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Inducible Nitric Oxide Synthase Activity Does Not Contribute to the Maintenance of Peripheral Vascular Tone in Patients with Heart Failure

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Abstract

Background: Enhanced iNOS activity may contribute to vascular dysfunction in patients with heart failure. We aimed to determine whether iNOS activity contributes to the maintenance of vascular tone in patients with symptomatic heart failure, with the use of the highly selective iNOS inhibitor, 1400W.

Methods: Bilateral forearm blood flow was measured using venous occlusion plethysmography in 12 patients with New York Heart Association class II-IV heart failure and 8 matched healthy control subjects during intra-brachial infusion of 1400W (0.1-1 $\mu\text{mol/min}$), N^{G} -monomethyl-L-arginine (a non-selective NOS inhibitor; 2-8 $\mu\text{mol/min}$) and norepinephrine (control vasoconstrictor, 60-480 pmol/min).

Results: In both patients and controls, intrabrachial infusion of N^{G} -monomethyl-L-arginine and norepinephrine caused dose-dependent reductions in infused forearm blood flow ($p < 0.05$ for both): peak reductions of $32 \pm 6\%$ and $37 \pm 4\%$ during N^{G} -monomethyl-L-arginine and $52 \pm 6\%$ and $49 \pm 5\%$ during norepinephrine respectively ($p = \text{ns}$, patients vs. controls). In contrast, 1400W had no effect on blood flow: $-3 \pm 4\%$ in patients (95% confidence intervals: -11 to 5%) and $3 \pm 8\%$ in controls at 1 $\mu\text{mol/min}$ ($p = \text{ns}$).

Conclusions: We have demonstrated that intrabrachial selective iNOS inhibition does not influence forearm blood flow in patients with heart failure. We conclude that iNOS activity does not contribute to peripheral vascular tone in patients with symptomatic heart failure.

Abbreviations and Acronyms

1400W	N-(3-(Aminomethyl) benzyl) acetamidine
eNOS	endothelial nitric oxide synthase
FBF	forearm blood flow
iNOS	inducible nitric oxide synthase
L-NMMA	N ^G -monomethyl-L-arginine
NE	norepinephrine
nNOS	neuronal nitric oxide synthase
NOS	nitric oxide synthase
NOx	nitrite/nitrate
NYHA	New York Heart Association
TNF α	tumour necrosis factor alpha

Introduction

Nitric oxide, a key mediator of vascular tone, is synthesised from L-arginine by a triad of isozymes, the nitric oxide synthases (NOS). Neuronal and endothelial nitric oxide synthase (nNOS and eNOS respectively) are both constitutively expressed under normal physiological conditions. In contrast, inducible nitric oxide synthase (iNOS) is activated in response to inflammatory mediators, is uncoupled from G-protein receptor activation, and is capable of sustained production of large quantities of nitric oxide within endothelial and vascular smooth muscle cells [1]. Excessive generation of nitric oxide by iNOS may account for the vascular dysfunction evident in a number of inflammatory conditions including sepsis and endotoxaemia [2,3].

Heart failure is a chronic inflammatory state [4] associated with endothelial dysfunction [5,6], and more specifically, impairment of nitric oxide mediated endothelium-dependent vasodilatation [7-11]. However, controversy remains as to whether there is altered basal nitric oxide release. Whilst some studies suggest that there is enhancement of basal nitric oxide release [8,12-14], others show evidence that there may be no change [15] or even a reduction [16-18]. Induction of iNOS in heart failure may account for alterations in vascular generation of nitric oxide [14].

Until recently, it has not been possible to distinguish clearly between constitutive and inducible NOS activity due to the lack of specific inhibitors. N-(3-(Aminomethyl) benzyl) acetamidine (1400W) is a novel selective inhibitor of human iNOS that has recently become available for clinical use [19,20]. It competes with L-arginine to bind irreversibly with iNOS and is at least 5000-fold more selective for iNOS than eNOS making it the most selective iNOS inhibitor to date [19]. The present study aimed to assess the contribution of functional iNOS activity to peripheral vascular tone in patients with symptomatic heart failure by determining the effect of direct local intra-arterial 1400W on peripheral blood flow.

Methods

Subjects and Patients

The protocol was undertaken with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki. The written informed consent of each subject was obtained before entry into the study. Twelve patients with stable New York Heart Association (NYHA) class II-IV congestive heart failure and objective evidence of left ventricular impairment (left ventricular ejection fraction <35%, shortening fraction <20% or left ventricular end-diastolic diameter >5.5cm) and 8 age- and sex-matched control subjects were enrolled in the study. Patients were excluded if they had significant valvular heart disease, hepatic or renal impairment. Subjects were non-smokers and abstained from alcohol for 24 hours before and from food and caffeine-containing drinks on the day of the study. Diuretics were withheld on the morning of the study for patient comfort. All studies were performed in a quiet, temperature-controlled room.

