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Section 1

L-Arginine transport in retinas from streptozotocin diabetic rats: correlation with the level of IL-1β and NO synthase activity

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Abstract

Several evidences suggest that the pro-inflammatory cytokines IL-1β and the radical NO are implicated as effectors molecules in the pancreatic β-cells dysfunction; an event preceding the pathogenesis of diabetes. IL-1β induces the expression of the inducible isoform of NO synthase (iNOS), which use L-arginine as substrate to overproduce NO. However, it is not known whether these events may participate in the development of diabetic retinopathy, which is the main cause of blindness. In this work, we found an increased level of IL-1β in retinas from streptozotocin-induced (STZ) diabetic rats. We also observed that the activity of the NO synthase (NOS) and the L-arginine uptake are enhanced in retinas from STZ-induced diabetic rats as compared to retinas from control rats. We found that the uptake of L-arginine in retinas from control and diabetic rats occurs through a transporter resembling the Y+ system, i.e. it is saturable, not affected over the pH range 6.5 to 7.4, and is independent of the extracellular Na^+. Nevertheless, the L-arginine transport in retinas from diabetic rats occurs through a carrier with lower affinity ($K_m = 25$ μM) and higher capacity ($V_{max} = 295 \pm 22.4$ pmol L-arginine/mg protein) than in retinas from control rats ($K_m = 5$ μM and $V_{max} = 158 \pm 12.8$ pmol L-arginine/mg protein) which is correlated with the increased NOS activity and consequent depletion of the intracellular pool of L-arginine.

Keywords: Streptozotocin-induced diabetes; Retina; L-arginine uptake; Nitric oxide synthase; Interleukin-1β

1. Introduction

L-Arginine is an essential amino acid, predominantly cationic at the physiological pH range. In most mammalian cells, the uptake of extracellular L-arginine occurs via a plasma membrane cationic amino acid (CAT) transporter system termed Y+ system. The transport activity of this Y+ system is sodium independent, saturable and pH insensitive (White, 1985; Simmons, Closs, Cunningham & Smith, 1996). Once inside the cells, L-arginine may be converted into L-citrulline and nitric oxide (NO) by two distinct isoforms of the enzyme NOS, the constitutive and the inducible isoforms. NO produced by the constitutive isoform acts as a signaling molecule whereas the excessive amount of NO produced through the inducible isoform functions as a cytotoxic molecule (Bogle, Baydoun, Pearson, Moncada & Mann, 1992; Schmidlin & Wiesinger, 1994; Aldridge & Collard, 1996). The overproduction of NO may cause nitrosylation of the Fe–S groups in mithocondrial enzyme aconitase there by inhibiting glucose oxidation and ATP production which leads to cell death and tissue damage, as seen in inflammatory disorders and in diabetes (Bogle et al., 1992, Sharma, Danoff, DePiero & Ziyadeh, 1995; Sobrevia, Nadal, Yudilevich & Mann, 1996).

In diabetes mellitus there is an increase in plasma concentration of both, glucose and pro-inflammatory cytokines, namely IL-1β and TNF-α (El Asrar, Mai-

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mone, Morse, Gregory & Reder, 1992; Welsh, Welsh, Bendtzen, Mares, Strandell, Öberg & Sandler, 1995). These cytokines induce the iNOS expression and consequent NO overproduction in different cell types (Welsh et al., 1995; Kuemmerle, 1998). Furthermore, high concentration of glucose, induces the iNOS expression, and increases the L-arginine uptake in murine mesanglial cells (Sharma et al., 1995), in murine macrophages (Sharma et al., 1995) and in endothelial cells (Sobrevia et al., 1996).

Although, the causes of diabetic retinopathy still remain incompletely understood changes in blood retinal barrier (BRB) permeability have been reported in experimental diabetes mellitus (Vinores, Niel, Swerdloff & Campochiaro, 1993). Additionally our recent findings have revealed that BRB permeability and retina glucose concentration are increased in eight days STZ-induced diabetic rats (Carmo, Ramos, Reis, Proença & Cunha-Vaz, 1998a; Carmo, Lopes, Santos, Proença, Cunha-Vaz & Carvalho, 1998b). Because both, glucose and cytokines, namely IL-1, have been implicated in the pathogenesis of diabetes mellitus (El Asrar et al., 1992; Rabinovitch & Suarez-Pinzon, 1998; Sjöholm, 1998), the aim of this study was to assess the levels of IL-1 and L-arginine concentration in retinas from STZ-induced diabetic rats, and to evaluate whether L-arginine uptake and the NOS activity are altered in comparison with control rats.

