**REGULAR ARTICLE**

**Endothelial Dysfunction, Nitric Oxide and Platelet Activation in Hypertensive and Diabetic Type II Patients**

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**Abstract**

Alterations in the synthesis or enhanced inactivation of nitric oxide (NO) and an increase in endothelin-1 production lead to an imbalance that can induce arterial hypertension. Type II diabetes is characterized by impaired endothelium-dependent vasodilation and vascular disease. NO is produced through l-arginine pathway by three different isoforms of nitric oxide synthase (NOS), an inducible form that can be activated by cytokines such as tumor necrosis factor α (TNFα). We evaluated NO plasmatic levels, endothelial damage markers such as von Willebrand factor (vWF), platelet activation, soluble P-selectin (sP-Sel), TNFα levels, insulinaemia (I), glycosylated haemoglobin (HbA1c), glycaemia and blood pressure in 32 hypertensive diabetic type II patients (Group A), 37 hypertensive patients (Group B) and 35 healthy subjects (Group C) matched in sex, age, body mass index and dietary habits. The level of I was increased in patients compared to the controls and correlated with their NO levels.

vWF plasmatic levels were increased in Group A compared to Groups B and C. We also found significant differences in platelet activation among all the groups. In diabetic patients, increased NO levels correlated with TNFα, HbA1c and platelet activation showed greater endothelial damage than in Group B. These parameters described a prothrombotic state associated with an insulin resistance state, an increased vWF release, raised sP-Sel and TNFα levels and, maybe, low NO bioavailability, which could lead to a higher risk of development of thrombotic events in hypertensive diabetic patients (Group A) than in the hypertensive patients in Group B.

**Key Words:** Endothelial dysfunction; Hypertension; Diabetes; Nitric oxide; Platelet activation; von Willebrand factor

**Dysfunction of the vascular endothelium has been implicated in the pathophysiology of several cardiovascular disorders, hypertension and diabetes [1], and has been defined as an imbalance between relaxing and contracting factors [2]. Nitric oxide (NO) is synthetized through the l-arginine pathway by the enzyme nitric oxide synthase (NOS). There are three different NOS isoforms: neuronal (nNOS), endothelial (eNOS), which are constitutives, and an inducible NOS form (iNOS) [3].**
Impairment of NO synthesis or increased inactivation contribute to the clinical consequence which includes vascular hypertrophy, increased platelet and monocyte adhesion to the endothelium, atherosclerosis, myocardial infarction, peripheral arterial occlusive disease and stroke [4,5].

Attenuated endothelium-dependent vasorelaxation has been demonstrated in patients with diabetes mellitus, hypercholesterolemia and hypertension even in absence of morphologically evident vascular lesions [6]. Arterial hypertension produces a shear stress effect on vascular endothelial cells, and this could lead to change in the production or release of vasoactive substances such as NO [7].

Micro- and macrovascular complications of diabetes have a complex pathogenesis involving dysfunction and damage of vascular endothelial cells [8], which are susceptible to stimulatory factors such as increased glucose (GI) concentrations, oxidative stress and advanced glycation end-products (AGEs) [9].

Although hyperglycaemia, dyslipidemia and hypertension can all independently cause vascular disease, endothelial dysfunction may be intrinsic to the insulin resistance syndrome described in type II diabetes and hypertension [10–13].

This can lead to an “activated state” characterized in part by platelet adhesion and aggregation increases, as well as the expression of P-selectin (P-Sel) on platelet membrane [14].

P-Sel (GMP-140, PADGEM, CD62P), a member of the selectin family, cellular adhesion molecules, is a 140-kDa glycoprotein, which is found in the platelet α granules and in the Weibel–Palade bodies of endothelial cells [15]. After platelet activation produced by agonist such as thrombin or histamin, P-Sel is translocated into the surface of plasmatic membrane and mediates adhesion of platelet or endothelial cells to neutrophils and monocytes [16] and can also contribute to the recruitment of leucocytes and oxidative injury in the vascular endothelium. P-Sel is considered a marker of platelet activation [14] and could have an important role in the development of diabetic atherosclerosis. Raised levels of soluble P-selectin (sP-Sel) in the plasma have been described in diabetes and, also, in hypertension [17,18].

On the other hand, von Willebrand factor (vWF), a multimeric glycoprotein synthesized by megakaryocytes and endothelial cells, helps platelet adhesion into the subendothelium and increased plasmatic levels have been related to endothelial cell dysfunction [19].

Inflammatory cytokines such as interleukin-1 and tumor necrosis factor α (TNFα) have multiple effects on the endothelium, can lead to prothrombotic and proinflammatory states and stimulate NO production through the iNOS isoform [20].

To better understand the mechanisms involved in the prothrombotic state of diabetes, we evaluate the relationship between metabolic parameters, arterial pressure, cytokines and NO levels in 69 patients.

1. Patients and methods

1.1. Patients

The study was conducted with three groups of subjects who gave informed consent. The study protocol was approved by the Ethics Committee of the Hospital.

