G protein-coupled receptors mediate coronary flow- and agonist-induced responses via lectin-oligosaccharide interactions

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Perez-Aguilar S, Torres-Tirado D, Martell-Gallegos G, Velarde-Salcedo J, Barba-de la Rosa AP, Knabb M, Rubio R. G protein-coupled receptors mediate coronary flow- and agonist-induced responses via lectin-oligosaccharide interactions. Am J Physiol Heart Circ Physiol 306: H699–H708, 2014. First published December 20, 2013; doi:10.1152/ajpheart.00481.2013.—Blood flow acts parallel to the coronary luminal endothelial surface layer (LESL) and modulates multiple parenchymal functions via the release of paracrine agents. Evidence indicates that the LESL may be a flow-sensing organelle and that perhaps through flow-induced lectin (L)·oligosaccharide (O) complex formation (L·O) participates in this process. LESL integrins and selectins are both lectinic and flow sensitive, but the L properties of flow-sensitive G protein-coupled receptors (GPCRs) are unknown. Therefore, we investigated the presence of L in the LESL and hypothesized that if flow-sensitive GPCRs are L, flow and O will determine their response to receptor activation. The LESL protein fraction isolated from guinea pig hearts was passed through an affinity chromatography column made of three sugars, mannose, galactose, and N-acetylgalucosamine, and the lectinic fraction was eluted. Immune dot blot was used to identify L proteins in the LESL fraction. Our results indicate the following. 1) Two-dimensional SDS-PAGE (2D-SDS-PAGE) of the LESL lectinic fraction revealed at least 167 Ls. 2) Among these Ls, we identified three selectins and the GPCRs: angiotensin II, bradykinin (B2-R), adenosine A1 and A2, prolactin, endothelin, α1-adrenergic (α1A-R), thromboxane A2, β1-adrenergic, β1-adrenergic, and insulin receptors; the first six GPCRs are known to be flow sensitive. 3) The amplitude of receptor-induced vascular responses by α1A-R and B2-R activation (phenylephrine or bradykinin, respectively) was a function of flow and O (hyaluronidate). Our results support a novel mechanism of GPCR-mediated responses to flow via L·O interaction.

flow sensitivity requirements; G protein-coupled receptors; lectins and sugars of glyocalyx; mechanosensors; regulatory extracellular matrix

One of the proposed mechanisms has been that some of the flow-sensing properties may reside in the glyocalyx of the luminal endothelial surface layer (LESL), which has an ideal location to be acted upon by flow (3a, 17, 19, 30, 35, 37, 38, 40, 41, 48, 49). The LESL is 0.2–0.7 µm thick and made of highly hydrated polyanions, numerous diversely glycosylated proteins, glycosaminoglycans, proteoglycans, and glycolipids (3, 12, 30, 47, 56, 57) that on the LESL are distributed in well-ordered microdomains (60). We and others have shown that flow-induced responses require the presence of LESL glycosidic, lectinic structures. Interestingly, several flow-sensitive G protein-coupled receptors (GPCRs) are present in the LESL (2, 7, 8, 9, 26, 33, 38, 42, 46, 54, 58, 61, 62).

Coronary flow (CF) regulation of diverse cardiac functions as well as other flow-regulated vascular functions requires the presence of various LESL oligosaccharides. These CF effects are specifically obliterated if LESL-specific glycans are enzymatically hydrolyzed or bound to selective plant lectins. For example, if N-linked β1,3 N-acetylgalucosamine (GlcNac) polymers are enzymatically hydrolyzed by endoglycanase-H or bound to the lectin of Lycopersicon esculentum, both act equally to depress CF-induced responses (40). Similar results were demonstrated with other enzyme-lectin pairs (40). The glycosaminoglycans hyaluronidate and heparinate are also important participants in flow-mediated responses (3a, 30, 35, 38, 40, 41, 49), but we do not know whether being glycosidic is obligatory to sense flow. Interestingly, the roles of LESL sugars in flow-induced responses are specific for diverse functions (3a, 15, 19, 30, 38, 40, 41), but our understanding of the mechanism behind this specificity is incomplete.

