Diabetologia (1998) 41: 101--104

Diabetologia

Decreased nitric oxide synthase activity in platelets from IDDM and NIDDM patients

R.A. Rabini[1], **R. Staffolani**[2], **P. Fumelli**[1], **B. Mutus**[3], **G. Curatola**[2], **L. Mazzanti**[2]

[1] Department of Diabetology, INRCA Hospital, Ancona, Italy
[2] Institute of Biochemistry, University of Ancona School of Medicine, Ancona, Italy
[3] Department of Chemistry and Biochemistry, University of Windsor, Windsor, Ontario, Canada

Summary Nitric oxide (NO) produced by platelet nitric oxide synthase (NOS) inhibits platelet activation by increased cytoplasmic cGMP levels. The aim of this study was to investigate platelet NOS activity in insulin-dependent (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM), which are characterized by enhanced platelet activation. HbA_{1c} levels, platelet NOS and platelet membrane Na^+/K^+ ATPase activity were determined in 19 IDDM patients, 21 NIDDM patients and 31 healthy control subjects. NOS activity was measured by a spectrophotometric method based on NO-dependent oxidation of oxyhaemoglobin to met-haemoglobin. Na^+/K^+ ATPase activity was measured by the method of Kitao and Hattori. Both NOS and Na^+/K^+ ATPase activity were significantly reduced in diabetic subjects compared with control subjects. NOS showed a significant negative relation with HbA_{1c} levels and a positive relation with Na^+/K^+ ATPase activity in diabetic patients. It is hypothesized that the decreased NOS activity might play a role in the pathogenesis of diabetic vascular complications. [Diabetologia (1998) 41: 101--104]

Keywords Nitric oxide synthase, platelet, diabetes mellitus, HbA_{1c}, Na^+/K^+ ATPase.

Nitric oxide (NO) has been shown to inhibit platelet activation through an increase in cytoplasmic cGMP levels [1,2]. Recent evidence suggests that the cGMP increase induced by insulin in human platelets, which accounts for the antiaggregatory effect of the hormone, is mediated by NO [3]. NO production within the platelet is made possible by the presence of a constitutive isoform of the enzyme nitric oxide synthase (NOS) , which has been recently isolated from human platelets [4]. Platelet NOS has a distinct molecular weight (150 kDa) and its amino acid sequence has been deduced from its mRNA sequence [4].

Low levels of intraplatelet cGMP have been described in insulin-dependent diabetic (IDDM) subjects and it has been hypothesized that this alteration might account for the platelet hyperreactivity during the disease [5]. However, no data are available at present in the literature directly concerning the study of platelet NOS in human diabetes, which is accompanied by an enhanced platelet activation.

The aim of the present study was to investigate directly the platelet NOS activity in patients affected by IDDM and NIDDM and to look for possible relations with metabolic control. Moreover, we determined in the same subjects the platelet Na^+/K^+ ATPase activity, as a relation has been hypothesized between this enzymatic activity and NO production in endothelial cells from diabetic rats [6].

Received: 30 June 1997 and in revised form: 3 September 1997

Corresponding author: Prof. L. Mazzanti,, Institute of Biochemistry, University of Ancona, via P. Ranieri 65, 60131 Ancona, Italy

Abbreviations: NO, Nitric oxide; NOS, nitric oxide synthase; IDDM, insulin-dependent diabetes mellitus; NIDDM, non-insulin-dependent diabetes mellitus.

Subjects and methods

The study was performed on 19 IDDM outpatients (9 men, 10 women, age 34 ± 8 years, duration of disease 7 ± 5 years, fasting glycaemia = 9.5 ± 2.9 mmol/l, HbA_{1c} 9.0 ± 1.3 %, range 6.3--11.3 %), 21 NIDDM outpatients (10 men, 11 women, age

61 ± 7 years, duration of disease 5 ± 4 years, fasting glycaemia = 8.9 ± 2.8 mmol/l, HbA_{1c} 8.8 ± 1.6 %, range 6.1--11.9 %), and 31 healthy volunteers (15 men, 16 women, age 56 ± 12 years, fasting glycaemia = 4.3 ± 0.5 mmol/l, HbA_{1c} 4.9 ± 0.7 %). Each subject gave informed consent before the investigation. The diabetic subjects were normoalbuminuric and normotensive. All the subjects showed plasma lipid levels and body mass index within the normal range. Three IDDM patients and two NIDDM patients were affected by background retinopathy. The patients were receiving the same medications (oral agents for the treatment of diabetes for NIDDM, insulin regimen for IDDM).

