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The neuropeptide galanin promotes an anti-thrombotic phenotype on endocardial endothelial cells from heart failure patients

Christina Tyrrell, Amanda Toyooka, Faiza Khan, Kent L. Thornburg, James O. Mudd, Wohaib Hasan*

Knight Cardiovascular Institute, Oregon Health and Science University, 3181 S.W. Sam Jackson Park Rd., Portland, OR 97239-3098, USA

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ABSTRACT

Thromboembolic complications are a significant cause of mortality and re-hospitalization in heart failure (HF) patients. One source of thrombi is the ventricular endocardial surface that becomes increasingly pro-thrombotic as HF progresses. Anticoagulation comes with bleeding risks so identifying therapeutic agents for improving cardiac endothelial health are of critical clinical importance.

Endocardial endothelial cells are closely apposed to cardiac sympathetic nerves. In HF, cardiac sympathetic nerves are dysregulated and promote disease progression. Whether endocardial endothelial health and function is impacted by sympathetic dysregulation in HF is unknown. Also unexplored is the impact of neuropeptides, such as galanin and neuropeptide Y (NPY), co-released from sympathetic nerve terminals, on endothelial health.

In this study we examined the effect of sympathetic nerve-released neurotransmitters and neuropeptides on the procoagulant phenotype of cultured human endocardial endothelial cells from HF patients. As a functional readout of procoagulant state we examined thrombin-mediated von Willebrand factor (vWF) extrusion and multimer expression. We demonstrate that vWF extrusion and multimer expression is promoted by thrombin, that isoproterenol (a beta-adrenergic receptor agonist) augments this effect, whereas co-treatment with the beta-blockers propranolol and carvedilol blocks this effect. We also show that vWF extrusion and multimer expression is attenuated by treatment with the neuropeptide galanin, but not with NPY.

Our results are consistent with a protective role of beta-blockers and galanin on endocardial endothelial health in heart failure. Improving endothelial health through galanin therapy is a future clinical application of this study.

1. Introduction

Thromboembolic complications in heart failure (HF) patients are the most frequent reason (up to 60%) for mortality or re-hospitalization (Beemath et al., 2006; Bettari et al., 2011; De Lorenzo et al., 2003; de Peuter et al., 2009; Ng et al., 2010; Roberts et al., 1987; Saric et al., 2016; Subramaniam et al., 2009; Uretsky et al., 2000; Zannad et al., 2013). However, anticoagulant therapy has serious bleeding risks (Ahnert and Freudenberger, 2008; Bettari et al., 2011; Cleland et al., 2004; De Lorenzo et al., 2003; Diet and Erdmann, 2000) and is only recommended when definite indications of a pro-thrombotic state exist (Lip et al., 2012; Massie et al., 2009; Prom et al., 2014). Ventricular thrombi are one such indication for anticoagulation; rates for these mural thrombi range from 10% to as high as 50%. However, these thrombi are small and immobile and therefore difficult to detect by conventional echocardiography. In addition to disturbing flow within the chamber, mural thrombi can break off and cause a stroke or embolization episode whose origins may be unclear (Abdelmoneim et al., 2014; Stratton and Resnick, 1987; Yokota et al., 1989); as such the clinical significance of these thrombi is likely underappreciated.

Several endothelium-specific mechanisms normally keep the endocardial surface thrombus free. These include generation of several anticoagulant factors on the endothelial surface including activated protein C (APC), tissue factor pathway inhibitor (TFPI), and platelet inhibitors nitric oxide and prostacyclins (Dahlback, 2007; Hackeng et al., 2009; Olas, 2015; Rahbar et al., 2015; Riewald and Schuepbach, 2008; Van de Wouwer et al., 2004; van Hinsbergh, 2012; Wood et al., 2014). Conversely, thrombin (FIIa) is generated on the endothelial luminal surface in prothrombotic states where it can convert fibrinogen to fibrin and promote release of von Willebrand factor (vWF) from

Abbreviations: EEC, endocardial endothelial cell; EPCR, endothelial protein C receptor; FIIa, thrombin; HF, heart failure; LVAD, left ventricular assist device; NGF, nerve growth factor; NPY, neuropeptide Y; TM, thrombomodulin; vWF, von Willebrand factor

* Corresponding author at: Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA
E-mail address: wohaib.hasan@cshs.org (W. Hasan).

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Table 1
Limited chart history of heart failure patients. Demographics and cardiac history of 8 heart failure patients whose cardiac tissue was used in this study. All medications are those current at the time of surgery for tissue removal. Six patients were tissue donors at time of left ventricular assist device (LVAD) implant. Two patients were donors at time of heart transplant. Tx = transplant, NIDCM = non-ischemic dilated cardiomyopathy.

	1	2	3	4	5	6	7	8
Age at donation	71	37	65	37	59	63	52	59
Gender	M	M	M	M	M	M	M	M
Tissue source	LVAD	LVAD	LVAD	LVAD	LVAD	LVAD	Tx	Tx
HF stage, NYHA class	D, Class III–IV	D, Class III–IV	D, Class III	D, Class III	D, Class IV	D, Class III–IV	D, Class III–IV	D, Class III
Etiology	NIDCM	NIDCM	Ischemic	NIDCM	NIDCM	Ischemic	Ischemic	Ischemic
TE history	DVT	LV mural thrombus	None	LV mural thrombus	None	None	None	None
Anticoagulants, antiplatelet agents	Warfarin	Warfarin, aspirin	Warfarin, aspirin	Warfarin	Aspirin	Aspirin	Warfarin, aspirin	Warfarin, aspirin, enoxaparin
Beta-blockers	None	Metoprolol	None	None	None	None	Metoprolol	Carvedilol
ACE inhibitors	None	Lisinopril	Lisinopril	None	Lisinopril	Lisinopril	Lisinopril	None

endothelial cells (Fukuchi et al., 2001; Kleber et al., 2015; Mourik et al., 2013; van Hinsbergh, 2012). Multimers of vWF aggregate on the endothelial surface and aid platelet adhesion.

