Plasma Glutathione Peroxidase Deficiency and Platelet Insensitivity to Nitric Oxide in Children With Familial Stroke

Gili Kenet, Jane Freedman, Boris Shenkman, Eskarave Regina, Frida Brok-Simoni, Fanny Holzman, Fotini Vavva, Nathan Brand, Alan Michelson, Maria Trolliet, Joseph Loscalzo, Aida Inbal

Abstract—In a previous report by Freedman et al (J Clin Invest. 1996;97:979–987), plasma from 2 brothers with stroke or transient ischemic attack inactivated the antiplatelet effects of nitric oxide (NO), and this effect was found to be a consequence of a deficiency of plasma glutathione peroxidase (GSH-Px). In this study, we attempted to define the generalizability of this deficiency by studying NO-mediated antiplatelet effects in 7 families with familial childhood stroke. Seven families with familial childhood stroke that consecutively presented to a large referral center were included in the study. We monitored ADP-induced aggregation of normal gel-filtered platelets (GFP) in platelet-poor plasma (PPP) from normal individuals and from patients in the presence or absence of an NO donor (S-nitroso–glutathione). Surface P-selectin expression of normal GFP in patients’ PPP was analyzed by flow cytometry after incubation with a P-selectin–specific monoclonal antibody in the presence or absence of the NO donor. We also measured GSH-Px activity in plasmas from family members and normal controls using standard methods. In 6 of 7 families, NO failed to inhibit platelet P-selectin expression and platelet aggregation in PPP from the affected family members and some of their relatives. Of 4 families studied, 3 probands and their corresponding affected parent had 50% decrease in plasma GSH-Px activity. In some patients with childhood stroke, impaired metabolism of reactive oxygen species as a result of reduced GSH-Px activity results in NO insufficiency that affects normal platelet inhibitory mechanisms and predisposes to arterial thrombosis. (Arterioscler Thromb Vasc Biol. 1999;19:2017–2023.)

Key Words: stroke ■ glutathione peroxidase ■ platelets ■ nitric oxide

The incidence of ischemic stroke in the pediatric population is relatively uncommon (0.63 to 1.20 per 100,000 children per year).1–3 The most common cause of ischemic stroke in children is thrombotic vessel occlusion; however, rare vasculopathies, such as Moya-Moya disease, metabolic disorders, or paradoxical embolism, have also been reported.4,5

The prothrombotic conditions that promote cerebrovascular thrombosis are normally counterbalanced by the actions of anticoagulant factors, including protein C, protein S, and antithrombin III (ATIII). Although deficiencies of protein S, protein C, and ATIII are well known risk factors for venous thromboembolism, their role in arterial thrombosis is controversial.6–11 Some reports showed an association between hereditary protein S or protein C deficiency and childhood stroke10,11; however, no association has been demonstrated in other studies.12

Resistance to activated protein C due to a substitution of arginine by glutamine at amino acid residue 506 in coagulation Factor V (nt G1691A) is the most common cause of venous thromboembolism.13–15 Martinelli and colleagues reported an increased prevalence of Factor V G1691A in adults with cerebral venous thrombosis16; however, this mutation was not shown to predispose to the development of ischemic stroke in adults, according to other studies.17–23 An increased prevalence of Factor V G1691A has been observed in a few reports on pediatric arterial thromboses and stroke,24–27 but this association has not been clearly delineated.28

Hyperhomocysteinemia, due to genetic or environmental factors, is now recognized as a risk factor for thrombotic stroke in adults.29–32 Elevated levels of homocysteine can be caused by reduced levels of folic acid, vitamin B12, or vitamin B6; renal failure; and hereditary defects in cystathionine β-synthase or 5,10 methylenetetrahydrofolate reductase (MTHFR), including a functionally important polymorphism in the MTHFR gene that involves a nt 677 C→T transition.30 The homozygous state for this polymorphism (C677T) is associated with increased plasma homocysteine concentrations, particularly when the folic acid level is reduced.31 A preliminary report of 108 juvenile (aged <45 years) stroke patients showed increased frequency of this polymorphism among patients compared with controls.33 A recent report by
De Stefano and colleagues showed that a homozygous MTHFR genotype (C677T) was not increased in frequency among stroke patients compared with controls.\(^3\)

