[Frontiers in Bioscience 5421-5431, May 1, 2008]

Involvement of endothelial Man and Gal-binding lectins in sensing the flow in coronary arteries

Alma Barajas-Espinosa[1], Juan Ramiro-Diaz[1], Erika Briones-Cerecero[2], Erika Chi-Ahumada[1], Ana Barba De la Rosa[2], Blanca Arroyo-Flores[1], Rafael Rubio[1]

[1]Departamento de Fisiologia y Farmacologia, Universidad Autonoma de San Luis Potosi, San Luis Potosi, Mexico, [2]Departamento de Biologia Molecular, Instituto Potosino de Investigacion Cientifica y Tecnologica, San Luis Potosi, Mexico

TABLE OF CONTENTS

1. ABSTRACT

The coronary endothelial luminal membrane (CELM) glycocalyx has diverse molecules involved in blood flow signal transduction. Evidence suggests that some of these structures may be lectinic. To test this, we synthesized two monosaccharide polymers (Mon-Pols) made of Mannose (Man-Pol) or Galactose (Gal-Pol) covalently coupled to Dextran (70kDa) and used them as lectin affinity probes. *In situ* intracoronary infusion of both polymers resulted in CELM-binding but only Man-Pol caused a reduction in flow-induced positive inotropism and dromotropism. To demonstrate that our lectinic probes could bind to CELM lectins, a representative CELM protein fraction was isolated via intracoronary infusion of a cationic silica colloid and either Mannose- or Galactose-binding lectins were purified from the CELM protein fraction using the corresponding Mon-Pol affinity chromatography resin. Resin-bound CELM proteins were eluted with the corresponding monosaccharide. 2D-SDS-PAGE (pH 4-7) revealed 9 Mannose- and approximately 100 Galactose-selective CELM lectins. In summary, the CELM glycocalyx contains Mannose- and Galactose-binding lectins that may be involved in translating coronary flow into a cardiac parenchymal response.

2. INTRODUCTION

It is known that coronary vascular endothelium is sensitive to hemodynamic stimuli that cause certain paracrine-parenchymal cell responses (1, 2), indicating that coronary flow and cardiac function are elements of a closed feedback loop (1, 3, 4). This flow-function association was first observed in 1912 by Knowlton and Starling (5). This interaction was defined as a "coronary vascular mechanism that induces an inotropic effect" (6). The phenomenon was rediscovered by Gregg (7, 8), confirmed and extended to other functions (1, 3, 4, 9-12). Futhermore, it is now accepted that diverse luminal glycocalyx membrane structures are involved in short (minutes) and long term (hours/days) flow-sensing mechanisms (3, 4, 12-20). The luminal endothelial glycocalix is a dense (0.2-0.5μm) layer of polyanions and a vast array of glycan-containing structures such as glucosaminoglycans and proteoglycans (21-24) distributed in microdomains on the luminal membrane (17, 23-28).

Amongst the proposed flow-sensing endothelial structures are: core proteins (29), receptor tyrosine kinases and integrins (3, 9, 30), caveolae structural components

(17, 31), lectin-like proteins (32) and glycosylated membrane proteins (2-4, 10, 13). Coronary perfusion of vascular-confined glycoside-hydrolyzing enzymes, lectins or antibodies directed against specific coronary endothelial luminal membrane (CELM) proteins affected the coronary flow-induced cardiac dromotropic and metabolic effects (2-4). For example, enzymatic digestion of glycosidic structures by O-, N-glicanase, Sialidase, Heparinase and Hyaluronidase abolish diverse cardiac and blood vessel effects observed with increased vascular blood flow (2-4, 10, 13). Furthermore, lectin-like proteins present on the luminal vascular membrane appear to be involved in a number of physiological processes such as cardiac function regulation (32), cell adhesion (33, 34), pain perception (35) and cardiac ischemia-reperfusion injury (26). Thus, it is important to isolate, identify, characterize and define the function of the elements that constitute the CELM.

Isolation of highly enriched endothelial luminal membrane (ELM) elements is now possible (27, 36-43) through the stabilization of the plasma membrane by coating the cells with a dense pellicle of cationic silica particles (20 nm – 50 nm) that strongly adhere to anionic cell surfaces. Once the silica is bound to the exposed cell surface, the silica particles are cross-linked by an anionic polymer (AP), restoring thus the net negative charge of the ELM. The cross-linked, ELM-silica-AP pellicle shields the luminal surface from chemical and enzymatic probes while retaining ELM integrity and composition during the following homogenization and centrifugation steps. This procedure has evolved over the past 20 years and has allowed for the generation of a highly enriched ELM protein fraction subject to stringent criteria by diverse researchers (26, 36-43). Furthermore, the cationic silica-pellicle methodology preserves transient physiological stages of the ELM and permits the biochemical comparison of these stages (37, 40).

Many researchers, including us, have suggested that in blood vessels, luminal glycosidic, glycosilated (1-4, 10, 13, 21, 32) and lectinic (32) proteins participate in flow-detection. The isolation of CELM glycosilated proteins has been described (27, 40, 41) but the lectinic elements need to be isolated and defined. Because of the possible participation of CELM lectinic proteins in flow sensing, we have decided to isolate them and to attempt to establish their sensor-role.

In vivo, oligosaccharide chains of glycoproteins are comprised of: mannose, galactose, N-acetyl-glucosamine, neuraminic acid, and fucose (44). Therefore, we synthesized two high molecular weight polymers (probes) made up of mannose or galactose. We hypothesized that these probes, *in vitro*, capable of specifically binding to mannose- or galactose-recognizing plant lectins, would also bind to specific mannose- or galactose-recognizing CELM lectinic proteins. Furthermore, because their large size confines them to the intravascular space, intracoronary administration of these probes would allow for *in vivo* studies of their intravascular-binding effects on flow-induced cardiac physiology.

Here we demonstrate that, upon specific binding of a Mon-Pol to the CELM in isolated Langendorff-perfused guinea pig hearts, there is a physiological effect on coronary flow-induced cardiac responses. Furthermore, we have isolated CELM proteins (27, 36-43) and affinity purified them into two types of lectin groups with our two specific probes. These mannose- or galactose-recognizing CELM lectins were further characterized according to their isolectric point and molecular weight by two dimensional electrophoresis.

3. METHODS

3.1. Isolated heart preparations

Male Dunkin Hartley guinea pigs (380-400g) were anesthetized with an intraperitoneal injection of Nembutal (50 mg/kg) and heparin (500 IU). Animals were artificially ventilated and the heart was removed, the ascending aorta was cannulated and retrogradely perfused according to the method of Langendorff (1, 3, 4, 32). Two types of heart preparations were utilized: one for physiological studies and the second for CELM isolation.

