Serum Neuregulin-1β as a Biomarker of Cardiovascular Fitness

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Abstract: *Purpose:* Neuregulins (NRG) are growth factors that bind to receptors of the erbB family, and are known to mediate a number of processes involved in diverse tissues. Neuregulin-1β is expressed in skeletal muscle and is activated by exercise. We hypothesized that NRG-1β might circulate in the bloodstream and increase as a consequence of physical activity. A study was conducted in healthy subjects to determine if NRG-1β is immunodetectable in human serum, and if so whether levels relate acutely or chronically to exercise.

Methods: Nine healthy men underwent three bouts of exercise of varying degrees of intensity on a bicycle ergometer over a period of three weeks. Cardio-respiratory fitness was determined by measurement of maximal oxygen uptake (VO_2max). Serum was sampled prior to and immediately after each session (up to 30 minutes post) and serum NRG-1ß was quantified utilizing an indirect sandwich ELISA assay developed in our lab.

Results: Across subjects, mean serum NRG-1β levels ranged from 32 ng/mL to 473 ng/mL. Individual subjects showed relatively stable levels during the study period that did not change acutely after exercise. Serum NRG-1β demonstrated a positive correlation with VO_2max (r2=0.49, p =.044).

Conclusions: These preliminary observations suggest that at least in healthy men, serum NRG-1β is an indicator of cardio-respiratory fitness and does not change acutely with exercise.

Keywords: Growth factor, exercise, heregulin, cardiopulmonary fitness.

INTRODUCTION

Neuregulins (NRGs) are a complex family of proteins characterized by structural similarity to epidermal growth factor and are known to activate growth, differentiation and survival signaling in multiple tissues including cardiac and skeletal muscle (for review see [1]). NRGs mediate their effects on cells *via* binding to receptors of the erbB family. NRG-1 isoforms are expressed as either soluble ligands or as transmembrane proteins that require proteolytic processing for activation [2]. NRGs are expressed as either α or β isoforms that differ in their affinity and potency, with β isoforms generally showing greater bioactivity [3]. In skeletal muscle NRG-1 activates myogenesis [4, 5], acetylcholine receptor expression [6-8] and glucose transport [9, 10]. When rats exercise on a treadmill, or when muscle contraction is activated by nerve stimulation, there is activation of skeletal muscle NRG-1, with processing of high molecular weight isoforms that results in local erbB receptor activation [11]. In human skeletal muscle, there is also expression of NRG-1, along with erbB2, erbB3 and erbB4 receptors [12]. Interestingly, exercise training in humans is associated with increased expression of erbB3 [12].

Skeletal and cardiac muscle NRGs are primarily type I transmembrane proteins that are inactive until proteolytic release. In the heart, NRG-1β is released from microvascular endothelial cells *via* a metalloproteinase [13]. Moreover, NRG-1ß is present in the coronary effluent from the mouse heart, and the amount increases in response to cardiac stress.

Collectively our work in cardiac and skeletal muscle led us to hypothesize that NRG-1β may be present in the circulation, and may be released into the circulation in response to physical activity. We developed and employed an ELISA assay to demonstrate that NRG-1β is detectable in the human serum. Then we conducted a study to test the hypotheses that 1) exercise causes an acute increase in circulating NRG-1ß levels; and 2) serum NRG-1ß concentrations are associated with cardiorespiratory fitness as measured by VO_2max.

MATERIALS AND METHODS

Subjects

The study protocol was approved by the Institutional Review Board at Boston University Medical Center. All subjects provided written informed consent prior to participation in this study. Healthy adult volunteers were recruited between the ages of 18 and 65 who were able to pedal a stationary bicycle, had no history of chronic disease currently requiring medical therapy, had no prior history of cardiac

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condition including syncope, arrhythmia, hypertrophic cardiomyopathy, valvular disease, congenital heart disease, hypertension, coronary artery disease or congestive heart failure, had no history of exertional light-headedness, chest pain or shortness of breath with minimal exertion, absence of a systolic murmur on physical exam, and resting systolic blood pressure >140 or diastolic >90, without history of easy bruising or known coagulopathy.

Exercise Protocol

Participants underwent three bicycle exercise sessions at distinct levels of intensity designated as "maximal", "high" and "low" intensity. Each exercise session was separated by at least one week. Participants were asked to refrain from exercise 48 hours prior to each exercise session and from any oral intake aside from water 2 hours prior to each session. All sessions took place in the morning between 7 and 10 AM.