Blood was drawn in the fasting state for the determination of glucose, HbA_{1c} levels, Na^+/K^+ ATPase activity of the platelet plasma membrane, platelet NOS activity. HbA_{1c} was measured by high-performance liquid chromatography according to the method of Akai [7].

Na^+/K^+ ATPase assay. The preparation of platelet plasma membranes was performed as we described previously [8]. Briefly, platelet-rich plasma was centrifuged for 15 min at $3000 \times g$. The pellet was washed twice in modified Tyrode's buffer (pH 7.5) containing (mmol/l) 130 NaCl, 5 KCl, 1 NaH_2PO_4, 24 $NaHCO_3$, 2 Na_2 EDTA, 12 glucose, 12.5 sucrose, and 0.35 % (w/v) bovine serum albumin. The cells were then lysed by ultrasonication and the lysate was centrifuged at $19\,000 \times g$ to eliminate unlysed platelets, mitochondria, and granules. The supernatant was centrifuged at $100\,000 \times g$ and the pellet obtained was layered over a 40 % (w/v) sucrose solution and centrifuged again at $100\,000 \times g$ for 120 min. The interface membrane subfraction, which consisted of plasma membranes, was used for the Na^+/K^+ ATPase assay.

Na^+/K^+ ATPase activity was determined by a modification of the Kitao method [9], as previously described [10]. Briefly, ATPase activity was assayed by incubating membranes at 37 °C in (mmol/l) 5 $MgCl_2$, 140 NaCl, 14 KCl, 40 Tris-HCl, pH 7.7. The ATPase reaction was started by the addition of 3 mmol/l Na_2ATP. Inorganic phosphate (Pi) hydrolysed from this reaction was measured by the method of Fiske and Subbarow [11].The ATPase activity assayed in the presence of 10 mmol/l ouabain was subtracted from the total Mg^{++}-dependent ATPase activity to calculate the activity of Na^+/K^+ -ATPase. Protein concentration was determined as described by Lowry et al. [12] using serum albumin as a standard.

NOS assay. NOS activity was measured spectrophotometrically by following the oxidation of oxyhaemoglobin (λ max 426 nm) to methaemoglobin (λ max 405 nm) by NO at 37 °C, as previously described [4]. Oxyhaemoglobin was prepared by reduction. Briefly, human haemoglobin (60 mg; Sigma, St. Louis, Mo., USA) and sodium dithionite (120 mg) were dissolved in distilled water (2 ml) and gently agitated in a flat dish for 15 min. The haemoglobin mixture was then chromatographed on a column of Sephadex G-25 (1.5 × 30 cm). The reduced fraction, with its characteristic bright red colour, was isolated and stored frozen (--20 °C). The NOS assay mixture consisted of 200 µmol/l $CaCl_2$, 1 mmol/l $MgCl_2$, 100 µmol/l L-Arg, 100 µmol/l NADPH, 1.6 µmol/l oxyhaemoglobin, 12 µmol/l (6R)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride. The reaction was initiated with 50 µl of platelet homogenate which was mixed with the reaction medium with the aid of a glass plumper. The amount of NO produced was calculated from the decrease in absorbance at 426 nm (with an estimated extinction coefficient of 98000 litres · mol $^{-1}$ · cm^{-1}). NOS activity was expressed in pmol NO produced · min^{-1} · mg $protein^{-1}$.

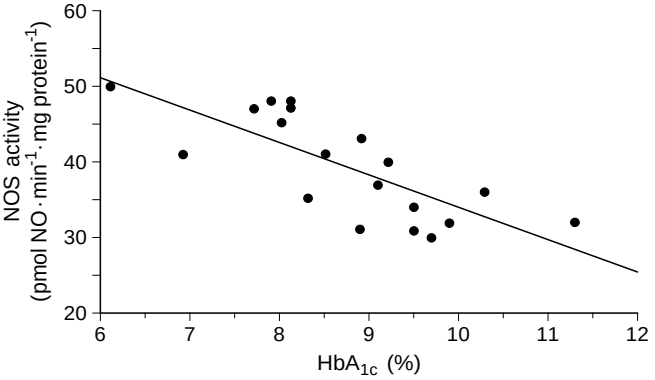

Fig. 1. Linear regression analysis of the relation between HbA_{1c} levels and platelet nitric oxide synthase (NOS) activity in IDDM patients ($y = 76.424 - 4.244x$; $r = -0.76$, $p < 0.001$)

