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Activation of Endothelial Cells in Conduit Veins of Dogs With Heart Failure and Veins of Normal Dogs After Vascular Stretch by Acute Volume Loading

Paolo C. Colombo, MD[1], **Sharad Rastogi, MD**[2], **Duygu Onat, PhD**[1], **Valerio Zacà, MD**[2], **Ramesh C. Gupta, PhD**[2], **Ulrich P. Jorde, MD**[1], and **Hani N. Sabbah, PhD**[2]

[1]Department of Medicine, Division of Cardiology, New York Presbyterian Hospital, Columbia University College of Physicians & Surgeons, New York, New York

[2]Department of Medicine, Division of Cardiovascular Medicine, Henry Ford Hospital, Detroit, Michigan

Abstract

Background—The venous endothelium is a key regulator of central blood volume, organ perfusion, and hemostasis in heart failure (HF). We previously reported activation of the inflammatory/oxidative program in venous endothelial cells collected from decompensated HF patients. The underlying causes are unknown. We tested the hypothesis that the pro-inflammatory state of HF and vascular strain associated with congestion can activate the endothelial inflammatory/oxidative and hemostatic programs.

Methods and Results—We studied 6 normal (NL) dogs (left ventricular ejection fraction [LVEF] >50%, central venous pressure [CVP] = 8 ± 2 mm Hg) and 6 dogs with HF (LVEF ~30%, CVP 8 ± 2 mm Hg) produced by intracoronary microembolizations. Normal dogs were studied at baseline and 1 hour after fluid load to a target CVP ≥20 mm Hg. Endothelial cells were scraped from jugular veins; mRNA expression was analyzed by reverse transcription polymerase chain reaction. The endothelial inflammatory/oxidative and hemostatic programs were significantly activated in HF dogs compared with NL. In NL dogs, fluid load significantly activated the endothelial inflammatory/oxidative and hemostatic programs, and, concurrently, caused a significant increase in plasma neurohumoral indices to levels that approached those of HF dogs.

Conclusions—The pro-inflammatory state of HF and vascular strain associated with congestion can both activate venous endothelial cells in dogs in a manner consistent with that seen in HF patients.

Keywords

Heart failure; endothelium; inflammation

Endothelial cells form the inner lining of a blood vessel with critical basal and inducible metabolic and synthetic functions that regulate vasomotor tone, hemostasis, angiogenesis,

Correspondence to Hani N. Sabbah, PhD, Cardiovascular Research, Henry Ford Hospital, 2799 West Grand Boulevard, Detroit, MI 4820. hsabbah1@hfhs.org.

and inflammatory responses. Endothelial cells in response to biomechanical (ie, vascular strain associated with intravascular congestion) and biochemical stimuli (ie, systemic neurohormonal activation) within the circulation may switch in their synthetic profile from quiescent toward an activated state that is vasoconstricting, pro-inflammatory, and pro-thrombotic.[1–3]

Endothelium nitric oxide (NO)-dependent vasodilation is impaired in both arteries and veins in patients with chronic heart failure (HF),[4,5] and is an independent determinant of poor prognosis.[6] Endothelium-mediated control of the venous tone is critical in HF. Veins are low-pressure reservoirs that contain > 70% of the systemic blood volume.[7] The marked capacity of this reservoir implies that relatively small volume changes in the peripheral veins are followed by substantial alterations in central blood volume, and, thereby, in cardiac filling pressures.[7] Venous pressure is also critical for kidney perfusion and sodium excretion. An increase in renal venous pressure, while arterial pressure is unchanged, decreases sodium excretion in a canine model of isolated perfused kidney.[8,9]

