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DUAL EFFECTS OF CYCLOSPORINE A ON ARACHIDONATE METABOLISM BY PERITONEAL MACROPHAGES

PHOSPHOLIPASE ACTIVATION AND PARTIAL THROMBOXANE-SYNTHASE BLOCKAGE

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Abstract—Because the oxygenated metabolites of arachidonate synthesized by macrophages, particularly prostaglandins (PG)I_2 and E_2, thromboxane (TX)A_2 and 12-hydroxyeicosatetraenoic acid (12-HETE) have been shown to modulate the immune response of T-cells, we tested the effect of cyclosporine A (CsA), a potent immunosuppressor agent, on arachidonate (AA) metabolism in cultured peritoneal rat macrophages. Endogenous AA release and 12-HETE synthesis were estimated by radiometric HPLC after prelabelling of macrophages with [^{3}H]AA whereas PG were determined either by radiometric HPLC or by direct radioimmunoassay in the culture mediums. Exposure of prelabelled cells for 16 hr to CsA led to a large increase in the release of AA itself and of its oxygenated metabolites, PG and 12-HETE, indicating stimulation of phospholipase activity. This effect was time- and dose-dependent at concentrations of CsA between 2 and 50 μM. There was also a marked increase in the ratio PGI_2/TX, suggesting, in addition to activation of phospholipase, a partial blockade of TX synthase. When macrophages were triggered by A 23187 calcium ionophore (2 μM) or opsonized zymosan (1 mg/ml), the only detectable effect of CsA was a strong and specific inhibition (50%) of TX synthesis. Addition of an excess of exogenous AA (5 μg/ml) to cells treated by CsA confirmed the fact that CsA acted by specifically blocking the transformation of AA into TX without affecting PGI_2 or 12-HETE synthesis. These results demonstrate that CsA acts at two different levels: it promotes phospholipase activation on resting cells but simultaneously induces a partial blockade of TX-synthase. This latter effect predominates when cells are stimulated. The resulting change in the ratio PGI_2/TX promotes immunosuppression to the expense of immunostimulation. This may represent one of the factors underlying the potent immunosuppressive role of CsA.

Macrophages are considered to mediate the response of T-lymphocytes to antigenic stimulation via secretion of a variety of factors including the oxygenated metabolites of arachidonic acid (AA). More precisely, macrophages not only release prostaglandins (PG)I_2 and E_2 which behave as inhibitory signals [1, 2] but also thromboxane (TX) and hydroxyeicosatetraenoic acids (HETE) which stimulate the lymphocyte response to mitogens [3, 4]. In addition, TXB_2, the stable metabolite of TXA_2, antagonizes the inhibitory effect of PGE_2 on Ia antigen expression [5] and 12- and 15-HETE exert a direct mitogenic role [6]. Therefore, any change in the amount or the repartition of the oxygenated metabolites of AA formed by macrophages may be crucial in the modulation of the cellular immune response.

Cyclosporine A (CsA), a potent immuno-suppressive fungal agent, is currently used to prevent and treat allograft rejection. The rejected transplant is infiltrated by a number of cell-types, particularly stimulated mononuclear cells [7, 8] which are chronically exposed to CsA present in the serum of treated patients. The influence of CsA on AA metabolism in macrophages has already been investigated but the results of these studies are conflicting. *In vitro*, Whisler *et al.*. [9] reported an increase of PGE_2 secretion by human monocytes in response to CsA which they attributed to phospholipase A_2 activation whereas Fan *et al.* [10] observed after the same treatment a decrease of PGI_2 secretion in zymosan-stimulated macrophages resulting from phospholipase A_2 inhibition. *In vivo*, there was an increase of TXB_2 secretion by peritoneal macrophages isolated from CsA-treated rats [11]. In none of these studies was the effect of CsA on 12-HETE synthesis examined although this lipoxygenase product represents the most abundant oxygenated AA metabolite secreted by macrophages under resting condition [12, 13].

The present study was designed to address the following questions: (1) Is CsA inhibitory or stimulatory for phospholipase activity of peritoneal macrophages *in vitro*?; (2) What is the effect of CsA on PG production under resting conditions and after stimulation?; (3) Does CsA influence the synthesis of lipoxygenase products?

We could demonstrate a dual action of CsA on peritoneal macrophages *in vitro* including stimulation of phospholipase activity affecting both the cyclooxygenase and the lipoxygenase products and, simultaneously, a partial inhibition of TX synthase activity.

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MATERIAL AND METHODS

Reagents. Cyclosporine A (CsA) was a generous gift from Sandoz (Rueil-Malmaison, France). CsA was dissolved in ethanol at a concentration of 10 mg/ml and this stock solution was used to prepare various concentrations of the drug in the culture medium. Ethanol concentration never exceeded 1%. A 23187 calcium ionophore was from Boehringer (Mannheim, F.R.G.), zymosan A from Sigma Chemical Co. (St Louis, MO, U.S.A), RPMI 1640 culture medium and fetal bovine serum (FBS) from Flow Laboratories (Irvine, U.K.).

