

**Background:** Our previous results showed that in retinas from streptozotocin (STZ)-induced diabetic rats there is an increased level of interleukin-1 $\beta$  (IL-1 $\beta$ ). This cytokine may be involved in the expression of the inducible isoform of the nitric oxide synthase (iNOS), with consequent synthesis of large amounts of NO and blood-retinal barrier (BRB) breakdown.

**Aims:** The aim of this work was to examine whether the administration of cyclosporin-A (Cs-A) to STZ-induced diabetic rats inhibits the synthesis of IL-1 $\beta$  and the expression of the inducible proteins, iNOS and cyclo-oxygenase-2 (COX-2) in retinal cells, and whether the activity of these proteins contribute to BRB breakdown.

**Methods:** The level of IL-1 $\beta$  was evaluated by ELISA and the NO production by L-[ $^3$ H]-citrulline formation. Expression of iNOS and COX-2 proteins was determined by two methods, western blot and immunohistochemistry. The permeability of the BRB was assessed by quantification of the vitreous protein.

**Results and discussion:** Our results indicated that the levels of IL-1 $\beta$  and NO in retinas from Cs-A-treated diabetic rats are significantly reduced, as compared to that in non-treated diabetic rats. The treatment of diabetic rats with Cs-A also significantly inhibited the expression of the inducible proteins, iNOS and COX-2. The evaluation of the vitreous protein content revealed that Cs-A also reduces the BRB permeability. Taken together, these results suggest that the increased production of the inflammatory mediators, IL-1 $\beta$  and NO, in diabetes may affect the BRB permeability and therefore contribute to the development of diabetic retinopathy.

**Key words:** Blood-retinal barrier (BRB), Nitric oxide, Interleukin-1 $\beta$  (IL-1 $\beta$ ), cyclosporin-A (Cs-A)

## Introduction

Homeostasis in the retina is regulated by the blood-retinal barrier (BRB) which, among other physiological functions, restricts the movement of molecules and minimizes leukocyte migration into the retina.<sup>1,2</sup> In diabetic retinopathy, the integrity of the BRB is altered,<sup>1,3,4</sup> which leads to visual loss by mechanisms that are not well understood.

Results from our previous work suggested that in streptozotocin (STZ)-induced diabetic rats the increased BRB permeability may be the consequence of an increased expression of the inducible isoform of the enzyme nitric oxide synthase (iNOS).<sup>5</sup> In addition we also found that, in retinas from diabetic rats, there was an increased level of interleukin-1 $\beta$  (IL-1 $\beta$ ) and prostaglandin E2 (PGE2).<sup>6,7</sup> These findings are in agreement with other studies showing that IL-1 $\beta$  induces the expression of iNOS and of cyclo-oxygenase-2 (COX-2) in  $\beta$ -cells from Langerhans islets of diabetic animals, leading to cell death and to islet destruction.<sup>8,9</sup> It was also observed that an intraocular

## Effect of cyclosporin-A on the blood-retinal barrier permeability in streptozotocin-induced diabetes

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injection of IL-1 $\beta$  in Wistar rats causes disruption of the BRB<sup>10</sup> and in the BB/Edinburgh rats, an animal model of insulin-dependent diabetes, cyclosporin A (Cs-A) reduces  $\beta$  cell destruction and prevents the development of diabetes.<sup>11</sup>

In this work, Cs-A, an anti-inflammatory drug that inhibits IL-1 $\beta$  production,<sup>12</sup> was used to investigate whether the increase of IL-1 $\beta$  synthesis that occurs in STZ-induced diabetic rats modulates both the expression of the inducible proteins iNOS and COX-2 in the retinal cells and the BRB permeability.