The vascular endothelium modulates several other physiologic and pathologic processes besides NO-mediated control of the vasomotor tone. Inflammation, oxidative stress, and hemostasis are all controlled by the vascular endothelium through transitions between quiescent and activated states.[10,11] The endothelial inflammatory/oxidative program (ie, inducible nitric oxide synthase [iNOS] and cyclooxygenase-2 [COX-2], and nitrotyrosine formation) is activated in patients with decompensated HF.[12,13] Venous endothelial activation partially subsides with diuresis and clinical improvement during the index hospitalization. At the time of clinical decompensation, the venous endothelium is typically exposed to *acute* strain from intravascular congestion,[14,15] in addition to *chronic* systemic inflammation and oxidative stress, which are typical of the HF syndrome.[13,16] Whether each of these prototypic environmental (biomechanical and biochemical) “hits” are individually sufficient to cause endothelial activation in vivo is unclear. In the present study, we used a dog model of HF produced by serial coronary microembolizations[17] to investigate the hypothesis that the pro-inflammatory state of HF characterized by systemic neurohormonal activation, and the vascular strain associated with congestion are both sufficient to cause endothelial activation. This animal model of HF manifests the disease syndrome in the absence of other comorbid conditions, often present in patients with HF, and is thus more amenable to mechanistic investigations.

The objectives of this study were: 1) to compare *chronic* levels of venous endothelial activation in normal (NL) dogs and dogs with HF; 2) to determine the *acute* changes in venous endothelial activation in response to intravascular volume expansion (to a target central venous pressure [CVP] ≥20 mm Hg) in NL dogs); and 3) to compare *chronic* levels of systemic neurohormonal activation in HF dogs with those in NL dogs before and after *acute* volume load. Selected markers of endothelial activation linked to the inflammation/oxidative program (ie, iNOS and COX-2, tumor necrosis factor-α [TNF-α], receptor for advanced glycation end products [RAGE], and CD40), and hemostasis (ie, tissue factor [TF] and plasminogen activator inhibitor-1 [PAI-1]) were measured. Superoxide dismutase (SOD) and glutathione peroxidase (GPX) and were also assessed to determine whether *chronic* HF or an *acute* increase in intravascular pressure may also affect compensatory expression of

antioxidant enzymes. Plasma markers of systemic neurohormonal activation linked to the HF syndrome (ie, norepinephrine [NE], TNF-α, interleukin-6 (IL-6), and endothelin-1 [ET-1]) were concurrently measured.

Methods

We studied 6 normal (NL) mongrel dogs and 6 mongrel HF dogs with systolic left ventricular (LV) dysfunction produced by serial intracoronary microembolizations.

Experimental Model

Chronic LV dysfunction was produced by multiple sequential intracoronary embolizations with polystyrene latex microspheres (77 to 102 g in diameter), as previously described.[17] Coronary microembolizations were performed during sequential cardiac catheterizations under general anesthesia, mechanical respiratory support, and sterile conditions. The anesthesia regimen in the present study consisted of a combination of intravenous injections of oxymorphone hydrochloride (0.22 mg/kg), diazepam (0.17 mg/kg), and sodium pentobarbital (150 to 250 mg to effect). In all dogs, coronary microembolizations were discontinued when LV ejection fraction (LVEF), determined angiographically, was ~30%. To achieve this target EF, dogs underwent an average of 5.3 microembolization procedures performed over an average period of 8.8 weeks. Microembolization procedures were performed 1 to 2 weeks apart. The first 3 embolizations consisted of 2 mL microsphere suspension injected subselectively into either the left anterior descending or left circumflex coronary artery in an alternating fashion. Subsequent embolizations consisted of 3 to 6 mL of microspheres divided equally between the left anterior descending or left circumflex coronary artery. Left ventriculograms were performed at baseline in all dogs and before each embolization in HF dogs to assess LVEF. The LVEF was calculated as the ratio of the difference of LV end-diastolic volume (LVEDV) and LV end-systolic volume (LVESV) to end-diastolic volume. LV volumes were measured using the area-length method as previously described.[17]

Hemodynamic measurements and endothelial sampling were performed 2 weeks after last embolization under general anesthesia, mechanical respiratory support with positive end-expiratory pressure, and sterile conditions (see previous section). Aortic pressure was invasively monitored throughout the procedure with a catheter-tip micromanometer (Millar Instruments, Houston, TX). Central venous pressure was measured by using a 6 Fr right Judkins catheter, introduced through the right femoral vein, with the distal edge positioned at the level of the right atrium, connected to a fluid-filled system. Heart rate was also monitored throughout the procedure. Normal dogs were studied at baseline and 1 hour after rapid fluid load (≥500 mL Dextran-40) resulting in a sustained increase in CVP to ≥20 mm Hg. The right jugular vein was excised and cut open in NL and HF dogs at baseline. The left jugular vein was excised and cut open in NL dogs 1 hour after rapid fluid load. Blood was carefully removed by washings with saline. The endothelium was collected by gentle scraping. Ten milliliters of blood were also withdrawn from the CVP catheter at the same time points.