Preparation of macrophages. Resident macrophages were obtained from Sprague–Dawley rats as described previously in details [13]. Ten ml of Hank's balanced salt solution, pH 7.3, containing penicillin (100 U/ml), streptomycin (50 μg/ml) and heparin (20 U/ml) were injected into the peritoneal cavity. The cell-enriched fluid was collected and the cells were washed using the same solution. To purify macrophages, peritoneal cells were suspended in RPMI 1640 medium containing 10% FBS and were allowed to adhere for 2 hr at 37° in multiwell dishes (12 wells) in an atmosphere of 5% CO_2-95% air. The non-adherent cells were then removed by three washings with FBS-free RPMI 1640 medium. Each well was plated with 1.5 to 2.0 × 10^6 cells and contained 1 ml of FBS-free RPMI 1640 medium.

Prelabelling of macrophages and study of the release of radioactivity in the medium. The cells present in each well were exposed to 5 μCi of [^{3}H] arachidonic acid (100 Ci/mmol; Centre d'Etudes Nucléaires, Saclay, France) in 1 ml of RPMI 1640 medium supplemented with 10% FBS. After 16 hr at 37°, the supernatants were discarded and the cells were gently washed twice with Krebs buffer containing 2 mg/ml fatty acid-free ablumin. One millilitre of fresh FBS-free RPMI 1640 medium was then added, containing or not the agent to be tested. Total ^{3}H radioactivity released in the medium was measured after 16 hr incubation. In some experiments, total ^{3}H radioactivity was also measured after disruption of the cells by sonication. Liquid scintillation counting was carried out on a 1211 Rackbeta (LKB, Bromme, Sweden) instrument using ACS-2 (The Radiochemical Centre, Amersham, U.K.) as a scintillator.

Cell incubation. Before each experiment, each well was washed twice with FBS-free RPMI 1640 medium and 1 ml of fresh medium was added with or without the drug to be tested. When used in combination with another drug (A 23187 calcium ionophore, zymosan), CsA was added 2 min before the addition of the stimulus. The cells were then incubated for the period of time indicated and the reaction was stopped either by decanting the supernatants for determination of PG or by adding 2 vol of ice-cold methanol for determination of AA and 12-HETE.

Radioimmunoassays. The two major PG synthesized by macrophages were determined by direct radioimmunoassay in the supernatant. Anti-TXB_2 and anti-6 keto-$PGF_{1\alpha}$ antibodies were generous gifts from Dr Hornych (Paris) and Prof. Dunn (Cleveland), respectively. The assay was performed as previously described (14). IC_{50} was 22 pg/tube for TXB_2 and 30 pg/tube for 6 keto-$PGF_{1\alpha}$. The samples were diluted at 1/5 and 1/10 for control cells, 1/50, 1/100 and 1/200 for stimulated cells or cells exposed to CsA for 16 hr.

Radiometric high performance liquid chromatography (HPLC) of AA and 12-HETE. In order to determine AA release and 12-HETE synthesis, we used radiometric HPLC. Macrophages which had been prelabelled with [^{3}H] AA, were incubated for the indicated time at 37° under 5% CO_2–95% air with the drugs to be tested and the reaction was stopped by adding 2 vol of ice-cold methanol to each well. Extraction of AA and 12-HETE was performed as previously described [15] using ether and water in an acidic medium. The ethereal supernatant was dried under nitrogen and resuspended in 100 μl of HPLC eluent. The products were then analysed by straight-phase HPLC on a Varian model 5000 chromatograph equipped with a micropack Si 5 column (4 mm i.d. × 30 cm). The eluting solvent was hexane/ethanol/acetic acid (993:6:1; v/v). The flow rate was 1.5 ml/min. Authentic standards were used to identify AA and 12-HETE peaks. Retention times were 6 and 12 min, respectively.

Radiometric HPLC of PG. In order to examine the complete spectrum of PG produced by macrophages, prelabelled cells were incubated for 16 hr with or without 50 μM CsA. The supernatants were then collected, acidified to pH 3, diluted to 3 ml with H_2O and purified through Sep-PAK C_{18} cartridges as described previously [16]. PG were eluted by 7 ml ethylacetate. After evaporation to dryness under nitrogen, PG were resuspended in the HPLC eluent (acetonitrile/H_2O/acetic acid; 250:750:1, v/v) and injected through a reverse-phase column (ODS 5, 4 mm i.d. × 30 cm). The flow rate was 1.9 ml/min. Retention times of authentic standards were 11, 24, 31 and 37 min for 6 keto-$PGF_{1\alpha}$, TXB_2, and $PGF_{2\alpha}$ and PGE_2, respectively.