These groups consisted of 32 hypertensive non-insulin-dependent diabetes mellitus (NIDDM) patients aged 64.9 ± 1.7 years, 93% males (Group A), in treatment with sulphonylureas, 37 hypertensive patients (Stages I and II, JNC VI) [21] aged 61.3 ± 1.7 years, 92% males (Group B), in treatment with calcium antagonist or diuretics and non-nitrate givers and 35 healthy controls aged 59.1 ± 2.0 years, 91% males (Group C) and were matched for body mass index and dietary habits.

All subjects were free of any acute clinical illness or coronary artery disease or stroke 6 months before the study.

Exclusion criteria were erythrocyte sedimentation rate > 20 mm/h, serological evidence of hepatitis or HIV infection, acute or chronic liver and kidney disease, malignancy and connective tissue disease.

Total cholesterol, LDL and HDL fractions, coagulation and hematological screening were done at the initial visit.
1.2. Methods

Blood samples were obtained following nontraumatic venipuncture. Gl was measured on a Vita Lab Selectra analyser (Merck, USA), and glycosylated haemoglobin (HbA1c) by glycated haemoglobin-capture Reagent Pack (IMX System, Abbott Diagnostic, USA). sP-Sel, vWF, TNFα and insulinaemia (I) were assayed by ELISA (R&D Systems, UK).

1.3. Nitric Oxide

NO was measured as nitrite + nitrate in heparinized plasma as described by Moshage et al. [22]. Nitrate was assayed as nitrite after enzymatic conversion by nitrate reductase from Aspergillus spp. (Sigma-Aldrich, USA), then nitrite was measured by using the Griess reaction (1% sulfanilamide, 0.1% naphthylethylenediamine in 2.5% phosphoric acid).

Diastolic and systolic blood pressure (DBP and SBP) were measured with a sphygmomanometer (in mm Hg).

1.4. Platelet activation

The expression of P-Sel on surface (CD62P) was performed by a modification of the Michelson [23] method. Platelets were isolated by differential centrifugation. Briefly, 77 mM EDTA anticoagulated blood was centrifuged at 200 × g for 20 min at room temperature (RT). The platelet-rich plasma from patients and normal controls was fixed with equal volume of 2% formaldehyde in phosphate-buffered saline pH 7.4 (PBS). The platelets were washed twice with 10 mM EDTA buffer and the concentration was adjusted to 10^7 platelets/ml. Aliquots of 50 μl of the fixed platelets suspension were incubated with 10 μl of monoclonal antihuman platelet glycoprotein IIIa (CD61) (clone Y2/51, Dako, Glostrup, Denmark), washed twice, and

![Fig. 1. NO plasmatic level (NO, μM). Measured as nitrite + nitrate in heparinized plasma. Statistical significance: Group A vs. B, *P < .01; Group A vs. C, **P < .001; Group B vs. C, *P < .05.](image-url)
then with 10 μl of monoclonal antibody CD62P (Immunotech, Marseille, France) for 30 min at RT followed by two washes with EDTA buffer and then underwent a second incubation with FITC-conjugated F(ab')2 fragment of goat antimouse immunoglobulins (F 479, Dako). Platelet activation was measured in 10000 CD61 positive events by determining the percentage of platelet expressing CD62P. Statistical significance: Group A vs. B, \( P < .001 \); Group A vs. C, \( ** P < .001 \); Group B vs. C, \( * P < .005 \).

Fig. 2. Platelet expression of CD62P (%). Platelet activation was measured in 10000 CD61 positive events by determining the percentage of platelet expressing CD62P. Statistical significance: Group A vs. B, \( P < .001 \); Group A vs. C, \( ** P < .001 \); Group B vs. C, \( * P < .005 \).

1.5. Statistical Analysis

Results was expressed as means ± S.E.M. Statistical significance was inferred at a two-tailed \( P < .05 \). The control and hypertensive groups were compared using two-sample \( t \) test and Pearson correlation analysis. All statistical tests

Fig. 3. vWF plasmatic level (IU/ml). Assayed by ELISA method. Statistical significance: Group A vs. B, \( P < .05 \); Group A vs. C, \( * P < .05 \); Group B vs. C, NS.

Fig. 4. CD62P and I correlation in Group A. Pearson’s \( r \) (correlation coefficient) = .9521. \( R^2 = .9066 \). \( P < .01 \).

Fig. 5. NO and I correlation in Group A. Pearson’s \( r \) (correlation coefficient) = .9226. \( R^2 = .8512 \). \( P < .01 \).
were performed using the statistical software Kwistat 4.

2. Results

Table 1 shows characteristics of patients and controls. A significant difference was noted between the three groups in NO plasmatic level (Fig. 1), in CD62P platelet expression (Fig. 2) and in the insulin levels. Asterisks in figures indicate differences between patients and normal controls. Moreover, there was a significant difference between patients, as well. A difference was also recorded when analysing plasmatic levels of vWF between Groups A and B ($P < .05$), similar to Groups A and C ($P < .05$). However, the vWF release from Group B was not different from that in normal controls (Fig. 3). TNF$_\alpha$ plasmatic levels were increased in Group A with respect to Groups B and C ($P < .05$ and $P < .001$, respectively), but not between Groups B and C. Plasmatic level of sP-Sel, which is considered to be an activation platelet marker [18], was increased in Groups A with respect to B ($P < .05$) and C ($P < .001$). As shown in Table 1, the values of I, Gl and HbA1c found in Groups A and B were significantly different from normal controls, although these values were within the normal range in Group B.