Endothelial mRNA Expression

Total RNA was isolated from endothelial tissue using RNAqueous-4PCR Kit according to the manufacturer's instructions (Ambion, Austin, TX). The isolated RNA in each sample was determined spectrophotometrically. RNA with an absorbance ratio (260 nm/280 nm) higher than 1.7 and that exhibited 3 major bands, namely 28S, 18S, and 5.8S on 2% agarose, with 28S being much stronger than 18S, was considered of good quality. Approximately 4 μg RNA was reverse-transcribed into cDNA in an assay volume of 80 μL. The assay contained (final concentration) 3.6 mM of each dNTP (dATP, dTTP, dGTP, and dCTP), 40 units recombinant RNasin (RNase inhibitor, Promega), 6 μM oligo (dT) primer, and 1 unit MMLV reverse transcriptase in 10 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM DTT, and 3.0 mM $MgCl_2$. Assay tubes were incubated at 42°C for 60 minutes and then at 96°C for 10 minute for denaturation. For each polymerase chain reaction (PCR), 2 μL first-strand cDNA was added to 18 μL of a reaction mixture containing 20 pmol of each gene-specific forward and reverse primer, 200 μM of each dNTP, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.1% Triton-X100, and 3.0 mM $MgCl_2$. After heating the tube to 95°C for 5 minutes, 1-unit platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA) was added and the PCR was allowed to proceed for 20 to 40 cycles. PCR products were analyzed by subjecting 20 μL of each reaction mixture to electrophoresis on 1% to 1.5% ethidium-bromide-agarose gels. Band size of the products was compared with standard DNA size markers. The primers were based on the gene sequences reported to Gene Bank. Gene accession numbers, primer sequences, and amplicon sizes are shown in Table 1. Band intensity was quantified in arbitrary densitometric units (du) using a Bio-Rad Gel densitometer.[18]

Measurements of Biomarkers in Plasma

TNF-α, IL-6, and ET-1 concentration was determined in plasma on the principle of the double antibody sandwich enzyme-linked immunosorbent assay[18–20] and NE based on competitive enzymelinked immunosorbent assay.[20,21] All biomarkers were assayed using commercially available assay kits. Kit for NE was purchased from ALPCO Diagnostics (Salem, NH) for IL-6 and TNF-α from Assay Designs Inc (Ann Arbor, MI), and for ET-1 from R&D Systems (Minneapolis, MN). Using standard curves and software, the concentration of each biomarker was determined and expressed as pg/mL.

Data Analysis

Data are presented as mean ± SD. Measurements were compared by Student's *t*-test. The study was approved by Henry Ford Health System Institutional Animal Care and Use Committee and conformed to the National Institute of Health "Guide and Care for Use of Laboratory Animals" and the "Position of the American Heart Association on Research Animal Use."

Results

At baseline, NL dogs had higher mean arterial pressure (MAP), heart rate (HR), and LVEF, and lower LVEDV and LVESV than HF dogs with severe LV dysfunction, whereas baseline CVP was identical (Table 2).

Acute intravascular volume expansion with Dextran-40 significantly increased MAP, HR, and CVP in NL dogs (Table 2). Fluid load also caused a significant increase in plasma NE, IL-6, ET-1, and TNF-α to levels that approached those of HF dogs (Table 2).

The vascular endothelium was successfully collected from internal jugular veins: eNOS (endothelial marker) was highly expressed, whereas leukocyte common antigen-1 (LCA-1) (leukocyte marker) was barely detected in all vascular samples (Fig 1).

Endothelial iNOS, COX-2 TNF-α, RAGE, CD40, TF, PAI-1, SOD, and GPx were significantly increased in HF dogs compared with NL (Fig 1, Table 3). In NL dogs, acute fluid load doubled iNOS, COX-2, TNF-α, RAGE, CD40, TF, SOD, and GPx expression to levels that approached those of HF dogs. This increase was statistically significant with the exception of TF where the *P* value was .07.

