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Inhibition of prostacyclin formation by cyclosporin is not due to reduced availability of arachidonic acid in membrane phospholipids of cultured human endothelial cells

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Cyclosporin (CS) is a potent immunosuppressive agent widely used in all forms of organ transplantation, however, the clinical effectiveness of cyclosporin is limited by its nephrotoxicity. Both the mechanism of nephrotoxicity and the action of CS at the molecular level remain unclear. Cyclosporin nephrotoxicity has been associated with altered renal haemodynamics, and suppression of prostaglandin systems [1–3]. Vasodilator prostaglandins, play a central role in the regulation of renal blood flow, and a major component of the haemodynamic changes might be explained by inhibition of prostacyclin (PGI_2).

We had previously shown that CS caused a dose and time-dependent inhibition of PGI_2 production, when cultured human umbilical vein endothelial cells (HUVEC) were stimulated with either exogenous arachidonic acid (AA) or thrombin, and that CS did not inhibit PGI_2-synthetase, indicating that inhibition of PGI_2 production was proximal to this enzyme [4, 5]. Our findings suggested that CS acts at the level of cyclo-oxygenase, but we wanted to exclude the possibility that it might act at the membrane level by altering the availability of arachidonic acid (AA) from membrane phospholipids.

We have therefore examined the effect of CS on the distribution of [^{3}H]AA in membrane phospholipids of HUVEC.

Materials and methods

Cell cultures. Endothelial cells were harvested from human umbilical veins by treatment with 0.1% collagenase according to the method described by Jaffe *et al.* [6]. Cells were established in tissue culture and characterized as endothelial cells according to the method described previously [4].

Cellular lipids. Cells (2×10^5/mL) were plated into six-well tissue culture plates. Confluent cells were incubated for 20 hr at 37° in the presence of 1 mL of medium 199 + 20% FCS containing 0.5 μCi of [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (sp. act. 210 Ci/mmol, Amersham, Bucks, U.K.). Monolayers were washed and cultured for a further 24 hr at 37° with either 1 mL of CS (5 μM), equivalent dilution of vehicle (absolute alcohol), or medium alone (M199 + 20% FCS). At the end of the incubation period, the cells were immediately scraped in 1 mL of ice-cold 10 mM hydrochloric acid. Cell lipids were extracted, separated and quantified using a method described by Emilsson and Sundler [7]. The TLC plates were sectioned and radioactivity determined by liquid scintillation spectrometry. Radioactivity incorporated into each lipid species is expressed as a % of total radioactivity incorporated. These values are then expressed as a % of vehicle control values.

Prostacyclin release. Endothelial cells (2×10^5/mL) were seeded in to 24 multiwell plates. Confluent cells were washed and treated for 24 hr at 37° with either 500 μl of CS (5 μM), vehicle (absolute alcohol), or medium alone (M199 + FCS). Incubations were terminated by washing the cells, monolayers were then either stimulated with 20 μM arachidonic acid or 2 units/mL human thrombin for 15 min. Culture supernatants were assayed for PGI_2 by measuring the stable end product 6-KetoPGF$_1\alpha$ using standard radioimmunoassay technique as described previously [4]. For each experiment the concentration of 6-KetoPGF$_1\alpha$ (ng/mL/10^5 cells) in supernatants was expressed as a percentage of vehicle control.

Cell toxicity was assessed by measuring uptake of [^{3}H]adenine uptake according to the method described by deBono *et al.* [8]. Results are expressed as the mean percentage (± SE) of control release.

Results and discussion

After 20 hr of incubation with 0.5 μCi of [^{3}H]arachidonic acid, 60 ± 6.3% of initially added radioactivity was recovered in endothelial cell lipids (mean ± SE of six separate experiments, each carried out in duplicate. 100% corresponds to mean ± SE of 419210 ± 22011 dpm over all experiments). The incorporated radioactivity was detected in the phospholipids with the following decreasing order: phosphatidylethanolamine (PE) (48.5 ± 5%), phosphatidylcholine (PC) (31 ± 2.5%), phosphatidylinositol (PI) (11 ± 2.5%) and phosphatidylserine (PS) (7.5 ± 1.5%). Only 2.1% of the incorporated radioactivity remained as unesterified arachidonate.

