Luminal endothelial lectins with affinity for N-acetylglucosamine determine flow-induced cardiac and vascular paracrine-dependent responses

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Many functions reside in the luminal endothelial glyocalyx: it limits the exchange of solutes between plasma, endothelial cell, and interstitium (54, 55) and mediates leukocyte rolling and extravasation (45) and maintenance of an anticoagulant environment (37). In addition, stimuli applied to the endothelial lumen modulate paracrymal functions through the release of paracrine effectors (18, 30, 35). One of these stimuli is blood flow (8, 9, 30, 35).

In 1963 Gregg described an increase in myocardial oxygen consumption induced by a rise in coronary blood flow (19, 27). Thereafter, others showed that augmented coronary blood flow stimulates heart glucose uptake (50); ventricular contraction (19, 47); auricular-ventricular transmission (47); spontaneous ventricular rhythm (47); and release of vasoactive agents such as nitric oxide (35, 40), prostaglandins (31, 38), endothelin (43), and auricular natriuretic peptide (41). All these cardioytic effects strongly suggest the existence of a paracrine interaction between coronary endothelium and cardiac parenchymal cells (18, 30, 35). However, the mechanism of flow sensation by the coronary vasculature nor the paracrine mediators are known.

Our laboratory has provided evidence that luminal endothelial glyocalyx structures are involved in flow detection. The endothelial glyocalyx is a 0.2- to 0.5-μm thick layer (54, 56) on the endothelial luminal membrane (ELM). This glycosilated and hydrated complex structure is composed of glycoproteins, proteoglycans, and glycolipids. They are anchored (glycoproteins and glycolipids) or absorbed directly (heparan sulphate, heparin, and hyaluronan) on the luminal membrane (56). The oligosaccharides that constitute the glyocalyx are mainly composed of galactose, mannose, N-acetylg glucosamine, fucose, xylose, and syalic acid. (52). Glyocalyx oligosaccharides are required for flow detection (22, 46, 47, 50).

We demonstrated the participation of coronary ELM (CELM) oligosaccharides in cardiac flow-induced responses. Intracoronary administration of lectins with specificity to oligosaccharides glycosidic moieties (46, 47, 50) and/or their selective enzymatic digestion both equally inhibited flow-induced cardiac responses (22, 46, 50). In addition, several publications have demonstrated the existence of stress-sensitive glycosilated proteins in the CELM glyocalyx (7, 14, 23, 33, 42, 61). Stress-sensitive Na+/Ca2+-dependent channels were demonstrated in endothelial cells by Lamsan et al. (36). Thereafter, in isolated blood vessels, Bevan and Joyce (8, 9) indicated that a Na+/Ca2+-dependent and amiloride-sensitive endothelial structure responded to flow. Recently, a family of highly glycosilated and amiloride-sensitive Na+/Ca2+ channels (ENaCs) have been proposed to be involved in mechanism transduction in vascular smooth muscle cells and in flow-induced endothelial responses (20, 36, 51, 55).
Lectins are a group of proteins widely distributed in nature that have high affinity for specific oligosaccharide sequences, a feature behind specific molecule-molecule recognitions (13, 45, 57). Our laboratory has shown that the CELM glycolalloy contains flow-sensitive lectins with affinity toward glucose and mannosae (4a, 28). Intracoronary infusion of various size dextrans in a concentration-dependent manner bind to CELM lectins altering flow-induced cardiac responses (26).

Similar results were obtained with mannose but not galactose probes of 460 kDa. These two probes were also used as affinity resins, and the corresponding CELM lectins were isolated (4a). Based on all these results we proposed that cardiac effects of coronary flow may result from a stoichiometric flow-modulated lectin-oligosaccharide interaction and that the number of these interactions is transduced to a quantitative release of endothelial paracrine messengers of parenchymal function.

Because N-acetylglucosamine (GlcNac) is one of the main components of glycolalloy oligosaccharides (i.e., hyaluronan [-4GlC(U/A)β1–3GlcNAcβ1–]) the aim of this article is to isolate and define CELM GlcNac-recognizing lectins and determine their functional role in cardiac and vascular flow-induced effects. For this purpose a GlcNac polymer (GlcNac-Pol) was synthesized and used as lectin probe. To study the vascular effects, we have adopted a well-accepted preparation: an isolated perfused blood vessel.

Our results show that the GlcNac probe upon binding to CELM lectins inhibits flow-induced cardiac and vascular responses, likely competing and displacing intrinsic hyaluronan from lectinic ENaC.