Intra-Arterial Drug Administration

The brachial artery of the non-dominant arm was cannulated with a 27-standard wire gauge steel needle (Cooper's Needle Works Ltd, Birmingham, UK) under local anesthesia. The cannula was attached to a 16-gauge epidural catheter (Portex Ltd) and patency was maintained by infusion of saline (0.9%: Baxter Health Care Ltd, Thetford, UK) via an IVAC P1000 syringe pump (IVAC Ltd). The rate of intra-arterial infusions was maintained constant throughout all studies at 1 mL/min. Pharmaceutical grade 1400W (Merck Biosciences AG, Läufelfingen, Switzerland), N^G-monomethyl-L-arginine (L-NMMA; Merck Biosciences AG) and norepinephrine (Levophed, Sanofi-Winthrop Ltd, Guildford, UK) were dissolved in saline on the day of the study.

Haemodynamic Measurements

Blood flow was measured in both forearms by venous occlusion plethysmography as described previously [21-23]. Blood pressure and heart rate were monitored in the non-infused arm at intervals throughout each study with a semi-automated non-invasive oscillometric sphygmomanometer.

Study Design

Subjects attended on a single occasion at 9 am and were recumbent throughout the study. Strain gauges and cuffs were applied, and the brachial artery of the non-dominant arm was cannulated. Forearm blood flow was measured every 6-10 minutes. Following a 30 minute equilibration period, subjects received intra-arterial infusions of 1400W (a selective iNOS inhibitor, at 0.1 and 0.3 $\mu\text{mol}/\text{min}$ for 6 minutes at each dose and 1 $\mu\text{mol}/\text{min}$ for 18 minutes), L-NMMA (a non-selective NOS inhibitor, at 2 and 4 $\mu\text{mol}/\text{min}$ for 6 minutes and 8 $\mu\text{mol}/\text{min}$ for 18 minutes) and norepinephrine (a control vasoconstrictor; at 60, 120, 240 and 480 pmol/min for 6 minutes at each dose) with 30 minute saline washouts periods between drugs.

Data Analysis and Statistics

Plethysmographic data were extracted from the Chart data files, and the last 5 linear recordings in each measurement period were averaged. Forearm blood flows were calculated from the plethysmographic data as described previously [24,25]. Percentage changes in the infused forearm blood flow were calculated [23,24] as follows:

$$\% \text{ Change in blood flow} = 100 \times (I_t/NI_t - I_b/NI_b) / I_b/NI_b$$

where I_b and NI_b are the infused and non-infused forearm blood flows at baseline (time 0) respectively, and I_t and NI_t are the infused and non-infused forearm blood flows at a given time point (t), respectively.

Data were examined, where appropriate, by ANOVA with repeated measures and 2-tailed Student's *t* test using Microsoft Excel 2002. Data are expressed as mean $\pm$ standard error of the mean. Statistical significance was taken at the 5% level.

Results

Patients and controls were generally well matched although patients had lower systolic and diastolic blood pressures (Table 1). Heart rate, blood pressure and non-infused forearm blood flow were unchanged throughout the study in both groups.

Forearm blood flow responses

Infusion of both L-NMMA and norepinephrine produced dose-dependent reductions in forearm blood flow in both patients (basal blood flow 2.3 ± 0.3 and 1.8 ± 0.2 mL/100mL/min respectively; peak reductions of $32 \pm 6\%$ and $52 \pm 6\%$ respectively) and healthy controls subjects (basal blood flow 1.7 ± 0.1 and 1.6 ± 0.3 mL/100mL/min respectively; peak reductions of $37 \pm 4\%$ and $49 \pm 5\%$ respectively; $p = \text{ns}$ patients vs controls; Figure 1). In contrast, infusion of 1400W had no effect on forearm blood flow in either group ($p = \text{ns}$): $-3 \pm 4\%$ (95% confidence intervals, -11 to 5%) in patients and $3 \pm 8\%$ (95% confidence intervals, -13 to 19%) in controls at 1 $\mu\text{mol/min}$.

Discussion

There has been controversy surrounding the importance of inducible nitric oxide synthase activity in the vasculature of patients with heart failure. Using the highly selective iNOS inhibitor 1400W, we have demonstrated for the first time that intrabrachial selective iNOS inhibition does not alter forearm blood flow in patients with heart failure. We therefore conclude that there is no evidence of iNOS activity in the peripheral vasculature of patients with symptomatic heart failure.

