

## Norepinephrine Transporter is Involved in Down-Regulation of $\beta$ 1-Adrenergic Receptors Caused by Adjuvant Arthritis

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**ABSTRACT - Purpose.** Inflammation in forms of rheumatoid and experimental arthritis cause not only joint pain but also excessive cardiovascular mortality. The condition also reduces response to calcium channel and  $\beta$ -adrenergic ( $\beta$ 1-AR) antagonists. For calcium channel inhibitors, the reduced response is shown to be due to the reduced expression of target proteins. Hydroxymethylglutaryl CoA reductase inhibitors (statins) restore response to propranolol and verapamil. We tested the effect of adjuvant arthritis on the norepinephrine (NE) transporter (NET) density since altered sympathetic nervous system innervation has been observed in rheumatoid arthritis. **Methods.** Male Sprague–Dawley rats were divided into the following groups: Healthy/Placebo, Healthy/Statin, Pre-AA/Placebo, and Pre-AA/Statin (n=7-8/group). On Day 0, to the Pre-AA and Healthy groups, was injected *Mycobacterium butyricum* or saline, respectively. On Days 4-8, Statin and Placebo groups received either pravastatin (6 mg/kg) or placebo twice daily, respectively. On day 8, heart and blood samples were collected. The density of NET and  $\beta$ 1-AR in heart homogenate; NE in plasma and heart and inflammatory mediators (nitrite and interferon- $\gamma$ ) in serum were determined. **Results.** Inflammation was associated with a significant reduction in both  $\beta$ 1-AR and NET density with a positive correlation between the two proteins ( $r=0.978$ ,  $p<0.0001$ ). The down-regulating effect of inflammation was not reversed by pravastatin. Inflammation had no significant effect on the plasma or heart NE concentration. **Conclusion.** The close relations of NET and  $\beta$ 1-AR implicates altered sympathetic innervation and/or local NE handling in pharmacotherapeutic desensitization observed in arthritis.

### INTRODUCTION

Rheumatoid arthritis, a disease with 1% prevalence in the western world (1), causes not only joint pain and disability but also excessive cardiovascular mortality (2, 3). The mechanism of increased mortality is unknown. However, amongst various potential causes, excessive atherosclerosis, arterial thickness, and elevated pro-inflammatory mediators have been suggested (4-8).

Compared to healthy volunteers, obese adults (9) and children (10), elderly subjects (11) and patients with active rheumatoid arthritis (12) have reduced sensitivity to the calcium channel antagonist verapamil. Similarly, in the rat, inflammation diminishes response to verapamil (13, 14), propranolol (15, 16) and sotalol (17). A lack of response to pharmacotherapy may contribute to the high morbidity and mortality of patients with inflammatory conditions. In both humans for verapamil (12) and in rats for propranolol (15) the diminished pharmacological response is observed despite substantial increases in plasma drug

concentration. For calcium channel antagonists in the rat, it has been reported that inflammation results in reduced expression of the target proteins (14), and hence reduced drug-receptor binding (13). An inflammation-induced reduced  $\beta$ -adrenergic receptor target protein ( $\beta$ 1-AR) has not been reported. In addition, the molecular mechanism behind these observations is unclear. Excessive sympathetic nervous system innervation has been observed in rheumatoid arthritis, and the latter is suggested to have a potential role in

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cardiovascular mortality(18-20). Further, norepinephrine (NE) transporter (NET) functionality has a strong role in dictating the distribution of sympathetic innervation (21)

Administration to rats of pravastatin,(16) a hydroxymethylglutaryl CoA reductase inhibitor (statin), and angiotensin II receptor blockers (ARB) (14, 22) with inflammation appears to restore response to propranolol and verapamil, respectively. The effect of statins (23-25) and ARBs (26) may be explained by their antiinflammatory properties. Similarly, patients with rheumatoid arthritis whose disease is in remission do not exhibit reduced response to verapamil(27).