Another newly described prothrombotic polymorphism in Factor II (FII) involves a G→A transition at position 20210 of the 3′ untranslated region of the FII gene.\(^3\) This substitution (G20210A) is associated with increased plasma levels of FII (prothrombin) and was found to confer an excessive risk of venous thrombosis.\(^3\)–\(^6\) No association of the G20210A polymorphism with cerebrovascular disease in adults was observed in 1 preliminary report.\(^3\) However, De Stefano and colleagues recently reported a strong association between G20210A and cerebral ischemia in patients aged <50 years.\(^3\) The relationship between this polymorphism and childhood stroke remains unknown at the current time. Taken together, these data suggest that the association of pediatric ischemic stroke with either inherited deficiencies of protein C, protein S, ATIII, or with prothrombotic polymorphisms, such as F V G1691A, MTHFR C677T, and FII G20210A, clearly needs to be evaluated in further studies.

Alternative prothrombotic factors may involve conditions that impair nitric oxide (NO) metabolism. NO or endothelial-um-derived relaxing factor (EDRF) is a potent vasorelaxant that stimulates soluble guanylyl cyclase, thereby elevating cyclic guanosine monophosphate in vascular smooth muscle cells.\(^3\) In addition to its vasorelaxant properties, NO inhibits platelet aggregation,\(^3\) fibrinogen binding to platelets,\(^3\) platelet adhesion to damaged endothelium,\(^9\)–\(^10\) dense granule secretion from platelets,\(^4\) and expression of platelet surface P-selectin.\(^4\) Oxidative reactions involving NO in plasma can attenuate its effect on platelet function and, subsequently, induce thrombosis.

It was recently reported by Freedman and colleagues\(^4\) that plasma from 2 young siblings with a cerebral thrombotic disorder attenuated NO-induced platelet inhibition. In this report, we attempted to define the generalizability of this observation by analyzing NO-mediated inhibition of platelet function in 7 families with familial childhood stroke.

### Methods

#### Patients

From 1993 to 1997, 43 children with stroke were referred to our tertiary referral center in Israel for evaluation of thrombophilia. The patients were referred by pediatric neurologists from throughout the country. Among the 43 families in 9 families, an additional member suffered stroke, thus defining them as familial stroke cases. In 2 out of these 9 families, protein C deficiency was diagnosed; therefore, they were excluded from the study. In the remaining 7 families, no underlying cause for stroke was found. These 7 families with familial childhood stroke were further evaluated in the present study. The pedigrees of the families are shown in Figure 1. The definition of familial stroke included the presence of an acute thrombotic cerebrovascular event in >1 member of a family. In the corresponding affected parent, the stroke event occurred in childhood or young adulthood. In family A, the affected mother suffered stroke at the time of second pregnancy at the age of 36. In family B, the mother suffered stroke at the age of 7 years. The corresponding mothers from families C and G suffered stroke at the ages of 17 and 18 years, respectively. In family F, the affected paternal uncle suffered stroke when he was <40 years old. The corresponding affected grandparent from the families suffered stroke when they were <55 years old. No risk factors for stroke were diagnosed in the affected adult members of the families. Clinical diagnosis of stroke was confirmed by CT or MRI in all the patients. There was no difference in ethnic origin, clinical features, CT or MRI localization, or outcome of stroke between the 7 families studied and all other families evaluated for thrombophilia. Twenty healthy, age-matched individuals served as a control group for plasma and platelet studies.

#### Coagulation Tests

Protein C and ATIII activities were measured by chromogenic assays (Baxter Dade), and free protein S antigen was measured by ELISA (Gladipore Elisa test kit). The APC resistance test was performed as described previously.\(^1\)

#### Determination of Prothrombotic Polymorphisms

Genomic DNA was isolated from 5 mL whole blood using standard procedures. The G1691A polymorphism in the Factor V gene was detected by PCR amplification of a 267 bp fragment and MnlI digestion, as previously described.\(^1\) The C677T substitution in the MTHFR gene was identified using HinfI cleavage of a 198 bp PCR-amplified product, as described by Frost and colleagues.\(^9\) For identification of the G20210A substitution in the FII gene, a slight modification of the method of Poort and colleagues was used.\(^3\) A 253 bp fragment of the 3′ untranslated region of the gene was amplified by PCR and digested simultaneously with HindIII and MspI. The A20210 and G20210 alleles were discernible by this procedure because the A20210 allele bears a restriction site for MspI.\(^3\)