## Material and methods

### Chemicals

STZ (60 mg/ml/kg) and the kit for protein quantification were purchased from Sigma Chemical Co., St Louis, USA. Cs-A (10 mg/kg/day) was from Sandoz and the kit for quantification of Cs-A was from Abbott Laboratories (Lisboa, Portugal). The kit for IL-1 $\beta$  quantification was purchased from R&D Systems,

Oxon, UK. L-[<sup>3</sup>H]-arginine was purchased from Amersham, Bucks, UK. Dowex AG 50W-X8 was obtained from BioRad, Herts, UK. The kit for glucose quantification was purchased from Boheringer, East Sussex, UK. The antibody anti-iNOS was purchased from Transduction Laboratories, Lexington KY, USA and the antibody anti-COX 2 was from Santa Cruz, CA, USA. The antibody anti-actin was from Boheringer, East Sussex, UK. The secondary antibodies anti-rabbit and anti-rat were from Dako, Copenhagen, Denmark and the anti-goat was from Boheringer, East Sussex, UK. All other chemicals used in this study were of analytical grade.

## Animals

Twenty male Wistar rats (250–300 g body weight) were anesthetized with ether and injected peritoneally with a freshly prepared solution of STZ (60 mg/ml/kg in 0.1 M citrate buffer, pH=4.5), ten of them started treatment with Cs-A (10 mg/kg/day) on the same day. Twenty normal rats were injected with an equal volume of 0.1 M citrate buffer and ten of them also started treatment with Cs-A (10 mg/kg/day).

All animals were assessed regularly for weight, blood glucose and glycosuria. The blood glucose levels were checked with a colorimetric method based on a standard method, and glycosuria was assessed with glucose oxidase reagent strips. The criteria for diabetes were persistent hyperglycemia (>350 mg/100 ml), glycosuria, polyuria and impaired growth according to the results previously obtained.<sup>4</sup>

The Cs-A-treated animals were also assessed daily for blood Cs-A which was determined according to the kit instructions.

Eight days after the induction of diabetes the animals were anesthetized, the eyes were removed and the retinas peeled off without the retinal pigment epithelium. Since the development of the retinal alterations due to diabetes is not symmetrical each eye was analysed independently. For each experiment five normal rats, five Cs-A-treated normal rats, five diabetic rats and five Cs-A-treated diabetic rats were used.

In all the experiments the animals were treated according to the statements of the 'Association for Research in Vision and Ophthalmology'.

## Assay for IL-1 $\beta$ measurement

To quantify the retinal levels of IL-1 $\beta$  the retinas were washed and sonicated in the diluent calibrator of the kit. The level of IL-1 $\beta$  was then determined by ELISA according to the kit instructions.

## Measurement of nitric oxide synthase activity

To determine the NOS activity the retinas were washed and sonicated in HEPES buffer (20 mM, pH = 7.4). The NOS activity was determined in the retinal

lysates by measuring the formation of L-[<sup>3</sup>H]-citrulline from L-[<sup>3</sup>H]-arginine, as previously described.<sup>5</sup> Briefly, retinal lysates (0.5 mg protein/ml) were incubated for 30 min, at 37°C, in a total volume of 200  $\mu$ l of an incubation medium containing 0.5 mM NADPH, 2 mM Ca<sup>2+</sup>, 1 mM EGTA and 10 nM L-[<sup>3</sup>H]-arginine (60 Ci/mmol). The enzymatic reactions were initiated by addition of retinal lysates to the incubation medium and the assays were terminated using 1.8 ml of STOP solution (50 mM HEPES, pH 5.5, 5 mM EDTA, at 4°C). The L-[<sup>3</sup>H]-citrulline was separated from L-[<sup>3</sup>H]-arginine on Dowex AG 50W-X8 (Na<sup>+</sup> form) columns previously equilibrated with STOP solution. The L-[<sup>3</sup>H]-citrulline was eluted with 2 ml of distilled water, and the radioactivity was quantified by liquid-scintillation counter.

## Western blot analysis

For immunodetection of iNOS and COX-2, retinas were sonicated in 200  $\mu$ l of lysis buffer (phosphate-buffered saline containing 10 mM EDTA, 1% Triton X-100 and the protease inhibitor cocktail). In brief, protein samples (25  $\mu$ g protein of retinal lysate) were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% dry milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h. The levels of iNOS protein were detected using a 1:2000 dilution of a rabbit polyclonal anti-mouse iNOS antibody for 1 h, followed by incubation with a 1:1000 dilution of the horseradish peroxidase-conjugated anti-rabbit antibody for 1 h. The levels of COX-2 were determined using a 1:500 dilution of goat polyclonal anti-rat COX-2 antibody for 1 h, followed by incubation with a 1:1000 dilution of horseradish peroxidase-conjugated anti-goat antibody for 1 h. The immunocomplexes were visualized by the ECL chemiluminescence method. Subsequently, the relative expression of iNOS was analysed by a computer program (Adobe Photoshop, IBM) and a densitometric analysis was performed using the ImageQuant software (Molecular Dynamics Inc., Sunnyvale, CA). To ensure that the loading of protein was equivalent, the membranes were stripped and reprobed with anti-actin antibody (1:10000).

