Transcriptional re-programming in rat central nervous system two weeks after burn trauma: the impact of nephrilin treatment on the expression of oxidative stress-related genes

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Abstract

Introduction: Survivors of severe burns suffer lifetime neuroinflammatory consequences manifested by higher incidence of major depression and neurodegenerative disease. In a scald model, nephrilin peptide has previously been shown to protect rats from loss of lean body mass, kidney function and glycaemic control, complications that have also been shown to endure in burn patient populations. Nephrilin’s mechanism of action has been suggested to involve protection from excessive oxidative stress.

Methods: Using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) amplification of transcripts in total RNA extracted from dorsal root ganglia of male rats 14 days after exposure to thermal insult, we query the relative levels of expression of 34 genes believed to be associated with oxidative stress biology in the central nervous system (CNS). We use these data to explore the central role of oxidative stress in astrogliosis, immunosuppression and mitochondrial homeostasis.

Results and Discussion: Rats that received nephrilin treatment (4 mg/kg by subcutaneous bolus injection once daily for seven days after scald injury) showed significantly reduced elevations in gene expression of some key genes such as NOX2, GFAP, AQP4 and RAC1, but not of others such as NOX4, STEAP4, ARG1 and CCL2.

Conclusion: The implications of these data with reference to nephrilin’s potential clinical utility for mitigating the enduring effects of burn trauma on the CNS are discussed. Nephrilin reduces the expression of some genes implicated in neurodegeneration after burn insult.

Lay Summary

Nephrilin peptide is a novel treatment for short- and long-term systemic effects of burn trauma. This study measures the capability of nephrilin to address post-traumatic neurodegenerative disease by looking at the expression of genes in the central nervous system, in a rat scald model. Nephrilin appears to have beneficial effects by reducing the expression of some key genes known to be relevant in neurodegenerative processes, but not others.

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Introduction

Nephrilin peptide is an inhibitor of Rictor complex that is actively transported into cells in vivo.\(^1\) Nephrilin peptide has been used to reverse the systemic effects of traumatic, metabolic and xenobiotic stress in a number of rodent models.\(^2\) In a well-characterised rat scald model, we previously demonstrated the pleotropic effects of nephrilin peptide in combating post-burn systemic neuroinflammation, loss of glycaemic control, lean body mass and kidney function, and impaired wound healing. These short-term effects mirror more enduring consequences of thermal injury in survivors of severe burns.\(^3,4\) Burn injury has been implicated in the development of long-term neurodegenerative conditions such as severe depression.\(^5,6\) In this study, we set out to look at the role of oxidative stress in nephrilin peptide’s protective mechanisms post-burn in the central nervous system (CNS) of rats. Nephrilin’s mechanism of action has previously been shown to involve protection from excessive oxidative stress in kidney.\(^7\)

In this study, using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) amplification of transcripts in total RNA extracted from dorsal root ganglia (DRG) of male rats 14 days after exposure to thermal insult, we query the relative levels of expression of 34 genes believed to be associated with oxidative stress biology in the CNS, with an emphasis on astrogliosis, immunosuppression and mitochondrial homeostasis. These phenomena appear to be inextricably linked to one another, and to CNS oxidative stress post-traumatic insult in a variety of contexts.\(^8,9\) Astrocytes are the most abundant cell type in the CNS. Activation of astrocytes and microglia is a key early step in the propagation of the defensive immune response from the CNS after insult.\(^8\) Upon traumatic injury, activated astrocytes participate in the protection of neural cells from excessive oxidative stress, but astrogliosis can proceed to pathology.\(^9\) Furthermore, immunosuppression mediated by myeloid-derived suppressor cells (MDSC)—a major complication of traumatic insult—is enhanced by oxidative stress.\(^10\) The context in which all these related dysfunctions occur is, in turn, believed to be inextricably linked to mitochondrial homeostasis.\(^11\) In this study, I analysed the expression of 34 genes selected based on their implication in the above processes, as described in the scientific literature.\(^8,9\) The analysis was performed with qRT-PCR on RNA templates extracted from DRG of male rats 14 days after exposure to thermal insult.

Materials and methods

Nephrilin peptide

Nephrilin peptide, a 40-mer peptide carrying a sequence ‘derived from PRR5/Protor’ (the sequence is conserved in human, rat and mouse species) was synthesised by Lifetein LLC (Hillsborough, NJ, USA) and purified to > 80% purity by HPLC. The design and synthesis of nephrilin have been previously described.\(^1\)

Dissection of dorsal root ganglia and extraction of RNA

In a subset analysis of a previously reported burn study,\(^4\) DRG were dissected from male adult Sprague Dawley rats (250–300 gm, Charles River Laboratories, Wilmington, MA, USA) using a procedure previously reported.\(^3\) As tissue was not available from all animals, DRG from three randomly selected animals in each treatment group (sham, burn + vehicle, burn + nephrilin 4 mg/kg/day) were pooled for further analysis by qRT-PCR. Experimental outcomes from the original study\(^4\) were re-calculated for this subset and are shown in Table 1. Total RNA was extracted from each pool using the RNeasy Midi Kit (Qiagen, Germantown, MD, USA). Yield was ~30 ug RNA per pool and A260/A280 ratio was in the range of 1.87–2.04 in all cases. The high quality of each RNA was further confirmed by electrophoresis (using Eukaryote Total RNA Nano).