Viability of macrophages. Cell viability was assessed by measurement of lactate dehydrogenase activity (LDH) in the medium. Total LDH activity was obtained after disruption of control cells by sonication. Results are expressed as percentage of specific LDH release and were calculated according to the following formula: (Sample LDH−Control LDH)/(Total LDH−Control LDH).

Statistical analysis. Although macrophages were plated at a similar range of 1.5–2 × 10^6 cells/ml, the number of adherent cells varied from one experiment to the other. For this reason, the results expressed in ng of PG produced per ml of medium were somewhat variable. We thus mostly used the Student's *t*-test for paired values. Covariance analysis or regression analysis were also used whenever necessary.

RESULTS

Effect of cyclosporine A on phospholipase activity of peritoneal macrophages

Measurement of the release of AA and its metabolites. Resting rat peritoneal macrophages which had been prelabelled with [^{3}H] AA were incubated for 16 hr under control conditions or in the presence of 50 μM CsA. We measured the release of total ^{3}H radioactivity and more specifically, using radiometric

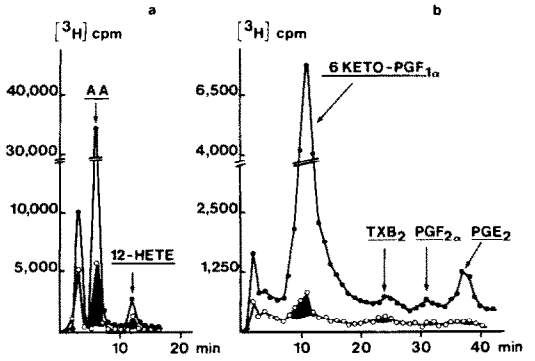

Fig. 1. Effect of CsA on phospholipase activity of peritoneal macrophages. Macrophages which had been prelabelled with [^{3}H] AA were incubated for 16 hr under control conditions (unfilled circles) or in the presence of 50 μM CsA (filled circles). After lipidic extraction, two methods of radiometric HPLC were used: straight phase HPLC to purify [^{3}H] AA and [^{3}H] 12-HETE present in the cells and their incubation medium (a), reverse phase HPLC to purify [^{3}H] PG released in the incubation medium (b). Each of the two elution profiles shown is representative of three different experiments. Arrows indicate the retention times of authentic standards and shaded areas the peaks which have been identified. The earliest peak in Fig. 1a is a non-enzymatic oxidation product of AA.

Table 1. Effect of increasing CsA concentrations on PG secretion by peritoneal macrophages

Added CsA (μM)	TXB_2 (ng/ml)	6 Keto-$PGF_{1\alpha}$ (ng/ml)
0	5.1 ± 1.5	14.3 ± 4.2
2	5.0 ± 1.6	29.0 ± 9.4
5	6.1 ± 1.8	46.1 ± 14.4
10	9.1 ± 2.1	61.0 ± 16.0
50	17.8 ± 4.9	89.2 ± 22.6

Macrophages which had been plated in multiwell dishes were incubated for 16 hr at 37° in RPMI medium in the presence of increasing concentrations of CsA. Supernatants were then collected for TXB_2 and 6 keto-$PGF_{1\alpha}$ radioimmunoassays. The effect of CsA on TXB_2 (6 experiments) and 6 keto-$PGF_{1\alpha}$ (5 experiments) productions was estimated using regression analysis ($r = 0.62$, $P < 0.001$ and $r = 0.59$, $P < 0.01$ for TXB_2 and 6 keto-$PGF_{1\alpha}$, respectively). Results represent means ± SE.

HPLC, that of [^{3}H] AA, both being taken as indexes of phospholipase activity. [^{3}H] 12-HETE, the major metabolite of the lipoxygenase pathway in resting macrophages, and [^{3}H] PG were also determined. In addition to CsA, we studied other agents such as A 23187 calcium ionophore and opsonized zymosan which have been shown extensively to stimulate phospholipase activity. These two products as well as CsA increased the release of ^{3}H radioactivity from the cells. Under control conditions, 109,140 ± 9372 cpm were released whereas 190,560 ± 5867, 299,010 ± 11835 and 259,330 ± 12386 cpm (mean ± SE, N = 8) were found in the medium after incubation of macrophages with CsA (50 μM), opsonized zymosan (1 mg/ml) and A23187 calcium ionophore (2 μM), respectively ($P < 0.01$ vs control). The stimulatory effect of CsA on phospholipase activity was confirmed by measuring AA itself and its metabolites after HPLC purification. As shown in Fig. 1a, control cells released little AA and 12-HETE. Treatment of the cells by CsA resulted in a marked increase of AA production associated with a significant, although more discrete, increase of 12-HETE synthesis.