We hypothesized that the expression of myocardial  $\beta$ 1-AR is reduced by inflammation through a mechanism that links to an altered neuromuscular junction NE concentration and/or changes in the density of NET. We further expected that the down-regulation of  $\beta$ 1-ARs would be restored by pravastatin. The animal model of inflammation that we used was pre-adjuvant arthritis (Pre-AA), i.e., rats in the early phase of adjuvant arthritis when systemic inflammatory changes have already taken place, yet without the manifestation of disabling physical symptoms of the disease (28). Additionally, adjuvant arthritis is a well-established model of rheumatoid arthritis that elevates cytokines and causes arthritic symptoms (29). The utility of Pre-AA as a model of inflammation associated with rheumatoid-like diseases in rats without causing pain and suffering has previously been established (28).

## METHODS

### Materials

Protease inhibitor cocktail for mammalian tissues (#P 8340) was obtained from Sigma-Aldrich (St. Louis, MO). Tris (0.025M)/glycine (0.192 M) buffer, and Tris (0.025 M)/glycine (0.192 M)/SDS (0.1%) buffer were purchased from ICN Biomedicals (Aurora, Ohio). Immun-Star horseradish peroxidase Chemiluminescent kit, Precision Plus Protein Standards Dual Color, Bio-Rad D<sub>C</sub> Protein Assay Sodium, dodecyl sulfate, TEMED, 10% Tween 20, and 40% acrylamide/Bis 29:1 solution were purchased from Bio-Rad (Hercules, CA). Pravastatin was acquired from Apotex (Toronto, Canada). Labor LDN

Norepinephrine (Noradrenaline) Ultra Sensitive enzyme link immunoassay kit was obtained from Rocky Mountain Diagnostics (Colorado Springs, CO, USA). Heat-killed, desiccated *Mycobacterium butyricum* was purchased from Difco Laboratories (Detroit, MI). Mouse monoclonal to  $\beta$ -actin-Loading control (#ab8226) was purchased from Abcam Incorporated (Cambridge, MA). Rabbit anti-NET (#NET-101AP) was purchased from FabGennix Inc. International (Frisco, Texas). Rabbit polyclonal to beta-1 adrenergic receptor (#PA1-049) was purchased from Affinity BioReagents (Golden, CO).

### Animals and Protocol

The study was approved by the Health Sciences Animal Policy and Welfare Committee of the University of Alberta. Adult male Sprague–Dawley rats (250±10 g) were used. They were housed in a 12 h light/dark cycle and always had free access to water.

Rats were divided into 4 groups: Healthy/Placebo, Healthy/Statin, Pre-AA/Placebo, and Pre-AA/Statin (n=8/ group). On Day 0, to the Pre-AA and Healthy groups, single injections of *Mycobacterium butyricum* in squalene (0.5 mL of 50 mg/ml ) or saline was administered, respectively. On Days 4-8, Statin and Placebo groups received either pravastatin (6 mg/kg) or vehicle (1% methylcellulose in water) twice daily, respectively through gastric gavage. On day 8, under anesthesia (halothane/O<sub>2</sub> mixture), incisions were made, hearts and blood sample collected. Plasma was immediately separated and along with the hearts stored at -70<sup>o</sup> C until analyzed. Plasma and cardiac NE concentrations were not measured in the Healthy/Statin groups due to technical problems.

### Western Blot

Western blot analysis was used to determine the density of NET and  $\beta$ 1-AR;  $\beta$ -actin was used as a loading control. Whole heart was triturated in the presence of liquid nitrogen. Approximately 75 mg of ground heart was diluted 1 in 5 with homogenate buffer (0.05 M tris buffer pH 7.4, 0.05 M, 2% protease cocktail inhibitor), followed by 1 min of trituration on ice. Following centrifugation, the supernatant was kept and assessed for protein concentration using the Lowry method. Heart

protein samples (loading, 45 µg) were prepared with Lane Marker Reducing Sample Buffer and put into boiling water for 60 s. Proteins were separated using SDS-polyacrylamide electrophoresis on a 7.5% gel. Blotting was done onto a nitrocellulose membrane (0.45 µm, Trans-Blot Transfer Medium). Non-specific antibody binding was reduced by blocking for 2 h at room temperature or overnight at 4°C in blocking solution (5% skim milk/ 2% bovine serum albumin/ 0.05% Tween in tris-buffered saline). Membranes were then incubated with primary antibody diluted with 0.05% Tween in tris-buffered saline at 1:750 and 1:1000 for NET and β1-AR, respectively. Horse radish peroxidase-conjugated secondary antibodies (GAR or GAM), were diluted 1:15000 in blocking solution and incubated at room temperature for 1 h. Secondary antibodies were visualized using chemiluminescence and captured using Kodak BioMax Light Film (Sigma-Aldrich, St. Louis, MO). Bands were assessed by densitometry using ImageJ software (National Institute of Health, Bethesda, MD). Density for the proteins of interest was corrected for loading variations by taking the ratio to loading control.

