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Original Article

Effects of the nephrilin peptide on post-burn glycemic control, renal function, fat and lean body mass, and wound healing

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Abstract: The mechanisms underlying the effects of severe burn trauma are not well understood. We previously demonstrated the ability of nephrilin peptide (an iron-binding peptide believed to enter cells through iron-uptake pathways) to suppress aspects of the neuroinflammatory response in a rat scald model, as well as sepsis mortality in a mouse model. This study explores the effect of nephrilin on other clinically relevant outcomes in the rat scald model. In a rat scald model, animals were treated with nephrilin either in week 1 or week 2 post-burn. Measurements were made of serum glucose and creatinine as well as wound area by planimetry and body composition by DEXA. Given the potential role of iron, results were analyzed both for the entire cohort of animals and for the normoferremic (>100 ug/dL serum iron) subset of animals. Nephrilin improved body composition, wound healing, kidney function, and glycemic control. The first two effects were significant in normoferremic but not in hypoferremic animals suggesting an effect of iron status on burn injury outcomes. Nephrilin treatment modulates a number of relevant variables in the rat scald model.

Keywords: Nephrilin, burn injury, iron, lean body mass, wound healing, eGFR, glycemic control

Introduction

Severe burn trauma is associated with a vast array of secondary effects including systemic neuroinflammation, loss of lean body mass, sepsis, organ failure, loss of glycemic control, delayed wound healing, neuropathies, and cognitive deficits. These serious and enduring complications can lead to substantial morbidity and mortality [1-6]. We previously showed the pleotropic effects of nephrilin peptide in combating post-burn systemic neuroinflammation and sepsis in rodent models [7]. We examine the hypothesis that nephrilin exerts positive effects upon other relevant variables in the rat scald model. In this work we extend our earlier study to a number of clinically significant variables such as glycemic control, wound healing, loss of lean body mass, and organ function.

In the earlier study, treatment effects with nephrilin were observed after animals received daily injections over a 14-day period, beginning immediately after injury. In this study we explore treatment with nephrilin either from Day 1-7 or Day 8-14. We also begin treatment of the first group 2 hours after scald injury to more appropriately mimic real life, where most admissions to burn trauma units occur within two hours post-injury.

Severe trauma is associated with inflammatory anemia mediated by hepcidin, an iron-regulatory protein in serum and a current target of therapy in critical illness [8, 9]. In this work we explore the possible role of hepcidin-controlled iron metabolism in modulating the effects of nephrilin, an iron-binding peptide [12]. Fourteen days after scald injury we observed that up to a

third to one half of rats in the model showed serum iron levels below 100 ug/dL. Given the potential role of iron in the effects of this peptide, results were analyzed both for the entire cohort of animals and for just the normoferremic (>100 ug/dL serum iron at Day 14) subset of animals, to see if persistent hypoferremic status might play a role in some or all of the peptide's effects.

Nephrilin is a 40-mer peptide designed as a competitive inhibitor of mammalian target of rapamycin complex 2 (mTORC2) binding to PRR5/Protor, and has previously been shown to modulate the neuroimmune response to a variety of xenobiotic and metabolic stressors in rodents [10, 11]. When injected into mice at high doses daily for 26 days, nephrilin generates no visibly differential pathology compared to vehicle [11]. Nephrilin contains a metal-binding domain known to bind ferrous ($Fe2^{+}$) and ferric ($Fe3^{+}$) iron [12]. Based on crosslinking studies, uptake of this metal-binding domain into mammalian cells significantly involved binding to integrin-beta-3, a component of the ferric uptake pathway [13] as well as to transferrin receptor [14].

In this study we used nephrilin in a well-characterized rat scald model [7] to examine its effects on clinically relevant variables. We paid particular attention to the possible impact of hepcidin status on these effects.

Materials and methods

Reagents

Nephrilin peptide, a 40-mer peptide carrying a sequence derived from PRR5/Protor (the sequence is conserved in human, rat and mouse species) was synthesized by Genemed Synthesis (San Antonio, TX) and purified to >80% purity by HPLC. The design and synthesis of nephrilin have been previously described [11]. BCA Protein Kit was from Pierce (Rockford, IL). Antibodies for ELISAs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). CelLytic M cell lysis reagent and the iron assay kit were obtained from Sigma (St. Louis, MO). Serum iron reference range was 60-170 ug/dL. The cutoff for allocation to the hypoferremic group was <100 ug/dL (n=12 per group). No animals in the cohort were hyperferremic (>170 ug/dL). Hepcidin was measured using a kit purchased from MyBiosource, Inc. (San Diego, CA). GDF15 ELISA kit was purchased from Biomatik (Wilmington, DE).

