

# Myotrophin in Human Heart Failure

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<b>OBJECTIVES</b>	The goal of this study was to investigate plasma levels of myotrophin in heart failure (HF) and their relationship to gender and disease severity.
<b>BACKGROUND</b>	Myotrophin is a myocardial hypertrophy-inducing factor initially demonstrated in hypertrophied and cardiomyopathic hearts. Recent evidence suggests an interaction with the transcription factor nuclear factor kappa B (NFκB), which is activated in HF and modulates myocardial protein expression. It is unknown whether this peptide has an endocrine/paracrine role in man. We hypothesized that it may have a role in HF and would be raised in plasma.
<b>METHODS</b>	We developed a competitive binding assay specific for human myotrophin. Myotrophin was measured in plasma extracts of 120 HF patients and 130 age- and gender-matched normal controls.
<b>RESULTS</b>	Myotrophin in plasma existed as the full-length 12 kD form with also a 2.7 kD form (possibly a degradation product). Log normalized myotrophin levels were significantly elevated in HF patients (mean ± SEM [geometric mean, range], 2.402 ± 0.021 [252, 72 to 933] vs. 2.268 ± 0.021 [185, 28 to 501] fmol/ml, $p < 0.0005$ ). There was no relationship between myotrophin and age or gender in controls. However, males with HF had higher levels of myotrophin than females ( $p < 0.001$ ). There was an inverse relationship of myotrophin levels with New York Heart Association class in patients with no gender difference in the relationship.
<b>CONCLUSIONS</b>	There is evidence of early activation of the myotrophin system in HF, which is more evident in males. This response is attenuated in more severe disease. The contribution of myotrophin to NFκB-mediated gene transcription and preservation of cardiac muscle mass remains to be investigated further. (J Am Coll Cardiol 2003;42:719–25) © 2003 by the American College of Cardiology Foundation

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A number of endocrine and paracrine systems are activated in human heart failure (HF). These include the renin-angiotensin system, catecholamines, endothelin, the natriuretic peptides, and cytokines such as tumor necrosis factor- $\alpha$  (TNF $\alpha$ ). In the early stages of HF, activation of some of these systems may serve to compensate for the reduced cardiac output, but, as the disease progresses, they may contribute to deterioration by affecting left ventricular (LV) remodeling.

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Myotrophin is a 12 kD protein initially isolated from hypertrophied hearts of spontaneously hypertensive rats (1). Subsequently, levels were found to be elevated in human cardiomyopathic hearts (2). In vitro, its effects on cultured cardiomyocytes include an increase in protein synthesis, cellular hypertrophy, gap-junction formation, increased sarcomere number, induction of early response genes such as c-myc, c-fos, c-jun, and, subsequently, of transcripts of skeletal  $\alpha$ -actin, total myosin, and atrial natriuretic peptide (3). The hypertrophic effects on cardiomyocytes are mediated via protein kinase C activation (4). Due to the presence of tandem ankyrin repeats in the myotrophin molecule and

their resemblance to those found in the IκB $\alpha$ /rel proteins, a role has been postulated for myotrophin binding to and modulating activity of the transcription factor nuclear factor kappa B (NFκB) (5). Recent evidence suggests that myotrophin disrupts the formation of the NFκB p50-p65 transactivating heterodimers while increasing the formation of repressive NFκB p50-p50 homodimers (6). Cyclic stretch leads to increased myotrophin levels in cardiomyocytes with increased translocation of the factor into the nucleus from the cytoplasm (7). Myotrophin and TNF $\alpha$  increase the expression of each other in cardiomyocytes (7). There is evidence of increased activity of NFκB in human HF, with a role postulated for induction of a number of inflammatory mediators (8,9).

Human myotrophin was cloned recently and found to be highly homologous to the rat protein (10). Its messenger ribonucleic acid (mRNA) is widely distributed in many tissues, with relatively high levels in heart, skeletal muscle, liver, and pancreas. In the cardiovascular system, myotrophin mRNA was expressed in monocytes, macrophages, vascular smooth muscle, and endothelial cells. The recombinant myotrophin protein stimulates protein synthesis, and increased mRNA was found in a small number of heart specimens with dilated or ischemic cardiomyopathy (10). To date, there has been no report of the presence of this peptide in human plasma and its levels in patients with HF. In view of its potential role in modulating NFκB activity, and the documented role of this transcription factor in HF, we investigated the myotrophin system in humans with HF.