3.2. Synthesis of monosaccharide polymers (Mon-Pols)

Polymers of two *in vivo* elements of mammalian glycosidic moieties (44), mannose and galactose, were synthesized using well-established procedures (45). For each Mon-Pol, 1.0 g of dialyzed 70 kDa dextran was dissolved in 40 mL of $NaCO_3$ (0.5M, pH 11), mixed with 1 mL of divinyl sulfone (DVS) suspended in 10 mL of $NaCO_3$ (0.5M, pH 11) and stirred for 1hr. Theoretically, one of the two vinyl moieties of each DVS molecule will react with one hydroxyl group of the dextran. The remaining free vinyl moiety was subsequently reacted with either: 1) the hydroxyl group of either mannose or galactose upon addition of 12 g of monosaccharide, the reaction was allowed to proceed for 4 hours and possible remaining free vinyl groups were neutralized upon reacting overnight with the hydroxyl group of ethanolamine added in excess; 2) the hydroxyl group of ethanolamine added in excess, leaving a free amino group (negative control 1); or 3) the hydroxyl group of methanol added in excess, leaving free a hydroxyl group (negative control 2). These three polymers were extensively dialyzed against water and dried at 60 ºC. The two monosaccharide polymers synthesized were labeled Man-Pol and Gal-Pol. The amount of monosaccharide linked to the polymer was quantified via the dinitrosalisylic acid technique (46). When fluorescein isothiocyanate (FITC)-labeled Mon-Pols (FITC-Man-Pol and FITC-Gal-Pol) were synthesized, 0.5. g of 70 kDa dextran was mixed with 0.5. g of 70 kDa FITC-dextran and the synthesis proceeded as described above.

Negative polymer controls did not have a physiological effect nor did they bind to the CELM (not shown), indicating that the presence of a monosaccharide moiety is necessary for Mon-Pol activity.

Similar Mon-Pols were constructed to generate a matrix capable of isolating lectins by affinity chromatography. After addition of the monosaccharide, the solution pH was raised to 11 with $NaCO_3$, resulting in

insoluble Mon-Pol particles. The Mon-Pol particles were used to pack affinity chromatography columns.

3.3. Physiological effects of Man-Pol and Gal-Pol

Hearts were perfused with oxygenated Kreb's Henseleit (K-H, 37ºC, pH 7.4). Initially, coronary flow was kept constant at 8 mL/min. A stimulatory pair of electrodes was placed on the right atria and the hearts were paced with electric square pulses of 2 ms duration, voltage 2x threshold, and a rate of 4.5. Hz. Following a 30 min stabilization period the experiments were conducted. For each individual heart, experimental values were expressed as a percent of its own (100%) control.

3.3.1. Ventricular contraction measurements

A latex balloon attached to the end of a fluid filled catheter was introduced into the left ventricle via the left atrium. The other end of the catheter was connected to a pressure gauge and 0.2. ml of water was injected into the balloon to adjust diastolic pressure to 10 mmHg. Developed ventricular pressure was continuously recorded. The frequency response of this pressure system may not be optimal to distinguish changes in diastolic time fraction and pressure development (dP/dt). Therefore, changes in amplitude of developed ventricular pressure were taken as a qualitative indicator of inotropic response. At the end of the stabilization period and at a coronary flow of 8 mL/min, the contraction amplitude was taken as control and defined as 100%. The mean contraction amplitude was 31 ± 8 mmHg.

3.3.2. A-V delay measurements

To record atrial and ventricular electrocardiograms, one electrode was placed in the left atrium and a second electrode on the apex of the left ventricle. These two electrodes were connected to an oscilloscope synchronized with the atrial pacing stimulator. The atrial-ventricular interval (A-V delay, in msec) was continuously monitored and measured as the time interval between the application of the stimulus to the auricle and the rising phase of the ventricular electrical signal. Our group and others' (1, 3) have shown that under a variety of experimental conditions, changes in the A-V delay are caused solely by changes in the delay in the A-V nodal region, as defined by the time interval between the electrocardiogram of the atrium and the bundle of His (A-H interval). The A-V delay is at all times a constant 11.3. ± 0.2. msec longer than the A-H interval (1). Therefore, changes in A-V delay were taken to indicate a dromotropic response. At the end of the stabilization period and at a coronary flow of 8 mL/min the A-V delay was taken as control and defined as 100%. The mean A-V delay value was 105 ± 12 msec.

3.3.3. Dromotropic and inotropic effects of Mon-Pol coronary infusion

Maximal effective concentrations for each of Mon-Pol were first determined. To define this concentration, hearts were perfused at a constant coronary flow (8 mL/min) and effects on A-V delay and contraction amplitude were monitored. Six hearts were used for each Mon-Pol. Effects of successive step-increases (1 order of magnitude= ten fold) of Mon-Pol concentrations (range $1x10^{-11}$ to $1x10^{-4}$ M) were determined. Each Mon-Pol concentration was administered intracoronarily for a total of 5 min. Effects were determined at 2 min and the responses remained persistent even after a 5 min washout period. This cycle was repeated for the next 10x higher concentration of Mon-Pol, a higher level of response was thus achieved until the maximal effect was reached. For these experiments the maximal increase of A-V delay for Man-Pol was 20 ± 5 msec and for Gal-Pol was 12 ± 4 msec. The response obtained during the infusion of each Mon-Pol concentration persisted during the subsequent wash period, indicating that the effect was not washable. Similar cumulative concentration-dependent effects have been shown using glucose polymers (31). For both Mon-Pols the concentration utilized to study their effect on function-coronary flow curves was $1x10^{-4}$ M.

To study the effects of a Mon-Pol on A-V delay-coronary flow and ventricular contraction-coronary flow curves, the control curves were first simultaneously determined in hearts perfused with K-H. Coronary flow was varied (range 6 to 12 mL/min), with step increases of 1 mL/min. Each step of flow was maintained constant for a period of 2 min and the steady state responses determined. A-V delay (105 ± 12 msec) and contraction amplitude (31 ± 8 mmHg) at a coronary flow of 8 mL/min were taken as controls and defined as 100%. Once these control coronary flow-curves were determined, coronary flow was maintained at 8 mL/min and either Mon-Pol ($1x10^{-4}$ M) was continuously administered for 10 min. Infusion of Mon-Pol was stopped, its excess washed out with K-H for 5 min and the experimental A-V delay-coronary flow and ventricular contraction-coronary flow curves were determined. Responses were expressed as percent of their corresponding 100 % control. A total of 6 hearts were used for each Mon-Pol.

3.3.4. Statistical analyses

Paired Student T-tests were performed on paired positive/negative variable values (%) and results with $p < 0.05$% were taken as statistically significant.

3.4. Mon-Pol adherence to CELM

Isolated hearts (n= 3) were coronary perfused for 10 min with K-H followed by a fixative solution of paraformaldehyde 10 % in 0.1. M phosphate buffer (pH 7.4, 1.2. mM of Ca^{2+} and 10 mM mannose, in order to preserve its recognition sites). Fixative solution was rinsed by perfusion K-H for 5 min, followed by a 5 min perfusion with K-H containing $1x10^{-4}$ M of FITC-labeled Man-Pol. Non-attached Man-Pol was washed out with K-H for 5 min. These experiments were repeated using galactose and FITC-labeled Gal-Pol (n = 3).

Hereafter, all tissue manipulations were carried out in the dark. 5 mm-wide blocks were cut from the mid-portion of the left ventricular wall and frozen at -20 °C. In a Cryostat (Reichert HistoSTAT), frozen tissues were cut into 20 µM thick sections and picked up on aminopropyltriethoxysilane (APES)-coated slides. Sections were air-dried overnight at room temperature,

fixed with acetone for 20 min, air-dried again, rinsed and rehydrated in Tris buffer saline (TBS) and immunostained.

Two criteria were utilized to identify arteries, arterioles, and capillaries: size of the blood vessel and selective immunolabeling. Arteries and arterioles were immuno-identified by labeling smooth muscle with anti-alpha-actin and endothelium with anti-VonWillebrand Factor. To immunolabel, tissue sections were incubated with selective primary antibodies (Santa Cruz, 1/100 dilution) and incubated for 30 min. Slides were then washed with TBS, and incubated in a rhodamine-coupled secondary IgG. Excess secondary antibody was washed with TBS (47). A drop of vectashield mounting medium for fluorescence (H-1000, Vector) was placed over the tissue and this was subsequently covered with a coverslip. FITC-fluorophore (Mon-Pol) was excited with λ_{ex}= 495 nm and its emission detected at λ_{em}= 519 nm. The rhodamine-fluorophore (Tritc) was excited with a λ_{ex}= 552 nm and its emission detected at λ_{em}= 578 nm.

