiments with similar results.

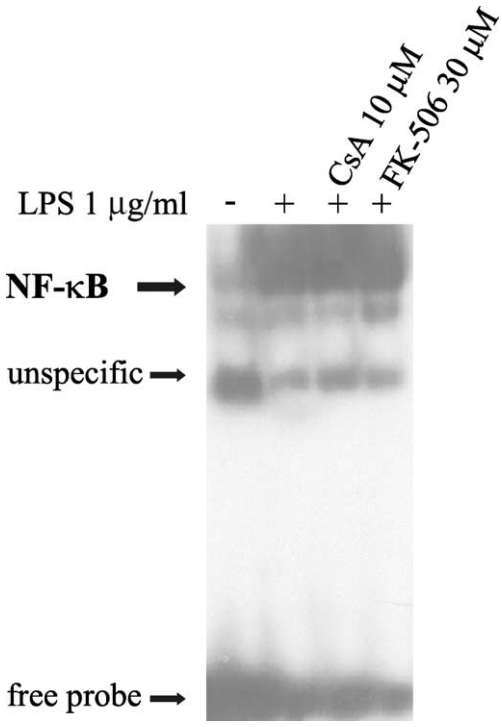

Fig. 4. The effects of cyclosporin A (CsA) and FK-506 on endotoxin (lipopolysaccharide, LPS)-induced NF-κB activation. Human T84 colon epithelial cells were incubated for 30 min with endotoxin, with endotoxin and cyclosporin A, or with endotoxin and FK-506. NF-κB binding activity was determined by electrophoretic mobility shift assay. The figure shows a representative of four separate experiments with similar results.

in T84 cells and by 21% in J774 cells (Table 1). Methotrexate, sulfasalazine and 5-aminosalicylic acid inhibited NO production by less than 20% at 100 μM concentrations and had no effect at concentrations of 30 μM or lower. TNF-α antagonists infliximab (10 μg/ml) and etanercept (100 μg/ml) did not significantly alter endotoxin-induced NO production in T84 cells. In murine J774 macrophages, the TNF-α antagonists had a slight (<20%) inhibitory action.

Table 1
Effects of methotrexate, sulfasalazine, 5-aminosalicylic acid, infliximab and etanercept on endotoxin (1 μg/ml)-induced NO production in T84 cells and in J774 cells

	T84 cells, nitrite (% of control)	J774 cells, nitrite (% of control)
Control	100	100
Methotrexate (100 μM)	83 ± 0.72 **	96 ± 1.5
Sulfasalazine (100 μM)	74 ± 4.9 **	79 ± 0.64 **
5-Aminosalicylic acid (100 μM)	90 ± 0.79 **	88 ± 1.5 **
Infliximab (10 μg/ml)	110 ± 2.5 **	89 ± 1.7 **
Etanercept (100 μg/ml)	105 ± 2.4	81 ± 1.3 **

Endotoxin and drugs tested were added to the cells at the beginning of the incubation, cells were incubated for 24 h and, thereafter, nitrite was measured from the culture medium as a marker of NO production. ** indicates $P<0.01$. Mean ± standard error of mean (S.E.M.), $n=6$.

4. Discussion

Cyclosporin A is a potent immunosuppressive agent. It acts primarily as an inhibitor of T-cell activation, but it has been reported to affect also other cell types (Schreiber and Crabtree, 1992). In T cells, cyclosporin A acts by suppressing T-cell receptor-activated signal transduction pathway. Cyclosporin A inhibits the action of Ca^{2+}/calmodulin-dependent phosphatase calcineurin resulting in a suppressed activation of transcription factor of activated T cells (NF-AT). This action leads to reduced production of interleukin-2 and other cytokines in activated T cells. The most important clinical use of cyclosporin A is in the prevention and treatment of allograft rejection. It is also used in autoimmune disorders such as rheumatoid arthritis and inflammatory bowel disease (Faulds et al., 1993).