## Immunohistochemical localization of iNOS

For immunohistochemical detection of iNOS, the animals were killed and the eyes removed and fixed for 24 h in 4% paraformaldehyde in 0.1 M Na phosphate buffer, pH 7.4 at 4°C. Eyes were then dehydrated through a graded series of alcohols, embedded in paraffin and 5- $\mu$ m thick sections were cut.

Immunohistochemistry was performed on sections pre-incubated in phosphate-buffer saline containing normal rabbit serum (1:20). For localization of iNOS,

eye sections were incubated for 2 h at room temperature with polyclonal anti-iNOS (2.5 µg/ml) diluted in phosphate-buffer saline and then incubated for 45 min at room temperature with fluorescein swine anti-rabbit (1:20) diluted in phosphate-buffer saline. Control procedures included the study of eye sections without the primary antibody.

### Evaluation of BRB permeability

The permeability of BRB was measured by quantification of the vitreous protein concentration according to the method described by Berkowitz and co-workers.<sup>13</sup> Briefly, the eyes were removed and vitreous samples dissected into 10 mM phosphate-buffer saline. After the removal of the samples they were frozen at -70°C. On the day of protein measurement, the vitreous was thawed to room temperature, sonicated and centrifuged for 30 min at 10,000 g. Protein levels were measured, using the Sigma protein assay kit with 25 µl of sample in 200 µl of assay reagent for 30 min at 37°C, before reading the absorbance at 550 nm. Protein concentrations were determined using the Sigma protein assay kit, and standards of albumin. The vitreous from all rats was weighted to determine whether it had been losing water due to the hyperosmolarity developed by hyperglycemia, but no differences were found.

### Statistics

All data are presented as mean ± SD of at least five separate experiments. Statistical analyses were performed using one-way ANOVA analysis and Dunnett's test. The confidence interval was 95%.

## Results

### Effect of Cs-A on IL-1β concentration

Eight days after the induction of diabetes by STZ all the diabetic animals, treated and non-treated with Cs-A, developed hyperglycemia (above 350 mg/100 ml), increased polyuria and decreased body weight. In both types of animals, normal rats treated and non-treated with Cs-A, the glycemia was lower than 95 mg/100 ml.

Quantification of the Cs-A concentration in serum and in retinas showed that in normal and diabetic rats treated with Cs-A, the serum concentration of Cs-A was higher than 1500 ng/ml and the retinal concentration of Cs-A was higher than 200 ng/ml, which have been considered therapeutical levels.<sup>14</sup>

As indicated in Fig. 1 the levels of IL-1β in retinal lysates from Cs-A-treated diabetic rats were significantly reduced ( $0.17 \pm 0.014$  pg/mg protein) compared to those in non-treated Cs-A diabetic rats ( $0.31 \pm 0.08$  pg/mg protein),  $p < 0.05$ . The level of

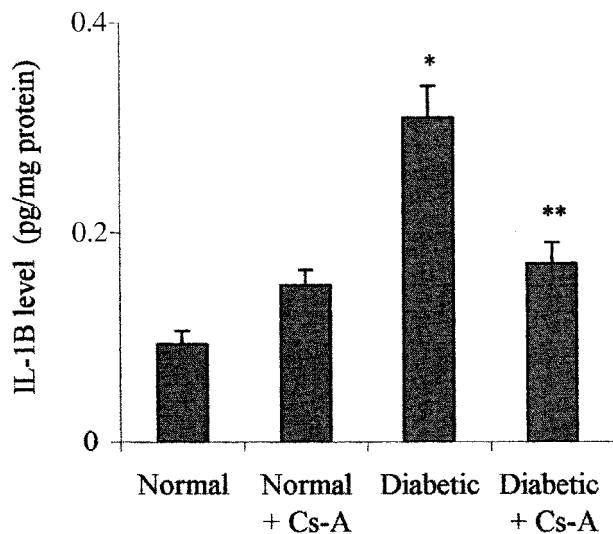


FIG. 1. Level of IL-1β in retina. The levels of IL-1β were determined in retinal lysates according to the kit instructions. Data are mean ± SD values from 5 rats from each group.