Immunolabeled blood vessels in tissue sections were visualized, laser scanned on all their depth and photographed using a laser scanning confocal microscope (Leica TCS SP2). Micrographs shown correspond to one confocal section. In 10 tissue sections, all observed arteries, arterioles and capillaries showed positive immunoreactions (positive controls) to the antibodies used. Other cardiac structures did not immunoreact (negative controls). Sole incubation with the secondary antibody resulted in no fluorescent signal.

3.5. Experiments to isolate CELM proteins

3.5.1. Solutions used for the silica pellicle technique

The following eight solutions were prepared: 1-3) Three different MES-Buffer Saline (MBS) solutions were prepared: 8.532g MES sodium salt (19.6. mM) and 17.532 g NaCl (150 mM) made up to 2 L, pH adjusted either to 5, 6, or 7. 4) Colloidal cationic silica (30 %, kindly donated by Dr. Beer Stolz, University of Pittsburgh Medical School) was dialyzed overnight against MBS, pH 5 and diluted to 1.5. % silica with the same buffer. 5) 3 mg/mL polyacrylic acid in MBS pH 6. 6) Lysis Buffer solution (LB), 5.9575 g HEPES and 85.575 g of sucrose made up to 1 L, pH 7.4. 7) Nycodenz I: 1.03 x *g* (~55 % w/v) were dissolved in 5.5mL of LB. 8) Nycodenz II: 70 % (w/v) 7.0 g Nycodenz up to 10 mL with LB.

3.5.2. Isolation and characterization of CELM proteins

The previously established (10, 27, 36-43, 48, 49) colloidal silica technique was utilized. Briefly, hearts were retrogradely perfused with MBS (50 mL, 10 mM EGTA, pH 6) at a constant pressure of 70 cm of water, followed by successive perfusions with MBS (pH 6 and pH 5, 10mL each). Thereafter, 1.5. % cationic colloidal silica (24 mL) was perfused and excess was washed with 20 mL of MBS (pH 5). CELM-bound silica was polymerized with anionic polyacrylic acid (AP, 40 mL, Aldrich), its excess washed with MBS (20 mL, pH 5) and LB (20mL). In three hearts we visualized via electron microscopy, as have others (27, 39-41, 49), a continuous uninterrupted film of polymerized silica bound to the CELM (electron micrographs not shown).

Ventricles were dissected, minced and suspended in 10 mL of LB containing 1 mM of phenylmethylsulphonyl fluoride (PMSF, SIGMA) and homogenized with a glass-teflon homogenizer, at 6000 RPM, 5 min, at 4 ºC. The homogenate was mixed with an equal volume of Nycodenz I solution, layered on top of 0.5. mL of Nycodenz II solution, and centrifuged for 20 min at 20,000 x *g*. The CELM-silica pellet was washed three times with MBS pH 6 to remove a variety of contaminating non-ELM proteins (36) and resuspended in 2 % SDS. This was sonicated, boiled for 5 min, centrifuged for 10 min at 14,000 x *g* and the supernatant was analyzed via SDS-PAGE and Western Blotting. CELM proteins were quantified using the Bicinchoninic Acid Kit (Sigma B9643).

Well-established data (10, 27, 36-43, 48, 49) supports the hypothesis that the resulting ELM-silica-AP pellicle is a representative sample of the ELM organelle, and we ruled out the possibility that alien non-CELM proteins contaminated the CELM-silica-AP fraction by adding a saturating concentration of a non-CELM alien protein, bovine serum albumin (Sigma, A-4503) to the homogenizing medium. The final concentration of albumin was 5x the calculated total tissue protein(5 %/w). Apart from being an exogenous protein, albumin is able to bind reversibly to at least three CELM albumin-recognizing proteins (43, 49) and non-specifically binds cationic silica (38); thus if contamination occurred during homogenization and centrifugation, the albumin was at least 5x more likely to be a contaminating non-CELM protein. However, protein electrophoresis profiles of the CELM were albumin-free (not shown), suggesting a low probability of alien protein contamination of the CELM fraction.

We also immunoprobed CELM proteins by Western Blotting with antibodies directed against proteins positive markers: Caveolin-1, vascular cell adhesion molecule (VCAM), VonWillebrand Factor and Mannose receptor, and negative markers: purinergic receptors, P_2Y_{1-4} (19, 27, 30, 31, 34, 35, 50-52). CELM proteins were visualized using 12 % bisacrylamide mini gels, and stained with either silver (53) or BioSafe Coomassie (BioRad, 161-0787). Preparative gels were run and proteins transferred onto Nitrocellulose membrane (NitroPure, Pierce 88018) for Western Blotting analyses. Blots were first rehydrated in 1 x TBS, blocked for 1 hr with 10 % non-fat powder milk made in TBS and incubated with primary antibody (produced in rabbit) 1/500 overnight at 4ºC with constant agitation. Next day, at room temperature, the blot was washed twice with 1 x TBS 0.1. % Tween20 and twice with 1x TBS, 5 min each, milk-blocked for 1 hr and incubated 1 hr with secondary anti-rabbit HRP-coupled antibody (dilution 1/5000). The washes were performed again and the western was then developed with SuperSignal West Pico Chemiluminscent Substrate (Pierce 34080) and visualized on Kodak photographic paper. All antigen-recognizing CELM proteins; VCAM, Caveolin-1, VonWillebrand Factor and Mannose Receptor immuno-

reacted positively while the non-CELM protein-antigens (P_2Y_{1-4}) did not. Only the results of the Mannose Receptor are shown because of its relevance to this paper.

Together, the evidence from our laboratory and from others supports the hypothesis that the final CELM-silica-AP pellicle pellet is a representative sample of the coronary luminal endothelial membrane organelle (10, 27, 36-43, 48, 49). This subject will not be a matter of further discussion in this manuscript.

3.6. Affinity-purified lectins from CELM lysates

3.6.1. Column construction and control lectins

Lectins are defined by their selective monosaccharide binding affinity, it is the basis for their chromatography separation. Affinity columns for lectins recognizing either mannose or galactose moieties were built by suspending a hydrated matrix of either Man-Pol or Gal-Pol in 0.1mM PBS (pH 7.4, 0.2mM $CaCl_2$) (54) and gravity-packing columns. The ability and specificity of the columns to specifically retain lectins with affinity for the Mon-Pol in question were determined using commercially available plant lectins. As a positive control for the Man-Pol column we used *Concanavalin A* (with affinity for Mannose) and for its negative control we used *Ulex europeaus II* (it has affinity to N-AcetylGlucosamine). For the positive control of Gal-Pol column we used *Bandeira simplifolia-1* (with affinity for Galactose) and for its negative control we used *Maackia amurensis* (with affinity for Sialic acid). Each lectin was dissolved in 2mL of 0.1. mM PBS, poured into the column and allowed to absorb onto the matrix for 1 hr at room temperature. Proteins not bound to the resin were washed off with 10 mL 0.1. mM PBS, while affinity-bound lectins eluted with 10 mL 0.1. mM PBS pH 7.4, 0.4. mM EGTA, 200 mM free monosaccharide (Man or Gal). Eluted proteins were visualized by SDS-PAGE.

