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Flow Cytometric Analysis of the In Situ Hybridization of Cyclooxygenase Isoforms in Mesangial Cells Treated with Cyclosporine A

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Background: Cyclosporine A increases oxidative stress in kidney and we hypothesized that cyclooxygenase (COX) may be involved in this effect.

Material and Methods: Mesangial cells of Cyclosporine A-treated (4, 7 or 10 days) rats were obtained to evaluate mRNA expression of COX-isoforms (COX-1, constitutive and COX-2, inducible) by "in situ" hybridization. Probes were labelled using "Gene Image Random Prime Labelling Protocol" and COX expression was measured by flow cytometry.

Results and Discussion: "In situ" hybridization by flow cytometry is an useful method to detect mRNA. We observed an increased COX-2 expression in a time-dependent manner in parallel with Reactive Oxygen Species synthesis. COX-1 expression increased only at 10 days.

Key terms: cyclosporine; cyclooxygenase; oxidative stress; reactive oxygen species; flow cytometry; in situ hybridization

Cyclosporine A (CsA) is an immunosuppressor widely used for prevention of organ rejection in transplant patients and in treatment of several diseases such as uveitis, rheumatoid arthritis, primary biliary cirrhosis, or psoriasis. Unfortunately, this drug has some toxic effects, with nephrotoxicity being its main adverse effect.

Several mechanisms and some mediators have been suggested to explain the renal damage, although it is accepted that the pathogenesis may be multifactorial. It has been demonstrated that oxidative stress is implicated in the impairment of renal function in patients or CsA-treated animals (1,2) and in cellular models of nephrotoxicity (3). Several experiments showed that renal damage caused by CsA is related to the increase in reactive oxygen species (ROS) (2–4). ROS can be synthesized by glomerular cells or may come from infiltrating blood cells such as neutrophils (PMN), one of the main sources of ROS in vivo.

ROS are highly reactive and can attack unsaturated bonds of membrane lipids inducing lipid peroxidation and subsequent alterations of cell membrane permeability and viscosity. These oxidated fatty acids are metabolized by phospholipase A_2, releasing arachidonic acid and malondialdehyde, an index of lipidic peroxidation. Degradation of arachidonic acid releases vasoconstrictor eicosanoids such as thromboxane that could explain the decrease in renal plasma flow and glomerular filtration rate caused by CsA.

Cyclooxygenases (COX) are crucial enzymes in arachidonic acid metabolism and as a consequence, our study was designed to analyze the expression of COX isoforms and their relationship with glomerular and leucocyte ROS synthesis in rats treated with CsA.

MATERIALS AND METHODS

Experimental Groups

Adult male Wistar rats weighing ~250–300 g were used. Our studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by US National Institutes of Health. They were kept at stable room temperature (21°C), with a 12-h cycle of dark–light. Animals had free access to water and food; they were fed with standard rat–mouse chow (IPM-R20, Letica, Barcelona, Spain). CsA (Sandimmun, Novartis, Basel, Switzerland) was administered at 9 a.m. at dose of 50 mg/kg/day for 4, 7, or 10 days (five rats per group, all experiments were performed in tri-

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plicate). Control animals (five animals) received the CsA vehicle ethanol (0.063 ml/kg/day) for 10 days.

Blood Samples

At the end of the study, the animals were sacrificed following ether anesthesia. A cannula was inserted into the abdominal aorta and blood samples were obtained in tubes containing 7.5% EDTA.

Glomeruli and Mesangial Cells

The aorta was clamped above the renal arteries and the vena cava was opened for drainage. After perfusion with ice-cold saline (0.9% NaCl), blanched kidneys were removed and immersed in ice-cold Tris-HCl buffer. The cortex from the kidneys was dissected and minced to a paste-like consistency. Glomeruli were obtained by differential sieving through a 105-μm metal sieve (Navalab, Madrid, Spain) that excluded the tubules and a 75-μm metal sieve that retained glomeruli (2). Purity of glomerular suspension was determined by microscopy, and the final tubular contamination was less than 5%. Glomeruli were incubated with collagenase and trypsin at 37°C and filtered through a 35-μm metal mesh to obtain a mesangial cells (MCs) suspension. MCs were washed twice in PBS/4°C and fixed 10′/22°C with 4% paraformaldehyde; 3×10^5 cells were treated with 10% diethyl pyrocarbonate (DEPC) in ethanol for 1 h at room temperature, centrifugated (12.000g/5′) and diluted in 50 μl PBS/0.5% Tween 20.