The endocardium is protected from thrombus formation by specialized endothelial cells known as the endocardial endothelial cells (EECs). Although similar to vascular (and coronary) endothelium, EECs have distinct developmental origins, have greater gap junction expression than vascular endothelium, and are critical for cardiomyocyte peak force development through secretion of cardioactive factors (e.g. endothelin-1 and nitric oxide) (Brutsaert et al., 1988; Li et al., 1993; Ostadal et al., 1975; Red-Horse et al., 2010; Sugi and Markwald, 1996). We have recently shown that activation of protein C, as well as expression of its associated receptors thrombomodulin (TM) and endothelial protein C receptor (EPCR), are attenuated on the endocardial surface of heart failure mice (Schoner et al., 2015). Further, we demonstrated increased FIIa generation and vWF extrusion from the endocardial surface of heart failure mice, suggesting a prothrombotic endocardial surface is key for development of endocardial thrombi in heart failure.

A key follow up question to our studies in heart failure mice was what normally regulates endocardial endothelial health and how this may be disrupted in heart failure. We identified cardiac autonomic nerves as a possible candidate for regulating endothelial health from the subendocardium. This was based on several pieces of evidence suggesting a close functional relationship between nerves and endothelial cells. It is known that cardiac sympathetic tone is increased early in the progression of heart failure (Hasan, 2013; Meredith et al., 1991; Rundqvist et al., 1997; Shivkumar et al., 2016), and is a better predictor of heart failure severity than ejection fraction (Miranda et al., 2013). This increased cardiac sympathetic tone results in excessive norepinephrine (NE) release from cardiac nerve terminals with limited re-uptake, resulting in catecholamine toxicity to cardiomyocytes and tachyarrhythmia promotion (Azevedo et al., 2001; Bohm et al., 1995; Du et al., 1999; Kreusser et al., 2008; Ramchandra et al., 2009; Rundqvist et al., 1997; Triposkiadis et al., 2009; Tsutamoto et al., 2008; Watson et al., 2006). Such a direct effect on cardiomyocytes likely means similar effects on EECs that express appropriate receptors and are closely spatially associated with the endocardial surface. Indeed, sympathetic nerves in the subendocardium make intimate (0.2–0.6 μm) connections with EECs (Marron et al., 1995; Marron et al., 1994) and both nerves and endothelial cells release factors to maintain these connections. Sympathetic nerves promote endothelial cell proliferation and nitric oxide release (Ferro et al., 1999; Gray and Marshall, 1992; Panjala and Steinle, 2011; Rider et al., 2009; Spindler and Waschke, 2011). These nerves also release neuropeptides, such as galanin and neuropeptide Y (NPY); the latter is involved in EEC regulation of intracellular free calcium as well as in endothelial cell migration following injury (Ghersi et al., 2001; Gu et al., 1984; Jacques et al., 2006). In turn, endothelial cells release the neurotrophin nerve growth factor (NGF) for maintaining sympathetic nerve connections (Lecht et al., 2010; Sornelli et al., 2010). A close functional relationship exists therefore between these nerves and EECs.

In heart failure it is unknown whether factors released from sympathetic nerves promote a prothrombotic phenotype on EECs, or have a positive effect on EEC health and anticoagulant function. In our previous study (Schoner et al., 2015), we identified vWF extrusion from the endocardial surface as being a key indicator of endothelial dysfunction in the progression of heart failure in the mouse. In this study, using vWF extrusion as a marker for EEC prothrombotic phenotype, we examine the role of norepinephrine and its antagonists (both alpha and beta), as well as neuropeptides galanin and NPY (and their antagonists), in promoting or curtailing FIIa-mediated vWF extrusion from human heart failure EECs in culture. We aim to identify potential therapeutic avenues for improving EEC health in heart failure and other prothrombotic states.

2. Methods

2.1. Human heart failure patient tissue collection

Left ventricular (LV) tissue from the free wall was obtained from 8 male heart failure patients (patient characteristics in Table 1). We were limited to male donors due to the limited donor pool at our institute. The tissue was either LV tissue obtained at the time of LV-assist device (LVAD) implantation, or the explanted heart at transplant. For the LVAD-core tissue, a roughly 2–3 cm diameter piece of the LV apex was obtained and an equivalent location was chosen for the explanted heart pieces. Tissues from transplant patients with a prior LVAD implantation were excluded, as these devices directly affect coagulation.

2.2. Human heart failure EEC culture and immunocytochemistry

Tissue was obtained in the operating room at time of LVAD implantation, or when heart was excised prior to cardiac transplantation. The tissue was immediately placed in cold HBSS solution, transported to the laboratory, and tissue trimmed to 1 cm^2 under BSL-2 conditions. The endocardial surface was dissected free from the underlying myocardium and placed in a 24-well plate with the endocardial surface exposed. Collagenase solution (2 mg/mL) was used to cover the endothelial surface then incubated for 45 min at 37 °C. After digestion, the endocardial surface was gently scraped with a scalpel blade and the cell suspension washed with endothelial growth medium-2 (EGM-2; Lonza), filtered through a 40 μm cell strainer (Fisher) and spun at 200*g* for 10 min. The resultant pellet was reconstituted in 500 μL of EGM-2 media and cells grown in T25 culture flasks coated with collagen to confluence with medium changed every 48 h. Endothelial cells