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Abbreviations and Acronyms

HF	= heart failure
ILMA	= immunoluminometric assay
LV	= left ventricle/ventricular
mRNA	= messenger ribonucleic acid
NFκB	= nuclear factor kappa B
NYHA	= New York Heart Association
streptavidin-MAE	= streptavidin labeled with methyl-acridinium ester
TNFα	= tumor necrosis factor-alpha

Although present within cells, extracellular myotrophin has effects on protein synthesis, cell hypertrophy, and induction of early genes, which may point to other effects of this peptide (3,4,7).

**METHODS**

**Study populations.** We studied 120 HF patients, all with echocardiographically confirmed LV systolic dysfunction (LV ejection fraction <45%). Age- and gender-matched normal controls, LV ejection fraction >50%, were recruited from the local community by advertisement. All subjects gave informed consent to participate in the study, which was approved by the local ethics committee. The study conforms to the principles of the Declaration of Helsinki.

**Blood sampling and plasma extraction.** A total of 20 ml of peripheral venous blood was drawn into pre-chilled Na-EDTA (1.5mg/ml blood) tubes containing 500 IU/ml aprotinin. After centrifugation at 3,000 rpm at 4°C for 15 min, plasma was separated and stored at -70°C until assay. Prior to assay, plasma was extracted on C18 Sep-Pak (Waters, Peninsular Labs, Liverpool, United Kingdom) columns and dried on a centrifugal evaporator.

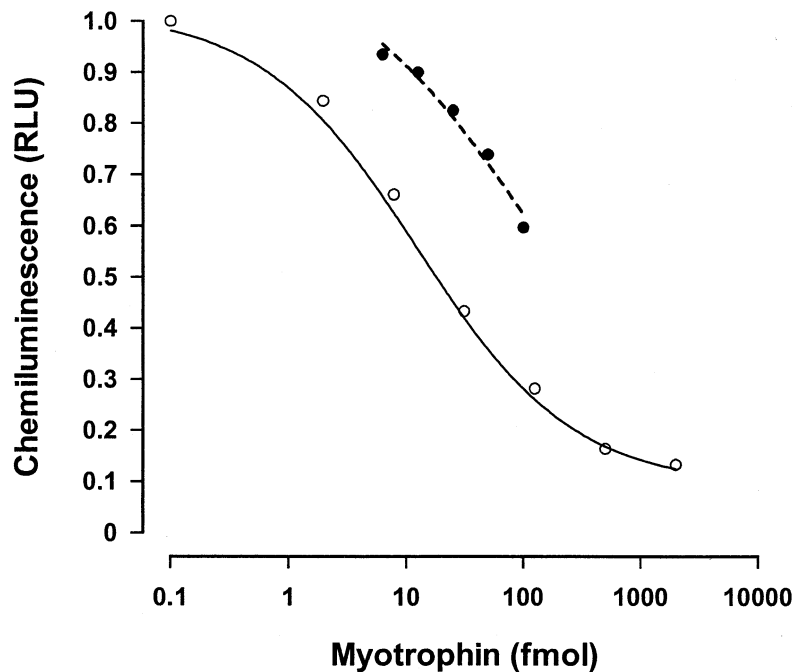
**Assay of myotrophin.** A peptide corresponding to a C-terminal domain (amino acids 102-113) of the human myotrophin sequence (LTAFEATDNQAI) (10) was synthesized in the Medical Research Council Toxicology Unit, University of Leicester. A rabbit was injected monthly with this peptide conjugated to keyhole limpet hemocyanin as previously described (11); IgG from the sera was purified on protein A sepharose columns. The above peptide was also biotinylated using biotin-X-N-hydroxysuccinimide ester (Calbiochem, Nottingham, United Kingdom) and the tracer purified on HPLC using an acetonitrile gradient. Plasma extracts and standards were reconstituted with immunoluminometric assay (ILMA) buffer consisting of (in mmol/l) NaH<sub>2</sub>PO<sub>4</sub> 1.5, Na<sub>2</sub>HPO<sub>4</sub> 8, NaCl 140, EDTA 1, and (in g/l) bovine serum albumin 1, azide 0.1; ELISA plates were coated with 100 ng of anti-rabbit IgG (Sigma Chemical Co., Poole, United Kingdom) in 100 μl of 0.1 mol/l sodium bicarbonate buffer, pH 9.6. Wells were then blocked with 0.5% bovine serum albumin in bicarbonate buffer. A competitive immunoluminometric assay was set up