Our laboratory has shown that LESL lectinic proteins also participate in flow sensing (3a, 17, 19, 38). Lectins have high affinity for specific oligosaccharide sequences, a requisite characteristic for specific molecule-molecule recognition (52, 59). LESL contains lectins with affinity toward glucose, mannose, galactose, GlcNac, and likely other sugars (3a, 17, 19, 37, 38). Intracoronary infusions of various polymers of galactose, mannose, or GlcNac that bind irreversibly to LESL lectins alter CF-induced cardiac responses in a concentration-dependent manner. Only the last two polymers inhibited irreversibly CF-induced inotropism and dromotropism and blood vessel vasodilation (3a, 38). These results indicate that their physiological effects are not the result of indiscriminate binding but imply a selective chemical interaction. Thus the identity and number of LESL proteins whose function derives from their lectinic nature need to be defined. These three monosaccharide polymers were also used separately as affinity resins to isolate and separate by two-dimensional SDS (2D-SDS) three appar-
ent groups of lectins: mannose-, galactose-, and GlcNac selective. The numbers of the three groups amounted to a total of at least 144 LESL lectinic proteins (3a, 38). Because lectins can have affinity for more than one monosaccharide, the total number of lectinic proteins may be less than 144 if a lectin binds to more than one sugar. To avoid this problem and because it is important to define the number of lectins in the LESL, we have now created an affinity resin with a combination of the three monosaccharide polymers. These results are shown in the present report.

Flow is a multifunctional stimulus of diverse cardiac functions, blood vessel tone, and release of multiple bioactive agents (4, 13, 18, 23, 25, 27, 29, 32, 37, 41, 49, 53, 60). Furthermore, these various flow-induced responses can be dissociated from one to another, suggesting the existence of multiple flow-sensing structures. This is possible because a number of molecules are affected by flow, although they are not necessarily direct flow sensors. These are core proteins (46), G proteins (33), caveola structural components (39, 45), receptor tyrosine kinases and integrins (31, 42, 46), the cell-adhesion proteins VCAM-1 and PECAM-1, αβ-integrin, sialyl-Lewis-selectins (24, 34, 41), ion channels, purinergic ion channel P2X4, and an increasing list of GPCRs (2, 9, 26, 28, 33, 38, 42, 54, 58, 62). With the exception of the first two, these are transmembrane glycosylated proteins and may be lectinic. It is important to determine whether lectinic properties are a necessary requirement for flow sensing.

The lectinic nature of receptors appears to be critical for their function. The ion channel-glutamate receptors are lectinic, and their conductivity and affinity for their agonist are properties that are a necessary requirement for flow sensing.

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METHODS

Isolated, Perfused Guinea Pig Heart Used for Protein Isolation from LESL

Heart preparation. All experimental protocols used in this work were approved by the Animal Research Committee of the Faculty of Medicine of the University of San Luis Potosi according to international guidelines. In Mexico the humane use of animals for experimentation is regulated by Federal and State laws modeled after international laws, which demand, define, and establish humane standards for maintenance and handling of animals identical to those defined in the Guide for the Care and Use of Laboratory Animals. Guinea pigs (1.0–1.2 kg body wt) were anesthetized with pentobarbital sodium. The heart was retrogradely perfused with MBS (50 ml, pH 7) at a constant pressure of 70 cmH2O, followed by successive perfusions with MBS (pH 6 and pH 5, 10 ml each). Thereafter, 1.5% cationic colloidal silica (24 ml) was perfused, and the excess was washed out with 20 ml of MBS (pH 5). LESL-bound silica was polymerized with anionic polyacrylic acid (AP, 40 ml), and its excess was washed out with MBS (20 ml, pH 5) and LB (20 ml).

Venricles were dissected, minced, suspended in 10 ml of LB containing 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma), and homogenized with a glass-Teflon homogenizer at 4°C. The homogenate was filtered, mixed with an equal volume of Nycodenz I solution, layered on top of the Nycodenz II solution, and centrifuged. The LESL-silica pellet was washed two times with MBS pH 6 to remove a variety of contaminating non-LESL proteins and resuspended in 500 μl of 2% SDS. This was sonicated, boiled, and centrifuged, and aliquots of the supernatant were frozen. LESL proteins were quantified.

The supernatant was analyzed via SDS-PAGE.

Isolation of LESL proteins (LESL fraction). Isolation of protein from the coronary luminal endothelial surface membrane and glyco-calyx (LESL) is possible thanks to the method developed and refined by different groups (3a, 5, 8, 10, 16). This procedure consists of coating LESL with cationic colloidal silica (20- to 50-nm diameters) due to binding to glyco-calyx anionic proteins, washing off excess silica, and polymerizing LESL-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 LESL. The LESL-silica-AP pellet produced is very stable, resists processes like tissue homogenization and centrifugation, and does not drag protein contaminants. All of these studies support this method as appropriate for isolation of LESL proteins.

Characterization of LESL lysate. Previously, many groups demonstrated that the silica pellet technique is appropriate for isolation of highly purified LESL proteins (3a, 5, 8, 10, 16). However, to ensure that our modified LESL extraction procedure does not contain non-lectinic proteins we performed the following tests.