**METHODS**

All experimental procedures are succinctly described here and were approved by the Universidad Autonoma de San Luis Potosi Committee for the Use of Animals for Experimentation. A detail description can be found in the online supplemental version of this article.

**Synthesis of Soluble and Matrix-Insoluble GlcNac-Pol**

GlcNac-Pol both nonfluorescent and fluorescently labeled with FITC were synthesized using well-established procedures (29). Dextran (70 kDa) was the starting material, which was polymerized with GlcNac via divinyl sulfone (DVS) bridges (3 GlcNac and DVS molecules per glucose dextran moiety). The final product GlcNac-Pol had a molecular mass of 460 kDa. Soluble GlcNac-Pol was utilized for functional and tissue microscopy studies. A matrix-insoluble GlcNac-Pol was synthesized and utilized as a chromatography affinity probe.

**Isolated Perfused Guinea Pig Heart**

Anesthetized male Dunkin Hartley guinea pigs (380–400 g) were utilized. The heart was retrogradely perfused at constant flow of 8 ml/min, according to the method of Langendorff (47) with oxygenated Krebs Henseleit (pH 7.4) 37°C. Heart rate was kept constant at 4.5 Hz by application of electric pulses to the right atria.

**Physiological Cardiac Studies**

Ventricular contraction measurements: inotropic effect. Developed ventricular systolic and diastolic pressures were continuously recorded by a fluid-filled balloon inserted in the left ventricle. Differences between these two pressures defined contraction amplitude. At a coronary flow of 8 ml/min the contraction amplitude was taken as control and defined as 100%. For each heart contraction amplitude values were expressed as a percentage of its own control. The mean control contraction amplitude was 31 ± 8 mmHg.

**Auricular-ventricular delay measurements: dromotropic effect.** Auricular-ventricular (A-V) delay (in ms) was recorded via two electrodes connected to an oscilloscope. One electrode was placed in the left atrium and a second electrode on the apex of the left ventricle. This interval directly and solely reflects the change in electrical conduction time through the A-V node (47).

At a coronary flow of 8 ml/min the A-V delay was taken as control and defined as 100%. For each heart A-V delay values were expressed as percentages of its own control. The mean control A-V delay value was 105 ± 12 ms.

**Dromotropic and isotropic effects of coronary flow under control condition and after GlcNac-Pol infusion and wash.** To study the effects of GlcNac-Pol, its maximal effective concentration was first determined from dose-response curve in six hearts and found to be 100 μM GlcNac-Pol. Control A-V delay-coronary flow and ventricular contraction-coronary flow (6 to 12 ml/min) curves were simultaneously performed. Each flow step was maintained constant for a period of 2 min, and the steady state responses were determined. Thereafter, GlcNac-Pol (100 μM) was administered for 10 min followed by a wash with K-H for by 5 min. Thereafter, A-V delay-coronary flow and ventricular contraction-coronary flow curves were repeated (n = 6).

**Statistical analyses.** Paired Student t-tests were performed on paired positive/negative variable values (in percentages), and results with P < 0.05% were taken as statistically significant.

**Binding of FITC-GlcNac-Pol to the CELM**

**Demonstration of binding of GlcNac-Pol to luminal wall of cardiac coronary vessels.** Isolated guinea pig hearts (n = 3) were coronary perfused with buffered paraformaldehyde, followed by a washoff by perfusion with K-H. Thereafter, a 5-min perfusion with K-H containing 100 μM of 50% FITC-GlcNac-Pol was followed by a washoff with a 5-min perfusion with K-H to remove nonattached 50% FITC-GlcNac-Pol. Frozen 20-μm tissue sections were cut and air-dried overnight at room temperature. To identify arteries, arterioles, and capillaries, vessel size was measured and the tissue selectively immunoabeled. Smooth muscle of arteries and arterioles was immunolabeled with anti-α-actin and endothelium labeled with von Willebrand factor, slices washed with TBS, and incubated with a rhodamine-coupled secondary IgG. Tissue images were taken at the following excitation and emission wavelengths (λ): FITC fluorophore (FITC-GlcNac-Pol) λex = 495 nm and λem = 519 nm. For rhodamine fluorophore (Trite) λex = 552 nm and λem = 578 nm. In 10 tissue sections, all observed arteries, arterioles, and capillaries showed their positive immunoreactions (positive controls). Incubation with the secondary antibody alone resulted in no fluorescence signal.