by preincubating 50 ng of the IgG with standards or samples within the wells. After overnight incubation, 50 μl of the diluted biotinylated myotrophin peptide tracer (1 μl/ml of the stock solution) was added to the wells. After another 24 h of incubation at 4°C, wells were washed three times with a wash buffer (NaH<sub>2</sub>PO<sub>4</sub> 1.5 mmol/l, Na<sub>2</sub>HPO<sub>4</sub> 8 mmol/l, NaCl 340 mmol/l, Tween 0.5 g/l, sodium azide 0.1 g/l). Streptavidin labeled with methyl-acridinium ester (MAE) was synthesized as described (12). Wells were incubated for 2 h with 100 μl of ILMA containing streptavidin-MAE (5 million relative light units per well). After further washes, chemiluminescence was detected by sequential injections of 100 μl of 0.1 M nitric acid (with H<sub>2</sub>O<sub>2</sub>) and then 100 μl of NaOH (with cetyl ammonium bromide) (11,12) in a Dynatech MLX Luminometer (Dynex Technologies Inc., Worthing, West Sussex, United Kingdom). The lower limit of detection was 11.6 fmol/ml. Within and between assay coefficients of variation were 3.5% and 7%, respectively. There was no cross-reactivity with peptides previously demonstrated to be elevated in HF such as atrial natriuretic peptide, brain natriuretic peptide, N-terminal pro-brain natriuretic peptide, C-type natriuretic peptide, cardiostrophin-1, or leukemia inhibitory factor.

**Size exclusion chromatography and gel electrophoresis of plasma extracts.** Plasma extracts were fractionated by isocratic size exclusion chromatography on a 300 × 7.8 mm Bio-Sep SEC S2000 column (Phenomenex, Macclesfield, Cheshire, United Kingdom) using 50 mmol/l NaH<sub>2</sub>PO<sub>4</sub> (pH 6.8) at a flow rate of 1 ml/min as the mobile phase. Standards used to establish molecular weights included IgG (150 kD), bovine serum albumin (68 kD), ovalbumin (44 kD), soybean trypsin inhibitor (20 kD), aprotinin (6.5 kD), and tryptophan (204D) (Sigma Chemical Co.). Fractions collected every 20 s were dried on a centrifugal evaporator before assaying for myotrophin as preceding text.

A total of 50 μg of rat tissue extracts (heart, brain, muscle, liver, kidney, spleen) were also resolved on 17% SDS polyacrylamide gels with colored molecular weight markers, with subsequent blotting of the proteins onto reinforced nitrocellulose (0.2 mm). Blots were blocked overnight in 1% dried low-fat milk powder in Tris buffered saline (TBS, composed of Tris 20 mmol/l, NaCl 135 mmol/l) containing 0.1% Tween-20. Detection was performed with the myotrophin antibody (1 μg/ml in TBS Tween) for 2 h at room temperature, followed by donkey anti-rabbit IgG horseradish peroxidase conjugate (Amersham, United Kingdom). The bands were visualized using enhanced chemiluminescence kits (Amersham, United Kingdom) onto pre-flashed X-ray films.

Human leucocytes were also fixed on glass slides with 2% paraformaldehyde in phosphate buffered saline for 20 min, before permeabilizing with 0.2% Triton X-100 for 15 min (7). Endogenous peroxidases were blocked by treatment with 0.5% H<sub>2</sub>O<sub>2</sub> for 10 min. After blocking with 10% goat serum overnight, slides were incubated in 10 μg/ml myotro-