The Man-Pol columns retained *Concanavalin A,* which eluted only with free mannose. This column did not retain *Ulex europeaus I,* its negative control. Similarly, the Gal-Pol column was able to retain *Bandeira simplifolia-1,* and this eluted with free Galactose. This column did not retain *Maackia amurensis,* its negative control. These control data are not shown.

3.6.2. Mannose- or Galactose-recognizing CELM lectins

CELM lectinic proteins were isolated according to their sugar-binding affinity towards either mannose or galactose. 0.5. mg/2 mL of previously dialyzed (dialyzed against 0.1. mM PBS pH 7.4, 0.2. mM $CaCl_2$) CELM protein was poured into either the Man-Pol or the Gal-Pol column and incubated for 1 hr (54). Non-bound proteins were eluted with PBS and bound lectins eluted with 200 mM of the corresponding free Monosaccharide (10 mL 0.1. mM PBS pH 7.4, 0.4. mM EGTA) (54). Eluates were extensively dialyzed against water for one week at 4°C, lyophilized, reconstituted in a small volume and examined via one (1D) and two-dimensional (2D) SDS-PAGE.

3.7. Two-Dimensional PAGE of CELM lectins

CELM lectin samples were desalted and rehydrated with buffer containing DTT and ampholytes pH 3-10 and 4-7 (2-D Clean Up Kit and buffer, Amersham) and protein concentrations were estimated via the Bradford Assay. 20 μg of protein was loaded on 11 cm strips, pH gradients used were either 4-7 or 3-10, (Immobiline Drystrips; Amersham). Focusing was carried out using the Ettan IPGphor Isoelectric Focusing System (Amersham Biosciences) at 20 ºC at the 3 step and hold voltage mode for a total of 17.6. V•hr. The second-dimension SDS-PAGE was performed in 12 % polyacrylamide gels, run at 20 ºC first with a current of 20 mA for 20 min and then for 2 hrs at 50 mA. Gels were fixed overnight in 50 % MetOH, 10 % Acetic Acid Sypro Ruby stained and photographed on an ultraviolet transiluminator.

4. RESULTS

4.1. Man-Pol and Gal-Pol induce changes in flow-regulated dromotropic and inotropic effects

The well-established coronary flow-stimulated inotropism and dromotropism under control conditions (38-40) are shown (Figures 1 and 2, squares). Once the control coronary flow-curves were determined, either Man-Pol or Gal-Pol ($1x10^{-4}$ M) were infused for 10 min. Thereafter, their excess was washed out for 5 min before an experimental set of A-V delay-coronary flow and ventricular contraction-coronary flow curves were repeated. The post infusion effects of the Mon-Pols are shown (Figures 1 and 2, circles). Man-Pol (Figure 1A) induced an upward displacement of the A-V delay-coronary flow curve, however, this effect was of lesser magnitude in the case of Gal-Pol (Figure 1B). Man-Pol induced a significant downward displacement of ventricular contraction-coronary flow curve for flows between 6 to 9 mL/min (Figure 2A). However, Gal-Pol did not have an inotropic effect (Figure 2B). Because our negative controls did not exert physiological effects and intracoronary infusion of the vascular-confined Mon-Pol molecules resulted in persistent, non-washable, concentration-dependent effects suggests that Mon-Pols exert their effect as a result of binding to CELM monosaccharide moieties. This reasoning prompted us to determine whether the Mon-Pols remained attached to the CELM *in situ* even after their wash out period.

4.2. *In situ* vascular adhesion of Man-Pol and Gal-Pol

Following coronary infusion of either FITC-Man-Pol or FITC-Gal-Pol their excess was washed out and their possible adherence to CELM was determined. Both FITC-Man-Pol (Figures 3A, 3D) and FITC-Gal-Pol were observed to bind to vascular endothelial structures in laser confocal images. Vascular vessels and their cell components were identified by the following two criteria: a) immunoreactivity of endothelial cells with anti-VonWillebrand Factor and arteriolar smooth muscle with anti alpha-actin (both in red), and b) the vessel diameter (capillaries diam < 10 μm, arterioles diam > 20 μm). In Figure 3B the same blood vessels (diameter < 10 μm) as in Figure 3 reacted positively to the cell membrane-directed antibodies. Because the vessel in Figure 3B was labeled with the cell-membrane anti- VonWillebrand Factor antibody and it had a diameter < 10 μm, it was defined as a capillary. Figure 3C results from overlapping of its

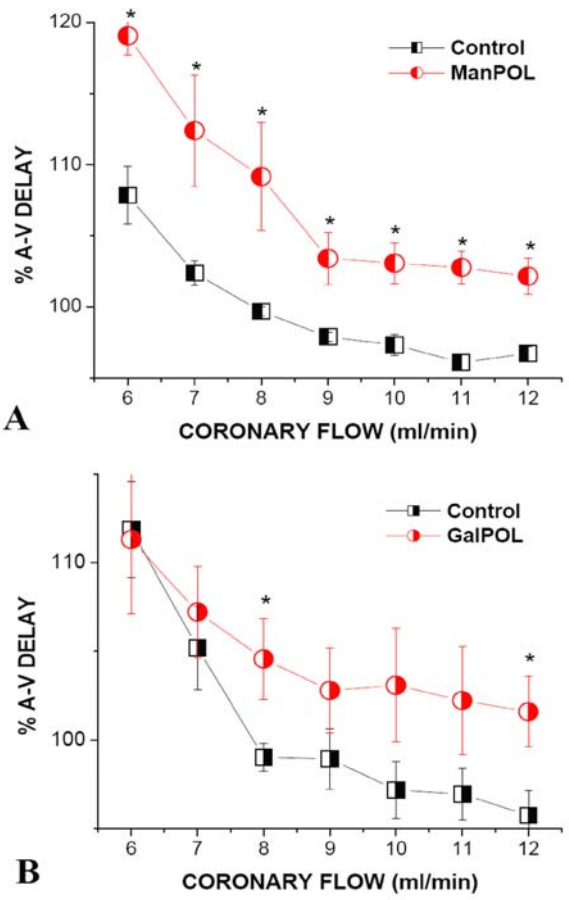

Figure 1. Positive dromotropic effect of coronary flow and its modulation by bound Mon-Pol to the CELM. The curves labeled Man-Pol (A) or Gal-Pol (B) were obtained after the Mon-Pol had been intracoronarily infused (1 x 10^{-4} M) and washed off. Man-Pol exerted a significant negative dromotropic effect but Gal-Pol's effects were of lesser magnitude. For both groups n=6.

corresponding Figure 3A (green) and Figure 3B (red) fluorescent images. The resulting dominant yellow fluorescence indicates both fluorescent probes (FITC-Man-Pol and anti-VIII factor) are in the same structure: the CELM. Binding of FITC-Man-Pol (Figure 3D) to endothelial cells of arterioles is also shown. Similar results were obtained for the FITC-Gal-Pol (not shown). The *in situ* binding of both Man-Pol and Gal-Pol to the CELM suggests the existence of monosaccharide-recognizing membrane structures, i.e. lectinic proteins.

