[Frontiers in Bioscience 10, 1050-1059, May 1, 2005]

ENZYMATIC HYDROLYSIS OF LUMINAL CORONARY GLYCOSIDIC STRUCTURES UNCOVERS THEIR ROLE IN SENSING CORONARY FLOW

Ivan Rubio-Gayosso [1], Alma Barajas-Espinosa [1], Jesús Castillo-Hernandez [1], Juan Ramiro-Diaz [1], Guillermo Ceballos [2], and Rafael Rubio [1]

[1] Depto. de Fisiologia, Facultad de Medicina UASLP. San Luis Potosi, SLP. Mexico 78210, [2] Seccion de Graduados, Escuela Superior de Medicina IPN. Mexico, DF. Mexico 11340

TABLE OF CONTENTS

1. ABSTRACT

Endothelial luminal glycocalyx (ELG) is a multifunctional complex structure made off of a diversity of glycosilated proteins, and glycosaminoglycans (GAG).Coronary ELG may participate as a sensor of coronary flow (CF) to induce inotropic and dromotropic effects. In isolated perfused guinea pig heart we tested the role of glycosidic groups of glycans bound to proteins and GAG of the ELG on CF-induced inotropic and dromotropic effects. To study the role of saccharide related groups of certain glycans, they were removed by selective enzyme hydrolysis or bound to a selective plant lectin. CF-induced positive inotropic and positive dromotropic control curves were obtained and the effects of intracoronary infusion of enzyme or lectin determined. The analyzed groups were as follow: 1) Fucosidase enzyme and Ulex europeasus lectin; hydrolysis and binding respectively (H&Br) to α-linked fucosyl related groups. 2). Endoglycanase-H and Lycopersicon esculentum (H&Br to N-linked beta-1,3GlcNAc related groups). 3) O-glycanase and Arachis hypogea (H&Br to O-linked beta-Gal1, 3GalNac related groups). 4) Sialidase and Maackia amurensis (H&Br to neuraminic acid related groups). In treatments 1-3 both. lectin and corresponding enzyme, equally depressed CF-positive dromotropic effects without affecting positive inotropic effects. In treatment 4 both lectin and enzyme equally depressed CF-positive inotropic effects without dromotropic effects. The differential role of GAG hyaluran or heparan groups on CF-positive inotropism and positive dromotropism respectively was shown. Infusing hyaluranidase removed hyaluran that solely inhibited CF-inotropism while removal of heparan with heparinase solely inhibited CF-dromotropism. Only the effects of hyaluronidase were reversed infusing hyaluronidate. Our results indicate glycans of ELG are elements of complex multimolecular sensors of coronary flow.

2. INTRODUCTION

Endothelial luminal glycocalyx is directly exposed to the blood stream and is a complex multimolecular structure composed by a large variety of glycosilated proteins and glycosaminoglycans. The endothelial luminal glycocalyx begins at the outer leaflet of the plasmalemma of vascular endothelial cell and extends into the vessel lumen to a depth estimated between 0.20 to 0.87 μm (1-9).

The luminal endothelial glycocalyx is multifunctional; has a differential permeability (4, 7, 10, 11), antithrombogenic and modulatory leukocyte-endothelial interaction properties (9, 12-14), participates in microvascular blood flow resistance (15) and as shown by us and others its glycosilated structures may translate hemodynamic stimuli into parenchymal function modulation (16-20). Target functions of the mechanical stimuli by coronary blood flow are: coronary resistance, myocardial oxygen and glucose consumptions, ventricular contraction, auricular-ventricular transmission and spontaneous ventricular rhythm (17, 18, 20-22). However, how physical stimuli are sensed, integrated and transduced, by endothelial cells into parenchymal function modulation is still unclear.

Diverse monosacharides constitute complex glycosidic chains (glycans) linked to proteins. Glycans are constituted by chain-linked; N-acetylglucosamine, N-acetylgalactosamine, galactose, mannose, fucose, xylose, neuraminic acid. Glycans are classified as N-linked and O-linked. In N-linked glycosidic chains a N-acetylglucosamine moiety is attached to a asparagine protein residue. In O-linked glycans commonly a N-acetylgalactosamine moiety is attached to a serine or threonine or hydroxylisine protein residues. The large glycosaminoglycans hyaluronic acid and heparinate are also important constituents of the luminal glycocalyx. Hyaluronic acid is not covalently linked to protein while heparinate it is (23).

We reasoned since coronary endothelial luminal glycans complexed to proteins contain; fucose (fuc), N-acetylglucosamine (GlcNac), N-acetylgalactosamine (GalNac) and neuraminic/sialic acid (NeuAc) moieties. These moieties can be hydrolyzed from the glycan chain by their respective glycanases; alpha-.fucosidase, endoglycanase-H, O-glycanase and sialidase. If certain glycans participate in flow-sensing, it would be expected that separate intracoronary administration of each glycanase the function-coronary flow curve would be altered. Furthermore, administration of a lectin that bind to a defined saccharide after its hydrolisis by the glycanase should be without effect, because binding sites have been removed. In addition, the participation of two densely distributed glycosaminoglycans; heparan sulfate and hyaluran on coronary flow-induced effects was tested upon their enzymatic removal. Our results further support the hypothesis that proteoglycans and glycosaminoglycans of the coronary endothelial luminal glycocalyx participate in flow sensing.

3. MATERIAL AND METHODS

3.1. Experimental model

Isolated, perfused guinea pig heart model was utilized as previously (17, 18, 20, 22). Guinea pigs 350-400 g were anesthetized with pentobarbital (50 mg/ml IP) and heparin (500 U IP). Under artificial respiration, the chest was opened, heart removed, placed into ice-cold physiological saline solution and ascending aorta cannulated. Retrograde non-recirculating constant flow perfusion with Krebs-Henseleit solution (K-H) was performed. Initially, for a period of 5 min at a flow of 15 ml/min. Thereafter coronary flow was set at 10 ml/min for a 25 min period of stabilization. All connective non-cardiac tissue was removed. K-H solution was equilibrated with 95% O_2, 5% CO_2, pH 7.4 at 37° C and contained glucose 5mM and pyruvate 5mM. The mean coronary perfusion pressure at a flow of 10 ml/min was 49 $\pm$ 1.4 mmHg. This value remained constant throughout all the maneuvers of the experiment. Perfusion pressure at different coronary flows also were not affected by the experimental manipulations.