Nephrilin administration

Treatment group sizes were as follows: group S (n=4) sham-treated; group B (n=8) burn + saline; group N1 (n=9) burn + nephrilin daily, days 1-7; group N2 (n=5) burn + nephrilin, days 8-14. Nephrilin was administered once daily by subcutaneous bolus at 4 mg/kg, with the first dose administered after completion of the scald procedure. Injection volume was 400 uL. Control animals received the same volume of saline. The 4 mg/kg daily dosage of nephrilin was selected based on its demonstrated safety and efficacy in eleven different rodent models tested to date [7, 10, 11, 14; and unpublished data]. In a non-GLP study, mice treated daily with 20 mg/kg nephrilin by subcutaneous bolus for 26 days showed no differential toxicology in major organs when compared to a saline control [11]. For analysis of the normoferremic subset, animals with <100 ug/dL serum iron were excluded from the analysis. Each of the treatment groups turned out to have exactly four normoferremic animals (>100 ug/dL serum iron) and these were used in the analysis of the normoferremic subset.

Rat scald model

The rat scald burn model [7] is a modified Walker-Mason model that induces inflammation and hypermetabolism in line with what severely burned patients experience. The model results in a mortality rate of <1%. Adult male Sprague Dawley rats (275-300 gm, Charles River Laboratories, Wilmington, MA, USA) were housed in clean cages on a 12 hrs light/dark cycle with access to food (standard chow) and water *ad libitum.* Animals were allowed to acclimate for one week prior to the experiment. All animal procedures were performed in adherence to the National Institute of Health's *Guide for Care and Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas Medical Branch at Galveston. All procedures were initiated in the morning between 7 and 10 a.m. Prophylactic analgesia (0.05 mg/kg body weight Buprenorphin) was administered 5 min before general anesthesia (40 mg/kg body weight ketamine/

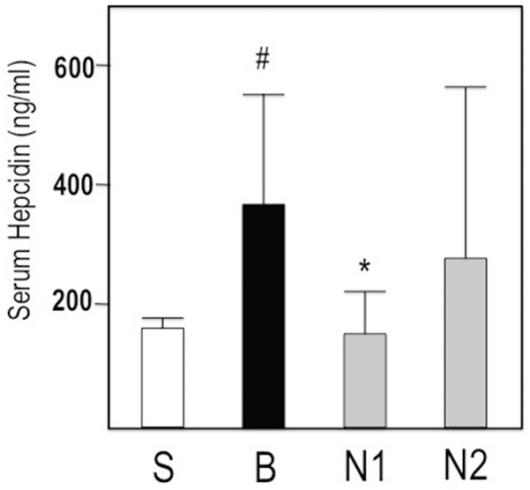

Figure 1. Serum hepcidin. #p<0.05 relative to S (sham) group. *p<0.05 relative to B (burn + saline) group.

xylazine). The dorsum of the trunk and the abdomen were shaved, and a 60% of total body surface area (TBSA) burn administered by placing the animals in a mold and immersing them in 98-100°C water for 10 seconds on the back and for 2 seconds on the abdomen. This method delivers a full-thickness cutaneous burn as confirmed by histological examination. Burned rats were immediately resuscitated with 40 cc/kg Ringer's Lactate injected intraperitoneally. Animals in the sham group were treated exactly as described above for burned animals except that the animals were not placed in hot water. Animals were randomly assigned to treatment groups, and nephrilin (4 mg/kg) or saline were administered by subcutaneous bolus daily. Each treatment group comprised 4-9 animals per time point. At the end of the study period animals were euthanized by decapitation as approved by the University of Texas Medical Branch IACUC guidelines, the NIH's Office of Laboratory Animal Welfare (OLAW), and the AVMA recommendations. Animals were euthanized on day 14. All tissues and organs of interest were rapidly dissected or collected and flash frozen in liquid nitrogen with subsequent storage at -80°C.

Glucose tolerance test

Fourteen days post-burn, the rat tail was snipped and the baseline glucose level measured using a BAYER contour blood glucose monitoring system. The rats were then injected with glucose and readings were performed at 15 min, 30 min, 45 min, and 60 min post-injection. The animals were then euthanized and the blood was collected for further analysis. Results were expressed as AUCs (ug/dL/hr) over baseline over the sampling period.

