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ORIGINAL ARTICLE

Attenuating Pulmonary Hypertension by Protecting the Integrity of Glycocalyx in Rats Model of Pulmonary Artery Hypertension

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Abstract— The endothelial glycocalyx has been proved to be a polysaccharide protein complex covering the surface of vascular endothelial cells, playing an important role in vascular permeability, blood flow shear stress induction, and prevention of endothelial cell adhesion. The pathogenesis of PAH includes pulmonary arterial endothelial cell dysfunction and pulmonary arterial smooth muscle cell (PASMCs) proliferation. Based on the physicochemical properties of endothelial glycocalyx involving pathogenesis of pulmonary hypertension. We hypothesized that the endothelial glycocalyx is involved in the development of pulmonary hypertension; pulmonary hypertension can be regulated by protecting the integrity of glycocalyx. Expression of glycocalyx markers including heparin sulfate proteoglycan (HSPG), hyaluronan (HA), and syndecan-1 (SDC-1) was detected in monocrotaline (MCT)-induced PAH in rats and these components were detected when the PAH rats were treated with heparin that protected the role of glycocalyx. Results showed that plasma levels of HSPG, HA, and SDC-1 were increased in MCT group when compared with control group. However, rats in treatment group showed reduced levels of HSPG, HA, and SDC-1. Expression of HSPG, HA, and SDC-1 in pulmonary arteries was also reduced in MCT group when compared with those in the control group. By contrast, expression of HSPG, HA, and SDC-1 in pulmonary arteries increased in treatment group. In conclusion, destruction of glycocalyx was involved in the development of pulmonary hypertension. Pulmonary hypertension can be regulated by protecting the integrity of glycocalyx.

KEY WORDS: glycocalyx; pulmonary; hypertension; pathogenesis.

INTRODUCTION

Pulmonary arterial hypertension (PAH) is a lethal syndrome characterized by increased resistance in the pulmonary circulation and occlusive vascular remodeling, with increased afterload until right ventricular failure. On the one hand, the traditional methods for treatment of PAH have no obvious efficacy. On the other hand, the new type of specific treatment for endothelin, prostacyclin, and

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phosphodiesterase cannot completely cure PAH. Therefore, novel approaches are urgently required for the treatment of PAH. The endothelial glycocalyx has been recognized to be a polysaccharide protein complex covering the surface of vascular endothelial cells. It is mainly composed of heparan sulfate proteoglycan (HSPG), hyaluronan (HA), and syndecan-1 (SDC-1) [1–3]. Glycocalyx performs significantly in vascular permeability, blood flow shear stress induction, NO production, and prevention of endothelial cell adhesion [4–7]. The pathogenesis of PAH includes pulmonary arterial endothelial cell dysfunction and pulmonary arterial smooth muscle cell (PASMCs) proliferation. We hypothesized that the destruction of glycocalyx is involved in the development of pulmonary hypertension. Pulmonary hypertension can be regulated by protecting the integrity of glycocalyx and protection of glycocalyx may have the possibility of prevention and treatment of PAH.

Extensive experimental and clinical data show that the integrity of the vascular endothelial glycocalyxes is inversely related to the degree of inflammation, and the more severe the inflammation is, the worse the glycocalyx structure is destroyed. Heparin is an ancient anticoagulant that has an anti-inflammatory effect in addition to anticoagulation. Heparin can downregulate the levels of inflammatory mediators TNF-a and IL-6 and relieving the inflammatory reaction by binding inflammatory cytokines and acute phase reactants and inhibiting the activation of nuclear factor-Kappa (NF-κB) through controlling transfer of NF-κB from the cytoplasm to the nucleus [8]. Heparin plays an indirect role in protecting the glycocalyx *via* the anti-inflammatory effect. Heparinase is the only endoglycosidase in glycosaminoglycans that can degrade HS side chains and can specifically recognize the specific sites on the HS side chains with HSPG cleavage. Heparin, as an inhibitor of heparanase, inhibits the activity of heparanase and prevents its destruction of glycocalyx.

In the present study, heparin is used to intervene in an animal model of pulmonary hypertension to demonstrate whether protection of glycocalyx can prevent pulmonary hypertension.

MATERIALS AND METHODS

Animal Preparation and Experimental Protocol

This study was approved by the Institutional Animal Care and Use Committee of Sichuan University, Sichuan, China. All animals received humane care in compliance with the guide for the Care and Use of Laboratory Animals published by the U.S. National Institute of Health. The experiments followed the Guidelines of the American Physiological Society.