Quantitative reverse transcriptase polymerase chain reaction

RNAs were diluted in RNase/DNase free water and aliquoted into wells in triplicate. Approximately 100 ng of RNA was used per well. A one-step qPCR method was performed using Luna Universal One-Step RT-qPCR kit (New England Biolabs, Ipswich, MA, USA) containing reverse
transcriptase enzyme mix. Primer pairs for each gene were synthesised for the SYBR assay. Primer sequences are listed in Table 2. The following standard qPCR cycling conditions were used: 55 °C for 10’ (for RT), 95 °C for 1’ followed by 40 cycles at 95 °C for 10 s, 58 °C for 30 s. Background was set at 3–10 cycles and the threshold was set at 0.02 for all runs. Ct values were collected and analysed using the ‘delta-delta Ct’ method. All samples amplified well within acceptable Ct range. Expression of each gene was normalised to GAPDH, a house-keeping gene. Comparisons between treatment groups in each case were done by setting the sham group value to 1.

**Statistical analysis**

Data are presented as mean ± SD unless otherwise indicated. Probability values (P values) were computed using Student’s t-test and expressed as relative values to sham or saline-treated group.

**Results**

**Astrocyte activation**

After CNS injury, formation of an astrocytic scar adjacent to the ‘lesion’ is a characteristic histopathologic feature that can be demonstrated by immunohistochemistry with primary antibodies against glial fibrillary acidic protein (GFAP) or connective tissue growth factor (CTGF).9,12–14 Aquaporin-4 (AQP4), a membrane-bound protein that regulates water permeability is expressed in the endfeet of astrocytes in the CNS. Recently, AQP4 has been extensively examined for its role as a neuroimmunological inducer.15 After insult, circulating monocytes and lymphocytes are known to enter the CNS. Peptidase Pi16 is a master regulator of T-cell subsets, a key function in the adaptive immune response in all tissues.16

Table 1. Clinically relevant variables in the three treatment groups of the study.

|Variable| Sham| Burn + vehicle| Burn + nephrilin|
|---|---|---|---|
|Lean body mass (DEXA)| 343.6 ± 15.7*| 304.9 ± 9.2| 328.5 ± 5.1*|
|Glycaemic control (GTT AUC mg.dL.h)| 44.7 ± 19.0†| 117.0 ± 19.1| 69.0 ± 18.1*|
|Kidney function (eGFR, calculated)| 1.21 ± 0.23*| 0.58 ± 0.18| 1.29 ± 0.32*|
|Urinary 8-isoprostone (ng/pg cystatin)| 5.02 ± 2.91†| 26.44 ± 1.60| 4.53 ± 1.21†|

* p<0.05, † p<0.01 versus Burn+Vehicle group

DEXA, dual-energy X-ray absorptiometry; eGFR, estimated glomerular filtration rate; GTT AUC, glucose tolerance test, area under the curve.

Figure 1a shows the results obtained by RT-PCR of GFAP, CTGF, AQP4 and Pi16 genes using RNA extracted from CNS of scalded rats (with or without nephrilin treatment), with sham-treated rats as a control. The results show that burn injury causes significant increase in the expression of all four genes; nephrilin treatment significantly reduces those elevations.

**MDSC-mediated immunosuppression**

The co-induction of chronic low-grade inflammation and MDSC-mediated immunosuppression is a hallmark of post-traumatic responses, aging and neurodegenerative disease.17–19 MDSC-mediated immunosuppression is dependent upon oxidative stress10 and is brought about by the interplay of a number of genes. NADPH oxidase 2 (NOX2) and neuropeptide Y (NPY) are both key players in this interplay and in the effects of oxidative stress in the CNS.20–22 S100A9 production is dependent upon reactive oxygen species (ROS) and is specifically exported by exosomes produced by MDSC in the inflammatory environment,23,24 while galanin (GAL) protects rat astrocytes from oxidative stress.25

Figure 1b shows the results obtained by RT-PCR of GAL, NPY and S100A9 expression in the CNS of scalded rats (with or without nephrilin treatment) and sham-treated rats. The results indicate that burn insult causes significant increase in the expression of all three genes, and nephrilin treatment significantly reduces those elevations.

