Therapeutic immunoglobulin reduces Ca\(^{2+}\) mobilization and von Willebrand factor secretion, and increases nitric oxide release in human endothelial cells

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Summary

Intravenous γ-immunoglobulin (IVIg) is commonly used in the treatment of autoimmune and inflammatory vascular disorders to prevent thrombotic complications. The mechanism of action of IVIg is, however, not yet elucidated. In view of this, we investigated the ability of IVIg to modulate i) Ca\(^{2+}\) signals of fura-2 loaded endothelial cells, and ii) the associated release of nitric oxide (NO) and von Willebrand factor (vWF). NO was measured either indirectly by radioimmunoassay of cGMP in unstimulated cells or directly by electrochemistry at the surface of stimulated endothelial cells from human umbilical cord veins (HUVEC). Short-term treatment of unstimulated HUVEC with intact IVIg decreased the basal cytosolic Ca\(^{2+}\) concentration by 20\% while it activated the NO/cGMP synthesis. Following IVIg treatment of HUVEC, the Ca\(^{2+}\) liberation from internal stores and the vWF secretion induced by ATP, thrombin or histamine were significantly reduced by 38 and 60\%, respectively. The effects on Ca\(^{2+}\) signals were observed with intact IVIg as well as with the F(ab’)\(^2\) or the Fc fragments indicating that both portions are involved in the mechanism of action. The IVIg treatment of HUVECs had no effect on the NO release induced by thrombin or histamine. By contrast, the IVIg treatment increased the ATP-activated NO release by amplifying the Ser1177-eNOS phosphorylation. The IVIg also activated the NO-dependent cGMP release in resting and collagen-stimulated platelets. Since NO is a potent inhibitor of platelet activation and vWF is a platelet adhesion cofactor, the beneficial effects of therapeutic IVIg may lie in the inhibition of platelet adhesion to damaged endothelium.

Keywords

Endothelial cells, IVIg, nitric oxide, platelets, von Willebrand factor

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Introduction

Intravenous immune globulin (IVIg) has broad therapeutic applications with beneficial effects in several autoimmune and inflammatory diseases, in particular in those associated with alterations of vascular endothelial functions (1, 2). The endothelium, which exerts anti-thrombotic functions under physiological conditions, may indeed become a pro-thrombotic surface (3). Thus, thrombotic complications are observed in the presence of activated complement components as a consequence of antigen-antibody interactions, of endothelial cell-reactive antibodies, of bacterial toxins or of pro-inflammatory cytokines (3-5). Treatment with IVIg prevents the thrombosis in experimental antiphospholipid and hemolytic uremic syndromes, and reduces the coronary aneurism in patients with Kawasaki disease (6-8). IVIg is also used in the treatment of systemic vascul-
litis associated with anti-endothelial cell or anti-neutrophil cytoplasmic antibodies (9, 10). Little is known, however, about the mechanisms of action of IVIg on the vessel wall.

Normal human immunoglobulins (IgG) bind to endothelial cells in vitro and are internalized (11). The infusion of IVIg could accelerate the degradation of pathogenic IgG by saturating the Fc receptor FcRn, a receptor highly expressed in endothelial cells and involved in the regulation of serum IgG levels (12, 13). In endothelial cells, IVIg blocks the expression of adhesion molecules and chemokines induced by pro-inflammatory cytokines, and inhibits cell proliferation and secretion of thromboxane A2 and endothelin (14, 15), all events directly controlled by cytosolic Ca\textsuperscript{2+} signalling (16). We previously showed, indeed, that IVIg inhibits the thrombin-induced Ca\textsuperscript{2+} response but does not modify that elicited by endothelin-1 in aortic endothelial cells from guinea pig (17).

An increase in cytosolic Ca\textsuperscript{2+} concentration is one of the earliest events of the endothelial cell activation by circulating blood stimuli which are locally released during injury to arteries and vessels, such as thrombin, histamine or ATP (18, 19). In cultured endothelial cells, this Ca\textsuperscript{2+} signal stimulates the synthesis of nitric oxide (NO) (20), cGMP and prostacyclin (PGI\textsubscript{2}) (21), as well as the acute secretion of von Willebrand’s factor (vWF) (22). Both NO and PGI\textsubscript{2} inhibit platelet aggregation thus as well as the acute secretion of von Willebrand’s factor (23).