Taken together, our results suggest that the increased level of IL-1β in retinas from STZ-induced diabetic rats may contribute to the development of inflammatory conditions, namely the increased production of NO which may lead to retinal cell death observed in diabetic retinopathy.

2. Material and methods

2.1. Chemicals

Streptozotocin (STZ) (60 mg/ml per kg), veratridine (50 μM), *N*-methyl-L-arginine (1 mM) monensin (50 μM) and the kit for protein quantification were purchased from Sigma, St Louis, MO. L-[^{3}H]arginine was purchased from Amersham, Bucks, UK. Dowex AG 50W-X8 was obtained from BioRad, Hertfordshire, UK. The kit for IL-1β quantification was purchased from R&D systems, Oxon, UK. The kit for glucose quantification was purchased from Boheringer, East Sussex, UK. All other chemicals used in this study were of analytical grade.

2.2. Animal model

Male Wistar rats (250–300 g body weight) were anesthetized with ether and injected peritoneally with a freshly prepared solution of streptozotocin (STZ) (60 mg/ml per kg in 0.1 M citrate buffer, pH 4.5). Control rats were injected with an equal volume of 0.1 M citrate buffer. All animals were assessed regularly for weight, blood glucose and glycosuria. Blood for these analyses was collected via tail clipping under ether anesthesia. 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 was persistent hyperglycemia ($>$ 350 mg/100 ml), glycosuria, polyuria and impaired growth according to the results obtained in Carmo et al. (1998a).

2.3. Quantification of IL-1β levels

To quantify the levels of IL-1β in retinas from control and STZ-induced diabetic rats, five animals from each group were killed, the eyes were removed and retinas without the pigment epithelium were gently peeled away, washed and sonicated in diluent calibrator of the kit. The level of IL-1β was then determined by ELISA according to the kit instructions.

2.4. Nitric oxide synthase activity and L-arginine concentration in retinas from control and diabetic rats

To determine the NOS activity in control and 8 days streptozotocin-induced diabetic rats, five animals from each group were killed, the eyes were removed and retinas without the pigment epithelium were gently peeled away, 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-[^{3}H]citrulline from L-[^{3}H]arginine. Retinal lysates (0.5 mg protein/ml) were incubated for 30 min, at 37°C, in a total volume of 200 μl of a incubation medium containing 0.5 mM NADPH, 2 mM Ca^{2+}, 1 mM EGTA, and 10 nM L-[^{3}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-[^{3}H]citrulline was separated from L-[^{3}H]arginine on Dowex AG 50W-X8 (Na^+ form) columns previously equilibrated with STOP solution. The L-[^{3}H]citrulline was eluted with 2 ml of distilled water, and the radioactivity was quantified by liquid-scintillation counter (Bredt & Snyder, 1989).

To determine the L-arginine concentration the isolated retinas from control and diabetic rats were sonicated in 500 μl of HEPES buffer (20 mM, pH 7.4) and were centrifuged at 20 000 rpm, for 20 min. The supernatant was collected and stored at -80°C until use. Amino acid concentrations were measured by high-performance liquid chromatography (HPLC) after deriva-

tization with *o*-phthalaldehyde/2-mercaptoethanol, as previously described (Santos, Moreno & Carvalho, 1996).

2.5. Uptake of L-arginine assays

Eight days after streptozotocin or citrate injection, five rats from each group were killed, and the eyes were removed. Retinas were gently peeled away from pigment epithelium, resuspended and washed in Krebs modified solution (1 mM $CaCl_2$, 132 mM Na^+, 1.2 mM H_3PO_4, 4.7 mM KCl, 1.5 mM Hepes–Tris, 5.6 mM glucose and 1.177 mM $MgSO_4$) according to the experiments. Each retina was divided in four portions and each portion was weighted to ensure an equalization of the samples.