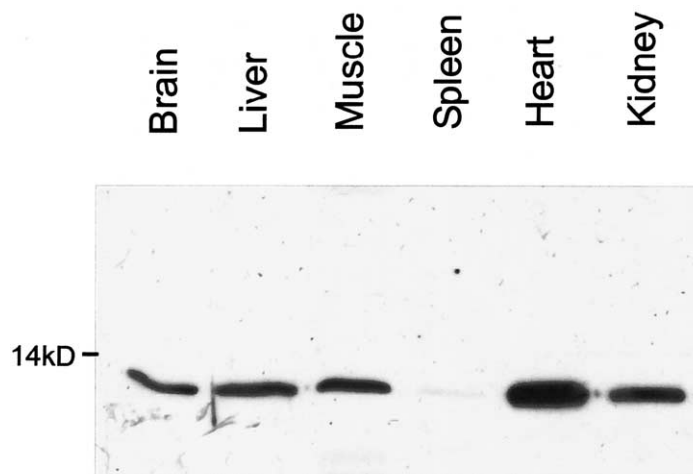


**Figure 1.** A standard curve for the myotrophin competitive immunoassay. A patient's plasma extract (solid circles joined by dotted line) was diluted in twofold steps, showing parallelism with the standard curve.

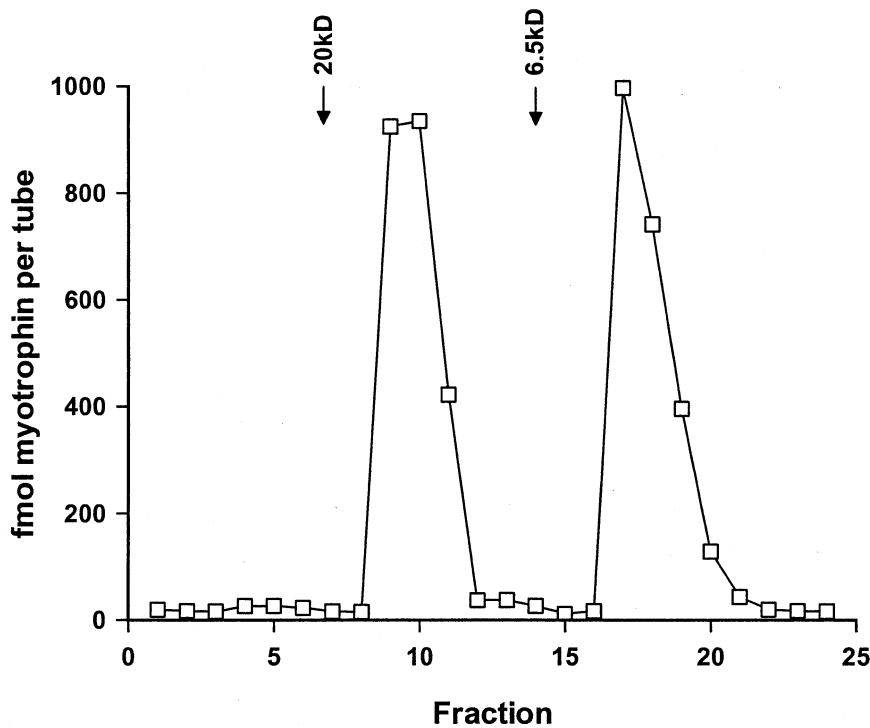
phin antibody in phosphate buffered saline Tween overnight. Control slides were not incubated with the myotrophin antibody. After extensive washes, detection was with donkey anti-rabbit IgG horseradish peroxidase conjugate (1:1,000) and visualized using 3, 3'-diaminobenzidine.

**Statistical analysis.** Statistical analysis was performed using SPSS Version 11.0 (SPSS Inc., Chicago, Illinois). Data are presented as mean  $\pm$  SEM or median (range) for data with non-Gaussian distribution, which were log-transformed before analysis. Normality of distribution

of data was confirmed by the Anderson-Darling test. For continuous variables, one-way analysis of variance (ANOVA) was used. The interaction of multiple independent variables was sought using the univariate general linear model procedure with p values from Bonferonni's test reported. Pearson correlation analysis was performed, and box plots were constructed consisting of medians, boxes representing interquartile ranges, and the whiskers representing the 2.5th to the 97.5th percentile. A p value below 0.05 was considered statistically significant.



**Figure 2.** A Western blot of rat tissue extracts (50  $\mu$ g per lane) resolved on 15% SDS-polyacrylamide gels, using the antibody (1  $\mu$ g/ml) to detect myotrophin. A 12 kD band was evident in all extracts, with the lowest levels in spleen.



**Figure 3.** Size exclusion chromatography with analysis of the fractions for myotrophin. The points of elution of markers for 20 kD and 6.5 kD are indicated by arrows. Two peaks of immunoreactivity for myotrophin are evident at 12 and 2.7 kD.