Surprisingly, Figure 1c shows no change in the expression of NRF2, a master regulator of the response to oxidative stress in other environments,26 but significant elevation of transcript levels for SLC7A11, ARG1, CD63 and CCL2. However, nephrilin treatment did not affect the elevation of these transcripts. SLC7A11, which codes for the...
Table 2. Genes and primers used in the study.

| RefSeqID     | Description                                                                 | Gene Symbol | Amplicon bp | Primer sequence         |
|--------------|-----------------------------------------------------------------------------|-------------|-------------|-------------------------|
| NM_134366    | Ras-related C3 botulinum toxin substrate 1                                 | RAC1        | 111 bp      | Fwd: TGCCTGCTCATCAGTTACACG  
                              |                                             |             |                         | Rev: GCCCAGATTCACTGGTTTTCCA |
| NM_023965    | NADPH oxidase 2                                                             | NOX2        | 121 bp      | Fwd: TCTTGTTCATTCTGTTGTGTTGG  
                              |                                             |             |                         | Rev: AGAGCCAGTGCTGACCACA |
| NM_017316    | Solute carrier family 23 (nucleobase transporters), member 2                | SLC23A2     | 136 bp      | Fwd: TCCCGGTGATCATCAATGGT  
                              |                                             |             |                         | Rev: CAGTGCTGCGGAGTTCTCT |
| NM_057194    | Phospholipid scramblase 1                                                  | PLSCR1      | 199 bp      | Fwd: CTCTTGGAGATACCGTTCTGA  
                              |                                             |             |                         | Rev: TGCATCTCAGGGGTCTCTCA |
| NM_031530    | Chemokine (C-C motif) ligand 2                                              | CCL2        | 152 bp      | Fwd: GCCAATCTCACTGGAGCCAG  
                              |                                             |             |                         | Rev: TGAATAGCAGGAGGTGAGTG |
| NM_134372    | Aminocarboxymuconate semialdehyde decarboxylase                            | ACMSD       | 118 bp      | Fwd: AGCAAGGCAAGGAGAAGCA  
                              |                                             |             |                         | Rev: ACTGTCACTCTTCTGTTCTT |
| NM_017073    | Glutamate-ammonia ligase (glutamine synthetase)                            | GLUL        | 153 bp      | Fwd: CATGTATATCTGGTGTAGTACC  
                              |                                             |             |                         | Rev: GGAGGTACATGCTGTTTG |
| NM_031347    | Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha      | PPARGC1A    | 151 bp      | Fwd: GTCTCCTTTTGAACGCTTCTG  
                              |                                             |             |                         | Rev: ATTTCTCATGAGTTCCTT |
| NM_001105919| Fission 1 (mitochondrial outer membrane) homolog (S. cerevisiae)           | FIS1        | 94 bp       | Fwd: GAGGAGCTGTTGCCAAAGG  
                              |                                             |             |                         | Rev: CTTTTCATATATTGAGCCGT |
| NM_130894    | Mitofusin 2                                                                 | MFN2        | 193 bp      | Fwd: TGGGGGCTCATCAAGCAGAG  
                              |                                             |             |                         | Rev: CTCTCCCATTGCCTGCC |
| NM_001106694| PTEN induced putative kinase 1                                              | PINK1       | 149 bp      | Fwd: CCTGTCAAGGAGATCCAGGCAA  
                              |                                             |             |                         | Rev: GGCTTCATACACAGCGGCA |
| NM_053517    | SHC (Src homology 2 domain containing) transforming protein 1              | SHC1        | 135 bp      | Fwd: GACTCAGGTCACAGGGAGG  
                              |                                             |             |                         | Rev: CCAGCAGTTCACTCGTACTCT |
| NM_031326    | Transcription factor A, mitochondrial                                       | TFAM        | 211 bp      | Fwd: AGAAACCTATGAGCTCATACCGATT  
                              |                                             |             |                         | Rev: AGCGTCTCTTTATACGCTCAG |
| NM_001044265| STEAP family member 4                                                      | STEAP4      | 119 bp      | Fwd: CTGGGGCTCTCCAGTCAGGAA  
                              |                                             |             |                         | Rev: CCAGTGGAGTGGCCAAAGA |
| NM_019354    | Uncoupling protein 2 (mitochondrial, proton carrier)                        | UCP2        | 165 bp      | Fwd: CAGAGCACTGCTGAGGCGGCTAC  
                              |                                             |             |                         | Rev: TGCATGAGGTTGCTTCTGAG |