Male Wistar rats weighing 250 to 300 g were purchased from HuaFu Kang BioScience Co. Inc. (Beijing, China). Fifteen animals were randomized into 3 groups: MCT-induced PAH group (MCT group, $n = 5$), in which animals received 50 mg/kg of MCT intraperitoneally (Aladdin, Shanghai); control group ($n = 5$), in which animals received an equivalent volume of saline solution; treatment group ($n = 5$), in which animals received heparin (Aladdin, Shanghai) injection into the cervical skin 180 U/mg/day for 3 weeks after PAH induction.

Determination of PAH Model

After MCT injection for 21 days, the experimental rats were anesthetized with 7% chloral hydrate (0.5 ml/100 g, Aladdin, Shanghai) and inserted with a 3F-Miller micro tip catheter *via* the right jugular vein into the right ventricle to obtain the right ventricular systolic pressure (RVSP). Results of the RVSP were used to evaluate whether the model of PAH was successfully established.

Enzyme-Linked Immunosorbent Assay

Plasma samples were frozen at − 80 °C until assayed. Specific enzyme-linked immunosorbent assay (ELISA) kits were used to measure plasma levels of HA, HSPG, and SDC-1 (Sangon, Shanghai), according to the manufacturer's protocol. Each sample was tested in duplicate.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was performed to assess HA and SDC-1 expression in pulmonary artery tissue. Proximal sections of the pulmonary artery were collected and stored at − 80 °C until assayed. Total RNA was extracted using TRIzol following the manufacturer's protocol (Qiagen, Valencia, Germany). The integrity and concentration of RNA were measured by the 2100 Bioanalyzer (Agilent, CA, USA). Reverse transcription reaction was conducted at 37 °C for 15 min and 85 °C for 5 s in a 20-μL mixture containing 1 μg of total RNA and PrimeScript™ RT Master Mix. Each real-time PCR was prepared in a 20-μL reaction mixture containing 10 μL SYBR Green PCR Master Mix, 1 μL cDNA, and 1.4 μL primers, and conducted on an ABI Prism 7900 sequence detector (Applied Biosystems, Carlsbad, CA, USA). Cycling conditions

consisted of an initial denaturation of 2 min at 95 °C, followed by 39 cycles of 5 s at 95 °C and 10 s at 60 °C. The following primers were used for pulmonary artery analyses: HA, 5′-GCAGTTTCGGTGATGATAGGC-3′ (forward), and 5′-ACTTGCTCCAACGGGTCTG-3′ (reverse). SDC-1, 5′-GATATGACTTTGTCACGGCA GAC-3′ (forward), and 5′-GGCAGGATAGAGGT GAGGGTG-3′ (reverse). GAPDH, 5′-ATCA AGGAAGCGGTGAAGAAGG-3′ (forward), and 5′-CGAAGATGGAGGAGTGGGTGTC-3′ (reverse). All samples were assayed in triplicate. Relative gene expression was determined by the $2^{-\Delta\Delta ct}$ method.

Western Blot

Pulmonary artery tissues were pulverized in liquid nitrogen. Cytoplasmic and nuclear proteins were extracted using NE-PER™ nuclear and cytoplasmic extraction reagents (Pierce, Rockford, USA). Protein concentrations were determined using a Bio-Rad protein-assay instrument. Equal amounts of protein were denatured and separated by sodium dodecyl sulfatepolyacryl-amide gel electrophoresis (SDS-PAGE). The protein was electrophoresed and transferred onto polyvinylidene fluoride membranes. The membranes were incubated with the primary antibodies and then were incubated with secondary antibodies. The protein blots were detected using an enhanced chemiluminescence kit (Pierce, USA) and exposed to X-ray film. The primary antibodies used in this study included rabbit anti-HSPG and rabbit anti-SDC-1(Abcam, Cambridge, UK), and the secondary antibody was anti-HRP-IgG (Sigma, NY, USA).

Statistical Analysis

Data were expressed as mean ± standard deviation (SD) and analyzed by SPSS l8.0 software. Comparisons of parameters between 2 groups were made with unpaired Student's *t* test. Comparisons of parameters among 3 groups were made with one-way analysis of variance (ANOVA), followed by multiple comparison test. $P < 0.05$ was considered statistically significant.