Measurement of cardiorespiratory exercise capacity (maximal oxygen uptake (VO_2max)) and performance of low and high intensity exercise protocols were done using an Ergoline electronically-braked cycle ergometer (Bitz, Germany). During the exercise sessions, breath by breath gas was analyzed using a Viasys Healthcare Vmax Encore Metabolic System (Yorba Linda, California) and heart rate was measured using a 5-lead EKG (Cardiosoft 4.2 software, GE Medical Systems Information Technology, Freiburg, Germany).

In the first exercise session, determination of VO_2max was performed using an individualized ramping protocol (26.67 ± 4.33 watts/min) and maintaining a cadence of 60 revolutions per minute (rpm) until volitional fatigue (mean test duration = 10:48 ± 2:10). All subjects met standard criteria for achievement of VO_2max; specifically, a plateau in VO_2 consumption despite an increase in work rate, achievement of age-predicted maximal heart rate and a respiratory exchange ratio equal to or greater than 1.15.

On subsequent sessions, low and high intensity exercise protocols were performed and consisted of cycling at a steady cadence (60 rpm) for 20 min. The target work rates for 'low' and 'high' intensity bouts were calculated based upon the anaerobic threshold (AT), defined as the inflection point at which VCO_2 increases nonlinearly with increasing exercise intensity, measured during the first exercise session. The low intensity protocol was performed at a VO_2 that was 80% of AT (20.21 ± 3.60 ml/kg/min). The high intensity protocol was performed at a VO_2 set above AT by 25% of the difference between VO_2max and AT (33.4 ± 7.85 ml/kg/min).

During low and high intensity protocols, work rate was adjusted to maintain the target VO_2.

Serum Sampling

Venous blood was drawn from brachial vein 15 minutes prior to the start of each exercise session and an additional three samples were drawn immediately (within 1 min), 15, and 30 minutes into recovery. Whole blood samples were stored at room temperature for one to two hours allowing for clot formation. Samples were then centrifuged at 1000 g for ten minutes at 4° C. Serum was removed and stored at -80 ° C.

NRG-1β Immunodetection in Serum

Samples were assayed for serum NRG-1β utilizing an indirect sandwich ELISA developed in our lab as well as immunoprecipitation and immunoblot. For the ELISA, microplates were coated with polyclonal anti-NRG-1ß antibody (polyclonal chicken IgY, contracted to Lampire Biological Laboratories, Inc) [13] diluted in PBS to a concentration of 118 mg/ml and incubated at 4° C overnight. All subsequent incubations were performed at 37° C. Non-specific binding was blocked using 5% BSA for one hour. Serum samples or standard (Recombinant NRG-1β Isoform (R& D Systems, Minneapolis, MN)) were diluted in 2% BSA and incubated for 2 hours. Serum samples were diluted 1:10 based on prior experiments demonstrating linearity at dilutions of less than 1:8. Detection of captured NRG-1β was performed using a monoclonal anti-NRG antibody (catalog # MS-272-P1, Labvision Co., Freemont, CA) diluted to a concentration of 2 μg/ml in 5% BSA and incubated for 2 hours. Following extensive washing, enzyme conjugated goat anti-mouse IgG at a dilution of 1:4000 (final concentration of 0.1 μg/ml) in 5% BSA after 1 h incubation was used for detection with fluorogenic substrate (Quantablu Fluorogenic Peroxidase substrate, Pierce Biotechnology, Inc., Rockford, IL) using a microplate fluorimeter (Molecular Devices).

An eight point standard curve was generated using recombinant human NRG-1β at concentrations ranging from 0 to 1600 ng/mL. Correlation coefficient for the standard curve was $R^2 = 0.9956$. There was no interaction between the assay and recombinant human epidermal growth factor or NRG-1α. All samples and standards were run in duplicate with a resultant mean coefficient of variability in samples of 8.8%.

Immunoblot and immunodetection of serum NRG-1β was performed using a modification of previously described protocol [13]. Serum sample (4 μl) was suspended in sample buffer directly, or after immunoprecipitation with monoclonal anti-NRG antibody (catalog # MS-272-P1, Labvision Co., Freemont, CA) (0.5 μg) on pre-cleared protein A/G beads (Santa Cruz). Following transfer of SDS-PAGE separated samples to PVDF membrane, blots were incubated overnight at 4C with biotinylated polyclonal anti-NRG-1β antibody (same antibody as for ELISA, biotinylated using Sulfo-NHS-Biotin from Pierce according to manufacturer's instructions) diluted 1:1000 in Tris-buffered saline (pH 7.4) with 0.1% Tween (v/v, TBST) and 5% milk (w/v). After washing in TBST, blots were incubated with streptavidin-conjugated horse radish peroxidase (Pierce) and detected with chemiluminescence (Pierce) and exposure to X-ray film.

