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Short communication

Homocysteine-induced inhibition of nitric oxide production in platelets: a study on healthy and diabetic subjects

B. Mutus[1], R. A. Rabini[2], R. Staffolani[2], R. Ricciotti[2], P. Fumelli[2], N. Moretti[3], D. Martarelli[3], L. Mazzanti[3]

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

Abstract

Aims/hypothesis. The molecular mechanisms involved in the platelet activation observed in hyperhomocysteinemia are not known. We aimed to discover if homocysteine concentrations are associated with abnormal platelet nitric oxide production in healthy and diabetic subjects.
Methods. The study cohort included 28 patients with Type I (insulin-dependent) diabetes mellitus, 30 patients with Type II (non-insulin-dependent) diabetes mellitus, and 34 healthy subjects. Homocysteine plasma concentrations were measured by high-performance liquid chromatography. Platelet nitric oxide production was measured using a nitric oxide meter before and after a 3-h incubation with 100 μmol/l homocysteine. Stimulation experiments were done in vitro by the addition of α-thrombin (0.2 U/ml).
Results. Basal platelet nitric oxide production was lower in diabetic patients than in healthy subjects. Nitric oxide release was reduced by in vitro homocysteine incubation, being lower in platelets from diabetic patients than in platelets from control subjects. Thrombin increased nitric oxide synthesis in platelets from healthy subjects both in the presence and absence of homocysteine. In diabetic subjects thrombin increased nitric oxide release in the absence of homocysteine. But in the presence of homocysteine the response was reduced. An inverse relation was found between plasma homocysteine levels and basal platelet nitric oxide release in diabetic and healthy subjects.
Conclusion/interpretation. Homocysteine could exert its atherogenic action in healthy and diabetic subjects partly by inhibiting platelet nitric oxide production with the subsequent increased platelet activation and aggregation. [Diabetologia (2001) 44: 979–982]

Keywords Diabetes mellitus, nitric oxide, platelets, homocysteine.

Moderate increases in the plasma concentrations of homocysteine are associated with an increased incidence of arteriosclerosis and cardiovascular disease [1]. Homocysteine could exert adverse vascular effects by a number of mechanisms: production of reactive oxygen species caused by auto-oxidation, reduced endothelial generation of nitric oxide (NO) and/or enhancement of vascular smooth muscle cell proliferation [2]. Recent data have shown that flow-mediated vasodilation is reduced in the presence of methionine-induced mild hyperhomocysteinemia and that this endothelial dysfunction is because of impaired NO activity without change of oxidative status [3]. Moreover, the exposure to homocysteine increases the interaction of leukocytes and endothelium through the expression of the endothelial cell adhesion molecules (P-selectin and ICAM-1) and the up regulation of CD18 expression in neutrophils [4]. A concomitant treatment with an NO donor reduces these homocysteine effects [4].

Platelet-rich thrombus formation has been found in homocysteine-induced atherosclerotic lesions but

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Corresponding author: Prof. Laura Mazzanti, Institute of Biochemistry, School of Medicine, University of Ancona, Via Ranieri 65, Ancona, Italy
Abbreviations: PRP, platelet rich plasma

there is little data on the molecular mechanisms involved in platelet activation [2]. Nitric oxide is a powerful inhibitor of platelet activation [5]. The increased risk of arteriosclerosis in diabetes mellitus is accompanied by enhanced platelet aggregation. The later could be partly explained by the reduced production of nitric oxide observed in platelets from Type I and Type II diabetic patients [6].

We aimed to determine in vivo whether increased plasma homocysteine levels are associated with abnormal platelet NO production in healthy and diabetic subjects and to study in vitro the effect of incubation with homocysteine on the release of NO from platelets.

Subjects and methods

The study was performed on 28 patients affected by Type I diabetes mellitus (14 men, 14 women, age = 38 ± 10 years, duration of disease = 12 ± 7 years, fasting glycaemia = 5.9 ± 0.9 mmol/l, HbA_{1c} = 7.9 ± 0.6 %), 30 patients affected by Type II diabetes mellitus (15 men, 15 women, age = 53 ± 9 years, duration of disease = 8 ± 5 years, fasting glycaemia = 5.4 ± 0.7 mmol/l, HbA_{1c} = 7.3 ± 0.6 %) and 34 healthy control subjects (18 men, 16 women, age = 46 ± 13 years, fasting glycaemia = 4.4 ± 0.5 mmol/l, HbA_{1c} = 5.0 ± 0.5 %). The subjects gave their informed consent. All the subjects were normoalbuminuric and normotensive and their plasma lipid concentrations and BMI were within the normal range. The diabetic patients did not show any microvascular or macrovascular complication and were receiving the same medications (oral agents for the treatment of diabetes for Type II and insulin regimen for Type I diabetic patients).