Preparation of Labeled Probe

COX-1 and COX-2 DNA probes (Applied Biosystem, Foster City, CA, USA) were diluted to a concentration of 25 ng/μl in TE buffer, denatured by heating 5′/95°C, and chilled on ice. Probes were labeled with "The Gene Image Random Prime Labeling Module" kit (Amersham Life Science, USA). Denatured COX probes (2 μl) were incubated with 10 μl nucleotide mix (dNTPs) that also contained Fluorescein-11-dUTP (FITC-dUTP), 5 μl primers (nonamers of random sequence), and 32 μl H_2O, in a reaction catalyzed by 5 units of exonuclease-free Klenow fragment of *E. coli* DNA polymerase I for 1 h at 37°C. Fluorescein-11-dUTP partially replaces dTTP so that FITC-labeled probes were generated.

Hybridization in Suspension

Probes (~250 ng) were denatured by boiling, snaped cool on ice, and mixed with 3×10^5 MCs and 20 μl hybridization solution that contained 10 μl formamide, 5 × SSC, 5 × Denhardt's, and 0.1 μl SDS (4). The hybridization was carried out at 48°C/12 h (overnight) in a shaking water bath. Then cells were centrifugated, incubated at 45′/48°C with stringency wash solution (100 μl, 0.1 × SSC), washed in PBS with 0.5% Tween 20, and resuspended in 300 μl of the same solution.

Flow Cytometry

Analysis of cell suspensions was performed with a FACScan™ flow cytometer (BD Biosciences, San José, CA) equipped with a 15-mW argon laser. Fluorescence excitation was at 488 nm, and 10,000 events per sample were acquired. MCs were identified by Thy 1.1-PE monoclonal specific antibody (Pharmingen, San Diego, CA) and their position in the forward/sideward scatter (FSC/SSC) dotplot was fixed by a gate. COX-1 and COX-2 expressions were measured in this gate by FITC fluorescence.

ROS in PMN were determined using 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA, Molecular Probes, San

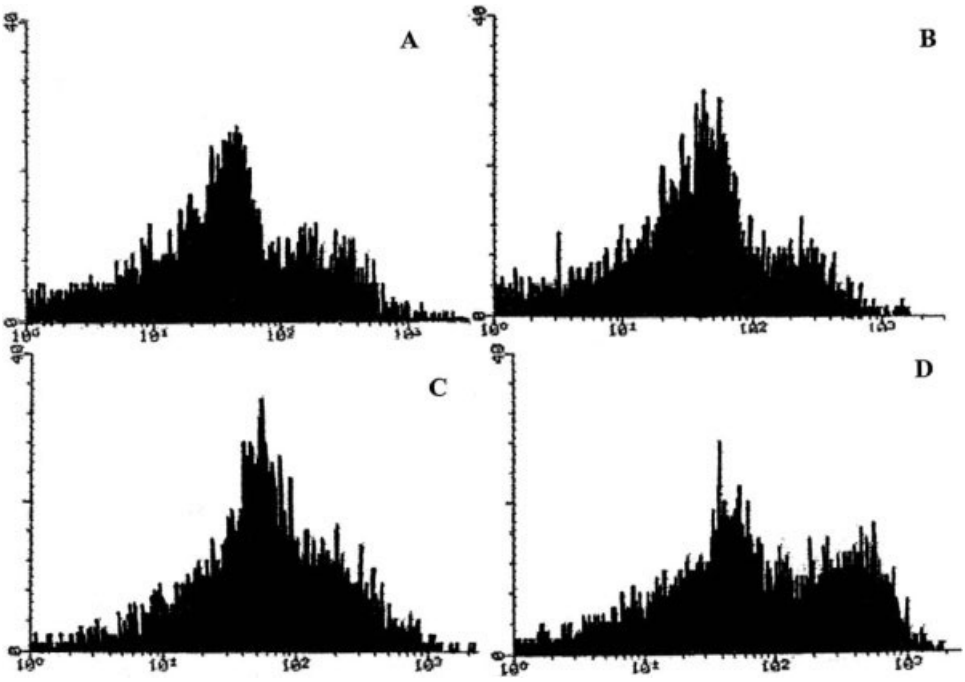

FIG. 1. ROS production in PMNs from control (**A**), four (**B**), seven (**C**), and 10-days CsA-treated rats (**D**). Data are displayed as DCFH fluorescence intensity in FL1 on a 4-decade log scale versus cell count.

José, CA) in accordance to Bass et al. (5). Flow cytometry data were analyzed using the CellQuest software (BD Biosciences), and the mean fluorescence intensities (MFI) of DCFH-DA and of COX-1 and COX-2 probes were determined. Histogram and data analysis were performed by gating in the FSC/SSC dot-plot on whole blood cells with high FSC and SSC (PMNs) for DCFH-DA (Fig. 1) or on Thy1.1 fluorescence for MC and COX-1 and COX-2 MFI ratio regard control (Fig. 2).