Body composition

Dual-energy-x-ray absorptiometry (DEXA) scans were performed at 14 days post burn to measure lean body mass (LBM), bone mineral content (BMC), and fat mass (FM) using DEXA (QDR-4500W model, Hologic, Waltham, MA). Using a small phantom, the machine is calibrated daily before use and the data were recorded using the small animal software package. The measurement of different tissues in the body using the DEXA is based on the principle that the intensity of a beam of x-rays passing through the rat body is related to the thickness, density, and chemical composition of the tissues traversed [15]. Each scan takes 90 seconds and is performed on isofluorane anesthetized rats.

Wound closure

The burn wounds of anesthetized rats were traced on a transparent film. Planimetric measurement of the surface area of the wound was performed using Image software.

Kidney function via eGFR

Kidney function was indirectly assessed by measuring serum creatinine. eGFR (ml/min/100 g animal body weight) was computed as previously described [16].

Tissue protein extracts and ELISAs

ELISAs were performed as previously described [7, 10, 11]. Serum iron, GDF15, hepcidin, CXCL5, and EGF and urinary hepcidin were measured. Owing to insufficiency of serum sample, EGF assay data are available for only a subset (n=6-7) of animals.

Statistical analysis

Data are presented as means ± SD. Probability values (*p* values) were computed using Student's *t*-test and expressed relative to sham or saline-treated group. Group size for treatment groups was 4-9 animals, depending on the experiment (actual number indicated in Methods, above).

Table 1. Tissues from hypoferremic (<100 ug/dL) and normoferremic (100-170 ug/dL) groups were analyzed as described in Methods

Category	Item	Hypoferremic	Normoferremic	*p* value
Controls	Total serum iron (ug/dL)	71.5 ± 17.1	147.5 ± 29.3	0.001
	Urinary hepcidin (ng/pg cys C)	115.4 ± 18.4	80.8 ± 31.1	0.044
	Spleen weight (grams)	1.65 ± 0.51	1.20 ± 0.20	0.046
Serum Cytokines/Growth Factors	GDF15 (ng/ml)	0.58 ± 0.18	1.03 ± 0.34	0.011
	CCL2 (ng/ml)	1.89 ± 0.67	2.02 ± 0.45	0.678
	CCL5 (ng/ml)	35.3 ± 62.1	2.20 ± 2.91	0.175
	CXCL5 (ng/ml)	3.34 ± 0.75	1.77 ± 1.37	0.024
	EGF (ng/ml)	1.37 ± 0.79	2.39 ± 0.58	0.022
Clinically Relevant	Glucose tolerance (mg/dL/hr)	72.5 ± 46.0	88.4 ± 35.6	0.464
	eGFR (ml/min/100 g)	0.86 ± 0.29	0.97 ± 0.63	0.693
	Dorsal wound area (% avg)	109.1 ± 13.6	320.7 ± 15.0	0.037
	Lean Mass (DEXA)	306.1 ± 16.9	320.7 ± 15.0	0.037
	Fat Mass (DEXA)	21.2 ± 5.8	16.1 ± 4.5	0.026

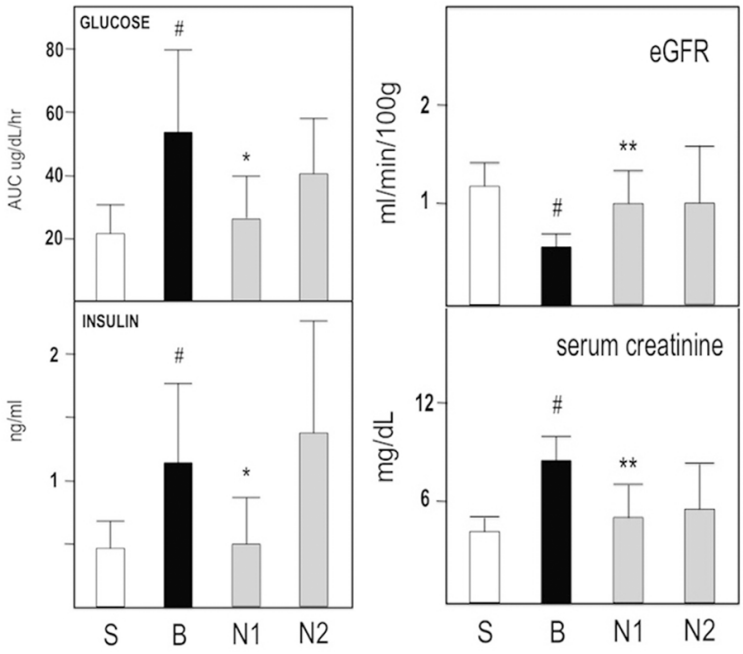

Figure 2. Left panels: glycemic control. Right panels: kidney function. #p<0.05 relative to S (sham) group. *p<0.05 relative to B (burn + saline) group.