Blood was drawn in the fasting state to measure plasma glucose and homocysteine concentrations, HbA_{1c} concentrations, and for the isolation of platelet rich plasma (PRP), which was prepared by centrifuging the freshly obtained blood at 1000 x g for 15 min. Platelets were counted in the PRP using a Coulter counter (Coulter-S-plus). The PRP was divided into 3 aliquots. These were used respectively for an immediate measurement of platelet NO production as well as for a 3-h incubation with or without 100 μmol/l homocysteine (DL-homocysteine, Sigma, St. Louis, Mo., USA) to measure NO production. Stimulation experiments were furtherly done by the addition of human α-thrombin (0.2 U/ml for 1 min) to PRP with and without previous incubation with homocysteine.

Plasma homocysteine was measured with high-performance liquid chromatography (HPLC) using a method previously described [7]. HbA_{1c} was measured using an automated HPLC analyzer (reference range :4.0–6.0 %) [6].

Platelet NO production. NO released by the platelets was directly measured in the PRP using an isolated NO meter and its associated probe (IsoNO Mk-II, World Precision Instruments, Sarasota, Fla., USA) equipped with the Duo.18 Data Acquisition System, as recently described [8]. NO gas diffuses through to the probe tip and is oxidized at the working electrode, resulting in an electrical current proportional to its concentration. NO production was measured in the PRP after the addition of 100 μmol/l L-Arg. This induced a rapid increase in NO release measurable at 30 s after stimulation with a peak between 60 and 90 s.

Statistical analysis. The significance of differences was assessed by variance analysis. The correlation studies were done by linear regression analysis, using Pearson's coefficient of correlation. A p value of less than 0.05 was considered to be statistically significant.

Results

Plasma homocysteine concentrations did not change in diabetic patients when compared with healthy control subjects (healthy control subjects: 9.4 ± 2.0, Type I diabetic patients: 9.9 ± 1.8, Type II diabetic patients: 10.8 ± 2.2 μmol/l).

NO production measured in platelets immediately after drawing blood was lower both in Type I (1.45 ± 0.31 nmol NO/min/10^6 cells, $p < 0.01$) and Type II diabetic subjects (1.56 ± 0.42 nmol NO/min/10^6 cells, $p < 0.01$) when compared with control subjects (2.85 ± 0.61 nmol NO/min/10^6 cells) (Fig. 1). No change in NO production was found after a 3-h incubation of PRP without homocysteine in comparison with basal NO release (Type I diabetic patients = 1.37 ± 0.37, Type II diabetic patients = 1.52 ± 0.41, control subjects = 2.92 ± 0.57 nmol NO/min/10^6 cells). On the contrary, after a 3-h incubation of PRP with 100 μmol/l homocysteine a reduction in NO release was observed in all the three groups of subjects ($p < 0.01$ vs basal levels; Fig. 1). NO production in platelets from diabetic patients after homocysteine incubation was lower than NO release from the platelets of control subjects under the same conditions (Fig. 1, $p < 0.001$ both Type I and Type II diabetic patients vs healthy subjects).

In control subjects the addition of thrombin to PRP caused an increase in NO synthesis both with and without homocysteine pre-incubation (Fig. 1; $p < 0.001$ vs production in the presence and in the absence of homocysteine without stimulation). In diabetic patients, thrombin stimulation markedly increased NO release in the absence of homocysteine (Type I: 4.04 ± 0.76, Type II: 3.92 ± 0.81 nmol NO/min/10^6 cells, $p < 0.001$ vs basal production both in the presence and in the absence of homocysteine). In the presence of homocysteine pre-incubation the response, however, was significantly reduced ($p < 0.01$ vs basal levels both in the presence and in the absence of homocysteine and vs thrombin-stimulated production without homocysteine).

After thrombin stimulation without homocysteine no difference was observed in platelet NO production between healthy subjects and diabetic subjects but in the presence of homocysteine the thrombin-stimulated increase in NO production was significantly lower in diabetic patients than in control subjects ($p < 0.001$, Fig. 1).

The correlation studies found a significant inverse relation between plasma homocysteine concentra-