RESULTS

Measurement of Right Ventricular Systolic Pressure

Right ventricular systolic pressure (RVSP) was used to evaluate the model of MCT-induced PAH. RVSP of MCT, control group, and treatment group were measured. The detailed results are shown in Fig. 1a.

HSPG, HA, and SDC-1 Expression on MCT, Control, and Treatment Groups

Plasma levels of HSPG, HA, and SDC-1 were determined using ELISA. The detailed results are shown in Fig. 1b.

Protein expression of HSPG and SDC-1 was analyzed using immunoblotting, and results are shown in Fig. 1c.

Relative mRNA expression of HA and SDC-1 were determined by qRT-PCR, and results are shown in Fig. 1d.

DISCUSSION

There was no special clinical manifestation of pulmonary hypertension in the early stage, so it failed to attract attention. However, right heart failure could occur along with the progress of the disease. The current drug treatment is limited and expensive, therefor end-stage patients can only rely on lung transplantation for survival. This situation often presents great blows to patient and patient's family members. The search for new therapeutic targets has become an urgent need for current. The glycocalyx covers the surface of all vascular endothelial cells and affects the function of these cells. The destruction of its structure and function is involved in the development of cardiovascular diseases [9]. In inflammatory conditions, a variety of inflammatory mediators resulted in the loss of glycocalyx, weakening its vascular protection [10]. Some studies have shown that in the mouse model of sepsis-induced acute lung injury, the pulmonary vascular endothelial glycocalyx is degraded along with structure and function destroyed, which exposes its masked vascular adhesion molecules ICAM-1 and VCAM-1, in which white blood cells are more likely to adhere to vascular endothelial cells, causing a series of subsequent pathological changes [11].

In our study, the rat model of pulmonary hypertension induced by MCT showed significantly higher levels of peripheral blood glycocalyx shedding ingredients, such as HSPG, HA, and SDC-1, when compared to control groups. The glycocalyx component of the pulmonary artery was reduced theoretically with glycocalyx shedding. Protein expression of HSPG and SDC-1 in rats of pulmonary arteries from the MCT group was significantly decreased. Furthermore, mRNA levels of HA and SDC-1 were also strongly decreased when compared with the control

