Functional Consequences of Endothelial Nitric Oxide Synthase Uncoupling in Congestive Cardiac Failure

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Background—Impaired endothelium-mediated vasodilatation (EMVD) in congestive cardiac failure (CCF) has been linked to decreased nitric oxide (NO) bioavailability because of its interaction with vascular superoxide (O$_2^-$), derived predominantly from NAD(P)H-dependent oxidases. When uncoupled from essential cofactors, endothelial nitric oxide synthase (eNOS) produces O$_2^-$. We studied the functional consequences of eNOS uncoupling in relation to EMVD in patients with CCF.

Methods and Results—We employed the platelet as a compartmentalized ex-vivo model to examine O$_2^-$ and NO production. When eNOS is functioning normally, incorporation of N$^\bullet$-Nitro-L-Arginine methyl ester (L-NAME, 1 mmol/L), results in increased O$_2^-$ detection, as inhibition of NO production prevents NO scavenging of O$_2^-$. This was observed in controls and 9 of the CCF patients, in whom O$_2^-$ detection increased by 63% and 101%, respectively. In the remaining 9 CCF patients, incorporation of L-NAME reduced O$_2^-$ production by 39%, indicating O$_2^-$ production by eNOS uncoupling. Detection of platelet-derived NO was significantly greater in eNOS-coupled platelets compared with the uncoupled group (2.8±1.4 versus 0.9±0.4 pmol/10^8 platelets, P=0.04). Endothelium-dependent and -independent vasodilator responses to acetylcholine and sodium nitroprusside recorded using venous occlusion plethysmography were significantly impaired in patients exhibiting eNOS uncoupling.

Conclusions—This study provides first evidence that platelet eNOS can become uncoupled in human CCF. Impaired endothelium-dependent and -independent vasodilator responses and diminished platelet-derived NO production occurred in association with enzyme uncoupling. (Circulation. 2003;107:1725-1728.)

Key Words: nitric oxide synthase • nitric oxide • platelets • acetylcholine • vasodilation

Endothelium-derived NO-dependent vasodilatation is markedly abnormal in congestive cardiac failure (CCF), contributing to elevated peripheral resistance and systemic vasoconstriction. An important mediator implicated in the control of vascular tone is NO. Diminished expression and activity of endothelial nitric oxide synthase (eNOS) and enhanced NO biodegradation by vascular superoxide (O$_2^-$) in CCF decreases NO bioavailability. O$_2^-$ and NO react rapidly to produce peroxynitrite, which further decreases NO bioavailability while promoting protein and lipid oxidation.

CCF is associated with increased levels of oxygen-derived free radicals, in particular O$_2^-$ produced by xanthine oxidase, cyclooxygenase, and mitochondrial oxidases, with vascular NAD(P)H oxidases being the principal source in CCF. Studies have demonstrated that eNOS, when uncoupled from the essential cofactor tetrahydrobiopterin (BH$_4$), can act as a source of O$_2^-$

Platelets contain all components of the L-arginine-nitric oxide pathway and represent an accessible alternative to endothelial cells for functional studies. Furthermore, as platelets share similar contractile systems with vascular smooth muscle, activity of the NO pathway and O$_2^-$ generation in platelets may relate to changes in smooth muscle tone. Using this model, we examined whether eNOS uncoupling occurs in human CCF and investigated the functional consequences of eNOS uncoupling in relation to changes in platelet-derived NO and forearm blood flow.

Methods

Participants and Procedures

We studied 18 patients (14 male, 4 female; mean age 74 [range 64 to 85]) with ischemic CCF and New York Heart Association (NYHA) symptom class II or III. All patients had a documented hospital admission for pulmonary edema and a left ventricular ejection fraction of <40%. Mean duration of symptoms was 23 months (range 6 to 62). Patients receiving warfarin were excluded because of the...
invasive nature of the study, as were those with diabetes mellitus (fasting glucose >7.0 mmol/L) or hypertension (blood pressure >160/90). Patients were taking maximal tolerated heart failure medication, with 100% receiving aspirin and angiotensin-converting enzyme inhibitors, 88% receiving β-blockade and statins, and 78% receiving spironolactone. Fifteen healthy volunteers were recruited as a control group (8 male, 7 female; mean age 72 [range 66 to 81]).