Statistical Analysis

All samples were assayed in duplicate and reported values represent averages. Two analyses were prospectively described in our application to the IRB: the effect of exercise on serum NRG-1β during each session, and the relationship between resting serum NRG-1β and maximal aerobic capacity. Baseline and post-exercise serum NRG-1β values were compared and analyzed by repeated measures ANOVA. Correlations between serum NRG-1β and VO_2max were assessed by linear regression analysis. A third analysis not prospectively described was performed examining whether there was any change in resting levels of NRG-1β over the three

Table 1. Subject Characteristics

Characteristic	Mean*	95% CI †
Age (years)	29.8 ± 1.5	26.3 - 33.2
Height (cm)	174.3 ± 2.5	168.6 - 180
Weight (kg)	74.1 ± 4.6	63.5 - 84.8
Body Mass Index (kg/m2)	24.4 ± 1.4	21.1 - 27.6
Absolute VO2Max (L/min)	3.6 ± 0.3	2.9 - 4.2
Relative VO2Max (ml/kg/min)	48.4 ± 3.6	40.9 - 55.9

*Plus-minus values represent means ± standard error.
†CI denotes confidence interval.

exercise sessions. A repeated measures ANOVA was used for this analysis.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

RESULTS

Subject Characteristics and Exercise Data

A total of nine subjects were enrolled and all completed the study. All participants were male with ages between 21 and 36. Subject descriptive characteristics are summarized in Table **1**. VO_{2Max} ranged from 35 to 63 ml/kg/min.

Detection of NRG-1β in Human Serum

Circulating levels of NRG-1β were present in the collected human serum as demonstrated by immunoblot of serum (Fig. **1**). Serum immunoprecipitated with an antibody to common extracellular domain of NRG-1 gene products followed by immunoblotting with the anti- NRG-1β antibody detected a single protein of reasonable abundance at ~40 kDa. Using the same antibody pair in an ELISA format,

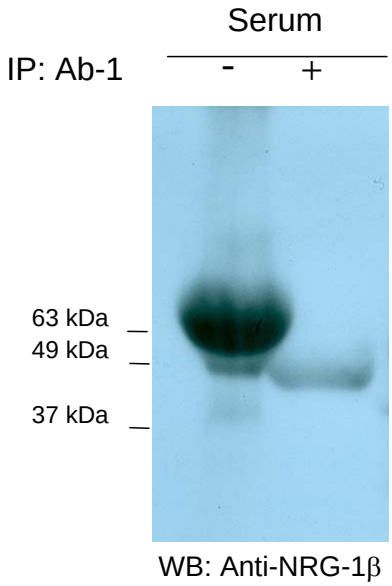

Fig. (1). Detection of NRG-1β in serum. Serum from a single subject was separated by SDS-PAGE and probed for NRG-1β before (left) or after immunoprecipitation (right) as described in Methods section. Blot demonstrates that the primary protein detected by this antibody pair is a ~40kDa protein.

NRG-1β levels had a wide range of distribution across subjects. Average resting values for each subject as determined from samples collected prior to the max, low and high intensity sessions ranged from 32 ng/mL to 473 ng/mL (mean = 217 ± 170 ng/mL). The distribution of mean serum NRG-1β levels among the study participants was large, whereas the distribution of samples from an individual over the course of the study was much more narrow (Fig. **2**). Interestingly there was a decline in the resting NRG-1β levels over the three sessions that reached statistical significance. This was not due to instability of the NRG-1β protein in serum, as all samples were processed in a two day period, and repeated measurements of NRG-1β from aliquots taken of the same samples do not show any consistent change over 6 months (data not shown).

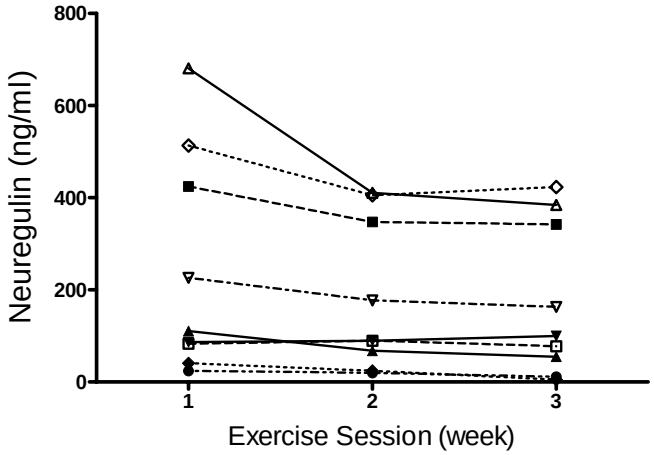

Fig. (2). NRG-1β in serum at three serial visits. $[NRG\text{-}1\beta]_{serum}$ was measured by ELISA in serial serum samples taken at rest during three visits over a period of 2-3 weeks. While levels remained relatively stable for individuals, there was a decrease in level over the three visits for a number of participants (p=0.019).