Control cells generated small amounts of PG (Fig. 1b). PGI_2, measured as its stable metabolite, 6 keto-$PGF_{1\alpha}$, appeared to be the major metabolite, a result already reported [12, 13]. Exposure of macrophages to 50 μM CsA for 16 hr produced an increase in the formation of all the PG detected by HPLC. 6 keto-$PGF_{1\alpha}$ was the most affected whereas TXB_2, the stable metabolite of TXA_2, increased very moderately.

Dose-dependency. The stimulatory effect of CsA on PG production was measured after exposure of the cells for 16 hr at 37° to increasing doses of CsA from 2 to 50 μM. The results, shown in Table 1, indicate that this effect depended upon the dose used. The smallest doses of CsA (2 and 5 μM) were unable to increase TXB_2 formation, but they promoted a significant increase of 6 keto-$PGF_{1\alpha}$ synthesis ($P < 0.05$). Higher doses of CsA (10–50 μM) significantly increased both TX and PGI_2 synthesis. Consequently, the ratio of 6 keto-$PGF_{1\alpha}/TXB_2$ first increased from 2.8 (control) to 5.8 (2 μM CsA) and 7.6 (5 μM CsA). Then, there was a decrease down to 6.7 (10 μM CsA) and 5.0 (5 μM CsA).

Time-dependency. In order to precise the time-dependency of this effect of CsA on phospholipase activity in resting macrophages, two types of experiments were performed: (1) Prelabelled cells were treated by CsA for a short (30 min) or a long (16 hr) period of time and the amounts of [^{3}H] AA and [^{3}H] 12-HETE produced were estimated by radiometric

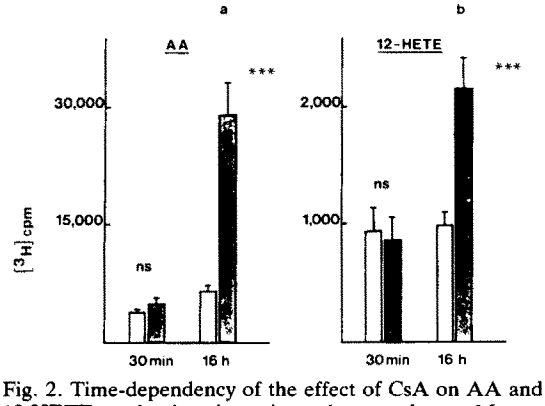

Fig. 2. Time-dependency of the effect of CsA on AA and 12-HETE productions in peritoneal macrophages. Macrophages which had been prelabelled with [^{3}H]AA were incubated for 30 min or 16 hr under control conditions (unfilled bars) or in the presence of 50 μM CsA (filled bars). After lipidic extraction, radiometric straight phase HPLC was used to purify and to measure [^{3}H] AA (a) and [^{3}H] 12-HETE (b). Means ± SE of six experiments are shown. The effect of CsA was estimated using Student's t-test for paired values. ns = non-significant; *** = $P < 0.001$.

Table 2. Time-course of TXB_2 and 6 keto-$PGF_{1\alpha}$ productions (ng/ml) by peritoneal macrophages in response to CsA

Time of exposure (min)	TXB_2		6 Keto-$PGF_{1\alpha}$		Ratio 6 keto-$PGF_{1\alpha}/TXB_2$	
	Control	CsA	Control	CsA	Control	CsA
0	0.4	—	1.4	—	3.2	—
30	0.9	0.8	3.4	6.2	3.8	7.8
60	1.4	1.4	5.2	13.4	3.7	9.6
180	2.5	4.9	8	37	3.2	7.6
240	2.3	5.8	7	42	3.0	7.2
360	2.4	10.4	7.5	60	3.1	5.8
960	2.8	10.8	7.4	59.4	2.6	5.5

Macrophages were incubated in RPMI alone (control) or supplemented with 50 μM CsA. The supernatants were removed at the indicated times and PG were assayed by RIA. Values are means of two determinations. The effects of CsA on the time-courses of TXB_2 and 6 keto-$PGF_{1\alpha}$ productions were estimated using covariance analysis ($P < 0.01$ for both parameters).

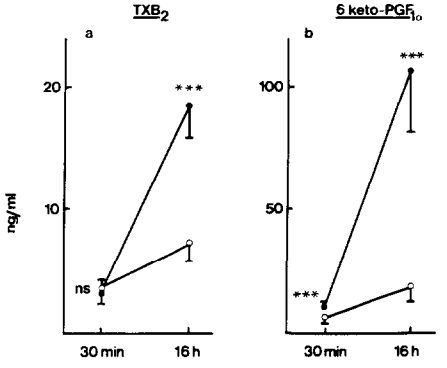

Fig. 3. Time-dependency of the effect of CsA on PG production in peritoneal macrophages. Macrophages were incubated for 30 min or 16 hr under control conditions (unfilled circles) or in the presence of 50 μM CsA (filled circles). TXB_2 (a) and 6 keto-$PGF_{1\alpha}$ (b) were measured in the incubation medium by direct RIA. Means ± SE (ng/ml of medium) of 9–12 experiments are shown. The effect of CsA was estimated using Student's t-test for paired values. ns = non-significant; *** = $P < 0.001$.