Hearts were electrically stimulated by placing in the right atria, a pair of electrodes 2 mm apart and applying square pulses of 2 msec of duration, 4.5 ± 0.1 Hz and a voltage 2-fold the threshold. To measure auricular-ventricular (A-V) delay an electrode was placed in left atria and other in the ventricular apex. Electrodes were connected to an oscilloscope synchronized with the electric stimulator. Atrial delay between stimulus application and left atria potential was 22.6 ± 1 msec and was not affected by any of the manipulations/agents utilized. A-V delay (msec) was determined as the time elapsed between the application of stimulus and the initiation of the QRS wave. As we and others have shown A-V delay changes are due to time delay solely in the auricular-ventricular nodal area (22). A-V delay changes were expressed as percent of the A-V delay value (100 %) determined for each heart at a coronary flow of 16 ml/min under control condition. The mean control A-V delay value was 99.2 ± 3 msec.

To estimate ventricular contraction, through mitral valve a latex fluid-filled balloon was introduced into left ventricle and connected to a pressure transducer. The latex balloon was inflated to a 10 mmHg of diastolic pressure. Left ventricular developed pressure was taken as an index of ventricular contraccion and an inotropic effect was calculated as percent of the contraction amplitude developed at 16 ml/min in control condition

3.2. Effects of coronary flow in ventricular contracccion and A-V delay

Coronary flow was initially set at 5 ml/min, thereafter, step increases of 1 ml/min each were performed up to a maximal level of 16 ml/min and each flow step lasted 2 min. ventricular contraccion and A-V delay were measured at each flow level to generate function-coronary flow curves. All enzymes or lectins treatments were made after control curves and each heart was its own control.

3.3. Infusion of glycan hydrolyzing enzymes and lectins

Enzymes utilized that hydrolyze different specific carbohydrate groups are shown in table 1. Also shown are the corresponding lectins with binding affinity for these different specific saccharide groups. In table 1 are shown the concentrations of enzymes and lectins infused and their corresponding substrates. Lectins and enzymes were extensively dialyzed (cutoff MW 10,000) against K-H before used. After control function-coronary flow curves were obtained, coronary flow was set at 6 ml/min and either enzyme or lectin were infused. To assure that during the infusion period either lectin or enzyme remain confined

Table 1. LECTINS, ENZYMES, THEIR CORRESPONDING AFFINITIES AND CONCENTRATIONS UTILIZED.

Lectins 10 μg/ml	Glycosidic affinity	Enzymes (U/ml)	Glycosidic substrate
Ulex europeaus I (Furze gorse)	alpha-Fuc	alpha-Fucosidase (0.5) (bovine kidney)	Fucα1—3,4R_n
Lycopersicon esculentum (Tomato)	(beta1,4GlcNAc)$_4$ >(beta1,4GlcNAc)$_3$	Endoglycanase-H (0.2) (Streptomyces plicatus)	GlcNAcbeta1,4GlcNAc—R_1
Arachis hypogea (Peanut)	Ga-beta1GalNAc >alpha and beta-Gal	O-Glycanase™ (0.1) (S. pneumoniae)	Ga-lbeta1—3GalNAc-alpha1—R_2
Maackia amurensis (Amur maackia)	NeuAc	Sialidase (0.2) (Arthrobacter ureafaciens)	NeuAc-alpha2—3,6,8R_n

GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; Gal, galactose; Fuc, fucose; NeuAc, N-acetylneuraminic acid; R_1: GlcNAc, GlcNAc—Fuc or Asn; R_2: Ser or Thr; R_n: any glycosidic residue.

intravascularly, they were infused for short periods of time (30 sec), followed by a washout period (2 min) and re-infused again (30 sec). This cycle was repeated six times, coronary flow was set a 10 ml/min (3min) and a experimental function-coronary flow curve was performed, To test that the enzyme-induced change on the function-coronary flow curve was due to removal of its respective glycosidic substrate, a third curve was performed after the infusion of the corresponding matching lectin expecting no effect by the lectin since its substrate have been removed. As negative controls, enzymes and lectins, denatured by heating at 100° C for 5 min, were intracoronarily infused as described above and function-coronary flow curves were performed. These heat denatured agent were ineffective. In addition, to test for the stability of the experimental set up the same protocol was followed infusing K-H without enzymes or lectins and function-coronary flow curves determined. This curve was the same as the control curve.

3.4 Infusion of glycosaminoglycan hydrolyzing enzymes and glycosaminoglycan restitution

Glycosaminoglycan hydrolyzing enzymes were extensively dialyzed (cutoff 10,000 MW) against K-H. Hyaluronidase 0.5 U/ml or heparinase 0.5 U/ml were infused with the same protocol described above and experimental function-coronary flow curve determined. Thereafter, in an attempt to restitute glycosaminoglycans (24, 25) hydrolyzed by these enzymes, heparan sulfate or hyaluronic acid or chondroitin sulfate were infused. Flow was set to 6 ml/min, and either substance (10 μg/ml) alone or the combinations of hyaluranic acid plus chondroitin sulfate or heparan sulfate plus chondroitin sulfate or the mixture of the three substances were infused intracoronarily for 6 minutes. After a 3 min washout a third function-coronary flow curve was done.

3.5 Infusion of a mixture of enzymes

Control function-coronary flow curves were determined. Thereafter, enzymes were infused at the same concentrations and following the same protocol just described. First; a mixture of O-glycanase, fucosidase, and endoglycanase-H was infused. This was followed by infusion of a mixture of hyaluronidase and heparinase. Thereafter, the experimental curves were determined.

3.6 Statistics

Data are shown as a mean ± SE. Each heart was its own control. Comparisons among treatments were made using ANOVA of repeated measures. Individual comparisons between treatments were performed by Bonferroni's posttest. A p value ≤ 0.05 was accepted as statistically significant difference.

4. RESULTS

4.1. Differential effects of enzymes on the positive dromotropic and inotropic effects induced by coronary flow

It is well established that in the isolated perfused guinea pig heart (17, 18, 22) under control conditions, step increases of coronary flow increases ventricular contraccion and decreases A-V delay with time constants of ~30 sec and ~15 sec, respectively and the plateau value is reached at ~45 sec and ~35 sec respectively. The plateau levels are maintained as long as the coronary flow level is maintained. A second and third control curves show reproducibility.

