Platelet nitric oxide production and IR: Relation with obesity and hypertriglyceridemia

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Abstract  Background and Aim: Three NOS isoforms are responsible for nitric oxide production in various tissues. Endothelial constitutive NOS is expressed in vascular endothelium and in platelets, contributing to vascular tone regulation and platelet aggregation.

The aim of the present work was to examine eNOS polymorphism, to find a correlation with platelet NO production and degree of insulin resistance (IR) in non-diabetic subjects and in patients affected by type 2 diabetes.

Methods and Results: Seventy-one non-diabetic subjects and 37 patients affected by Type 2 diabetes were recruited. The subjects were subdivided into 3 groups as cut-off for the definition of an insulin resistant state: IR non-diabetic subjects, insulin sensitive subjects, and insulin-resistant patients affected by Type 2 diabetes.

Plasma glyco-metabolic parameters, platelet nitric oxide production, endothelial nitric oxide synthase (eNOS) gene polymorphism were measured in all subjects enrolled. Significant differences between groups were found in BMI, fasting glycaemia, fructosamine and HbA1c, triglycerides and HDL cholesterol levels.

Evaluating all the subjects, platelet NO production was significantly related with BMI, waist circumference, and triglycerides concentrations, thus suggesting an association between increased platelet NO production, obesity and hypertriglyceridemia, independent of the degree of insulin-resistance.

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Conclusion: The modified platelet NO synthesis does not seem to be due to eNOS Glu298Asp polymorphism, while it can be hypothesized that it is caused by an iNOS induction, present in obesity, hypertriglyceridemia and in type 2 diabetes.
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Introduction

Nitric oxide (NO) has been identified as a mediator of different physiological processes. In particular, its role in the endothelium-mediated vasodilatation and in platelet activation focused the attention of researchers on the study of NO metabolism during atherosclerosis and in diseases characterized by an increased prevalence of atherosclerosis, such as diabetes mellitus.

An endothelial dysfunction is present in diabetes and in different pathologies that are included in the insulin-resistance (IR) syndrome, such as hypertension and hyperlipidemia. Therefore, it might be hypothesised that endothelial dysfunction, due to an altered NO cellular metabolism, is associated with the IR syndrome [1].

Insulin induces the release of NO from endothelial nitric oxide synthase (eNOS) in endothelial cells [2] and causes a dose-dependent induction of eNOS in human aortic cells (and possibly arterial/endothelial cells) [3]. Both effects may contribute to the overall vasodilatory effect of insulin.

NO is physiologically produced by platelets and decreases platelet adhesion and aggregation. It has been demonstrated that insulin stimulates the formation of NO in human platelets through an increased production of cGMP [4,5]. In recent years, it has been shown that insulin is able to exert anti-inflammatory, antithrombotic and profibrinolytic actions [6]. These biological effects include inhibition of platelet aggregation by prompting both the synthesis of NO in platelet and of prostacyclin in endothelial cells [7–9]. On the contrary, IR conditions, such as type 2 diabetes mellitus, are characterized by increased platelet aggregation [10].

A correlation between nitric oxide metabolism and insulin action is also demonstrated by recent experimental data. Rats with alterations of the eNOS and nNOS genes show a reduction in the glucose utilization rate secondary to the development of IR, suggesting that nitric oxide plays a role in the modulation of insulin sensitivity [11]. Duplain and coll. demonstrated that rats with a deletion of the eNOS gene not only show a reduction of the insulin-induced glucose uptake, but also the development of hypertension, early hyperinsulinemia and hyperlipidemia [12].

On the basis of these observations it might be hypothesized that the relation between NO pathway and insulin action may be one of the links between insulin-resistance and atherosclerotic disease.

Three isoforms of NOS are responsible for NO production in various tissues. Endothelial constitutive NOS (eNOS or NOS3) is expressed in vascular endothelium and in platelets and contributes to the regulation of the vascular tone and of platelet aggregation. Its gene is located on chromosome 7q35–36.

