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Inhibitory effect of cyclosporin A and FK506 on nitric oxide production by cultured macrophages. Evidence of a direct effect on nitric oxide synthase activity

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SUMMARY

Casein-elicited peritoneal macrophages from mice were cultured either alone or with interferon-γ (IFN-γ) and bacterial lipopolysaccharide (LPS), and the effect of cyclosporin A (CsA) and FK506 on NO_2^- production (due technical difficulties NO_2^- was taken as the index for NO) was analysed. We observed an inhibitory effect of CsA and FK506 on NO_2^- production. The IC_{50} for NO_2^- production by casein-elicited macrophages was 0·1 μg/ml for CsA and 0·3 μg/ml FK506. The effect of both drugs was dose-dependent and was more clear in non-stimulated macrophages. The presence of IFN-γ and LPS in the culture increased NO_2^- production by casein-elicited macrophages and partially eliminated the inhibition exerted by CsA and FK506. Both drugs acted directly on the nitric oxide synthase (NOS), since CsA and FK506 reduced by 35% and by 17%, respectively, NOS activity in the crude cytosolic fraction. However, CsA and FK506 did not alter $^{14}CO_2$ production from [1-^{14}C]glucose, suggesting that the pentose monophosphate pathway activity was not modified. These data add new insight into the interpretation of the immunosuppressive properties of both drugs.

INTRODUCTION

Nitric oxide (NO) has been identified as a potent and pleiotropic mediator in several processes such as endothelium-dependent vasodilation, neurotransmission and defence against parasites.NO is synthesized by the oxidation of one of two chemically equivalent guanidinium nitrogens of L-arginine.[1] The reaction is catalysed by nitric oxide synthase (NOS). Two isoforms of the enzyme have been found in mammalian cells. One is constitutively expressed, requires Ca^{2+}/calmodulin for its activation and is present in endothelial cells[2] and nervous system.[3] The other is an inducible form of the enzyme, is Ca^{2+}/calmodulin-independent and is present mainly in macrophages. Exposure of macrophages to cytokines and microbial products induces the expression of the enzyme.[4] The simultaneous production of NO and anion superoxide may also form peroxynitrite, which is a much stronger oxidant than NO.[5]

Cyclosporin A (CsA), a lipophilic undecapeptide of fungal origin is a potent immunosuppressor agent widely used to prevent rejection of transplanted organs.[6] The immunosuppressive effect of CsA 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.[7] Recently, we have shown that CsA also reduces the phorbol myristate acetate (PMA)-dependent O_2^- production by macrophages, and that this inhibitory capacity depends of the activation state of macrophages.[8]

The present study analyses the impact of CsA on NO_2^- production (which is formed by the spontaneous oxidation of NO) by cultured macrophages. We also studied a new immunosuppressor agent, FK506,[9] in order to compare their effects. It has been described that both agents bind specific cytoplasmic proteins (immunophylins) and that both complexes interact with calcineurin.[10]

Present data demonstrate that both drugs inhibit, at different rates, NO production by macrophages. This suggests that inhibition of macrophage functions is involved in the immunosuppressive action of CsA and FK506.

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Abbreviations: BH_4, (6R)-tetra-hydro-L-biopterin; CsA, cyclosporin A; DTT, dithiothreitol; IFN-γ, interferon-γ; IL, interleukin; LPS, bacterial lipopolysaccharide; NO, nitric oxide; NOS, nitric oxide synthase (EC 1.14.23); PMFS, phenylmethylsulphonyl fluoride.

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MATERIALS AND METHODS

Materials

Male CD-1 mice (6–10 weeks of age) from IFA-CREDO (Barcelona, Spain) were used. Chemicals were of analytical grade and were obtained from Merck (Barcelona, Spain).

Biochemical reagents and enzymes were from Boehringer Mannheim (Barcelona, Spain) or from Sigma (Alcobendas, Spain). L-[2,3,4,5-^{3}H]arginine monohydrochloride, [6-^{14}C]glucose and [1-^{14}C]glucose were obtained from Amersham Int. (Amersham, UK). Murine interferon-γ (IFN-γ) was purchased from Boehringer Mannheim. Culture media were from ICN (Barcelona, Spain). CsA was kindly provided by Dr J. F. Borel (Sandoz Ltd, Basel, Switzerland). FK506 was a gift from Fujisawa Gmbh (München, Germany).