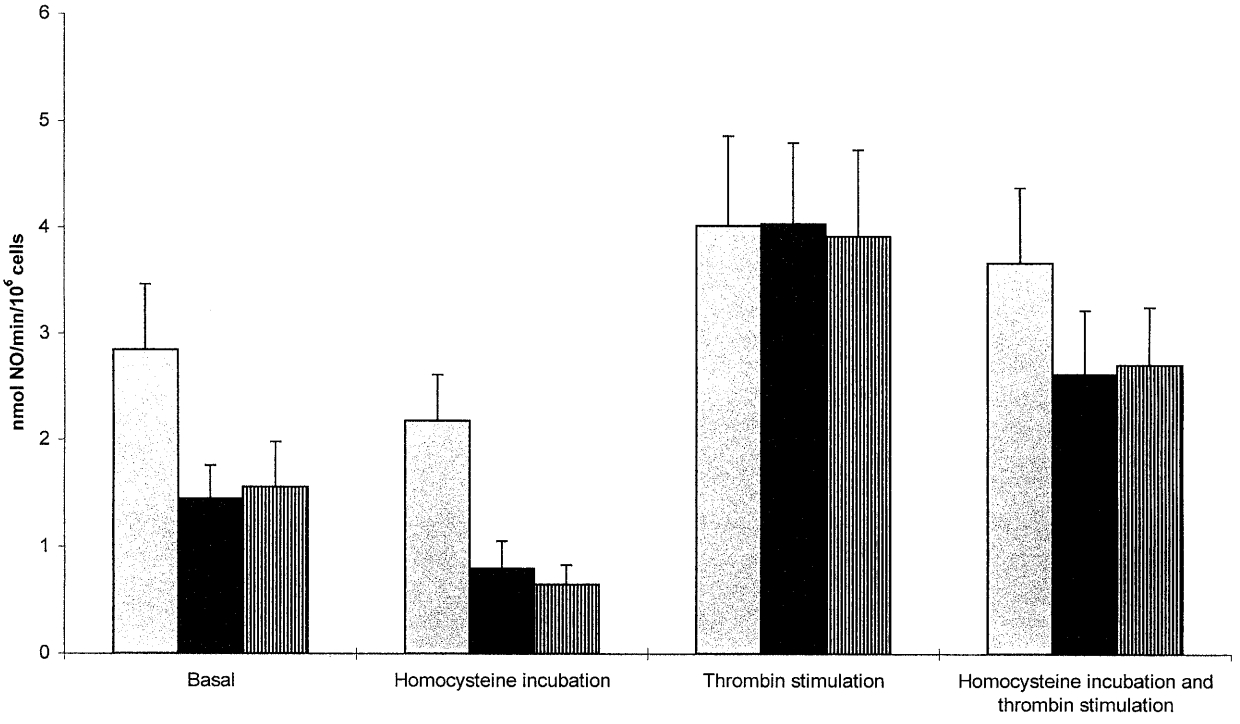

Fig. 1. Nitric oxide production in platelets from healthy control subjects (▒) and from Type I (■) and Type II (▥) diabetic patients in the basal state (basal), after in vitro incubation with homocysteine (homocysteine incubation), after thrombin stimulation in the absence of homocysteine (thrombin stimulation), after thrombin stimulation in the presence of homocysteine (homocysteine incubation and thrombin stimulation). Mean and standard deviation are shown

tions and basal platelet NO release in Type I ($y = -0.119x + 2.65$, $r = -0.774$, $p < 0.001$) and Type II diabetic patients ($y = -0.155x + 3.23$, $r = -0.830$, $p < 0.001$), and in healthy subjects ($y = -0.190x + 4.85$, $r = -0.508$, $p < 0.005$). Plasma homocysteine concentrations and basal platelet NO release were not related with glycaemia or HbA_{1c} concentrations in any of the groups studied.

Discussion

We recently reported a reduced amount of activity of the enzyme nitric oxide synthase in platelets from both Type I and Type II diabetic patients, suggesting that reduced NO production might cause enhanced platelet activation in diabetes mellitus [6]. Our results support this observation because a direct measurement of NO production showed it was significantly reduced in resting platelets from diabetic patients compared with healthy subjects.

There is no data on the possible relation between homocysteine and platelet NO production, although the action of homocysteine on NO release from endothelial cells has been widely studied. This study shows that an association exists between homocysteine levels and platelet nitric oxide (NO) production both in vivo and in vitro in healthy and diabetic subjects. The inverse relation, found in vivo in both diabetic and healthy subjects, between plasma homocysteine concentrations and basal platelet NO production is consistent with previous observations showing a strong relation between changes in plasma homocysteine concentrations and endothelial function, probably occurring by modifications in endothelial NO activity [3, 4].

The results of our in vivo study agree with an in vitro investigation, where we found platelet NO production to be reduced by homocysteine by about the same absolute amounts in all the three groups, so that cellular NO release after homocysteine incubation was again lower in diabetic patients than in healthy control subjects. Patients affected by other diseases with impaired cellular NO release, such as hypercholesterolaemia, might also have a reduced NO production related to homocysteine.

Recent epidemiological studies have validated the relation between high plasma homocysteine levels and increased incidence of atherosclerosis and cardiovascular disease [1, 2]. Our study suggests that homocysteine might exert its atherogenic action in diabetic patients partly by further reducing the basally impaired platelet NO release with a subsequent increased activation and aggregation. The blunted in vitro response of platelet NO production to thrombin confirms that homocysteine is associated with an alteration in NO metabolism in diabetic patients in the presence of physiological stimuli as well.

Our in vitro observations could be physiologically relevant because the homocysteine concentrations used were close to the plasma levels of mild hyperhomocysteinemia (15 to 30 μmol/l) [1, 2]. Moreover, the short incubation time might mimic the in vivo conditions because it is probable that homocysteine plasma levels rise for short period of times to concentrations higher than in the fasting state (for example, in the post-prandial state after methionine intake).

Homocysteine could lower platelet NO production in several ways: direct binding to NO with the formation of S-nitrosohomocysteine, generation of superoxide anion radicals and hydrogen peroxide with subsequent reduced bioavailable NO [9, 10], induction of inducible NOS (iNOS) with concomitant production of NO and $O_2^{\cdot-}$ reacting together to form peroxynitrite. Further studies are necessary to clarify the molecular mechanisms involved in the inhibiting effect exerted by homocysteine on platelet NO release which could play a crucial part in the pathogenesis of atherosclerosis.

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