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

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Vascular complications are the leading causes of morbidity and mortality in diabetic patients. The contribution of platelets to thromboembolic 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 B_2, the stable catabolite of thromboxane A_2, significantly increased in resting platelets from both type 1 and type 2 diabetic patients (58 and 88%, 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 Ca^{2+}-sensitive arachidonoyl-selective 85-kDa cytosolic phospholipase A_2 ($cPLA_2$) (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 A_2 (TxA_2), a potent proaggregatory and vasoconstricting substance (7) that rapidly breaks down to form the stable and inactive end product thromboxane B_2 (TxB_2).

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,

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BHT, butylated hydroxytoluene; cGPx, cytosolic glutathione peroxidase; $cPLA_2$, cytosolic phospholipase A_2; 12-HETE, 12-hydroxy-eicosatetraenoic acid; 12-HpETE, 12-hydroperoxy-eicosatetraenoic acid; HPLC, high-performance liquid chromatography; MAP, mitogen-activated protein; MDA, malondialdehyde; PC-OOH, phosphatidylcholine hydroperoxides; PHGPx, phospholipid hydroperoxide glutathione peroxidase; TLC, thin-layer chromatography; TxA_2, thromboxane A_2; TxB_2, thromboxane B_2.

TABLE 1
Clinical characteristics of the patients studied

	Type 1 diabetic patients	Type 2 diabetic patients
n (M/F)	2/8	6/4
HbA_{1c} (%)	9.3 ± 1.0	7.2 ± 0.3
Glycemia (mmol/l)	11.9 ± 2.0	9.0 ± 0.8
Total cholesterol (mmol/l)	5.0 ± 0.2	5.4 ± 0.2
HDLc (mmol/l)	1.6 ± 0.1	1.3 ± 0.1
LDLc (mmol/l)	3.1 ± 0.3	3.5 ± 0.2
Triglycerides (mmol/l)	0.8 ± 0.1	1.5 ± 0.1

HDLc, HDL cholesterol; LDLc, LDL cholesterol.

the effect of hyperglycemia on platelet antioxidant defenses and arachidonic acid metabolism in relation to platelet function.

RESEARCH DESIGN AND METHODS

We studied 10 nonsmoking type 1 diabetic patients (8 women and 2 men, mean age 35.8 ± 3.6 years) with a duration of disease >10 years. They were matched to 10 healthy subjects for sex and age (8 women and 2 men, age 34.6 ± 3.1 years). Type 2 diabetic patients (6 men and 4 women, mean age 55.4 ± 2.5 years) and 10 matched healthy subjects (6 men and 4 women, age 51.7 ± 1.3 years) were also studied. Subjects with cardiovascular complications were excluded. The clinical characteristics of the diabetic patients are summarized in Table 1. All subjects entered the study voluntarily and gave informed written consent. The protocol of the study was approved by our local ethics committee.

Platelet preparation. Blood samples were collected into tubes containing acid-citrate-dextrose (ACD; 0.8% citric acid, 2.2% sodium citrate, and 2.45% dextrose; 6:1 vol/vol). Platelet-rich plasma was prepared as previously described (13). Briefly, platelet-rich plasma obtained by centrifugation of the blood at 100*g* for 15 min was acidified to pH 6.4 with citric acid and centrifuged at 900*g* for 20 min. The resulting platelet pellet was resuspended into a Tyrode-HEPES buffer solution (in mmol/l: 137 NaCl, 2.6 KCl, 11.9 $NaHCO_3$, 0.46 NaH_2PO_4, 1 $MgCl_2$, and 5.5 dextrose, pH 7.35).

Platelet aggregation studies. Aggregations were induced by thrombin (Sigma, St. Louis, MO) and performed in a Chronolog dual-channel aggregometer (Coulter, Margency, France) according to the turbidimetric method of Born (14).

Arachidonic acid metabolism

Metabolism of exogenous arachidonic acid. The oxygenation of exogenous arachidonic acid through the cyclooxygenase and lipoxygenase pathways was determined by incubating 2.2 μmol/l of [1-^{14}C] arachidonic acid (specific activity 2.07 GBq/mmol) with platelets for 4 min at 37°C. Reactions were terminated by the addition of ethanol (3 vol), and the samples were extracted twice with chloroform (6 vol) in the presence of butylated hydroxytoluene (BHT) as an antioxidant. Lipid residues were submitted to thin-layer chromatography (TLC), and oxygenated products were visualized and quantified with a Berthold TLC analyzer radioscanner (Berthold LB511) (15).