Cyclosporin had a complex effect on the distribution of AA in membrane lipids of HUVEC, which resulted in increased amounts of free arachidonic acid (132 ± 10.5%) and marked decrease in PC (81.5 ± 2.5%) (Fig. 1). The source of the AA appeared to be PC, indicating activation of phospholipase A_2 (PLA_2), an effect which has been attributed to the involvement of calcium activated PLA_2 [9, 10]. Cyclosporin on its own, in a dose range of 0.1 to 5 μM did not cause PGI_2 release from HUVEC at 24, 48 or 96 hr. The current data demonstrate that although there is abundant free AA in CS treated HUVEC, PGI_2 production is decreased when thrombin was used as an agonist (Table 1), indicating that CS would appear to act distal to phospholipase A_2. This was further corroborated by our finding that CS was able to inhibit PGI_2 production even when AA was

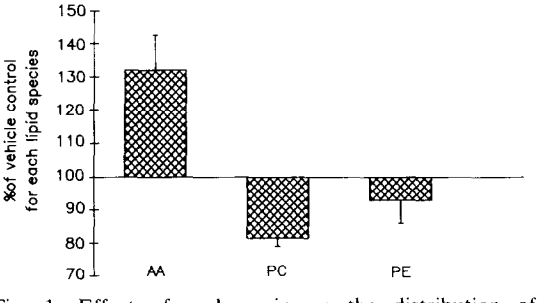

Fig. 1. Effect of cyclosporin on the distribution of [^{3}H]arachidonic acid-associated radioactivity in membrane lipids of unstimulated HUVEC. The [^{3}H]AA labelled cells were treated with cyclosporin (5 μM), vehicle or media alone for 24 hr. Each point represents the mean of 4–6 experiments. Radioactivity incorporated into each lipid species is expressed as a percentage of total ratioactivity incorporated. These values are then expressed as a percentage of vehicle control values.

Table 1. Effect of cyclosporin on 6-KetoPGF$_1\alpha$ formation and endothelial cell cytotoxicity

Concentration of drug	Agonist	6-KetoPGF$_1\alpha$ (%)	[^{3}H]Adenine (%)
5 μM CS	20 μM AA	68 ± 4	109 ± 10
5 μM CS	2 units/mL TH	60 ± 6	90 ± 13

Assays were performed as described under Materials and methods. Results represent mean ± SE from 4–6 separate experiments, each carried out in duplicate and are expressed as the percentage of vehicle control.

used as an agonist (Table 1). The latter observation is in agreement with those of Voss *et al.* [11]. Cyclosporin at 5 μM concentration induced no detectable cell damage when compared with control cells as measured by [^{3}H]adenine uptake assay (Table 1).

Studies with lymphocytes have suggested that CS interfers with the activation of T lymphocytes by inhibiting the activation of acyltransferase [12]. Although inhibition of acyltransferase by CS may explain the increase in free AA observed, it does not account for the reduced PGI_2 production seen when exogenous AA is provided. Thus CS may exert its inhibitory effect at more than a single site. The alternative site of inhibition inferred from our data is cyclo-oxygenase, and, indeed, Gordon *et al.* [13] have reported that CS inhibits PGI_2 formation in HUVEC by reversibly inhibiting cyclo-oxygenase.

In rat mesangial cells, however, CS, in a dose-dependent manner, inhibited A23187 and angiotensin II induced release of [^{3}H]-labelled arachidonic acid [14]. Suggesting that the observed reduction in PGE_2 formation was probably due to inhibition of AA release rather than an effect on cyclo-oxygenase activity. The apparent discrepancy between our study and that of Stahl *et al.* [14], might reflect differences in cell type as well as variations in the experimental procedure used. Our study allows us to determine the effects of CS on phospholipid hydrolysis and on AA metabolism in the absence of an agonist. In contrast, mesangial cell experiments were performed after 3 hr of incubation with radioactive arachidonate, and measured the release of [^{3}H]AA and PGE_2 following agonist stimulation. Thomas *et al.* [15] have demonstrated that a longer labelling time is required in order to reach an intracellular distribution of the tracer which reflects the endogenous distribution of AA between the various cell lipids.