Plasmatic levels of cholesterol were increased in hypertensive NIDDM patients (data not shown). Hematological screening and plasmatic coagulation studies were within normal range in all the subjects studied.

Correlations (Group A): We observed highly significant positive correlations between CD62P and HbA1c, Gl and I ($P < .001$) (an example of these correlations is shown in Fig. 4) and I with sP-Sel and NO ($P < .006$, $P < .01$, respectively). Fig. 5 provides information about the relationship of NO and I. A positive correlation was also obtained between TNF$_\alpha$ levels and HbA1c and NO ($P < .001$, $P < .05$, respectively).

Correlations (Group B): Only one positive correlation was observed between I and NO ($P < .001$) (Fig. 6).

3. Discussion

Products of advanced protein glycosilation (AGES) accumulate in tissues as a function of time and sugar concentration and induce permanent abnormalities in the extracellular matrix component function, stimulate cytokine release and reactive oxygen species production through AGE-specific receptors and modify intracellular proteins [24]. In diabetic animals, accumulated AGEs correlate with defect in the vasodilatory response to NO [25].

Some authors reported that arterial hypertension could be an insulin resistance state with raised insulin values to keep relatively normal glycaemia levels [26,27]. A primary defect in the vascular action of insulin may be an intermediate mechanism that links endothelial dysfunction with reduced insulin-mediated cellular Gl uptake in metabolic and cardiovascular disorders. There is a functional coupling between insulin action and basal endothelial NO production in humans [28]. Basal levels of NO are produced by the action of eNOS in endothelial cells [3]. The expression of iNOS can be induced by inflammatory mediators as lipopolysaccharide (LPS) and cytokines such as TNF$_\alpha$, and this isoform produces larger amounts of NO than eNOS [29,30]. Experimental studies in animals with endothelial dysfunction, showed that there

![Fig. 6. NO and I correlation in Group B. Pearson’s $r$ (correlation coefficient) = .8536. $R^2 = .7287$. $P < .001$.](image-url)
was a compensated relationship between both enzymes and increased plasmatic levels of NO, which would display less biological activity or impaired bioavailability in hypertension [31,32]. The increased level of NO could also be cytotoxic and related to the pathophysiology of myocardial infarction, cardiomyopathy and septic shock [33].

Ferlito and Galina [5] reported that in hypercholesterolemic diabetic patients, the NO-enhanced levels could reflect a compensatory response to a continuous inactivation of NO involved in a protective competition toward damaging factors and mainly against oxidized LDL.

In spite of the increased levels of NO found in our diabetic patients (Fig. 1), the hypertensive state remained; in these situations, NO bioavailability may be reduced by a mechanism involving superoxide anion (O$_2^-$) in the vascular wall [34] but not cyclooxygenase derivatives [35]. NO and I levels correlated in diabetic and in hypertensive patients (Figs. 5 and 6).

Among other authors, we demonstrated that elevated percentage of activated platelet circulate in diabetic patients [12,14,15,19] (Fig. 2). High levels of HbA1c and G1 corresponded with an increased CD62P expression in all the patients of Group A. There were important differences respect to the type of diabetes and the clinical status. In NIDDM patients, there was a constant increase in the fraction of activated platelets, and there also was a correlation between CD62P and I (Fig. 4). The increased subpopulation of CD62P positive platelets seems of particular interest for the pathogenesis of vascular lesions, since CD62P-related antigen is a specific cytoadhesive molecule for the attachment of neutrophils to activated platelets and endothelial cells [14,15].

Several clinical and experimental reports suggested that high vWF levels reflect damage to the endothelium or endothelial dysfunction (for review, see Ref. [19]). Abnormalities of vWF were demonstrated in diabetics and could be involved in the pathogenesis of diabetic vasculopathy. As Lip and Blann [19] previously reported, despite the fact that the vWF levels were significantly increased in diabetic, we were unable to correlate it with HbA1c. vWF release was increased in patients with hypertension but was normalized in patients in whom hypertens-

sion was successfully treated with antihypertensive drugs (Fig. 3).

Raised sP-Sel levels were found in disease such as diabetes and related with platelet activation and thrombosis [17]. We observed that there was no significant correlation between sP-Sel and vWF suggesting that this molecule have different release mechanism from that of vWF [18]. We found a positive correlation between sP-Sel and I levels in diabetic patients. (Group A). sP-Sel may be the product of both platelet and endothelial cells in these patients.

We showed that levels of I correlated with values of CD62P, NO and sP-Sel in NIDDM and with levels of NO in hypertensive patients, these relationships could be associated with their extent of endothelial dysfunction [26–28].

Our results foster the idea that the greater the platelet activation, vWF release, sP-Sel and inflammatory cytokines such as TNF$\alpha$ levels, the higher the risk of the patients for development of thrombotic events is.

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References