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Inducible nitric oxide synthase in rat neutrophils: role of insulin

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Abstract

Defective leukocyte–endothelial interactions are observed in experimental diabetes mellitus. Endogenous substances, including nitric oxide (NO), have anti-inflammatory effects within the vasculature by reducing leukocyte adherence to post-capillary venules. The purpose of this study was to examine the activity and expression of NO synthase in neutrophils from alloxan-induced diabetic rats. Glycogen-elicited peritoneal neutrophils were obtained from diabetic rats and matching controls 10, 30, and 180 days after alloxan (42 mg/kg, i.v.) or saline injection. NO synthase activity was determined by the [^{3}H]L-citrulline assay method. Expression of the enzyme was investigated by western blot analysis. Relative to controls, neutrophils obtained from diabetic rats presented a 2-fold increase in the activity of inducible NO synthase (iNOS), accompanied by an increase in the expression of the enzyme depicted by western blot. Treatment of diabetic animals with NPH insulin (2 IU/day, for 3 days) reduced both the activity and expression of iNOS to normal levels. Results presented suggest that overexpression of the inducible isoform of NO synthase by neutrophils may be responsible, at least in part, for the defects in leukocyte–endothelial interactions in diabetes mellitus.

Keywords: Neutrophils; Nitric oxide synthase; Diabetes mellitus

1. Introduction

Evidence has shown that insulin is involved with the development of the inflammatory process [1,2]. The early local exudative cellular reaction in an inflammatory lesion is impaired in alloxan-induced rats due to a reduced migration of neutrophils to the inflamed area [3]. This finding is not dependent on the number of circulating leukocytes, hyperglycaemia alone, or hyperosmolality secondary to hyperglycaemia. A complete recovery is attained by treatment of the rats with insulin [3]. The number of leukocytes rolling along the venular endothelium of the microcirculation network is reduced markedly in diabetic rats. If a noxious stimulus is applied to induce a local lesion, leukocytes adhere to the vessel wall and accumulate in the connective tissue of normal rats in a pattern characteristic of the inflammatory reaction, whereas in diabetic rats the number of adhered leukocytes is reduced and only a few cells are found in the perivascular tissue. Reversal of the defective leukocyte–endothelial interactions is observed after treatment of diabetic rats with insulin [4,5].

Several lines of evidence indicate that NO is an endogenous modulator of leukocyte adhesion in postcapillary venules [6], due to its ability to inhibit the expression of cell adhesion molecules, including E-selectin, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 [7]. NO is synthesized by a variety of cells from L-arginine, by a family of NO synthase enzymes. The endothelial [8] and neuronal [9] enzymes are constitutive isoforms dependent upon calcium and calmodulin. The inducible isoforms, calcium-independent, are present in macrophages and others cells [10]. NADPH and cofactors, such as H_4B, are required for enzyme activation [11,12].

The aim of the present study was to evaluate the activity and expression of NO synthase in neutrophils from alloxan-induced diabetic rats.

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Abbreviations: NO, nitric oxide; iNOS, inducible nitric oxide synthase; Tris, Trizma base; FMN, flavin mononucleotide; H_4B, (6*R*)-tetrahydro-l-biopterin; DTT, dithiothreitol; L-NAME, N^G-nitro-L-arginine methylester; L-NMMA, N^G-monomethyl-L-arginine; NBT, 4-nitroblue tetrazolium chloride; and BCIP, 5-bromo-4-chloro-3-indolyl-phosphate.

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2. Materials and methods

2.1. Materials

Alloxan monohydrate, oyster glycogen, Tris, HEPES, EDTA, NADPH, FMN, FAD, H_4B, DTT, L-NAME, L-NMMA, Dowex AG 50Wx8–400 resin, SDS, glycerol, bromophenol blue, and β-mercaptoethanol were purchased from the Sigma Chemical Co. [^{3}H]L-Arginine was from NEN Products. Liquid scintillation Ultima Gold was from Packard. NPH insulin (Iolin®) was from Biobrás. NBT and BCIP were obtained from Bio-Rad. Nitrocellulose membranes were from Amersham. Anti-NO synthase antibody (mouse IgG_1 anti-rat iNOS), alkaline-phosphatase conjugated rabbit anti-mouse IgG, and the positive control (mouse macrophage iNOS) were from Transduction Laboratories.