In hearts during tissue homogenization, a saturating concentration of a non-LESL 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, it can bind reversibly to at least three LESL albumin-recognizing proteins (3a), and it unspecifically binds strongly to cationic silica. The final electrophoretic profiles of isolated LESL did not contain albumin. This indicates that during the
homogenization and centrifugation procedure albumin did not contami

We have also immunoprobbed LESL proteins by SDS-PAGE and Western blotting with antibodies directed against luminal endothelium-positive protein markers (3a, 8): caveolin-1, VCAM, and PECAM-1. All antigen-recognizing LESL proteins (VCAM, PECAM-1, caveolin-1, von Willebrand factor, and mannose receptor) reacted positively, while the non-LESL protein antigens (P2Y1–4) did not react; results are not shown.

Our established controls indicate that the LESL-silica-AP pellicle is representative of the luminal endothelial membrane organelle.

**Synthesis of Insoluble Matrixes of N-Acetylglucosamine, Mannose, or Galactose**

Polymers synthesized to isolate LESL lectinic proteins. Well-established procedures were used for polymer synthesis (3a, 21, 38). Dextran (70 kDa) was the starting material that was polymerized with N-acetylglucosamine, mannose, or galactose via divinyl sulfone (DVS) bridges (3 monosaccharide and DVS molecules per glucose dextran moiety). The final products of polymerization, GlcNAc-Pol, Man-Pol, and Gal-Pol, with a molecular weight of 460,000, were loaded independently into columns and utilized as chromatography affinity probes to isolate lectins with affinity toward the corresponding sugar (3a, 38). To isolate lectins with affinities toward one, two, or three sugars, an equal amount of each of the three sugar-polymers [(GlcNAc-Pol + Man-Pol + Gal-Pol) = Σ-Pol] was mixed and the mixture was added to a chromatography column. The four affinity columns were GlcNAc-Pol, Man-Pol, Gal-Pol, and Σ-Pol.

**LES L Lectins: Affinity Chromatographic Isolation, SDS-PAGE, 2D-SDS-PAGE, Patterns, and Immunoblot Identification**

Selectivity of affinity chromatography columns for separation of lectins. Lectin separation was based on selective monosaccharide binding affinity to the Glc-Nac-Pol, Man-Pol, or Gal-Pol columns. These columns selectively retained only their specific lectins: *Ulex europaeus II* (with affinity for GlcNac), concanavalin A (with affinity for mannose), and *Bandeira simplifolia*-1 (with affinity for galactose). Each column selectively retained only one of these three lectins (positive control). The retained lectins were eluted with the corresponding 200 mM free monosaccharide. The affinity chromatography columns are selective and have the ability to retain lectinic proteins with affinity for specific monosaccharides (3a, 38).

**Isolation of LESL lectins with affinity for specific monosaccharides.** Columns were washed with 0.1 mM PBS pH 7.4; 0.2 mM CaCl2 and 2 ml of LESL protein (200 μg/ml in 0.1 mM PBS) were poured into each column and washed with 0.1 mM PBS. The bound lectins were eluted with 200 mM of the corresponding sugar or, in the case of the Σ-Pol resin elution, with a mixture of 200 mM of each of three sugars in 0.1 mM PBS pH 7.4, 0.4 mM EGTA. All elutes were extensively dialyzed against water for 1 wk at 4°C, lyophilized, reconstituted in a small volume, and examined via SDS-PAGE.

**SDS-PAGE of lectins.** For SDS-PAGE, 30 μg of each of the four LESL lectin fractions was desalted and rehydrated with buffer containing DTT. Each LESL lectin fraction was loaded and subjected to SDS-PAGE. The gels were silver stained and visualized.

**2D-SDS-PAGE of lectins.** 2D-SDS-PAGE was performed only for the LESL lectin fraction with affinity for the three sugars. Two hundred fifty micrograms of LESL lectin sample was desalted and rehydrated with buffer and ampholytes pH 3–10. The protein was loaded on strips 11 cm long with a pH gradient of 3–10. Focusing was carried out with an isoelectric focusing system at 20°C at the 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 fixed overnight in 50% methanol, 10% acetic acid. The gels were silver stained and visualized. This experiment was performed three times, and the results were the same. The results for the lectins with affinity to individual sugars have been reported previously (3a, 38).