**Isolation of CELM Proteins**

Isolation of protein from the coronary endothelial luminal membrane (CELM) is possible thanks to the method developed and refined by different groups (5, 6, 15, 25, 48, 49). This procedure consists of coating CELM with cationic colloidal silica (20–50 nm diameter) due to binding to glycolalloy anionic proteins, washing off excess silica, and polymerizing ELM-bound silica with an anionic polymer (AP). The AP cross-links the silica particles and also neutralizes all of its free positive charges. This charge neutralization prevents binding of proteins foreign to CELM. The CELM-silica-AP pellicle produced is very stable, resists ulterior processes like tissue homogenization and centrifugation, and does not drag protein contaminants (5, 6, 15, 25, 48, 49). All these studies support this method as appropriate for isolation of CELM proteins.
Characterization of CELM Lysate

Previously, many groups demonstrated that the silica pellicle technique is appropriate to isolate highly purified ELM proteins (5, 6, 15, 25, 48, 49). However, to ensure that our modified CELM extraction procedure does not contain nonluminal proteins we performed the following tests.

In three separate hearts during tissue homogenization a saturating concentration of a non-CELM alien protein bovine serum albumin (5× total tissue protein) was added; this protein was chosen because it is not present in isolated saline perfused hearts and it can bind reversibly to at least three CELM albumin-recognizing proteins (48) and unspecifically binds strongly to cationic silica (15). The final electrophoretic profiles of isolated CELM did not present albumin (not shown). This indicates that during the homogenization and centrifugation procedure albumin did not contaminate/bind to CELM fraction or to silica.

We also immunoprobed CELM proteins by SDS-PAGE and Western blot analysis with antibodies directed against luminal endothelial positive protein markers caveolin-1, VCAM, and PECAM-1. All antigen-recognizing CELM proteins (VCAM, PECAM-1, caveolin-1, von Willebrand factor, and mannose receptor) reacted positively, whereas the non-CELM protein-antigens (P2Y1–4) did not react; results are not shown.

This evidence indicates that the resulting final CELM-silica-AP pellicle is representative of luminal endothelial membrane organelle.

Separation of Lectins from CELM Lysate by Affinity Chromatography

Affinity chromatography columns: construction and affinity tests. Lectins were separated based on their selective monosaccharide binding affinity. Affinity columns for lectins that recognize GlcNac (GlcNac-Pol) were built. These columns selectively retained the lectin Ulex europeaus II (GlcNac-Pol) and did not retain Concanavalin A with affinity for mannose. The retained lectin needed to be eluted with buffer 200 mM free GlcNac. Eluted protein was identified by SDS-PAGE. These results indicate that our affinity chromatography columns were selective and have the ability to retain lectinic proteins with affinity for N-acetylglucosamine.

Separation of N-Acetylglucosamine-Recognizing Lectins from CELM Lysates

CELM proteins (0.5 mg in 2 ml) were previously dialyzed against 0.1 mM PBS (pH 7.4) and 0.2 mM CaCl2 and were poured into a GlcNac-Pol affinity column (52). Thereafter, nonbound proteins were eluted with PBS and bound lectins eluted with 10 ml 200 mM of free N-acetylglucosamine in 0.1 mM PBS (pH 7.4) and 0.4 mM EGTA (52). All eluates were extensively dialyzed against water for 1 wk at 4°C, lyophilized, reconstituted in a small volume, and examined via one- and two-dimensional electrophoresis.

Two-Dimensional PAGE of CELM Lectins

Protein (20 μg) was loaded on 11-cm strips with a pH gradient 4–7. Focusing was carried out using an isoelectric focusing system at 20°C at the three step and hold voltage mode for a total of 17.6 V-h. The second dimension SDS-PAGE was performed in 12% polyacrylamide gels, run at 20°C first with a current of 20 mA for 20 min and then for 2 h at 50 mA. Gels were SyproRuby stained and photographed on an ultraviolet transiluminator.

Determination of β-ENaC in Whole CELM Lysate and CELM Lectinic Fraction

To test the existence of β-ENaC in the luminal membrane of coronary endothelium and determine their lectinic characteristics we perform dot blots of whole CELM extract and GlcNac lectinic fraction. The dot blot was blocked with 3% albumin and incubated for 2 h with primary antibody to β-ENaC wash and incubated with the secondary antibody and revealed.

Flow-induced Vasodilation Response

An isolated blood vessel preparation was selected because it is an accepted model for studies of endothelial-parechymal (smooth muscle) interactions (8, 9, 28, 44).

Isolated guinea pig aortas perfused with oxygenated K-H at 37°C containing 10 μM phenylephrine were utilized. The thoracic aorta was dissected and excised from the end of the aortic arch to the diaphragm. A cannula was placed in both ends of the vessel. The cannula in the aortic arch side was connected to a pump, and through the cannula at the distal end a small diameter fluid-filled catheter was inserted with its tip located at mid-length of the blood vessel. The opposite end of this catheter was connected to a pressure gauge.