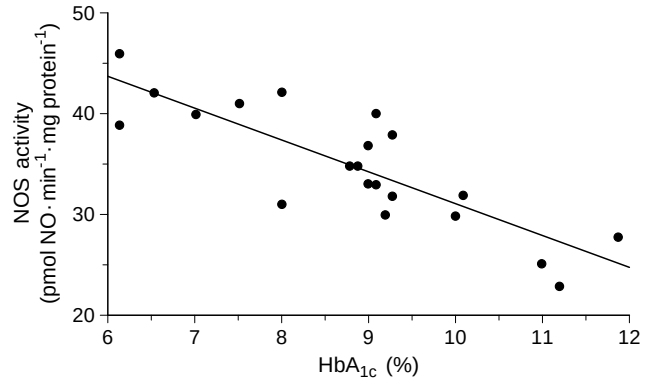

Fig. 2. Linear regression analysis of the relation between HbA_{1c} levels and platelet nitric oxide synthase (NOS) activity in NIDDM patients ($y = 62.739 - 3.163x$; $r = -0.84$, $p < 0.001$)

Statistical analysis. The significance of differences was assessed by variance analysis. The correlation studies were performed by linear regression analysis, using Pearson's coefficient of correlation.

Results

Platelet NOS activity was significantly reduced in both IDDM and NIDDM patients compared with control subjects (Table 1, $p < 0.01$). A significant negative correlation was found between HbA_{1c} levels and NOS activity in IDDM ($r = -0.76$, $p < 0.001$, Fig. 1) and NIDDM subjects ($r = -0.84$, $p < 0.001$, Fig. 2), while no significant relation was observed between these parameters in healthy subjects. Fasting glycaemia and NOS activity were not related in any of the groups studied.

The Na^+/K^+ ATPase activity of the platelet membrane was significantly lower both in IDDM and in NIDDM patients than in healthy subjects (Table 1, $p < 0.01$). No statistically significant relation was found between Na^+/K^+ ATPase activity and HbA_{1c} levels in the groups studied ($r = -0.41$ in NIDDM,

Table 1. Platelet nitric oxide synthase (NOS) activity and platelet membrane Na^+/K^+ ATPase activity in healthy control subjects, IDDM, and NIDDM patients

	Control subjects ($n = 31$)	IDDM ($n = 19$)	NIDDM ($n = 21$)
NOS activity (pmol NO · min^{-1} · mg $protein^{-1}$)	115 ± 34	40 ± 11[a]	35 ± 6[a]
Na^+/K^+ ATPase activity (μmol Pi · h^{-1} · mg $protein^{-1}$)	1.62 ± 0.23	1.18 ± 0.08[a]	1.09 ± 0.14[a]

Data are mean ± SD
[a] $p < 0.01$

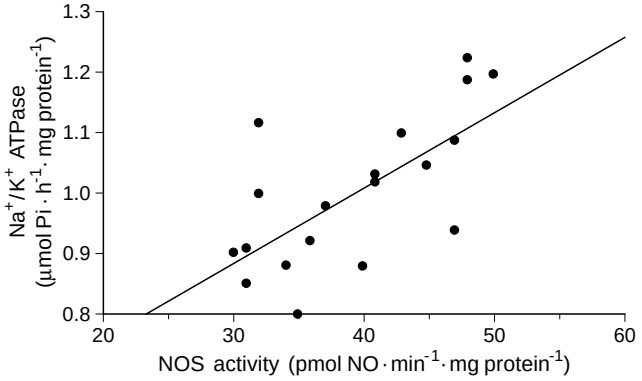

Fig. 3. Linear regression analysis of the relation between platelet nitric oxide synthase (NOS) activity and platelet membrane Na^+/K^+ ATPase activity in IDDM patients ($y = 0.506 + 0.013x$; $r = 0.68$, $p < 0.001$)