**Imune dot blots for LESL fraction and the lectinic LESL with affinity to the three sugars.** The purpose of these studies was to detect the presence or absence of a given protein. Each LESL fraction and the three LESL sugar affinity lectins were denatured in loading buffer (0.5:3) and boiled for 10 min. Sixty micrograms of protein was loaded on nitrocellulose membranes (in the form of a dot) and air dried. Dots were blocked in 5% powdered nonfat milk, 0.05% Tween 20 (TBS-T-L) for 1 h and washed three times in TBS-T (10 min each wash). The dot blots were incubated with the primary antibody (1:250) in TBS-T-L overnight at 4°C. Thereafter, the blots were washed three times with TBS-T (10 min per wash). Blots were reacted with peroxidase-coupling secondary antibody (1:500) in TBS-T-L for 1 h, washed twice with TBS, developed with chemiluminescence reagent, and visualized on Kodak photographic paper.

**Physiological Coronary Resistance Studies**

*Isolated, perfused guinea pig heart.* Dunkin-Hartley guinea pigs (300–400 g body wt) were anesthetized intraperitoneally with pentobarbital sodium (50 mg/kg) and sodium heparin (500 U). Under artificial respiration the thorax was opened and the heart was excised and retrogradely perfused at a constant flow of 8 ml/min, according to the Langendorff method, with oxygenated Krebs-Henseleit (K-H; in mM: 127 NaCl, 6 KCl, 1.8 CaCl2, 1.2 NaH2PO4, 1.2 MgSO4, 25 NaHCO3, 5 dextrose, 2 pyruvate; pH 7.4, 37°C, 95% O2-5% CO2). Heart rate was kept constant at 4.5 Hz by application of electric square pulses to the right atria. Thirty minutes of stabilization was allowed before any of the treatments. The coronary perfusion pressure (CPP) was recorded via a pressure transducer connected to a side branch of the perfusion cannula and was continuously measured (3a, 8, 17, 38, 40, 49).

**Receptor activation at two different coronary flows: effects on CPP.** Extensive work from our laboratory has established that CF within a range of 5–14 ml/min regulates diverse cardiac functions (3a, 8, 17, 38, 40). To study the effects of flow on the vascular responses induced by agonist receptor activation we choose two flows within this range, a low flow of 6 ml/min and a higher flow of 10 ml/min. At each flow four different concentrations of the agonists phenylephrine (adrenergic α1-R) and bradykinin (B2-R) were infused as a bolus and the peak value of the transient CPP was determined. Dose-response effects were measured. Stock K-H solutions of agonists at high concentrations were infused as a bolus (50-μl volume, 0.5-s duration), and the concentration at the peak of the bolus was estimated from the dilution. The concentrations ranged from 0.1 to 20 μM for phenylephrine and from 0.01 to 20 μM for bradykinin. To determine the concentration-response curve the peak value of CPP transient (response) was plotted against the estimated agonist concentration (x-axis). First a concentration response for an agonist was determined in a heart at a flow of 6 ml/min. Thereafter, in the same heart the flow was increased to 10 ml/min and the concentration-response curve determined. For the two agonists concentration-response curves were compared at the two flows. Each heart was its own control. A group of three hearts was used for each treatment.

**Concentration-response curves at a constant flow before and after in situ hydrolysis of hyaluronic acid: effects on CPP.** Hyaluronic acid participates in diverse flow-induced responses (30, 38, 40, 49). LESL hyaluronic acid can be exclusively and selectively hydrolyzed with hyaluronidase by restricting its presence to the vascular lumen by minimizing its transendothelial diffusion with a low concentration hyaluronidase gradient and a very short time of exposure (11, 38, 40, 49). LESL in situ hydrolysis of hyaluronic acid: effects on CPP.
subsequently treated with hyaluronidase, and the procedure was repeated. At a given flow, concentration-response curves before and after hyaluronidase treatments were compared. Each heart was its own control. A group of four hearts was used for each treatment.

In all concentration-CPP curve experiments each heart was its own control. To determine the linear relationship between CPP and logarithm of concentration (x-axis), a straight line was obtained from semilogarithmic plots. The slope value of this straight line defines the gain of the response; it expresses the change of CPP in millimeters of mercury induced per every 10-fold change in the concentration of the agonist.

Statistical Analyses

Paired Student’s t-tests were performed on paired positive/negative variable values, and results with \( P < 0.05 \) were taken as statistically significant.

RESULTS

Isolation of LESL Lectins with Affinity to \( \Sigma \)-Pol and 2D-SDS-PAGE

We have previously established that each of the lectin probes here mentioned, GlcNac-Pol, Man-Pol, and Gal-Pol, as a result of lectinic binding to the LESL, inhibits flow-induced inotropic, dromotropic, and coronary vascular effects (3a, 38). In addition, when each of these probes was used as an affinity resin, it bound selectively to LESL lectinic proteins that could be separated by 2D-SDS-PAGE. Three groups of LESL lectins with apparent affinity for N-acetylgalosamine, mannose, or galactose were identified. However, since a lectin may have affinity for more than one monosaccharide, it is likely that a given lectin would appear in more than one of the three groups. This would create the appearance of the existence of a number of LESL lectins larger than the actual number. To avoid this problem, we used \( \Sigma \)-Pol as an affinity resin and proceeded to identify some of these molecules.