The retinas isolated from control and diabetic animals were incubated in 200 μl of Krebs modified solution containing also 10 μl of L-[^{3}H]arginine (63 Ci/mmol) for 30 min, at 37°. After incubation, each retina portion was washed three times with cold Krebs modified solution, resuspended in 200 μl of cold medium and sonicated. The accumulated radioactivity was estimated by liquid scintillation counting, and the concentration of the accumulated L-arginine was calculated from its specific activity. In some experiments *N*-metil-arginine (NMA) (1 mM) which inhibits the L-[^{3}H]arginine uptake by a competitive mechanism (Lopes, Cardoso, Schousboe & Carvalho, 1994) was added simultaneously with the L-[^{3}H]arginine to the incubation medium.

The effect of extracellular pH on the L-[^{3}H]arginine uptake was studied by incubating retinas from control and diabetic rats for 30 min, at 37°, on Krebs modified solution at different pH values (4, 6.5, 7.4 and 8.5). The extracellular pH was changed by addition of NaOH 1 M or HCl 1 M to the Krebs modified solution.

To study the effect of intracellular pH on the L-[^{3}H]arginine uptake, retinas from control and diabetic rats were pre-incubated for 30 min, at 37°, in *N*-metil-glucamine (NMG), which is impermeable to the cellular membrane, in Krebs modified sodium free solution with monensin (5 μM) an ionophore that transports Na^+ into the cell in exchange for H^+, at different pH (4, 6.5, 7.4 and 8.5) without L-[^{3}H]arginine. Retinas were then incubated for 30 min, at 37°C, in a medium with the same composition in the presence of 10 μl of L-[^{3}H]arginine.

To study the effect of extracellular sodium on the L-[^{3}H]arginine uptake into retina from control and diabetic rats two sets of experiments were performed. In one the extracellular sodium of the Krebs modified solution was replaced by NMG (132 mM) and in the other veratridine (50 μM) which in low concentration induce Na^+ influx, was added to the Krebs modified solution.

2.6. Statistics

All data are presented as mean ± S.E.M. of at least five separate experiments. Statistical analysis was performed using Stastistica W/5.0 from Statsoft. Student's *t*-test for independent samples was used to compare differences between the control and the diabetic groups and Student's *t*-test for dependent samples was used to compare differences between the same group (control or diabetic).

3. Results

We measured the levels of IL-1β in retinal lysates from control and STZ-induced diabetic rats and we correlated them with both the NOS activity and the L-arginine uptake. We found a significant increase ($P < 0.05$) in the levels of IL-1β in retina lysates from diabetic rats (0.31 ± 0.08 pg/mg protein) as compared to that in control rats (0.093 ± 0.013 pg/mg protein) (Fig. 1).

In parallel experiments, as depicted in Fig. 2, we observed that the NOS activity in retinal lysates from diabetic rats was significantly increased, $P < 0.05$ (270 ± 21 pmol L-[^{3}H]citrulline/mg protein/30 min) as compared to the NOS activity in retinal lysates from control rats (125 ± 19 pmol L-[^{3}H]citrulline/mg protein/30 min). The L-arginine concentration in retinal lysates from diabetic rats was significantly lower, $P < 0.05$ (560 ± 30 pmol/mg protein) than that in retinal lysates from control rats (3010 ± 340 pmol/mg protein) (Fig. 2), which is in agreement with an increased activity of NOS.

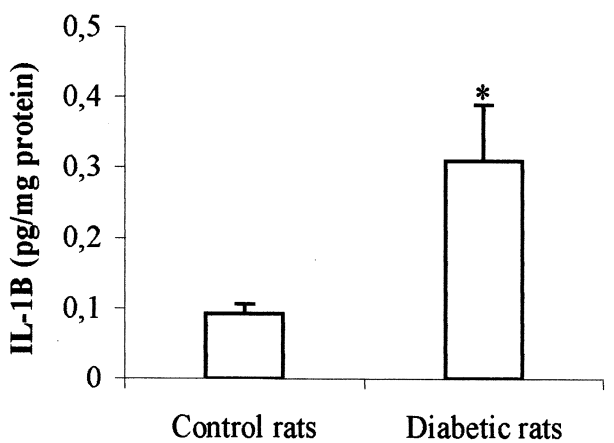

Fig. 1. Levels of the cytokine IL-1β in retinal lysates from control and STZ-induced diabetic rats. The level of IL-1β was determined by ELISA according to standard method. Data are mean ± S.E.M. from five separate experiments each made in triplicate with retinas from five control and five STZ-induced diabetic rats. * Significant differences from control rats ($P < 0.05$).