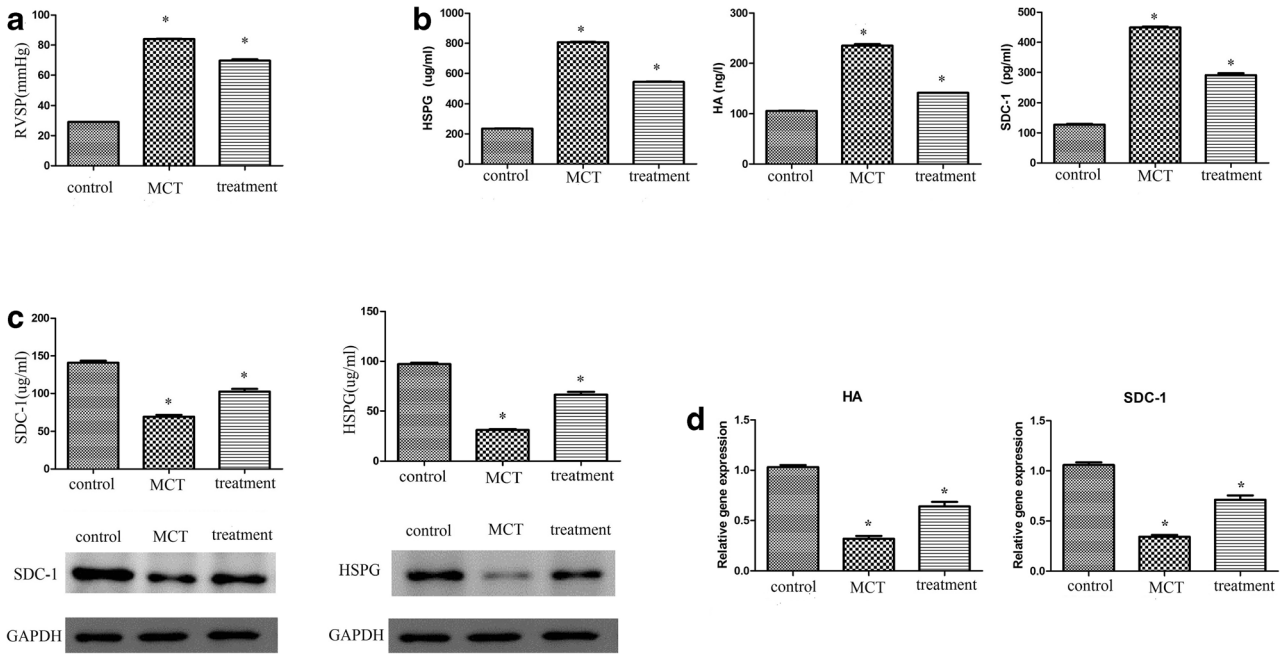

Fig. 1. Measurement of right ventricular systolic pressure and HSPG, HA, and SDC-1 expression in each group. **a** There was significantly increased RVSP in MCT group (84.009 ± 0.393) than the control group (29.159 ± 0.149). By contrast, the RVSP of rats in treatment group (69.727 ± 1.787) was significantly lower than that in MCT group $*P < 0.01$. **b** Rats in MCT group had higher plasma HSPG, HA, and SDC-1 levels than the control group, respectively. On the contrary, rats in the treatment group showed reduced plasma levels of HSPG, HA, and SDC-1 when compared with the MCT group, respectively $*P < 0.01$. **c** The protein expression of HSPG and SDC-1 was significantly reduced in pulmonary arteries in rats from MCT group than control group, respectively. Interestingly, the levels of HSPG and SDC-1 protein from rats in treatment group were significantly higher than those from MCT group, respectively $*P < 0.01$. **d** The mRNA expression of HA and SDC-1 was reduced in rats pulmonary arteries from MCT group than control group, respectively. However, the mRNA expression of HA and SDC-1 was significantly increased in rats from treatment group than MCT group, respectively $*P < 0.01$.

groups. The increase of the glycocalyx composition in the blood and the decrease in blood vessel may suggest that the glycocalyx was destroyed in the MCT group. Indeed, our study also found that pulmonary arterial pressure of the MCT group was significantly higher than that of the control group. When the PAH rats model treated with heparin, expression of HSPG, HA, and SDC-1 in the peripheral blood of the treatment group was significantly decreased when compared with those in the MCT group. Because of the decreased glycocalyx shedding components, heparin treatment showed significantly higher protein expression of HSPG, SDC-1 in the treatment group compared to the MCT group. Similarly, heparin treatment increased the mRAN expression of HA and SDC-1 in the treatment group when compared to the MCT group. Results of the decrease of glycocalyx composition in the peripheral blood and the increase in the blood vessel in the treatment group suggested that the glycocalyx was effectively protected. Moreover, we found decreased pulmonary arterial pressure in the treatment group. Although not reduced to normal level, the difference was statistically significant comparison with MCT group. Our results for the first time demonstrated that glycocalyx is involved in the process of PAH. The protection of glycocalyx significantly attenuates MCT-induced pulmonary hypertension. Hence, how does the glycocalyx participate in the development of pulmonary hypertension? Whether it plays a key role in the pathogenesis of this disease?

As a barrier between blood and endothelial cells, the physiological function of glycocalyx is closely related to the pathogenesis of pulmonary hypertension. Firstly, the glycocalyx has negative charges, which can adsorb plasma proteins around glycocalyx. The glycocalyx and the adsorbed plasma protein can form a selective natural barrier, which restricts macromolecular transport to endothelial cell membrane, maintaining the body fluid balance and endothelial cellular homeostasis [2]. The destruction of glycocalyx structural function directly leads to the loss of endothelial cell barrier function. Secondly, glycocalyx mediates shear stress and induces the production of nitric

