

Autonomic Contribution to Endothelin-1 Increase during Laboratory Anger-Recall Stress in Patients with Coronary Artery Disease

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In coronary artery disease (CAD), endothelin-1 (ET-1) is released by activated macrophages and thereby contributes to coronary plaque rupture and triggered cardiac events. The multifactorial regulation of ET-1 includes stimulated release by cytokines and autonomic factors. Laboratory stress provokes alteration in autonomic tone and prolonged ET-1 mediated endothelial dysfunction. The objective of the study is to determine the autonomic contribution to an increase in ET-1 in response to laboratory stress in patients with CAD. Patients (n = 88) with chronic stable CAD instrumented with hemodynamic monitor, digital electrocardiogram (ECG) monitor and indwelling catheter for blood sampling completed a laboratory protocol that included initial rest (30 min), baseline (BL: 10 min), and anger recall stress (AR: 8 min). Change from BL to AR was determined for (a) parasympathetic activity (by spectral analysis of ECG); (b) sympathetic activity (by circulating catecholamines); and (c) ET-1. AR provoked increases from BL in catecholamines, and a decrease in parasympathetic activity. Multivariate analysis with change in parasympathetic activity and catecholamines, while controlling for age and use of β -blockers, revealed a significant odds ratio (OR = 3.27, 95% CI 1.03, 10.41 $P = 0.04$) for an increase in ET-1 associated with parasympathetic withdrawal; no other variables were significant. The predominant influence of parasympathetic activity on anger/stress-provoked increase in ET-1 is consistent with the cholinergic antiinflammatory pathway. Future examination of autonomic influences on atherosclerotic leukocytes, endothelial cell function and the dynamics of ET-1 are warranted.

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INTRODUCTION

Endothelin-1 (ET-1) is a potent endogenous vasoconstrictor that can be released rapidly by activated macrophages (1) and the vascular endothelium (2–4) in response to stimuli of short duration (5), thereby contributing to coronary plaque rupture (6,7) and triggered cardiac events (8,9), including those triggered by emotional stress (10). The multifactorial regulation of ET-1 includes stimulated release by cytokines (11–14) and autonomic factors. Acetylcholine (ACh), the primary messenger of the parasympathetic nerv-

ous system is known to influence ET-1 secretion and action directly (15), and may furthermore influence ET-1 release indirectly, as described by Tracey *et al.* (16), through cytokine release (11,12,14). In addition, bi-directional and synergistic effects between ET-1 and norepinephrine secretion, and vasomotor modulation have been reported (17,18). Hence, influences on ET-1 release are multifaceted and include both the direct and indirect contribution of autonomic pathways.

Given the demonstrated role of emotional stress as a trigger of catastrophic

cardiac events (19,20), we and others have used laboratory probes to study the pathophysiology underlying the link between stress and acute coronary syndromes. With this paradigm, the response to cognitive and emotional stressors has been characterized by heightened sympathetic nervous system activity, indexed by an increase in circulating levels of catecholamines (21), and reduced parasympathetic activity, indexed by reduced heart rate variability in the high frequency domain (22,23). Each of these effects contributes to elevations in heart rate, and systolic and diastolic blood pressure (24), and in patients with coronary artery disease (CAD), to reduced myocardial blood flow due to both epicardial vasoconstriction (25,26) and microvascular dysfunction (27). Laboratory emotional stress also provokes endothelial dysfunction that can persist for more than 90 min

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after the stress has been terminated (28), with a central role for endothelin-1 (ET-1) recently demonstrated when ET_A receptor blockade abolished this effect (29).

The modulation of ET-1 during laboratory emotional stress, while likely to involve autonomic pathways, remains to be described. Laboratory administration of anger-related stress—a stimulus with a demonstrated contribution to provoked ischemic syndromes, and with ET-1 an apparently important component in the overall pathophysiology—provides a useful paradigm for an exploration of these dynamics. The current study therefore sought to examine the contribution of changes in autonomic activity provoked by this laboratory stressor to concurrent changes in circulating ET-1 among patients with CAD.