Statistics

Data were analyzed in the RSIGMA BABEL 2000 software (Horus Hardware S.A., Madrid, Spain). Differences in fluorescence intensities were tested with the Friedman test and regarded as statistically significant if $P < 0.05$(*).

RESULTS

CsA increased time-dependent production of ROS by PMN (Figs. 1A–1D). Control DCFH-fluorescence was 44.6 ± 5.6 MFI (mean ± standard deviation) and 4-, 7-, and 10-day treatment were 72.3 ± 15.8*, 112.4 ± 14.6*, and 153.5 ± 20.5* MFI, respectively. When we analyzed PMN in rats treated with CsA, we could identify two different populations, one with markedly increased fluorescence (ROS "high" producers), and other with lower fluorescence (ROS "low" producers). Population of ROS "high" producers was only 5% of the PMN in control rats, but increased up to 32% in animals treated for 10 days (Figs. 1A and 1D).

CsA increased COX-2 expression (inducible COX) with time compared to control values. Results were statistically different in the 7- and 10-day treatment groups versus control (Fig. 2): Control = 112 ± 23 MFI; and 151 ± 31, 176 ± 47*, and 193 ± 56* MFI in the 4-, 7-, and 10-day groups, respectively.

The expression of COX-1 (constitutive COX) measured by the same method was only increased in the 10-day treatment group compared to the control group (212 ± 60 MFI versus 170 ± 45* MFI) (Fig. 2).

DISCUSSION

It has been demonstrated that CsA induced the increase of ROS in numerous experimental models. Previous studies have shown that CsA increased ROS in glomeruli and MCs (1,3). Also, antioxidant treatment in animals prevented nephrotoxicity and increase of ROS (6). The source of ROS in kidney disease is not always well defined but hypothetically they can be formed in kidney resident cells (such as MC) or they can come from circulating cells.

Here we demonstrate that CsA increased ROS synthesis in neutrophils of rats. The presence of these cells in the kidneys could explain, at least in part, the drug's nephrotoxicity comparable to other kidney inflammatory diseases accompanied by neutrophil infiltration.

COX is a rate-limiting enzyme in prostaglandins and thromboxane synthesis; two isoforms have been identified in cells: the constitutive (COX-1) and the inducible form (COX-2). COX-2 may increase in response to a wide variety of stimuli such as lypopolysaccharide, IL-1, TNF-α, Platelet activating factor and others (7).

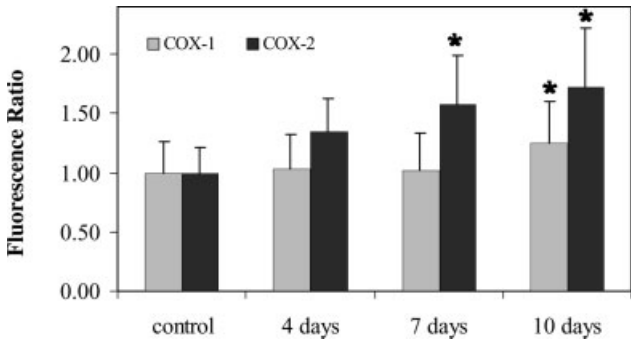

FIG. 2. COX expression as ratio with regard to control. Expression of inducible COX isoform (COX-2) was increased in a dependent-time way in CsA-treated groups. The constitutive isoenzyme (COX-1) only increased in 10-days treated group. (*) Significant difference ($P < 0.05$) compared with the control group.

Also, it has been demonstrated (8) that hydrogen peroxide and superoxide generation systems increased the expression of COX-2 in MCs. The increased expression of COX-2 which we have described in MC may explain the increase of the vasoconstrictor thromboxane that can mediate nephrotoxicity of CsA (2).

However, in several experiments in cellular systems it has been shown that CsA could inhibit the overexpression of COX-2 induced by stimuli like IL-1, angiotensin II or endothelin-1 (9).

Taking into account all these results it is possible that CsA may exert its effects in several ways. In stimulated cells, usually as a result of inflammatory mechanisms, the nuclear effects of CsA through its binding to calcineurin and further regulation of transcriptional factors, may decrease COX-2 expression. However, as we have demonstrated previously in cellular models, CsA may exert toxic direct effects in cells inducing the synthesis of ROS (3). ROS, through its influence on transcriptional factors such as NFkB may induce the increase in COX-2 expression (7).

In summary, our results showed an increase in ROS production (neutrophils) and COX-2 expression (MC) in rats treated with CsA. It is possible that ROS, through mechanisms still not properly defined, may increase COX-2 expression.

Our experimental design allowed us the study of mRNA expression of COX in suspension cells by flow cytometry. This method is sensitive and reproducible and can be applied to analyze modifications in the expression of COX isoforms in different clinical settings (10,11) and the expression of other molecules * (12).

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