### Enzyme-linked Immunosorbent Assay of Norepinephrine

NE concentrations were determined using a commercially-available, ultrasensitive, competitive enzyme-linked immunosorbent assay (Rocky Mountain Diagnostics, Colorado Springs, CO, USA). Plasma or heart homogenate samples were assessed by adding samples directly to the 96 well plate. For whole heart samples, 150 µl of ice-cold 0.01 N hydrochloric acid was added to 100 µg of triturated tissue, followed by homogenization on ice using the pellet pestle for 1 min. Following 5 min of centrifugation at 19 000 G, 100 µl of supernatant was added to the NE enzyme immunoassay 96 well plate. The following simplified steps were applied to the immunoassay plate as per the kit instructions: a) NE is extracted using a cis-diol specific affinity gel; b) acetylation to N-acetylnorepinephrine; c) enzymatic conversion to N-acetylnormetanephrine; d) Acetylated NE from the sample and solid-phase well-bound NE compete for a fixed number of antiserum binding sites; e) upon system equilibration, free acetylated NE, which was

derived from the sample, and free acetylated NE-antiserum complexes are removed from the wells; f) the antibody that is bound to the kit's NE (solid phase) is detected using an anti-rabbit IgG-peroxidase conjugate using the substrate tetramethylbenzidine. Using a plate reader at 450 nm gives a reading inversely proportional to the NE content of the sample since the peroxidase is bound to the solid phase NE.

### Serum Interferon Gamma and Nitrite

Interferon gamma (IFN-γ) was determined using a commercially available enzyme-linked immunosorbent assay. Aliquots of 50 µl of serum were transferred to an anti-rat-IFN-γ coated 96 well plate, followed by 2 h incubation and 5 washings. Horseradish peroxidase-conjugated anti-rat-IFN-γ was added to the wells followed by 2 h incubation then 5 washings. Substrate solution containing stabilized hydrogen peroxide and stabilized chromogen (tetramethylbenzidine) was added to each well, followed by 30 min incubation at room temperature. The reaction was stopped with diluted HCl then read at 450 nm with a 543 nm correction. The standard curve was linear over the range of 31.2 to 2000 pg/ml ( $r^2=0.997$ ) and the lower detectable limit was set at 10 pg/ml, while the lower limit of quantification was 31.2 pg/ml.

As a marker of plasma NO, we assessed the concentration of its stable breakdown product, nitrite (NO<sub>2</sub>) using a previously described Griess reaction (30). First, 10 µl *Aspergillus* nitrate reductase (10 U/ml) was added to 100 µl of plasma, and incubated for 30 min at 37°C in the presence of 25 µl of 1 M HEPES buffer (pH 7.4), 25 µl of 0.1 mM FAD, 50 µl of 1mM NADPH. Then 5 µl of 1500 U/ml lactate dehydrogenase and 50 µl of 100nM pyruvic acid was added and incubated for an additional 10 min at 37°C. Nitrite levels were quantified by adding 1 ml of Griess reagent and incubating for 10 min at room temperature for 10 min prior to an absorbance scan at 543 nm. Calibration curves were used for both nitrite and nitrate, confirming a nitrate reductase efficiency of >99%. The standard curve was linear over the range of 12.5 to 200 µM ( $r^2>0.99$ ), and the lower limit of quantification was 12.5 µM (coefficient of variation<15%).

## Statistics

For the effect of inflammation and treatments on the density of  $\beta$ 1-AR and the concentration of NE, nitrite and IFN- $\gamma$ , ANOVA, and when necessary, Tukey's post hoc test were used. The association between NET and  $\beta$ 1-AR was assessed using the linear Pearson regression analysis. Data is presented as the mean $\pm$ SEM, and p-values less than 0.05 were considered significant.