4.3. CELM lectinic proteins

Our results and others' suggest that the CELM-silica-AP pellicle is representative of coronary luminal endothelial membrane organelle (10, 27, 36-43, 48, 49). SDS-PAGE of CELM protein extracts showed a diverse number of bands (Figure 4). To define which of these proteins were lectinic, the CELM protein extracts were separated according to their monosaccharide-binding affinities. Each of our affinity chromatography columns selectively bound specific plant lectins. CELM lysates were loaded onto either a Man-Pol or Gal-Pol affinity chromatography column (for each Mon-Pol n = 5), rinsed and eluted with the corresponding free monosaccharide. Our lectin output was 4-7 % of total CELM protein loaded onto the affinity column, as we had a yield of 20-38 µg of purified lectins from 500 µg of CELM proteins loaded into the affinity columns. The SDS-PAGE patterns of the affinity chromatography eluates from Man-Pol column are shown in Figure 5A and those from Gal-Pol column are in Figure 5B. Approximately 7 protein bands were eluted from the Man-Pol affinity purification column and a band of approximately 60 kDa immunoreacted with the mannose receptor antibody, a mannose-binding endothelium c-type lectin (Figure 5C) (19, 52). In the case of the Gal-Pol affinity purification column near 20 protein bands were eluted. It is likely that these lectinic protein bands may be composed of several proteins.

The Man- and Gal-binding CELM lectinic proteins were subjected to 2D SDS-PAGE for further separation into individual elements. We observed nine protein spots in the Man-Pol eluant (Figure 6) and over 100 spots in the Gal-Pol eluant (Figure 7).

5. DISCUSSION

Our results support the concept that CELM composition comprises lectinic proteins, some of which participate in coronary flow-induced parenchymal responses. We have synthesized two polymers, one of mannose (Man-Pol) and another of galactose (Gal-Pol). Both polymers bind to specific mannose- and galactose-recognizing lectins *in vitro*, and both are able to discriminate against lectins with affinity towards other monosaccharides. *In situ*, when each of the Mon-Pols are administered intracoronarily, their monosaccharide moieties bind to the CELM, forming a non-washable complex. Furthermore, Man-Pol selectively alters the dromotropic and inotropic effects of coronary flow. Gal-Pol exerted a small dromotropic effect but lacked any inotropic effect. We have isolated a CELM protein fraction and purified from it two lectin protein groups: a mannose- and a galactose-binding group. These two groups were isolated using affinity chromatography matrices made up of the two different functional Mon-Pols and further separated via 2D-SDS-PAGE. The CELM was demonstrated to contain a number of Mannose- and Galactose-recognizing proteins that selectively bind our Mon-Pols and alter the coronary flow-induced parenchymal response.

5.1. Role of lectins on parenchymal function-coronary flow responses

Both of our Mon-Pols bound *in situ* to the CELM of ventricular vasculature and once bound Man-Pol and Gal-Pol exerted major and lesser, respectively, negative dromotropic effects and altered the coronary flow-A-V delay curve. Man-Pol but not Gal-Pol demonstrated inotropic activity. Interestingly, a greater number of Gal-binding lectins were isolated from the CELM lysate as compared to Man-binding lectins. These results indicate that the *in situ* effects of Man-Pol and Gal-Pol and their

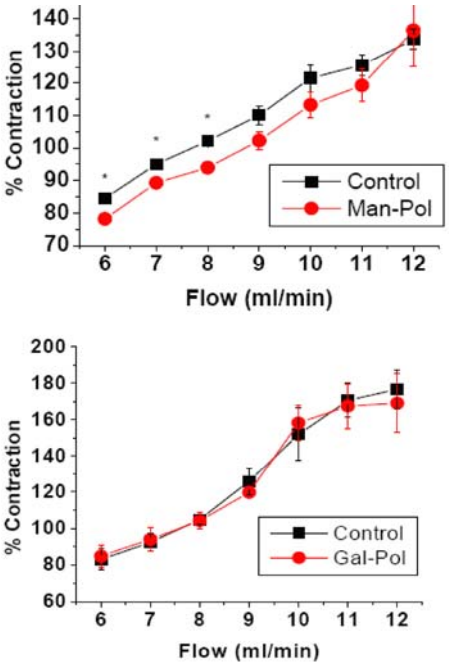

Figure 2. Inotropic effects of Mon-Pols. Positive inotropic effect of coronary flow and its selective modulation by bound Man-Pol to the CELM (upper figure). Gal-Pol lacked of an effect (lower figure). These curves were obtained simultaneously with those of figure 1. Both groups n=6.

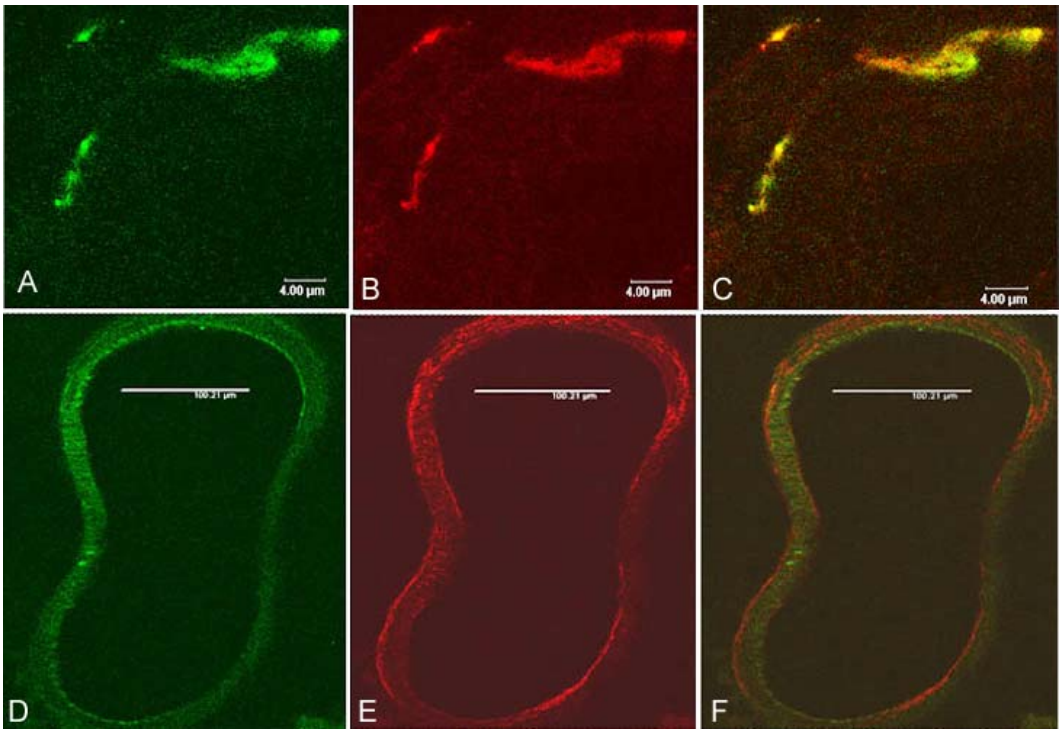

Figure 3. Mon-Pol adheres to CELM. FITC-Man-Pol remains adhered to CELM of capillary (green, a) and to an arteriole (green, d). Capillary endothelium was identified with luminal anti- VonWillebrand Factor red, b), overlapped images of a and b result in a yellow fluorescence (c). In order to identify the arteriole smooth muscle it was labeled with anti-alpha-actin (red, e), FITC-labeled Man-Pol is bound to endothelium (d). (f) overlapped images of d and e.

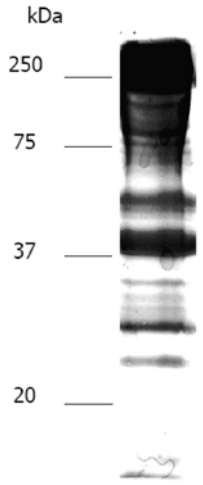

Figure 4. SDS-PAGE patterns of proteins extracted from CELM-silica-AP. Proteins in gels were stained with BioSafe Coomassie.