#### EDRF/NO Congeners

The EDRF congeners S-nitroso-N-acetylcysteine (SNAC) and S-nitrosothiol (SNO-Glu) were prepared by reacting freshly prepared solutions of N-acetylcysteine or reduced glutathione (GSH) with NaNO₂ at acidic pH, as previously described.\(^6\) The C677T substitution in the MTHFR gene was identified using HinfI cleavage of a 198 bp PCR-amplified product, as described by Frost and colleagues.\(^9\) For identification of the G20210A substitution in the FII gene, a slight modification of the method of Poort and colleagues was used.\(^3\) A 253 bp fragment of the 3′ untranslated region of the gene was amplified by PCR and digested simultaneously with HindIII and MspI. The A20210 and G20210 alleles were discernible by this procedure because the A20210 allele bears a restriction site for both MspI.\(^3\)

#### P-Selectin–Specific Monoclonal Antibody

S12 (provided by Dr Rodger P. McEver, University of Oklahoma, Norman, Okla) is a murine monoclonal antibody directed against P-selectin\(^1\) that is a component of the α-granule membrane of resting platelets and is only expressed on the platelet surface membrane after degranulation and secretion. Phycoerythrin-conjugated monoclonal antibody against P-selectin (AK 4) was purchased from Pharmingen (San Diego, CA). Normal mouse IgG1 conjugated to phycoerythrin was purchased from Becton Dickinson (San Jose, CA). Control IgG1 (DAK-601) was obtained from Dako A/S (Glæstrup, Denmark).

#### Preparation of Platelet-Rich Plasma

Nine volumes of peripheral blood from family members and controls were drawn into 1 volume of 0.129 mol/L trisodium citrate. After
centrifugation (150g, 15 minutes, 22°C), the upper two-thirds of the supernatant platelet-rich plasma (PRP) was removed. Platelet-poor plasma (PPP) was prepared by centrifugation of PRP at 1200g for 10 minutes.

Preparation of Gel-Filtered Platelets

Gel-filtered platelets (GFP) were obtained by passing PRP over a Sepharose-2B column (Pharmacia Biotech) in Tyrode’s-Hepes–buffered saline, as previously described. Platelet counts were determined using a Coulter Counter, model Technicon H2 (Bayer Diagnostics). Platelets were adjusted to 1.5×10^9 platelets/mL by the addition of Tyrode’s-Hepes–buffered saline.

Platelet Aggregation

Aggregation of normal GFP with patients’ PPP was monitored using a PACKS-4 aggregometer (Helena Laboratories). GFP from healthy donors were mixed with PPP from the patients and their family members in the presence or absence of 1 μmol/L SNO-Glu. Aggregations were induced by adding 5 μmol/L ADP, as previously described.

Flow-Cytometry

GFP in PPP were analyzed after incubation with phycoerythrin-conjugated MAb in a Coulter FACS (Coulter EPX XL, Coulter Corp), as previously described. Platelet activation was induced by the addition of 20 μmol/L thrombin receptor activating peptide. The fluorescence of phycoerythrin-conjugated MAb was detected using 525- and 575-nm band pass filters. After 3 minutes incubation at 22°C, the reaction was stopped by 20-fold dilution with cold buffered saline, as previously described. Platelet counts were determined using a Coulter Counter, model Technicon H2 (Bayer Diagnostics). Platelets were adjusted to 1.5×10^9 platelets/mL by the addition of Tyrode’s-Hepes–buffered saline.

Determination of Glutathione Peroxidase Activity

Endogenous plasma glutathione peroxidase (GSH-PX) was assayed by coupling the peroxidase reaction with the reduction of oxidized glutathione by glutathione reductase and NADPH. t-Butyl-hydroperoxide (hydrogen peroxide or tert-butyl hydroperoxide) reduction was followed by the decrease in absorbance of NADPH at 340 nm. Activity was evaluated using GSH as the cosubstrate.

Results

Patients’ clinical and laboratory data are presented in Table 1. Protein C, ATIII, protein S, and APC resistance ratios obtained from proband plasmas were essentially within normal limits, with the probands from families E and F having low normal values for protein C activity (adjusted for 6 months, see Reference 45) and the proband from family G having a low normal value for free protein S antigen (Table 1). Analysis of patients’ DNA showed that none of them carried either Factor V G1691A or FII G20210A polymorphisms. Two probands belonging to 2 unrelated families (Table 1), were found to be homozygous for the MTHFR C677T polymorphism.