Analysis of basal TxB_2. In the absence of specific stimulation, platelet suspensions supplemented with BHT as an antioxidant (5.10^{-5} mol/l) were immediately frozen until analysis. TxB_2 was quantified by enzyme-linked immunosorbent assay according to the manufacturer's recommendations (Amersham, Buckinghamshire, U.K.).

Malondialdehyde determination. The malondialdehyde (MDA) content of unstimulated platelets was measured by the high-performance liquid chromatography (HPLC) technique of Therasse and Lemonnier (16) with fluorimetric detection (excitation and emission λ of 515 and 553 nm, respectively).

Cytosolic glutathione peroxidase activity and protein detection. Measurement of total cytosolic glutathione peroxidase (cGPx or GPx-1) activity was carried out according to the method described by Paglia and Valentine (17) as modified by Chaudière and Gérard (18). Briefly, tert-butylhydroperoxide (0.2 mmol/l) and reduced glutathione (3 mmol/l) were used as substrates at 37°C in the presence of 0.14 mmol/l NADPH and 0.7 units/ml glutathione reductase. The rate of NADPH oxidation at 340 nm was used as an index of hydroperoxide reduction. Proteins were assayed according to the Bradford method (19) using bovine albumin as a standard. cGPx quantities were assessed by Western blotting using a rabbit polyclonal antibody raised against cGPx from bovine erythrocytes (20,21).

Phospholipid hydroperoxide glutathione peroxidase activity and protein detection. Total phospholipid hydroperoxide glutathione peroxidase (PHGPx or GPx-4) activity was assayed by the procedure of Roveri et al. (22) using phosphatidylcholine hydroperoxides (PC-OOH) generated by the hydroperoxidation of phosphatidylcholine using soybean lipoxygenase type V (Sigma). PC-OOH (30 μmol/l) and reduced glutathione (3 mmol/l) were used as substrates in the coupled test with 0.2 mmol/l NADPH and 0.6 units/ml glutathione reductase. Similarly to cGPx activity, the rate of NADPH oxidation at 340 nm was used as an index of hydroperoxide reduction. PHGPx quantities were assessed by Western blotting, using a rabbit anti-human antibody raised against antigenic peptides of human PHGPx (CovalAb, Lyon, France).

Vitamin E. Determinations of α- and γ-tocopherols were performed according to a previously described method (23). Briefly, after extractions with hexane, tocopherol isomers were separated by reverse-phase HPLC and detected by fluorimetry (excitation and emission λ of 295 and 340 nm, respectively).

Statistics. Data are presented as means ± SE. Statistic evaluation was performed using the Mann-Whitney *U* test.

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 ($P < 0.03$) 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 formation of 12-HETE, the lipoxygenase end product, was similar in diabetic and control groups (results not shown). Similarly, the production of the main products of cyclooxygenase, namely 12-hydroxyheptadecatrienoic acid (HHT) and TxB_2 (the stable catabolite of TxA_2), was not modified in diabetic patients compared with matched healthy control subjects (results not shown).

Basal formation of TxB_2. 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-

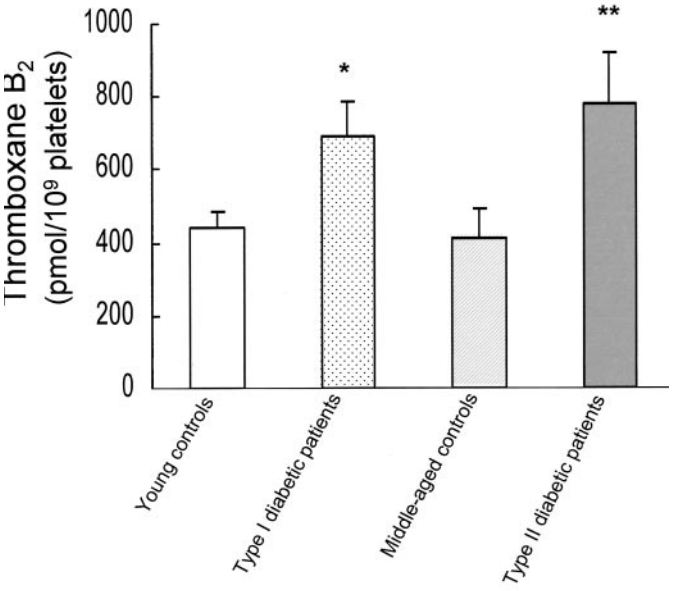

FIG. 1. Basal formation of TxB_2 in unstimulated platelets from both type 1 and type 2 diabetic patients and from respective control subjects. Results are expressed as the means ± SE (n = 9). *P < 0.05; **P < 0.03.

