Diabetic Patients Without Vascular Complications Display Enhanced Basal Platelet Activation and Decreased Antioxidant Status

Evelyne Véricel,1 Caroline Januel,1 Martine Carreras,1 Philippe Moulin,1,2 and Michel Lagarde1

Vascular complications are the leading causes of morbidity and mortality in diabetic patients. The contribution of platelets to thrombembolic complications is well documented, but their involvement in the initiation of the atherosclerotic process is of rising interest. Thus, the aim of the present study was to evaluate basal arachidonic acid metabolism in relation to the redox status of platelets in both type 1 and type 2 diabetic patients, in the absence of vascular complications, as compared with respective control subjects. For the first time, we show that basal thromboxane B2, the stable catabolite of thromboxane A2, significantly increased in resting platelets from both type 1 and type 2 diabetic patients (58 and 58%, respectively), whereas platelet malondialdehyde level was only higher in platelets from type 2 diabetic subjects (67%). On the other hand, both vitamin E levels and cytosolic glutathione peroxidase activities were significantly lower in platelets from diabetic patients as compared with respective control subjects. We conclude that platelet hyperactivation was detectable in well-controlled diabetic patients without complications. This abnormality was associated with increased oxidative stress and impaired antioxidant defense in particular in type 2 diabetic patients. These alterations contribute to the increased risk for occurrence of vascular diseases in such patients. Diabetes 53: 1046–1051, 2004

Diabetes is associated with accelerated rates of thrombosis, circulation dysfunction, and atherosclerosis, and it is fully recognized that long-term macrovascular complications are the main cause of morbidity and mortality associated with diabetes. The Diabetes Control and Complications Trial (DCCT) and U.K. Prospective Diabetes Study (UKPDS) (1,2) indicate a consistent relationship between hyperglycemia and the incidence of chronic vascular complications in patients with type 1 (insulin-dependent) and type 2 (non–insulin-dependent) diabetes. One of the consequences of hyperglycemia is oxidative stress resulting in the generation of free radicals, glycation, and advanced glycation end products. On the other hand, it is generally admitted that abnormally accelerated platelet functions contribute to the increased incidence of thrombotic and atherosclerotic diseases. Several factors can contribute to platelet activation, and one of them could be oxidative stress (3), considering that hyperglycemia increases the production of reactive oxygen species, leading to oxidative stress (4).

Several factors are involved in the platelet activation process, including the platelet shape change, release of intracellular organelles (in particular, components of the blood coagulation pathway), and aggregation. The molecular steps of platelet activation are numerous and complex, including the release of arachidonic acid from membrane phospholipids by the Ca2+-sensitive arachidonoyl-selective 85-kDa cytosolic phospholipase A2 (cPLA2) (5). Once arachidonic acid is released, it can be oxygenated by the lipoxygenase and cyclooxygenase pathways. The former pathway oxygenates arachidonic acid into 12-hydroperoxy-eicosatetraenoic acid (12-HpETE), which is then reduced into 12-hydroxy-derivative (12-HETE) by a cytosolic glutathione-dependent peroxidase (6). The latter pathway converts arachidonic acid into prostaglandin endoperoxides further isomerized into thromboxane A2 (TxA2), a potent proaggregatory and vasoconstricting substance (7) that rapidly breaks down to form the stable and inactive end product thromboxane B2 (TxB2).

There is abundant literature on enhanced platelet sensitivity to a variety of aggregating agents, especially in diabetic patients with vascular complications (8–10). Given that some differences in platelet responses appeared between type 1 and type 2 diabetes (11), the mechanisms of platelet dysfunction may be different. Nevertheless, one of the mechanisms involved in platelet dysfunction could be caused by glycoxidative stress leading to an increased level of lipid peroxides. Such an increase might activate the release of arachidonic acid from phospholipids and subsequently amplify platelet activation (12). The present study was undertaken to determine, in both type 1 and type 2 diabetic patients without any cardiovascular complications as compared with respective control subjects,
immediately frozen until analysis. TxB₂ was quantified by enzyme-linked
immunosorbent assay according to the manufacturer's recommendations
(Stago, France).

**RESULTS**

**Platelet aggregation.** Platelets from diabetic patients were found to be hypersensitive to thrombin, especially platelets from type 2 diabetic patients. Indeed, platelets from type 1 diabetic patients were hypersensitive to 0.01 units/ml thrombin as compared with young control subjects (65.8 ± 6.5 vs. 38.8 ± 8.7% of aggregation, respectively; n = 10, P < 0.03). Platelet aggregation induced by 0.01 units/ml was also significantly higher (68.4 ± 3.7% of aggregation, n = 10) in type 2 diabetic patients than in middle-aged subjects (46.7 ± 6.9% of aggregation, n = 10), and a significant increase (P < 0.01) of aggregation was observed when thrombin was used to 0.1 units/ml in patients versus that in age-matched control subjects (91.7 ± 1.2 vs. 87.5 ± 0.8%, respectively; n = 10).