\*Significant differences from normal rats. \*\*Significant differences from Cs-A non-treated diabetic rats ( $p > 0.05$ ) (one-way ANOVA analysis, Dunnett's test).

IL-1β did not differ significantly between the Cs-A-treated and non-treated normal rats.

### Effect of Cs-A on nitric oxide synthase activity

The activity of NOS in retinal lysates was evaluated by the conversion of L-[<sup>3</sup>H]-arginine to L-[<sup>3</sup>H]-citrulline. As indicated in Fig. 2, in Cs-A-treated diabetic rats the production of L-[<sup>3</sup>H]-citrulline was significantly reduced ( $175 \pm 19.5$  pmol L-citrulline/mg protein/30 min).

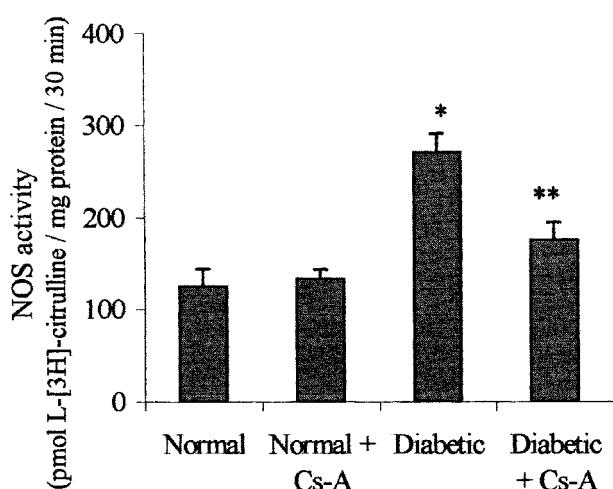


FIG. 2. Measurements of NOS activity in retina. Retinal lysates (0.5 mg protein/ml) were incubated in a medium containing 1 mM EGTA, 0.5 mM NADPH and 10 nM L-[<sup>3</sup>H]-arginine in the presence or absence of 2 mM Ca<sup>2+</sup>, in a total volume of 200 µl. The NOS activity was measured by the formation of L-[<sup>3</sup>H]-citrulline. Data are mean ± SD values from 5 rats from each group. \*Significant differences from normal rats. \*\*Significant differences from Cs-A non-treated diabetic rats ( $p < 0.05$ ) (one-way ANOVA analysis, Dunnett's test).

30 min) compared to that in non-treated diabetic rats ( $270 \pm 215$  pmol L-citrulline/mg protein/30 min),  $p < 0.05$ . The production of L-[<sup>3</sup>H]-citrulline did not differ significantly between the Cs-A-treated and non-treated normal rats.

### Effect of Cs-A on iNOS expression

To determine whether Cs-A reduces NOS activity by inhibiting iNOS expression we examined the amount of iNOS protein content by Western blot analysis in retinal lysates (Fig. 3) and the localization of iNOS protein by immunohistochemistry in retinal sections.

As indicated in Fig. 3A, the Western blot analysis showed that in retinas from Cs-A-treated diabetic rats (Fig. 3A, Lane 4) the amount of iNOS protein (130.6 kDa) decreased compared to that in retinas from Cs-A non-treated diabetic rats (Fig. 3A, Lane 3). Similar amounts of iNOS protein were detected in both treated and non-treated Cs-A normal rats (Fig. 3A, Lanes 1 and 2). These results indicate that in retinas from normal rats iNOS protein is constitutively expressed. As indicated in Fig. 3B the densitometric analysis revealed a significant decrease of iNOS protein content in retinas from Cs-A-treated diabetic