4.1.1. Enzymes and "matching" lectins affecting solely A-V delay-coronary flow curve

Infusion of lectin Ulex europeaus I (binding preferentially to alpha-L-fucose related groups) on A-V delay-coronary flow curve was tested. Intracoronary Ulex europeaus caused also an upward displacement of the A-V delay-coronary flow curve, as compared to the control curve (figure 1A). In a separate group of hearts intracoronary infusion of the enzyme alpha-fucosidase caused the same negative dromotropic effect (figure 1B), however, infusion of Ulex europeasus after alpha-fucosidase was without additional effect (figure 1B).

Similarly, previously (28) we show that a series of lectins among them Lycopersicum esculentum (binding preferentially to beta1,4GlcNAc related groups) and Arachis hypogea (binding preferentially to Galbeta1GalNac related groups) exerted a negative dromotropic effect; an upward displacement of the A-V delay-coronary flow curve (18). In the present publication intracoronary infusion of the corresponding hydrolyzing enzymes; endoglycanase-H (figure 2A) and O-glycanase (fig 2B) both caused also an upward displacement of the A-V delay-coronary flow curve, as compared to the control curve, however, after enzyme infusion administration of the corresponding lectin were without additional effect (figures 2A and 2B). These results suggest that after an enzyme has hydrolyzed its glycosidic substrate there are not available binding sites for the lectin as a result there is not an effect
None of these particular enzymes nor the lectins affected the ventricular contraction-coronary flow curve (not shown).

4.1.2. Enzyme and "matching" lectin affecting solely ventricular contraction-coronary flow curve

Maackia amurensis (binding to NeuAc related carbohydrates) and its corresponding enzyme,

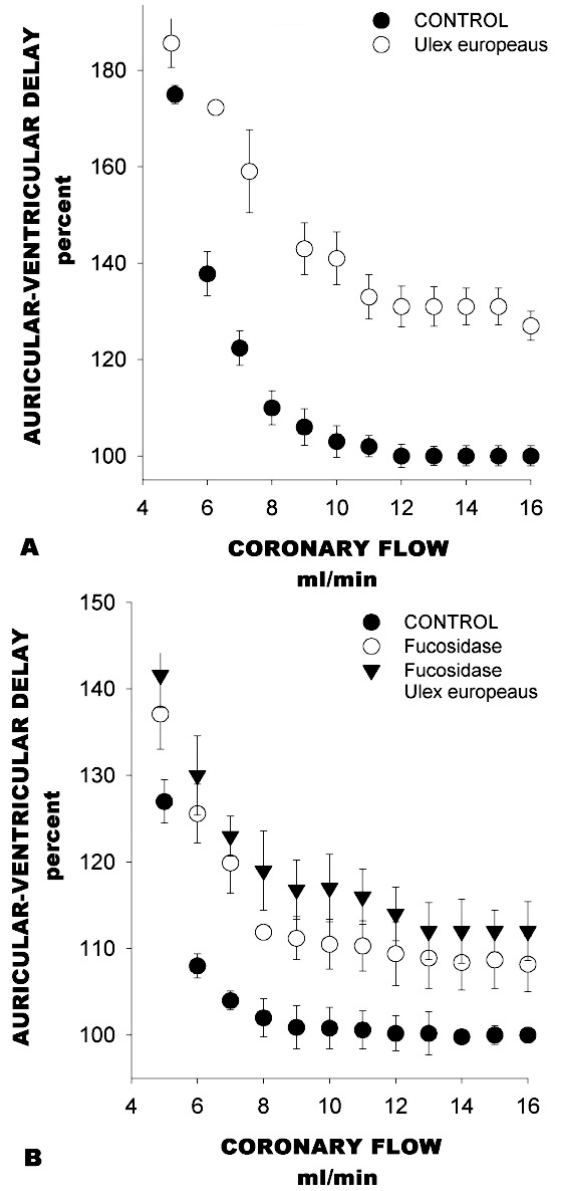

Figure 1. Effects of the Ulex europeaus and its matching glycosidic hydrolyzing enzyme Fucosidase on the coronary flow-induced positive dromotropic effect. **A**: Control (solid circles) and an upward displacement of the curve following lectin infusion (open circles). **B**: controls (solid circles), upward displacement of the curve following infusion of Fucosidase (open circles). After treatment with fucosidase infusion of Ulex europeaus was without additional effect (triangles) indicating alpha-L-Fucose functional groups have been removed by the enzyme (triangles). Differences between circles and triangles was not significative. Values are expressed as mean ± SE (each treatment, n=6 hearts). * P<0.05.

Sialidase, caused an upward displacement of the ventricular contraction-coronary flow control curve (figures 3A, 3B). After the enzyme was infused and its positive inotropic effect determined, intracoronary infusion of Maackia amurensis did not have any additional effect (figure 3B, triangles). These results suggest that after sialidase have hydrolyzed neuraminic acid residues there are not available binding sites for the matching Maackia amurensis as a result there is not an additional effect. Neither Maackia amurensis nor sialidase affected the A-V delay-coronary flow curve (not shown).

4.2. Differential effects of coronary infused glycosaminoglycan hydrolyzing enzymes and respective glycosaminoglycans on ventricular contraction- and A-V delay-coronary flow curve

Hyaluronidase infusion caused a downward displacement of the ventricular contraction-coronary flow curve (figure 4A). This depression of the coronary flow-induced inotropism was restored completely following infusion of the mixture hyaluran and chondroitin sulfate (figure 4A). It was not restored upon infusion of hyaluran alone or chondroitin sulfate alone. Neither hyaluronidase nor hyaluran, chondroitin nor hyaluran plus chondroitin affected A-V delay-coronary flow curve (not shown).
Heparinase infusion caused an upward displacement of the A-V delay-coronary flow curve (figure 4B). This depression of coronary flow-induced dromotropism caused by heparinase was not restored following infusion of either a mixture of heparan sulfate and chondroitin sulfate (figure 4B), or heparan sulfate alone, or of the mixtures heparan sulfate/hyaluran, or heparan sulfate/hyaluran/chondroitin sulfate (not shown). These results contrasted with the effects of hyaluronidase. Neither heparinase nor heparan sulfate nor chondroitin sulfate affected ventricular contraction-coronary flow curve (not shown).