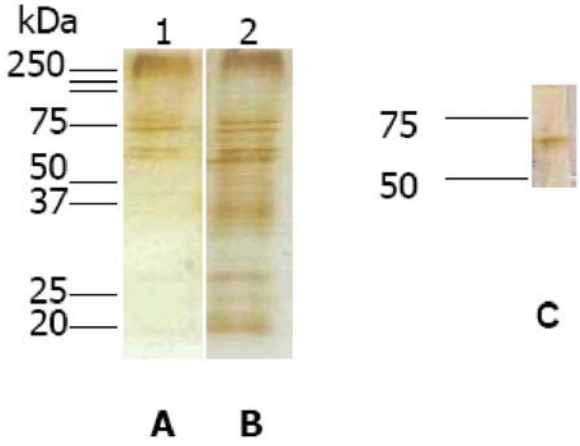

Figure 5. SDS-PAGE patterns of lectin proteins extracted from CELM-silica-AP. Lectins were run on 12 % SDS-PAGE and stained with silver nitrate. (A) Mannose-recognizing lectin protein bands of eluates from Man-Pol affinity column. At least seven bands are visible. (B) Galactose-recognizing lectin protein bands of eluates from Gal-Pol affinity column. Aproximately 20 protein bands are now seen. (C) The endothelial luminal C-type lectin Mannose-receptor was detected using antibodies: primary anti-MPR, (1/500) and secondary anti-goat IgG (1/5000) coupled to horseradish peroxidase.

distinct numerical differences in binding sites are not due to unspecific massive binding to the CELM but rather to a specific recognition of a given Mon-Pol by luminal lectin proteins.

It is important to note that a Mon-Pol is a lectin probe which, upon binding to CELM lectins, affects parenchymal function-coronary flow response. This raises the question of whether a flow sensor's lectinic properties are a flow-sensing prerequisite or whether lectinic properties are only a fortuitous way to identify these flow sensors. It is possible that the same effects could be observed if the sensor protein was bound to any other molecule different from Mon-Pol. These questions remain to be investigated.

The lack of inotropic effect of Gal-Pol could be due to a number of reasons. First, our Mon-Pols differ considerably from the natural lectin substrates (oligosaccharide branching chains) that are made up of combinations of: mannose, n-acetyl-glucosamine, galactose, fucose, and sialic acid. Lectins in general have greater selectivity and affinity for dimers/trimers of these sugars than for single monosaccharides. Thus, it is likely that the Mon-Pols may bind loosely or not bind to the function-relevant lectins. If the Mon-Pol were made up of dimers/trimers of galactose or mannose in addition to other monosaccharides, this structurally more complex Mon-Pol would likely bind more function-related CELM lectins and have more pronounced and diverse cardiac effects. Second, the apparent greater action of Man-Pol and Gal-Pol on the CELM of capillaries in the AV-nodal region suggests the existence of Mon-Pol-recognizing lectins involved in coronary flow-transduction in this region. This would imply that not only is there heterogeneity in CELM molecular composition throughout the coronary vascular network (22, 34, 41, 50) but also physiological region-specificity. Indeed, there is evidence that binding of plant lectins to CELM oligosacharides or hydrolysis of glycocalix glycosidic chains with specific glycanases, can selectively modulate either the A-V delay-coronary flow curve or the ventricular contraction-coronary flow curve or both, as well affect other coronary flow-parenchymal function responses (2-4, 10, 13, 32). The change in a defined coronary flow-parenchymal function response therefore appears to depend on a specific oligosacharide either binding to a lectin or being hydrolyzed by a glycanase. This further suggests that there is a non-homogenous distribution of both lectins and glycosylated structures in the coronary vasculature. Conversely, it is possible that lectins and glycosylated structures are distributed homogeneously and the observed action selectivity of the Mon-Pols could be due to a site-specific function of CELM components.

5.2. Characterization of CELM lectinic proteins

In the methods section (3.5.2.) we thoroughly described a number of tests conducted by our group and others that together support the conclusions that the final CELM-silica-AP pellicle is a representative sample of the CELM organelle and its protein components (10, 27, 36-43, 48, 49). Our CELM-silica-AP pellet was not contaminated by a large concentration of an exogenous protein (albumin) added during its extraction. Furthermore, in Western Blot analyses all CELM-positive antigens: VCAM, Caveolin-1, VonWillebrand Factor and Mannose Receptor reacted positively while the non-CELM protein-antigens: P_2Y_{1-4} failed to do so (19, 27, 30, 31, 33, 34, 49-52).

CELM lectins were isolated using polymers of mannose and galactose as binding matrices in affinity purification columns. These affinity chromatography columns were tested in the presence of Ca^{++} for specificity

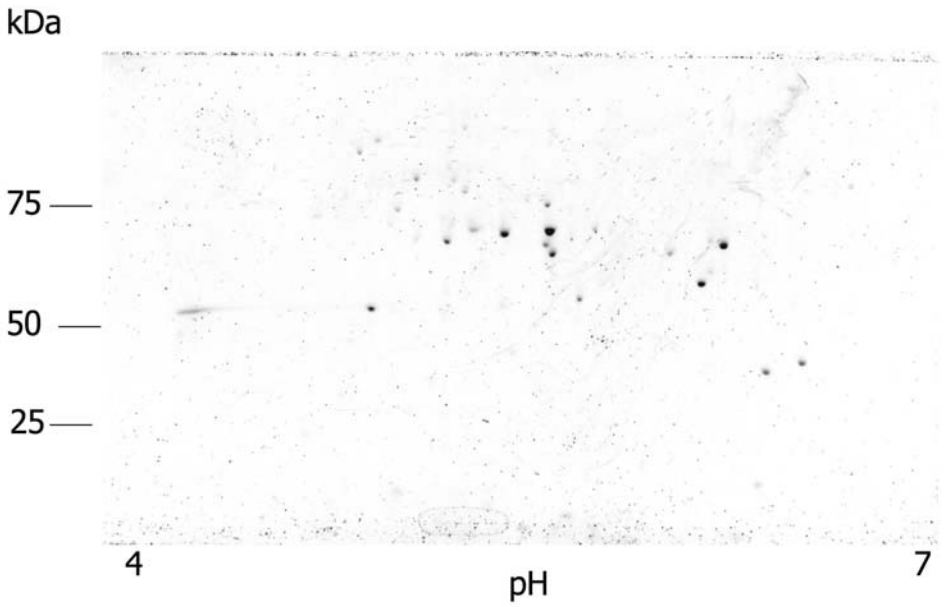

Figure 6. Two dimension SDS-PAGE of CELM mannose-selective lectins. 20 ug of protein were loaded, first dimension on pH range 4-7, SyproRuby-stained. At least 9 lectins (spots) can be visualized.

