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RAPID COMMUNICATION

Inhibitory Effect of FK 506 and Cyclosporin A on Nitric Oxide Production by LPS-Treated Cultured Rat Macrophages

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Summary

We analyzed the effect of FK 506 on the production of nitric oxide by macrophages. Isolated rat peritoneal macrophages were cultured for 24 h with or without lipopolysaccharide (LPS) (5 μg/ml) and in the absence or presence of FK 506 (0.1 and 1 μg/ml). The concentration of NO_2^- in culture supernatants was taken as a measure of nitric oxide production. FK 506 (0.1 and 1 μg/ml) reduced the LPS-induced increase of NO_2^- levels by 68 % and 81 %, respectively. The impact of cyclosporin A (CsA) was studied in order to compare their effects. CsA (0.1 and 1 μg/ml) decreased the levels of nitrites by 39 % and 69 %, respectively. The results obtained suggest that both immunosuppressive drugs exhibit a dose-dependent inhibitory effect on nitric oxide production and that FK 506 is a more potent agent than CsA in this respect.

Key words

Nitric oxide • NO inhibitor • Activated macrophages • FK 506 • Cyclosporin A

Macrophages play an essential role in immunity responses to many microbial pathogens *in vivo*. Their exposure to cytokines and microbial products induces the generation of nitric oxide (NO).

Nitric oxide is a recently identified messenger with diverse functions throughout the body. It is widely distributed in various tissues and plays a number of physiological functions in immune, cardiovascular, central nervous and other systems (Ignarro 1990, Li *et al.* 1999). NO is formed *via* oxidation of terminal guanidino nitrogen of L-arginine through a reaction catalyzed by nitric oxide synthase (NOS). The synthesis of NO requires NADPH, tetrahydrobiopterin and other cofactors and results in the formation of citrulline (Knowles and Moncada 1994).

Constitutive isoforms of NOS (cNOS) are expressed in neurons, the endothelium and also in other tissues. These enzymes produce small amounts of NO over several minutes in response to agonists that elevate intracellular Ca^{2+}. Other cell types (macrophages, tumor cells, smooth muscle cells, cardiac myocytes, glial cells, keratinocytes) express an inducible isoform of NOS (iNOS) that produces relatively large amounts of NO during hours or days following exposure to cytokines or

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bacterial lipopolysaccharides (Lorsbach and Russell 1992).

The macrolide FK 506 (tacrolimus) is a novel selective immunosuppressive drug which was isolated from *Streptomyces tsucubaensis*. It is gaining wide applications in transplantation surgery and in the treatment of several autoimmune diseases. The immunosuppressive effect of FK 506 results from its action on T lymphocytes inhibiting T-helper cell-dependent production of lymphokines and the transcription of the interleukin-2 (IL-2) gene in several T-cell lines (Dumont *et al.* 1998).

The present study was directed to investigate the possible modulatory effect of FK 506 on NO production induced in isolated macrophages by the addition of the exogenous stimulant *Escherichia coli* lipopolysaccharide (LPS). We also studied the impact of cyclosporin A (CsA), a lipophilic undecapeptide of fungal origin, in order to compare their effects. It has been reported that both agents bind to cytoplasmic proteins (immunophilins) and that both complexes interact with calcineurin (calmodulin-dependent protein phosphatase) to hinder its enzymatic activity. Both agents act at an early stage of the T cell activation process by interrupting the signal transduction pathway (Hutchinson *et al.* 1998).

The present data demonstrate that both drugs decrease NO production by LPS-stimulated macrophages, where FK 506 is more potent in this regard.

Animals. The experiments were performed on male Wistar rats (Velaz-Lysolaje). Eight animals, 7-10 weeks old, were used. All animals were kept under conventional conditions and were acclimatized in our facility for at least 5 days prior to the experiment. They had free access to water and commercial pelleted food.

Materials. Chemicals were obtained from Sigma (Prague, Czech Republic) and from Sevapharma (Prague, Czech Republic). FK 506 was a gift from Fujisawa GmbH (München, Germany), CsA was from Sandoz Ltd. (Basel, Switzerland).

Macrophage cultures. Peritoneal macrophages were isolated by i.p. lavage with 15 ml of sterile saline and resuspended in an incomplete culture medium (RPMI-1640, 7.5 % $NaHCO_3$), plated in flat-bottomed 96-well culture plates and incubated for 2 h hat 37 °C, 5 % CO_2 to allow them to adhere. The number of cells per well was $2x10^5$ and the amount of culture media was 100 µl. Non-adherent cells were removed and macrophage monolayers were then cultured in a complete culture medium (RPMI-1640, fetal bovine serum, 7.5 % $NaHCO_3$, 50 µg/ml gentamicin, 2 mM L-glutamin, 5×10^{-5} M 2-mercaptoethanol) for 24 h. Macrophages were cultured with or without LPS (5 µg/ml) and in the absence or presence of FK 506 (0.1 and 1 µg/ml) or CsA (0.1 and 1 µg/ml). The immunosuppressive drugs were added to the medium containing LPS.

Determination of NO production. Nitric oxide production was determined by measuring the concentration of nitrites (the stable end product of NO) in culture supernatants. This was detected colorimetrically in individual cell-free samples incubated for/ 10 min at room temperature with an aliquot of Griess reagent (1 % sulfanilamide, 0.1 % naphtylethylendiamine, 2.5 % H_3PO_4). The absorbance at 550 nm was recorded using a microplate reader. The NO_2^- concentration was calculated from a $NaNO_2$ standard curve.

Statistical analysis. Data are expressed as means ± S.D. Comparisons were analyzed by Student's test for unpaired data. The differences were considered significant at $P<0.05$.