4.3. The effects of enzymes mixtures on the ventricular contraction- and A-V delay-coronary flow effects.

An increase in coronary flow (dF) causes an increase in ventricular contraction (dP) and a decrease A-V delay (-dT). The first derivative of the ventricular contraction-coronary flow curve (dP/dF) or that of A-V delay-coronary flow curve (-dT/dF) could be adopted to define the "response" at a given coronary flow. This "response" could be compared with another under different experimental conditions. Since each of the enzymes utilized acted on a different group of saccharides and with the exception of sialidase, they caused a depressive effect, we decided to explore the possibility that hearts, infused with a mixture of only the depressive enzymes, resulted in first derivative values lower than controls and ideally approaching zero. dP/dF and -dT/dF at various coronary flows under control and after enzyme mixture infusion are shown in figure 5.

–dT/dF control (full squares) was highest at low coronary flows and decreased approaching zero as coronary flow increased. In contrast –dT/dF after enzyme mixture treatment (squares) was not statistically different from zero in all the coronary flow range.

dP/dF control (full circles) at low flow was small, then reached a maximun, thereafter, it gradually decreased approaching zero at a flow value of ≅ 15 ml/min. dP/dF values after enzyme treatment (circles) at flows within the

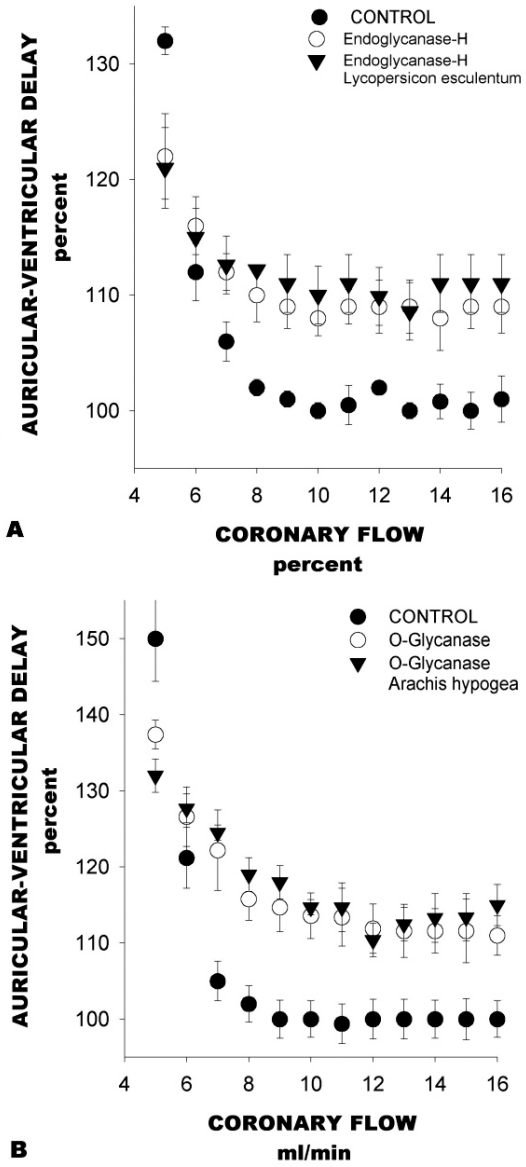

Figure 2. A: Effects of the glycosidic hydrolyzing enzyme Endoglycanase-H on coronary flow-induced positive dromotropic effect. Controls (solid circles). Infusion of Endoglycanase-H caused an upward displacement of the curve (open circles). Following Endoglycanase-H treatment infusion of matching lectin Lycopersicon esculentum was without additional effect (triangles) indicating GlcNAcbeta1,4GlcNAcbeta1 functional groups have been removed by the enzyme. **B:** Effects of the glycosidic hydrolyzing enzyme O-Glycanase on the coronary flow-induced positive dromotropic effect. Controls (solid circles), Infusion of O-Glycanase caused an upward displacement of the curve (open circles). Infusion of lectin Arachis hypogea following O-glycanase treatment infusion was without additional effect (triangles) indicating Galbeta1,4GalNAc functional groups have been removed by the enzyme. Values are expressed as mean ± SE (each treatment, n=6 hearts). * $P<0.05$.

range of 5 to 9 ml/min were significantly lower than control (full circles) and as flow increased this difference was reduced.

5. DISCUSSION.

Our results show that intracoronary infusion of any of the hydrolyzing enzymes; alpha-Fucosidase or Endoglycanase-H or O-Glycanase or the "matching" lectins; Ulex europeaus or Lycopersicum esculentum or Arachis hypogea, selectively depressed the coronary flow-induced positive dromotropism. Furthermore, following an enzyme treatment, infusion of lectin that preferentially binds to the hydrolyzed saccharide or related carbohydrates, was without effect. These results suggest that enzymatic removal or lectinic binding of either alpha-L-fucose or beta1,4GlcNAc or Gal-beta1GalNac groups in certain luminal coronary glycocalyx structures selectively depress A-V delay-coronary flow curve; an effect likely exerted at the level of capillaries adjacent to A-V nodal myocytes (18, 22).

On the other hand, coronary flow-induced positive inotropism was selectively increased by infusion of the enzyme sialidase or the lectin Mackia amurensis which hydrolyze or bind respectively to sialic acid related groups. Furthermore, following enzyme treatment, infusion of the lectin Mackia amurensis was without effect showing specificity of neuraminic acid related groups on inotropic function. These results indicate that enzymatic removal or lectinic binding of neuraminic acid groups bound to certain luminal coronary glycocalyx structures selectively enhance coronary flow-induced positive inotropism; an effect likely exerted at the level of capillaries adjacent to contractile ventricular myocytes.

Glycosaminoglycans also participate in the function-coronary flow regulated processes because their enzymatic hydrolysis by; hyaluronidase or heparinase, which hydrolyze hyaluronidate and heparinic luminal groups differentially, depress the coronary flow-induced positive inotropism and dromotropism respectively. Infusion of hyaluronidate/chondroitin groups after their enzymatic removal restored the ventricular contraction-coronary flow effect to control implying their reincorporation into the glycocalix. On the other hand, infusion of heparinate after their enzymatic removal did not restored the A-V delay-coronary flow effect to control.

All these results indicate that there is a differential anatomical distribution of glycans composition in the coronary endothelial luminal glycocalyx. The differential pattern found suggest that monosaccharides related groups that compose glycans involved in coronary flow translation into function show heterogeneity between the endothelium at A-V nodal area and ventricular contractile myocardium. The result being different molecular structures participate in coronary flow-induced modulation of specific cardiac functions; A-V delay or ventricular contraction.