**Cyclooxygenase and lipoxygenase enzymes.** Incubation of platelets with exogenous arachidonic acid allowed us to measure the specific oxygenation of this fatty acid by the dioxygenases cyclooxygenase and lipoxygenase. The production of 12-HETE, the lipoxygenase end product, was compared with middle-aged control subjects (Fig. 2).

**Basal formation of TxB₂.** In the absence of specific stimulation, higher amounts of this metabolite were found in “resting” platelets from either type 1 or type 2 diabetic patients when compared with the respective control subjects (Fig. 1).

**Lipid peroxidation.** Finally, to assess the overall lipid peroxide level in platelets, MDA content of platelet suspensions was determined. Compared with young control subjects, the formation of MDA was not significantly higher in type 1 diabetic subjects. In contrast, a marked increase of MDA level (90%) was found in type 2 diabetic patients compared with middle-aged control subjects (Fig. 2).

**Platelet antioxidant status.** To evaluate the antioxidant status, three markers were measured. First, α-tocopherol, an effective lipophilic antioxidant and free radical scavenger, was determined in platelets from diabetic and control subjects. Significant decreases of α-tocopherol levels in platelets from type 1 and type 2 diabetic patients were observed when compared with their respective control subjects (Fig. 3). No significant differences were observed for γ-tocopherol (results not shown). Then, the two cellu-
lar glutathione peroxidases cGPx and PHGPx were investigated. Figure 4A shows cGPx activities in platelets from diabetic and control groups. cGPx activity was significantly lower (22.6%) in platelets from type 1 diabetic patients versus young control subjects. In the same way, cGPx activity was significantly lower (24.6%) in platelets from type 2 diabetic patients compared with middle-aged control subjects (Fig. 4A). Based on Western blot analysis, expression of cGPx was also lower in the two groups of diabetic patients compared with their respective control subjects (Fig. 4B). Finally, we also studied PHGPx activity and expression. As shown in Table 2, no significant alteration could be observed with both types of measurement in both populations, except for a tendency to decreased activity in platelets from type 2 diabetic patients.

**DISCUSSION**

Cardiovascular complications are recognized risks in patients with either type 1 or type 2 diabetes. Platelet hyperactivation could play a key role in the pathogenesis of diabetic micro- and macroangiopathies, and among the well-characterized events associated with platelet activation, there is the release of arachidonic acid from membrane phospholipids. Indeed, platelets are efficient cells in processing arachidonic acid with the formation of both cyclooxygenase and lipoxygenase bioactive metabolites. Thus, one of our aims was to evaluate platelet function both in type 1 and type 2 diabetic patients, in the absence of vascular complications, in relation to arachidonic acid metabolism. First, our purpose was to define the level of platelet aggregation in such patients. Indeed, platelet hyperactivity has been reported in the literature, and this is supported by numerous studies in diabetic patients with vascular complications (10,24). Our present study shows an increased sensitivity to thrombin in diabetic patients, even in the absence of vascular complications. In accordance with the known importance of surface glycoprotein, this hypersensitivity could be caused by a greater expression of its thrombin receptor, the fibrinogen-binding glycoprotein IIb/IIIa receptor, as it has been previously reported (25). In addition, increased plasma thrombin generation has been described in diabetic patients (26), and one of the mechanisms involved could be the release of arachidonic acid from membrane phospholipids. In other experiments, we have also shown that p38 mitogen-activated protein (MAP) kinase phosphorylation was significantly higher in platelets from type 2 diabetic patients (27). Such an increase indicates an activation of p38 MAP kinase. Given that activated p38 MAP kinase is involved in the phosphorylation and activation of cPLA2, the key

**FIG. 1.** Basal formation of TxB₂ in unstimulated platelets from both type 1 and type 2 diabetic patients and from respective control subjects. Results are expressed as the means ± SE (n = 9). *P < 0.05; **P < 0.03.

**FIG. 2.** Basal formation of MDA in unstimulated platelets from both type 1 and type 2 diabetic patients and from respective control subjects. Results are expressed in percent of control values and are means ± SE (n = 9). The value from young control subjects was 378 ± 40 pmol/10⁹ platelets, and that from the middle-aged control subjects was 451 ± 79 pmol/10⁹ platelets. *P < 0.05.

**FIG. 3.** Platelet vitamin E (α-tocopherol) level from both type 1 and type 2 diabetic patients and from respective control subjects. Results are expressed as means ± SE (n = 10). *P < 0.05.
enzyme in arachidonic acid release, cPLA2 activation could be possible in platelets from diabetic patients, in agreement with previous observations (28,29). However, and in contrast to cPLA2, we show that cyclooxygenase, thromboxane synthase, and lipoxygenase enzymes were not activated, which is consistent with an earlier report (28). Thus, the increased basal TxB2 (the stable catabolite of TxA2) that we found in diabetic platelets could be caused by increased arachidonic acid release (induced by activation of phospholipases) and not by its increased oxygenation. This higher endogenous generation of TxA2 indicates that “resting” platelets of diabetic patients, even without vascular complications, are prone to activation. The higher susceptibility of platelets to produce TxA2 could be of pathophysiological relevance because TxA2 has been shown to exert potent biological actions (30) with vasoconstricting and proaggregatory effects relevant to the risk of developing atherothrombogenesis occurring in diabetes.