**RESULTS**

A typical standard curve for myotrophin peptide is illustrated in Figure 1, showing a fall in chemiluminescence with increasing concentrations of the peptide. Dilutions of patients' plasma extracts showed parallelism with the standard curve.

Figure 2 shows the reactivity of the antibody towards rat tissue extracts, with a clear band detected in all tissues at 12 kD, the lowest expression being found in spleen. We also localized myotrophin in human leucocytes using the myotrophin antibody and goat horseradish peroxidase con-

jugated anti-rabbit IgG, with staining present entirely within these cells.

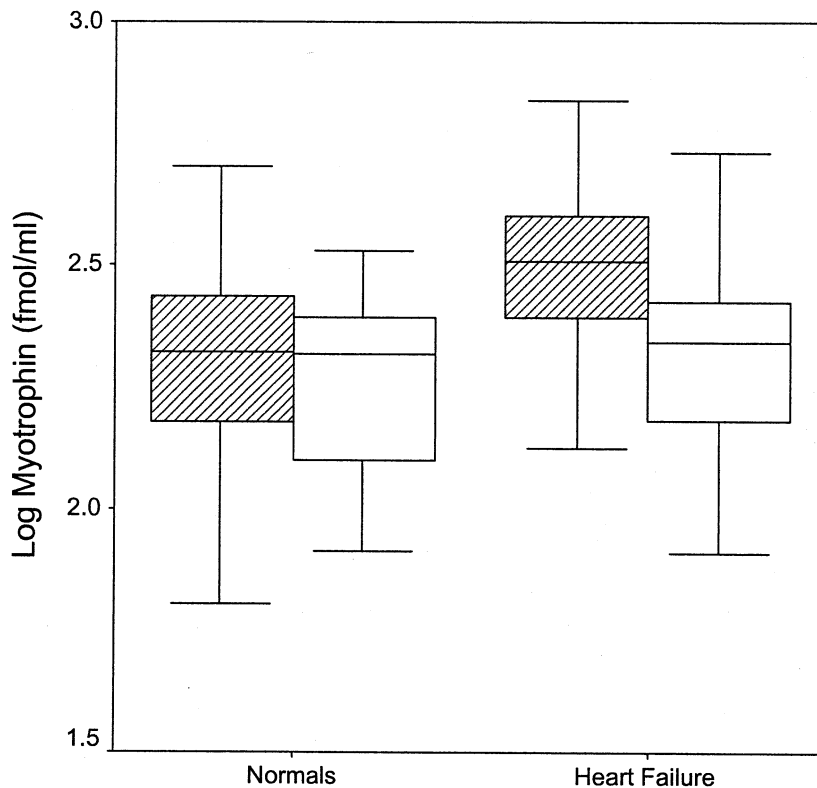
Isocratic size exclusion chromatography was performed on human plasma extracts (Fig. 3). This was resolved into two main immunoreactive fractions, one at 12 kD (which is the expected molecular weight of human myotrophin) and another fraction at 2.7 kD, which is likely to be a degradation product consisting of the C-terminal of myotrophin (an epitope to which the antiserum was directed).

The characteristics of the normal and HF patients are shown in Table 1. Groups were well matched for age and

**Table 1.** Patient Characteristics in the Study

	Normal Controls	HF Patients	p Value
Number	130 (41 [31%] female)	120 (35 [29%] female)	NS for gender
Age (yrs)	60.4 (26-81)	63 (20-87)	NS
Drug therapy	None		
Diuretics		98	
Beta-blockers		47	
ACE inhibitors		99	
Etiology of cardiomyopathy			
Ischemic		80	
Dilated		29	
Hypertensive		7	
Valvular		4	
NYHA class			
I		29	
II		34	
III		30	
IV		27	

Medians (ranges) are reported, and p values were computed using analysis of variance (comparing normal and HF patients). ACE = angiotensin-converting enzyme; HF = heart failure; NYHA = New York Heart Association.



**Figure 4.** Box plot of log-transformed plasma myotrophin levels in normal subjects and HF patients of both genders. **Hatched bars** = male; **open bars** = female.