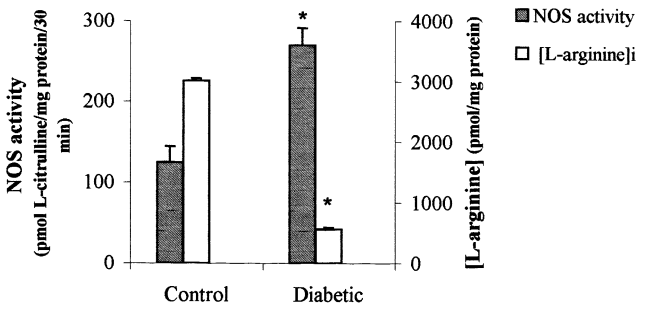

Fig. 2. NOS activity and intracellular L-arginine concentration in retinas from control and STZ-induced diabetic rats. NOS activity was assayed in an incubation medium containing 0.5 mg protein/ml, 2 mM Ca^{2+}, 1 mM EGTA, 0.5 mM NADPH and 10 nM L-[^{3}H]arginine in a total volume of 200 μl in the absence and in the presence of the NOS inhibitors, as indicated in Table 1. The intracellular concentration of L-arginine was determined by HPLC as described in materials and methods. Data are mean ± S.E.M. values of five separate experiments in triplicate. * Significant differences from control rats ($P < 0.05$).In the figure [L-arginine]i means intracellular concentration of L-arginine.

Since the production of NO is mainly dependent on the availability of L-arginine, we studied the characteristics of the L-arginine uptake in retinas isolated from control and diabetic rats. In Fig. 3 is indicated the effect of the temperature on L-arginine uptake, we observed that at 4°C, there was no difference between the uptake of L-[^{3}H]arginine into retinas from control (38.5 ± 4.2 pmol/mg protein/30 min) and diabetic rats (39.7 ± 5.3 pmol/mg protein/30 min). However, at 37°C, the uptake of L-[^{3}H]arginine was significantly increased in any groups of the retinas, and it was significantly higher in retinas from diabetic rats (239 ± 27.6 pmol/mg protein/30 min) than in retinas from control rats (186 ± 14.3 pmol/mg protein/30 min) (Fig. 2), $P < 0.05$. Further, the addition of the L-arginine

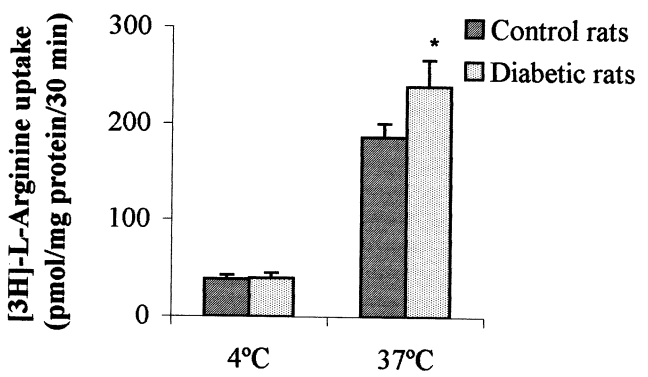

Fig. 3. Effect of the temperature on L-[^{3}H]arginine uptake into the rat retinas isolated from control and STZ-induced diabetic rats. Intact retinas were incubated for 30 min in Krebs modified medium, containing L-[^{3}H]arginine (10 nM) and cold L-arginine (10 μM). The retinas were washed, sonicated and the radioactivity measured as described in material and methods. Data are mean ± S.E.M. of five separate experiments in duplicate. * Significant differences from the control rats ($P < 0.05$).

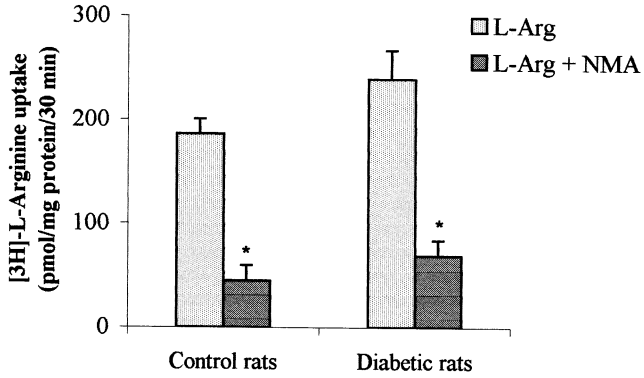

Fig. 4. Effect of methyl-L-arginine (1 mM) on L-[^{3}H]arginine uptake into the rat retinas isolated from control rats and STZ-induced diabetic rats. Intact retinas were incubated at 37° for 30 min in Krebs modified medium, containing L-[^{3}H]arginine (10 nM), cold L-arginine (10 μM) and NMA (1 mM). The retina was washed, sonicated and the radioactivity measured as described in material and methods. Data are mean ± S.E.M. of five separate experiments in duplicate. * Significant differences from the results obtained without NMA ($P < 0.05$).