Evidence for iNOS in the peripheral vasculature

Inducible nitric oxide synthase expression [26] and activity [27] has been demonstrated in the vasculature of rodent models of heart failure. Vascular iNOS activity is associated with functional changes and, more specifically, altered vascular responsiveness to adrenergic stimulation, which can be reversed by selective iNOS inhibition [26,28].

In patients, iNOS is present in a number of tissues including the myocardium, intramyocardial vasculature and skeletal muscle [29-35]. However, the evidence for functional iNOS in the peripheral vasculature remains unconvincing. Despite the presence of iNOS in the aorta [27] and mesenteric vessels [26] of rodent models of heart failure, there are no animal or human studies of heart failure that have isolated either iNOS messenger ribonucleic acid or protein from the peripheral vasculature.

Indirect evidence of functional iNOS activity in the peripheral vasculature of patients with heart failure comes from an *in vivo* study. Ishibashi and colleagues reported a modest reduction in forearm blood flow during infusion of aminoguanidine, a NOS inhibitor with some selectivity for iNOS [14]. Aminoguanidine is, however, an extremely weak inhibitor of iNOS and complete inhibition of iNOS is only achieved with high (approaching millimolar) concentrations [20]. Furthermore aminoguanidine is only nine-fold more selective for iNOS compared with eNOS [20,36]. A reduction in forearm blood flow during aminoguanidine infusion only becomes evident at higher doses (10-20 $\mu\text{mol/min}$) when effective end-organ concentrations would be in the order

of between 400-800 μM [14]. During infusion of such high doses, aminoguanidine would be expected to have considerable action on eNOS [20,36].

In contrast to patients with heart failure, aminoguanidine has no effect on forearm blood flow in healthy control subjects [14]. This suggests that patients with heart failure have altered NOS activity as compared with controls. Aminoguanidine has an augmented inhibitory action on eNOS in the presence of excess tetrahydrobiopterin, the requisite cofactor for NOS [37]. Inflammatory stimuli enhance tetrahydrobiopterin synthesis, by induction of GTP cyclohydrolase I [38,39]. As heart failure is an inflammatory disorder, there may be increased availability of tetrahydrobiopterin as a result of increased activity of GTP cyclohydrolase I. We therefore speculate that, consistent with the work of Vallance's group [40], the purported *in vivo* inhibition of iNOS seen with aminoguanidine simply reflects an enhanced inhibition of eNOS in the presence of upregulated GTP cyclohydrolase I. The possibility that these patients may have augmented (or "induced") eNOS activity as a consequence of enhanced tetrahydrobiopterin availability merits further investigation.

Considering the lack of specificity of aminoguanidine as an inhibitor of iNOS, and the possibility that eNOS activity may be upregulated during inflammation, it is likely that the effects of aminoguanidine are a consequence of inhibition of eNOS rather than iNOS. In the present study, we have been able to use a highly selective inhibitor of iNOS to demonstrate that functional iNOS activity does not appear to play a major role in the regulation of peripheral vascular tone in heart failure patients.

1400W is a highly selective inhibitor of iNOS

The present study is the first to use the iNOS inhibitor 1400W in patients with heart failure *in vivo*. 1400W is a monoamidine monoamine analogue that competes with L-arginine to bind tightly and irreversibly to iNOS with a rapid onset of action (<5 min) [19]. It is > 5000-fold more potent against purified human iNOS than eNOS and thus is the most selective inhibitor of iNOS reported to date [19]. The selectivity of 1400W, both *in vitro* and *in vivo*, makes it an attractive tool for assessing the contribution of

iNOS [20,36]. As the primary aim of the study was to determine the contribution of iNOS activity to the maintenance of vascular tone, and considering that 1400W irreversibly inhibits iNOS whilst L-NMMA inhibits both iNOS and eNOS, intra-arterial drug order was not randomised. Instead, the effects of selective iNOS inhibition on forearm blood flow were observed first, followed by non-selective NOS inhibition with L-NMMA and finally, intra-arterial administration of the control vasoconstrictor norepinephrine. As a consequence, norepinephrine was noted to have a rather blunted (albeit significant) effect on forearm blood flow which is probably explained by a persistent vasoconstrictive effect of L-NMMA.

An important concern regarding this study is the possibility that the lack of effect seen with 1400W on forearm blood flow reflects a failure of 1400W to inhibit iNOS effectively *in vivo* at the chosen dose range (0.1-1 $\mu\text{mol/min}$). 1400W inhibits iNOS with an 8-fold higher potency than L-NMMA (EC_{50} values of 0.8 μM and 6 μM respectively) [19]. During infusion of 1400W at 1 $\mu\text{mol/min}$, we would predict effective end organ concentrations to be between 40-50 μM . This is > 50-fold higher than the EC_{50} , and 10-fold higher than concentrations that we have demonstrated to have vascular effects *in vitro* in rodents [26]. It would therefore be anticipated that this dose is sufficient to achieve inhibition of functional iNOS activity. Indeed, we have recently demonstrated that intra-arterial infusion of 1400W, at the same doses used in the present study, results in a reduction in forearm blood flow in patients with liver cirrhosis [41], an inflammatory condition associated with induction of iNOS [42].