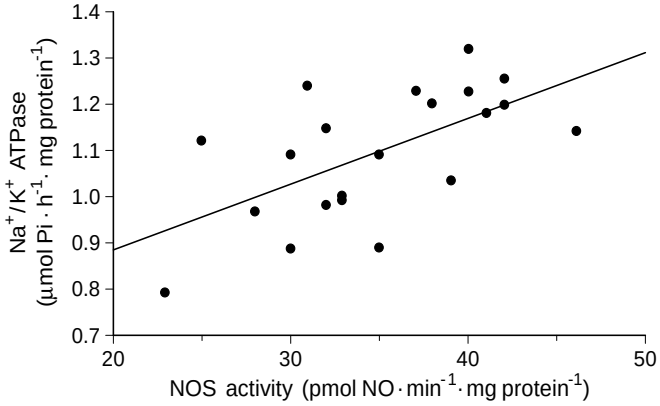

Fig. 4. Linear regression analysis of the relation between platelet nitric oxide synthase (NOS) activity and platelet membrane Na^+/K^+ ATPase activity in NIDDM patients ($y = 0.592 + 0.014x$; $r = 0.61$, $p < 0.01$)

$r = -0.43$ in IDDM, $r = 0.03$ in healthy subjects). NOS and Na^+/K^+ ATPase activity showed a significant positive relation in IDDM ($r = 0.68$, $p < 0.001$, Fig. 3) and NIDDM subjects ($r = 0.61$, $p < 0.01$, Fig. 4), while no significant statistical relation was found in healthy subjects.

Discussion

The inhibition of platelet aggregation seems to be one of the primary biological roles of NO [13], which can be synthesized within the platelets by NOS from L-arginine. In fact, the addition of L-arginine to a platelet suspension has been demonstrated to inhibit collagen-induced aggregation [1].

In the present study a significant decrease in NOS activity was found in both IDDM and NIDDM patients. This observation is consistent with previous studies reporting an increased platelet aggregability in diabetes and a decreased cGMP concentration in IDDM patients [5]. Moreover, it confirms in human platelets the reduced capacity to synthesize NO that has been observed in other cell types, i.e. endothelial cells, in experimental [14] and human [15] diabetes.

The relation observed between glycated haemoglobin concentration and intraplatelet NOS activity in diabetic subjects suggests that a poor glycaemic control is accompanied by a decreased platelet NO production. Such a result seems consistent with the increased micro and macro angiopathic complications observed in poorly controlled diabetes, possibly related to platelet activation [16]. The lack of correlation between NOS activity and HbA_{1c} in healthy subjects might be explained by the narrow range of HbA_{1c} levels in this group, which would require a larger number of observations to detect a statistical significance.

The relation between HbA_{1C} and NOS suggests that glycation is involved in the reduced NOS activity in diabetes, while the lack of a correlation between glycaemia and NOS seems to indicate that the chronic exposure to high glucose levels is more relevant than the acute effects of hyperglycaemia. Both the hypotheses of a direct glycation of NOS and of an effect mediated by the glycation of calmodulin, which is known to modulate NOS activity [4], must be taken into consideration. However, a possible role of insulin resistance or insulin deficiency cannot be excluded on the basis of the present data and of the reported stimulatory effect of insulin on NO production in platelets from healthy subjects [3].

In addition, it must be underlined that high glycaemic levels might exert a direct action on NO produced by cells. In fact, during glucose autooxidation reactive oxygen species are formed, such as superoxide, hydrogen peroxide, and hydroxyl radicals [17]. NO is rapidly inactivated by superoxide anion which reacts to produce peroxynitrite ($ONOO^-$), which is a strong oxidant able to cause lipid peroxidation and protein modifications [18]. The hypothesis that in diabetic patients NO is rapidly inactivated by the glucose-induced oxidative stress must therefore also be suggested.

Previous studies by our group reported a derangement in platelet ion transport through the plasma

membrane during IDDM and NIDDM, with modifications of calcium cytosolic levels related to changes in the active sodium transport [19]. The activity of NOS is modulated by intracellular levels of calcium ions by means of changes in calmodulin concentrations [4]. It might therefore be hypothesized that the correlation observed in IDDM and NIDDM subjects between platelet NOS and Na^+/K^+ ATPase activity is dependent on their common relation with intracellular calcium. The relation observed in the present work between these enzymatic activities is consistent with a previous study reporting that the decrease in vascular Na^+/K^+ ATPase activity induced by hyperglycaemia in rabbit aorta is related, at least in part, to a decrease in the basal release of endothelium-derived NO [6].