MATERIALS AND METHODS

Subjects

Patients with chronic stable CAD (n = 88) documented by history of acute coronary syndrome (ACS), surgical or percutaneous revascularization and/or positive exercise myocardial perfusion study were recruited from the cardiology outpatient clinics at Yale University Medical Center and the Veterans Administration Connecticut Healthcare System. Patients with a diagnosis of myocardial infarction or unstable angina within 3 months of the study, surgical or percutaneous revascularization within 6 months of the study, major cardiac arrhythmia or presence of a pacemaker or implantable cardioverter defibrillators, uncompensated congestive heart failure, incapacitating or life-threatening illness, diabetes mellitus (due to effects on endothelial function and vessel wall inflammation), major psychiatric or substance abuse disorder (by history), cognitive impairment, pregnancy and/or inability to speak or read English, were excluded. Medical chart review and patient interview were used to obtain demographic information and to determine cardiovascular risk profile. The population was homogeneous with regard to severity of CAD as determined

Table 1. Patient characteristics.

Variable	Overall cohort, % or mean (SD) ^a	Group 1 (n = 46), % or mean (SD) ^{a,b}	Group 2 (n = 42), % or mean (SD) ^{a,b}
Age	67.1 (8.8)	67.9 (8.6)	66.2 (9.0)
Female	3.4	6.5	0.0
Nonwhite race	10.2	8.7	11.9
History of hypertension	84.1	80.4	88.1
Active smokers	14.8	13.0	16.7
LVEF	52.3 (9.3)	52.8 (8.8)	51.7 (9.8)
Medications			
Ace inhibitors	52.3	52.2	52.4
β-Blockers	78.4	73.9	83.3
Aspirin	68.2	71.7	64.3
Calcium channel blocker	28.4	32.6	23.8
Statins	89.8	89.1	90.5

^aValues are percentage of group, or group mean (SD).

^bGroup 1 patients demonstrate increase in ET-1 from BL to AR. Group 2 patients do not demonstrate increase in ET-1 from BL to AR. There were no significant differences between groups on any variable.

by myocardial perfusion study. Participants with a recent history of systolic pressure > 140 mmHg or diastolic pressure > 90 mmHg or currently taking medication for high blood pressure were classified as hypertensive, while those with total cholesterol ≥ 200 mg/dL, LDL ≥ 130 mg/dL or taking cholesterol lowering medications were classified as having hypercholesterolemia. Tobacco use also was determined. The study was approved by the Institutional Review Board at both medical facilities. Patient characteristics are provided in Table 1.

Procedures

Participants reported to the Cardiovascular Behavioral Medicine research laboratory at 9:00 AM on the morning of study. They were instructed to eat a light breakfast and take their normal medications. Upon arrival, they were instrumented with 5-lead digital holter monitor and automated hemodynamic monitor. An indwelling intravenous (i.v.) catheter was placed for collection of blood samples. Participants then relaxed in a recumbent position, and a 30-min rest period was initiated to allow for return of catecholamines to resting levels after catheter placement.

After completion of the 30-min rest period, participants underwent a standard

mental stress protocol (24,25,29) defined by a 15-min resting baseline (BL) and subsequent 8-min anger recall (AR). The time of initiation and end for each of these conditions (rest, BL, AR) was recorded. During BL, participants were instructed to close their eyes and imagine being in a restful setting. Approximately 10 min into this condition, a 4-mL blood sample was collected into refrigerated tubes containing reduced glutathione (for catecholamine assay) and placed on ice. A second blood sample was collected into a refrigerated vacuum tube containing potassium EDTA as an anticoagulant, and mixed by gently inverting the tube for 30 sec (for ET-1 assay) and placed on ice as well.