Discussion

The present data provide the first in vivo evidence that the pro-inflammatory state of HF itself, and vascular stretch from intravascular pressure overload, are both sufficient to activate venous endothelial cells in dogs in a fashion consistent with that seen in patients with HF. Molecular data in dogs expand our previous findings in humans[13] by showing that 1) increased COX-2 and iNOS expression are manifestations of a coordinated and comprehensive activation of the inflammatory/oxidative and hemostatic programs with adaptive expression of antioxidant enzymes and that 2) this activation takes place in response to both biochemical (ie, systemic neurohormonal activation) and biomechanical (ie, vascular strain associated with intravascular congestion) environmental stressors, the latter being itself associated with an acute surge in plasma neurohormonal indices.

In HF patients and in experimental canine models of HF, excessive systemic inflammation and oxidative stress may result in progressive tissue injury when endogenous defenses and repair processes are overwhelmed.[22–25] After completion of the embolization protocol, HF dogs continue to deteriorate as evidenced by a progressive reduction of LVEF, increase in LVEDV and filling pressures.[17] As cardiac function declines, plasma neurohormonal indices increase[25] in a manner consistent with that seen in patients with CHF.

In the midst of this hemodynamic and humoral turmoil, endothelial cells, can integrate and transduce biomechanical (eg, vascular strain[3]) and biochemical stimuli (eg, circulating cytokines,[26] advanced glycation end products [AGEs],[27,28] oxidized lipoproteins[29]) into a series of key physiologic and pathologic signaling programs that modulate not only vascular tone, but also, among the others: inflammation, oxidative stress, and hemostasis.[11,30]

In vitro studies have shown that cytokines,[31] AGEs,[27] oxidative products,[32] and vascular strain[3,33] may promote overlapping patterns of endothelial activation (through an increase in oxidative stress or nuclear translocation of transcription factor NF-*k*B)[34] characterized by increased expression of iNOS,[35,36] COX-2,[37,38] TNF-α,[35,39,40] RAGE,[27,39] CD40,[32,41] (inflammatory/oxidative program), TF,[41,42] and PAI-1[43,44] (hemostatic program). In the present study, endothelial inflammatory/oxidative and hemostatic programs were activated in *chronic* HF dogs, and in NL dogs after *acute* intravascular volume expansion as compared

with euvolemic NL dogs. Our experimental design specifically tested the effects of acute intravascular volume expansion on endothelial activation since venous pressures are typically increased in HF patients at the time of clinical decompensation.[45] Unfortunately, because pulmonary artery catheters were not inserted, we cannot provide objective data on pulmonary congestion (ie, pulmonary artery and wedge pressures).

Inducible NOS has recently been shown to bind, nitrosylate, and activate COX-2, a key observation that links 2 major human regulatory systems, NO and prostaglandins, in their response to environmental stressors.[46] iNOS may also shift from NO to superoxide production. This shift occurs when nitric oxide synthases are uncoupled because of oxidation of tetrahydrobiopterin (a critical cofactor for NO synthases)[47,48] or from high levels of asymmetric dimethylarginine (ADMA),[49] a competitive inhibitor that is increased in several chronic inflammatory disease states including *chronic* HF.[50] TNF-α,[51] RAGE,[52] and CD40[53] may also increase superoxide production by promoting NAD(P)H oxidase activity. Oxidative injury itself may cause endothelial cells to transition from a quiescent to an activated state where, in a vicious cycle, oxidative stress promotes inflammation, and inflammation, in turn, increases oxidative stress. In this context, antioxidant enzymes are the primary defense mechanism against damage, counteracting a system that has lost internal control. SOD and GPx inactivate reactive oxygen species, thereby protecting cells from the pleiotropic detrimental effects of inflammation and oxidative stress.[54–56] Our observation that expression of antioxidant enzymes is increased in the HF state, and in response to increased vascular strain likely represents a compensatory attempt to counteract this bidirectional and harmful axis that links oxidative stress to inflammation.

We also observed that *chronic* HF dogs and NL dogs after *acute* intravascular volume expansion exhibit increased expression of pro-coagulant genes. Patients with HF are at an increased risk of venous thromboembolism, a risk that escalates with HF progression.[57,58] Our data provide the first in vivo mechanistic evidence that both biomechemical and biomechanical stressors may independently contribute to this clinical complication of HF.