Polymorphisms of the eNOS gene might directly or indirectly involve the regulatory site of transcriptional activity or modify the three-dimensional structure of the protein, therefore altering the NOS enzymatic activity and the cellular NO production. Wang et al. [13] have recently demonstrated that the variable nucleotide tandem repeats (VNTR) polymorphism is related to the variations of the plasma levels of NO by-products (NOx) in healthy subjects. On the contrary, no data are currently available in the literature on the relation between Glu298Asp polymorphism, located within exon 7 in the intermediate portion of the heme-binding and calcium-calmodulin binding sites, and the cellular NO synthesis in vivo. Only a few studies reported significant associations between eNOS polymorphisms and cardiovascular disease and conflicting results were often found [14]. Studies of genetic association between the Glu298Asp variant and human diseases found a significant association with myocardial infarction [15], while no association with cerebrovascular diseases or a relation with ischemic stroke have been reported [16].

The aim of the present work was to analyze platelet NO production and its relation with alterations in the eNOS gene in IR conditions. We chose to examine the eNOS polymorphism that was more frequently reported to be associated with cardiovascular diseases, in order to find a correlation with platelet nitric oxide production and with the IR degree in non-diabetic subjects (insulin sensitive and insulin resistant non-diabetic subjects) and in patients affected by type 2 diabetes.

Methods

Patients

The study was performed on 108 men (age of 51.5 ± 12.7 years): 71 healthy non-diabetic subjects (age 52.8 ± 6.6 years) and 37 patients affected by Type 2 diabetes (age 55.9 ± 8.7 years). All participants gave their informed consent prior to drawing the peripheral venous blood. The study was approved by the local ethics committee and was carried out in accordance with the principles of the Declaration of Helsinki as revised in 2000.

Diabetes was diagnosed by OGTT. Control subjects were classified as non-diabetic by means of OGTT. No subject showed the presence of autoantibody (GAD or IA-2). The diabetic patients were not on pharmacological therapy, but only on diet therapy, and particularly they were not taking drugs able to modify insulin-resistance. No chronic complication of the disease was present.

The presence of insulin resistance was investigated by iv insulin tolerance test (ITT). The following parameters were measured before the test: fasting glycemia, HbA1c levels, plasma fructosamine, total and HDL cholesterol, and triglyceride concentrations, plasma concentrations of A1

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and B100 apolipoproteins, fibrinogen levels and platelet NO production. The G894T genetic polymorphism of eNOS was studied in the same subjects.

The plasma concentrations of total and HDL cholesterol, triglycerides, fructosamine, A1 and B100 apolipoproteins, and glyceremia were measured by standard laboratory methods, as previously described [17]. HbA1c was measured using an automated HPLC analyzer (reference range: 4.0—6.0%).

Insulin-resistance assay

Insulin resistance was assessed by the iv insulin tolerance test (ITT), based on the measure of the plasma glucose disappearance rate in the first 15 min after the injection of 0.1 IU regular insulin/kg of body weight as a single bolus. The insulin sensitivity index was calculated according to the formula 0.693/t/2 (half-life of plasma glucose) and defined as KITT [18]. KITT values have been demonstrated to be a reliable measurement of insulin sensitivity [18].

Platelet nitric oxide production

NO released by the platelets was directly measured in the platelet rich plasma (PRP) using an isolated NO meter and its associated probe (IsoNO Mk-II, World Precision Instruments, Sarasota, FL) equipped with the Duo.18 Data Acquisition System, as recently described by Chakravarthy et al. [19]. NO gas diffuses through to the probe tip and is oxidized at the working electrode, resulting in an electrical current proportional to its concentration. The NO probe was calibrated according to the chemical calibration method of Tsukahara [20]. NO production was determined in the PRP after addition of 100 μM L-Arg, which induced a rapid increase in NO release measurable 30 s after stimulation, with a peak between 60 and 90 s. Cell-free controls (media only) were also included and subtracted from the other readings. The intra- and inter-assay variation (CV) were also included and subtracted from the data. The G894T genetic polymorphism of eNOS was previously described [17]. HbA1c was measured using an automated HPLC analyzer (reference range: 4.0—6.0%).

