Human Immunoglobulins Inhibit Thrombin-induced Ca\textsuperscript{2+} Movements and Nitric Oxide Production in Endothelial Cells*

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Olivier Schussler‡, Frédérique Lantoine§, Marie-Aude Devynck, Denis Glotz‡, and Monique David-Dufilho¶

From the Department of Pharmacology, URA CNRS 1482, Paris V University, Necker University School of Medicine, 156 rue de Vaugirard, 75015, Paris, France, the Laboratoire d’Electrochimie et de Chimie Analytique, CNRS URA 216, Ecole Nationale Supérieure de Chimie Paris, 11 rue Pierre et Marie Curie, 75231 Paris Cedex 05, France, and §INSERM U430, Laboratoire d’Immunologie-Pathologie Humaine, Hôpital Broussais, 96 rue Didot, 75674 Paris, France

Binding of natural antibodies to endothelial cell plays an important role in hyperacute xenograft rejection between discordant species. Human intravenous immunoglobulins (IVIg) delay this hyperacute rejection, but their mechanisms of action on endothelial cells have to be defined. Here we demonstrate that IVIg dose-dependently prevent thrombin from eliciting cytosolic Ca\textsuperscript{2+} movements and nitric oxide (NO) production in aortic endothelial cells from guinea pig. The Ca\textsuperscript{2+} response to thrombin was similarly affected by IVIg whether they were removed or not from the incubation medium before stimulation. Pretreatment by rat natural antibodies also suppress the thrombin-induced Ca\textsuperscript{2+} peak corresponding to Ca\textsuperscript{2+} release from intracellular stores but stimulate the subsequent sustained increase in [Ca\textsuperscript{2+}], and the release of NO. The action of human intravenous immunoglobulins seems to be selective for the thrombin receptor because they do not affect [Ca\textsuperscript{2+}], and NO responses to endothelin-1 or thapsigargin. However, these antibodies also suppress the first phase of the cytosolic Ca\textsuperscript{2+} response to ATP, which does not release NO under our experimental conditions. These observations raise the possibility that IVIg selectively interact with targets localized on plasma membrane of endothelial cells for controlling receptor-activated Ca\textsuperscript{2+} pathways and NO release.

Vascular endothelial cells respond to various neurohumoral and physical stimuli by increasing cytosolic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]), and releasing substances such as nitric oxide (NO).\textsuperscript{1}

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\‡ To whom correspondence should be addressed: Dept. of Pharmacology, URA CNRS 1482, CHU Necker, 156 rue de Vaugirard, 75015 Paris, Tel.: 33-1-40-61-53-34; Fax: 33-1-40-61-55-84.

\¶ The abbreviations used are: NO, nitric oxide; IVIg, immunoglobulins for intravenous use; PBS, phosphate-buffered saline; NOS, nitric oxide synthase; ET-1, endothelin-1; TG, thapsigargin.
assessed by performing similar experiments in the presence of equivalent quantities of human albumin. Only alive cells as defined by propidium iodide staining exclusion were considered. In the range of IVIg concentrations studied, the presence of antibodies did not alter cell viability estimated as the percentage of cells excluding trypan blue or propidium iodide (>95%). Specific binding of IVIg to endothelial cells was obtained after subtraction of nonspecific binding from the total.

**Determinations of Cytosolic Ca\(^{2+}\) Concentrations**—Cytosolic free Ca\(^{2+}\) concentrations were determined as described previously (21). Briefly, cells cultured on coverslips were transferred into buffer A (in mM: NaCl 136, KCl 5, NaH\(_2\)PO\(_4\) 2, MgSO\(_4\) 0.4, NaHCO\(_3\) 4, glucose 8, Hepes 25, pH 7.4 at 37°C) containing 20% fetal calf serum, a cocktail of amino acids, and 2 mM glutamine. They were first equilibrated in buffer A for 30 min at 37°C and then loaded with 2 µM fura-2 (acetoxyethylxylester) for 25 min. De-esterification was completed in fresh buffer A containing 20% of fetal calf serum for 25 min at 37°C. To record fluorescence signals, cells were washed twice with buffer A, and the coverslip was inserted in a special quartz-suprasil cuvette thermostated at 37°C (22). Fura-2 was excited at 340 and 380 nm, and emission was collected at 505 nm on a spectrofluorimeter SPEX CMII (ISA-Jobin-Yvon, Longjumeau, France). Each measurement was systematically corrected for autofluorescence of unloaded cells incubated or not with different concentrations of human immunoglobulins.

[Ca\(^{2+}\)]\(_i\), was calculated from the 340/380 ratios of fluorescence intensities using the equation of Grynkiewicz et al. (23) and the previously determined \(K_i\) and calibration parameters (21).