2.2. Animals

Male Wistar rats weighing 180–200 g at the beginning of the experiments were used. The rats were allowed a standard pellet diet and free access to water and maintained at 23° under a 12/12 hr light/dark cycle. All experiments were in accord with the ethical principles in animal research adopted by the Biomedical College of Animal Experimentation (COBEA) and approved by the Ethical Committee for Animal Research (CEEA) of the Biomedical Sciences Institute/University of São Paulo.

2.3. Induction and treatment of diabetes mellitus

Diabetes mellitus was induced by the i.v. injection of alloxan (42 mg/kg) dissolved in physiological saline. Control rats were injected with physiological saline alone. Ten, thirty, or one hundred eighty days thereafter, the presence of diabetes was verified by blood glucose concentrations $>$ 200 mg/dL, determined with the aid of a blood glucose monitor (Advantage®, Eli Lilly), in samples obtained from the cut tip of the tail. A group of diabetic rats were treated with NPH insulin (2 IU/day, s.c.) for 3 days before measurements or assays were performed.

2.4. Isolation of peritoneal neutrophils

Neutrophils were obtained from the peritoneal cavity of the rats 4 hr after the injection of 20 mL of sterile 1% oyster glycogen in physiological saline [13]. Erythrocytes were removed by hypotonic lysis. The final cell suspension contained 95–98% neutrophils. Cell viability was assessed by the Eosin Y exclusion method.

2.5. NO synthase activity

NO synthase activity was measured by the [^{3}H]L-citrulline assay method as described previously [14] with slight modifications. Neutrophils (1×10^8 cells/mL) resuspended in HEPES buffer (20 mM HEPES, 2 mM $CaCl_2$, 0.32 M sucrose, 1 mM DTT, pH 7.4) were lysed with the aid of a sonifier (Thorton), and centrifuged at 500 *g* for 10 min at 10°. Aliquots (100 μL) from the supernatant were incubated with 200 μL HEPES buffer (50 mM HEPES, 1 mM EDTA, 1.25 mM $CaCl_2$, pH 7.4), containing 4 μM FAD, 4 μM FMN, 4 μM H_4B, 11 mM NADPH, and 1 μCi [^{3}H]L-arginine for 60 min at 37°. The reactions were stopped by the addition of 20 μL buffer containing 20 mM L-arginine and 50 mM EDTA. The reaction mixtures were applied to Dowex AG 50Wx8–400 (Na^+ form) resin columns and eluted with HEPES buffer (20 mM, pH 5.5) and water (v/v). The radioactivity corresponding to [^{3}H]L-citrulline content in the samples was measured by liquid scintillation counting. Protein concentration was determined by the Bio-Rad protein assay [15]. Results are presented as picomoles citrulline per milligram protein per minute.

Tests were performed in the absence of NADPH and calmodulin and in the presence of NO synthase inhibitors, such as L-NAME and L-NMMA, at concentrations of 10, 100, and 1000 μM.

2.6. Western blot

Samples obtained from lysed neutrophils, as described above, were diluted (1 mg protein/mL) in Tris buffer [62.5 M Tris–HCl (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.001% bromophenol blue] and heated at 100° for 5 min. Proteins (10–20 μg) were separated on 7% gels by SDS–PAGE [16] and transferred to nitrocellulose membranes during 2 hr (150 mA) in Tris–glycine buffer (25 mM Tris, 192 mM glycine) containing 0.1% SDS and 20% methanol. Membranes were incubated with Tris-buffered saline [TBS; 200 mM Tris, 1.37 M NaCl (pH 7.6)] containing 5% skim milk, followed by overnight incubation at 4° with the primary monoclonal antibody (mouse IgG_1 anti-rat iNOS, 1:2500). After extensive washings in TBS, the nitrocellulose membranes were incubated with the secondary antibody (rabbit anti-mouse IgG conjugated to alkaline phosphatase, 1:1500) for 2 hr at room temperature. The immunoreactive proteins were visualized with NBT/BCIP. The densitometric quantitation of iNOS expression was performed using Sigma Gel, Gel Analysis Software, Jandel Scientific (Sigma Chemical Co.). Results are presented as arbitrary units.