The SDS electrophoresis patterns from each of these three columns, Man-Pol, Gal-Pol, GlcNac-Pol, or \( \Sigma \)-Pol (Fig. 1) show there are bands that are common to more than one affinity resin (columns M, G, N and 3, respectively). In the SDS electrophoresis pattern of \( \Sigma \)-Pol (Fig. 1, column 3) bands tend to be denser and there are \( \sim 25 \) bands.

To determine the total number of individual LESL lectinic proteins, the eluate from the \( \Sigma \)-Pol affinity column was separated by 2D-SDS-PAGE, revealing at least 167 individual lectinic proteins (Fig. 2). To identify “clusters” of proteins, we have circled them in Fig. 2. Also, from reported molecular weights and corresponding isoelectric points for diverse luminal endothelial membrane proteins, several proteins were identified and are listed in Table 1.

Identification of 10 GPCRs, the Insulin Receptor, and 3 Selectins in the LESL Protein Fraction and the Lectinic \( \Sigma \)-Pol Fraction

The dot immunoblot results to establish the presence or absence of specific proteins from the LESL fraction (column C) or lectinic proteins from the \( \Sigma \)-Pol affinity resin (column 3) are shown in Fig. 3. The immune-identified peptides from the LESL and the lectinic \( \Sigma \)-Pol fractions were the GPCRs: endothelin (ETAR), prolactin (PRL-R), thromboxane A2 (TXA2R), angiotensin II (AT1), adenosine A1 (A1R), adenosine A2 (A2R), bradykinin (B2-R), \( \alpha \)-adrenergic (\( \alpha \)1A-R), \( \beta \)-adrenergic (\( \beta \)1A-R), and \( \beta \)-adrenergic (\( \beta \)3A-R). Also identified were the insulin receptor and selectins ICAM-1, VCAM-1, and PECAM-1. Dot intensities varied, but in general intensities tend to be higher in the lectinic \( \Sigma \)-Pol group (Fig. 3, column 3) than in the corresponding LESL protein group (Fig. 3, column C). This test establishes the lectinic nature of the immune-identified proteins.
Table 1. 2D-SDS coordinate values for proteins found in coronary luminal membrane

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Molecular Weight (×10^3)</th>
<th>Isoelectric Point (pI)</th>
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<tr>
<td>ICAM-1</td>
<td>58.85</td>
<td>5.79</td>
</tr>
<tr>
<td>8ENaC</td>
<td>72.2</td>
<td>5.72</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>75–77</td>
<td>5.0</td>
</tr>
<tr>
<td>ETAR</td>
<td>48.58</td>
<td>8.68</td>
</tr>
<tr>
<td>PRL-R</td>
<td>66</td>
<td>5.66</td>
</tr>
<tr>
<td>TXA2R</td>
<td>37.1</td>
<td>10.00</td>
</tr>
<tr>
<td>Caveolin a</td>
<td>20.54</td>
<td>5.64</td>
</tr>
<tr>
<td>A1-R</td>
<td>36.64</td>
<td>8.77</td>
</tr>
<tr>
<td>A2A-R</td>
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</tr>
<tr>
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</tr>
<tr>
<td>B2R</td>
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</tr>
<tr>
<td>PECAM-1</td>
<td>80</td>
<td>6.45</td>
</tr>
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2D-SDS, 2-dimensional SDS.

Vascular Effects of Phenylephrine and Bradykinin Are Stimulated by Flow and Are Inhibited by Hyaluronidase

It has been demonstrated that the α1A-R agonist phenylephrine and the B2-R agonist bradykinin, when given intravascularly, act on their corresponding endothelial luminal membrane receptor (7, 42).

CPP changes by phenylephrine at two different coronary flows before and after hyaluronidase treatment. Figure 4 shows the effect of intravascular administration of the α1A-R agonist phenylephrine on the coronary flow response to phenylephrine stimulation at 10 ml/min.

The four CPP responses were induced by the same concentration of phenylephrine (0.1 μM) at a CF of 6 ml/min before (Fig. 4A, left) and after (Fig. 4A, right) hyaluronidase treatment and at a CF of 10 ml/min before (Fig. 4B, left) and after (Fig. 4B, right) hyaluronidase treatment. These traces clearly show that the phenylephrine-induced CPP change at 6 ml/min (Fig. 4A, left) is smaller than that at 10 ml/min (Fig. 4B, left), a flow-magnifying effect. After hyaluronidase treatment at both flows the CPP responses (Fig. 4, A and B, right) are markedly reduced.