HPLC; (2) unlabelled macrophages were exposed to CsA for the same periods of time and PG were directly determined in the supernatants by radioimmunoassay. Although the amount of AA released by macrophages exposed for 30 min to CsA (Fig. 2a) tended to increase, the admitted degree of statistical significance ($P < 0.05$) was not reached. However, after 16 hr of treatment, macrophages released a much greater ($P < 0.001$) amount of AA than control cells, a result consistent with phospholipase activation. The results in Fig. 2b demonstrate that, after 30 min of treatment, 12-HETE production was not modified whereas it increased significantly after 16 hr of exposure to CsA ($P < 0.001$).

The results for PG secretion are shown in Table 2 and Fig. 3. In a preliminary experiment, a complete time course study was achieved and showed that CsA-induced increase of PG production was time-dependent. However, it appeared clearly that CsA affected differently TXB_2 and 6 keto-$PGF_{1\alpha}$ levels. Whereas an increase in 6 keto-$PGF_{1\alpha}$ was already observed at 30 min, TXB_2 level remained stable until 60 min and increased only after 3 hr of CsA treatment. Accordingly, the ratio 6 keto-$PGF_{1\alpha}/TXB_2$ which was fairly constant for control cells (2.6–3.8) varied with time for CsA-treated cells. From 3.2 at zero time, it peaked at 9.6 at 60 min and then slowly decreased to 5.5 at 16 hr, while remaining higher than in control cells (5.5 vs 2.6).

To further confirm these preliminary results, suggesting a different time-dependent effect of CsA on TXB_2 and 6 keto $PGF_{1\alpha}$ levels, a short (30 min) and a long (16 hr) incubation time were chosen and the experiment was repeated (N = 9–12). As shown on Fig. 3a, if addition of CsA to the cells had no effect on TX synthesis at 30 min, it promoted a significant increase (× 2.5; $P < 0.001$) after 16 hr. In contrast (Fig. 3b), a small but significant increase already occurred after 30 min for 6 keto-$PGF_{1\alpha}$ (× 1.6; $P < 0.001$) and was followed by a six-fold increase after 16 hr ($P < 0.001$). Consequently, the ratio 6 keto-$PGF_{1\alpha}/TXB_2$ increased from 1.9 to 3.3 and from 2.3 to 5.5 at 30 min and 16 hr, respectively. Thus, subsequently to the endogenous release of AA, 6 keto-$PGF_{1\alpha}$ was much more stimulated by CsA than TXB_2, a result which suggests a partial but specific blockade of TX synthase.

LDH release. It was verified by assessing LDH release, chosen as an index of cytolysis, that the effects of CsA on PG production were not due to cell death. LDH in the medium of macrophages which had been incubated for 30 min with CsA was not different from that found under control conditions. It averaged 9 IU/ml, i.e. 3% of the total LDH whatever the dose of CsA used. After 16 hr of exposure to CsA, there was a dose-dependent increase in LDH release that did not exceed 30% of the total LDH content (2 μM: 4.8%, 5 μM: 9.3%, 10 μM; 22.1%, 50 μM: 29.9%). Thus, although CsA definitely exerts, as already reported [19], a cytotoxic effect on the cells, there was still 95% of viable cells after 16 hr of exposure to 2 μM CsA. ^3H radioactivity release from prelabelled macrophages exhibited a parallel relationship with CsA concentration (2 μM = 5.3%; 5 μM = 7.95%; 10 μM = 16.8%; 50 μM = 28.7%) and there was a significant correlation ($r = 0.98$; $P < 0.05$) between ^3H radioactivity and LDH releases.

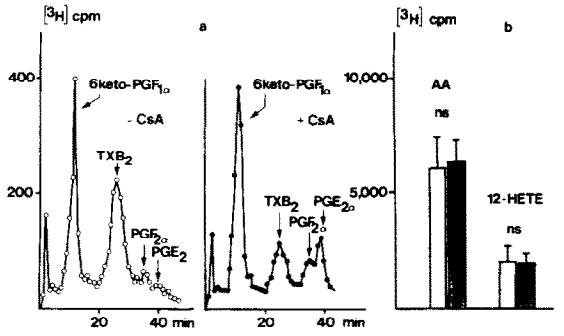

Fig. 4. Effect of CsA on the production of AA, 12-HETE and PG in stimulated peritoneal macrophages. Macrophages which had been prelabelled with [^{3}H] AA were stimulated by 2 μM A 23187 calcium ionophore for 30 min with or without 50 μM CsA. [^{3}H] PG were purified by reverse phase HPLC. Two elution profiles (a) are shown corresponding to incubations with (filled circles) or without (unfilled circles) CsA. [^{3}H] AA and [^{3}H] 12-HETE were purified by straight phase HPLC. Means ± SE of six experiments are given (b). Filled and unfilled bars correspond to incubations with and without CsA respectively. The effect of CsA was estimated using Student's *t*-test for paired values. ns = not-significant.