Macrophage culture

Mice were injected with 1 ml of 2% sterile casein in phosphate-buffered saline (PBS) 3–5 days before harvest of macrophages (referred to as casein-elicited macrophages). In some experiments macrophages were isolated from non-treated mice (referred to as resting macrophages). The cells were resuspended in culture medium (RPMI-1640, 20 mM HEPES, 2 mM L-glutamine, 10% fetal calf serum and antibiotics), plated (5×10^5 cells/300 μl) in flat-bottomed 96-well culture plates and incubated at 37° for 2 hr. Non-adherent cells were removed by washing three times with RPMI-1640, and macrophage monolayers were then cultured in culture medium. The amount of adherent protein per well was 53·21 ± 5·1 μg and the different treatments used did not alter this parameter. The duration of culture as well as the components added to the culture medium are indicated in the figures and tables.

NOS activity assay

The enzyme preparation was obtained from macrophages cultured with 100 U/ml IFN-γ and 0·1 μg/ml lipopolysaccharide (LPS) for 24 hr. Macrophage monolayers were detached using a rubber policeman and resuspended in 50 mM Tris-HCl pH 7·4, 1 mM dithiothreitol (DTT), phenylmethylsulphonyl fluoride (PMFS) (0·1 mg/ml), trypsin inhibitor (0·01 mg/ml) and leupeptin (0·01 mg/ml), at a cell density of 1×10^7 cells/ml. The cells were disrupted by sonication and centrifuged at 100 000 for 30 min at 4°. The supernatant was collected to assay the enzyme activity.

NOS activity was assayed following [^{3}H]citrulline formation from [^{3}H]arginine, as described previously[3] with minor modifications. Briefly, the reaction mixture (100 μl) contained 100 μM L-[^{3}H]arginine (2 μCi/μM), 500 μM NADPH, 20 μM BH_4, 20 μM FAD, 1 mM DTT and 100 000 *g* supernatant in 50 mM Tris-HCl pH 7·4. After 60 min incubation at 37°, the reaction was finished by addition of 400 μl of cold-stop buffer (10 mM EGTA, 100 mM HEPES, pH 5·5, and 1 mM L-citrulline). The reaction mixture was then applied to a 1-ml column of Dowex AG 50W-X4 from BDH (England) (Na^+ form), pre-equilibrated with stop buffer, which was eluted with 2 ml of water. L-[^{3}H]Citrulline was quantified by liquid scintillation.

Nitrite determination

The accumulation of NO_2^- in the culture supernatant was measured with Griess reagent.[11] The NO_2^- concentration was calculated from a $NaNO_2$ standard curve.

Other analytical procedures

$^{14}CO_2$ released from [1-^{14}C]glucose or [6-^{14}C]glucose was measured by collecting $^{14}CO_2$ in 0·2 ml of hyamine in impregnated Whatman filter papers placed in the centre of an Erlenmeyer flask. The radioactivity recovered in hyamine was counted with Biofluor as scintillant in a β-counter, and the flux through the pentose phosphate cycle was estimated from specific $^{14}CO_2$ yields from both [1-^{14}C]glucose and [6-^{14}C]glucose, as described Katz & Wals.[12] Lactate released was measured using a standard spectrophotometric assay.[13] Protein concentration was determined by the Lowry method.[14]

RESULTS

Kinetics of NO_2^- production—effect of CsA and FK506

In studies on macrophages, three states can be considered: Resting macrophages, characterized by a low metabolic activity and low physiological functionality (e.g. production of reactive species); activated macrophages, characterized by an increase in specific activities (e.g. respiratory burst) that allow cells to perform a complex function (e.g. anti-tumour or anti-microbial effects) (this state may be achieved *in vitro* by culturing macrophages with IFN-γ and LPS); an intermediate state termed elicited macrophages, in which the cells are primed to respond to foreign agents or cells.[15]

In the present study NO production by elicited macrophages was investigated, either without additions or with IFN-γ/LPS in the culture media. We analysed the NO_2^- production by cultured macrophages as an index for NO synthesis by these cells. NO is reactive in oxygenated aqueous solution and decomposes to NO_2^- and NO_3^-. Due to the technical difficulties of trying to measure NO, most laboratories use NO_2^- as an index for NO synthesis.[16]

NO_2^- production was dependent of the activation state of the cells. Resting macrophages, after 72 hr of culture, produced the lowest levels of NO_2^- (0·65 ± 0·15 nmol NO_2^-/well). When resting macrophages were cultured in the presence of 100 U/ml IFN-γ and 0·1 μg/ml LPS per well, the NO_2^- production increased 70-fold (45·2 ± 4·8 nmol NO_2^-/well). Intermediate NO_2^- production was observed by casein-elicited macrophages, which oscillated at 15–18 nmol NO_2^-/well. In this case, further addition of IFN-γ/LPS to the culture media also enhanced the NO_2^- production (36–46 nmol NO_2^-/well), and achieved a similar level to that of resting macrophages cultured with IFN-γ/LPS.