CONCENTRATION-RESPONSE CURVES TO PHENYLEPHRINE AT TWO DIFFERENT FLOWS BEFORE AND AFTER HYALURONIDASE TREATMENT. The graphs in Fig. 4 illustrate the concentration-response curves obtained at CFs of 6 ml/min and 10 ml/min (Fig. 4C), at a CF of 6 ml/min before and after hyaluronidase treatment (Fig. 4D), and at a CF of 10 ml/min before and after hyaluronidase treatment (Fig. 4E).

CONCENTRATION-RESPONSE CURVES AT TWO DIFFERENT CORONARY FLOWS. In Fig. 4C, the basal CPP values (no agonist present) are indicated by the horizontal lines. The top and bottom horizontal lines correspond to the CPP basal values at flows of 10 ml/min and 6 ml/min, respectively. The higher CPP basal value at 10 ml/min is simply because 10 ml/min > 6 ml/min (P = F × R). At each flow, administration of increasing concentrations of phenylephrine increased CPP above its corresponding basal value, yielding a pair of concentration-response curves. A plot of these results as the logarithm concentration (x-axis) against CPP yielded straight lines with significantly different slopes of 4.9 ± 0.22 (for 6 ml/min) and 7.6 ± 0.30 (for 10 ml/min) (Table 2). This indicates that CF increases the gain of phenylephrine stimulation.

CONCENTRATION-RESPONSE CURVES AT THE SAME CORONARY FLOW BEFORE AND AFTER HYALURONIDASE TREATMENT. In Fig. 4D, the basal CPP values (no agonist present) are indicated by the horizontal lines. The bottom and top horizontal lines correspond to the CPP basal values at a flow of 6 ml/min before (control) and after hyaluronidase treatment, respectively. The higher CPP basal value after hyaluronidase indicates a higher resistance to flow whose mechanism is unknown. Under both conditions, control and after hyaluronidase, administration of increasing concentrations of phenylephrine increased CPP above its corresponding basal value, yielding a pair of different concentration-response curves. Plots of these results as the logarithm concentration (x-axis) against CPP yielded straight lines with significantly different slopes of 4.9 ± 0.22 (control) and 1.8 ± 0.14 (after hyaluronidase) (Table 2). This indicates that hyaluronidase pretreatment reduces the gain of phenylephrine stimulation at 6 ml/min.

Figure 4E shows the same concentration-response curves except at a CF of 10 ml/min (see description of Fig. 4D). Under both conditions, control and after hyaluronidase, administration of increasing concentrations of phenylephrine increased CPP above its corresponding basal value, yielding a pair of different concentration-response curves. A plot of these results as the logarithm concentration (x-axis) against CPP yielded straight lines with significantly different slopes of 7.6 ± 0.30 (control) and 4.5 ± 0.24 (after hyaluronidase) (Table 2). This indicates that hyaluronidase pretreatment reduces the gain of phenylephrine stimulation at 10 ml/min.

Fig. 3. Immune dot blots of specific proteins in the LESP fraction (column C) and the lectinic fraction eluted from the Σ-Pol affinity column, lectins with affinity toward the 3 sugars mannose, galactose, and GlcNac (column 3). The 2 columns at left correspond to the 2 negative controls (Blank), which show no reaction (background), and, in descending order, 3 selectins [intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and platelet endothelial cell adhesion molecule-1 (PECAM-1)] and the insulin receptor. The 4 columns inside the box correspond to 10 G protein-coupled receptors. C and 3 correspond to LESP protein and LESP lectinic protein fractions, respectively.

The identified receptors are endothelin receptor (ETAR), prolactin receptor (PRL-R), thromboxane A2 receptor (TXA2R), ANG II receptor 1 (AT1R), adenosine receptors 1 and 2A (A1R and A2AR, respectively), bradykinin receptor (B2R), and adrenergic receptors (α1A-R, β1A-R, and β3A-R). Note that the lectinic dots tend to have a higher density than the corresponding control.
CPP changes by bradykinin at two different coronary flows before and after hyaluronidase treatment. Figure 5 shows that the CPP effects of intravascular administration of the B2-R agonist bradykinin differ significantly in magnitude depending on CF and hyaluronidase pretreatment.