TABLE 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ir C (32)</th>
<th>Is C (39)</th>
<th>Ir D2 (37)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI</td>
<td>30.48 ± 5.22</td>
<td>26.13 ± 3.87</td>
<td>28.41 ± 7.21</td>
<td>0.03</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>100.4 ± 12.1</td>
<td>93.3 ± 14.1</td>
<td>96.6 ± 12.1</td>
<td>NS</td>
</tr>
<tr>
<td>KITT</td>
<td>1.41 ± 0.41</td>
<td>3.34 ± 1.25</td>
<td>1.16 ± 0.47</td>
<td>0.001</td>
</tr>
<tr>
<td>HOMA IR</td>
<td>2.91 ± 1.24</td>
<td>1.54 ± 0.96</td>
<td>4.72 ± 1.30</td>
<td>0.001</td>
</tr>
<tr>
<td>Fasting glycemia (mmol/L)</td>
<td>5.38 ± 0.61</td>
<td>5.10 ± 0.55</td>
<td>9.16 ± 3.71</td>
<td>0.001</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.1 ± 0.8</td>
<td>4.7 ± 0.6</td>
<td>6.5 ± 1.8</td>
<td>0.001</td>
</tr>
<tr>
<td>Fructosamine (μmol/L)</td>
<td>207.82 ± 17.83</td>
<td>199.35 ± 40.29</td>
<td>249.65 ± 37.29</td>
<td>0.001</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.47 ± 1.15</td>
<td>5.39 ± 0.99</td>
<td>5.63 ± 1.12</td>
<td>NS</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.03 ± 0.22</td>
<td>1.19 ± 0.36</td>
<td>0.93 ± 0.16</td>
<td>0.002</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.39 ± 0.64</td>
<td>1.09 ± 0.46</td>
<td>1.52 ± 0.68</td>
<td>0.03</td>
</tr>
<tr>
<td>A1 Apolipoprotein (mg/dL)</td>
<td>156.58 ± 27.35</td>
<td>161.48 ± 29.56</td>
<td>156.39 ± 27.49</td>
<td>NS</td>
</tr>
<tr>
<td>B100 Apolipoprotein (mg/dL)</td>
<td>112.65 ± 32.86</td>
<td>106.95 ± 29.83</td>
<td>126.96 ± 32.02</td>
<td>NS</td>
</tr>
<tr>
<td>NO production (nmol/10⁶ cells)</td>
<td>2.73 ± 0.93</td>
<td>2.72 ± 1.11</td>
<td>2.85 ± 0.81</td>
<td>NS</td>
</tr>
</tbody>
</table>

Means ± standard deviations (SD) are shown. P < 0.05 for the following comparisons: *Ir C vs. Is C and Ir D2; †Is C vs. Ir D2 and Ir C; ‡Ir D2 vs. Ir C and Is C.

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Endothelial nitric oxide synthase (eNOS) gene polymorphism

The endothelial nitric oxide synthase (eNOS) gene is located on chromosome 7q35—36. A variant within exon 7 of the eNOS gene has been studied consisting in a GAG to GAT substitution due to the replacement of glutamic acid by aspartic acid in position 894 (Glu298Asp) [21].

Genomic DNA was prepared from peripheral blood by a standard extraction technique with phenol-chloroform. A 248-base pair (bp) fragment encompassing the polymorphic region has been amplified in vitro by polymerase chain reaction (PCR) according to the protocol described by Yoshimura et al. [21] with the following primers:

5’-AAG GCA GGA GAC AGT GGA TGG A-3’
5’-CCA AGT CAA TCC TGG TGC TCA-3’

Samples were amplified for 30 cycles, consisting of denaturation at 95°C for 3 min, annealing at 68°C for 1 min, and extension at 72°C for 1 min. The amplified fragment was digested by the BanII restriction enzyme. The restriction produces two fragments of 158 bp and 90 bp, analyzed by electrophoresis on 2% agarose gels. The fragment containing the restriction site is defined G allele, while the mutant allele without the BanII cutting site is defined as T allele [22].

