

## REQUIREMENT OF A PLASMA FRACTION FOR THE LOSS OF LARGE VON WILLEBRAND FACTOR MULTIMERS INDUCED BY HIGH WALL SHEAR RATE

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**Summary** The perfusion of serum, citrated whole blood and citrated plasma, through a simple tube system resulted in a significant loss of large von Willebrand factor (vWf) multimers, without decrease in antigen levels. Maximum loss of large multimers was observed at a shear rate of  $15,000\text{ s}^{-1}$  for 15 min. Heparin, aprotinin, soybean trypsin inhibitor, phenylmethylsulphonylfluoride, N-ethylmaleimide, leupeptin or calpain inhibitor peptide could not prevent the loss of large vWf multimers in citrated plasma. The addition of EDTA calcium salt partially prevented it, and it was totally prevented by EDTA without calcium. Perfusion of purified vWf did not induce the loss of large multimers, but this did happen after the addition of either whole serum or a plasma fraction. The activity of this plasma fraction disappeared at  $\text{pH} < 6.8$ . Besides, we have found that the binding to subendothelium of purified vWf diluted in dialized serum was lower at  $\text{pH} 7.2$  than at  $\text{pH} 6.0$ . Chromatographic studies demonstrated that the loss of large vWf multimers, induced by high shear rates, involves a plasma substance(s) of molecular weight larger than 200 kD; calpain and granulocyte or cysteine proteases do not seem to be this plasma substance(s).

**Key words:** von Willebrand, shear rate, multimers

Von Willebrand Factor (vWf) is a multimeric molecule constructed of a series of dimers of identical subunits (270kD)<sup>1,2</sup>. It is well known that the binding of vWf to platelets and to subendothelial collagen depends on the multimeric size<sup>3,4</sup> and occurs at high shear rates<sup>5,6</sup>. The vWf molecule, not only mediates platelet adhesion to subendothelium, but it is also involved in platelet-platelet interaction. In fact, vWf can support shear stress-induced platelet aggregation<sup>7-10</sup>. All these findings support the role of vWf as a crucial molecule in initiating the hemostatic or thrombotic process; much less is known about the mechanisms involved in the down regulation of this phenomenon.

In preliminary studies<sup>11</sup> we have observed that high shear rates induced a loss of large vWf

multimers and a reduction of ristocetin cofactor (RiCof) activity in plasma samples. It has been found that a substance(s) from plasma cryosupernatant regulates accumulation of unusually large vWf multimers from endothelial cells<sup>12</sup>. Phillips et al<sup>13</sup> have shown that a disulfide bond reductase, present in plasma, reversibly decreases unusually large multimers by a vWf processing activity. Tsai et al<sup>14</sup> have shown that shear stress can enhance the susceptibility of vWf to proteolytic cleavage. It promotes vWf proteolysis in normal plasma at a site that generates the 140kD-176kD fragments, leading to a decrease in multimeric size.

In the present study we have analyzed the effect of different shear rates and perfusion times on vWf multimeric size. We also examined the potential effect of sample volume, perfusion temperature, pH and different substances that inhibit the loss of large vWf multimers. Finally, we have partially characterized the plasma substance(s)

Received: 29-V-1996

Accepted: 7-I-1997

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that promotes the loss of large vWf multimers at high wall shear rate.

## Materials and methods

### Reagents

Leupeptin, EDTA disodium salt, EDTA calcium salt, N-ethylmaleimide (NEM), aprotinin, trypsin type I, soybean trypsin inhibitor type I-S, phenylmethylsulphonylfluoride, agarose type V, Tween 20, Sephacryl S-1000, Sephacryl S-200, DEAE-Sephadex G-50, polyethylene glycol 4,000 and 20,000, calpain inhibitor peptide, bovine serum albumin (BSA) and 4-Chloro-1-naphthol were from Sigma (St. Louis, MO). Molecular weight (MW) markers were from Pharmacia. Heparin was from Immuno, Viena. Rabbit antibody to human vWf, biotinylated antiserum to rabbit immunoglobulin and ABCComplex/HRP were from Dako Corp (Carpinteria, CA). Fibrinogen assay kit was from MeDiTech (Ventura, CA). FVIIIc assay kit was from STAGO (France). Silastic, medical grade tubing, was from Dow Corning Corp. (Midland, MI).