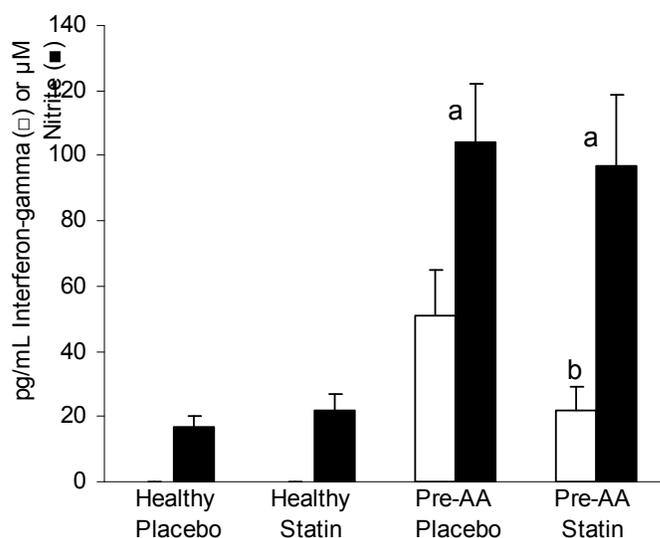
## RESULTS

**Inflammation.** The inflammatory condition was confirmed as the mediators measured postmortem were significantly higher in Pre-AA groups (Figure 1). IFN- $\gamma$  which was undetectable in the serum of Healthy groups was elevated to 51.3 $\pm$ 14.1 pg/mL and 21.6 $\pm$ 7.1 pg/mL in Pre-AA/Placebo and Pre-AA/Statin groups, respectively. The difference in IFN- $\gamma$  between Pre-AA/Placebo and Pre-AA/Statin was significant ( $p < 0.05$ ). Nitrite concentration was 17.4 $\pm$ 3.3 and 21.8 $\pm$ 4.6  $\mu$ M in Healthy/Placebo and Healthy/Statin, respectively. It was significantly elevated to 104 $\pm$ 18 and 97 $\pm$ 22  $\mu$ M in Pre-AA/Placebo and Pre-AA/Statin, respectively

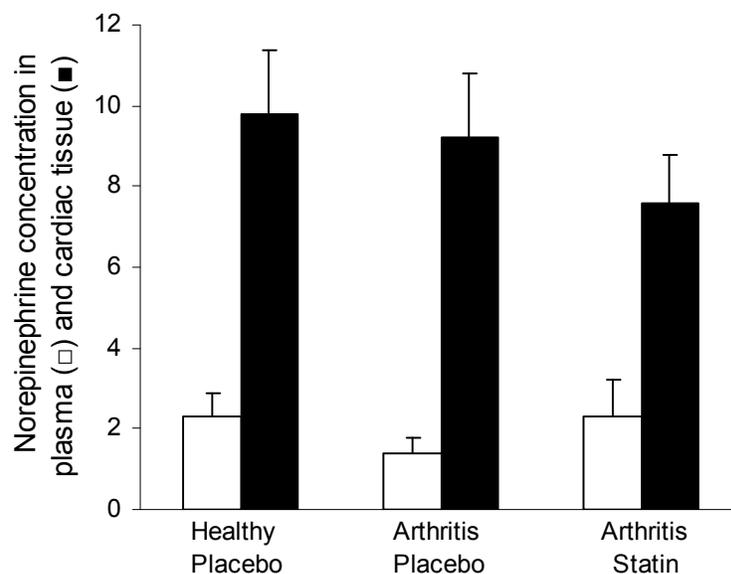
( $p < 0.01$ ). There was no significant difference between rats within either Healthy or Statin groups.

**Norepinephrine Concentrations.** NE concentrations in plasma or heart were not significantly different among the groups (Figure 2): Plasma (ng/mL): Healthy/Placebo, 2.3 $\pm$ 0.6; Pre-AA/Placebo, 1.4 $\pm$ 0.4; Pre-AA/Statin, 2.3 $\pm$ 0.9, Cardiac (ng/g): Healthy/Placebo, 9.8 $\pm$ 1.6; Pre-AA/Placebo, 9.2 $\pm$ 1.6; Pre-AA/Statin, 7.2 $\pm$ 1.7.