analog, *N*-methyl-L-arginine (NMA), to the incubation medium inhibited the uptake L-[^{3}H]arginine by 75.8% (from 186 ± 14.3 to 45.0 ± 14.8 pmol/mg protein/30 min) in retinas from control rats and by 71.2% (from 239 ± 27.6 to 68.8 ± 15 pmol/mg protein/30 min) in retinas from diabetic rats (Fig. 4).

In order to evaluate the time course of the L-[^{3}H]arginine uptake, the freshly isolated retinas were continuously incubated for as long as 60 min (from 1 to 60 min), in the presence of L-[^{3}H]arginine (10 nM) and unlabeled L-arginine (10 μM). The uptake of L-arginine into retinas from control and diabetic rats followed a linear time course over 5 min (Fig. 5). The steady state was reached at 30 min in retinas from control rats (186 ± 14.3 pmol L-arginine/mg protein) and at 45 min in retinas from diabetic rats (251 ± 30.4 pmol L-arginine/mg protein). The initial rate of the L-arginine transport was higher in retinas from diabetic rats (70.7 ± 10 pmol L-arginine/mg protein 1 min of incubation) than in retinas from control rats (58.6 ± 5.71 pmol L-arginine/mg protein 1 min of incubation) (Fig. 5).

The maximal transport rate (V_{max}) and half saturation constant (K_m) of L-[^{3}H]arginine transport were determined by the Lineweaver–Burk plot over the concentration range of 0.5–600 μM of L-arginine (Table 1). The transport of L-[^{3}H]arginine by retinas from both control and diabetic rats was saturable (data not shown). The V_{max} value for L-arginine transport is higher in retinas from diabetic rats (295 ± 22.4 pmol/mg protein) than in retinas from control rats (158 ± 12.8 pmol/mg protein). The K_m value is five times higher in retinas from diabetic rats (25 μM) than in retinas from control rats (5 μM) (Table 1). These findings indicate that the L-arginine transport in retinas

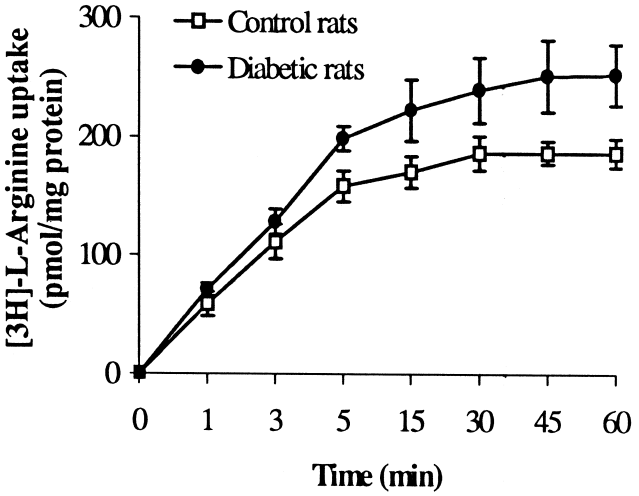

Fig. 5. Effect of incubation time on L-[^{3}H]arginine uptake into the rat retinas isolated from control rats and STZ-induced diabetic rats. Intact retinas were incubated at 37° for different times in Krebs modified medium, containing L-[^{3}H]arginine (10 nM) and cold L-arginine (10 μM). The retinas were washed, sonicated and the radioactivity measured as described in material and methods. Each value is the mean ± S.E.M. of five separate experiments in duplicate.