### Preparation of whole blood

Blood for whole blood studies was collected into plastic tubes containing citrate to achieve a final concentration of 12.9 mM.

### Preparation of serum

Blood for serum studies was incubated at 4°C for 24 h. Serum was obtained by centrifuging blood samples at 2,500 g at 4°C for 1 h. Serum was dialyzed against 0.15M NaCl pH 6 or pH 7.2 for some experiments.

### Preparation of platelet poor plasma (PPP)

Blood for plasma studies was collected into plastic tubes containing the following different anticoagulants or protease inhibitors: a) citrate b) citrate plus 14 U/mL heparin, c) citrate plus 60 mM NEM, d) citrate plus 1,500 KIU/mL aprotinin, e) citrate plus 4 mM phenylmethylsulphonylfluoride (PMSF), f) citrate plus soybean trypsin inhibitor 33 mg/ml, g) citrate plus 5 mM EDTA, h) citrate plus 5 mM EDTA calcium salt, i) citrate plus 1 mM leupeptin and j) citrate plus 0.032 mM calpain inhibitor peptide. Each blood sample was centrifuged at 2,500 g for 1 h at 4°C and the plasma was transferred to another tube and centrifuged at 40,000 g for 30 min or filtered through a syringe filter with 0.2 µm pore sizes.

### Preparation of purified vWf

vWf was isolated as previously described<sup>15</sup>. Briefly, vWf concentrate was purified by gel filtration on Sephacryl

S-1000. Only the void volume fractions, free of fibrinogen contamination, were collected and pooled. The sample obtained was then concentrated using 20% Polyethylene glycol 20,000 and dialyzed against 0.15M NaCl, pH 7.2.

### Separation of plasma components

Different citrated plasma samples were obtained by the following methods: a) gel filtration (elution buffer: NaCl 0.15M pH 7.2) on Sephacryl S-200 column (flow rate: 4 mL/h cm<sup>2</sup>) and b) stepwise gradient elution on DEAE-Sephadex G-50, eluent 0.1 M Tris-HCl buffer, pH 6.5, with a salt gradient from 0 to 0.3 M NaCl. Flow rate: 12 mL/h cm<sup>2</sup>. The fractions obtained were concentrated using 20% Polyethylene glycol 20,000 and dialyzed against 0.15M NaCl pH 7.2. The protein standards employed in chromatographic studies for MW determinations were: blue dextran 2000, and BSA.

### Perfusion system

The simple tubing perfusion model was made using a peristaltic pump with speed controller. The sample (1.3 mL) was perfused through a system composed of two silastic tubes (A and B): A) 26 cm and B) 19 cm in length, with different internal diameter (id); A) id = 0.076 cm and B) id = 0.158 cm that were connected end-to-end. The tubes A and B were changed in each experiment. Before and after each perfusion, the total tubing system was perfused consecutively with 0.1 M NaOH (1 min), distilled water, bovine serum albumin (BSA) (30 mg/mL) (5 min) and an aliquot of the sample. The different shear rates (9,000 to 15,000 s<sup>-1</sup>) were obtained varying the pump speed (flow rates from 390 µL.s<sup>-1</sup> to 670 µL.s<sup>-1</sup>). The wall shear rate was calculated by the following formula<sup>16</sup>:

$$G = \frac{32Q}{\pi \cdot id^3}$$

G = wall shear rate in s<sup>-1</sup>, Q = flow rate in mL s<sup>-1</sup>, id = internal diameter of the tube in cm. Since the shear rate of the tube B was very low compared to the tube A, it was not considered.