Statistical analysis

All results are shown as means ± standard deviation (SD). Data were analysed with SPSS-Win program (version 11.5, SPSS Inc. Chicago, IL). The differences among the groups were estimated by the analysis of variance (ANOVA) followed by Bonferroni t multiple comparisons test to reduce the probability of significant differences arising by chance. The correlation studies were performed by linear regression analysis, using Pearson’s coefficient. A value of p < 0.05 has been considered statistically significant.

Table 1 Body mass index (BMI), waist circumference, insulin sensitivity index (KITT), HOMA IR index, and clinical laboratory parameters of insulin-resistant (IR) non-diabetic subjects (Ir C), insulin sensitive subjects (Is C), and IR patients affected by Type 2 diabetes (Ir D2)
Results

For statistical analysis the subjects studied have been initially subdivided into 3 groups by using a $K_{IT}$ = 2 as a cut-off for the definition of an insulin resistant state: IR non-diabetic subject (Ir C), insulin sensitive subjects (Is C), and insulin-resistant patients affected by Type 2 diabetes (Ir D2). Table 1 shows the data on body mass index, waist circumference, $K_{IT}$, plasma parameters, and platelet NO production of the 3 groups studied.

Statistically significant differences among the groups were found in BMI (Ir C vs. Is C and Ir D2, $p < 0.03$), fasting glycaemia (Ir D2 vs. Ir C and Is C, $p < 0.0001$), fructosamine (Ir D2 vs. Ir C and Is C, $p < 0.0001$) and HbA1c, levels (Ir D2 vs. Ir C and Is C, $p < 0.0001$), plasma levels of HDL cholesterol (Is C vs. Ir C and Ir D2, $p < 0.002$) and triglycerides (Is C vs. Ir C and Ir D2, $p < 0.03$). On the contrary, waist circumference, plasma total cholesterol, A1 and B100 apolipoprotein concentrations did not differ statistically among the groups nor were statistically significant differences found in platelet NO production in the three groups (Table 1).

Three genotypes associated with the Glu298Asp polymorphism were found: GG, TG and TT. The eNOS/GG, TG and TT genotypes were found in 48%, 34% and 15% of all the subjects studied, respectively. The distribution of the polymorphism among the groups is shown in Fig. 1. The statistical study did not find significant differences in the frequency of the eNOS variants among the groups.

When all the subjects studied were divided into three groups according to the presence of a different eNOS genotype, no significant difference in the platelet NO production was found among these three groups (Fig. 2) (GG = 2.81 ± 0.99 nmol NO/10^6 cells, TG = 2.75 ± 0.93 nmol NO/10^6 cells, TT = 2.46 ± 0.54 nmol NO/10^6 cells).

When insulin resistance expressed as $K_{IT}$ was considered as a continuous variable, no significant correlation was found between the degree of IR and platelet NO production either examining all the subjects as a single group ($r = 0.041$, NS) or examining separately non-diabetic subjects ($r = 0.099$) and subjects affected by Type 2 diabetes ($r = -0.143$). Also when subjects were divided into groups according to the eNOS genotype, no significant correlation was present between $K_{IT}$ and NO production (GG: $r = -0.085$, TG: $r = -0.159$, TT: $r = 0.010$). Additionally, no significant correlation was found between the HOMA IR index and platelet NO production either examining all the subjects as a single group ($r = 0.150$, NS) or examining separately non-diabetic subjects ($r = 0.211$, NS) and subjects affected by Type 2 diabetes ($r = -0.198$, NS).

When all the subjects of this study were evaluated as a whole, platelet NO production was significantly related with BMI ($r = 0.378$, $p = 0.008$), waist circumference ($r = 0.259$, $p = 0.023$) and plasma triglycerides concentrations ($r = 0.310$, $p = 0.008$), while no significant correlation was found with other parameters.

When the subjects were divided into two groups according to the presence or absence of Type 2 diabetes, platelet NO production was significantly related with BMI ($r = 0.378$, $p = 0.008$), waist circumference ($r = 0.259$, $p = 0.023$) and plasma triglycerides concentrations ($r = 0.310$, $p = 0.008$), while no significant correlation was found with other parameters.