With this as background information, we investigated the role of IVIg in the regulation of cytosolic Ca\textsuperscript{2+} signals and NO/cGMP metabolism and vWF release in human endothelial cells. We demonstrated that human therapeutic immunoglobulins reduced basal cytosolic Ca\textsuperscript{2+} concentration and activated the release of NO-dependent cGMP. In activating conditions, IVIg also decreased agonist-activated Ca\textsuperscript{2+} release from internal stores and vWF secretion in endothelial cells. The results suggest that IVIg may prevent adhesion of platelets to damaged endothelium.

Materials and methods

Materials

Fura-2 acetoxyethyl ester (fura-2 AM) was obtained from Molecular probes (Eugene, OR), native collagen fibrils from equine tendons from Nycomed (Munich, Germany) and N\textsuperscript{6}-monomethyl-L-arginine monooacetate (L-NMMA) from Alexis Corporation (Paris, France). The polyclonal antibody against phospho-Ser\textsuperscript{1177}-eNOS was from Cell Signaling Technology, and the monoclonal antibody anti-eNOS was from Transduction Laboratory. All other products were purchased from Sigma-Aldrich Chimie (St Quentin Fallavier, France) Biorad laboratories (Hercules, CA, USA). Lyophilised IVIg were provided by Baxter (Lessines, Belgium), and were dialyzed for 24 h at 4°C against reaction buffers. The absence of degradation of IVIg was checked by electrophoresis on a SDS-polyacrylamide gel.

Cell cultures

Endothelial cells were isolated from segments of human umbilical cord vein by collagenase digestion. Human umbilical vein endothelial cells (HUVEC) were cultured in medium 199 (40%) and RPMI 1640 (40%) supplemented with 20% fetal calf serum as previously described (24). At confluence (4-5 days), cells were detached by incubation for 1-2 minutes at room temperature with 0.01% trypsin and 0.004% EDTA, washed and grown until confluence. Cells were used at first passage. For vWF and [Ca\textsuperscript{2+}]\textsubscript{i} measurements, HUVEC cultured onto six-wells plates or glass coverslips (9 x 35 mm) were equilibrated for 30 minutes with buffer A (in mM: NaCl 136, KCl 5, Na\textsubscript{2}PO\textsubscript{4} 2, MgSO\textsubscript{4} 0.4, NaHCO\textsubscript{3} 4, CaCl\textsubscript{2} 1, glucose 8, glutamine 2, a cocktail of amino acids, and HEPES 25, pH 7.4). Cells were pre-incubated for 5 min at 37 °C in buffer A with or without IVIg, IVIg fragments (Fab\textsuperscript{b}, Fc) or albumine either directly or after loading with 2 µM fura-2 AM, as previously described (24). For NO and cGMP determinations or for western blot analysis, HUVEC cultured onto 35 or 60 mm plastic culture dishes were pre-incubated for 5 min at 37 °C in PBS containing 5 mM glucose, 0.5 mM MgCl\textsubscript{2} and 1 mM CaCl\textsubscript{2} (PBS-MgCa) in the presence or absence of IVIg or albumin. Unbound antibodies were removed by washing the cells with respective incubation buffers.

Preparation of platelets

Venous blood from healthy subjects was collected into tubes containing 0.1 vol. anticoagulant (2.73% citric acid, 4.48% trisodium citrate and 2% glucose) and used within 3h of blood sampling. Platelet-rich-plasma (PRP) was obtained by centrifugation for 15 min at 530g at 20°C. PRP was incubated for 10 min at 37°C with vehicle, IVIg, fragments of IVIg or albumin either directly or after loading with 2 µM fura-2 AM as previously described (25). Platelets were immediately washed by centrifugation at 270 x g for 15 min at 20°C. They were then suspended in buffer B (in mM: NaCl 145, KCl 5, MgCl\textsubscript{2} 0.5, glucose 5 and HEPES 10, pH 7.4 at 37°C) at a density of 2.5x10\textsuperscript{10} or 10\textsuperscript{9} platelets/ml for [Ca\textsuperscript{2+}]\textsubscript{i} or cGMP determinations, respectively.