**Target Proteins.** Inflammation reduced the relative density of  $\beta$ 1-AR protein from 2.19 $\pm$ 0.67 to 0.88 $\pm$ 0.21 ( $n=8$ ,  $p < 0.05$ ), however pravastatin treatment did not restore this down regulating effect of inflammation (0.971 $\pm$ 0.30,  $n=8$ ) (Figure 3). NET was also significantly down-regulated by inflammation from 1.57 $\pm$ 0.55 to 0.67 $\pm$ 0.25 ( $n=8$ ,  $p < 0.05$ ). The effect was not reversed by pravastatin (0.78 $\pm$ 0.32,  $n=8$ ) (Figure 3). Statin treatment had no significant effect of the relative density of either  $\beta$ 1-AR (2.22 $\pm$ 0.71,  $n=8$ ) or NET (1.71 $\pm$ 0.71,  $n=8$ ) protein in healthy rats. There existed a strong positive correlation between these two proteins ( $n=16$ ,  $r=0.971$ ,  $p < 0.001$ ) (Figure 4).



**Figure 1.** The effect of Pre-AA inflammation on serum nitrite and IFN- $\gamma$  in the presence or absence of 4-day pravastatin treatment 8 days after adjuvant injection ( $n=8$ /group, mean $\pm$ s.e.m); a, significantly different from the healthy groups; b, significantly different from Pre-AA/Placebo group ( $p < 0.05$ ). IFN- $\gamma$  was not detectable in the healthy groups.



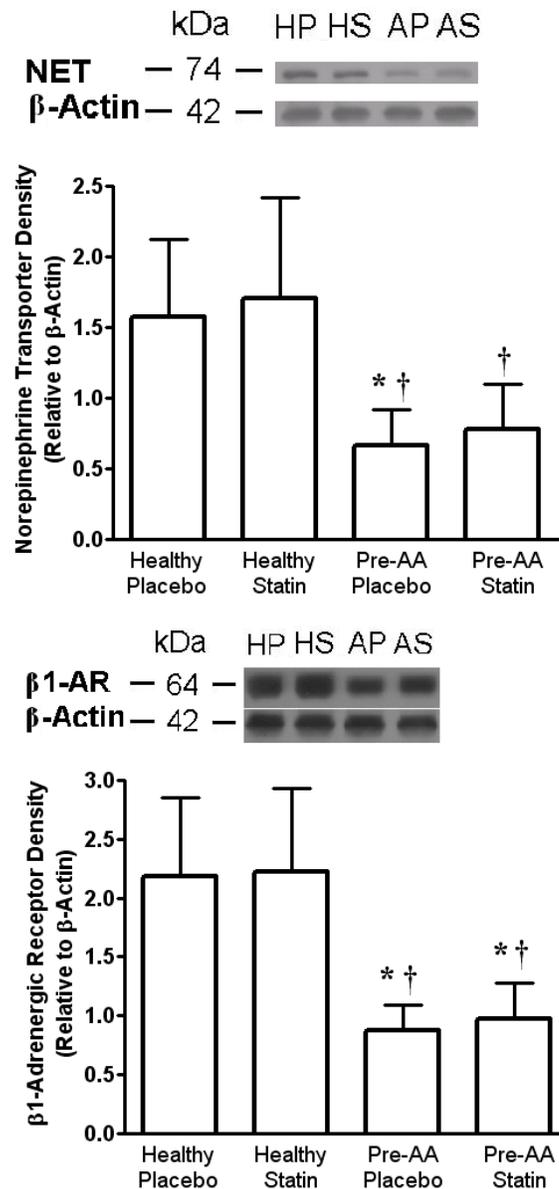
**Figure 2.** The effect of Pre-AA inflammation on plasma (ng/mL) and cardiac (ng/g) norepinephrine in the presence or absence of 4-day pravastatin treatment 8 days after adjuvant injection ( $n=8/\text{group}$ ,  $\text{mean}\pm\text{s.e.m}$ ). The groups were not significantly different from each other.