After completion of BL, the AR condition was initiated. Participants were instructed to recall a recent incident that had made them aggravated or angry. They were then instructed to describe this incident to the interviewer in detail. Follow-up questions designed to promote the reexperience of anger vividly were asked throughout the condition. Approximately 2 min into the condition, a 4-mL blood sample was collected into refrigerated tubes containing reduced glutathione (for catecholamine analysis) and placed on ice. The timing of the 2-min sample was based on previously

published data from our laboratory concerning onset and peak of cardiovascular effects associated with laboratory emotional stress (24). At the end of AR, an additional sample was collected into a refrigerated vacuum tube containing potassium EDTA (for ET-1 assay), mixed by gently inverting the tube for 30 sec, and placed on ice. Upon completion of the condition, the catheter was removed, and patients were de-instrumented and released.

Measures

Heart rate (HR), and systolic and diastolic blood pressure (SBP and DBP, respectively) were collected every 2 min during BL and every min during AR. Averages were calculated for these parameters separately for the last three readings of BL and for all readings during AR. Change in HR and SBP/DBP from BL to AR was calculated by subtracting the BL average from the AR average.

Following standardized procedures (30,31) that we have used previously (32,33) for the determination of high frequency power (0.15 to 0.40-Hz frequency band), the Holter recorded continuous electrocardiogram (ECG) was scanned by an experienced technician who then edited the RR interval data to remove ectopic beats and noise. Gaps were filled by interpolated linear splines. Holter recordings with >20% interpolated RR intervals were excluded from further analysis. The RR interval time series was sampled using a boxcar window to obtain 1,024 samples per 5 min (3.41333 Hz). The power spectrum was computed using a fast Fourier transform with a Parzen window on 4-min segments with a 1-min sliding window, corrected for attenuation owing to windowing and sampling and integrated over five standard frequency bands. High-frequency power (hf-HRV) in Ln msec^2 , a demonstrated index of parasympathetic activity (34), was determined separately for BL and AR conditions through averaging of HF values for 4-min segments within each phase, and change was calculated (AR minus BL).

Blood samples for catecholamine analysis were brought to the Yale General Clinical Research Center within 60 min where they were spun (centrifugation at 3000g for 15 min) and stored at -70°C until analysis. Samples from each patient were assayed in the same batch. Catecholamines were analyzed by high-performance liquid chromatography, (ESA Inc, Chelmsford, MA, USA) using electrochemical detection (Coulchem II, ESA Inc) after alumina extraction. Sensitivity of the assays for both epinephrine and norepinephrine were at least 5 pg/mL.

Blood samples for ET-1 assay were centrifuged at 3000g for 15 min to separate plasma. Aliquots of plasma were then stored at -70°C until analysis. Enzyme-linked immunosorbent assay (ELISA) was used for assessment of ET-1 using a colorimetric sandwich kit generating absorbance at 450 nm (Biomedica Gruppe, Austria). The kit has a detection limit of 0.02 fmol/mL (0.05 pg/mL). Specificity of the antibody used in this kit has been described previously (35). This kit has been used to obtain results in a previous study of peripheral ET-1 levels in humans (36). All assays from a single subject were analyzed in one assay to ensure against interassay variation.

Statistical Analysis

For the primary analysis, change from BL to AR was computed for ET-1, hf-HRV, epinephrine and norepinephrine. The distributions of ET-1 at BL and AR were highly skewed, as was the change, and multiple transformation efforts did not alter this substantially (all Shapiro-Wilks P values were < 0.0001). Given the nature of the population, defined as having chronic stable coronary disease and in almost all cases taking at least one vasoactive medication, this was not unexpected. Change in ET-1 (fmol/mL) was therefore treated as a dichotomous variable (increase versus no change/decrease), an approach further justified by our interest in the factors that contribute to an increase in this protein under conditions of emotional stress; changes in hf-HRV, epinephrine and norepinephrine were treated as

continuous variables. Multivariate logistic regression was used to determine the odds of an increase in ET-1 from BL to AR versus a decrease/no change associated with these variables, adjusting for age and β -blocker use, each known to influence levels of ET-1, dynamically.