Exercise and Serum NRG-1β

To determine whether exercise influences serum NRG-1β, resting and post-exercise values were compared. There was no significant difference between pre- and post-exercise (0,15 or 30 minutes) levels of serum NRG-1β during max (p = 0.48, Fig. **3**), high (p = 0.52, data not shown) or low (p = 0.18, data not shown) exercise intensities. Hence exercise at low or high intensity does not acutely change levels of serum NRG-1β.

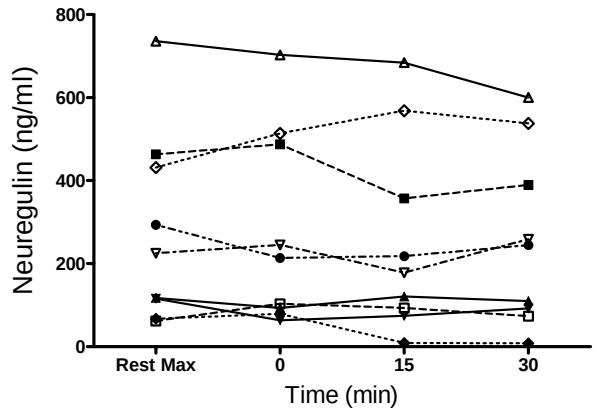

Fig. (3). NRG-1β in serum in response to exercise acutely. $[NRG\text{-}1\beta]_{serum}$ values from the maximal exercise session where subjects are exercised to exhaustion are shown. Resting sample was drawn immediately prior to exercise. Time 0 sample was drawn immediately upon termination of exercise. There was no effect of exercise on $[NRG\text{-}1\beta]_{serum}$.

VO_2max and Circulating NRG-1β

To examine the hypothesis that exercise determines serum NRG-1β over longer time periods, its relationship with cardiorespiratory exercise capacity measured as VO_2max was examined. There was a significant relationship between levels of $[NRG\text{-}1\beta]_{serum}$ and VO_2max (Fig. **4**). This relationship was similar regardless of whether we used resting values measured the day of the maximal aerobic capacity, the mean of the resting values over the three time points, or the mean of all serum NRG-1β values.

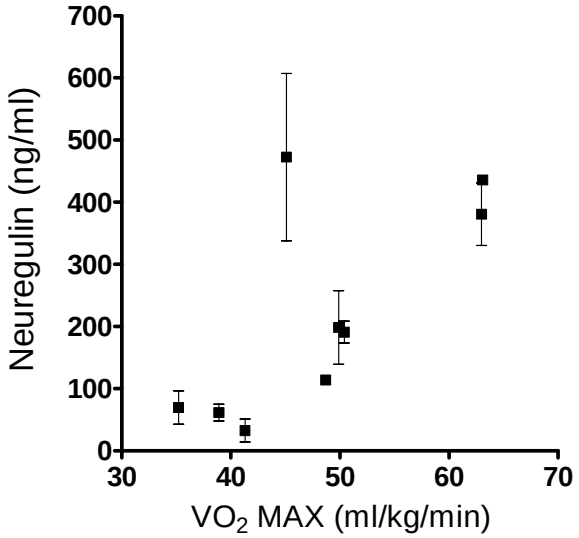

Fig. (4). NRG-1β in serum and maximal aerobic capacity. Mean +/- standard error of resting $[NRG\text{-}1\beta]_{serum}$ (from each three sessions) was plotted against aerobic capacity measured by VO_2max. There was a positive correlation between serum NRG-1β and aerobic capacity ($R^2 = 0.49$, $p=0.044$).

DISCUSSION

In this study we introduce a novel assay for serum NRG-1β and demonstrate that its levels in human serum correlate with cardiopulmonary exercise capacity. While these results are preliminary, to our knowledge this is the first study to demonstrate the presence of immunodetectable NRG-1β in serum. The levels of NRG-1β did not change acutely after a single bout of exercise. The correlation between serum NRG-1β and fitness suggests that serum NRG-1β is a function of aerobic capacity. Whether this is due to chronic training or genetics is unclear. This raises many interesting questions about the biological function of serum NRG-1β.