In conclusion, the present study demonstrates a decreased NO production in platelets from IDDM and NIDDM patients and suggests a relation between this alteration and glycaemic control. The modification in NOS activity might play a central role in the development of the increased platelet reactivity in human diabetes mellitus and in the pathogenesis of diabetic micro- and macroangiopathy.

Acknowledgements. The present work was supported by a grant of the Ferrero Foundation to L.M. and by the grant n. 96.03293 of the Italian National Research Council (CNR) to G.C.

References

1. Radomsky MW, Palmer RMJ, Moncada S (1990) An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. Proc Natl Acad Sci USA 87: 5193--5197
2. Mellio BT, Ignarro LJ, Ohlstein EH, Pontecorvo EG, Hyman AL, Kadowitz PJ (1981) Evidence for the inhibitory role of guanosine-3',5'-cyclic monophosphate in ADP-induced human platelet aggregation in the presence of nitric oxide and related vasodilators. Blood 57: 946--955
3. Trovati M, Massucco P, Mattiello L, Piretto V, Cavalot F, Mularoni E, Anfossi G (1996) The insulin-induced increase of guanosine-3',5'-cyclic monophosphate in human platelets is mediated by nitric oxide. Diabetes 45: 768--770
4. Muragandam A, Mutus B (1994) Isolation of nitric oxide synthase from human platelets. Biochim Biophys Acta 1200: 1--6
5. Amado JA, Salas E, Botana MA, Poveda JJ, Berrazueta JR (1993) Low levels of intraplatelet cGMP in IDDM. Diabetes Care 16: 809--811
6. Gupta S, Sussman I, McArthur CS, Tornheim K, Cohen RA, Ruderman NB (1992) Endothelium-dependent inhibition of Na^+-K^+ -- ATPase activity in rabbit aorta by hyperglycemia. Possible role of endothelium-derived nitric oxide. J Clin Invest 90: 727--732
7. Akai T (1982) On the measurement of glycosylated hemoglobin In: Baba S, Okuda K (eds) Vensonic Seminar, Proceedings 1. Kyoto Daiichi Kagaku Co, Kobe, Tokyo pp 1117--1125
8. Rabini RA, Ferretti G, Galassi R et al. (1994) Modified fluidity and lipid composition in lipoproteins and platelet membranes from diabetic patients. Clin Biochem 27: 381--385
9. Kitao T, Hattori K (1983) Inhibition of erythrocyte ATPase activity by aclacinomycin and reverse effect of ascorbate on ATPase activity. Experientia 39: 1362--1364
10. Mazzanti L, Staffolani R, Rabini RA et al. (1992) Modifications in platelet membrane transport functions in insulin-dependent diabetes mellitus and in gestational diabetes. Biochim Biophys Acta 1139: 65--69
11. Fiske C, Subbarow Y (1925) The colorimetric determination of phosphorus. J Biol Chem 66: 375--400
12. Lowry OH, Rosenbrough NJ, Farr AL, Randall RT (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 192: 265--275
13. Moncada S, Palmer RMJ, Higgs EA (1991) Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 43: 109--141
14. Durante W, Sen AK, Sunahara FA (1988) Impairment of endothelium dependent relaxation in aortae from spontaneously diabetic rats. Br J Pharmacol 94: 463--468
15. Saenz de Tejada I, Goldstein I, Azadzoi K, Krane R, Cohen R (1989) Impaired neurogenic and endothelium-dependent relaxation of human penile smooth muscle: the pathophysiological basis for impotence in diabetes. N Engl J Med 320: 1025--1030
16. Colwell JA, Lopez-Virella MF (1988) A review of the development of large vessel disease in diabetes mellitus. Am J Med 85 [Suppl 5A]:113--118
17. Baynes JW (1991) Perspectives in diabetes: role of oxidative stress in development of complications in diabetes. Diabetes 40: 405--412
18. Francescutti D, Baldwin J, Lee L, Mutus B (1996) Peroxynitrite modifications of glutathione reductase: modeling studies and kinetic evidence suggest the modification of tyrosines at the glutathione disulfide binding site. Protein Engineering 9: 189--194
19. Mazzanti L, Rabini RA, Faloia E, Fumelli P, Bertoli E, De Pirro R (1990) Altered cellular calcium and sodium transport in diabetes mellitus. Diabetes 39: 850--854