Adoption of the first derivative of the ventricular contraction-coronary flow curve (dP/dF) or that of A-V delay-coronary flow curve (-dT/dF) to define "response" at

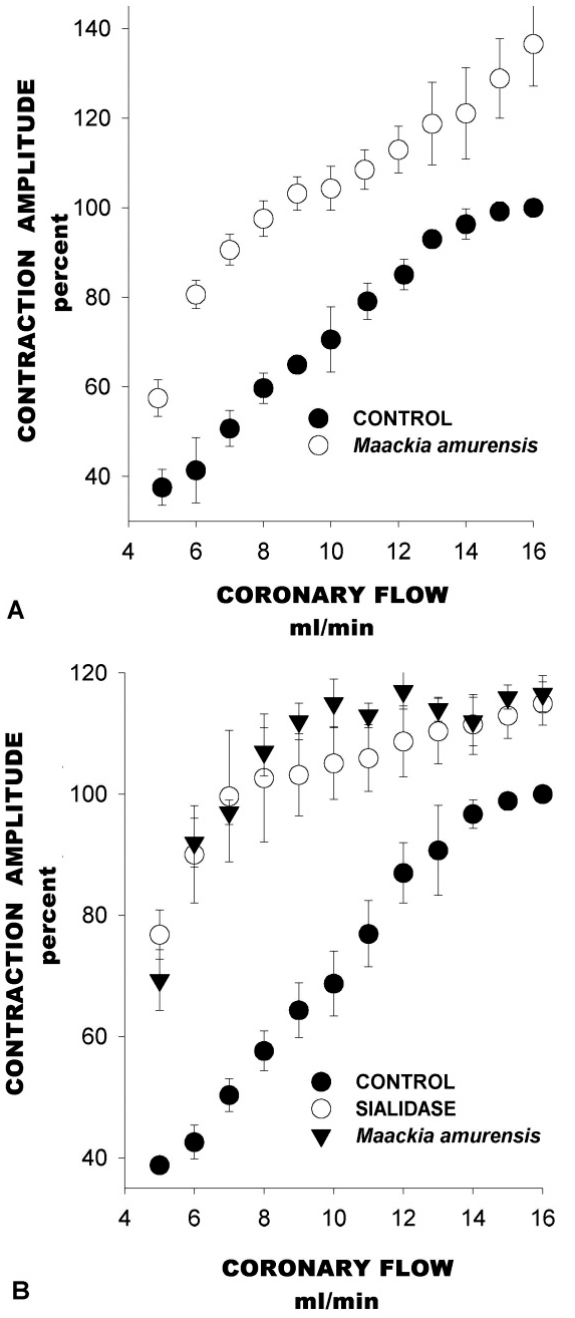

Figure 3. Effects of lectin Maackia amurensis and the matching glycosidic hydrolyzing enzyme sialidase on the coronary flow-induced positive inotropic effect. **A**: Control (solid circles) an upward displacement of control curve was produced by infusion of Maackia amurensis (open circles). **B**: controls (solid circles). Infusion of Sialidase caused an upward displacement of curve (open circles). Following sialidase treatment Infusion of Maackia amurensis was without additional effect (triangles) indicating neuraminic acid functional groups have been removed by the enzyme. Differences between circles and triangles was not significative. Values are expressed as mean ± SE (each treatment, n=6 hearts). * $P<0.05$.

a given coronary flow, show that in hearts infused with a mixture of the depressive enzymes the "response" was depressed as compared to control. –dT/dF after enzyme treatment was statistically equal to zero in all the flow range; i. e. flow insensitive. dP/dF values after enzyme treatment at flows within the range of 5 to 9 ml/min were lower than control; i. e. less flow sensitive, but, at higher flows they were equal. In contrast to –dT/dF, dP/dF after enzyme treatment was not reduced to zero. This may indicate that coronary flow exerts its inotropic effect via two stimuli: a flow effect; glycan/glycosamineglycan dependent, and a pressure-stretching effect; not glycosidic dependent, on the endothelial membrane (26-28).

Our results support the hypothesis that certain coronary endothelial luminal glycosidic structures participate in flow-sensing. If a certain glycosidic chain is site hydrolyzed by an enzyme or at this site a lectin is bound, either alteration will equally modify the function-coronary flow curve. Our results suggest that specific glycan structures and glycosaminoglycans of luminal endothelial glycocalyx form part of the molecular complex for transduction of coronary flow into parenchymal function regulation.

The irreversibility of the Lectinic and enzymatic treatments on function-coronary flow are likely due to selective molecular targeting rather than an effect on the viability of the isolated perfused guinea pig heart because if this was the case, one would expect a decay of all functions and not a differential effect on one without an effect on the other function.

The effects of infused lectins and enzymes were restricted to actions on luminal surface structures of the coronary endothelium because these agents during infusion remain intravascularly, Pore theory modeling studies in dog myocardium (29), predict that the infused lectins and enzymes (ranging from 25 kDa to 70 kDa) require close to one hour of continued infusion to cross the endothelial wall in a significant amount (6, 30). Nevertheless, in order to prevent or minimize transendothelial diffusion of these agents they were infused intermittently; 30 seconds infusion followed by 2 minutes washout up to six cycles. Lectins when intracoronarily infused are well known to bind discriminately with high affinity solely to saccharides of luminal endothelial proteins, as a result are not likely to diffuse transendothelially (1, 18, 30-35).

It is evident that A-V delay-coronary flow as compared to ventricular contraction-coronary flow respond differently to the manipulations we have used. Furthermore, another ventricular function; spontaneous ventricular rhythm, that reflects the discharge of a dominant pace-maker in Purkinje fibers within the Purkinje-muscular myocyte region (36, 37), is also coronary flow stimulated and in contrast to contraction, is inhibited by infusion of lectins (18). These three different coronary flow modulated responses reflect functions of three different types of myocyte, which are in distinct anatomical locations on the heart. Each type of myocyte has distinct functional and structural properties and they are