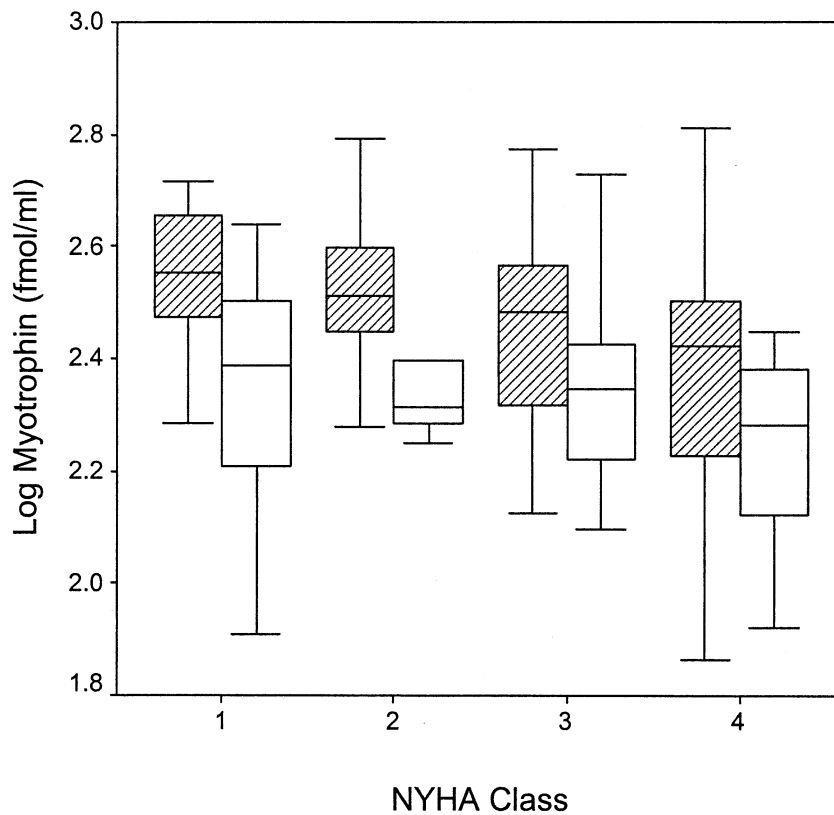
gender. In the normal population, there were no significant gender differences in plasma myotrophin levels (Fig. 4), and there was no correlation of myotrophin with increasing age. In patients with HF, plasma myotrophin levels were very significantly elevated ( $p < 0.0005$  by ANOVA). Homogeneity of variance was confirmed on all comparisons. Using the univariate general linear model procedure with log myotrophin as the dependent variable, and gender and presence of HF as predictor variables, male gender (mean  $\pm$  SEM log myotrophin [geometric mean, range] in males  $2.382 \pm 0.016$  [241, 28 to 933] vs. females  $2.288 \pm 0.024$  [194, 81 to 537] fmol/ml,  $p < 0.001$ ) and presence of HF (mean  $\pm$  SEM [geometric mean, range] HF  $2.402 \pm 0.021$  [252, 72 to 933] vs. normal  $2.268 \pm 0.021$  [185, 28 to 501] fmol/ml,  $p < 0.0005$ ) were significant as predictors. There was also a gender/presence of HF interaction ( $p < 0.007$ ) suggesting that the plasma myotrophin response in males with HF is different to that in females (Fig. 4). The adjusted  $r^2$  for this model was 0.174. There was no significant relationship of myotrophin with plasma creatinine levels.

The most common etiologies for HF were ischemic and dilated cardiomyopathy, and, for both, log myotrophin levels were significantly higher than normal controls (mean  $\pm$  SEM [geometric mean, range], ischemic cardiomyopathy  $2.443 \pm 0.023$  [277, 83 to 933], dilated cardiomyopathy  $2.479 \pm 0.034$  [301, 81 to 691] fmol/ml, both  $p < 0.0005$  compared with normal controls). There was no significant difference in myotrophin levels between the two etiologies.