Measurement of endothelial nitric oxide

NO concentration was measured at the surface of HUVEC by differential pulse amperometry with a three-electrode potentiostatic bipuise system (Tacussel, Villeurbanne, France) and a NO-selective microsensor, as previously described (20). For each experiment, the NO sensor was calibrated by addition of
NO standard solutions as detailed previously (26). The calibration curves were similar for control and IVIg-treated cells. Results were expressed as the maximum of the agonist-induced oxidation current.

**Measurement of endothelial vWF release**
Cells were incubated for 10 min in the absence or presence of agonist. Transferring incubation media to separate tubes that were stored at -20°C until determinations of vWF by ELISA (Diagnostica Stago, Asnières, France) terminated the reaction. Results were expressed as % of values determined in unstimulated HUVEC.

**Determination of cytosolic Ca²⁺ concentration**
Fluorescence intensities of fura-2 loaded HUVEC were recorded on a spectrofluorimeter SPEX CMIII (ISA-Jobin-Yvon, Longjumeau, France). Each measurement was systematically corrected for autofluorescence of unloaded cells and the calibration parameters of fura-2 signals were determined as previously detailed (24).

**Western blot analysis**
Cells were washed and harvested in an ice-cold buffer consisting of 150 mM NaCl, 50 mM Tris, 1 mM EDTA, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonide, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin and 1% Nonidet P-40. The lysates were cleared by centrifugation at 12000g for 15 minutes at 4°C. The different samples containing equal amount of protein were then resolved by electrophoresis on 8% SDS-polyacrylamide gels. The separated proteins were transferred electrophoretically onto nitrocellulose membranes, and non specific binding sites were blocked in Tris-buffered saline containing 0.1% Tween 20 and 5% non-fat dry milk. Membranes were incubated overnight with primary antibody against phospho-Ser1177-eNOS or eNOS. The washed membranes were then incubated with horseradish peroxidase-conjugated secondary antibody. The immunoreactive bands were detected by an enhanced chemiluminescence system (Super Signal West Pico-Pierce) and quantified by densitometry using NIH Image 1.61 software.

**Determination of cGMP contents**
Following incubation of HUVEC for 10 min at 37°C in PBS-MgCa, adding ice-cold ethanol (1/2.5 v/v) stopped the reaction. Cells were then scraped with a rubber policeman and the suspension was submitted to 30s sonication. Platelets in buffer B were equilibrated for 5 min at 37°C and incubated for 2 min in the presence of 1 mM CaNO₃ as previously described (27). The reaction was started by addition of vehicle or agonist and stopped after 20, 30 and 60s. Lysates from HUVEC and platelet were deproteinized by heating at 100°C for 10 min and centrifuged. Supernatants were evaporated in a speedvac concentrator (Savant Instrument, Hicksville, NY) and dried extracts stored at -80°C. Detection of cGMP was performed after resuspension in acetate buffer (50 mM, pH 6.2) and acetylation step to increase the sensitivity by radio-immunoassays (NEN, Chicago, IL).

**Data analysis**
Results are expressed as means ± S.E.M. Multiple comparisons and dose-dependent effects were examined by one-way analysis of variance and Fisher’s test or two-tailed paired Student’s t-test. The time-dependent effects of IVIg treatment was assessed by a 2-way ANOVA.

**Results**

**Action of IVIg on [Ca²⁺]i and release of NO and vWF in unstimulated HUVEC**
Treatment of HUVEC with 0.1 mM IVIg significantly decreased by 20% the cytosolic Ca²⁺ concentration (Fig. 1A). To investigate whether the IVIg-induced decrease in basal [Ca²⁺]i led to alterations of endothelial functions, the release of NO and vWF from unstimulated HUVEC were determined. Detection of basal NO was not possible by electrochemistry; it was thus evaluated by the measure of cGMP. Indeed, the guanylate cyclase is directly activated by NO. The marginal NO release in control HUVEC was confirmed by the non significant effect of the NO synthase inhibitor, L-NMMA (Fig. 1B, control). Treatment with IVIg increased the cGMP levels in L-NMMA-sensitive manner while it had no effect on the basal vWF release (Fig. 1C). Treatment with albumin had no effect either on [Ca²⁺]i or on cGMP pointing out the specific effects of IVIg.