Flow-induced vasodilation determinations. Phenylephrine was required to produce a sustained state of smooth muscle tone. Aortas were perfused at 8 ml/min for 10 min (basal period), and the resulting aortic pressure was defined as basal pressure. Mean basal pressure for all experiments was 35.3 ± 6.2 mmHg. To determine flow-induced vasodilation (FIV), flow was step increased from basal (8 ml/min) either 25 or 50 or 75 ml/min and each step sustained for 10 min. In all cases, during each basal period 10 μM of l-arginine was perfused for 5 min and washed for 2 min.

For each flow step, aortic pressure rapidly rose to a peak value and gradually decreased to a lower plateau value. We measured the difference between the peak pressure and the basal pressure (Δpeak) and difference between the plateau pressure and basal pressure (Δplateau). The difference, Δpeak − Δplateau/Δpeak × 100 was taken as flow-induced FIV response.

Effects of GlcNac-Pol on FIV Response

Once the control FIV curve was obtained, 10 μM of GlcNac-Pol was infused during a basal period and washed for 2 min. Thereafter, a new FIV curve was determined. Control and experimental FIV curves were compared. Each aorta was its own control (n = 6, paired t-test, P < 0.05).

Effect of Amiloride on FIV Response

To test for the participation of nitric oxide and prostaglandins on FIV a control curve was performed. The curve was repeated with 100 μM of nitro-l-arginine methyl ester (l-NAME) and 10 μM indomethacin in the perfusion media (n = 6, paired t-test, P < 0.05) (1, 59).

In another set of experiments the effects of GlcNac-Pol during l-NAME plus indomethacin were tested. The control FIV curve was performed and thereafter 100 μM of l-NAME and 10 μM indomethacin plus 10 μM of GlcNac-Pol were infused for 10 min. GlcNac-Pol was then washed for 2 min while continuing l-NAME plus indomethacin infusion, and a new FIV curve was determined (n = 6, paired t-test, P < 0.05). This curve was not different from those with l-NAME and indomethacin alone or with GlcNac-Pol alone (results not shown). These results indicate that the independent effects of l-NAME and indomethacin and those of GlcNac-Pol are via the same mechanism. Consequently, when applied together there is no addition of their effects.

Effect of Amiloride on FIV Response

ENaCs are very sensitive to amiloride blockade (20). To test the possible participation of ENaC in FIV we tested the effects of amiloride on FIV. A control FIV curve was performed followed by a curve with 30 μM amiloride in the perfusion media (n = 6, paired t-test, P < 0.05).
Effect of Hyaluronan Hydrolysis on FIV

Hyaluronan participates in flow sensing (46, 47). To determine its participation on FIV we perform FIV response at 75 ml/min during control and after enzymatic hydrolysis of hyaluronan. To administer hyaluronidase, a bolus of 1 ml K-H containing hyaluronidase (0.5 U/ml) was administrated, and 30 s later the aorta was washed for 1 min (8 ml/min) and hyaluronidase administration was repeated six times followed by a second FIV determination. Thereafter, hyaluronidate (10 µg/ml) was added to perfusion media and administered for 6 min and washed and a third FIV determination was performed \textit{(n = 3, paired t-test)}.

RESULTS

Studies in Isolated Heart

GlcNac-Pol alters flow-induced inotropic and dromotropic effects. It is well established (19, 46, 47) that increments of coronary flow cause positive inotropic and dromotropic effects (Fig. 1). Once the control curves were obtained, 100 µM GlcNac-Pol was infused. Because its size (460 kDa) and the short period of perfusion (10 min) this polymer remains confined in the intravascular space (17, 54). Thereafter, GlcNac-Pol excess was washed out for 5 min and a new set of function-flow curves was obtained. The results show that GlcNac-Pol cause a significant \textit{(P < 0.05)} shift of the inotropic curve downward (Fig. 1A) and dromotropic curve upward (Fig. 1B) and reveal that its inhibitory effects persist after it has been washed away. These results suggest that GlcNac-Pol binds to the luminal endothelial surface and upon binding exerts its inhibitory effects on flow-induced responses.