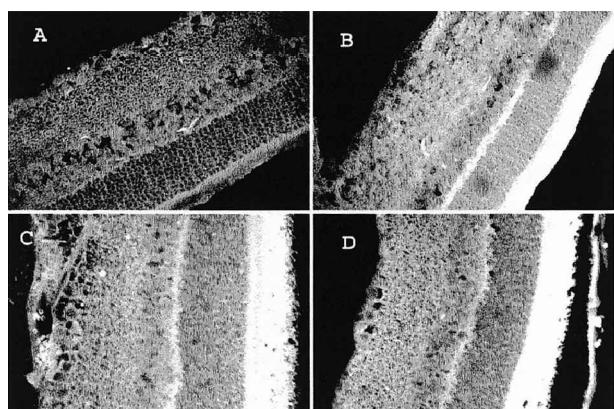


FIG. 4. Immunohistochemical localization of iNOS in intact retina. Retinal sections from normal and diabetic rats were incubated with the same anti-iNOS antibody used for the Western blot analysis. In control experiments retinal sections were only incubated with the secondary antibody. (A) retinal section from normal rats; (B) retinal section from Cs-A-treated normal rats; (C) retinal section from diabetic rats; (D) retinal section from Cs-A-treated diabetic rats. Magnification 20x.

rats ( $154.45 \pm 22.8$  optical density) compared to that in retinas from Cs-A non-treated diabetic rats ( $96.0 \pm 10.5$  optical density),  $p < 0.05$ .

The immunolocalization of iNOS showed that in non-treated Cs-A diabetic rats, iNOS protein was clearly detected in the outer nuclear layer, inner nuclear layer, inner plexiform layer and also at the ganglion cell layer (Fig. 4C). In Cs-A-treated diabetic rats (Fig. 4D), iNOS protein remained localized at the inner nuclear and at the ganglion layer although the number of cells stained for iNOS in both retinal cell layers decreased compared to that in Cs-A non-treated diabetic rats. In retinas from Cs-A treated and non-treated normal rats iNOS protein was slightly expressed at the inner nuclear layer which explains the reduced amount of iNOS protein detected by Western blot (Fig. 4A, B).

### Effect of Cs-A on COX-2 expression

To determine whether Cs-A reduces the expression of COX-2 protein we evaluated the amount of COX-2 protein by Western blot (Fig. 5). The results revealed that in retinas from Cs-A-treated diabetic rats (Fig. 5A, Lane 4) the amount of COX-2 protein decreased compared to that in retinas from Cs-A non-treated diabetic rats, (Fig. 5A, Lane 3). Similar amounts of COX-2 protein were detected in treated and non-treated Cs-A normal rats (Fig. 5A, Lanes 1 and 2). The data from the densitometric analysis of the blots confirmed that in Cs-A-treated diabetic rats there was a significant decrease ( $103.5 \pm 9.7$  optical density) in COX-2 protein content compared to that in retinal lysates from Cs-A non-treated diabetic rats ( $143.7 \pm 16.4$  optical density),  $p < 0.05$ , Fig. 5B.