2.7. Data analysis

Results are presented as means $\pm$ SEM. When appropriate, the data were statistically analyzed by Student's *t*-test or ANOVA followed by the Tukey-Kramer multiple comparisons test. $P < 0.05$ was considered significant.

Table 1
General characteristics of the rats and NO synthase activity in neutrophils

Rats	Body weight gain (g)	Blood glucose (mg/dL)	NO synthase activity (pmol/mg protein/min)	N
Diabetic (10 days)	5 ± 3*	444 ± 12*	343.86 ± 16.29**	7(12)
Matching controls	55 ± 3	105 ± 4	171.98 ± 19.88	7(11)
Diabetic (30 days)	23 ± 13*	367 ± 24*	191.17 ± 4.26*	3(6)
Matching controls	120 ± 4	97 ± 5	133.30 ± 1.02	3(6)
Diabetic (180 days)	104 ± 14***	371 ± 11*	268.10	1(3)
Matching controls	232 ± 11	108 ± 3	105.80	1(3)

Rats were rendered diabetic by the injection of alloxan (42 mg/kg, i.v.) 10, 30, or 180 days before measurements were made. Values are means ± SEM (N = the number of assays performed.). Figures in parentheses indicate the number of rats used in each group.
*–*** Statistically significant vs matching control: * $P < 0.001$, ** $P < 0.0001$, and *** $P < 0.01$.

3. Results

3.1. NO synthase activity in alloxan-induced diabetic rats

Relative to controls, the activity of NO synthase in neutrophils was increased markedly in rats rendered diabetic by the injection of alloxan. Estimates made 10, 30, or 180 days after alloxan administration showed that the increase in NO synthase activity was comparable in all groups of rats tested. These findings were not dependent on the number of neutrophils recovered from the peritoneal cavity of the rats [$1.30 \pm 0.07 \times 10^8$ and $1.40 \pm 0.11 \times 10^8$ cells (means ± SEM) in control and diabetic rats, respectively]. Blood glucose concentrations were elevated sharply and a significant reduction in body weight gain was observed in diabetic rats during these intervals (Table 1).

Tests performed in the absence of some cofactors showed that the activity of the enzyme did not change in the absence of calmodulin, but the absence of calcium reduced NO synthase activity by 30–35% in neutrophils from both diabetic and control rats. In the absence of NADPH, enzyme activity was abolished almost completely. Irrespective of the type of treatment, NO synthase activity in neutrophils from diabetic rats was twice that observed in the controls. Results are illustrated in Fig. 1. Similar results were observed upon inhibition of NO synthase by L-NAME and L-NMMA. Both substances caused a concentration-dependent inhibition of NO synthase, the remaining enzyme activity levels being higher in diabetic rats than in the controls (Fig. 2).

3.2. Role of insulin on NO synthase activity

To verify the relationship between NO synthase activity and the circulating level of insulin, rats that were rendered diabetic by i.v. injection of alloxan, 10 days earlier, were given 2 IU of NPH insulin, s.c, each evening for the last 3 days of the experimental period. Complete normalization of NO synthase activity was observed in diabetic rats treated with insulin in this manner (Fig. 3). The values attained matched those observed in the controls. In addition, blood glucose levels decreased from (mean ± SEM) 447 ± 15 mg/dL before treatment to 285 ± 28 mg/dL after insulin treatment ($P < 0.0001$).

Western blot analysis performed on the lysates from neutrophils showed a strong immunoreactivity for iNOS in the diabetic group. Expression of iNOS decreased after treatment of the diabetic animals with insulin (Fig. 4).