### Perfusion experiments

The effects of shear rate (9,000, 12,000, 15,000 s<sup>-1</sup>) and perfusion time (5, 15, 30 min) on vWf structure were evaluated in 1.3 mL of citrated plasma samples at 37°C. Besides, we analyzed the influence of sample volume (1.3, 2.6 and 6.5 mL) and perfusion temperature (4.25 and 37°C) on vWf structure modification, at a shear rate of 15,000 s<sup>-1</sup> for 15 min. The chosen perfusion conditions were: shear rate of 15,000 s<sup>-1</sup> during 15 min.

To evaluate the effect of pH, plasma substances of MW larger than 200 kD were obtained on Sephacryl S-200 column and eluted with 0.15M NaCl pH 6. The sam-



ple was assayed in the same buffer followed by pH adjustment between 5.5 and 7.2. Enzyme-immunoassay for vWf binding to human arterial subendothelium was performed as previously described<sup>17</sup>. The perfusion was carried out in a perfusion chamber, with everted human umbilical arterial segments. The perfusate was purified vWf (1 U/mL) diluted in dialyzed serum in 0.15M NaCl pH 6 or 7.2 containing BSA. The vessel segments perfused and control segments were washed and incubated with a rabbit antibody to human vWf. Then, the nonbound rabbit antibody to human vWf from all samples was used to determine vessel bound vWf by indirect enzyme-immunoassay. The amount of bound vWf for each experiment was calculated by subtracting the value of the respective control sample.

We evaluated the effect of substances that inhibit or promote the loss of large vWf multimers in serum, whole blood (citrate) and plasma obtained with different anticoagulant mixtures. In some experiments we assayed the effect of perfusion on purified vWf diluted in: a) 0.15 M NaCl, b) human serum, c) human serum incubated 1 h at 37°C with 5 mM EDTA, and d) fractions obtained by purification of plasma by stepwise gradient elution on DEAE-Sephadex G-50.

All the samples were perfused containing 30 mg/mL of BSA.

#### *Laboratory tests to evaluate perfusion experiments*

A multimeric analysis of vWf was performed on SDS-1% agarose gel electrophoresis and visualized by immunoenzymatic stain<sup>19</sup>. vWf multimers were measured by densitometrical scanning of the stained gels with an Ultrascan-Laser Densitometer (LKB 2202) equipped with an Integrator (LKB 2221). The length of each curve starting from the cathodal origin of each gel was expressed in cm.

vWf antigen (vWf:Ag) was measured by electroimmunoassay (EIA)<sup>20</sup> and in some samples, to discard the measurement of spurious high levels of the protein, by enzyme-linked immunosorbent assay (ELISA) using a biotinylated antibody to human vWf<sup>21</sup>.

Fibrinogen concentration was determined by Clauss clotting time method with a commercial kit. FVIIIc was assayed using a commercial kit.

#### *SDS-polyacrylamide gel electrophoresis*

Plasma fractions eluted from Sephacryl S-200 and DEAE-Sephadex G-50 column were prepared for electrophoresis by solubilizing in 2% SDS. These samples were immediately immersed in a boiling bath for 5 min. All samples were electrophoresed on 9% and 6% SDS-polyacrylamide gels, as previously described<sup>22</sup>. Besides, the samples were diluted in 0.09 Tris, 0.08 M Boric Acid, 0.93 g/l EDTA buffer at pH 8.4 and were

electrophoresed on 6% polyacrylamide gels. The protein standards employed in this study for MW determinations were: thyroglobulin (669 kD), ferritin (440 kD), catalase (232 kD), lactate dehydrogenase (140 kD), and albumin (66 kD). The gels were visualized by staining with coomassie blue.

#### *Statistical analysis*

The effects of shear rate, perfusion time and temperature, sample volume and pH on vWf multimeric structure and the presence of substances that inhibit or promote the loss of large vWf multimers were evaluated by paired t-test.