To date, NRGs are thought to work primarily in a paracrine and autocrine manner *via* secretion or shedding of the active transmembrane portion into the extracellular matrix [1]. The presence of immunoglobulin-like and kringle domains on the extracellular portion of neuregulin are thought to interact with proteoglycans of the extracellular matrix preventing its entry into the circulation. Our findings demonstrate that NRG-1β circulates and suggests the intriguing possibility that it is involved in tissue-to-tissue interactions at a distance. Determination of precise NRG-1β isoform(s) present in serum requires further investigation. The ~40 kDa protein detectable by immunoprecipitation and western blotting using the same antibody pair in our ELISA could represent any number of NRG-1β isoforms. In preliminary experiments, treatment of rodent cardiac myocytes with human serum results in phosphorylation of the erbB4 receptor (unpublished observation), consistent with there being some biologically active NRG-1β isoform(s) in serum. Moreover the antibody used in the ELISA is one that binds the beta EGF-like domain of NRG-1β that is critical for the biological activity of NRG-1β *in vitro* [13]. Thus one might speculate that serum NRG-1β is not merely an indicator of muscle activity/aerobic capacity, but possibly an active humoral factor that mediates the beneficial effects of physical activity to other organ systems.

Levels of serum NRG-1β did not change acutely with exercise. If proteolytic processing of NRG-1β does occur in response to exercise in humans similar to what occurs in rodents [11], the current findings suggest that the acutely activated forms of NRG-1β in skeletal muscle are not released into the circulation over the time-course of 30 minutes. This is in contrast to other growth factors such as VEGF, IGF-1 and growth hormone, the serum levels of which have all been shown to increase within minutes after intense exercise [14, 15]. Studies were performed at three levels of exercise intensity, including maximal aerobic capacity, so it is unlikely that an effect that occurs only at a specific exercise intensity was missed.

The positive correlation between aerobic capacity and serum NRG-1β does support the hypothesis that NRG-1β is released by skeletal and/or cardiac muscle over some longer time-scales. In that context it is interesting that the serum NRG-1β levels fell over the course of the three weeks of participation. In designing this study participants were asked to refrain from exercising for 48 hours prior to each session, over concern for the effect of recent exercise on our ability to detect acute effects of exercise on serum NRG-1β levels. We speculate that these instructions may have resulted in a decline in exercise participation over the period of the study.

Although the subject number was small, it appears that low levels of $[NRG\text{-}1\beta]_{serum}$ were found in subjects with VO_2max below 45 ml/kg/min, and levels increase in propor-

tion to VO_2max above this level, with the exception of one outlying individual. It is also worth noting that this subject with the highest mean and widest variance for serum NRG-1β levels was a self-reported body builder. This suggests that lean body mass may in fact be a more important determinant of serum NRG-1β than cardiopulmonary exercise capacity. Larger subject numbers and more detailed body composition analysis are necessary to test this hypothesis. In addition, a more formal examination of what happens to serum NRG-1β during various forms of exercise training will be necessary to specifically measure the effect of chronic exercise intervention on serum NRG-1β.

NRG-1β is critical for cardiovascular development [16], and in the adult heart appears to act as a cardioprotective factor *via* activation of cardioprotective signaling [13, 17], as well as modulation of sympathetic/parasympathetic balance [18]. Systemic NRG-1β administration protects the heart [19] and promotes skeletal muscle growth/repair [20]. It is also interesting that local delivery of NRG-1β to isolated skeletal muscle [10] or cardiac myocytes [3] activates glucose homeostasis. The strong relationship between physical activity and many of these known NRG-1β activated biological responses lead us to speculate that serum NRG-1β may in fact be a mediator of the beneficial effects of exercise on cardiovascular health. Interestingly, NRG-1β is bioactive in many other organs besides the cardiovascular system [21], and therefore levels of serum NRG-1β may have other equally important implications beyond cardiovascular fitness.

LIMITATIONS AND FUTURE WORK

The small number of subjects enrolled into this preliminary study limits the strength of our conclusions. Moreover, this study was performed in healthy subjects, and these were all males. In this small sample set there was no correlation between subject body mass index and serum NRG-1β. Further work is clearly needed to examine the relationship between serum NRG-1β and other clinical parameters, for example gender, age, and lean body mass. Moreover, measurement of serum NRG-1β in well-characterized epidemiologic populations or clinical cohorts will be necessary to determine whether there is any prognostic value of serum NRG-1β.

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