Numerous studies support the conclusion that there is an association between diabetes and oxidative stress (31). A higher production of reactive oxygen species has been attributed to protein glycation and/or autooxidation caused by an hyperglycemic environment (32), and lipid peroxidation of cellular structures (a consequence of free radical activity) is thought to play an important role in diabetic complications. MDA is considered as a marker of oxidative stress, and plasma MDA has been found to be increased in some studies (33,34). However, divergent data have been reported on this marker, specifically in type 1 diabetic patients. Indeed, some studies have shown an increased level of plasma MDA (34,35), whereas others did not find any alteration of this marker (36,37). Concerning platelet MDA, little data are available. However, given that MDA is generated both by lipid oxidation and as a byproduct of prostaglandin and thromboxane synthesis, platelet MDA is considered as a global oxidative stress index. Interestingly, platelet MDA levels were significantly elevated in type 2 but not in type 1 diabetic patients. Thus, overall lipid peroxidation could be more pronounced in platelets from type 2 than from type 1 diabetic patients, but enzymatic lipid peroxidation (as assessed by basal platelet TxB2) was higher in platelets from both types of diabetic patients studied.

Phosphatidylserine is normally located in the inner bilayer of platelet membrane. During the final stages of activation, platelets express the aminophospholipid phosphatidylserine at the platelet outer membrane surface, and this process can be induced by MDA. Indeed, previous studies (38,39) have shown that part of phosphatidylserine can move from the inner to the outer side of the membrane when cells are treated with MDA. Considering that the exposure of phosphatidylserine in the outer plasma membrane plays an important role in thrombin generation (40), this is in agreement with the increased plasma thrombin generation described in diabetic patients (41) and could contribute to the enhanced basal activation observed in the present study.

Interestingly, the relationship between platelet MDA level and platelet vitamin E concentration, which exists in healthy subjects ($r = -0.59, P = 0.007$), was lost both in type 1 and type 2 diabetic patients. This lack of any relationship has already been found in erythrocytes from type 1 diabetic patients (42). These findings suggest that the relationship between antioxidants and oxidants is altered in diabetic patients.

The augmented oxidative stress seen in diabetic patients may be either the result of greater free radical production...
and/or caused by decreased antioxidant defenses. Oxidative defense is provided by vitamins, including the chain-breaking scavenger vitamin E, and by a number of enzymes, such as glutathione peroxidases. Data are conflicting regarding plasma tocopherol status in type 1 (35–37) as well as type 2 (43,44) diabetic subjects. Concerning platelets, the present study shows a significant lower vitamin E level in both type 1 and type 2 diabetic patients, which supports findings by other studies (45,46). In platelets, two glutathione peroxidases are present: cGPx and PHGPx. cGPx plays a key role in the protection of cells from oxidative damage and also regulates the formation of eicosanoids, since their formation depends on the peroxide tone of the cells (47,48). According to the literature, the glutathione peroxidase response to diabetes has been conflicting, but, as previously reported (49,50), cGPx activities were found to be lower in the present study. Lower cGPx activity can lead to a relative accumulation of 12-HpETE, the main hydroperoxide formed from arachidonic acid, and such an increase could activate signal transduction pathways leading to arachidonic acid release (12,27), thus amplifying platelet activation. Platelet PHGPx activity was also measured for the first time in diabetic patients. Interestingly, this enzyme activity tended to decrease in platelets from type 2 but not type 1 diabetic patients, in agreement with a strong oxidative stress in platelets. Given that PHGPx is able to directly reduce both phospholipid and cholesterol hydroperoxides in cell membranes (51), and given that PHGPx exists as mitochondrial and nonmitochondrial forms (52), a tendency to decreased enzyme activity could increase both the intracellular peroxide level and oxidative damage in mitochondria, as previously reported (53). Altogether, our results show a low platelet antioxidant status in diabetic patients that would favor the generation of radical species.

In summary, the present data show that increased platelet aggregation is already detectable in diabetic patients who do not suffer from vascular complications, an alteration that is associated with increased basal arachidonic acid metabolism, possibly linked with impairment of antioxidant mechanisms. These results are in agreement with the major role played by platelets in the initiation of atherogenetic process as was recently reported (54). These platelet alterations could contribute to increase the risk for the occurrence of vascular diseases in diabetic patients.

ACKNOWLEDGMENTS

This work was supported by INSERM. The Région Rhône-Alpes is acknowledged for financial support.

We are grateful to the patients and control subjects for their cooperation and to the nurses of the Department of Endocrinology for their assistance. We thank Dr. J.F. Pageaux for advice in statistical analysis.

REFERENCES