4. Discussion

Results presented here suggest that the activity of NO synthase in neutrophils is regulated by insulin. This suggestion is supported by the following observations: (a) the activity of iNOS was increased markedly in neutrophils obtained from alloxan-induced diabetic rats; (b) this was accompanied by an increase in the level of enzyme expres-

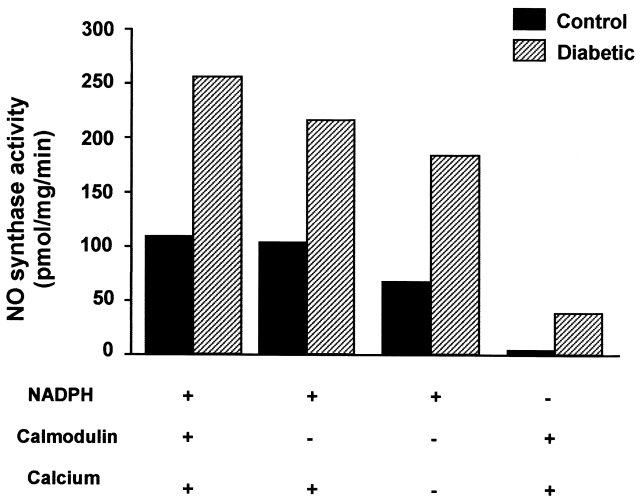

Fig. 1. Effect of calmodulin, Ca^{2+}, and NADPH on the activity of NO synthase in neutrophils of diabetic rats and matching controls. Rats were rendered diabetic by injection of alloxan (42 mg/kg, i.v.) 10 days before measurements were done. Glycogen-elicited peritoneal neutrophils from 3 to 4 rats were pooled, each animal yielding approximately 1×10^8 cells. Calmodulin was used at 400 U/mL; Ca^{2+} at 2 mM; and NADPH at 11 mM. Results are presented as pmol citrulline/mg protein/min. Values are the means of 2 independent experiments.

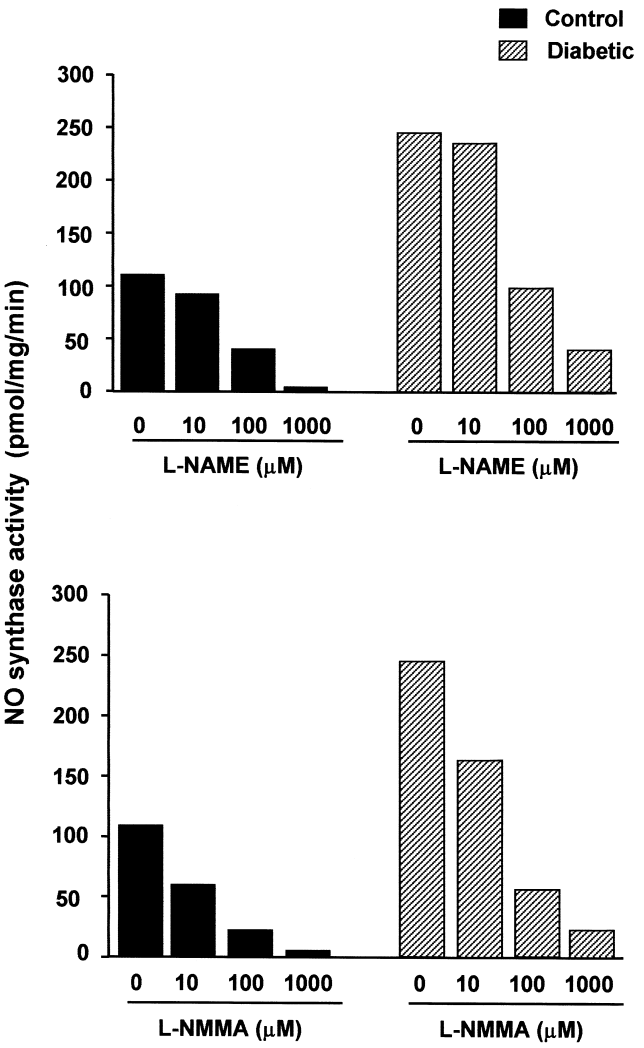

Fig. 2. Inhibition by L-NAME and L-NMMA of NO synthase activity in neutrophils from diabetic rats and matching controls. Animals were rendered diabetic by the injection of alloxan (42 mg/kg, i.v.) 10 days before assays were done. Glycogen-elicited peritoneal neutrophils from 3 to 4 rats were pooled, each animal yielding approximately 1×10^8 cells. Results are presented as pmol citrulline/mg protein/min. Values are the means of 2 independent experiments.