**Effects of IVIg on Ca²⁺ signals and vWF release from agonist-stimulated HUVEC**
The stimulation of HUVEC by thrombin, histamine or ATP elicited a biphasic rise in [Ca²⁺]: a peak occurring 15-30s after addition of the agonist followed by a long lasting plateau (Fig. 2 tracings). Irrespective of the agonist used to stimulate HUVEC, the pretreatment with IVIg significantly reduced the amplitude of Ca²⁺ peak: from 148±12 to 88±10 nM (P<0.01) for thrombin, from 320±43 to 213±23 nM (P<0.05) for histamine and from 112±10 to 72±15 nM (P<0.05) for ATP. In contrast, the amplitude of plateau was unchanged: 34±5 vs 33±5, 99±14 vs 75±9 or 22±6 vs 20±7 nM in thrombin-, histamine- or ATP-stimulated HUVEC, respectively. The agonist-induced Ca²⁺ response was associated with the release of vWF (Figure 2, white bars). The pre-treatment with IVIg resulted in an inhibition of vWF secretion of 40 to 60% (Fig. 2, black bars).
In order to investigate the mechanism of action of IVIg, HUVEC were also treated with the different IgG fragments. The effects of F(ab')2 fragments on \([\text{Ca}^{2+}]_i\) were similar to those of the intact IVIg (Table 1). Treatment of cells with F(ab')2 fragments reduced the basal \([\text{Ca}^{2+}]_i\) values and the amplitude of the \([\text{Ca}^{2+}]_i\) peak without changing that of the plateau in both thrombin- and histamine-stimulated HUVEC. The Fc fragments also reduced the agonist-induced \([\text{Ca}^{2+}]_i\) peak. By comparison, albumin had no effect. The results suggest that the effects of IVIg are dependent on both the F(ab')2 fragments and the constant region of the antibody molecule.

**Action of IVIg on NO release and eNOS phosphorylation**

Thrombin, histamine or ATP induced a transient NO release at cell surface (Fig. 3). The release of NO reached a maximum within 15-30 s and reverted to baseline within two minutes. Pre-treatment with IVIg had no effect on thrombin- or histamine-induced NO release (Fig. 3, upper and medium panel). In contrast, IVIg pre-treatment increased ATP-stimulated NO release from 34±3 to 49±6 nM (+44 %) (Fig. 3, lower panel).
The specific effect of IVIg on HUVEC was confirmed by the observation that human albumin had no effect either on Ca\(^{2+}\) or on NO signals.

That various stimuli activate NO synthesis by phosphorylating eNOS at the Ser\(^{1177}\) residue prompted us to investigate eNOS phosphorylation (28). In unstimulated HUVECs, there is no significant effect of IVIg on basal phosphorylation of Ser\(^{1177}\) eNOS. Following cell stimulation by ATP, a transient phosphorylation of Ser\(^{1177}\) eNOS was observed, which was maximal at 1 min and progressively decreased (Fig. 4). The time-course of ATP-induced phosphorylation in IVIg-treated cell, was similar, but the amount of phosphorylated eNOS was significantly increased at 1 and 2 minutes. The results suggest that IVIg increase the ATP-induced NO release by activating Ser\(^{1177}\) eNOS phosphorylation.

**Table 1**: Effects of F(ab\(^{1}\))\(_{2}\) and Fc fragments of IVIg on [Ca\(^{2+}\)]

Cells were incubated in buffer A in the presence or absence (Control, n=12 or 14) of 0.1 mM IVIg (n=12 or 14), F(ab\(^{1}\))\(_{2}\) (n=12 or 4), Fc (n= 9 or 3) or albumin (n=8 or 4). They were then stimulated by 2 U/ml thrombin or 10 µM histamine, respectively. \(^{*}P<0.05\) and \(^{**}P<0.01\) when compared to control.