In the present study we analyzed the modulatory effect of FK 506 and CsA on NO production induced by LPS treatment in cultured macrophages. NO is reactive in oxygenated aqueous solutions and is decomposed to NO_2^- and NO_3^-. A pilot experiment showed that the amount of NO_3^- formed in culture supernatants was negligible compared to NO_2^-. Therefore NO production was determined by measuring NO_2^- levels only.

Macrophage cultures stimulated with LPS (5 µg/ml) produced high levels of nitrites (46.19±13.11 µM). Unstimulated cells exhibited low activity of NOS (5.88±1.91 µM). As is shown in Figure 1, FK 506 (1 µg/ml) significantly decreased NO production by 81 % (8.58±1.44 µM). The inhibitory effects of FK 506 (0.1 µg/ml) and CsA (1 µg/ml) were similar (14.72±4.69 and 14.22±2.54 µM, respectively). CsA (0.1 µg/ml) reduced the production of nitrites by 39 % (28.14±6.26 µM).

Activated macrophages produce large amounts of NO, which are essential for their function. The metabolism of L-arginine to L-citrulline and NO production by activated peritoneal macrophages are well-established (Marletta *et al.* 1988). It was described that the amount of NO produced in the absence of previously activated cells is very low. In agreement with this, our data have demonstrated that macrophages increase their production of NO in response to LPS, a major component of bacterial endotoxin.

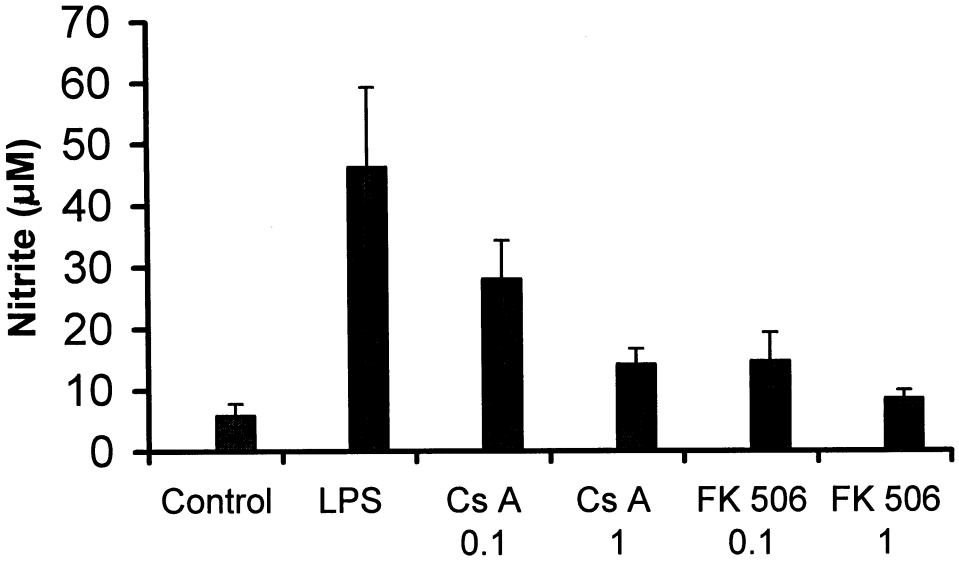

Fig. 1. *Modulatory effect of immunosuppressive drugs on production of nitrites induced by LPS treatment in cultured rat peritoneal macrophages. Macrophages were stimulated with LPS (5 μg/ml) in the absence or presence of cyclosporin A (0.1 or 1 μg/ml) or FK 506 (0.1 or 1 μg/ml). Unstimulated cells served as controls. NO release was determined after 24 h of incubation. Data are shown as means ± S.D. The significance of the difference (calculated by the unpaired t-test) is indicated as $P<0.05$.*

It has been shown that NO production is highly inhibited by 1 μg/ml CsA in activated macrophages (Buckart *et al.* 1992). This concentration is used routinely and is not toxic for macrophages. We compared the effects of CsA and FK 506 on NO production. Both drugs directly inhibit NOS activity, however, FK 506 is a more effective drug than CsA. Some relevant characteristics of iNOS in macrophages concern the fact that its activation is Ca^{2+}-independent and that it binds calmodulin tightly without requiring elevated Ca^{2+}(Nathan 1992, Nathan and Xie 1994). On the other hand, CsA and FK 506 exert their effects by interacting with intracellular binding proteins and thereby forming a molecular complex that binds to the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin (CaN) and hinders its enzymatic activity. The inhibition of CaN function by this complex prevents the dephosphorylation and subsequent nuclear translocation of cytoplasmic components of the nuclear factor of activated T-cells (NF-AT). The activity of this transcription factor is known to correlate with the level of cytokine production (Hutchinson *et al.* 1998). FK 506 and CsA significantly decreased in a dose-dependent manner the generation of NO by LPS-activated macrophages. This fact suggests that both drugs also inhibit the important functions of accessory cells in addition to a direct effect on T lymphocytes.

Although the significance of NO production modulation by FK 506 and CsA needs further study, some recent findings have demonstrated that chronic inhibition of iNOS prevented inflammation (Nussler and Billiar 1993), it probably prolongs graft survival after organ transplantation (Koglin *et al.* 1998, Szabolcs *et al.* 1998). Observations obtained with the novel type of iNOS inhibitors support these assumptions (Chester *et al.* 1998). In spite of the fact that inhibitors of NOS and their effect on physiological functions are intensively studied (Gerová 1999, Pecháňová *et al.* 1999), more work is needed to justify the assumption that NO inhibition or supplementation are involved under diseased conditions.

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