## DISCUSSION

Using the Pre-AA animal model of arthritis, we have shown for the first time that inflammatory disease model-based inflammation results in reduced cardiac  $\beta_1$ -AR density (Figure 3), and the latter is strongly and positively correlated with cardiac NET density (Figure 4). This observation, is suggestive of a potential mechanism responsible for reduced propranolol responsiveness in the presence of inflammation (15, 16). Reductions in NET, the primary component of NE uptake-1 (the NE uptake system that removes NE from neuronal junctions), so closely related to reductions in  $\beta_1$ -AR, implicates altered sympathetic innervation and/or local NE handling in pharmacotherapeutic desensitization observed in arthritis.

Excessive sympathetic nervous system innervation is a well established component of inflammatory conditions, possibly contributing to excessive cardiovascular mortality in RA (18-20, 31, 32). Reactive oxidase species, mediated by angiotensin II and NADPH in the rostral ventrolateral medulla of the brain, stimulate sympathetic nervous system outflow (33).

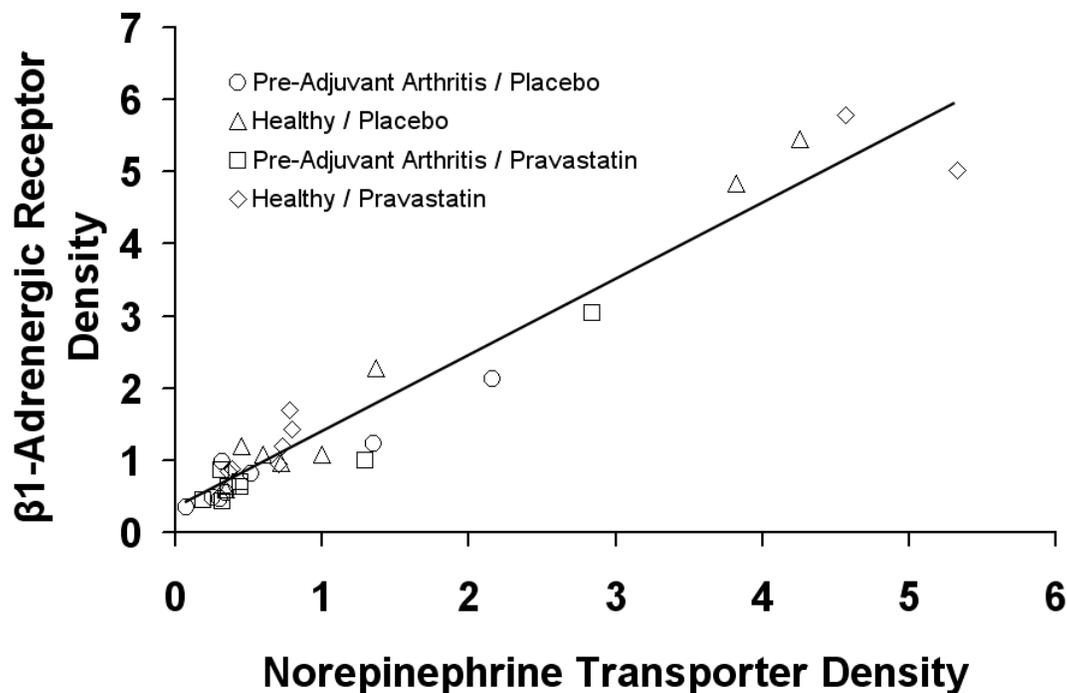
The observed down-regulation of NET in heart tissue did not appear to alter NE concentration in plasma or in the heart tissue as, in this regard, we observed no significant difference between Pre-AA and control groups (Figure 2). It is possible that the stress of anesthesia induction during the sample recovery blunted potential differences between the two groups. The effect of human arthritis on plasma NE concentration is unclear if not controversial. Biasi et al (34) measured higher NE in plasma of RA patients as compared with the previously reported normal range while Kuis et al (35) have reported lower NE plasma concentration in juvenile arthritis patients as compared with control subjects after 5 min in the tilt position. Changes in NET density and/or function may influence local NE concentration in the neuronal-cardiomyocyte interface, however, the corresponding effect on circulating NE and/or tissue NE levels may be difficult to pinpoint due to complicating factor such as dilution in the body and/or metabolism.