Effect of SNO-Glu on Platelet Aggregation

PPP from family members or normal controls were mixed with GFP from a normal control, incubated with the NO donor SNO-Glu for 1 minute, and aggregation induced with ADP. Significant attenuation of platelet aggregation is normally observed after the addition of the NO donor to normal GFP/PPP mixture in the presence of ADP (see curves 1 and 4, Figure 2A). However, when normal GFP was mixed with PPP from the affected family member from each family in the presence of SNO-Glu, no inhibition of aggregation was observed (curves 2 and 3, Figure 2A). This lack of inhibition by NO was observed in each proband’s plasma and in plasma from some proband parents or siblings in 6 of 7 families (Table 2). The mean values for the maximal extent of aggregation of GFP in patients’ plasmas in the presence of the NO donor was significantly higher than that observed in plasma from 20 healthy controls (55±20% versus 20±5%, P<0.01) (Table 2).

Effect of NO on Platelet Surface Expression of P-Selectin

Figure 2B shows the effect of the NO donor on platelet surface P-selectin expression. Plasma from probands prevented the NO-induced decrease in P-selectin expression compared with normal controls. When normal GFP were added to plasma from every proband and some family members, no NO-mediated attenuation of P-selectin expression was observed in the same members in 6 of 7 families who showed limited attenuation of ADP-induced aggregation by NO. These results on NO inhibition of platelet P-selectin expression are presented in Table 2. Mean expression of platelet P-selectin in the presence of the NO donor was significantly higher in plasma from affected individuals compared with control plasma (68±20% versus 17±4%, P<0.005) (Table 2).

The effect of NO on platelet aggregation and P-selectin expression was also studied in 5 children with perinatal nonfamilial stroke 7 to 12 months after the event. In contrast to healthy controls, platelet aggregation and P-selectin expression were increased in some of the children with perinatal nonfamilial stroke.
to the results obtained with familial stroke probands, NO-induced inhibition of ADP-induced platelet aggregation and P-selectin expression was demonstrated in every affected child (data not shown).

**Plasma GSH-Px Activity**

The levels of GSH-Px activity in PPP from the family members or normal controls were measured by a coupled spectrophotometric assay using H$_2$O$_2$ and GSH as cosubstrates. Plasmas from only 4 families were available for these studies. GSH-Px activity was decreased in plasma samples of 3 probands from 4 unrelated families (families A, B, and D, Table 3) as compared with normal, pooled pediatric control plasma. In addition, reduced GSH-Px activity was also observed in the plasma of the affected parent of each proband from these families (Table 3). Normal GSH-Px activity was observed in the plasma of the fourth proband studied (family G, Table 3). Interestingly, the magnitude of the average reduction in activity was approximately 50%, similar to that observed in the originally described family$^{42}$ (0.26±0.30 versus 0.47±0.026 μmol·min$^{-1}$·mL$^{-1}$ plasma). Plasma from the proband of family C also had decreased GSH-Px activity that was approximately 50% less than that observed in plasmas from other probands from families A, B, and D. Similarly, the normal sibling from family C also had a 50% decrease in GSH-Px activity as compared with normal members from families A, B, and D. Because plasma samples from these members of family C underwent repeated freeze-

**TABLE 2. NO Mediated Platelet Aggregation and Surface P-Selectin Expression of Normal Platelets With Plasma From Family Members and Controls**