**Measurements of NO Production**—NO release was monitored with a NO-selective microprobe (24). The working electrode was carbon microfiber (8-µm diameter, approximately 1-mm length), coated by tetrakis (3-methoxy-4-hydroxyphenyl) nickel (II) porphyrin and nafion films. Differential pulse amperometry was performed with a three-electrode potentiostatic biopulse system (Tacussel, Lyon, France) as described previously (24).

The cells were incubated in PBS medium complemented with 5 mM glucose and 0.5 mM MgCl\(_2\) (buffer B). The use of a micromanipulator (Biologic Instruments, Claix, France) attached to the stage of an inverted microscope (Zeiss, Germany) allowed to set the probe 10–15 µm above the cell surface, all the apparatus being enclosed in a Faraday's chamber. At the end of each experiment, internal calibration of the electrochemical sensor was performed by the addition of NO standard solutions as detailed previously (25). The sensitivity of electrodes varied from 0.5 to 2.6 nA/nM NO\(_2\)/pA, (1.6 ± 0.4 nM NO\(_2\)/pA, n = 9). Under our experimental conditions, the detection limit was 2–5 nM NO.

Identification of NO as responsible for the amperometric signal induced by 2 units/ml thrombin was checked by preincubating the cells for 5 min at 37°C with 10–3 M l-arginine or 10–3 M N\(_6\) monomethyl-l-arginine monooacetate. Thrombin-induced NO production was enhanced from 46 ± 15 up to 94 ± 10 nM by l-Arg pretreatment (n = 3) and decreased to 6.7 ± 3.3 nM in N\(_6\) monomethyl-l-arginine monooacetate-treated cells (n = 3).

**RESULTS**

In aortic endothelial cells from guinea pig, thrombin induced a biphasic increase in [Ca\(^{2+}\)]\(_i\); a transient peak occurring 15–20 s after stimulation followed by a long lasting plateau (Fig. 1A). NO production paralleled the rise of [Ca\(^{2+}\)]\(_i\); NO concentration reached a maximum within 15–20 s after thrombin addition (32 ± 6 nM, n = 9) and remained above basal level for a few minutes (Fig. 1A). Incubation of cells for 5 min at 37°C with 10 mg/ml IVIg or rat IgG followed by a washing to remove unbound antibodies from the incubation medium did not change basal [Ca\(^{2+}\)]\(_i\), values (Fig. 1, B and C) but altered the Ca\(^{2+}\) response to thrombin. This IVIg pretreatment prevented thrombin from significantly increasing [Ca\(^{2+}\)]\(_i\), and producing NO (Fig. 1B). Rat IgG pretreatment also suppressed the [Ca\(^{2+}\)]\(_i\), peak elicited by thrombin but increased the amplitude of the sustained [Ca\(^{2+}\)]\(_i\), rise from 56 ± 13 to 138 ± 27 nM (n = 5 and 12) and the NO production from 32 ± 4 to 52 ± 4 nM (n = 5) (Fig. 1C).

For [Ca\(^{2+}\)]\(_i\), measurements only, we could compare the response to thrombin in cells pretreated by 10 mg/ml IVIg whether they were removed or not from the incubation medium before stimulation. Under these two conditions, the amplitude of [Ca\(^{2+}\)]\(_i\), rise at peak was reduced by 85% on average and that at plateau by 70%. This indicates that the IVIg effects we saw were mainly due to their binding on endothelial cells and not to their direct interactions with thrombin in the extracellular medium. This is confirmed by the observation that the dose-dependent inhibitions of IVIg on thrombin-induced Ca\(^{2+}\) move-
Human IVIg prevented both the biphasic Ca\(^{2+}\) response and the NO production, whereas the rat natural IgG suppressed the first phase of the Ca\(^{2+}\) response but increased the sustained [Ca\(^{2+}\)] response and the NO concentration ([NO]). These distinct actions on Ca\(^{2+}\) pathways and NO production suggest the presence of different binding sites for IVIg and rat IgG on endothelial cell membrane. This is supported by the lack of effect of IVIg pretreatment on rat IgG binding to guinea pig endothelial cells (results not shown). Our results also underline the functional difference between rat IgG and human IVIg. The binding of antidonor antibodies to blood vessels of the xenograft occurs early in the rejection process (16), whereas purified human immunoglobulins reduce the cytotoxic activity of recipient serum in different discordant xenograft models (17, 18). From the present observations it may be proposed that binding of rat IgG to guinea pig endothelial cells induce cell contraction by amplifying the sustained Ca\(^{2+}\) response to thrombin. In contrast, the binding of IVIg to guinea pig endothelial cells may delay the cell activation and retraction by reducing the thrombin-induced Ca\(^{2+}\) movements. Thrombin has indeed been demonstrated to cause a marked retraction of confluent endothelial cells coincident with the sustained phase of [Ca\(^{2+}\)] response and paralleled by the formation of gaps in F-actin distribution (4). The retraction of endothelial cells leads to disruption of the cell monolayer thereby altering the vascular function.