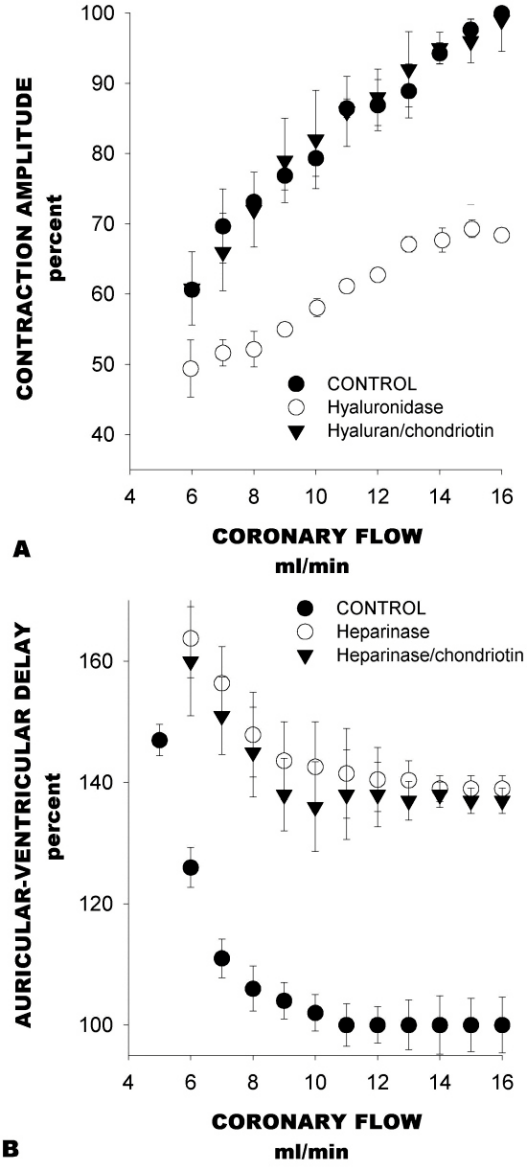

Figure 4. Differential roles of glycosaminoglycans; hyaluronidate and heparinate on the coronary flow-induced inotropism and dromotropism respectively. **A**: Coronary flow-induced positive inotropic effect; controls (solid circles). Following infusion of hyaluronidase caused a downward displacement of the curve (open circles). Following hyaluronidase treatment Infusion of hyaluran/chondroitin sulfate fully restored the curve back to control (full triangles). This indicates that hyaluran/chondroitin sulfate functional groups were reintroduced to the glycocalyx after their enzymatic removal. **B**: Coronary flow-induced positive dromotropic effect; controls (solid circles). Infusion of heparinase caused an upward displacement of the curve (open circles). Following heparinase treatment infusion of heparan sulfate failed to restore the curve back to control (closed triangles). This indicates that heparan sulfate functional groups cannot be reintroduced to the glycocalyx after their enzymatic removal.

closely apposed to their own endothelial capillary network (38), thus, forming a parenchymal-endothelial functional junction that responds to coronary flow.

The functional distinctiveness of each parenchymal-endothelial junction could be a consequence of: a different myocyte phenotype and/or an endothelial cell type with a chemically, structurally and functionally different intravascular luminal surface. This conclusion is derived from our observations showing that the coronary flow actions upon these three functional regions were affected differentially by lectins, enzymes and antibodies (18). Additional support that endothelial cells from various preparations are heterogeneous and that luminal glycosilated structures participate in flow-induced paracrine release of active substances like nitric oxide, has been shown. In isolated canine femoral artery flow-induced release of nitric oxide is blocked by pretreatment with hyaluronidase (39). In bovine aortic endothelial cells in culture flow-induced release of nitric oxide is blocked by pretreatment with heparinase (40). Finally, in saline perfused rabbit mesenteric arteries flow-induced release of nitric oxide is blocked by pretreatment with sialidase (41). Three different endothelial preparations, each require a different glycosidic hydrolyzing enzyme to block the same response to flow; nitric oxide release.

Lectins upon binding to specific glycosidic residues form very stable complexes (28, 30, 42). As we previously proposed (18, 20) lectins may cause cross-linking between neighboring intraluminal glycoproteins by binding to specific glycosidic groups. This lectin-induced glycoprotein polymerization could make these proteins less susceptible to flow deforming forces, resulting in inhibition of A-V delay-coronary flow effect. Another alternative could be simply that lectins by engulfing glycosidic groups prevent a flow-induced interaction with other proteins. Whatever the mechanism; flow-induced glycoprotein deformation or a glycosidic chain-protein interaction or both, could result in either an endothelial-mediated stimulatory mechanism of function or in a reduction of an endothelial-mediated inhibitory mechanism of function which expresses as a stimulation. However, the two opposing mechanisms may co-exist, particularly in the ventricular contraction-coronary flow response.

If the two opposing mechanisms co-existed we could explain the effects of maackia amurensis, that binds to sialic acid residues and sialidase that removes them, both agents causing an upward displacement of the ventricular contraction-coronary flow curve. Neuraminic acid containing glycans in the lumen of capillaries adjacent to contractile ventricular myocytes when acted upon by coronary flow may trigger endothelial-mediated negative inotropic mechanisms. This mechanism upon removal or binding of sialic acid residues could express as positive. In fact, Pohl, Herlan, Huang and Bassenge (41) in small rabbit mesenteric arteries reported that treatment with neuraminidase inhibited a flow induced relaxation that expressed as an enhanced contraction. Our findings imply that besides a negative inotropic mechanism a flow-