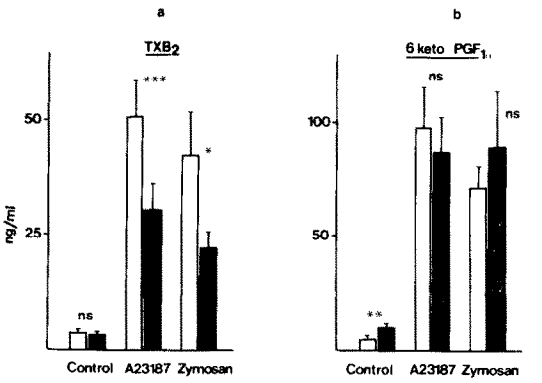

Fig. 5. Effect of CsA on TXB_2 and 6 keto-$PGF_{1\alpha}$ synthesis in stimulated peritoneal macrophages. Macrophages were exposed to two stimuli (2 μM A 23187 calcium ionophore or 1 mg/ml opsonized zymosan) for 30 min at 37° in the absence (unfilled bars) or the presence (filled bars) of 50 μM CsA. The supernatants were collected for TXB_2 (a) and 6 keto-$PGF_{1\alpha}$ (b) assays. Means ± SE of 4–10 experiments are shown. The effect of CsA was estimated using Student's *t* test for paired values. ns = non-significant; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

Effect of cyclosporine A on TXB_2 formation

Selective blockade of TXB_2 formation. To further document the partial blockade of TX synthase in resting cells exposed to CsA, we used stimulated macrophages. Macrophages which had been prelabelled with [^{3}H] AA, were triggered by 2 μM A 23187 calcium ionophore, a soluble agent stimulating phospholipase A_2. It was verified that, at this concentration, the drug enhanced AA release as well as 12-HETE and PG synthesis (unshown results). After 30 min at 37°, the [^{3}H] AA metabolites produced by stimulated cells which had been or not exposed to CsA were determined by radiometric HPLC. The profile of PG synthesized by CsA-treated cells (Fig. 4a) showed a selective inhibition of TXB_2 synthesis, the other PG being unaffected (6 keto-$PGF_{1\alpha}$) or even stimulated (PGE_2, $PGF_{2\alpha}$) by the drug. Results in Fig. 4b demonstrated that neither 12-HETE formation nor AA release were significantly modified by CsA treatment. When opsonized zymosan, a particulate stimulus also known to stimulate phospholipase A_2, was tested, similar results were obtained. There was a specific inhibition of [^{3}H] TXB_2 synthesis (266 vs 107 cpm for controls cells vs CsA-treated cells, respectively), no inhibition of 12-HETE synthesis (1284 vs 1030 cpm for control cells vs CsA-treated cells, respectively) and no change in AA release (41970 vs 37232 cpm for control cells vs CsA-treated cells, respectively). To quantify better the selective inhibition of TXB_2 observed in stimulated macrophages, we studied the effect of treatment by CsA on unlabelled macrophages which had been stimulated by A 23187 or opsonized zymosan, and we measured the level of PG in the supernatants by direct radioimmunoassay. The results in Fig. 5 show that both soluble and particulate stimuli, capable of stimulating phospholipase A_2, strongly enhanced PG synthesis by macrophages. Pretreatment of the cells by CsA resulted in a selective inhibition of TXB_2 (Fig. 5a) whereas 6 keto-$PGF_{1\alpha}$ was unaffected (Fig. 5b).

Time-dependency. In contrast with the effect of CsA on phospholipase activity which increased with time, TX inhibition fully occurred at 30 min. When ionophore- or zymosan-stimulated macrophages were exposed to CsA for a longer period (16 hr), the inhibition observed at 30 min was still apparent. In order to differentiate the short-term from the long-term effect of CsA on stimulated macrophages, the productions of TXB_2 over two successive periods of 0–30 min and 30 min–16 hr were measured (Table 3). In resting macrophages, CsA stimulated TXB_2 synthesis only during the second period (30 min–16 hr). Two agents, A 23187 calcium ionophore and opsonized zymosan, were used to stimulate macrophages. The effect of the former was immediate but did not persist whereas that of the latter was as much marked during the early period as later on. CsA inhibited considerably both ionophore- and zymosan-dependent TXB_2 syntheses. Therefore, it can be concluded that the inhibitory effect of CsA on TX synthase is immediate but long-lasting.