Results

Iron status impacts post-burn variables in the rat scald model

Hepcidin-regulated anemia has been documented in human trauma [8]. We measured serum hepcidin (**Figure 1**) and observed a significant elevation of hepcidin levels in the burn group, as expected. This elevation was significantly reduced by early (N1) nephrilin treatment, but not with late treatment (N2).

Based on the above observations we assessed serum iron status on Day 14 as a co-variable in a pooled analysis (**Table 1**). For this preliminary analysis all treated animals in the study (i.e. except for sham-treated) were pooled to look beyond the effects of treatment. Significant differences were found between hypoferremic and normoferremic cohorts in a number of control variables as expected (serum iron, urinary hepcidin, spleen weight), serum biochemistry (GDF15, CXCL5 and EGF), and three clinically-relevant variables (wound healing, lean mass, and fat mass). For these latter three variables we subsequently used only the normoferremic subset of animals (see Methods) in assessing group differences in order to exclude the possible confounding effects of iron metabolism.

Nephrilin administration reduces post-burn insulin resistance

Glycemic control is dysregulated in burn trauma [3, 4]. We assessed glucose area under the curve over a two-hour period following bolus injection of glucose. In addition, we measured glycemic control in treated animals by measur-

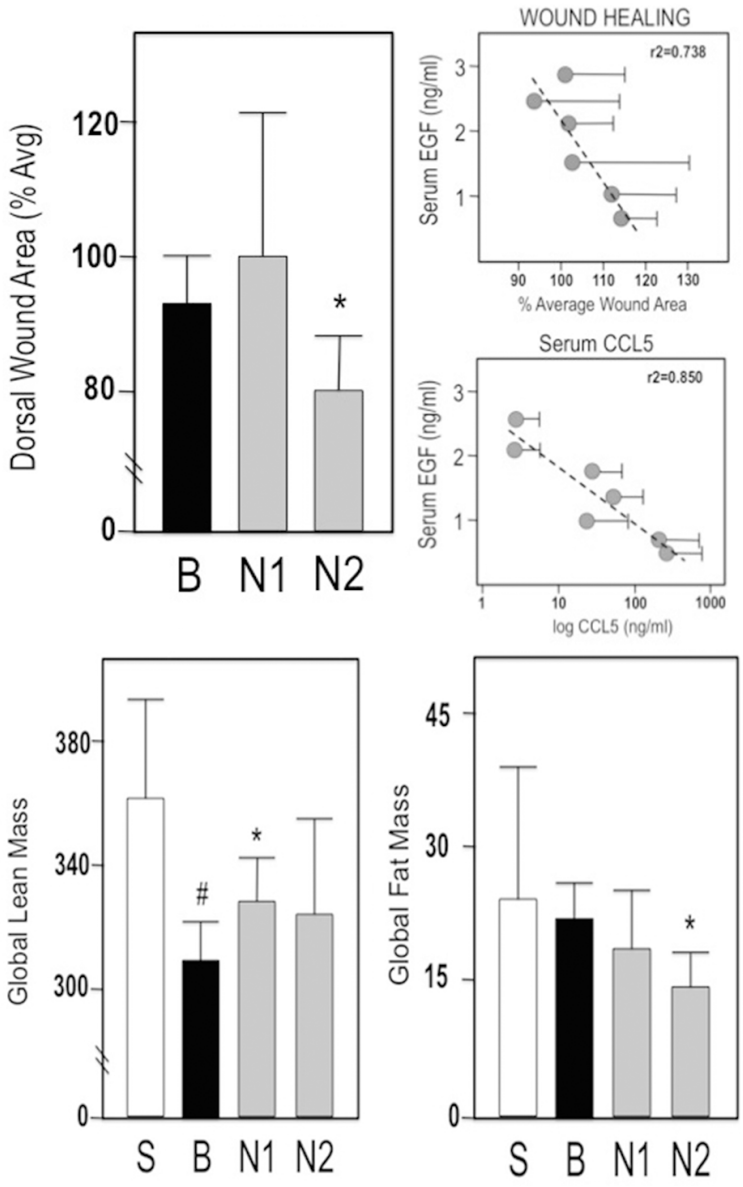

Figure 3. Top left: wound area as percent of average area for entire cohort (note that sham group has no wound). Top right: serum markers EGF versus wound area or CCL5 in all animals. Bottom panels: DEXA data. #p<0.05 relative to S (sham) group. *p<0.05 relative to B (burn + saline) group.

ing serum insulin levels. The results are shown in **Figure 2** (left panel). Glucose and insulin are significantly elevated in burned animals. This elevation is significantly reduced by early (N1) treatment with nephrilin, but not by late (N2) treatment.