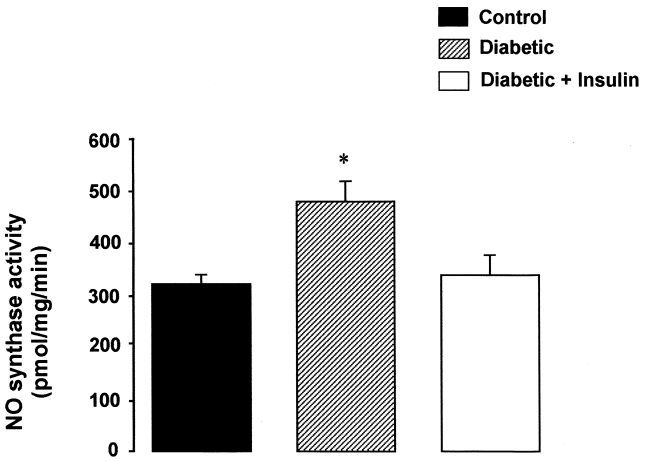

Fig. 3. NO synthase activity in neutrophils of diabetic rats, diabetic rats treated with insulin, and matching controls. Rats were rendered diabetic by the injection of alloxan (42 mg/kg, i.v.) 10 days before the administration of insulin. Insulin (NPH, 2 IU/day, s.c.) was given for the last 3 days before testing. Glycogen-elicited peritoneal neutrophils from 2 to 3 rats were pooled, each animal yielding approximately 1×10^8 cells. For diabetic rats, N = 6; diabetic rats + insulin, N = 8; matching controls, N = 7. Results are presented as pmol citrulline/mg protein/min. Values are means ± SEM of 3–4 experiments in each group. Key: (*) $P < 0.05$ vs other values.

sion; and (c) both the activity and expression of iNOS returned to normal after treatment of diabetic rats with insulin.

As determined by the [^{3}H]L-citrulline assay method, iNOS activity in peritoneal neutrophils from diabetic rats was approximately twice that observed in the controls. Differences between diabetic and control groups ranged from 1.5 to 2.5 times. This difference in activity was observed from the early stages of diabetes and persisted up to 180 days after alloxan injection. Similar results were obtained from western analysis of iNOS expression. The lysates prepared from neutrophils of diabetic rats showed strong immunoreactivity for the inducible isoform of NO synthase (130 kDa).

Tests performed to characterize NO synthase in peritoneal neutrophils showed that the enzyme activity is dependent on calcium but not on calmodulin. NO synthase purified from rat neutrophils is known to be dependent on calcium, NADPH, and H_4B [17,18]. A 130-kDa protein and a 22-kDa linked protein were purified by SDS–PAGE from human neutrophils [19] and were characterized in these cells upon stimulation with interleukin-1, tumor necrosis factor-α, and interferon-γ [20]. These proteins were also characterized in rat neutrophils in a model of endotoxic shock [21]. Furthermore, we clearly demonstrated a concentration-dependent effect on NO synthase activity in rat neutrophils using two inhibitors, L-NAME and L-NMMA.

In both series of experiments, testing enzyme activity in the absence of some cofactors or in the presence of L-NAME and L-NMMA, the activity of NO synthase in neutrophils from diabetic rats was approximately twice the activity observed in matching controls. Values returned to normal levels after the diabetic rats were treated with insulin. Daily injections of NPH insulin had to be administered for at least 3 days before the assays were done. Insulin treatment clearly was not sufficient to maintain normal blood glucose levels in these rats. Accordingly, the increase in NO synthase activity might be primarily linked to continuing insulin deficiency rather than to secondary hyperglycaemia occurring in the diabetic rats. Adequate concentrations of insulin seem to be required for the normal function of endothelial cells and neutrophils during the course of the inflammatory process. The local exudative cellular reaction in an inflammatory lesion, including carrageenan-induced pleurisy [3], allergic pleurisy [22], and