The description of Fig. 5 is similar to that of Fig. 4, except that bradykinin is a vasodilator and phenylephrine is a vasoconstrictor. Figure 5 shows the original traces from one heart (Fig. 5, A and B) and the concentration-response curves of bradykinin at two different CFs (Fig. 5C) and the concentration-response curves at the same CF before and after hyaluronidase treatment (Fig. 5, D and E).

A plot of the results in Fig. 5, C–E, as the logarithm of concentration (x-axis) against CPP yielded straight lines with significantly different negative slopes (Table 2). In the case of the effects of bradykinin at two different flows (Fig. 5C), the slopes were $-2.04 \pm 0.22$ (for 6 ml/min) and $-4.30 \pm 0.32$ (for 10 ml/min) (Table 2), indicating that CF increases the gain of bradykinin stimulation. In the cases of effects of hyaluronidase at two different CFs (Fig. 5, D and E) the slopes of the straight lines from semilogarithmic plots were for flow of 6 ml/min (Fig. 5D) $-2.04 \pm 0.22$ (control) and $-1.26 \pm 0.17$ (after hyaluronidase) and for flow of 10 ml/min (Fig. 5E) $-4.30 \pm 0.32$ (control) and $-1.73 \pm 0.15$ (after hyaluronidase) (Table 2). This indicates that hyaluronidase pretreatment reduces the gain of bradykinin stimulation.

These results (Fig. 4, Fig. 5, and Table 2) establish that the agonist effects on these two GPCRs depend on the level of flow and its oligosaccharide environment.

DISCUSSION

Our results further support the concept that blood vessel luminal glycocalyx components, oligosaccharides (O) and signaling lectinic transmembrane protein (L), respond to flow via interaction with each other (O·L) due to their proximity and constitute the true flow sensors, the structures that initiate and directly absorb the impact of flow. Furthermore, our results suggest that the signaling L is not an insular molecule but one whose function is determined by its interaction with adjacent O.

Briefly, in this report we 1) identify by immune dot blot 10 lectinic GPCRs, among them phenylephrine (α1A-R) and bradykinin (B2-R) receptors; 2) provide initial support for the concept that the vasoactive hormonal effects (gain) of these two lectinic receptors depend on flow and on their O environment; and 3) show that at least 167 lectins are found in the LSEL with affinity toward three sugars: mannose, galactose, and N-acetylglucosamine.

We previously demonstrated that lectins from the LSEL could be isolated when each of the probes Man-Pol, GlcNac-Pol, and Gal-Pol (Mon-Pols) is used as an affinity resin. To determine the number of individual lectinic proteins in each
fraction, eluates were separated by 2D-SDS-PAGE. We found at least 9, 35, and >100 protein spots in the Man-Pol, GlcNac-Pol, and Gal-Pol affinity column eluates, respectively, which sum to at least 144 lectinic protein spots (3a, 38). This number could be greater, because the 2D-SDS-PAGE methodology is not sensitive enough to detect individual proteins that are present in small amounts without overloading the gel. Alternatively, the total number may be less, because a given lectin may bind to more than one sugar, leading to overlap between the three gels. To minimize these two problems, we created an Σ-Pol affinity column and the amount of protein loaded was almost twice that used in previous studies. The number of lectinic proteins detected in the 2D-SDS-PAGE was 167 spots. The significance of these results is that lectinic binding is a common characteristic for LESL proteins and not the exception. The functional and structural implications of the lectinic nature of these numerous LESL proteins remain unknown. Furthermore, in in situ studies, the probes Man-Pol, GlcNac-Pol, and Gal-Pol were fluorescently labeled, administered by intracoronary infusion, washed extensively, and found to irreversibly bind to the LESL of coronary vasculature (3a, 19, 38). These monosaccharide polymers, upon binding to LESL, differentially inhibited flow-induced cardiac effects with an inhibitory potency sequence of GlcNac-Pol > Man-Pol >> Gal-Pol. Thus our results with these sugar polymers demonstrate both in vitro isolation of LESL lectins and in situ differential binding to functional LESL signaling lectins that serve as flow sensors. These flow sensors are capable of inducing endothelial paracrine secretion, similar to a GPCR when agonist activated (6, 8, 42, 43, 61). The fact that a given flow-induced function can be experimentally and independently modulated from other functions supports the existence of multiple LESL flow-sensing structures (22, 33, 35). These structures are not insular molecules but, more likely, molecular complexes consisting of both a lectinic protein (L) and oligosaccharide (O) molecules.