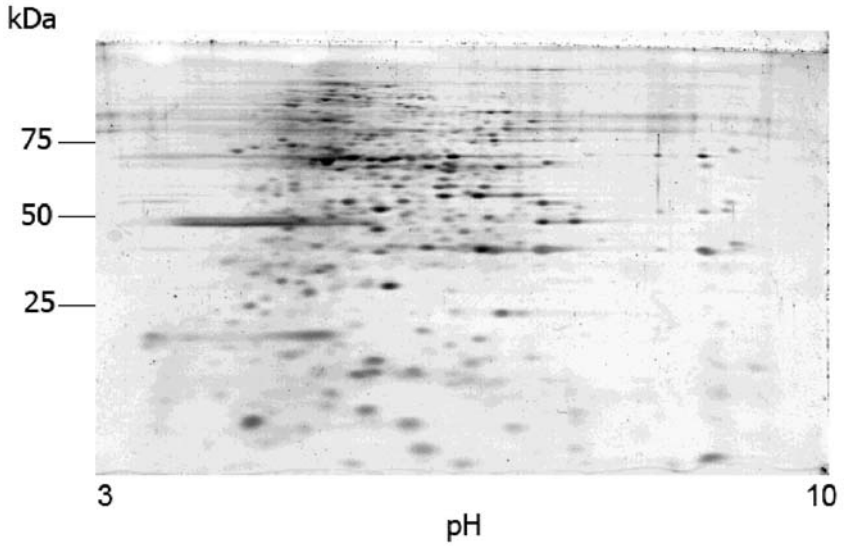

Figure 7. Two dimension SDS-PAGE of CELM galactose-selective lectins. Approximately 38 ug of protein were loaded, first dimension on pH range 3-10, SyproRuby-stained. At least 100 lectins can be seen.

and discriminative lectin binding (53). This was done because one of the CELM proteins isolated (mannose-binding protein) belongs the C-type lectin-like domain peptide family (11, 48, 55), thus suggesting the possibility of other C-type lectins amongst the CELM peptides extracted. When CELM lectins were visualized on 2D-SDS-PAGE there was a larger number of CELM galactose- than mannose-recognizing lectins. However, which of these individual CELM lectins are involved in parenchymal function-blood flow responses remain to be elucidated, yet their individual isolation constitutes the first necessary step. It is important to know what the functional significance behind the lectinic nature of these numerous proteins might be.

In summary, we are the first to demonstrate that our lectin affinity probes, Man-Pol and Gal-Pol, bind *in situ* to the CELM and that upon binding they alter the coronary flow-function curves. We isolated a CELM lysate that is representative of the coronary endothelial luminal membrane organelle protein constituents. From this organelle, using our functional affinity probes we purified CELM lectinic proteins according to their sugar-binding affinity towards

mannose or galactose and further separated them into individual protein elements. Our results support the concept that the CELM glycocalyx contains flow-sensing structures that are lectinic in nature.

6. ACKNOWLEDGEMENTSS

This research was supported by CONACyT SEP-42567, CONACyT-SALUD 2004-C01-156. We would also like to thank Ms. Maria Lourdes Palma Tirado at the Microscopy Unit of the Instituto de Neurobiologia, Campus UNAM Juriquilla, Queretaro, México for her help in the processing of the samples for visualization in the electron microscope.

7. REFERENCES

1. Rubio R, Ceballos G, Suarez J: Coronary flow stimulates auricular-ventricular transmission in the isolated perfused guinea pig heart. *Am J Physiol* 269, H1177-H1185 (1995)
2. Suarez J, Rubio R: Regulation of glycolytic flux by coronary flow in guinea pig heart. Role of vascular endothelial cell glycocalyx. *Am J Physiol* 261, H1994-H2000 (1991)
3. Rubio R, Ceballos G: Role of endothelial glycocalyx in dromotropic, inotropic, and arrythmogenic effects of coronary flow. *Am J Physiol* 278, H106-H116 (2000)
4. Rubio-Gayosso I, Barajas-Espinosa AR, Castillo-Hernandez J, Ramiro-Diaz J, Ceballos G, Rubio R: Enzymatic hydrolysis of luminal coronary glycosidic structures uncovers their role in sensing coronary flow. *Front Biosci* 10, 1050-1059 (2005)
5. Knowlton FP and Starling EH: The influence of variations in temperature and blood pressure on the performance of the isolated mammalian heart. *J Physiol London* 44, 206-219 (1912)
6. Anrep GV: On the part played by the suprarenals in the normal vascular reactions of the body. *J Physiol London* 45, 307-317 (1912)
7. Gregg DE: Effect of coronary perfusion pressure or coronary flow on oxygen usage of the myocardium. *Circ Res* 13, 497-500 (1963)
8. Sabiston DC, Gregg DE: Effect of cardiac contraction on coronary blood flow. *Circulation* 15, 14-20 (1957)
9. Chen KD, Li YS, Kim M, Li S, Yuan S, Chien S, Shyy JYJ: Mechanotransduction in response to shear stress. *J Biol Chem* 274, 18393-18400 (1999)
10. Florian JA, Kosky JR, Ainslie K, Pang Z, Dull RO, Tarbell JM: Heparan sulfate proteoglycan is a mechanosensor on endothelial cells. *Circ Res* 93, e136-e142 (2003)
11. Hansen S, Thiel S, Willis A, Holmskow U, Jensenius JC: Purification and characterization of two mannan-binding lectins from mouse serum. *J Immunol* 164, 2610-2618 (2000)
12. Rubanyi GM, Romero JC, Vanhoutte PM: Flow-induced release of endothelium-derived relaxing factor. *Am J Physiol* 250, H1145-H1149 (1986)
13. Mochizuki S, Vink H, Hiramatsu O, Kajita T, Shigeto F, Spaan JAE, Kajiya F: Role of hyaluronic acid glycosaminoglycans in shear-induced endothelium-derived nitric oxide release. *Am J Physiol* 285, H722-H726 (2003)
14. Pahakis MY, Kosky JR, Dull RO, Tarbell JM: The role of endothelial glycocalyx components in mechanotransduction of fluid shear stress. *Biochem Biophys Res Commun* 355, 228-233 (2007)
15. Pries AP, Secomb TW, Jacobs H, Sperandio M, Osterloh K, Gaehtgens P: Microvascular blood flow resistance: role of endothelial surface layer. *Am J Physiol* 273, H2272-H2279 (1997)
16. Rizzo V, McIntosh DP, Oh P, Schnitzer JE: *In situ* flow activates endothelial nitric oxide synthase in luminal caveolae of endothelium with rapid caveolin dissociation and calmodulin association. *J Biol Chem* 273, 34724-34729 (1998)
17. Rizzo V, Morton C, DePaola N, Schnitzer JE, Davies PF: Recruitment of endothelial caveolae into mechanotrasduction pathways by flow conditioning *in vitro*. *Am J Physiol* 285, H1720-H1729 (2003)
18. Shin J, Jo H, Park H: Caveolin-1 is transiently dephosphorylated by shear stress-activated protein tyrosine phosphate mu. *Biochem Biophys Res Commun* 339, 737-741 (2006)
19. Takahashi K, Donovan MJ, Rogers RA, Ezekowitz RAB: Distribution of murine mannose receptor expression from early embryogenesis through to adulthood. *Cell Tissue Res* 292, 311-323 (1998)
20. Tzima E, Irani-Tehrani M, Kiosses WB, Dejana E, Schultz DA, Engelhart B, Cao G, Delisser H, Schwatz MA: A mechanosensory complex that mediates the endothelial cell response to fluid shear stress. *Nature* 437, 426-431 (2005)
21. van den Berg BM, Vink H, Spaan JAE: The endotelial glycocalyx protects against myocardial edema. *Circ Res* 92, 592-594 (2003)
22. Ausprunk DH, Boudreau CL, Nelson DA: Proteoglycans in the microvasculature. *Am J Pathol* 103, 383-366 (1981)
23. Ausprunk DH, Boudreau CL, Nelson DA: Proteoglycans in the microvasculature. *Am J Pathol* 103, 367-375 (1981)
24. Holthofer H, Virtanen I, Kariniemi AL, Hormia M, Linder E, Miettinen A: *Ulex europeaus* I lectin as a marker for vascular endothelium in human tissues. *Lab Invest* 47, 60-66 (1982)
25. Sten O, Chajek T, Stein Y: Ultrastructural localization of concanavalin A in the perfused rat heart. *Lab Invest* 38, 103-110 (1976)
26. Lefer AM, Campbell B, Scalia R, Lefer DJ: Synergism between platelets and neutrophils in provoking cardiac dysfunction after ischemia and reperfusion: role of selectins. *Circulation* 98, 1322-1328 (1998)
27. Schnitzer JE, Oh P, Jacobson BS, Dvorak AM: Caveolae from luminal plasmalemma of rat lung endothelium: microdomains enriched in caveolin, Ca+2-ATPase, and inositol trisphosphate receptor. *Proc Natl Acad Sci USA* 92, 1759-1763 (1995)
28. Simionescu M, Simionescu N, Palade GE: Differentiated microdomains on the luminal surface of capillary endothelium: distribution of lectin receptors. *J Cell Biol* 94, 406-413 (1982)
29. Weinbaum S, Zhang X, Han Y, Vink H, Cowin SC: Mechanotransduction and flow across the endothelial glycocalyx. *PNAS* 100, 7988-7995 (2003)