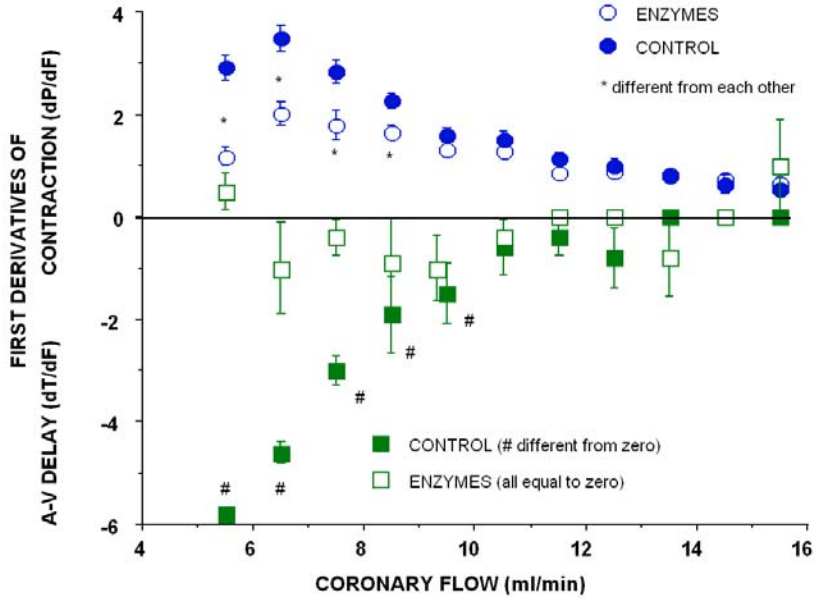

Figure 5. Effects of a mixture of enzymes; alpha-Fucosidase, Endoglycanase-H, O-Glycanase, Hyaluronidase and Heparinase on the coronary flow-stimulatory inotropic (dP/dF) and domotropic (-dT/dF) effects. Upper portion of the graph corresponds to dP/dF (mmHg/ml). Control (full circles). After treatment with enzyme mixture (open circles). From a coronary flow of 5 to 9 ml/min the experimental values were lower than the corresponding controls. Lower portion of the graph corresponds to –dT/dF (msec/ml). Control (full squares). After treatment with enzyme mixture (open squares). Control; as coronary flow increased –dT/dF gradually decayed toward zero. In contrast, after enzyme treatment all -dT/dF values at all flow values were not different from zero.

triggered positive inotropism should exist i. e. that a proper lectin treatment should inhibit the coronary flow-induced inotropism. We previously reported that simultaneous infusion of two or three lectins caused this effect even though individually each lectin had no action (18), furthermore, we now show that removal of luminal hyaluronidate depresses ventricular contraction-coronary flow curve.

The glycosaminoglycans; hyaluronidate and heparan are also participants of coronary flow sensing complex and are also function-specific. Our studies show that hyaluronidate participates in the transduction of coronary flow solely into inotropism, while heparan into dromotropism, i. e. site and function specific. The function targeting of these glycosaminoglycans is further illustrated by heparinase inhibiting the coronary flow-induced cardiac glycolytic flux with no inotropic effect, while hyaluronidase has a inotropic effect without effect on cardiac glycolytic flux (20). Heparinic groups have also been implicated in determining the permeability properties of some capillary beds (10).

Hyaluronidate forms a mesh with other structures within the luminal endothelial glycocalyx through non-covalent binding (23, 25). Thus, we anticipated that after its removal with hyaluronidase, intracoronarily infusion of hyaluran plus chondroitin sulfate should restore the depressed Myc-coronary flow effect, indicating that hyaluran have been restituted to the luminal endothelial mesh. In contrast, heparan sulfate is found forming covalent bonds (39) and its related-function was not expected to be restored simply adding heparan sulfate, our results confirmed this prediction. Heparinic and hyaluronidate residues are known to be present in high concentrations at the endothelial surface of the glycocalyx (23, 33).

In summary, our results further support the hypothesis that proteoglycans and glycosaminoglycans of the endothelial luminal glycocalyx form part of diverse multimolecular coronary flow sensors involved in modulation of specific functions of the subjacent parenchymal cardiac cells. On the other hand, other studies show that abluminal integrins and laminins receptors also participate in flow sensing (16, 18, 19, 26). These findings and ours indicate that at a given site of the vascular tree endothelial luminal structures, endothelial abluminal proteins and cellular signaling molecules together, constitute diverse flow sensing multimolecular complexes that are function specific.

6. ACKNOWLEDGEMENT.

This work was supported by Consejo Nacional de Ciencia y Tecnologia Grant G34998-N.

7. REFERENCES

1. Ausprunk D. H, C. L. Boudreau & D. A. Nelson: Proteoglycans in the microvasculature I. Histochemical localization in microvessels of the rabbit eye. *Am J Pathol* 103, 353-366 (1981)