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<th>[Ca(^{2+})] (nM)</th>
<th>Control</th>
<th>IVIg</th>
<th>Treatment F(ab(^{1}))(_{2})</th>
<th>Fc</th>
<th>Albumin</th>
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**Effect of IVIg on cGMP contents and [Ca\(^{2+}\)] of platelets**

The fact that IVIg increased NO release and inhibited vWf release -two events inhibiting platelet adhesion- prompted us to investigate the effect of IVIg on platelets. Treatment of platelets with IVIg resulted in an increase in cGMP level. The latter enhanced with increasing concentration of IVIg (Fig. 5A). This dose-dependent effect of IVIg reached a plateau at 0.1 mM IVIg. When the PRP was treated with IVIg together with L-NMMA (Fig. 5B), the IVIg-induced increase in cGMP levels was totally abolished, confirming that the IVG-induced increase in cGMP was due to NO production.

Activation of platelets by agonists also increases the cGMP level. This release of cGMP is, however, differently dependent...
on NO according to the agonist. Indeed, L-NMMA totally inhibited the collagen-induced cGMP release (n=3) whereas it reduced that induced by thrombin or ADP by 50±17 (n=7) and 35±30% (n=3), respectively. The increase in cGMP content induced by the IVIg pretreatment (Fig. 5) was suppressed by further stimulation of platelets by thrombin or by ADP but was maintained when platelets were activated by collagen (Fig. 6).

As for Ca²⁺ mobilization in platelets, neither the basal [Ca²⁺]i nor the biphasic Ca²⁺ response induced by thrombin was altered by pre-treatment with IVIg (56±5 vs 59±4 nM and 268±38 vs 277 ±74 nM at peak, 152±29 vs 156±34 nM at plateau, n=5).

The results show that IVIg is able to activate the synthesis of NO/cGMP the degradation of which occurs during thrombin- or ADP-induced activation but not after collagen.

**Discussion**

With the aim to better understand the beneficial action of therapeutic IVIg to prevent thrombotic complications, we investigated the effects of IVIg on endothelial cells and platelets. Our results demonstrate that IVIg inhibits the Ca²⁺ mobilization and the release of vWF from human endothelial cells and increases the synthesis of NO/cGMP and eNOS phosphorylation. In platelets, IVIg also increases the NO/cGMP level, a high level which was maintained following stimulation by collagen. The results suggest that IVIg could inhibit the platelet activating effect of damaged endothelium.

The IVIg interaction with endothelial cell is unclear and several mechanisms have been put forward to account for the IVIg beneficial action. They may modulate the production of cytokines (29), and interfere with the activated complement (30). They could also block the Fc receptors. We observed that both F(ab’)2 fragments and the Fc portion of IVIg inhibited the Ca²⁺ peak induced by agonists of G protein-coupled receptors in HUVECs. This agrees with previous studies showing that cell proliferation and secretion of thromboxane A₂ and endothelin are inhibited by both F(ab’)2 and Fc portion of IVIg (14, 15). Furthermore, both IVIg and F(ab’)2-IVIg, have been reported to inhibit the Ca²⁺ signals induced by anaphylatoxins in mast cells (31).

The therapeutic use of IVIg is, however, targeted to the inhibition of the FcR (32). Endothelial cells of human placenta villi express the FcγRIB (33). The FcγRIB bears the immunoreceptor tyrosine-based inhibitory motif (ITIM) that inhibits Ca²⁺ mobilization.
mobilization through the phospholipase C cascade initiated by other receptors especially those containing immunoreceptor tyrosine-based activation motif (ITAM) and in particular, FcγRIIA (34). In stimulated HUVEC, we showed that such phospholipase C-mediated Ca\(^{2+}\) mobilisation corresponds to the initial Ca\(^{2+}\) peak, i.e. release from internal stores (35). During activation of G protein-coupled receptors, the acute release of vWf is mainly dependent on Ca\(^{2+}\) release from intracellular stores (22). In agreement with that, we showed that IVIg inhibited the thrombin- and histamine-induced Ca\(^{2+}\) peak and vWf secretion. On the other hand, activation of the endothelial NO synthase (eNOS) requires Ca\(^{2+}\) entry to synthesize NO (20, 36), i.e. the plateau following the Ca\(^{2+}\) peak. This agrees with our observation that IVIg did not alter the Ca\(^{2+}\) entry and the NO release stimulated by thrombin or histamine.