The next experiments analysed the effect of two immunosuppressors (CsA and Fk506) on NO_2^- production by elicited macrophages and IFN-γ/LPS-activated macrophages. In Fig. 1, the dose-dependent inhibitory effect of CsA and FK506 on NO_2^- production, by elicited macrophages in culture with or without IFN-γ/LPS, is shown. This inhibition was clear for non-stimulated production, whereas for IFN-γ/LPS-stimulated production it was necessary to increase the concentration of CsA or FK506 10-fold in order to produce a significant decrease in the amount of NO_2^- accumulated in the culture media. The IC_{50} calculated for both drugs showed that the inhibitory effect of CsA was threefold more potent than FK506. Under the conditions used the maximal inhibitory effect was observed at 6×10^{-6} g/ml of CsA and FK506. At this concentration no effect on cell viability was detected. However, concentrations higher than 10^{-5} g/ml significantly reduced the viability of the macrophages, as estimated by trypan blue exclusion.

Figure 2 shows that NO_2^- production progressed very quickly in the first 24 hr of culture, and then it increased more slowly. Figure 2 also illustrates that CsA had inhibited the

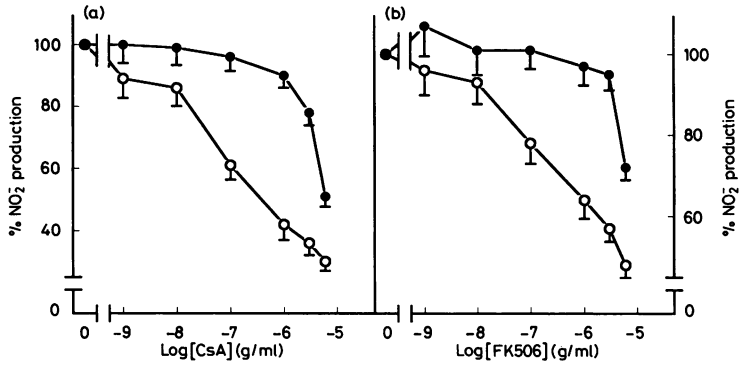

Figure 1. Dose-dependent curves for NO_2^- production by cultured macrophages in the presence of CsA and FK506. Casein-elicited macrophages were cultured in the absence (○) or presence (●) of 100 U/ml IFN-γ plus 0·1 μg/ml LPS. CsA (a) or FK506 (b) was added to the culture medium at the indicated concentrations. After 72 hr the media were collected for analysis of nitrite. The NO_2^- accumulated in the absence of CsA or FK506 was 15·99 ± 0·96 nmol/well in the absence of IFN-γ/LPS, and 46·17 ± 2·16 nmol/well in their presence. These values were normalized to 100% to calculate the percentage of inhibition observed in the presence of CsA or FK506. Data shown are means for four independent experiments.

NO_2^- production already at 24 hr of incubation, whereas FK506 inhibited NO_2^- production only after 48 hr of incubation. In the presence of these agents the NO_2^- production remained constant after 24 hr of culture.

The following experiments were designed to analyse whether *in vivo* CsA administration affected NO_2^- production by macrophages. Table 1 illustrates that macrophages from CsA-treated mice (25 mg CsA/kg body weight) produced less NO_2^- compared to control cells. It is interesting to note that CsA in these conditions was unable to inhibit the IFN-γ/LPS-induced NO_2^- production. These effects of CsA were observed at both 24 hr and 72 hr of culture. This finding reveals the stability of the CsA effect. Due to the low amount of FK506 available, *in vivo* experiments with this drug were not performed.

Effect of CsA on pentose monophosphate shunt and lactate production

In the search for the mechanism responsible for the CsA inhibition on NO_2^- production, two possibilities were analysed. In the first, the effect on NO_2^- production could be the inhibition of NADPH production. It is known that NOS requires NADPH as a cofactor.[17] To investigate this possibility, casein-elicited macrophages were incubated in the absence or presence of IFN-γ/LPS and 10^{-6} g/ml CsA. Flux through the pentose monophosphate shunt was estimated by measuring the amount of $^{14}CO_2$ released from [1-^{14}C]glucose and [6-^{14}C]glucose.[12] The glycolytic pathway was estimated as lactate production. PMA, 100 nM, was used to stimulate the cells. Table 2 shows that CsA did not modify the $^{14}CO_2$ production by elicited and IFN-γ/LPS-activated cells. It is noteworthy that the flux through the pentose monophosphate shunt was greater in IFN-γ/LPS-stimulated than in non-stimulated cells. The pentose monophosphate shunt is the most important pathway to support NADPH levels in the cell.[18] The present data show the failure of CsA to inhibit this