## **Results**

### *Effects of shear rate and perfusion time on vWf multimeric structure*

Perfusion of citrated plasma resulted in a very significant reduction of higher vWf multimers (Fig. 1). There were no changes in vWf: Ag concentration (Table 1). The loss of large vWf multimers depends on the shear rate and the perfusion time employed. The most important differences ( $p < 0.001$ ) were observed at 15,000  $s^{-1}$  during 15 min of 12,000  $s^{-1}$  during 30 (Table 1). We also evaluated the effect of perfusion on fibrinogen levels. Table 1 showed that fibrinogen concentrations decreased but they remained within the normal range. FVIIIc levels showed a significant reduction ( $n = 4$ ,  $p < 0.05$ ) in citrated plasma perfused at 15,000  $s^{-1}$  during 30 min (data not shown).

### *Effect of sample volume and perfusion temperature on vWf multimeric structure*

An increase in sample volume of 5 fold did not modify the vWf molecule present in citrated plasma that was perfused at high shear rate (data not shown). Modification of vWf multimers was similar whether perfusion was done at room temperature, 4°C or 37°C. Variations of temperature or sample volume perfusion did not modify vWf: Ag (data not shown).

### *Effect of pH on vWf multimeric structure*

Fraction of MW larger than 200 kD ( $V_0$ ) was more significantly modified at pH 6.8 ( $p < 0.001$ ) than at pH 6.5 ( $n = 4$ ) (data not shown).



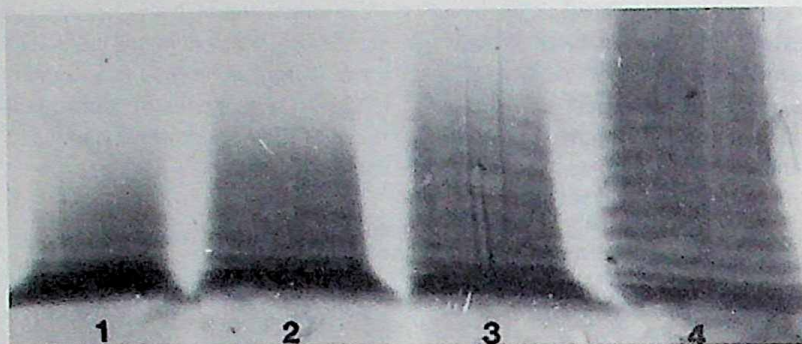


Fig. 1.— SDS-1% agarose gel electrophoresis of normal plasma vWf. Samples of citrated plasma were perfused at a shear rate of  $15,000 \text{ s}^{-1}$  during 30 min (lane 1), 15 min (lane 2), 5 min (lane 3). Unsheared normal plasma was considered as control (lane 4). Discernible differences were seen in the multimers of plasma vWf at different perfusion times. The anode was at the bottom.

TABLE 1.— Changes in laboratory measurements after citrated plasma perfusion at different shear rates ( $\text{s}^{-1}$ ) and times (min)

Test	T	Shear			Rate		
		C	9,000	C	12,000	C	15,000
vW	5	$4.6 \pm 1.3$	$3.8 \pm 1.4$	$5.8 \pm 1.0$	$4.6 \pm 1.4$	$5.2 \pm 0.5$	$4.2 \pm 0.7^*$
multimers (cm)	15	$5.8 \pm 0.8$	$4.9 \pm 1.3$	$6.0 \pm 0.3$	$4.6 \pm 1.1^*$	$6.1 \pm 0.5$	$3.1 \pm 1.0^+$
	30	$5.2 \pm 0.5$	$4.2 \pm 0.7^*$	$6.7 \pm 0.1$	$4.1 \pm 0.1^+$	$6.0 \pm 0.9$	$2.9 \pm 1.0^+$
vWf:Ag	5	$80 \pm 11$	$78 \pm 13$	$89 \pm 20$	$80 \pm 30$	$90 \pm 17$	$91 \pm 25$
EIA (U/dL)	15	$97 \pm 23$	$95 \pm 23$	$101 \pm 17$	$100 \pm 17$	$84 \pm 20$	$84 \pm 12$
	30	$89 \pm 16$	$85 \pm 26$	$100 \pm 10$	$107 \pm 12$	$107 \pm 20$	$108 \pm 15$
vWf:Ag	30	nd	nd	nd	nd	$80 \pm 16$	$84 \pm 22$
ELISA (U/dL)							
Fibrinogen	30	$340 \pm 35$	$277 \pm 12$	$400 \pm 10$	$280 \pm 14^*$	$352 \pm 47$	$278 \pm 53^*$
(Mg/dL)							

Values are: mean  $\pm$  SD,  $n = 4$  to 40

\*  $p < 0.05$ , +  $p < 0.001$ , paired t-test, relative to respective controls.