**Figure 3.** The effect of Pre-AA inflammation on cardiac NET (top) or  $\beta$ 1-adrenergic receptor (bottom) density in the presence or absence of 4-day pravastatin treatment, 8 days after adjuvant injection (n=8/group, mean $\pm$ s.e.m); inset is a representative Western blot sample. \* Significantly different compared to Healthy/Placebo, p<0.05; †Significantly different compared to Healthy/Statin, p<0.05.

Previously we have shown that Pre-AA significantly reduced propranolol response despite a 10-fold increase in plasma drug concentrations, and pravastatin restored the response (16). The NET is a Na<sup>+</sup>/Cl<sup>-</sup>-dependent neurotransmitter transporter that limits neurotransmitter activity, and whose inhibition can lead to increases in extracellular NE at central and peripheral noradrenergic synapses

(36). We were, therefore, expecting to observe a statin-induced recovery of both  $\beta$ 1-AR and NET density in Pre-AA rats. Statins reduce sympathetic nervous system outflow in rabbit by inhibiting superoxide pathways in the brain (37) and in stroke-prone spontaneously hypertensive rats can reduce 24-h urinary NE excretion in conjunction with elevated brain eNOS and iNOS (38).



**Figure 4.** The correlation between cardiac  $\beta 1$ -adrenergic receptor and NET density;  $r=0.971$ ,  $p<0.001$ .

However, we observed no differences either in  $\beta 1$ -AR or NET density between pre-AA animals that were treated with pravastatin and those that were treated with vehicle (placebo) (Figure 3). Neither did pravastatin treatment reduce serum nitrite in pre-AA to normal levels; it only normalized IFN- $\gamma$ , the hallmark helper-inducer T-lymphocyte (Th)-1 proinflammatory cytokine (39). Our data cannot explain this observation. The improving effect of pravastatin on response to propranolol (16), therefore, may be independent of our measured  $\beta 1$ -AR and NET densities as we did not assess the local post-synaptic NE concentrations or sympathetic nerve innervation.

Little is known about the regulation of NET in the heart during inflammation. Cultured sympathetic neurons incubated with the proinflammatory cytokine cardiotrophin-1, but not IL-6 or tumor necrosis factor alpha (TNF- $\alpha$ ), simultaneously suppress NE uptake and NE content (40). This suggests that IL-6 and TNF- $\alpha$ , seemingly, are not involved in the regulation of catecholamine synthesis or re-uptake (40). In contrast to other proinflammatory mediators, angiotensin II appears to up-regulate NET density (41). However, oxidative stress may increase sympathetic outflow

via the angiotensin II receptor type 1 in the brain (33). NE in arthritis is antiinflammatory at lymph tissue, and interaction with immune cells decreases TNF- $\alpha$  production (42). Reducing inflammatory mediator formation by reduction in NET may help limit inflammation in RA, but also may be related to the reduced  $\beta 1$ -AR density observed in our study, which is also associated with diminished propranolol response. In adrenal medullary cells, interferon- $\alpha$  administration results in reduced NET density and reduced [ $H^3$ ]NE uptake (43). Also, nitrogen oxides inhibit the uptake of NE (41). While the effects of inflammation on NE handling have been investigated in this paper, it is also important to note that NE itself has antiinflammatory properties. This is demonstrated by the fact that mice treated with desipramine, which elevates synaptic NE concentrations, have reduced TNF- $\alpha$  (proinflammatory) production and elevated IL-10 production (antiinflammatory) (42). One of the strengths of the current paper is that the in vivo nature of the Pre-AA model tests the effects of the constellation of changes in inflammatory mediators against NET density. The present data demonstrate inflammation-induced decreases in  $\beta 1$ -AR and NET density. However, exactly how the

alteration in the sympathetic nervous system of animals with arthritis influences drug response needs further investigation. The main limitations of this work are the lack of data on the local post-synaptic NE concentrations and the characterization of sympathetic nerve innervation. Further insight could be gained by investigating NET and  $\beta$ 1-AR mRNA levels or by assessing internalization-degradation of the respective proteins. Membrane preparations, biotinylation, or immunofluorescent staining could help in this observation.

## CONCLUSIONS

We provide novel information that suggests the reported reduced response to  $\beta$ 1-adrenergic antagonists under inflammatory conditions is likely due to a down-regulation of the target protein with a significant link to norepinephrine transporter density.

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