In summary, our studies have shown that CS inhibits PGI_2 production in HUVEC, that this inhibition is not overcome when exogenous AA is supplied, that the inhibitory action of CS is proximal to PGI_2 synthetase and finally that there is abundant free AA available in membrane phospholipids of CS treated HUVEC [4, 5]. In conclusion, CS does not appear to inhibit PGI_2 synthesis by reducing the availability of free AA in the endothelial cell membrane. Although CS appears to inhibit cyclo-oxygenase, we can not exclude an additional effect on acyltransferase.

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Bovine albumin–HABA interaction: re-analysis of earlier observations indicates that ligand-induced dimerization and a competitive contaminant operate simultaneously

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The carboxylic acid 2-(4′-hydroxybenzeneazo)benzoic acid (HABA) binds to human and bovine albumin and this dye is a useful model ligand for the investigation of ligand–acceptor interactions. There are a substantial number of reports which indicate that the number of binding sites (n) and/or the apparant association constant (K_a) are dependent upon protein concentration for a variety of ligand–acceptor systems [1, 2]. Under appropriate experimental conditions the results can give a Scatchard [3] plot with a positive slope [4, 5]. This is the case for HABA [6, 7] and a variety of other systems including some containing receptors for drugs and hormones [2]. The interaction of HABA with *human* albumin showed the phenomenon of inverse dependence [6, 7] and a quantitative analysis of these observations indicated that the most likely explanation for this dependence was that the high affinity binding site on human albumin for HABA was contaminated to the extent of about 95% with a competing ligand [2]. The contaminant in this context was probably an endogenous ligand retained by the albumin molecule during its isolation and purification [2].

The binding of HABA to *bovine* albumin also exhibited anomalous binding behaviour which included an effect of protein concentration. Scatchard plots of some of the data obtained by variation of the ligand concentration appeared to exhibit a maximum near the ordinate [7]. These experiments covered a much larger range of ligand concentrations than did the earlier work with human albumin [6] and the protein concentration dependence appeared to differ from that observed with the latter. No explanation was provided by Clegg and Lindup [7] for their results with bovine albumin and HABA and so these earlier data have been subjected to quantitative analysis with the intention of understanding the molecular basis for the observed protein concentration dependence. It is hoped that this approach will be applied more generally in due course to systems, such as those involving drug and hormone receptors, which have more immediate biological importance.

Materials and methods

Binding of HABA to bovine albumin. The three sets of data which were analysed were for the binding of a range of HABA concentrations (50 to 5000 μM) to bovine albumin at each of three different concentrations (1, 2 and 4% w/v) measured by equilibrium dialysis at 37° and pH 7.4 (see Fig. 1 in Ref. 7).

Analysis of experimental data. Each set of data was fitted separately to two model equations, by a non-linear least squares regression method. The Adair equation (no. 5 in Ref. 8) was applied because it is the most general description of the binding of a single ligand to a non-interacting protein. Polysteric effects are not included but given this restriction it covers all binding models for a single ligand and the best one can be derived from the Adair constants β_j [8]. The Adair constants β_j where j goes from one to the total number of sites needed in the description are the equilibrium constants for the j-th overall reaction of j ligands X with the protein P.

$$P + jX \rightleftarrows PX_j\ (\beta_j).$$

It is the Adair constants that are estimated by the fitting procedure, but the results are often given more conveniently as the stepwise (or consecutive) association constants K_j which are the equilibrium constants for the reactions:

$$PX_{j-1} + X_j \rightleftarrows PX_j\ (K_j).$$

These two equivalent sets of equilibrium constants are related by

$$\beta_j = K_1, K_2 \ldots . K_j.$$

The Adair equation does not contain the protein concentration however and so cannot explain the effect of this on binding. The success or failure of the Adair equation to describe the experimental data, the shape of the binding curve and the dependence of the Adair constants upon protein concentration can nevertheless all provide information or clues about the molecular binding mechanism.

The data were also fitted to the competitive contaminant model [1] since this model provided a quantitative explanation for the protein concentration dependence observed in the interaction of HABA with human albumin [2]. This model is based on the proposition that some of the binding sites for ligand X on protein P are contaminated by another ligand Y. The competition for these sites is assumed to be