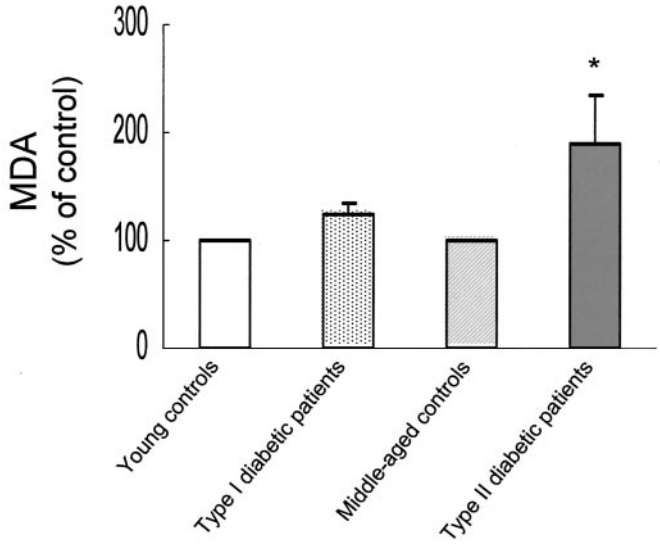

FIG. 2. Basal formation of MDA in unstimulated platelets from both type 1 and type 2 diabetic patients and from respective control subjects. Results are expressed in percent of control values and are means ± SE (n = 9). The value from young control subjects was 378 ± 40 pmol/10^9 platelets, and that from the middle-aged control subjects was 451 ± 79 pmol/10^9 platelets. *P < 0.05.

lar glutathione peroxidases cGPx and PHGPx were investigated. Figure 4*A* 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. 4*A*). 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. 4*B*). 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 $cPLA_2$, the key

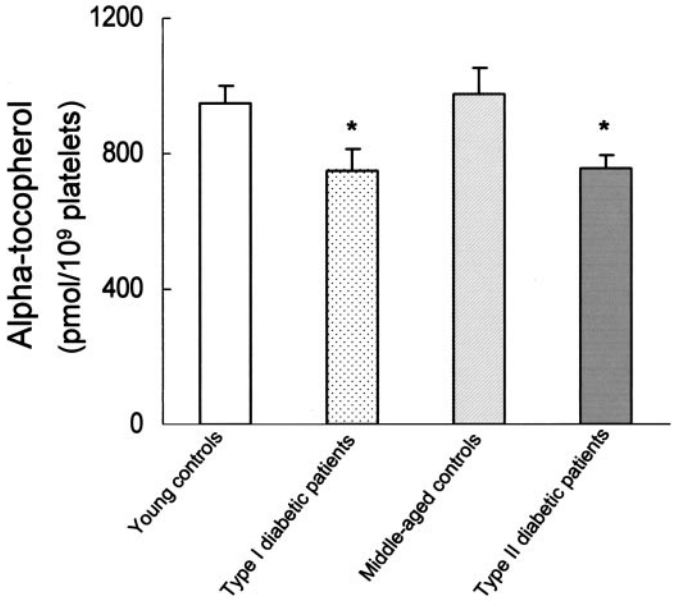

FIG. 3. Platelet vitamin E (α-tocopherol) level from both type 1 and type 2 diabetic patients and from respective control subjects. Results are expressed as means ± SE (n = 10). *P < 0.05.