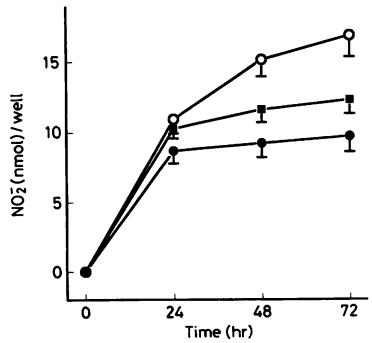

Figure 2. Time-course of the effect of CsA and FK506 on NO_2^- production by cultured macrophages. Casein-elicited macrophages were cultured with the following additions: no additions (○), 3×10^{-7} g/ml CsA (●) and 3×10^{-7} g/ml FK506 (■). The cultures were finished at the indicated times. NO_2^- accumulated in the media was analysed. Values shown are means of four different experiments.

Table 1. Effect of *in vivo* treatment with CsA on NO_2^- production by cultured macrophages

	NO_2^- (nmol/well)	
Additions to culture	24 hr	72 hr
Control mice (n = 12)		
None	11·22 ± 0·36	16·62 ± 0·54
100 U/ml IFN-γ + 0·1 μg/ml LPS	13·80 ± 0·48	37·44 ± 4·38
CsA-treated mice (n = 12)		
None	7·74 ± 0·55*	10·13 ± 0·6*
100 U/ml IFN-γ + 0·1 μg/ml LPS	12·45 ± 0·94	35·56 ± 3·88

After 3 days of i.p. injection of 1 ml 2% sterile casein in PBS, mice were injected with CsA (25 mg/kg weight) dissolved in oleic acid (intradermal injection) or with the solvent alone (control mice) for 3 consecutive days. Macrophages were then harvested and cultured as described in the Materials and Methods, with the indicated additions. Values shown are mean ± SEM of two independents experiments.

* The statistical significance of the difference calculated by unpaired t-test, indicated as $P < 0{\cdot}01$. n, number of animals.

Table 2. Effect of CsA on the flux through the pentose phosphate, and lactate release by cultured macrophages

Additions	(nmol/10^6 cells)	
	$^{14}CO_2$	Lactate
None	6·1 ± 0·3	15·4 ± 1·6
1 μg/ml CsA	6·0 ± 1·3	13·7 ± 0·9
100 U/ml IFN-γ + 0·1 μg/ml LPS	11·9 ± 1·4	30·3 ± 4·7
100 U/ml IFN-γ + 0·1 μg/ml LPS + 1 μg/ml CsA	11·6 ± 1·2	18·4 ± 0·7

Casein-elicited macrophages were cultured in Erlenmeyer flask with the indicated additions, at 37° for 48 hr. After this time, the culture medium was removed and macrophage monolayers were washed three times with Krebs–Ringer bicarbonate (KRB) buffer. Then, 2 ml of KRB containing 1 mM [1-^{14}C]glucose (0·2 μCi/μmol) or 1 mM [6-^{14}C]glucose (0·2 μCi/μmol) was added into the flask and the cells were further incubated for 3 hr at 37°. $^{14}CO_2$ and lactate release were measured as described in the Materials and Methods.

metabolic pathway, and consequently to modify the NADPH levels. The glycolytic flux, measured as lactate released to the incubation medium, was increased twofold by IFN-γ/LPS. This agrees with a previous report showing that glycolysis is an activation signal in macrophages.[19] In the presence of IFN-γ/LPS, CsA exerted an inhibitory effect on lactate production, lowering lactate production near to basal levels. This effect of CsA did not appear in resting cells (Table 2).

Effect of CsA and FK506 on NOS activity

A second hypothesis explaining the inhibitory effect of both drugs on NO production is direct inhibition of the NOS activity. To assess this hypothesis, we assayed NOS activity in the cytosolic fraction of activated macrophages. We observed that activated macrophages presented the maximal activity of NOS. Figure 3 illustrates the inhibitory effect of both drugs on NOS activity, expressed as L-[^{3}H]citrulline formed from L-[^{3}H]arginine. Under these conditions, the inhibitory effect of CsA was higher than that of FK506 on NOS activity, and for both drugs the effect was dose dependent. These data are consistent with those observed in cultured macrophages (Fig. 1). It is noteworthy that the inhibition of NOS activity was greater than that of NO_2^- production by activated macrophages and similar to that by elicited macrophages.