Binding of GlcNac-Pol to CELM. To test whether GlcNac-Pol binds and remains bound to CELM, FITC-GlcNac-Pol was infused and washed and confocal images were obtained. Capillaries or arteries were identified by their diameter and immunoreactivity to specific markers. These results revealed the polymer bound to the endothelium in capillaries (Fig. 2A) and small arteries (Fig. 2D). Capillaries show immunoreactivity to the endothelial marker von Willebrand factor and have diameters of 5 to 10 µm (Fig. 2B). Arteries were characterized by the vascular smooth muscle α-actin reactivity in the media (Fig. 2E), and their diameters were 300 µm. Superposition of images 2A and 2B (Fig. 2C) shows the combined fluorescence for both labels. This indicates that FITC-GlcNac-Pol and von Willebrand are coincident in the endothelial cell. In the case of the artery, there is a very poor degree of overlap of FITC-GlcNac-Pol and α-actin (Fig. 2F) fluorescence, indicating the two markers are in different cells. The apparent overlap of the two probes in parts of the tissue section is the result of the plane of the tissue section, which is not normal to the blood vessel axis, and the limited resolution of light microscopy, which is below the average endothelial cell thickness (0.2 µm). These microscopic and functional results described above indicate that the 460-kDa probe GlcNac-Pol binds with high affinity to CELM structures and is not easily washed away. These results together suggest the presence of GlcNac-recognizing lectins in the coronary luminal membrane.

Isolation of the CELM lectins with affinity to GlcNac-Pol. Highly purified whole CELM proteins were first obtained using the silica pellicle technique (5, 6, 15, 25, 48, 49). The electrophoretic pattern of the resulting proteins (Fig. 3A) shows a large number of bands. These CELM proteins were poured into an affinity chromatography column of GlcNac-Pol; unbound proteins were washed off, and retained proteins were eluted with free GlcNac. The SDS electrophoresis pattern shows 20 lectinic protein bands. To determine the number of individual peptides, the lectinic fraction was submitted to two-dimensional SDS-PAGE, revealing at least 35 individual lectinic peptides (Fig. 3C).

Determination of ENaC in the CELM and characterization of their lectinic properties. Dot blots of whole CELM extracts (Fig. 5A) demonstrate the existence of β-ENaC in the luminal membrane of coronary endothelium. Dot blot assay of the lectins eluted from the GlcNac affinity column
(Fig. 5AII) reveals that CELM β-ENaC have affinity toward GlcNac-Pol, i.e., is lectinic.

**Studies Performed in Isolated Blood Vessel**

*Modifications in aortic FIV by GlcNac-Pol.* FIV response was measured at different flows in control and postinfusion of GlcNac-Pol. Traces from a representative individual experiment are shown in Fig. 4A. In control conditions, it is seen as flow rises from a basal flow of 8 ml/min either to 25, 50, or 75 ml/min; there is a perfusion pressure increase (left traces), which gradually drops (FIV). The drop in pressure (FIV) increases with flow. After the blood vessel was infused with GlcNac-Pol, washed, and subjected to the same step flow increases (right traces), the FIV responses were diminished. Similar experiments, in other aortas, were performed during control and after treatment with L-NAME and indomethacin (Fig. 4B), which are nitric oxide and prostaglandin synthesis inhibitors, respectively (1, 59) and amiloride (Fig. 4C), a β-ENaC inhibitor (3, 8, 9, 20, 51). GlcNac-Pol, L-NAME, and indomethacin and amiloride all inhibited FIV. The mean values of these three different experiments are shown in Fig. 4D. At flow rates of 50 and 75 ml/min there were significant differences between control and experimental group (*P* < 0.05). The inhibitory effects of GlcNac-Pol and L-NAME plus indometh-
Hyaluronan plays an important role in FIV. Release of nitric oxide and prostaglandins, the mediators of FIV, are also very sensitive to amiloride blockade. Our FIV responses were blocked by the same concentration of amiloride used by these investigators (3, 8, 9, 20, 51); in addition, in the CELM we demonstrate the existence of β-ENaC and that β-ENaC has affinity for GlcNac. All of these results taken together indicate that GlcNac-Pol inhibits FIV, a paracrine-mediated process, by competing and displacing intrinsic hyaluronan bound to a lectinic structure that is amiloride sensitive ENaC. Nitric oxide and prostaglandins are the paracrine mediators of FIV.

**DISCUSSION**

Blood flow is an important hemodynamic stimulus that affects the cardiac parenchymal and smooth muscle cell function (19, 27, 31, 38, 40, 41, 43, 47, 50). We and others have proposed that flow acts directly on the luminal membrane of vascular endothelium, and this results in parenchymal effects due to release of paracrine mediators. It is accepted that glycosylated and lectinic structures are participants in the flow sensing process (18, 19, 30, 31, 35, 38, 41, 43).