Cyclosporin A was reported to inhibit endotoxin-induced NO production in macrophages and it was proposed to do this by suppressing iNOS induction rather than by inhibiting NOS activity (Hattori and Nakanishi, 1995). In another study, cyclosporin A was found to inhibit NOS activity in a dose-dependent manner in casein-elicited murine peritoneal macrophages (Conde et al., 1995). In murine macrophage cell line RAW 264.7, endotoxin-induced NO production was found to be inhibited at iNOS mRNA level by cyclosporin A (Attur et al., 2000). In the present study, we found that cyclosporin A inhibits endotoxin-induced NO production in a dose-dependent manner in human T84 cells and in murine J774 macrophages. Time course of this inhibitory action suggests that cyclosporin A inhibits iNOS expression but not NOS activity since 1 h before to 4 h after the endotoxin stimulation, the suppressive action of cyclosporin A on NO production was most efficient declining thereafter. The Western blot analysis confirmed the inhibitory action of cyclosporin A on iNOS protein expression after endotoxin challenge. NF-κB is an important transcription factor in the induction of iNOS gene (Xie et al., 1994). Therefore, we tested if cyclosporin A has any effect on the nuclear translocation and DNA-binding of NF-κB on endotoxin-induced T84 cells. Low basal activity of NF-κB was significantly increased after endotoxin challenge, but cyclosporin A had no effect on NF-κB activity. This result suggests that cyclosporin A mediates its effect on endotoxin-induced NO production by an NF-κB-independent manner.

Tacrolimus (FK-506) is another immunosuppressive compound that has a very similar mechanism of action as cyclosporin A. In activated T cells, cyclosporin A and FK-506 exert their effect on the transcription factor NF-AT (Schreiber and Crabtree, 1992). When bound to their cytosolic immunophilin receptors, cyclosporin A and FK-506 inhibit the action of a protein phosphatase, calcineurin, resulting in reduced dephosphorylation of the cytosolic component of transcription factor NF-AT. As a consequence, the nuclear translocation of NF-AT is inhibited. In our experiments, FK-506 had a very similar inhibitory action as cyclosporin A on NO production and iNOS expression both in human T84 colon epithelial cells and in murine J774 macrophages. These data suggest that the effect of cyclosporin A and FK-506 is mediated through inhibition of calcineurin either by NF-AT-dependent or -independent manner. The role of NF-AT in the regulation of iNOS expression is not known. We did a computer survey (MatInspector V2.2, Quandt et al., 1995) and found that there are several potential binding sites for NF-AT in human iNOS promoter region, but we did not find in the literature convincing data on any significant role of NF-AT in the regulation of iNOS transcription. On the other hand, our time–response curve of the effect of cyclosporin A on inducible NO production suggest that the effect is not a clear transcriptional one but more likely takes place at post-transcriptional level. Further studies are needed to specify the target of cyclosporin A and FK-506 in this process.

We also tested the effects of methotrexate, sulfasalazine, 5-aminosalicylic acid, infliximab and etanercept on endotoxin-induced NO production in T84 and in J774 cells. Methotrexate inhibits the reduction of dihydrofolate to tetrahydrofolate, which is needed in the synthesis of tetrahydrobiopterin, a cofactor of iNOS (Alderton et al., 2001). In T84 cells, a slight inhibition on endotoxin-induced NO production by high concentrations (100 μM) of methotrexate was detected, but not in J774 cells. These results suggest that in our experimental conditions, the synthesis of tetrahydrobiopterin is not a rate-limiting factor for inducible NO production. In macrophages from rats with adjuvant-induced arthritis, methotrexate has been shown to suppress NO production (Omata et al., 1997). 5-Aminosalicylic acid, when used at millimolar concentrations, has been shown to inhibit iNOS transcription in human intestinal epithelial cell lines DLD-1 and Caco-2BBe (Kennedy et al., 1999). We used lower concentrations (up to 100 μM) of 5-aminosalicylic acid and found a slight effect on endotoxin-induced NO production with the highest concentration used. Wahl et al. (1998) have reported that sulfasalazine, a combinatory molecule of 5-aminosalicylic acid and sulfapyridine, is a specific inhibitor of NF-κB activation when used at 2–5 mM concentrations. We used 100 μM of sulfasalazine and a 26% and 21% inhibition was found in endotoxin-induced NO production in T84 and in J774 cells, respectively. These data suggest that with concentrations we used, sulfasalazine does not significantly inhibit the activation of NF-κB since NF-κB is a critical transcription factor for iNOS (Xie et al., 1994). TNF-α antagonists infliximab and etanercept had no effect (T84 cells) or a slight inhibitory effect (J774 cells) on NO production, indicating that TNF-α is not significantly mediating or enhancing endotoxin-induced NO production in these cell types.

In summary, two calcineurin inhibitors, cyclosporin A and FK-506 (tacrolimus), inhibited endotoxin-induced iNOS expression and subsequent NO production in human intestinal T84 cells and in murine J774 macrophages by an NF-κB-independent manner. These results are implicated

in the anti-inflammatory action of cyclosporin and tacrolimus.

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