Measurement of Superoxide Production
Platelet O$_2^-$ production was measured by lucigenin-enhanced chemiluminescence, as described by McVeigh et al.$^{11,12}$ Samples were analyzed in the presence of the eNOS inhibitor L-NAME (1 mmol/L) to allow for NO scavenging of O$_2^-$, superoxide dismutase (200 u/mL) to confirm the identity of O$_2^-$, and diphenyliodonium (DPI); 100 μmol/L and quinacrine (1.0 mmol/L), potent inhibitors of flavoprotein C containing oxidoreductases including NAD(P)H oxidoreductase and eNOS.

Measurement of NO Release
Platelet NO release after stimulation with fibrinogen and ADP was recorded using a NO-selective microelectrode, as previously described in detail.$^{11,12}$

Venous Plethysmography
Forearm blood flow (FABF) was measured by venous occlusion plethysmography.$^{13}$ Acetylcholine (Ach) was infused in incremental doses (120, 180, and 240 nmol/min) for 3 minutes to assess endothelium-dependent vasodilatation. After a washout period of 30 minutes, sodium nitroprusside (SNP) was infused in incremental doses (6, 9, and 12 nmol/min) for 3 minutes to assess endothelium-independent vasodilatation.

Data Analysis
Biochemical characteristics were compared by use of independent samples t test. FABF with increasing concentrations of Ach and SNP was analyzed using ANOVA to assess intra-group and inter-group responses to the vasoactive substances. Data are expressed as mean±standard error of mean unless otherwise stated. Differences were considered significant at a value of P<0.05.

Results

Participant Characteristics
Patients and controls were matched for age (73±1.3 versus 72±4.6 years) and body mass index (25±2.3 versus 26±3.1 kg/m$^2$). Patients had significantly lower systolic and diastolic blood pressures (118±4.2 versus 142±2.6 mm Hg and 62±2.6 versus 76±2.4 mm Hg, respectively) and lower total cholesterol, high-density lipoprotein, and low-density lipoprotein as compared with controls (4.2±0.2 versus 5.5±1.1, 1.0±0.4 versus 1.4±0.7, and 2.5±0.2 versus 3.3±0.4 mmol/L, respectively).

The patients were subsequently segregated into groups A and B on the basis of their directional response in O$_2^-$ production as outlined below. There were no significant differences in age, sex, total cholesterol, or medications between the 2 groups (Table).

Superoxide Production
The O$_2^-$ production in patients was significantly greater than in controls. (101±24 versus 1±0.3 pmol/min per 10$^6$ platelets, P<0.01). The directional change in stimulated O$_2^-$ production (to 100 μL of phorbol 12-myristate 13-acetate, [10 μmol/L]) in response to the incorporation of L-NAME was used to designate patients into groups A and B (Figure 1). The control group and a subset of 9 patients designated group A exhibited increases in O$_2^-$ (63% and 101%, respectively) in response to L-NAME. This expected response is consistent with less O$_2^-$ scavenging by eNOS-derived NO. In the remaining 9 patients, designated group B, incorporation of L-NAME reduced O$_2^-$ detection by 39%, in keeping with
L-NAME inhibition of eNOS mediated \( \cdot \)O\(_2\)/H\(_2\)O\(_2\) production, confirming eNOS uncoupling and \( \cdot \)O\(_2\)/H\(_2\)O\(_2\) production in human CCF. Superoxide dismutase significantly decreased \( \cdot \)O\(_2\)/H\(_2\)O\(_2\) detection by a mean of 51% in each group, whereas DPI and quinacrine abolished \( \cdot \)O\(_2\)/H\(_2\)O\(_2\) production in all groups. In group B, in whom eNOS acted as an \( \cdot \)O\(_2\)/H\(_2\)O\(_2\) source, incorporation of L-NAME did not completely inhibit \( \cdot \)O\(_2\)/H\(_2\)O\(_2\) production, thereby implicating NAD(P)H oxidase as an additional \( \cdot \)O\(_2\)/H\(_2\)O\(_2\) source.