oxide (NO). Blood is able to produce shear stress on endothelial cells through the vessels. Through endothelial glycocalyx perception and conduction, the shear stress can upregulate endothelial NO synthase levels and then increase NO production [12, 13]. Structural disruption of glycocalyx leads to the NO disorder induced by shear stress, affecting the release of NO and its bioavailability. It is accepted that NO is important for vascular tension. The disorder may subsequently result in enhanced contraction. Which is caused by vasoconstrictor factors and an imbalance between the vasoconstrictor and the diastolic. Vasomotor dysfunction is the important pathophysiological basis of PAH. Thirdly, the glycocalyx structure contains many cell adhesion molecules. Because the size of the adhesion molecule is smaller than the thickness of the glycocalyx, it is shielded by glycocalyx, so that blood cells have no chance to interact with these adhesion molecules [7, 14]. When the structure of glycocalyx is destroyed, it will subsequently expose the concealed adhesion molecules, which makes it easier for the white blood cells to recognize and adhere to the surface of the endothelium. The adhesion and interaction between blood cells and endothelial cells is an important initial step for the dysfunction or loss of endothelial cells. Many studies suggest that endothelial cell injury and dysfunction plays a central role in the pathogenesis of PAH [15, 16]. Fourthly, endothelial cells display anticoagulant function under physiological conditions, which benefited from the glycocalyx containing many anti-clotting substances, such as antithrombin and heparin cofactor II, thrombomodulin. These anticoagulant substances are necessary for normal blood flow [9, 10]. Damage of glycocalyx affects the adhesion and activity of numerous anticoagulant factors and then affects thrombosis. Pulmonary artery embolism is one of the common pathological changes in pulmonary arterial hypertension. The authors believe that the destruction of glycocalyx can lead to the disappearance of endothelial cell barrier function, endothelial cell adhesion, thrombosis, and so on, which initiate the occurrence and development of pulmonary hypertension.

In theory, the higher the degree of inflammation in an inflammatory disease, the greater the degree of glycocalyx shedding. Recent studies in humans have shown that the degree of loss of glycocalyx is associated with the severity of the disease and the level of glycocalyx shedding in the blood [17]. Donati et al. showed that in patients with critically ill patients, the thickness of the vascular endothelium glycocalyx was reduced and the thickness of the glycocalyx reduced significantly in the more severe sepsis patients [18]. Other researchers also showed an increasing amount of HS and SDC-1 in the blood of infectious shock patients [11, 19–21], which was positively correlated with the increase in mortality [21]. Potter et al. indicated that in the acute cytokine-mediated glycocalyx destruction, the original thickness is recovered after 5–7 days and the acceleration of this process can limit the process of vascular inflammation [22].

In conclusion, the destruction of glycocalyx is involved in the development of pulmonary hypertension. Pulmonary hypertension can be attenuated by protecting the integrity of glycocalyx. The study of glycocalyx targeting therapy has important clinical significance. The insufficiency of this study: Endothelial injury is an important pathological basis of pulmonary hypertension. This study failed to discuss the relationship between glycocalyx destruction and the index of endothelial injury. Further studies will add to the detection of pulmonary hypertension endothelial cell function or smooth muscle cell function and its correlation with glycocalyx.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflicts of interest.

REFERENCES

1. Gouverneur, M., B.V.D. Berg, M. Nieuwdorp, et al. 2006. Vasculoprotective properties of the endothelial glycocalyx: Effects of fluid shear stress. *Journal of Internal Medicine* 259: 393–400.
2. Reitsma, S., D.W. Slaaf, H. Vink, M.A.M.J. van Zandvoort, and M.G.A. oude Egbrink. 2007. The endothelial glycocalyx: Composition, functions, and visualization. *Pflügers Archiv / European Journal of Physiology* 454: 345–359.
3. Vanteeffelen, J.W., J. Brands, et al. 2007. Endothelial Glycocalyx: Sweet shield of blood vessels. *Trends in Cardiovascular Medicine* 17: 101–105.
4. Levick, J.R., and C.C. Michel. 2010. Microvascular fluid exchange and the revised Starling principle. *Cardiovascular Research* 87: 198–210.
5. Zeng, Y., M. Waters, A. Andrews, et al. 2013. Fluid shear stress induces the clustering of heparan sulfate viamobility of glypican-1 in lipid rafts. *American Journal of Physiology. Heart and Circulatory Physiology* 305: H811–H820.
6. Tarbell, J.M. 2010. Shear stress and the endothelial transport barrier. *Cardiovascular Research* 87: 320–330.