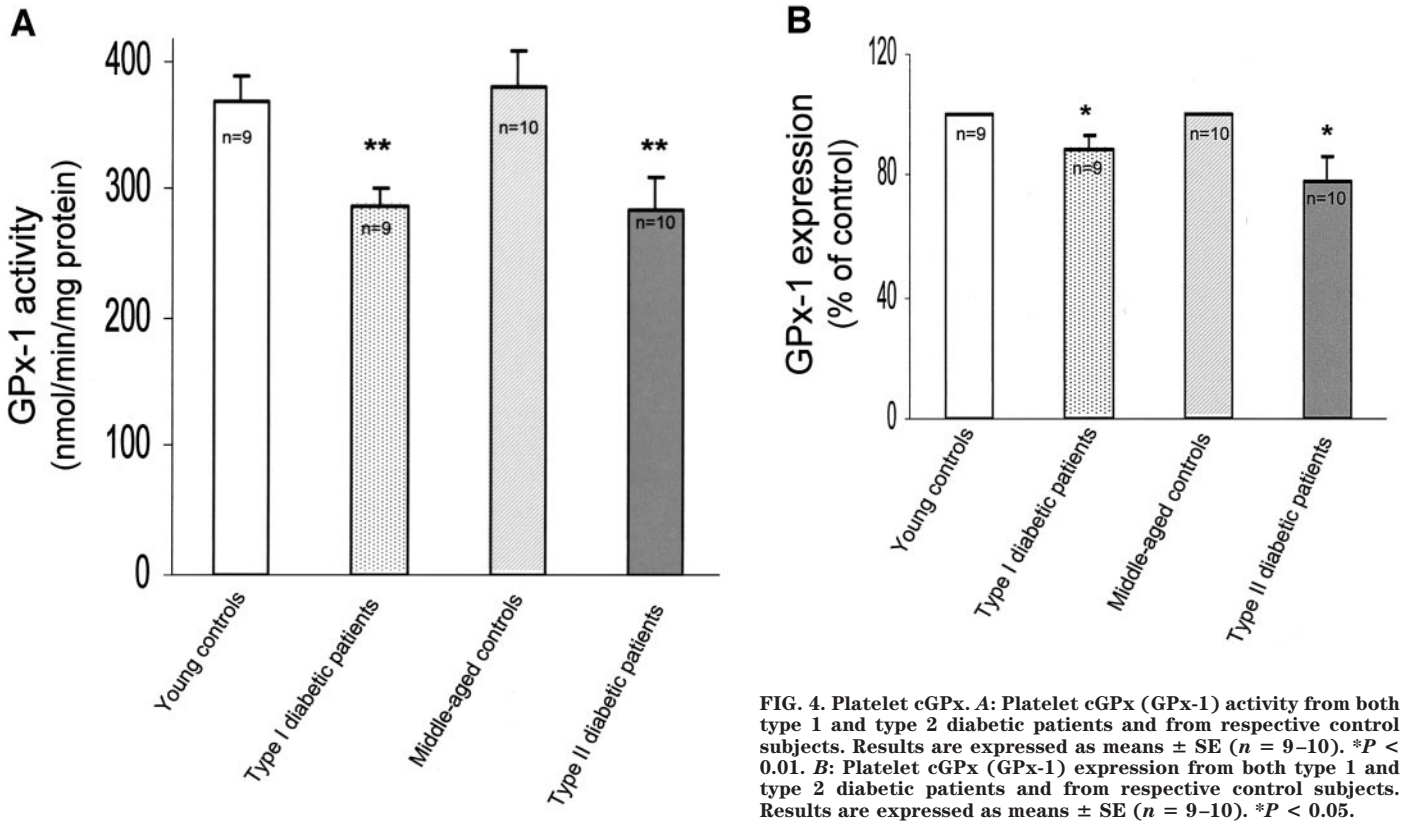

FIG. 4. Platelet cGPx. *A*: Platelet cGPx (GPx-1) activity from both type 1 and type 2 diabetic patients and from respective control subjects. Results are expressed as means ± SE (n = 9–10). *P < 0.01. *B*: Platelet cGPx (GPx-1) expression from both type 1 and type 2 diabetic patients and from respective control subjects. Results are expressed as means ± SE (n = 9–10). *P < 0.05.

enzyme in arachidonic acid release, $cPLA_2$ activation could be possible in platelets from diabetic patients, in agreement with previous observations (28,29). However, and in contrast to $cPLA_2$, we show that cyclooxygenase, thromboxane synthase, and lipoxygenase enzymes were not activated, which is consistent with an earlier report (28). Thus, the increased basal TxB_2 (the stable catabolite of TxA_2) 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 TxA_2 indicates that "resting" platelets of diabetic patients, even without vascular complications, are prone to activation. The higher susceptibility of platelets to produce TxA_2 could be of pathophysiological relevance because TxA_2 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 autoxidation 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 TxB_2) 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

TABLE 2
PHGPx activity and expression

	Young control subjects	Type 1 diabetic patients	Middle-aged control subjects	Type 2 diabetic patients
n	10	10	8	8
Activity (nmol · min^{-1} · mg protein^{-1})	7.07 ± 0.57	6.03 ± 0.53	8.3 ± 0.47	6.8 ± 0.59*
Amount (%)	100	94 ± 7	100	98 ± 9

Amount is % of respective control subjects. *$P = 0.09$.

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.

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