Study Limitations

The discordant findings in previous reports on nitric oxide generation in heart failure may, in part, be explained by differences in the severity of heart failure, with basal nitric oxide production increasing, and stimulated release decreasing, with progression of disease [43]. In the present study, inclusion criteria were deliberately chosen to encompass a broad range of patients, so that the response to selective iNOS inhibition could be correlated with disease severity. Almost half of the patients (5 out of 12) were

in NYHA class III or IV and thus had evidence of severe disease. Subgroup analysis confirmed that infusion of 1400W had no effect on forearm blood flow in patients in NYHA classes III or IV: $-3 \pm 5\%$ in at $1 \mu\text{mol/min}$ (95% confidence intervals: -6 to 12% , $n=5$; $p=\text{ns}$). It is therefore unlikely that the failure to detect iNOS activity was due to inadequate severity of disease in this group. It is noteworthy that almost all patients who took part in this study were prescribed diuretic, angiotensin converting enzyme inhibitor and beta blocker therapy that may have modified vascular nitric oxide effects.

Clinical Implications

In clinical conditions where iNOS activity is enhanced, one would intuitively expect inhibition of iNOS to result in vasoconstriction, as a consequence of reduced bioavailability of nitric oxide, a potent vasodilator. Vasoconstriction, in the context of heart failure, would result in reduced tissue perfusion and increased afterload, and hence a deterioration in cardiac function. However, the failure to demonstrate functional iNOS activity in the peripheral vasculature may provide an exciting opportunity to explore other potential benefits of iNOS inhibition in heart failure with less concern about the adverse effects of vasoconstriction.

Data from animal models suggest that modulation of iNOS activity may have therapeutic benefits in heart failure. Selective iNOS inhibition improves myocardial oxygen consumption and left ventricular function in dogs [44] and reverses the beta-adrenergic hyporesponsiveness of papillary muscle from rats [45] with congestive heart failure. iNOS is present in the myocardium [30-32] and skeletal muscle [34,35] of patients with heart failure and excessive generation of nitric oxide in these tissues may in part explain the impaired myocardial contractility [46] and exercise intolerance [47]. Further clinical studies are necessary to investigate whether systemic inhibition of iNOS is a therapeutic target for improving cardiac function and exercise tolerance in heart failure patients.

Conclusions

We conclude that in patients with symptomatic congestive heart failure, functional iNOS activity does not contribute to the maintenance of peripheral vascular tone.

Further studies may reveal exciting therapeutic potential for the inhibition of both GTP cyclohydrolase I and iNOS in patients with heart failure.

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Figure 1

Infused forearm blood flow during incremental doses of (a) N-(3-(Aminomethyl) benzyl) acetamidine (1400W), (b) N^G-monomethyl-L-arginine (L-NMMA) and (c) norepinephrine in patients with congestive heart failure (open circles; n=12) and controls subjects (closed squares; n=8).

* $p < 0.05$ (ANOVA) for dose response in both patients and control subjects.

Dover *et al.*, Figure 1.

Table 1.**Subject Baseline Characteristics**

	Patient Group (n=12)	Control Group (n=8)
Age (years)	64±3	57±3
Sex (Male/Female)	8/4	7/1
Etiology (DCM/IHD)	4/8	-
NYHA Class (II/III/IV)	7/4/1	-
Medications (n):		
Aspirin	11	-
ACE inhibitor	11	-
Diuretic	11	-
Beta Blocker	10	-
Spironolactone	5	-
Angiotensin receptor antagonist	3	-
Nitrate	3	-
LV Ejection Fraction (%)	31±4	-
LV Shortening Fraction (%)	15±2	-
LV End Diastolic Diameter (mm)	60±3	-
Heart Rate (beats/min)	58±2	59±3
Systolic Blood Pressure (mm Hg)	118±6*	143±5
Diastolic Blood Pressure (mm Hg)	67±3*	81±3
Baseline Infused Forearm Blood Flow (mL/100mL/min)	2.5±0.3	2.1±0.1
Baseline Non-Infused Forearm Blood Flow (mL/100mL/min)	2.2±0.2	2.2±0.3

Data are presented as mean±SEM.

* p < 0.01 compared with control subjects.

DCM = Dilated cardiomyopathy; IHD = Ischaemic heart disease; NYHA = New York Heart Association; LV = left ventricular.