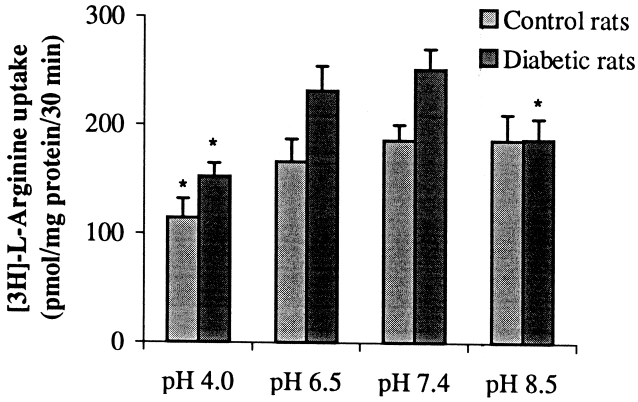

Fig. 6. Effect of extracellular pH on L-[^{3}H]arginine uptake into the rat retinas isolated from control rats and STZ-induced diabetic rats. Intact retinas were incubated at 37° for 30 min in Krebs modified medium, containing L-[^{3}H]arginine (10 nM) and cold L-arginine (10 μM) at different pH values (4, 6.5, 7.4 and 8.5). The retinas were washed, sonicated and the radioactivity measured as described in material and methods. Each value is the mean ± S.E.M. of five separate experiments in duplicate. * Significant differences from the results obtained at pH 7.4 ($P < 0.05$).

from diabetic rats occurs through a transporter with lower affinity and higher capacity than that in retinas from control rats.

In additional studies, we examined the effect of the extracellular and the intracellular pH, as well as the participation of Na^+ ion, on the L-[^{3}H]arginine uptake into the retinas. At extracelular pH values between 6.5 and 7.4 the uptake of L-[^{3}H]arginine was higher in retinas from diabetic rats than in retinas from control rats (Fig. 6). However, in that pH range, the L-[^{3}H]arginine uptake did not change significantly either in retinas from control rats or in retinas from diabetic rats (Fig. 6). At extracelular pH values 4.0 and 8.5, the uptake of L-[^{3}H]arginine was significant reduced as compared to the uptake at pH 7.4 either in retinas from control rats or in retinas from diabetic rats. Nevertheless, in both groups of retinas (control and diabetic) the decrease rate of L-[^{3}H]arginine uptake at pH 4 was more significant than that at pH 8.5 (Fig. 6).

Table 1
K_m and V_{max} values for the uptake of L-[^{3}H]arginine into rat retina isolated from control rats and eight days STZ-induced diabetic rats[a]

	V_{max} (pmol/mg protein)	K_m (μM)
Control rats	158 ± 12.8	5
Diabetic rats	295 ± 22.4	25

[a] Intact retinas were incubated for 5 min, at 37° in Krebs modified medium, containing L-[^{3}H]arginine (10 nM) and different concentrations of cold L-arginine (0.5–600 μM). The retina was washed, sonicated and the radioactivity measured as described in material and methods. Each value is the mean ± S.E.M. of five separate experiments in duplicate.

We also examined the effect of intracellular pH on the L-[^{3}H]arginine uptake in a Krebs modified sodium free medium at pH ranging from 4.0 to 9.0 (data not shown). For this, we used the ionophore monensin, that transports Na^+ into the cell in exchange for H^+. The results indicate that no change in the L-[^{3}H]arginine uptake occur when retinas were exposed to extracellular or intracellular pH ranging within the pH range 6–8.

The effect of extracellular Na^+ on the uptake of L-[^{3}H]arginine was studied by replacing the Na^+ by NMG (132 mM), or by the addition of veratridine (50 μM) to the Krebs modified solution. When sodium was replaced by NMG or when veratridine was added to the standard Krebs modified solution the uptake of L-[^{3}H]arginine into retinas of control and diabetic rats did not change (Fig. 7).

4. Conclusions

We found that the level of IL-1β in retinal lysates from diabetic rats was significantly increased as compared to that in retinal lysates from control rats (Fig. 1). Additionally we also observed that in retinas isolated from STZ-induced diabetic rats, there was an increased activity of the enzyme NOS and a decrease in the intracellular concentration of L-arginine in comparison to the values in control animals (Fig. 2). These results are consistent with our previous results showing that iNOS is expressed in retinal cells from STZ-induced diabetic rats (Carmo et al., 1998b). Recent data also revealed that IL-1 induced overproduction of NO is dependent on the transport of the extracellular L-arginine into the cells (Gill, Low & Grigor, 1996;