Nephrilin administration reduces post-burn renal dysfunction

Renal dysfunction has been documented in trauma [6]. We measured serum creatinine and calculated eGFR in the treatment groups (**Figure 2**, right panel). Serum creatinine is significantly elevated, and eGFR depressed, in burn trauma. These perturbations are normalized by early (N1), but not late (N2) treatment with nephrilin.

Improvements in post-burn body composition and wound healing result from administration of nephrilin

Loss of lean mass in burns is proportional to glycemic dysregulation [4]. **Figure 3** shows the results obtained in the normoferremic subset of treated animals. Early treatment with nephrilin (N1) significantly ameliorated the loss of lean body mass (bottom left panel). Conversely, late treatment (N2) significantly improved wound healing (top left) and reduced fat mass (bottom right). Fat storage is modified in burn patients, with reduction in peripheral fat mass and increases in central fat mass frequently reported [5]. The top right panels show the negative linear relationships between serum EGF and wound area (r^2=0.738) on the one hand, and with serum CCL5 (r^2= 0.85) on the other. EGF and CCL5 have been associated with platelet counts [17]. EGF may mediate wound healing while CCL5 may play a part in the newly discovered role of platelets in inflammation [18].

Discussion

In this small, preliminary study, we elucidated the pleotropic effects of nephrilin peptide in a rat burn model. The results of this study demonstrate the importance of two overarching variables: iron status and timing of nephrilin administration. An initial global evaluation of outcomes based on iron status revealed that

some, but not all, of the clinically relevant effects of nephrilin in this model were significantly dependent on iron status. As a result, we restricted the analysis of those effects to the normoferremic subset of animals in the study.

We examined the timing of the administration of nephrilin, but these results need to be confirmed in larger studies as the differences between the treatment groups may be solely due to the small number of animals per group. Early (post-burn week one, N1) treatment with nephrilin significantly reverses the effects of burn trauma on glycemic control, renal function, and loss of lean body mass. Late treatment (post-burn week two, N2) with nephrilin has significant and positive effects on wound healing and fat mass. Taken together, these observations may have relevance to the temporal disruption of clinical variables in the morbidity and mortality associated with burn trauma [1].

Coupled with earlier observations on the effects of nephrilin on neuro-inflammation in this rat model and sepsis in a murine model [7], the pleotropic efficacy of nephrilin peptide on some of the most significant negative consequences of burn trauma suggests an effect of this peptide on the underlying mechanism by which burn trauma produces its pleotropic sequelae. As the biochemical nature of this mechanism has never been fully elucidated, nephrilin offers a tool for studying this question further. In work to be published elsewhere we have made a detailed study of possible mechanisms, with a focus on oxidative metabolism (Mascarenhas D, unpublished). In this context it is worthy of mention here that the previous study showed elevations in inflammatory markers IL-6 and TNF-alpha in plasma at early times post-burn (first 24 hours). Treatment with nephrilin suppressed those elevations. That earlier observation is consistent with the effects of early (N1) treatment observed in the current study.

Our observations suggest a possible dual role for platelets in the wound-healing process in burns. Strong correlations between the anabolic growth factor EGF and the rate of wound healing, on the one hand, and between EGF and CCL5, on the other, suggest the possibility of a push-pull role for platelets in this process. Platelets secrete both EGF and CCL5, the former a growth factor, the latter a pro-inflammatory chemokine [17]. Platelets are known to express and release elements that promote tissue repair and promote wound healing. The role of platelets as regulators of inflammation has only recently been more fully appreciated [18].

The effect of serum iron on the efficacy of nephrilin in this model is intriguing. At face value, the fact that the metal-binding domain (MBD) in the nephrilin sequence binds both ferrous and ferric iron [12], coupled with the fact that cross-linking studies in live cells [14] have implicated uptake of the MBD with transferrin receptor, as well as integrin beta-3, a component of the ferric ion uptake system, these present observations suggest the possibility of exploring the administration of nephrilin as a peptide:iron complex in future studies.

The effect of timing of administration on the efficacy of nephrilin in burn trauma also raises some obvious questions for future study. Is a seven-day treatment beginning immediately post burn necessary to achieve the observed early effects in this model, or will a shorter treatment window suffice? Does the time of initiation of therapy impact outcomes? Will effective treatment of burns with nephrilin optimally involve a regimen of separate early and late windows of treatment? We intend to address these questions in future experiments.

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Disclosure of conflict of interest

None.

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