2. Constantinescu A. A, H. Vink & J. A Spaan: Elevated capillary tube hematocrit reflects degradation of endothelial cell glycocalyx by oxidized LDL. *Am J Physiol Heart Circ Physiol* 280, H1051-H1057 (2001)
3. Desjardins C & B. R. Duling: Heparinase treatment suggests a role for the endothelial cell glycocalyx in regulation of capillary hematocrit. *Am J Physiol Heart Circ Physiol* 258, H647-H654 (1990)
4. Henry C. B. S, & B. R. Duling: Permeation of the luminal capillary glycocalyx is determined by hyaluran. *Am J Physiol Heart Circ Physiol* 277, H508-H514 (1999)
5. Simionescu M, N. Simionescu, J. E. Silbert & G. E. Palade: Differentiated microdomains on the luminal surface of the capillary endothelium II. Partial characterization of their anionic sites. *J Cell Biol* 90, 614-621 (1981)
6. Simionescu M, N. Simionescu & G. E. Palade: Differentiated microdomains on the luminal surface of the capillary endothelium: Distribution of lectin receptors. *J Cell Biol* 94, 406-413 (1982)
7. Vink H & B. R. Duling: Identification of distinct luminal domains for macromolecules, erythrocytes, and leukocytes within mammalian capillaries. *Circ Res* 79, 581-589 (1996)
8. Vink H & B. H. Duling: Capillary endothelial surface layer selectively reduces plasma solute distribution volume. *Am J Physiol Heart Circ Physiol* 278, H285-H289 (2000)
9. Zhao Y, S. Chien & S. Weinbaum: Dynamic contact forces on leukocytes microvilli and their penetration of the endothelial glycocalyx. *Biophys J* 80, 1124-1140 (2001)
10. Huxley V. H & D. A. Williams: Role of a glycocalyx on coronary arteriole permeability to proteins: evidence from enzyme treatments. *Am J Physiol Heart Circ Physiol* 278, H1177-H1185 (2000)
11. Osterloh K, U. Ewert & A. R. Pries: Interaction of albumin with endothelial cell surface. *Am J Physiol Heart Circ Physiol* 283, H398-H405 (2002)
12. Araki H, J. Muramoto, K. Nishi, M. Jougasaki & M. Inoue: Heparin adheres to the damaged arterial wall and inhibits its thrombogenicity. *Circ Res* 71, 577-584 (1992)
13. Mulivor AW & H. H. Lipowsky: The role of glycocalyx in leukocyte-endothelial cell adhesion. *Am J Physiol Heart Circ Physiol* 283, H1282-H1291 (2002)
14. Sabri S, M. Soler, C. Foa, A. Pierres, A-M. Benoliel, P. Bongrand: Glycocalyx modulation is a physiological means of regulating cell adhesion. *J Cell Sci* 113,1589-1600 (2000)
15. Secomb T. W, R. Hsu & A. R. Pries: A model for red blood cell motion in glycocalyx-lined capillaries. *Am J Physiol Heart Circ Physiol* 274, H1016-H1022 (1998)
16. Gloe T & U. Pohl: Laminin binding conveys mechanosensing in endothelial cells. *NIPS* 17: 166-169, 2002.
17. Gonzalez-Castillo C, R. Rubio & T. Zenteno-Savin: Coronary flow-induced inotropism is modulated by binding of dextrans to the endothelial luminal surface. *Am J Physiol Heart Circ Physiol* 284, H1348-H1357 (2003)
18. Rubio R & G. Ceballos: Role of the endothelial glycocalyx in dromotropic, inotropic, and arrythmogenic effects of coronary flow. *Am J Physiol Heart Circ Physiol* 278, H106-H116 (2000)
19. Shyy JY-J & S. Chien: Role of integrins in endothelial mechanosensing of shear stress. *Circ Res* 91, 769-775 (2002)
20. Suarez J & R. Rubio: Regulation of glycolytic flux by coronary flow in guinea pig heart. Role of vascular endothelial cell glycocalyx. *Am J Physiol Heart Circ Physiol* 261, H1994-H2000 (1991)
21. Gregg D. E: Effect of coronary perfusion pressure or coronary flow on oxygen usage of the myocardium. *Circ Res* 13, 497-500 (1963)
22. Rubio R, G. Ceballos G & J. Suarez: Coronary flow stimulates auricular-ventricular transmission in the isolated perfused guinea pig heart. *Am J Physiol Heart Circ Physiol* 269, H1177-H1185 (1995)
23. Varki A, R. Cummings, J. Esko, H. Freeze, G. Hart & J. Marth: Essentials of glycobiology. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, New York. (1999)
24. Gandley R. E, M. K. McLaughlin, T. J. Koob, S. A. Little SA & L. J. McGuffee: Contribution of chondroitin-dermatan sulfate-containing proteoglycans to the function of rat mesenteric arteries. *Am J Physiol Heart Circ Physiol* 273, H952-H960 (1997)
25. Gill P. J, J. Adler, C. K. Silbert & J. E. Silbert: Removal of glycosaminoglycans from cultures of human skin fibroblats. *Biochem J* 194, 299-307 (1981)
26. Davies P. F: Flow–mediated endothelial mechanotransduction *Physiol Rev* 75, 519-560 (1995)
27. Praetorius H. A & K. R. Spring: Bending the MDCK cell primary cilium increases intracellular calcium. *J Membrane Biol* 184, 71-79 (2001)
28. Praetorius J, P. Backlund, A. L. Yergey & K. R. Spring: Specific lectin binding to beta1 integrin and fibronectin on the apical membrane of Madin-Darby canine kidney cells. *J Membrane Biol* 184, 273-281 (2001)
29. Taylor A. E & D. N. Granger: Exchange of macromolecules across the microcirculation. In Handbook of Physiology. The Cardiovascular System. Microcirculation. Bethesda, MD: Am. Physiol. Soc. sect 2, vol. IV, part 1, chapt. 11, (1984)
30. Stein O, T. Chajek T & Y. Stein: Ultrastructural localization of Concanavalin A in the perfused rat heart. *Lab Invest* 35, 103-110 (1976)
31. Ghitescu L, & M. Robert: Diversity in unity: The biochemiacl composition of th endothelial cell surface varies between the vascular beds. *Microscopy Res Tech* 57, 381-389 (2002)
32. Holthöfer H, I. Virtanen, A-L. Kariniemi, M. Hormia, E. Linder & A. Miettinen: *Ulex europaeus* I lectin as a marker for vascular endothelium in human tissues. *Lab Invest* 47, 60-66 1986
33. Pino R. M: Pertubation of the blood-retinal barrier after enzyme perfusion. A cytochemical study. *Lab Invest* 56, 475-480 (1987)
34. Porter G. A, G. Palade & A. J. Milici: Differential binding of the lectins *Griffonia simplicifolia I* and *Lycopersicon esculentum* to microvascular endothelium: organ-specific localization and partial glycoprotein characterization. *Eur J Cell Biol* 51, 85-95 (1990)
35. Schnitzer J. E, C-P. J. Shen & G. E: Palade: Lectin analysis of common glycoproteins detected on the surface of continuos microvascular endothelium in situ and in culture: identification of sialoglycoproteins. *Eur J Cell Biol* 52, 241-251 (1990)
36. Berenfeld E & J. Jalife: Purkinje muscle reentry as a mechanism of polymorphic ventricular arrytyhmias in a 3-

dimensional model of the ventricles. Circ. Res. 82, 1063-1077 (1998)
37. Carmeliet E & J. Vereecke: Electrogenesis of the action potential and automaticity. In: Handbook of Physiology. The cardiovascular System. The heart. Betehsda, MD: Am. Physiol. Soc. sect 2, vol. I, chapt 7, (1979)
38. McNutt N. S, & D. W. Fawcet: Myocardial ultrastructure. In: The mammalian myocardium. Ed. Langer GA, and Brady AJ. New York: Wiley, Pages. 1-49 (1974)
39. Mochizuki S, H. Vink, O. Hiramatu, T. Kajita, F. Shigeto, J. A. E. Spaan & F. Kajiya: Role of hyaluronic acid glycasominoglycans in shear-induced endothelium-derived nitric oxide release. Am. J. Physiol Heart Circ Physiol 285, H722-H726 (2003)
40. Florian J. A, J. R. Kosky, K. Ainslie, Z. Pang, R. O. Dull & J. M. Tarbell: Heparan sulfate proteoglycans is a mechanosensor on endotehlial cells. Circ. Res. 93, e136-e142 (2003)
41. Pohl U, K. Herlan, A. Huang & E. Bassenge: EDRF-mediated shear-induced dilation opposes myogenic vasoconstriction in small rabbit arteries. Am J Physiol Heart Circ physiol 261, H2016-H2023 (1991)
42. Sharon N & H. Lis: Legume lectins – a large family of homologous proteins. *FASEB J* 4:3198-3208, (1990)

Key Words: Endothelial luminal glycocalyx, Glycosidic enzymes, Coronary Flow, Multimolecular flow sensor

Send correspondence to: Dr Rafael Rubio, Depto de Fisiologia, Facultad de Medicina UASLP, Av. Venustiano Carranza 2405, San Luis Potosi, S. L. P., Mexico 78210, Tel/Fax: +52-444-8262355, E-mail: rrubio@uaslp.mx