7. Lipowsky, H.H. 2012. The endothelial glycocalyx as a barrier to leukocyte adhesion and its mediation by extracellular proteases. *Annals of Biomedical Engineering* 40: 840–848.
8. Thourani, V.H., S.S. Brar, T.P. Kennedy, L.R. Thornton, J.A. Watts, R.S. Ronson, Z.Q. Zhao, A.L. Sturrock, J.R. Hoidal, and J. Vinten-Johansen. 2000. Nonanticoagulant heparin inhibits NF-kappaB activation and attenuates myocardial reperfusion injury. *American Journal of Physiology. Heart and Circulatory Physiology* 278: H2084–H2093.
9. Broekhuizen, L.N., H.L. Mooij, J.J. Kastelein, E.S.G. Stroes, H. Vink, and M. Nieuwdorp. 2009. Endothelial glycocalyx as potential diagnostic and therapeutic target in cardiovascular disease. *Current Opinion in Lipidology* 20: 57–62.
10. Kolářová H, Ambrůzová B, Svihálková Š L, et al. 2014.Modulation of endothelial glycocalyx structure under inflammatory conditions. Mediators of Inflammation 2014:694312, 1, 17.
11. Schmidt, E.P., Y. Yang, W.J. Janssen, A. Gandjeva, M.J. Perez, L. Barthel, R.L. Zemans, J.C. Bowman, D.E. Koyanagi, Z.X. Yunt, L.P. Smith, S.S. Cheng, K.H. Overdier, K.R. Thompson, M.W. Geraci, I.S. Douglas, D.B. Pearse, and R.M. Tuder. 2012. The pulmonary endothelial glycocalyx regulates neutrophil adhesion and lung injury during experimental sepsis. *Nature Medicine* 18: 1217–1223.
12. Koo, A., D. Nordsletten, R. Umeton, B. Yankama, S. Ayyadurai, G. García-Cardeña, and C.F. Dewey Jr. 2013. In silico modeling of shear-stress-induced nitric oxide production in endothelial cells through systems biology. *Biophysical Journal* 104: 2295–2306.
13. Ebong, E.E., S.V. Lopez-Quintero, V. Rizzo, D.C. Spray, and J.M. Tarbell. 2014. Shear-induced endothelial NOS activation and remodeling via heparan sulfate, glypican-1, and syndecan-1. *Integrative Biology Quantitative Biosciences from Nano to Macro* 6: 338–347.
14. Becker, B.F., D. Chappell, and M. Jacob. 2010. Endothelial glycocalyx and coronary vascular permeability: The fringe benefit. *Basic Research in Cardiology* 105: 687–701.
15. Schermuly, R.T., H.A. Ghofrani, M.R. Wilkins, and F. Grimminger. 2011. Mechanisms of disease: Pulmonary arterial hypertension. *Nature Reviews Cardiology* 8: 443–455.
16. Pietra, G.G., F. Capron, S. Stewart, O. Leone, M. Humbert, I.M. Robbins, L.M. Reid, and R.M. Tuder. 2004. Pathologic assessment of vasculopathies in pulmonary hypertension. *Journal of the American College of Cardiology* 43 (12-supp-s): 25S–32S.
17. Nelson, A., I. Berkestedt, A. Schmidtchen, L. Ljunggren, and M. Bodelsson. 2008. Increased levels of glycosaminoglycans during septic shock: Relation to mortality and the antibacterial actions of plasma. *Shock* 30: 623–627.
18. Donati, A., E. Damiani, R. Domizi, R. Romano, E. Adrario, P. Pelaia, C. Ince, and M. Singer. 2013. Alteration of the sublingual microvascular glycocalyx in critically ill patients. *Microvascular Research* 90: 86–89.
19. Sallisalmi, M., J. Tenhunen, R. Yang, et al. 2012. Vascular adhesion protein-1 and syndecan-1 in septic shock. *Acta Anaesthesiologica Scandinavica* 56: 316–322.
20. Steppan, J., S. Hofer, B. Funke, T. Brenner, M. Henrich, E. Martin, J. Weitz, U. Hofmann, and M.A. Weigand. 2011. Sepsis and major abdominal surgery lead to flaking of the endothelial glycocalix. *Journal of Surgical Research* 165: 136–141.
21. Johansson, P.I., J. Stensballe, L.S. Rasmussen, and S.R. Ostrowski. 2011. A high admission syndecan-1 level, a marker of endothelial glycocalyx degradation, is associated with inflammation, protein C depletion, fibrinolysis, and increased mortality in trauma patients. *Annals of Surgery* 254: 194–200.
22. Potter, D.R., J. Jiang, and E.R. Damiano. 2009. The recovery time course of the endothelial cell glycocalyx in vivo and its implications in vitro. *Circulation Research* 104: 1318–1325.