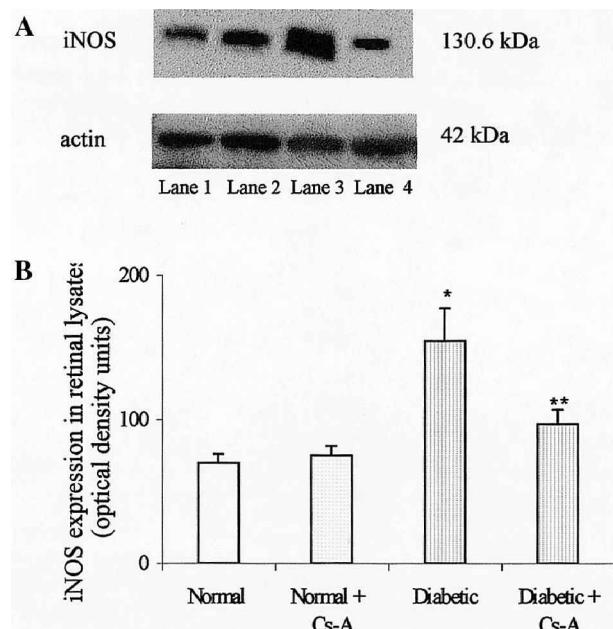


FIG. 3. Study of the expression of iNOS protein. (A) Western blot for detection of iNOS protein expression in retina. Retinal lysates were subjected to electrophoresis followed by immunoblotting with a polyclonal antibody against iNOS. Lane 1: retinal lysate from normal rats; Lane 2: retinal lysate from Cs-A treated normal rats; Lane 3: retinal lysate from diabetic rats; Lane 4: retinal lysate from diabetic rats. This figure is representative of 5 identical experiments. (B) Densitometric analysis of iNOS expression. Anti-actin was used to ensure that the protein loading was equivalent. Data are mean  $\pm$  SD values from 5 rats from each group. \*Significant differences from normal rats. \*\*Significant differences from Cs-A non-treated diabetic rats ( $p < 0.05$  one-way ANOVA analysis, Dunnett's test).

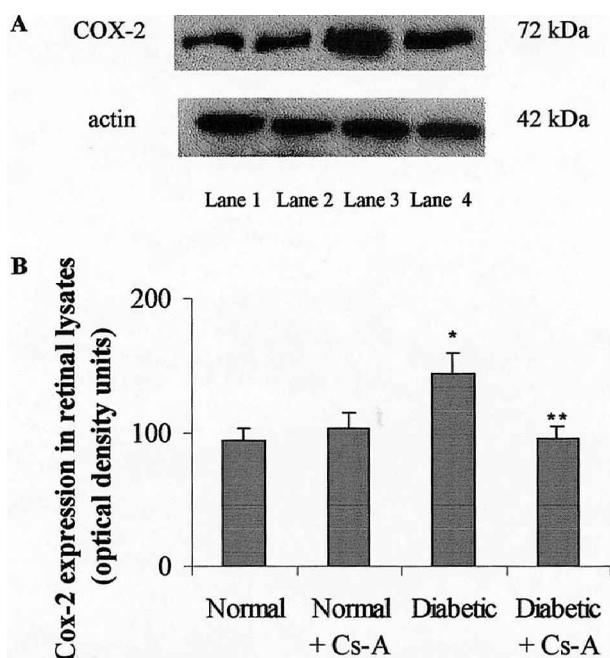


FIG. 5. Study of the expression of COX-2 protein. (A) Western blot for detection of COX-2 protein expression in retina. Lane 1: retinal lysate from normal rats; Lane 2: retinal lysate from Cs-A-treated normal rats; Lane 3: retinal lysate from diabetic rats; Lane 4: retinal lysate from diabetic rats. This figure is representative of 5 identical experiments. (B) Densitometric analysis of COX-2 expression. Anti-actin was used to ensure that the protein loading was equivalent. Data are mean  $\pm$  SD values from 5 rats from each group. \*Significant differences from normal rats. \*\*Significant differences from Cs-A non-treated diabetic rats ( $p<0.05$ ) (one-way ANOVA analysis, Dunnett's test).

### Effect of Cs-A on BRB permeability

In Cs-A non-treated diabetic rats there was a significant increase in vitreous protein concentration ( $3.10 \pm 0.28$  mg/ml of vitreous protein) compared to that in Cs-A non-treated normal rats ( $1.1 \pm 0.17$  mg/ml of vitreous protein),  $p<0.01$  (Fig. 6). These results indicated that in Cs-A non-treated diabetic rats the permeability of BRB is significantly increased.

Cs-A-treated diabetic rats showed a significant decrease in vitreous protein concentration ( $1.31 \pm 0.17$  mg/ml of vitreous protein) compared to that in Cs-A non-treated diabetic rats ( $3.10 \pm 0.28$  mg/ml of vitreous protein),  $p<0.01$  (Fig. 5), which indicated that Cs-A reduces the BRB permeability in diabetic rats.

### Discussion

In our previous studies, we observed that there was a breakdown of the BRB in STZ-induced diabetic rats which appeared to be correlated with an increased level of NO and IL-1 $\beta$  in retinas from STZ-induced diabetic rats.<sup>4-6</sup> In this study, we investigate whether the inhibition of IL-1 $\beta$  synthesis by Cs-A affects the expression of iNOS and COX-2 proteins, and we correlate those results with the BRB permeability.