DISCUSSION

The metabolism of L-arginine to L-citrulline and NO production by activated murine peritoneal macrophage are well established. Although this pathway has been associated with killing of tumour cell targets and parasites by macrophages,[20] the role for the physiological interaction of macrophages with other cells has not been established. It has been described that in the absence of a previous activation of cells, the amount of NO produced is very low. In agreement with this, we show here that NO production from activated macrophages (with IFN-γ/LPS) was greater than in resting or elicited macrophages. Recently, it has been shown that *in vivo* administration of

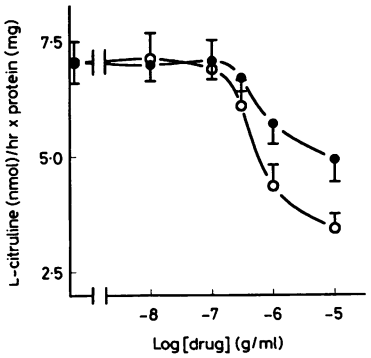

Figure 3. Effect of CsA and FK506 on NOS activity. NOS activity was assayed in macrophage cytosol preparations as described in the Materials and Methods section in the presence of the indicated concentrations of CsA (○) or FK506 (●). The results are the mean ± SEM of three separate experiments performed in triplicate.

glycogen, as an inflammatory stimulus, induces NOS activity in rat peritoneal neutrophils.[21] The use of casein as an inflammatory stimulus and further culture of mouse peritoneal macrophages resembles the above situation with neutrophils. However, other authors have found that elicited macrophages express NOS activity only after treatment with LPS or cytokines.[4,22]

Recently it has been shown that NO production is highly inhibited by 10^{-6} g/ml CsA in activated macrophages.[23] However the present data show very little inhibition at the same CsA concentration in activated macrophages (Fig. 1). As yet there is no easy explanation for this discrepancy. It may be that the experimental conditions were different, since the other authors used only LPS to activate the macrophages, whereas we utilized IFN-γ plus LPS. We also describe for the first time that CsA and FK506 directly inhibit NOS activity (Fig. 3). Some relevant characteristics of NOS induced from macrophages is that activation is Ca^{2+}/calmodulin independent[24] and that it binds calmodulin tightly without a requirement for elevated Ca^{2+}.[25] On the other hand, CsA also binds to calmodulin.[26] This association between calmodulin and inducible NOS could explain the inhibition of NOS activity by CsA and FK506. An analogous example is the inhibition of cyclic nucleotide phosphodiesterase by CsA in a non-competitive form.[27] Phosphodiesterase is another enzyme that binds calmodulin constitutively, in a Ca^{2+}-independent manner.[28] Thus, a similar mechanism for both enzymes, NOS and phosphodiesterase, is suggested.

CsA treatment *in vivo* suggested that CsA alters only the NO production from non-activated cells, having no effect when the cells were activated *in vitro* with IFN-γ/LPS. These experiments did not permit us to characterize *in vivo* the relation between the mechanism of NOS induction and repression. A more complete analysis may come through the study of mRNA NOS induction after *in vivo* treatment with CsA and IFN-γ/LPS simultaneously.

It is noteworthy that the different effects of the immunosuppressors depended on whether the cells were activated or elicited (Fig. 1). It is apparent that activated cells display a greater resistance to the inhibition of NO formation than resting cells. This fact could be explained by a different

structure and/or different amounts of NOS enzyme in the different cell states. A more complex mechanism, in which CsA operates through some target protein(s) (e.g. calmodulin),[26] could be hypothesized.

While the capacity for endogenous nitrate production in humans has been shown clearly,[29] no involvement of L-arginine-dependent NO production in human macrophages has been demonstrated.[30] Since NOS activity has been found in human platelets,[31] human neutrophils[32] and human chondrocytes,[33] it seems unlikely that L-arginine-dependent NO production simply does not exist in human macrophages (reviewed in ref. 34).

Initially, the study of the immunosuppressive effect of CsA focused on its actions on T cells. Today there is sufficient information to include its effects on other immunological cells, as is the case in the inhibition of respiratory burst and IL-1 production by macrophages.[35,36] Together with the respiratory burst, the NO production represents an important pathway for phagocytic cells to respond against host invasion and tumour cells. Thus a more complex mechanism of CsA action could be devised, in which CsA also inhibits the important function of accessory cells in addition to a direct effect on T lymphocytes.

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