To demonstrate the role in flow sensation by CELM GlcNac-recognizing lectins, we synthesized a large nondiffusible 460-kDa lectin binding probe GlcNac-Pol, which due to its large size and the short period of infusion cannot cross the endothelial wall (17) and only binds to and acts on CELM structures. In the heart intracoronary administration of GlcNac-Pol upon CELM binding diminishes the flow-dependent positive inotropism on FIV were the same. In addition, we infused GlcNac-Pol jointly with L-NAME and indomethacin (n = 6), which did not cause additive effects (data not shown).

Hyaluronan is a very large polysaccharide composed of subunits of [-4GlcUAβ1-3GlcNAcβ1-]n (52) that enriches the endothelial luminal membrane environment with GlcNac (50% of hyaluronidate mass). Hyaluronan has been demonstrated as an important participant on endothelial flow sensing (40, 46); consequently, we decided to explore its role in FIV. We found that the enzymatic digestion of this polysaccharide abolishes the FIV (P < 0.05, Fig. 5B) as well as GlcNac-Pol, L-NAME-indomethacin, or amiloride. But when hyaluronan was restored to the endothelium by perfusion, the FIV was reverted toward control (Fig. 5B) (46). These results indicate ELM hyaluronan plays an important role in FIV.

Lansman et al. (36) demonstrated stress-activated Na+/Ca++ ion channels in vascular endothelial cells. Later Bevan and Joyce (8, 9) demonstrated FIV to be dependent of extracellular Na+ and Ca++ and to be very sensitive to blockade by amiloride. More recently, the existence of Na+/Ca++ stress-sensitive channel family was demonstrated, ENaCs (3, 20, 51), which also are very sensitive to amiloride blockade. Our FIV responses were blocked by the same concentration of amiloride used by these investigators (3, 8, 9, 20, 51); in addition, in the CELM we demonstrate the existence of β-ENaC and that β-ENaC has affinity for GlcNac. All of these results taken together indicate that GlcNac-Pol inhibits FIV, a paracrine-mediated process, by competing and displacing intrinsic hyaluronan bound to a lectinic structure that is amiloride sensitive ENaC. Nitric oxide and prostaglandins are the paracrine mediators of FIV.

![Flow-induced vasodilation (FIV) in the aorta. GlcNac-Pol, nitro-L-arginine methyl ester (L-NAME; LN) and indomethacin (Indo), and amiloride equally block FIV. Control traces from 3 different experiments (A, B, and C) show how aortic flow increases from 8 to 25, 50, or 75 ml/min, which causes a rise in perfusion pressure followed by a gradual relaxation FIV. Thereafter, application of GlcNac-Pol (A) or L-NAME plus indomethacin (B) or amiloride (C) resulted in blockade of FIV. D: mean results from these experiments. Differences between control and corresponding blocking effects were significant for flows of 50 and 75 ml/min (P < 0.05). These results suggest that FIV results from activation of amiloride/stress-sensitive Na+/Ca++ channels (ENaC) and GlcNac-Pol effects are mediated by the inhibition of production or release of nitric oxide and prostaglandins, the mediators of FIV.](http://ajpheart.physiology.org/)

![Isolation of CELM lectinic ENaC and identification of their possible polisaccharidic ligand. Dot blot of whole CELM protein extract demonstrated the existence of β-ENaC in the luminal membrane of coronary endothelium (A). β-ENaC was also identified in the fraction of CELM lectins with affinity for GlcNac (A), indicating that ENaC have lectinic characteristics. To identify the possible glycosidic ligand of ENaC, the effects of hydrolysis of hyaluronan ([−4GlcUAβ1−3GlcNAcβ1−]n polysaccharide) on FIV were measured. Hyaluronan hydrolysis causes a significative diminution in FIV (B, white column). P < 0.05. Reconstitution of hyaluronan adding hyaluronidate to perfusion media is able to recover FIV.](http://ajpheart.physiology.org/)
pic and dromotropic effects. It is known that GlcNac-Pol cannot cross the endothelial wall due to its molecular size of 460 kDa (17); consequently, it can only bind and act in CELM structures such as lectins. This interpretation is supported by our microscopy studies that show that after perfusion and washout of FITC-GlcNac-Pol, it remains strongly bound to the lumen of coronary endothelium. This agrees with what is known about the interaction between lectins and their specific ligands (52). In addition, we use GlcNac-Pol as an affinity probe and isolated GlcNac-recognizing lectins from the CELM. The purified CELM whole fraction when passed through the GlcNac-Pol affinity column retained GlcNac-lectins that when eluted and developed in one- and two-dimension SDS-PAGE revealed at least 20 lectinic bands and 35 individual peptidic spots, respectively. β-ENaC was present in the CELM and is lectinic because it has affinity toward GlcNac.