<table>
<thead>
<tr>
<th>Families</th>
<th>Extent of ADP-Induced Platelet Aggregation (%)</th>
<th>Platelet P-Selectin (% Total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III-1*</td>
<td>68±1</td>
<td>61±2</td>
</tr>
<tr>
<td>III-2</td>
<td>40±2</td>
<td>43±1</td>
</tr>
<tr>
<td>IV-1*</td>
<td>53±1</td>
<td>56±7</td>
</tr>
<tr>
<td>IV-2</td>
<td>25±2</td>
<td>28±2</td>
</tr>
<tr>
<td>Family B</td>
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<td></td>
</tr>
<tr>
<td>I-1*</td>
<td>65±3</td>
<td>81±3</td>
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<tr>
<td>I-2</td>
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<td>10±1</td>
<td>10±3</td>
</tr>
<tr>
<td>II-2</td>
<td>12±2</td>
<td>8±2</td>
</tr>
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<td>II-3</td>
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<td>14±2</td>
</tr>
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</tr>
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<td></td>
</tr>
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<td>II-1*</td>
<td>40±5</td>
<td>69±6</td>
</tr>
<tr>
<td>II-2</td>
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<td></td>
</tr>
<tr>
<td>II-1</td>
<td>5±1</td>
<td>30±2</td>
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<tr>
<td>II-2</td>
<td>5±2</td>
<td>28±1</td>
</tr>
<tr>
<td>III-1</td>
<td>70±2</td>
<td>nd</td>
</tr>
<tr>
<td>III-2</td>
<td>20±1</td>
<td>nd</td>
</tr>
<tr>
<td>III-3</td>
<td>10±1</td>
<td>25±4</td>
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<tr>
<td>III-4*</td>
<td>60±2</td>
<td>87±3</td>
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<tr>
<td>Family E</td>
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</tr>
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<td>II-1</td>
<td>70±3</td>
<td>94±2</td>
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<td>Family G</td>
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<td>54±3</td>
<td>89±1</td>
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<td>I-2</td>
<td>15±2</td>
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<tr>
<td>II-1*</td>
<td>80±6</td>
<td>90±4</td>
</tr>
<tr>
<td>Controls (N=20)</td>
<td>20±5</td>
<td>17±4</td>
</tr>
</tbody>
</table>

*nd indicates not done. *Affected family member; values represent mean±SEM of 3 determinations.
The correlation between values of platelet aggregation and P-selectin expression from all family members and between P-selectin expression and GSH-Px activity are shown in Figure 3A. A strong positive correlation was observed between platelet aggregation and P-selectin expression in the presence of NO ($R = 0.90$). Similarly, a strong inverse correlation was observed between P-selectin expression and GSH-Px activity measured in members from families A, B, and D ($R = 0.79$).

**Discussion**

The present study provides evidence for a new prothrombotic mechanism that is associated with familial childhood stroke in a larger referral population. In the 7 families studied, stroke was diagnosed in >1 family member, thus suggesting a familial prothrombotic disorder. The precise prevalence of this familial disorder is difficult to estimate because of the small number of families studied and the selection bias of the referral process. In 4 of 7 families studied, the stroke of the proband occurred during the perinatal period. In these 4 families, 2 probands were found to be homozygous for the C677T MTHFR. Perinatal stroke is a unique condition of unknown etiology, and the association with known prothrombotic states has not been delineated.20 Thus, it is unknown if homozygosity for MTHFR is a risk factor for stroke in these 2 probands.

Platelet activation and aggregation play an essential role in the pathogenesis of stroke. Several studies reported the inhibitory effect of NO on platelet adhesion, aggregation, and surface P-selectin expression.38–41 This inhibitory effect of NO was studied in plasmas from the members of 7 families. All affected children and some of their family members demonstrated resistance to the inhibitory effect of NO on platelet aggregation and P-selectin expression in the presence of NO ($R = 0.90$). Similarly, a strong inverse correlation was observed between P-selectin expression and GSH-Px activity measured in members from families A, B, and D ($R = 0.79$).
patients alteration of the normal platelet inhibitory effect was caused by an interaction between NO and reactive oxygen species that would otherwise have been reduced by normal levels of GSH-Px activity in plasma. Impaired metabolism of reactive oxygen species reduces the bioavailability of NO in plasma and impairs normal platelet inhibitory mechanisms.

The previous report by Freedman and colleagues first identified a decrease in plasma GSH-Px activity as a cause for a prothrombotic state in 2 brothers with childhood cerebrovascular thrombotic disease. The authors observed in that study that a deficiency in plasma GSH-Px leads to an increase in plasma peroxides that can lead to inactivation of NO (via peroxyl-mediated formation of [lipid] peroxynitrites). We studied GSH-Px activity in plasma from the members of 5 families and found that in 4 families every affected member showed reduced GSH-Px activity compared with age-matched controls. It is possible that these patients suffered strokes due to a similar mechanism as that described by Freedman and colleagues in the originally described family. In the fifth family, the stroke may have resulted from a different mechanism that reduces the antiplatelet effects of NO.

Our data suggest that familial childhood stroke is a multifactorial disorder. In some patients, a reduction of GSH-Px activity may promote oxidative reactions that reduce NO bioavailability. Functional insufficiency of NO will result in attenuation of NO-mediated platelet inhibition, thereby predisposing to a thrombotic disorder. We suggest that the assessment of NO-mediated inhibition of platelet function should be added to the screening tests of familial, "idiopathic" childhood stroke.

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References


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