In vascular endothelial cells, the temporal and causal relationships between rise in [Ca\(^{2+}\)] and NO production have mostly been investigated with indirect methods. The production of eNOS, considered to be the cyslosolic effector of NO, appears to be directly related to the cytosolic Ca\(^{2+}\) level (7, 27, 28). This was explained by the Ca\(^{2+}\)/calmodulin dependence of

**DISCUSSION**

The vascular endothelium is more than a physical and selectively permeable barrier between blood and surrounding tissues; it maintains an anticoagulant environment, prevents blood cells adhesion, and secretes vasoactive substances (2). Activation of the endothelial cells have been recently shown to be a major mechanism at play in the genesis of hyperacute xenograft rejection (26). The present study demonstrates that natural antibodies alter the metabolism of Ca\(^{2+}\) ions, one of the most important second messengers for cell activation. Human and rat natural antibodies did not modify per se the cytosolic Ca\(^{2+}\) level in aortic endothelial cells from guinea pig but did modulate the Ca\(^{2+}\) movements elicited by thrombin, a protease generated at sites of vascular injury. The observation that these effects still appeared after removal of antibodies from the incubation medium suggests interactions with specific targets on endothelial cells rather than immunological interferences with thrombin itself.

**Fig. 4.** ATP-, endothelin-1-, and thapsigargin-activated NO production in control and IVIg-treated cells. Cells were preincubated for 5 min at 37 °C with either vehicle or 10 mg/ml IVIg and then washed to record the electric signal resulting from NO. Cells were stimulated by 0.5 mM ATP, 1 μM ET-1, or 1 μM TG as indicated by the arrows. Left panels, representative recordings of NO produced at surface of control cells. Right panels, means ± S.E. of the differences between maximal activated and basal signals in control (C) and IVIg-treated cells (IVIg) (n = 3 with ATP; n = 5 with ET-1; and n = 4 with TG).
NO synthase (NOS) activity (for review see Ref. 29). A few direct measurement of [NO] has investigated the relations of NO production to [Ca\(^{2+}\)], rises. The participation of Ca\(^{2+}\)/calmodulin in a step of NO synthesis has been also demonstrated by detection of [NO] with an amperometric NO sensor similar to the Clark type electrode for oxygen (30). Simultaneous measurements of [Ca\(^{2+}\)], and [NO] with a porphyrinic microsensor have indicated that the increase in [NO] outlasted the [Ca\(^{2+}\)], transient (8). Our study showed by real time measurement of [NO] with a porphyrinic microelectrode that thrombin, ET-1, and thapsigargin parallely increased [Ca\(^{2+}\)], and NO in accordance with the activation of NOS by Ca\(^{2+}\)/calmodulin. However, ATP elicited a clear biphasic Ca\(^{2+}\) response without any detectable [NO], indicating that this Ca\(^{2+}\) rise was nonsufficient for triggering NO production. The [NO] we detected here were in the same range as those previously reported with the Clark type electrode (30) but 10-fold lower than those obtained by Malinski's group in response to supraphysiological bradykinin concentration (8).

A parallel between [Ca\(^{2+}\)], rises; cGMP formation and nitrite/nitrate production has been clearly demonstrated for thrombin/thrombin for review see Ref. 34). The inhibition action of human IVIg and rat IgG on NO production is dependent on Ca\(^{2+}\)/calmodulin and protein tyrosine kinase (33). This suggests that interactions of IVIg with Ca\(^{2+}\) pathways depend on phospholipase C activation. This proposal agrees with the suppression by IVIg of the first phase of Ca\(^{2+}\) response to thrombin and ATP. This first step has been clearly demonstrated to be mediated by phospholipase C (for review see Ref. 34). The inhibitory action of human IVIg and rat IgG on this first phase of Ca\(^{2+}\) signals could therefore be mediated at least in part through the interactions of natural antibodies with phospholipase C. Furthermore, our results also indicated that the action of human IVIg and rat IgG on NO production is more likely related to their effects on the sustained phase of agonist-induced [Ca\(^{2+}\)], elevations. This agrees with a recent study demonstrating a clear relationship between nitrite production and sustained [Ca\(^{2+}\)], (32). The inhibition of the initial Ca\(^{2+}\) response to agonists seems to be a common feature of natural antibodies, whereas their action on the sustained [Ca\(^{2+}\)], rise and NO production appears to reveal their specificity.

The present study indicates that natural antibodies are capable of modulating Ca\(^{2+}\) homeostasis and NO production in stimulated endothelial cells. Their mechanisms of action remains to be better defined. Future studies must be directed toward the action of natural antibodies on the metabolism of intracellular messengers associated to phospholipase C activation and on the production of vasoactive factors of endothelial origin.

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