Finally, we adopted a well-accepted flow-responsive model of endothelial-parenchymal paracrine interaction (8, 9, 28, 44): isolated blood vessels perfused at controlled flow rates. We demonstrated that FIV is blocked by ELM-bound GlcNac-Pol, l-NAME and indomethacin, and amiloride, as well by hyaluronan enzymatic digestion.

All of these results taken together indicate that GlcNac-Pol inhibits FIV, a paracrine-mediated process, by competing and displacing intrinsic hyaluronan bound to a lectinic structure that is amiloride sensitive: ENaC. Nitric oxide and prostaglandins are the paracrine mediators of FIV (34, 59, 60).

**Characterization of N-Acetylglucosamine-Recognizing CELM Lectins**

Well-established data (5, 6, 15, 25, 48, 49) indicate that the resulting ELM-silica-AP pellicle is a representative sample of the ELM organelle. We have further ruled out the possibility that during homogenization and centrifugation alien proteins contaminate the CELM-silica-AP fraction (4a). Thus there is a low probability of alien protein contamination of the silica-CELM fraction.

N-acetylglucosamine-recognizing lectins were isolated from CELM using GlcNac-Pol as binding matrix in affinity purification columns. These affinity chromatography columns were tested in the presence of Ca$^{2+}$ for specificity and discriminative C-type lectin binding (52). When the CELM peptides with GlcNac affinity were visualized on two-dimensional SDS-PAGE, there were 35 peptide spots. However, which of these individual CELM lectins are involved in parenchymal function-blood flow responses remains to be elucidated; yet their individual isolation constitutes a first necessary step for their molecular and functional identification. The functional implication of the lectinic nature of these numerous CELM proteins remains unknown.

**Role of Lectins on Parenchymal Function-Coronary Flow Responses**

As described here for GlcNac-Pol, our laboratory previously (4a, 28) synthesized two polymers, one of mannose (Man-Pol) and another of galactose (Gal-Pol). These three monosaccharide-polymers (Mon-Pol) respectively bind to specific N-acetylglucosamine-mannose- and galactose-recognizing plant lectins in vitro. Our Mon-Pols bind in situ to the CELM of ventricular vasculature and, once bound, exert differential cardiac effects. The orders of inhibitory potency of Mon-Pols on flow-induced positive dromotropism were GlcNac-Pol ≳ Man-Pol ≳ Gal-Pol ≳ 0. However, of the three Mon-Pols, only GlcNac-Pol exerted an inhibitory effect on flow-induced positive inotropism: GlcNac-Pol ≳ Man-Pol ≳ Gal-Pol ≳ 0. The apparent dromotropic activity sequence GlcNac-Pol ≳ Man-Pol ≳ Gal-Pol ≳ 0. The apparent dromotropic activity sequence GlcNac-Pol ≳ Man-Pol ≳ Gal-Pol ≳ 0 could result from Mon-Pols differential binding to capillaries in the A-V-nodal area and adjacent to contractile ventricular myocytes, respectively. This suggests that there is heterogeneity in CELM molecular composition throughout the coronary vascular network (2, 4, 12, 25) that results in a region-specific physiology.

On the other hand, the number of lectin spots on two-dimensional SDS-PAGE from affinity chromatography fractions of different Mon-Pol differs considerably (GlcNac-Pol ≳ Man-Pol ≳ Gal-Pol). With Gal-Pol, 100 lectins were isolated, with Man-Pol 9 and 35 with GlcNac-Pol. These results indicate that in situ effects of Gal-Pol, Man-Pol, and GlcNac-Pol and their distinct numerical differences in binding sites are not due to unspecified massive binding to the CELM but rather to a specific recognition by luminal lectin proteins.

However, it is important to consider that Mon-Pol is a lectin probe that binds to CELM lectins and affects parenchymal function-coronary flow responses, but it could also act on CELM flow sensors of a nonlectinic structure. This possibility raises the questions of whether it is a prerequisite property that a flow sensor is lectinic or whether lectinic properties are only a fortuitous way to identify these flow sensors. These questions remain to be investigated.