30 .Shyy JYJ, Chien S: Role of integrins in endothelial mechanosensing of shear stress. *Circ Res* 91, 769-775 (2002)
31. Sun RJ, Muller S, Stoltz JF, Wang, X: Shear stress induces caveolin-1 translocation in cultured endothelial cells. *Eur Biophys J* 30, 605-611 (2002)
32. Gonzalez-Castillo C, Rubio R, Zenteno-Savin T: Coronary flow-induced inotropism is modulated by binding of dextrans to the endothelial luminal surface. *Am J Physiol* 284, H1348-H1387 (2003)
33. Blankenberg S, Banbaux S, Tiret L: VCAM adhesion molecules and atherosclerosis. *Atherosclerosis* 170, 191-203 (2003)
34. Brouland JP, Gilbert MA, Bonneau M, Pignaud G, Bol Dit Solier C, Drouet L: Macro and microheterogeneity in normal endothelial cells: differential composition of luminal glycocalyx and functional implications. *Endothelium* 6, 251-262 (1999)
35. Machelska H, Cabot PJ, Mousa SA, Zhang Q, Stein C: Pain control in inflammation governed by selectins. *Nature Med* 4, 1425-1428 (1998)
36. Beer Stolz D, Jacobson BS: Examination of transcellular membrane protein polarity of bovine aortic endothelial cells *in vitro* using the cationic colloidal silica microbead membrane-isolation procedure. *J Cell Science* 103, 39-51 (1992)
37. Beer Stoltz D, Ross MA, Salem HM, Wendy MM, Michalopoulus GK, Enomoto K: Cationic colloidal silica membrane perturbation as a means of examining changes at the sinusoidal surface during liver regeneration *Am J Pathol* 155, 1487-1498 (1999)
38. Chaney LK, Jacobson BS: Coating cells with colloidal silica for high yield isolation of plasma membrane sheets and identification of transmembrane proteins. *J Biol Chem* 258, 10062-10072 (1983)
39. Ghitescu LD, Crine P, Jacobson BS: Antibodies specific to the plasma membrane of rat lung microvascular endothelium. *Exp Cell Res* 232, 47-55 (1997)
40. Ghitescu LD, Gugliucci A, Dumas F: Actin and annexins I and II are among the main endothelial plasmalemma-associated proteins forming early glucose adducts in experimental diabetis. *Diabetis* 50, 1666-1674 (2001)
41. Ghitescu L, Robert M: Diversity in unity: the biochemical composition of the endothelial cell surface varies between the vascular beds. *Microsc Res Tech* 57, 381-389 (2002)
42. Murciano JC, Harshaw DW, Ghitescu L, Danilov SM, Muzykantov V: Vascular immunotargeting to endothelial surface in specific macrodomain in alveolar capillaries. *Am J Respir Crit Care Med* 164, 295-1302 (2001)
43. Schnitzer JE, Oh P, Pinney E, Allard J: Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytsosis, and capillary permeability of select macromolecules. *J Cell Biol* 127, 1217-1232 (1994)
44. Taylor ME, Drickamer K: Concepts of glycobiology. In: *Introduction to* Glycobiology, Oxford University Press, NY (2006)
45. Hermanson GT, Mallia AK, Smith PK: Immobilized affinity ligand techniques. Academic Press, San Diego California (1992)
46. Miller G: Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem* 31, 426-428 (1959)
47. Beesley JE: Immunolabelling techniques for light microscopy. In: *Immunocytochemistry,* edited by IRL Press, NY (1993)
48. Fornstedt N, Porath J: Characterization studies on a new lectin found in seeds of *VICIA ERVILLA*. *FEBS Letters* 57, 187-191 (1975)
49. Jacobson BS, Schnitzer JE, McCaffery M, Palade GE: Isolation and partial characterization of the luminal plasmalemma of microvascular endothelium from rat lungs. *Eur J Cell Biol* 58, 296-306 (1992)
50. Ahn M, Kim H, Matsumoto Y, Shin T: Increased expression of caveolin-1 and -2 in the hearts of Lewis rats with experimental autoimmune myocarditis. *Autoimmunity* 39, 489-495 (2006)
51. Boeynaems JM, Communi D, Gonzalez NS, Robaye B: Overview of the P2 receptors. *Semin Thromb Hemost* 31, 139-149 (1998)
52. Fiete D, Beranek MC, Baenziger JU: The macrophage/endothelial cell mannose receptor cDNA encodes a protein that binds oligosaccharides terminating with SO_4-4-GalNAcBeta1, 4GlcNAcBeta or Man at independent sites. *Proc Natl Acad Sci USA* 94, 11256-11261 (1997)
53. Gooderham K: High-sensitivity silver staining of proteins following polyacrylamide gel electrophoresis. In: Walker JM (ed) Methods in Molecular Biology. Humana Press , Clifton, N J (1984)
54. Wurzburg BA, Tarchevskaya SS, Jardetzky TS: Structural changes in the lectin domain of CD23, the low-affinity IgE receptor, upon calcium binding. *Structure* 14, 1049-1058 (2006)
55. Zelensky AN, Gready JE: The C-type letin-like domain superfamily. *FEBS J* 272, 6179-6217 (2005)

Abbreviations: ELM: endothelial luminal membrane, CELM: coronary endothelial luminal membrane, Mon-Pol(s): monosaccharide polymer(s), Gal: galactose, Man: mannose, Gal-Pol: galactose polymer, Man-Pol: mannose polymer, A-V: auricular-ventricular, A-H: auricular-bundle of His, SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis, K-H: Kreb's Henseleit, PMSF: phenylmethylsulphonyl fluoride, LB: lysis buffer, MBS: MES-buffer saline, FITC: fluorescein isothiocyanate, AP: polyacrylic acid.

Key Words: Coronary, Endothelium, Flow Sensation, Lectin, Monosaccharide

Send correspondence to: Dr. Rafael Rubio, Departamento de Fisiologia y Farmacologia, Facultad de Medicina, Universidad Autonoma de San Luis Potosi, Av. Venustiano Carranza #2405, San Luis Potosi, Mexico, C.P. 78210 Mexico, Tel: 444-826-2355, Fax: 444-826-2355, E-mail: rrubio@uslp.mx

http://www.bioscience.org/current/vol13.htm