T: time; C: unsheared samples; EIA: electroimmunoassay; ELISA: enzyme-linked immunosorbent assay; nd: not done.

The results of indirect enzyme-immunoassay for vWf binding to human arterial subendothelium were:  $1.7 \pm 0.5 \times 10^{-3}$  U of vessel bound vWf diluted in dialyzed serum/ $\text{cm}^2$  subendothelium pH 7.2 ( $n = 4$ );  $4.6 \pm 0.7 \times 10^{-3}$  U subendothelium of

vessel bound vWf in dialyzed serum/ $\text{cm}^2$  pH 6 ( $n = 4$ );  $4.6 \pm 0.7 \times 10^{-3}$  U subendothelium of vessel bound vWf in dialyzed serum/ $\text{cm}^2$  pH 6 ( $n = 4$ ). It means that at pH 7.2, vWf lost its large multimers decreasing its binding to subendothelium.



*Effect of substances that inhibit or promote the loss of large vWf multimers*

Loss of large vWf multimers was evident when perfusion experiments were performed using serum, citrated whole blood or citrated plasma ( $p < 0.001$ ,  $n = 40$ ). The same results were obtained with a mixture of citrate and heparin, aprotinin, NEM, PMSF, soybean trypsin inhibitor, leupeptin or calpain inhibitor peptide. It did not happen when samples were obtained using a mixture of citrate and EDTA ( $n = 7$ ). In samples anticoagulated with

mixture of citrate and EDTA calcium salt, we observed a partial loss of multimers ( $n = 3$ ) (Fig. 2).

Interestingly, the perfusions carried out with purified vWf showed an important loss of multimers ( $p < 0.001$ ,  $n = 6$ ), when it was diluted in serum (Table 2) or when it was diluted in either the 0.2 M NaCl peak (protein concentration: 5.7 mg/mL) or in the 0.3 M NaCl peak (protein concentration: 0.9 mg/mL) eluted from citrated plasma purification on DEAE-Sephadex G-50 column (Table 2). This 0.3M NaCl peak analyzed on 6% SDS-PAGE showed four major protein bands

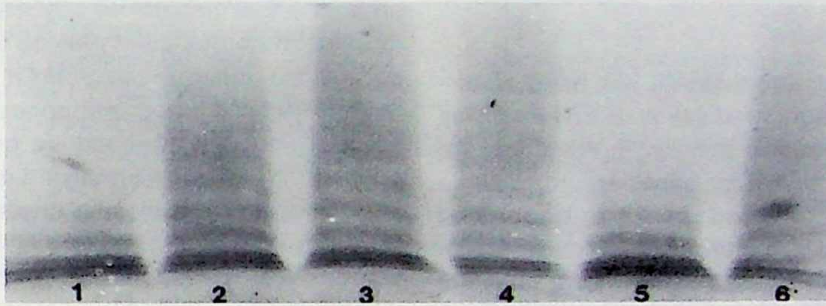


Fig. 2.— SDS-1% agarose gel electrophoresis of normal plasma vWf. The plasma samples used were obtained from whole blood anticoagulated with: citrate (c) (lane 3 and 5), c plus 1mM leupeptin (lane 1), c plus 5mM EDTA calcium salt (lane 2), c plus 5mM EDTA plus 6mM NEM plus 200 kallikrein inhibitor U/mL of aprotinin (lane 4), c plus 5mM EDTA (lane 6). Lane 1, 2, 4, 5, 6 samples were perfused at a shear rate of  $15000 \text{ s}^{-1}$  during 15 min. Unsheared sample was considered as control (lane 3) in lane 2 larger bands are partially diminished.