**NO Production**

NO detection was significantly lower in group B (eNOS uncoupled) than group A (eNOS coupled) (0.9±0.4 versus 2.8±1.4 pmol/10^8 platelets; \( P=0.04 \)), indicating decreased NO production and increased NO scavenging by eNOS-derived \( \cdot \)O\(_2\)/H\(_2\)O\(_2\) in the eNOS uncoupled group.

**Forearm Blood Flow Studies**

FABF increased significantly in all groups in response to Ach and SNP (\( P<0.05 \)). For Ach, the vasodilator response observed in group A (eNOS coupled) was significantly greater than group B (eNOS uncoupled), but was impaired when compared with controls (6.8±0.7, 3.9±0.6, and 10.1±0.5, respectively; \( P<0.05 \)) (Figure 2). For SNP, the dilator response observed in group A (eNOS coupled) was significantly greater than that in group B (eNOS uncoupled) (7.5±0.6 versus 4.8±0.4; \( P<0.05 \)), but was not different than controls (8.9±0.4) (Figure 2).

**Discussion**

This study provides the first evidence that eNOS can reside in the uncoupled state acting as a \( \cdot \)O\(_2\)/H\(_2\)O\(_2\) source in human CCF. eNOS uncoupling was associated with impaired platelet NO production and endothelium-dependent and -independent vasodilatation contributing, in part, to the impaired NO-mediated vasodilator responses described in CCF.

The mechanisms underlying eNOS uncoupling remain the subject of intense investigation. When eNOS becomes uncoupled from essential cofactors, \( \cdot \)O\(_2\)/H\(_2\)O\(_2\) is produced instead of NO. Peroxynitrite, the product of the NO/\( \cdot \)O\(_2\)/H\(_2\)O\(_2\) interaction, plays a pivotal role in eNOS uncoupling. Oxidation of the essential cofactor BH\(_4\) to the inactive pterin, 7,8-dihydrobiopterin (BH\(_2\)), and removal of zinc from the zinc-thiolate cluster by peroxynitrite results in dissociation of the cofactors from eNOS and facilitates \( \cdot \)O\(_2\)/H\(_2\)O\(_2\) production.

BH\(_4\) modulation of free radical activity clearly extends to the platelet, as BH\(_4\) regulates platelet-derived NO\(^-\) and \( \cdot \)O\(_2\)/H\(_2\)O\(_2\) generation by eNOS, an event of functional importance in influencing thrombosis formation. Platelet-derived NO release and endothelium-derived NO-mediated vasodilatation is
impaired with conventional cardiovascular risk factors, a fact that supports a link between the l-arginine–NO pathway in platelets and endothelial cells.\textsuperscript{17}

We have previously used the platelet as a compartmentalized tissue model to study the interaction of NO and O\textsubscript{2}\textsuperscript{−} and the functional consequences associated with altered platelet reactivity.\textsuperscript{11,12} All components of the L-arginine–NO pathway present in endothelial cells are found in platelets, and eNOS protein has been identified in platelets by Western blotting.\textsuperscript{18}

NO detection was decreased in the eNOS uncoupled group, as was endothelium-dependent and -independent vasodilation. The fact that NO could be detected, albeit in reduced amounts, suggests that eNOS uncoupling occurred only in a subset of platelets in the sample. The increased O\textsubscript{2}\textsuperscript{−} production in patients exhibiting enzyme uncoupling would facilitate the accelerated degradation of NO derived from the endothelium or from the direct acting donor SNP. This may account for the impaired endothelium-independent and -dependent responses in these patients. It is difficult to dissect out the independent contribution of CCF, as impaired NO-mediated vasodilator responses are well described in both atherosclerotic vascular disease and CCF. Uncoupling of the eNOS enzyme could not be explained by age, sex, or medication differences between the groups, but it may relate to differences in antioxidant defenses not addressed in this study.

In conclusion, uncoupling of platelet eNOS in human CCF was associated with decreased platelet-derived NO production, impaired endothelium-dependent and -independent vasodilatation.

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References


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