The NO synthesis indeed occurs through Ca\(^{2+}\)-dependent or -independent eNOS activation according to the stimuli applied (28). The present report shows that, depending on the agonist used, the IVIg treatment amplified or not the NO release at the endothelial surface. The IVIg has no effect on the NO release induced by thrombin or histamine, two agonists which activate the phosphorylation of the cytosolic tyrosine kinase, Pyk2, through a Ca\(^{2+}\)-dependent process (37) and phosphorylate eNOS at Ser-1177 (not shown). In contrast, the IVIg increased both the Ser\(^{1177}\)-eNOS phosphorylation and NO release from ATP-stimulated HUVECs. Bradykinin and ATP phosphorylate eNOS at Ser\(^{633}\) through a Ca\(^{2+}\)-independent way with the participation of the cAMP-dependent protein kinase (PKA) (38). The PKA indeed activates eNOS in a Ca\(^{2+}\)-independent manner by phosphorylating three eNOS residues, i.e. Ser\(^{1177}\), Ser\(^{633}\) and Thr\(^{695}\) (39). The AMP-dependent protein kinase (AMPK) also activates eNOS by phosphorylating the Ser\(^{1177}\) residue (40). Thus, it is not unlikely that the IVIg-treatment increased the ATP-induced NO release through a Ca\(^{2+}\)-independent way by interfering with the PKA or AMPK signaling. The Ca\(^{2+}\)-independent eNOS activation by IVIg is also supported by our observation that these natural antibodies decreased the basal Ca\(^{2+}\) concentration while it activated the NO-dependent cGMP synthesis in unstimulated endothelial cells.

The IVIg treatment also increased the synthesis of NO/cGMP in human platelets. This increase in cGMP level was maintained following stimulation by collagen but not during activation by thrombin- or ADP-activation. We previously demonstrated that thrombin and ADP evoked a biphasic increase in [Ca\(^{2+}\)], but did not produce significant NO release, whereas low collagen concentrations did not increase [Ca\(^{2+}\)], and yet activated NO release (45). Collagen binds to a receptor constitutively associated with ITAM and regulated by ITIM-bearing receptor (41). Thrombin and ADP bind to G-protein coupled receptors, the signal of which interact with that of the immunoglobulin Fc domain receptor, FcγRIIA, either by tyrosine phosphorylation of FcγRIIA (42) or by a FcγRIIA-mediated activation of phospholipase C (43). Thus, in IVIg-pretreated platelets, thrombin- and ADP-induced signals could counterbalance that of FcγRIIA thereby resulting in the degradation of the cGMP. Among the signalling kinases involved in the latter, the phosphatidylinositol 3-kinase (PI3-K) phosphorylates Akt and PKA which in turn activate NO synthesis at basal Ca\(^{2+}\) concentrations (28). It has previously been reported that IVIg increase the NO production through a PI3-K dependent mechanism in rat microglia stimulated by IFNγ (44). In endothelial cells and platelets, the IVIg could similarly activate the NO and cGMP synthesis through a Ca\(^{2+}\)-independent and PI3-K-dependent signalling pathway.

The therapeutic effects of IVIg in inflammatory vascular pathologies can be related to the observed stimulatory effects on NO release and inhibitory effect on vWf. Indeed, NO is a potent inhibitor of platelet aggregation and adhesion and vWF is the main mediator of platelet adhesion to the subendothelium. The loss of anti-thrombotic mechanisms together with the activation of pro-thrombotic ones and/or lyses of endothelial cells is critical steps of various inflammatory vascular pathologies with thrombotic events. Thrombosis is observed in vasculitis disorders and hyperacute rejection (3). Beneficial effects of IVIg have been reported in both systemic vasculitis and acute rejection of allogeneic graft where endothelial damage is mediated by endothelial cell-reactive or host antigraft antibodies.

In conclusion, our results show that natural antibodies are able to modulate Ca\(^{2+}\) signalling and vWf release in endothelial cells and to activate NO synthesis in both platelets and endothelial cells. The results provide an insight into events underlying the control of endothelial cell activation by IVIg in inflammatory vascular diseases, and hence the control of excessive platelet activation.

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References