RESULTS

The average age of the study cohort was approximately 67 years, with 3.4% female and 10% nonwhite. Most patients had a history of hypertension (84%) and less than 15% were active smokers. Mean LVEF was greater than 50%. Over half were on ACE inhibitors, over 78% on β -blockers, over 28% on calcium channel agents, almost 90% on statins, and almost 70% on aspirin. There was no significant difference in these variables between those individuals who demonstrated an increase in ET-1 from BL to AR (group 1) and those individuals who did not (group 2) (Table 1).

AR Stress and Change in ET-1

Consistent with previous research (24,25), there was overall a significant increase in HR, SBP, DBP, epinephrine and norepinephrine, and a significant decrease in hf-HRV from BL to AR. In addition, the average resting level of ET-1 for the total cohort was $1.58 (\pm 2.69)$ fmol/mL; the group defined by an increase in ET-1 from BL to AR demonstrated an 11% increase while the remainder demonstrated a 7% decrease (see Table 2). The multivariate model revealed an odds ratio (OR) of 3.27 (95% CI 1.03, 10.41 $P = 0.04$) for the risk of an increase in ET-1 associated with a decrease in hf-HRV. There was no associated effect for change in epinephrine or norepinephrine in this model that controlled for age and β -blockers, nor were the differences in epinephrine and norepinephrine between the groups at BL or AR significant (Table 3).

Effect of β -Blockers

In secondary analyses, we examined whether use of β -blockade affected the hemodynamic and autonomic response to AR. In these comparisons, both patients taking and not taking β -blockers

Table 2. Physiologic response to AR.^a

Measure	Overall (n = 88)		Group 1 ^b (n = 46)		Group 2 ^b (n = 42)	
	BL	AR	BL	AR	BL	AR
HR (bpm)	56.2 (8.6)	66.1 (10.6) ^c	55.5 (7.7)	65.8 (10.4) ^c	56.9 (9.5)	66.4 (10.9) ^c
SBP (mmHg)	131.7 (15.6)	161.1 (20.2) ^c	132.7 (14.5)	162.4 (19.0) ^c	130.7 (16.8)	159.6 (21.6) ^c
DBP (mmHg)	74.3 (8.5)	87.3 (10.5) ^c	74.0 (7.8)	88.0 (9.8) ^c	74.7 (9.3)	86.5 (11.4) ^c
hf-HRV ^d	4.8 (1.1)	4.5 (0.9) ^c	5.0 (0.9)	4.5 (0.8) ^c	4.6 (1.3)	4.5 (1.1)
Epinephrine (pg/mL)	27.2 (27.9)	30.8 (40.3) ^c	32.2 (36.5)	44.8 (49.7) ^c	22.2 (14.6)	30.7 (25.4) ^e
Norepinephrine (pg/mL)	336.1 (270.8)	350.0 (202.0) ^c	375.0 (309.9)	378.5 (215.1)	293.6 (216.1)	318.5 (184.0) ^f
Endothelin-1 (fmol/mL)	1.58 (2.69)	1.54 (2.70)	1.00 (1.79)	1.11 (2.04)	2.18 (3.31)	2.04 (3.25)

^aValues are mean (SD).

^bGroup 1 patients demonstrate increase in ET-1 from BL to AR, Group 2 patients demonstrate no change/decrease in ET-1 from BL to AR.

^cChanges significant at $P < 0.0001$.

^dHigh frequency power in Ln msec².

^eChange significant at $P < 0.001$.

^fChange significant at $P < 0.01$.

demonstrated a significant increase in HR and SBP/DBP (all $P < 0.0001$), with no differences between these groups at BL or AR. Those taking and not taking β -blockers also demonstrated a significant increase in epinephrine ($P < 0.01$) and norepinephrine ($P < 0.001$). Again, there were no significant differences between these groups at BL or AR. β -blockers also had no effect on the parasympathetic response to AR, with the pattern the same for both groups of patients, and no differences at BL or AR.