One limitation of the present study is that our experimental design included only 2 time points, baseline and 1 hour after fluid load, and, because of that, acute activation of the venous endothelium in NL dogs was not followed through its resolution. Besides, because protein and protein activity were not measured, we can only assume that protein and protein activity were also increased. On the other hand, 1-hour fluid load was chosen for mechanistic and practical purposes, rather than for clinical reasons, because fluid accumulation in decompensated patients, takes place and resolves over days (or weeks), rather than hours, in a setting of decompensation.[59,60] Therefore, monitoring the resolution of endothelial activation in dogs after 1 hour of intravascular volume expansion, even if described, would not have had any direct clinical correlates.

Importantly, our study cannot identify the source of increased neurohormonal synthesis in response to intravascular volume expansion. It is possible that the endothelium itself contributes to this neurohormonal surge in response to a mechanical stressor. The strongest arguments that favor an “endothelial” contribution are: 1) ET-1 is primarily an endothelial cytokine; 2) we have documented an increase in TNF-α expression in the ECs sampled from

NL dogs after volume load; and 3) previous reports indicate that ET-1,[61] IL-6,[62] and TNF-α[63] can be secreted by the endothelium within hours in response to mechanical stress.

In conclusion, we demonstrated that *chronic* pro-inflammatory state of HF and *acute* vascular strain associated with congestion promote a common pattern of endothelial activation with coordinated expression of pro-inflammatory, pro-oxidative, and pro-coagulant genes. Compensatory upregulation of antioxidant enzymes was also observed. Future longitudinal studies may test the effect of intermittent bouts of intravascular volume expansion in experimental models of HF to determine whether they may in fact act as secondary "hits" to augment baseline levels of endothelial activation, and, eventually, promote progression of *chronic* HF syndrome.

Activation of the venous endothelium is a further manifestation of the systemic inflammatory/oxidative and hypercoagulable state that accompanies the syndrome of advanced HF. Chronic inflammation and acute increase in intravascular volume appear sufficient to trigger endothelial activation. Future investigations are warranted to clarify whether the venous endothelial phenotype may ultimately allow us to track in a serial manner, in the unique individual, the vascular impact of subsequent environmental (ie, biomechanical and biochemical) stressors, as well as the potential response to therapeutic interventions.