TABLE 2.— *Effect of substances that inhibit or promote the loss of large vWf multimers. Changes in vWf multimers following samples perfusion*

Samples	vWF multimers (cm) Unsheared	vWF multimers (cm) Sheared
Citrated whole blood	$5.5 \pm 0.8$	$2.9 \pm 0.7^*$
Serum	$5.6 \pm 0.9$	$2.2 \pm 1.0^*$
Purified vWf (1U/mL) in saline-BSA- pH 7.2	$5.3 \pm 0.7$	$5.0 \pm 0.8$
Purified vWf (5U/mL) in serum	$5.6 \pm 1.0$	$2.8 \pm 1.1^*$
Purified vWf (1U/mL) in 0.3M NaCl peak DEAE-Sephadex	$5.1 \pm 0.2$	$3.8 \pm 0.3^*$

Values are: mean  $\pm$  SD,  $n = 6$

\*  $P < 0.001$ , paired t-test, relative to respective unsheared samples



with the following MW: a)  $> 400\text{kD}$ , b)  $322 \pm 14$ , c)  $210 \pm 11$ , d)  $99 \pm 12$  ( $n = 5$ ). When this 0.3M NaCl peak was diluted in 0.09 Tris, 0.08 M Boric Acid, 0.93 g/l EDTA buffer at pH 8.4 and was electrophoresed on 6% polyacrylamide gels we observed five major bands with the following MW: a)  $> 700$ , b)  $565 \pm 75$ , c)  $339 \pm 30$ , d)  $207 \pm 50$ , e)  $91 \pm 40$ .

The void volume ( $V_0$ ) peak eluted from Sephacryl S-200 analyzed on 9% SDS-PAGE showed protein bands with MW larger than 264kD (data not shown).

## Discussion

As the molecular size of vWf is a major determinant of its function, modulation of vWf size may be critical in its biologic activity. In this study we have shown that vWf from either serum, citrated whole blood or citrated plasma, undergoes multimeric structure modification. A decrease of the largest multimers associated with a constant quantity of vWf: Ag was achieved by using a tube perfusion model. The degree of loss of large vWf multimers depended on shear rate, perfusion time, sample volume, pH and the presence of a plasma fraction.

Polymethyl siloxane (silastic) has been extensively employed for material tubing because of its relative biocompatibility and favorable mechanical properties. However, when it was exposed to blood, adsorption of proteins such as fibrinogen, fibronectin and platelet retention were significant<sup>23</sup>. In fact, we also observed a reduction in fibrinogen levels in perfused plasma samples, but they were never fell below the normal value range. FVIIIc levels decreased due to an adsorption mechanism or to FVIII carrier (vWf) changes. In addition, in all cases the vWf: Ag level was not modified. Therefore, we consider that the observed decrease in large multimers was not related to an adsorption mechanism but to an alteration of the vWf molecule. Our results suggest that high shear rate by itself did not induce changes on purified vWf, but that significant changes required a plasma substance (s).

It is known that the enzymatic activity of calpains derived from platelets is the main cause of the natural occurring fragmentation of vWf<sup>24, 25</sup>. The prevention of the most significant decrease of

large vWf multimers in our systems depended on the presence of EDTA in plasma. However, since other inhibitors of calpain activity, such as NEM, leupeptin and calpain inhibitor peptide did not have the same effect, we ruled out the role of calpain(s) as a possible mediator of this phenomenon. It has been proposed that granulocyte proteases are capable of decreasing the size of either the largest plasma vWf multimers<sup>26</sup> or unusually large vWf (ULvWf) multimers derived from cultured human umbilical vein<sup>27, 28</sup>. However, Phillips et al<sup>29</sup> suggested that granulocyte proteases do not process ULvWf multimers in vivo, because they are rapidly cleared in the presence of either heparin or soybean trypsin inhibitor, also in normal adult plasma that contains  $\alpha_1$ -antiplasmin and  $\alpha_2$ -macroglobulin. Tsai et al<sup>14</sup> suggested that the shear enhances the proteolytic susceptibility of vWf, because prior exposure of purified vWf to shear enhanced its cleavage adding cathepsin G or plasma cryosupernatant.