DISCUSSION

Moderate to extreme anger and associated emotions can provoke transient (18,19) and catastrophic ischemic syndromes (24,25), mediated in part by dysregulated vasomotor tone (25–27). In the current study with CAD patients, AR stress, as expected, provoked an increase in sympathetic and a decrease in parasympathetic activity, however, only the latter predicted an associated increase in ET-1. Specifically, for each unit decrease in high frequency power, representing reduced parasympathetic activity, consequent to anger recall stress, there was a 3.27-times-greater likelihood of an associated increase in ET-1; increased sympathetic activity as demonstrated by circulating catecholamines showed no relationship to change in circulating levels of this peptide. ET-1 is a

potent vasoconstrictor, acting in both the peripheral and cardiac circulations (2–4,37,38). Furthermore, ET-1 has been identified as a key component in the triggering of acute coronary syndrome (10) and the dysregulated vascular response to mental stress (29). While data indicate that both sympathetically and parasympathetically mediated processes are involved in ET-1 regulation and effect, the specific processes by which mental stress modulates ET-1 release have not been identified previously. The current exploratory study more specifically supports a role for parasympathetic pathways.

CAD is a disease of chronic inflammation characterized in part by macrophage infiltration of atherosclerotic plaques (39). These macrophages continuously release inflammatory cytokines that, among other things, provoke endothelial cell dysfunction (36). Endothelial dysfunction contributes to the paradoxical vasoconstriction observed during mental

stress and Ach infusion in studies of patients with CAD (26). While endothelial function in general and increased vasomotor tone in particular are in part due to the availability of endogenous nitric oxide (NO), the vascular smooth muscle (along with macrophages) also produce ET-1, which, as a result, is abundantly present in the intima of atherosclerotic coronary arteries (3,4,37,38). A recent study found that at the site of stenotic coronary segments, ET-1 accounts almost totally for resting vasomotor tone, and contributes significantly to reactive tone in the coronary microvascular bed (38). Thus, ET-1 may play a prominent role in dynamic coronary vasomotor tone under conditions of emotional stress.

The role of vagal pathways in the modulation of cytokine secretion by macrophages has been demonstrated in a series of studies on a rat model of inflammation by Tracey *et al.* (16) where stimulation of these pathways attenuated

Table 3. Logistic regression predicting ET-1 increase from BL to AR.

Variable	Point estimate	95% Wald confidence limits
Change in hf-HRV ^a	3.270	1.027, 10.413
Change in epinephrine	1.029	0.993, 1.066
Change in norepinephrine	0.997	0.989, 1.004
Age	1.035	0.960, 1.116
Use of β -blockers	0.455	0.140, 1.482

^aHigh frequency power in Ln msec².

the release of inflammatory cytokines from macrophages during endotoxemia, while vagotomy eliminated this attenuation. Emerging data also shows a role for vagal pathways in the modulation of inflammatory processes in human populations. For example, in a study of 757 CARDIA participants, Sloan *et al.* (40) found that hf-HRV derived from 10 minutes resting ECG was correlated inversely with levels of both C-reactive protein and interleukin-6 (IL-6), two markers of chronic inflammation. In another study (41) it was found that aerobic training, known to increase resting peripheral nervous system (PNS) activity (42,43), significantly reduced the production of tumor necrosis factor (TNF)- α by stimulated macrophages. TNF- α can provoke release of ET-1 from macrophages (11,12), and has been observed in combination with ET-1 to promote constriction in the coronary microvascular bed (44). During mental stress in patients with CAD, disinhibition of macrophages consequent to withdrawal of vagal efferent activity may thereby accelerate the production and release of ET-1 from macrophages and/or adjacent endothelial cells that secrete the peptide (17,45). Furthermore, Ach may affect the synthesis and release of ET-1 through action on endothelial cells, since it is known to stimulate the synthesis of NO and, in turn, NO inhibits the synthesis and release of ET-1 (46,47). Hence, withdrawal of vagal stimulation on the vascular endothelium would reduce levels of NO, and thus disinhibit the production and release of ET-1, thereby providing an additional pathway for vagal influence on ET-1 production and release. Dynamic assessment of inflammatory markers, of nitric oxide synthesis and release, and of their combined effects with ET-1 on vasomotor tone during the experience of mentally and emotionally provocative stimuli was beyond the scope of the current investigation and awaits future research efforts.