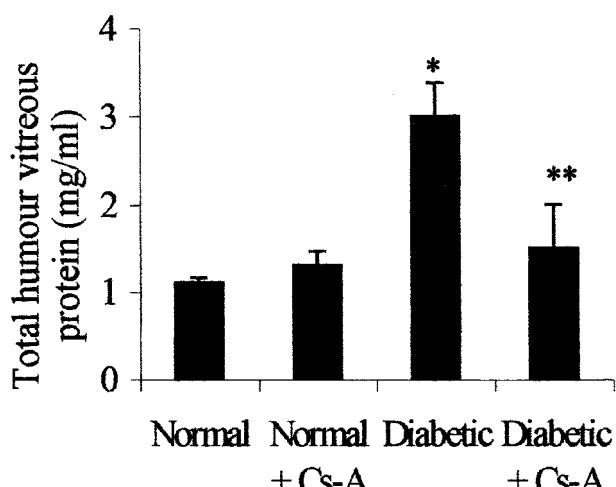


FIG. 6. Vitreous protein concentration. The protein concentration was determined according to the kit instructions. Data are mean  $\pm$  SD values from 5 rats from each group. \*Significant differences from normal rats ( $p<0.05$ ). \*\*Significant differences from Cs-A non-treated diabetic rats ( $p<0.05$ ) (one-way ANOVA analysis, Dunnett's test).

Cs-A reduced the level of IL-1 $\beta$  in retinas from Cs-A-treated diabetic rats. This result is in agreement with previous studies reporting that Cs-A inhibits IL-1 $\beta$  synthesis either *in vitro*, such as in rat renal mesangial cells<sup>15</sup> or *in vivo* as evaluated in atyemic rats.<sup>12</sup>

The treatment of STZ-induced diabetic rats with Cs-A significantly reduced the NO production in retinas, which seemed to be associated with a decreased expression of iNOS protein. Other studies report that in mesangial cells, Cs-A inhibits the IL-1 $\beta$ -dependent transcriptional up-regulation of the iNOS gene by inhibiting the activation of the transcription factor NF- $\kappa$ B.<sup>15</sup> Therefore, the decreased production of NO in retinas from Cs-A-treated diabetic rats, as indicated in Fig. 2, may be due to the inhibition of the cellular mechanism that controls the expression and the activity of iNOS.

We have previously observed that in eight-days STZ-induced diabetic rats the main alteration of the BRB permeability was localized at the retinal vessels and that the iNOS protein was mainly expressed at the inner nuclear layer.<sup>4,16</sup> Since the treatment of diabetic rats reduces the expression of iNOS protein which is accompanied by a significant reduction of the BRB permeability, it appears that the increased production of NO around the retinal vessels may affect retinal vessel permeability.

The COX-2 protein level in retinas from diabetic rats non-treated with Cs-A is increased compared to that in normal rats whereas in retinas from diabetic rats treated with Cs-A it is significantly reduced (Fig. 5). These results are in agreement with previous studies reporting that the expression of COX-2 could be induced by the cytokine IL-1 $\beta$  and by NO<sup>17,18</sup> and that in rat mesangial cells, IL-1 $\beta$ -induced COX-2 expression was suppressed by Cs-A.<sup>19</sup> The up-regulation of COX-2

expression leads to an increased production of prostaglandins, which may act as vasodilatory and inflammatory mediators.<sup>20,21</sup> Therefore, in the animal model of diabetes used in this work, COX-2 protein expression together with increased production of NO may be involved in the BRB breakdown.

Cs-A decreases the vitreous protein concentration in diabetic rats compared to that in diabetic rats non-treated with Cs-A (Fig. 6), which indicates that Cs-A causes a reduction of the BRB permeability.

In conclusion, the results from our study indicate that Cs-A reduces the BRB permeability in STZ-induced diabetic rats. The effect of Cs-A in the BRB permeability appears to be related to a down-regulation of pro-inflammatory cytokines IL-1 $\beta$  and NO as well as to the down-regulation of the expression of iNOS and COX-2 proteins.

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