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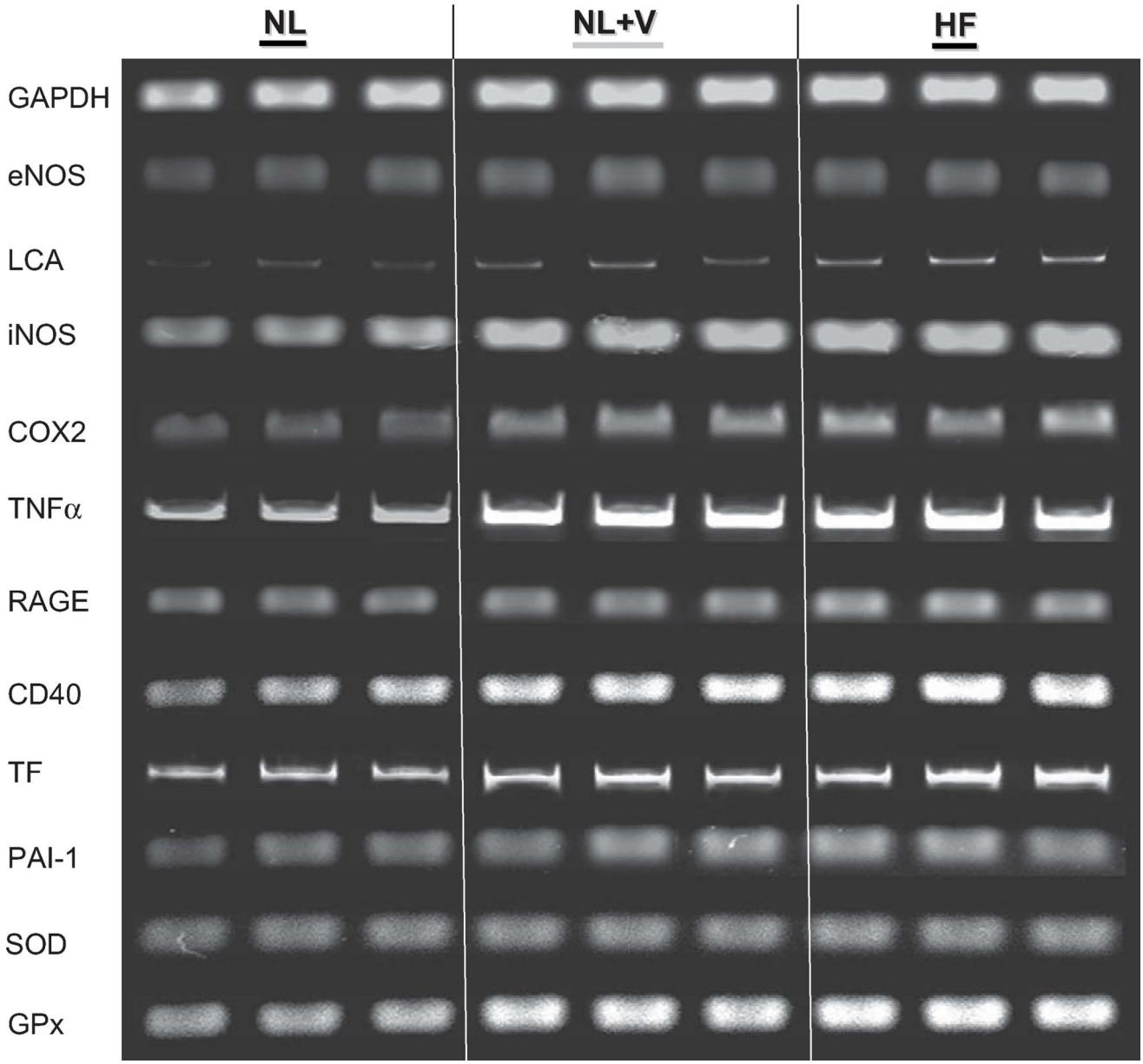

Fig. 1.

Representative ethidium-bromide-agarose gels of reverse transcriptase-polymerase chain reaction amplification products from vascular samples in normal (NL) dogs before and after acute volume (V) load, and in heart failure (HF) dogs. Endothelial nitric oxide synthase and leukocyte common antigen expression (LCA) confirmed the endothelial nature of the sample with minimal leukocyte contamination. GADPH served as an internal control for loading conditions. Band intensity of inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2), tumor necrosis factor-alpha (TNF-α), receptor for advanced glycation end products (RAGE), CD40, tissue factor (TF), plasminogen activator inhibitor-1 (PAI-1), superoxide dismutase (SOD), and glutathione peroxidase (GPx) mRNA expression was quantified in arbitrary densitometric units (du) using a Bio-Rad Gel densitometer. VO.

Table 1

Primer Sequences, Amplicon Sizes, and Gene Accession Numbers

Genes	Sequences	Product Size	Accession Number
GAPDH	F: 5′-ACCACCATGGAGAAGGCTGG-3′ R: 5′-CTCAGTGTAGCCCAGGATGC-3′	528	AB038240
eNOS	F: 5′-CGCTGTCTAGGCTCTCTGGT-3′ R: 5′-ATCAAACACCTGCAGCTTCC-3′	300	AF077821
LCA	F: 5′-GCGGTTACTCCCAGTTTTCA-3′ R: 5′-CAGAGCTACAGCAGGACACG-3′	301	CD215065
iNOS	F: 5′-GGCTCAAATCACAACGGAAT-3′ R: 5′-GAATAGGGAGCTGGGAGAGC-3′	82	AF143503
COX-2	F: 5′-TGAAACCCACTCCAAACACA-3′’ R: 5′-AACTGATGCGTGAAGTGCTG-3′	385	AY462100
TNF-α	F: 5′-TGACGGGCTTTACCTCATCT-3′ R: 5′-GGCGATGATCCCAAAGTAGA-3′	313	AF348421
RAGE	F: 5′-GGACTCTTAGCTGGCACTGG-3′ R: 5′-CCAGTGGTCATGGGCTTACT-3′	382	AY836509
CD40	F: 5′-ATATGTGCCCACCAGGAGAG-3′ R: 5′-AATGATGGGGACCACCACTA-3′	488	AY333789
TF	F: 5′-CCTCAGGCACTGCAGATGTA-3′ R: 5′-GGCTGTCCAAGCTTTGTCTC-3′	325	AB200288
PAI-1	F: 5′-CTCTCTCTGCCCTCACCAAC-3′ R: 5′-TCCAGTGTTGTCCCAGATGA-3′	442	XM_844252
SOD	F: 5′-TGCAGATGACCTGTCAGAGC-3′ R: 5′-GTGCCGGTCAGAGTCCTTAG-3′	440	XM_533218
GPx	F: 5′-GGCATCAGGAAAACGCTAAG-3′ R: 5′-CATGGATGAAATCCCCAGAG-3′	430	XM_533828