**Effects of GlcNac-Pol on Flow-induced Relaxation of the Aorta**

Isolated blood vessels perfused at controlled flow rates are a well-accepted flow-responsive model of endothelial-parenchymal paracrine interaction (8, 9, 28, 44). We demonstrated that FIV is blocked by ELM-bound GlcNac-Pol, l-NAME and indomethacin, amiloride, and enzymatic hydrolysis of the hyaluronan of ELM. Reinfusion of hyaluronan restores FIV. All of these results indicate that GlcNac-Pol inhibits FIV, a paracrine-mediated process, by competing and displacing intrinsic hyaluronan bound to a lectinic structure that is amiloride sensitive: ENaC. Nitric oxide and prostaglandins are the paracrine mediators of FIV (24, 32, 34, 59, 60).

The work of Bevan and Joyce (8, 9) indicated that FIV was Na$^+$/Ca$^{2+}$ dependent and very sensitive to amiloride. The existence of an endothelial stress-sensitive Na$^+$/Ca$^{2+}$ channel was previously demonstrated by Lansman et al. (36). These results suggested ENaC be ELM protein responsible for FIV (3, 20, 51). ENaC have a very long extracellular domain in the extracellular matrix (3, 20, 51). This property makes possible the interaction of ENaC with other glyco-calyx structures. Here we establish that β-ENaC is present in the CELM and is lectinic with affinity for GlcNac. Our work suggests GlcNac-Pol and hyaluronan compete for binding with the ELM ENaC. Furthermore, since amiloride and GlcNac-Pol inhibit equally FIV, we propose both agents act on ENaC. However, since amiloride is a small molecule able to extravasate and act upon parenchymal cells, we cannot rule out the participation of ENaC in smooth muscle. In this case the observed results must be an algebraic sum of effects in endothelium and smooth muscle.
Possible Role of CELM Oligosaccharide-Lectin Interaction on Parenchymal Function-Coronary Flow Responses

We and others have demonstrated that CELM glycosilated and lectinic structures also are involved in flow sensing processes (4a, 22, 26, 28, 40, 44, 46, 47, 50). Concerning the lectinic structures of CELM, only our laboratory has shown that administration of polymers either of glucose, mannose, galactose, and now GlcNac-Pol bind to CELM lectins and induce differential changes in cardiac flow-induced responses. These probes also allow for the isolation of CELM lectins (4a, 26, 28).

These findings suggest that a requirement for a molecule to participate in flow sensation is glycosylation or glycosylation recognition: lectinic. But perhaps these two chemical properties are only fortuitous and not required for flow sensing (4a, 22, 26, 28, 44, 46, 47, 50). It is possible that any molecule upon binding to a flow sensor would disturb the flow effects. For example, glycosylation may be necessary to sense blood flow because it assures a coat layer of hydration in the structure. Does this bound water continuously exchange with adjacent flowing water resulting in structure deformation? Or does blood flow modulate complexing of CELM glycosylated moieties with constitutive lectins?

A number of ELM transmembrane proteins have been identified as mechanosensors: VCAM-1, ICAM-1, PECAM-1, α,β, G protein-coupled receptors (B subunit, β, and γ subunits, α,β, and γ subunits, α,β, and β subunits, α, and β subunits, and ENaC, are glycosylated transmembrane proteins; some are lectinic whereas for others it is unknown if they are lectinic. Lectinic interactions regulated by mechanical stress are highly probable in the luminal endothelial membrane-glycocalyx where we have identified more than one hundred lectins (4a, 22, 28, 40, 44, 47, 50). Our evidence suggests that extracellular domains of ENaC could be lectinic and in situ interacts with glycosilated molecules such as hyaluronan.

In the case of integrins and ENaC it is known that anchor groups are required for stress sensing. It may be that the degree of complexing between these two strucutures is stoichiomertically regulated by mechanical stress. If this were the general principle that governs stress biological sensing, glycosylation and lectin properties are requirements for stress sensors to function, i.e., regulated and reversible stoichiometric complexing between anchor and lectin induced by flow/stress could be of an oligosaccharide-lectin nature. We propose the following hypothesis where O is oligosaccharide and L is lectin:

\[
O + L \rightarrow O \cdot L
\]

Cell signaling could result from either formation of O-L or its dissociation into O and L. This hypothesis implies that the extracellular domain of the receptor, a transmembrane protein, interacts with specific endothelial glycocalyx structures. The specific effect of different glycanases, lectins, and Man-Pol on flow-induced responses supports this idea (4a, 22, 28, 40, 44, 47, 50).

In summary, we demonstrated the existence of a broad spectrum of CELM lectins with affinity for GlcNac, one of them a β-ENaC. These CELM lectins strongly bind our polymer and some of these proteins mediate the cardiac and vascular flow-induced responses. In the aorta, GlcNac-Pol may compete and displace hyaluronan from lectinic ENaC.

GRANTS

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DISCLOSURES

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REFERENCES