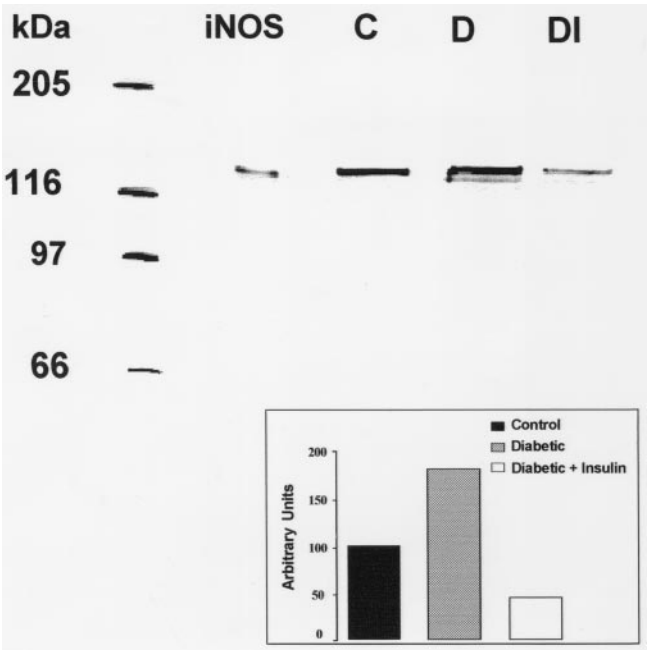

Fig. 4. Western blot analysis on lysates from neutrophils. Cells were obtained from diabetic rats (D), diabetic rats treated with insulin (DI), and matching controls (C). Animals were rendered diabetic by the injection of alloxan (42 mg/kg, i.v.) 10 days before the administration of insulin. Insulin (NPH, 2 IU/day, s.c.) was given for the last 3 days before testing. Glycogen-elicited peritoneal leukocytes from 2 to 3 rats were pooled, each animal yielding approximately 1×10^8 cells. Inducible NO synthase (iNOS) from mouse macrophages was used as a positive control. Densitometric analysis (inset) was performed using the Sigma Gel, Gel Analysis Software, Jandel Scientific (Sigma).

allergic airway inflammation [23] depends on the availability of insulin.

Recent studies support the idea that insulin may regulate the activity of NO synthase. NO production (NO_2 levels) and NO synthase mRNA expression are elevated in macrophages of rats and mice made diabetic by streptozotocin injection [24]. Increased expression of endothelial cell NO synthase in the kidneys of diabetic rats was prevented by treatment with L-NAME or insulin [25]. In the present study, we have demonstrated that the activity and expression of the inducible isoform of NO synthase in neutrophils are increased in alloxan-induced diabetic rats. The ability of insulin to normalize enzyme activity and expression is an indication that the alteration observed is a consequence of the diabetic state.

Several lines of evidence implicate NO as an endogenous inhibitor of leukocyte adhesion in venules. It has been shown that inhibition of NO synthase elicits recruitment of adherent leukocytes [26,27]; NO donors (nitroprusside) attenuate or prevent leukocyte adherence induced by different inflammatory stimuli [28]; and superoxide, by reacting with NO, promotes leukocyte adherence [29]. In addition, previous studies of experimental diabetes mellitus demonstrated defects in leukocyte–endothelial interactions and leukocyte chemotaxis [3–5,30]. Any dysfunction in the sequence of events leading to the ability of leukocytes to migrate efficiently in response to inflammatory stimuli could result in an increased susceptibility of the host to infectious diseases [31,32]. It is well established that certain infections occur almost exclusively in diabetic patients, and many diabetic patients have a worse prognosis once infection is established [1–2,33].

Results presented here suggest that overexpression of the inducible isoform of NO synthase by neutrophils may be responsible, at least in part, for the defects in leukocyte–endothelial interactions in diabetes mellitus, which represents an aggravating factor for host defence in the first stages of infection.

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