Mechanism of action. To define the level at which CsA exerted its inhibitory effect on TX production, we added an excess of AA (5 μg/ml) to cells which were incubated with or without CsA so as to by-pass the step of phospholipase activation. The results are shown on Fig. 6 and demonstrate that TX inhibition occurs further than AA release. Indeed, TXB_2, but not 6 keto-$PGF_{1\alpha}$ concentration, was significantly reduced in the medium of cells treated by CsA even when a large excess of AA was added. This inhibition in TX synthesis was selective since we verified that 12-HETE formation measured in the presence of an excess of [^{14}C] AA (5 μg/ml) was not affected by CsA treatment (9,481 cpm for CsA-treated cells vs 9921 cpm for control cells, N = 2).

Table 3. Effect of CsA on TXB_2 production (ng/ml) by resting or stimulated peritoneal macrophages

Addition	Period of production	
	0–30 min	30 min–16hr
None	3.0 ± 0.7	3.0 ± 1.1
CsA (50 μM)	2.5 ± 0.7	13.5 ± 1.9*
A 23187 calcium ionophore (2 μM)	42.5 ± 10.3	2.8 ± 2.6
A 23187 calcium ionophore (2 μM) + CsA (50 μM)	21.3 ± 5.7*	3.9 ± 2.6
Opsonized zymosan (1 mg/ml)	36.8 ± 6.9	59.1 ± 13.6
Opsonized zymosan (1 mg/ml) + CsA (50 μM)	18.6 ± 0.8*	27.8 ± 10.8*

Macrophages were exposed to CsA and to various stimuli for 16 hr. TXB_2 production was measured in the same medium after 30 min and 16 hr incubations. The amount of TXB_2 synthesized between 30 min and 16 hr was calculated by subtracting from the total amount of TXB_2 found at 16 hr that measured after 30 min. Results are means ± SE of five experiments. The effect of CsA on TXB_2 production was estimated under control conditions and in the presence of each of the various stimuli using Student's t-test for paired values. * $P < 0.05$.

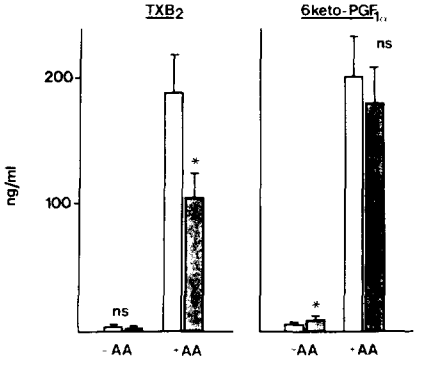

Fig. 6. Effect of an excess of AA on CsA dependent-PG production in peritoneal macrophages. Macrophages were incubated with (filled bars) or without (unfilled bars) 50 μM CsA in the presence or in the absence of 5 μg/ml AA. The supernatants were collected for TXB_2 (left) and 6 keto-$PGF_{1\alpha}$ (right) assays. Means ± SE of 5–6 experiments are shown. The effect of AA was estimated using Student's *t*-test for paired values. ns = non-significant; * = $P < 0.05$.

DISCUSSION

Our results demonstrate that CsA affects arachidonate metabolism in resting peritoneal macrophages at two different levels. First, the availability of endogenous AA is augmented by CsA, as judged by the increase of ^3H radioactivity and, more specifically, of ^3H AA itself from the cells which had been prelabelled with ^3H AA after treatment of the cells. ^3H radioactivity release reached 1.75 times the control value. It was less marked than that obtained in the presence of well-known stimulatory agents of phospholipase activity such as opsonized zymosan (× 2.74) or A 23187 calcium ionophore (× 2.38). Secondly, the profile of PG resulting from the conversion of AA is considerably modified to the detriment of TX, as shown by the following results: (i) The ratio 6 keto-$PGF_{1\alpha}$/TXB_2 was considerably increased (from 2.5 to 5.8) after exposure of the cells to CsA; (ii) CsA markedly blocked (50% inhibition) the synthesis of TXB_2 in macrophages stimulated by A 23187 calcium ionophore or zymosan; (iii) CsA inhibited the transformation of exogenously supplied AA into TXB_2. The effect of CsA on phospholipase activity not only affected PG but also the lipoxygenase product, 12-HETE, which considerably increased. In contrast to what was observed for TX, there was no inhibitory effect of CsA on the transformation of AA into 12-HETE. Taken together, it can be concluded that the treatment of resting macrophages by CsA promotes the release of free AA into the cytosol. The greater availability of AA results in the enhanced synthesis of its oxygenated metabolites, particularly 6 keto-$PGF_{1\alpha}$, TXB_2 and 12-HETE. Because of the specific blockade exerted by CsA on the conversion of endogenous AA into TX, the level of TXB_2, although augmented, cannot increase at the same extent as those of the other prostanoids, which results in an elevated 6 keto-$PGF_{1\alpha}$/TXB_2 ratio.