Our results show that the α1A receptor (phenylephrine) and the B2 receptor (bradykinin) have affinity for sugars and their concentration-response curve to intravascular administration of their corresponding agonist is determined by the level of flow and the oligosaccharide environment (±hyaluronidate). Similarly, the agonist response of glutamate ion channel receptor is dependent on the oligosaccharide environment (20, 50, 55). An increasing number of GPCRs have been shown to be activated by flow; so far these are AT1R, A1R, B2-R, PRL-R, ETAR, α1A-R, H1R, V1R, and M5R (2, 9, 22, 28, 33, 36, 62), and we now show that the first six GPCR are lectinic. In addition, we found that an additional four GPCRs, A2R, TXA2R, β1A-R, β3A-R, and the insulin receptor are lectinic, but their flow sensitivity remains to be demonstrated as well as the lectinic nature of H1R, V1R, and M5R.

A number of LESL transmembrane proteins have been identified as possible “mechanosensors”: VCAM-1, ICAM-1, PECAM-1, αβ3, some GPCRs, ENaC, and polycystin-2, a Ca2+ channel (1, 2, 9, 14, 22, 24, 26, 31, 33, 34, 38, 41, 45, 46, 48, 54, 62), but the direct absorption of flow impact by these molecules needs to established and explained. It is intriguing to speculate how these LESL proteins detect flow/mechanical stress and what biochemical properties make them mechanosensors (2, 38, 54). The integrin and ENaC families could be good examples of some of the molecular requirements for a structure to be a mechanosensor (14, 24, 46, 58). In the case of the abluminal integrin αβ3, flow causes its association with the adapter protein Shc (its anchor) present in focal adhesions. The formation of the integrin-Shc complex appears necessary for integrin physical activation (24, 46). Similarly, ENaC...
requires an extracellular anchor molecule to respond to stress (14, 58), and we have provided evidence that extracellular domains of ENaC likely are lectinic and interact in situ with glycosylated anchor molecules such as hyaluronan (38). Thus it appears that for flow-induced cellular signaling processes formation of a protein-anchor molecular complex is required.

On the basis of our results, we propose that GPCRs are not insular molecules but function as molecular complexes composed of both a lectinic moiety (L) and LESL oligosaccharide (O) molecules that, as a result of direct stoichiometric absorption of flow’s energy, form a “true mechanosensor.” It is implied that an extracellular lectinic domain (L) of a transmembrane protein, a receptor, binds to specific endothelial glycosalyx structures (O) and the level of complexing determines the level of activation for signaling. As a result, agonist-induced receptor activation would depend on both the level of flow and the oligosaccharide environment. However, it is still unknown whether cell signaling results from formation of O·L, its dissociation into O + L, or both. Our results suggest that when O is present flow stimulation would cause formation of O·L that results in amplified receptor “signaling.”

In contrast, removal of hyaluronidate leads to greater availability of free L with a reduced response to the agonist stimulus. This view is supported by previous results demonstrating that ANG II amplifies the stimulatory effects of flow on the AT1R (28).

Further support for this hypothesis is provided by the specific inhibitory effects of flow-stimulated functions by different exogenous 1) glycanases (hydrolyze specific O), 2) plant lectins (bind to specific O and compete with endogenous L), and 3) monosaccharide polymers (bind to endogenous L and compete with endogenous O). These results support the notion that an agonist and flow-sensitive molecular complex is composed of an intravascular glycosalyx moiety, an intravascular L recognition site, and a lectinic counterpart, an intravascular O recognition site (3a, 17, 19, 30, 35, 37, 38, 40, 41, 49).

It is well accepted that the intracellular side of a transmembrane signaling protein has specific anchor structures (G proteins, phospholipase C, kinases, etc.) and the binary coupling of signaling protein and anchor is required for activation of a pathway. In contrast, despite a large body of evidence, the same transmembrane signaling process on the extracellular side of the membrane has been overlooked. Data analyses in studies of effects of flow, hormone, and electrical stimuli on transmembrane effector molecules frequently are made assuming a cell membrane (a lipid bilayer) denuded of extracellular specific anchor structures: a “baldheaded” membrane (9, 33, 54). Thus we propose that “anchor” molecules located symmetrically on the intra- and extracellular sides of the membrane regulate the signaling capability of transmembrane proteins.

In summary, 1) we have demonstrated the existence of a large number of transmembrane LESL lectins with affinity for mannose, GlcNac, and galactose. 2) Among these lectins 3 selectins, 10 GPCRs, and the insulin receptor were identified, and many of these molecules are activated by flow. 3) We provide initial support that the vasoactive agonist effects (gain) of two lectinic receptors, α1A-R and B2-R, depend on flow and on their O environment because flow triggers the formation of the binary structure O·L complex that constitutes the most active transmembrane signaling structure. In other words, a GPCR is not an insular molecule; instead, it complexes with saccharides in response to flow that regulates GPCR and saccharide coupling.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