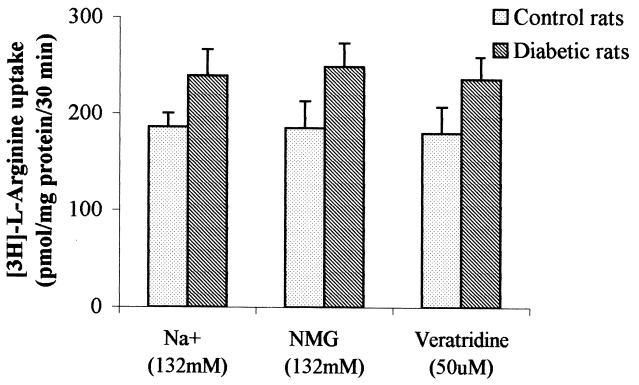

Fig. 7. Effect of sodium, *N*-metil-glucamine and veratridine on L-[^{3}H]-arginine uptake into the rat retinas isolated from control rats and STZ-induced diabetic rats. Intact retinas were incubated at 37° for 30 min in Krebs modified medium, in the presence or absence of Na^+ (132 mM), NMG (132 mM), and veratridine (50 μM). In all the experiments the incubation medium contained also L-[^{3}H]arginine (10 nM), and cold L-arginine (10 μM). The retinas were washed, sonicated and the radioactivity measured as described in material and methods. Each value is the mean ± S.E.M. of five separate experiments in duplicate.

Simmons et al., 1996), which constitutes the main source of the intracellular pool of L-arginine (White, 1985; Schmidlin & Wiesinger, 1994; Simmons et al., 1996).

Since the intracellular pool of L-arginine was depleted and iNOS activity was increased in retinas from STZ-induced diabetic rats (Fig. 2), we then examined the biochemical charactheristics of the L-arginine uptake. Our results indicated that the uptake of L-arginine into retinas isolated from control and diabetic rats occurs via a system with characteristics similar to those described for the CAT Y+ system (White, 1985; Stoll, Wadhwani & Smith 1993; Baydoun & Mann 1994). Furthermore, the time course of L-arginine uptake revealed that the uptake of L-arginine into retinas from STZ-induced diabetic rats was increased as compared to the uptake into retinas of control rats (Fig. 5) which may be explained by the up-regulation of iNOS and by the recruitment of the intracellular pool of L-arginine in retinas from STZ-induced diabetic rats.

The uptake of L-arginine into retinas from control and STZ-induced diabetic rats was not affected by extracellular Na^+ either when extracellular sodium was replaced by NMG, or when veratridine was added to the sodium medium (Fig. 7). These results indicate that in retinal cells, as was observed in other cell systems, the CAT Y+ system is not coupled to a co-transport with Na^+, and that the uptake of L-arginine does not require extracellular sodium for the full activity (White, 1985; Baydoun & Mann, 1994; Lopes et al., 1994).

The analysis of the kinetic parameters (K_m and V_{max}) for the L-arginine transport revealed that, in retinas from STZ-induced diabetic rats, the transporter of L-arginine has a lower affinity, and a higher capacity than that of retinas from control rats (Table 1).

It was recently reported that in cytokine-pretreated myocytes the expression of iNOS is correlated with the induction of the CAT family of transporters, CAT1, CAT-2B and CAT-2A (Simmons et al., 1996).

Experiments reported in our work show an increased production of NO by the retinas from STZ-induced diabetic rats (Fig. 2). This NO overproduction may be due to the IL-1β (Fig. 1) and glucose-induced iNOS expression (Carmo et al., 1998b). These results together with the comparative kinetic studies of L-arginine transport in retinas from STZ-induced diabetic and control rats (Table 1; Fig. 5) appear to indicate that the changes in L-arginine uptake in STZ-induced diabetic rats are correlated with the expression of the CAT family transporters different from those expressed in the retinas from control rats.

Since previous studies report an increased BRB permeability after a post intravitreal injection of IL-1β (Claudio, Martiney & Brosnan, 1994) it seems possible that in retinas from STZ-induced diabetic rats, the increased level of IL-1β together with the increased glucose concentration may contribute to the increased BRB permeability and consequently to the development of diabetic retinopathy.

Further studies will be necessary in order to study the inhibition of IL-1β, NO production and L-arginine uptake and its implications in blood-retinal barrier permeability.

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