On the contrary, an acute and durable inhibitory effect of CsA on TX synthesis occurs when cells are stimulated (Table 3). All these observations may contribute to explain some discrepancies of the literature about the *in vitro* effect of CsA on PG production. When stimulated cells were used as in the studies of Kurtz *et al.* [18] with vascular smooth muscle cells or Fan *et al.* [10] with macrophages, CsA was considered to be an inhibitory agent of PG synthesis. In these studies, FBS, which was present in the culture medium of the cells exposed to CsA, may have triggered PG production by providing more AA [19]. Under these conditions, the stimulatory effect of CsA cannot be demonstrated; only its inhibitory effect is apparent. On the contrary, if resting cells are used, CsA behaves as a strong stimulatory agent of PG synthesis by increasing arachidonate availability for transformation via the cyclooxygenase and lipoxygenase pathways. Such a

stimulatory effect of CsA has been already reported by Whisler *et al.* [9] on human monocytes and Zoja *et al.* [17] on endothelial cells. The question as to whether this effect only reflects CsA toxicity on the cells may be raised. It is clear that a long exposure of the cells to CsA was associated with an increased index of LDH release which indicates the cytotoxic effect of the drug. Moreover, ^3H radioactivity and LDH releases were closely correlated. Thus, the stimulation by CsA of phospholipase activity in cultured macrophages could, in part, reflect a toxic side-effect as already reported on endothelial cells [17]. However, after 30 min of exposure to CsA, no additional LDH release was observed, even for the highest dose tested (50 μM) although 6 keto-$PGF_{1\alpha}$ was already augmented.

How our *in vitro* results may be interpreted in comparison with those obtained in rats treated *in vivo* by CsA has to be considered. Indeed, several investigators reported that chronically *in vivo* administered CsA stimulated the urinary output of TXB_2 with [20, 21] or without [22–24] any change in 6 keto-$PFG_{1\alpha}$ and PGE_2 excretion. In addition, Rogers *et al.* demonstrated an increased production of TXB_2 both in the renal cortex and peritoneal macrophages of rats treated *in vivo* by CsA. No information was given on the level of 6 keto-$PGF_{1\alpha}$ synthesis. Taken together, these results indicate that the partial blockade of TX synthesis by macrophages *in vitro* is not sufficient to conceal the activation of phospholipase and blunt the resulting increase in PG production observed *in vivo*. Moreover, our data provide no information on the effects of CsA on PG production by the resident renal cells which are likely to be the major source of urinary PG. Finally, the blockade of TX synthase may occur acutely and thus not influence the urinary excretion of TXB_2 in chronically treated rats. Interestingly, the lowest active concentration of CsA used in this study (2 μM) corresponds to the peak level observed in the blood of patients in the course of renal transplantation 4 hr after oral administration of the drug [25].

The mechanism whereby CsA blocks TX formation is still imprecise. Inhibition was observed on cells stimulated by A 23187 calcium ionophore or opsonized zymosan, two agents which increase intracellular calcium. Ca^{2+}-movements could be implied in the mechanism of this blockade. Their role was already suggested by McMillen *et al.* [26] who reported that verapamil, a Ca^{2+}-channel blocking agent, potentiated the effect of CsA on the proliferation of human lymphocytes. It has also been shown that CsA binds to calmodulin [27] and it has been proposed that this drug could increase the total calcium content of the cell beyond the capacity of the intracellular organelles to maintain a normal cell homeostasis [28]. This would lead to irreversible cell injury via secondary activation of the Ca^{2+}-sensitive phospholipases. This hypothesis would be in accordance with the lag-period observed for the effect on phospholipase activity. Thus, the proposed mechanism of the dual effects of CsA, phospholipase activation and TX blockade, could imply Ca^{2+}-signalling.

CsA has been reported to inhibit lymphocyte proliferation [29, 30, 31], particularly after A 23187 ionophore stimulation. Mesangial cell growth can be also affected [32]. It is possible that the specific blockade of TX synthesis documented in our study is implied in this effect since TX has been shown to counteract the inhibition of cell proliferation observed in the presence of PGE_2 or PGI_2 [3, 5]. The partial blockade of TX synthesis by CsA could reinforce the acute immunosuppressive effect of this drug which, via its effect on phospholipase activity, stimulates the production of PGE_2 and PGI_2. The consequence would be an inbalance to the advantage of the immunosuppressive mediators.

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