Considering the predictors of log myotrophin levels in the HF patients, we found that gender ( $p < 0.0005$ ) and New York Heart Association (NYHA) class ( $p < 0.001$ ) were significant contributors to the variance,  $r^2$  for this model being 0.198. Males had higher levels than females ( $p < 0.001$ , Fig. 5), and myotrophin levels fell with increasing NYHA class (NYHA I vs. NYHA IV,  $p = 0.005$ ; NYHA II vs. NYHA IV,  $p = 0.002$  by Bonferonni's test) (Fig. 5). Myotrophin levels were not affected by therapy with diuretics, angiotensin-converting enzyme inhibitors, or beta-blockers. Similarly, stepwise linear regression analysis for predicting log myotrophin levels using age, gender, and NYHA class as independent variables confirmed gender and NYHA class (standardized  $\beta$  coefficients  $-0.318$  and  $-0.275$ , respectively,  $p < 0.001$  for both) as significant predictors.

## DISCUSSION

This is the first report documenting the presence of myotrophin in human plasma and describing an increased level in the plasma of patients with HF. The specificity of our in-house antibody was demonstrated by blotting tissue extracts from a variety of rat organs, demonstrating the ubiquitous presence of myotrophin. Myotrophin in human plasma appears to be in two forms, one the full-length myotrophin and the other a C-terminal fragment that is



**Figure 5.** Relationship of plasma myotrophin with severity of heart failure in males and females. **Hatched bars** = male; **open bars** = female. NYHA = New York Heart Association.

likely to be a degradation product. Immunoassay of extracts would detect both forms.

There is a clear gender difference in myotrophin plasma levels in HF, with higher concentrations in males. In addition, as the severity of the condition progresses, there is a reduction in plasma myotrophin. From the current investigation, it is unclear whether changes in plasma myotrophin are due to differences in secretion or in clearance of the peptide, although altered clearance is unlikely in view of the observed fall in plasma levels with increasing HF severity. Previous work on a limited number of cardiomyopathic hearts suggests increased myocardial synthesis of the peptide in HF (2,10). Human myotrophin RNA is widely distributed, being found in a wide variety of tissues including heart and skeletal muscle, liver, pancreas, and other cardiovascular tissue such as endothelial and vascular smooth muscle cells (10). All of these tissues could be potential sources of myotrophin, and it is uncertain which tissues contribute towards circulating myotrophin, and whether the peptide is actively secreted or leaking from damaged cells. The difference in response between males and females with HF is striking, as is the fall in plasma levels with disease severity. This paradoxical response of myotrophin may suggest that at least some of the peptide could have an extra-cardiac origin.

With regard to the latter point, it remains to be established whether myotrophin levels influence prognosis or

degree of cachexia. Myotrophin has also been suggested to be an NFκB binding protein (5,6). Its translocation from cytoplasm to nucleus during cell activation is also consistent with this role of modulating NFκB transactivating function (7,10). Activation of the NFκB system is widely documented in HF (8,9); NFκB mediates the induction of gene programs transcribing proinflammatory factors such as leucocyte adhesion molecules, cytokines, and chemokines such as TNFα (9), which may be important in the progression of HF. Tumor necrosis factor-α and myotrophin also mutually induce the transcription of each other (7), although these are acute effects demonstrated in vitro in the absence of other factors that may induce TNFα; TNFα rises with increasing HF severity, but its transcription is dependent on other factors other than myotrophin. The effect of myotrophin on altering the balance from highly activating p65-p50 heterodimers to repressive p50 homodimers and less active p65 homodimers suggests that it may overall reduce NFκB-activated DNA transcription (6). It is, therefore, of interest that we found the highest myotrophin plasma levels in early HF, perhaps suggesting an adaptive role in disrupting NFκB-mediated DNA transcription. As the disease progresses, less myotrophin was found, which would permit unopposed activation of TNFα by other factors. Myotrophin, when administered extracellularly, also has undoubted effects on protein synthesis and inducing early response genes and cell hypertrophy (3,4,7). Whether this adaptive

role is present in early HF but fails in later stages of the disease remains to be established in prospective studies of HF. Few hormones or cytokines show this paradoxical response to disease severity, but another example is urocortin, which is a stress-induced peptide capable of inducing diuresis and natriuresis in HF (13) and which we have demonstrated to fall with increasing disease severity (unpublished observations).

In conclusion, we have reported the elevation of a novel cardiac hypertrophy-inducing factor myotrophin in the plasma of patients with HF, particularly in males. Further investigations are warranted to elucidate the relationship of myotrophin to the status of the NF $\kappa$ B transcription system in HF patients at different stages of the disease and its impact on progression of the disease.

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