When the subjects were divided into two groups according to the presence of IR patients affected by Type 2 diabetes (Ir D2), NO production was not statistically related with any other parameter examined in the present study.
Our data show for the first time in vivo the lack of a statistically significant correlation between the Glu298Asp polymorphism and the basal platelet NO production in human subjects. Therefore, this genetic variant does not seem relevant in the regulation of the basal NOS activity in platelets.

Moreover, the present results did not find any statistically significant association between the Glu298Asp polymorphism and the presence of type 2 diabetes and/or insulin-resistance, conditions characterized by an increased prevalence of atherosclerotic lesions and cardiovascular disease.

Nitric oxide inhibits platelet activation through an increase of intracellular cGMP. Recent data demonstrated that the cGMP increase induced by insulin in human platelets is mediated by NO.

Previous studies by our group showed a reduced activity of platelet NOS in patients affected by type 1 and type 2 diabetes [17], suggesting that this biochemical alteration might be at the basis of the increased platelet aggregation in diabetes. The present results did not find significant modifications of the platelet NO synthesis in diabetic patients compared with non-diabetic insulin sensitive and insulin resistant subjects. This discrepancy might be due to the choice of patients, which in the present work were only on dietetic therapy and in better metabolic control than in the previous study (HbA1c = 6.4 ± 1.8, fructosamine levels = 249.65 ± 37.29 μmol/L vs HbA1c = 8.8 ± 0.8, fructosamine levels = 353.28 ± 51.16 in the previous work) [17].

No significant correlation has been found between platelet NO synthesis and IR evaluated either by KITT or by HOMA-IR index. This lack of a statistical association between insulin-resistance and NO synthesis might be due to the simultaneous presence of a significant direct correlation between NO production and BMI, waist circumference, and plasma triglycerides levels. The direct association between BMI and platelet NO production confirms recent data, reporting in healthy adolescents a relation between obesity and plasma NOx levels, with an increase in the estimated NO production in subjects with BMI > 25 kg/m² [23]. On the contrary, it is not consistent with the hypothesis that in obesity the bioavailability of NO might be reduced because of increased chronic oxidative damage [24].

Obesity has been recently defined as an inflammatory disease [25]. Adipocytes produce a number of peptides involved in the pathogenesis of insulin-resistance and/or inflammation, such as C reactive protein, interleukin 6, TNF-α and leptin, which are also related with cardiovascular diseases. This observation might explain the increased risk of diabetes and cardiovascular mortality in obese subjects.

The rat adipose tissue expresses the NOS inducible isoform [26]. Bacterial lipopolysaccharides (LPS) in vivo increase the expression and activity of iNOS both in the white and in the brown adipose tissue. The iNOS induction can be reproduced in vitro by treating adipocytes in culture with LPS and pro-inflammatory cytokines (TNF-α, interferon γ).

It might be hypothesized that the reduced NO synthesis by eNOS caused by a lower insulin action in the presence of insulin-resistance may be counterbalanced by the simultaneous iNOS activation caused by obesity and by the latent pro-inflammatory condition present during IR. iNOS has a specific activity for NO production 6 times greater than eNOS [27] and therefore its expression and activation in
platelets could completely mask the eNOS inhibition due to a reduced insulin action.

As concerns the correlation found between triglyceridemia and platelet NO production, data in the literature suggest that an important role in the modulation of endothelium-mediated vasodilation is played by the different content in fatty acids [28].

Experiments performed on in vitro cultured endothelial cells demonstrated that the NO release is modulated by the presence of different fatty acids. Fatty acids inside the cell can activate proteins of the PPAR (peroxisome proliferator-activated receptor) family, with induction of the iNOS expression, or can remain within the membrane lipid bilayer, determining alterations in the binding of proteins usually interacting with the membrane, such as eNOS. The finding of the strong statistical association between platelet NO production and triglyceridemia in all the subjects studied and in particular in Type 2 diabetes suggests an involvement of lipid metabolism in the regulation of NO synthesis. Further studies are needed to clarify the relations between NO pathway and dyslipidemia.

In conclusions, the results of this work suggest the presence of an association between increased platelet NO production, obesity and hypertriglyceridemia, independently of the degree of insulin-resistance. The modified platelet NO synthesis does not seem to be due to eNOS Glu298Asp polymorphism.

References