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; LCA, leucocyte common antigen; COX-2, cyclooxygenase 2; TNF-α, tumor necrosis factor-alpha; RAGE, receptor for advanced glycation end products; TF, tissue factor; PAI-1, plasminogen activator inhibitor-1; SOD, superoxide dismutase; GPx, glutathione peroxidase.

Table 2

Hemodynamics and Plasma Neurohormones in 6 NL Dogs before and after Acute Volume Load, and in 6 HF Dogs

	NL	NL + VO	HF
MAP, mm Hg	90 ± 21	130 ± 18 *	70 ± 10 *
HR, beats/min	88 ± 19	125 ± 13 *	64 ± 4 *
CVP, mm Hg	8 ± 2	22 ± 4 *	8 ± 2
LVEDV, mL	52 ± 1		67 ± 2 *
LVESV, mL	23 ± 2		48 ± 2
LVEF, %	56 ± 3		28 ± 1
NE, pg/mL	130 ± 11	491 ± 128 *	825 ± 83 *
IL-6, pg/mL	3.3 ± 1.2	15.8 ± 4.3 *	27.9 ± 7.7 *
ET-1, pg/mL	0.2 ± 0.1	1.8 ± 0.2 *	2.6 ± 0.8 *
TNF-α, pg/mL	1.1 ± 0.7	2.7 ± 0.3 *	4.3 ± 0.8 *

NL, normal; VO, volume; HF, heart failure; MAP, mean arterial pressure; HR, heart rate; CVP, central venous pressure; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; LVEF, left ventricular ejection fraction; NE, norepinephrine; IL-6, interleukin-6; ET-1, endothelin-1; TNF-α, tissue necrosis factor-alpha.

* $P < .05$ vs. NL dogs.

Table 3

Endothelial Gene Expression in 6 NL Dogs before and after Acute Volume Load, and in 6 HF Dogs

	NL (CVP = 8 ± 2 mm Hg)	NL+VO (CVP = 22 ± 4 mm Hg)	HF (CVP = 8 ± 2 mm Hg)
iNOS (du)	0.26 ± 0.09	0.48 ± 0.13*	0.54 ± 0.18*
COX-2 (du)	0.44 ± 0.14	0.67 ± 0.06*	0.76 ± 0.09*
TNF-α (du)	0.29 ± 0.02	0.47 ± 0.11*	0.54 ± 0.09*
RAGE (du)	0.35 ± 0.12	0.48 ± 0.07*	0.56 ± 0.08*
CD40 (du)	1.26 ± 0.30	1.91 ± 0.10*	2.29 ± 0.04*
TF (du)	0.57 ± 0.05	0.69 ± 0.12	0.88 ± 0.17*
PAI-1 (du)	0.62 ± 0.16	1.05 ± 0.20*	1.12 ± 0.25*
SOD (du)	1.05 ± 0.08	1.23 ± 0.04*	1.37 ± 0.11*
GPx (du)	1.33 ± 0.12	2.10 ± 0.01*	2.34 ± 0.12*

NL, normal; VO, volume; HF, heart failure; CVP, central venous pressure; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; TNF-α, tumor necrosis factor-alpha; RAGE, receptor for advanced glycation end products; TF, tissue factor; PAI-1, plasminogen activator inhibitor-1; SOD, superoxide dismutase; GPX, glutathione peroxidase.