In the current study, AR stress also increased sympathetic nervous system activity, as evidenced by elevations in circulating epinephrine and norepinephrine, with attendant elevations in hemo-

dynamic parameters. The increase in norepinephrine was not significant among patients who demonstrated an increase in ET-1 consequent to AR stress. Furthermore, compared with patients who did not demonstrate an increase in ET-1, this group of patients evidenced higher circulating levels of norepinephrine at both BL and AR stress, though again the differences were not significant statistically. The lack of statistical significance may reflect sample size. While the observed catecholamine response to anger recall stress was not associated with an increase in ET-1, the findings overall have implications for transient and catastrophic cardiac outcomes. Catecholamines increase heart rate and cardiac contractility via cardiac β -receptors, and promote vasoconstriction through vascular smooth muscle α -adrenergic receptors (48). Norepinephrine also has been found to potentiate the vasoconstrictive effects of ET-1 in diseased segments of coronary arteries (18). Therefore, parasympathetic withdrawal during mental stress may generate increased levels of ET-1, with sympathetic arousal accentuating its vasoactive effects. This suggests a mechanism through which sympathovagal imbalance during emotional stress promotes vasoconstriction of atherosclerotic vasculature, leading to stress-induced myocardial perfusion defects that have been reported previously (24). The combined effects of ET-1 and catecholamines on vascular performance during AR stress were beyond the scope of the current study. Furthermore, our findings that patients who demonstrated an increase in ET-1 with AR stress had both higher levels of catecholamines and lower levels of ET-1 at BL and in response to stress—even though not significantly so—indicates the complexity of vascular regulation during, and in response to, emotional stress. Future research should be directed toward a greater elucidation of these pathways, with markers of vasomotor tone included.

This exploratory study is not without limitation. The population was small and

close to 95% male, which may skew the results, in part because levels of ET-1 are generally lower in premenopausal females, possibly owing to protective effects of estrogen on endothelial function (49). Change in ET-1 from BL to AR stress was modeled as a dichotomous variable in analyses rather than as a continuous variable. Support for this approach, however, was provided by both our larger interest in factors responsible for an emotional stress provoked increase in ET-1, and the skew of the distributions of ET-1 at BL and AR, particularly given the exploratory nature of this study. Furthermore, since the dynamics of ET-1 synthesis and release, and its vascular effects are complex and multifactorial, our findings remain preliminary and warrant further investigation. It also is important to note that we observed a very small degree of parasympathetic withdrawal among the group of patients who did not demonstrate an increase in ET-1 consequent to AR stress. This raises a question regarding the more complete nature of the relationship between parasympathetic activity and ET-1 secretion, whether it is more fully dynamic and bi-directional, and whether threshold affects are operational. These important questions also were beyond the scope of the current exploration and remain to be addressed in a larger and more fully powered study.

Another potential issue concerns the effect of chronic β -blockade on the dynamics by which sympathetic pathways may influence ET-1 release during emotional stress. In the current study, 73.9% of those showing an increase in ET-1 from BL to AR stress, and 83.3% of those not demonstrating this effect were taking β -blockers. In a secondary analysis, we examined whether use of these agents affected the relationship of autonomic factors to change in ET-1 and found no such effect. Furthermore, despite β -blockade, significant elevations in hemodynamics and circulating catecholamines were realized with AR stress, as we have reported previously (33). Thus while exploratory, the present findings provide

preliminary support for a greater role for parasympathetic mediation of emotional stress-provoked elevation in circulating ET-1.

In summary, the recall of a previously anger-provoking incident by CAD patients induced sympathetic arousal and parasympathetic withdrawal, the latter of which predicted an associated increase in circulating ET-1. We speculate that reduced vagal inhibition of atherosclerotic macrophages, and/or direct effects of acetylcholine on endothelial cell function, may accelerate the release of ET-1 from these sources. Future basic and clinical experiments designed to discern the effects of vagal withdrawal versus sympathetic changes on atherosclerotic leukocytes, endothelial cell function and the dynamic regulation of ET-1 are warranted.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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