According to Tsai et al<sup>30</sup> it is doubtful that cathepsin G is responsible for the loss of large vWf multimers, considering that neither phenylmethylsulfonylfluoride nor heparin inhibited this physiologic effect; besides, leupeptin, NEM or iodoacetamide failed to inhibit the loss of large vWf multimers. In our studies, heparin, soybean trypsin inhibitor, aprotinin, PMSF and NEM (at high concentrations) did not prevent the loss of large vWf, so that we ruled out the action of granulocyte and cysteine proteases. Furthermore, our results differ from those of Tsai et al because we found that granulocytic proteases were not responsible for the loss of large vWf multimers.

The partial characterization showed that the substance(s) would fall within a plasma fraction MW  $> 200$  kD. Further studies are necessary to characterize and to identify the substance(s).

**Acknowledgements:** This study was supported by grants from CONICET and Fundación Alberto J. Roemmers. We are grateful to Dr. María E. Albertengo and her colleagues from Bromatology and Pharmacology National Institute, for provision of equipments and technical contributions and to Sandra Amoros and Juan Pablo Frontroth for their technical assistance. We appreciate the statistical advice provided by Dr. Norma Maugeri. We thank the Photography Department of the National Academy of Medicine and Verónica Rodríguez for word processing.



## Resumen

*Requerimiento de una fracción plasmática para la pérdida de los multímeros grandes del factor von Willebrand inducida por alta fuerza de cizallamiento*

La perfusión de suero, sangre entera y plasma citratado, a través de un sistema de tubuladura simple, provocó una pérdida significativa de los multímeros grandes del factor von Willebrand (vWf), sin disminución de los niveles de antígeno. La pérdida máxima de multímeros grandes, se observó realizando la perfusión a una fuerza de cizallamiento de  $15.000\text{ s}^{-1}$  durante 15 min. El agregado al plasma citratado de: heparina, aprotinina, inhibidor de tripsina soybean, fenilmetilsulfonilfluoruro, N-etilmaleimida, leupeptina o péptido inhibidor de la calpaína no previno la pérdida de los multímeros grandes. El agregado de EDTA cálcico la neutralizó parcialmente, mientras que la adición de EDTA sin calcio lo hizo totalmente. La perfusión de vWf purificado no evidenció la pérdida de los multímeros, pero ésta ocurrió cuando se agregó suero entero o una fracción de plasma de peso molecular (PM) mayor de 200kD. La actividad en esta fracción desapareció a  $\text{pH} < 6,8$ . Además, hemos encontrado que el enlace al subendotelio del vWf purificado diluido en suero dializado es menor a  $\text{pH} 7,2$  que a  $\text{pH} 6$ . Los estudios cromatográficos sugirieron que la pérdida de los multímeros grandes, inducida por la alta fuerza de cizallamiento, involucra una sustancia(s) plasmática de PM mayor de 200kD. Además, creemos que la calpaína y las proteasas granulocíticas o las cisteínoproteasas no parecen ser la sustancia en estudio.

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*A scientist lives with all reality. There is nothing better. To know reality is to accept it and eventually to love it. A scientist is in a sense a learned child. There is something of the scientist in every child. Others must outgrow it. Scientists can stay that way all their lives.*

Un investigador vive la realidad. No hay nada mejor. Apreciar la realidad es aceptarla y eventualmente quererla. Un investigador es en cierto sentido un niño instruido. Hay algo del investigador en cada niño. Otros deben madurar. Los investigadores pueden mantenerse así durante toda su vida.

George Wald (1906-1997)

...al recibir el Premio Nobel en 1967. *Nature* 1997; 387: 356