(Continued)
| RefSeqID      | Description                                    | Gene Symbol | Amplicon bp | Primer sequence      |
|--------------|-----------------------------------------------|-------------|-------------|----------------------|
| NM_017312    | BCL2-related ovarian killer                   | BOK         | 151         | Fwd: CGCTTGGGAGATGAGCTGGA  
                          |                  |             | Rev: TGCCCCCATGTGATACCTGCT  |
| NM_001142366 | Aquaporin 4                                   | AQP4        | 178         | Fwd: CACACCGGTTCATGGAACCTC  
                          |                  |             | Rev: ATTGAATTGCCAAMAATGTCACATAC  |
| NM_019204    | Beta-site APP cleaving enzyme 1               | BACE1       | 222         | Fwd: GGAGATGTGGAACACCTGAGG  
                          |                  |             | Rev: CCCCAGTTGAGGGCAGTAC  |
| NM_017125    | Cd63 molecule                                 | CD63        | 97          | Fwd: GTCTCATGATTACATTGCAACACATC  
                          |                  |             | Rev: GACTTCACCTGTCTCTAAACATAC  |
| NM_013064    | Hypocretin (orexin) receptor 1                | HCRTR1      | 231         | Fwd: TYCTCATAGCCTTGGTGGAAC  
                          |                  |             | Rev: CTGCCACTGACACCAACAC  |
| NM_134363    | Solute carrier family 12 member 5             | SLC12A5     | 204         | Fwd: CCTGTGAGGGAGAGATGGAAC  
                          |                  |             | Rev: ACACAAAAAGATTCTGCAAGC  |
| NM_031798    | Solute carrier family 12 member 2             | SLC12A2     | 132         | Fwd: CGATGAGCCTGGAAAGACCT  
                          |                  |             | Rev: ACACCGTCTGGTTGCAAC  |
| NM_031789    | Nuclear factor, erythroid 2-like 2            | NRF2        | 116         | Fwd: CTACTCCAGGTGTGGCCACCA  
                          |                  |             | Rev: TATCACGGGCAACGACTCA  |
| NM_031588    | Neuregulin 1                                  | NRG1        | 154         | Fwd: ACTGGGACCAGCCATCTCAT  
                          |                  |             | Rev: CGTATGGGGCCTGGGACTCA  |
| NM_001197332 | Oxidation resistance 1                       | OXR1        | 170         | Fwd: ACCCAATGACCTTACTGCCC  
                          |                  |             | Rev: CACCACATGCACCCGGAGTGT  |
| NM_001170481 | Peptidase inhibitor 16                       | Pi16        | 111         | Fwd: ACTACACTCAGGTAGTGAGGAGCA  
                          |                  |             | Rev: AGTTCACACCGGAGAGATG  |
| NM_001107673 | Solute carrier family 7 (cationic amino acid  | SLC7A11     | 137         | Fwd: CTGGAGTTATACAGCTAAATGGG  
                          | transporter), member 11 |             | Rev: GTGAGGTAACCCACCGCAGCA  |
| NM_053524    | NADPH oxidase 4                               | NOX4        | 110         | Fwd: GGATACAGAAGGTCCCTAGCA  
                          |                  |             | Rev: GCTACATGCACACCTGAGGAATATAC  |
| NM_022266    | Connective tissue growth factor               | CTGF        | 138         | Fwd: CAAGCAGCTGGGAGAAGCTG  
                          |                  |             | Rev: CCACGGAAGACACAGGAGT  |
| NM_053587    | S100 calcium binding protein A9               | S100A9      | 136         | Fwd: TGAGCATTGAGACGCCTGGA  
                          |                  |             | Rev: GTCTGTTCGAGGTGCTCAAGGTC  |
| NM_017009    | Glial fibrillary acidic protein               | GFAP        | 150         | Fwd: CCTAGAGGACAAAGCTCAAG  
                          |                  |             | Rev: AAGACTGGATCTCTCCCTCCAC  |

(Continued)
cystine/glutamate antiporter, modulates T-cell activation by depleting local cysteine.\textsuperscript{27,28} ARG1, an archetypal marker of immunosuppression, helps MDSCs deplete local arginine,\textsuperscript{29} while CD63 is a marker for exosome production (see SI00A9, above). C-C motif chemokine ligand 2 (CCL2) is a powerful recruiter of MDSCs.\textsuperscript{30}

**Mitochondrial homeostasis**

Dysfunction in genes key to mitochondrial homeostasis, such as TFAM,\textsuperscript{31} UCP2,\textsuperscript{32} NOX2 and NOX4,\textsuperscript{33} HCRTR1,\textsuperscript{34} OXR1,\textsuperscript{35} BOK\textsuperscript{36} and RAC1\textsuperscript{37} result in profound disruption of mitochondrial dynamics, oxidative stress and, in the context of the CNS, severe gliosis. Figure 1d shows the results obtained by RT-PCR of TFAM, UCP2, BOK, RAC1, NOX2, NOX4, OXR1 and HCRTR1 genes. The results demonstrate that burn injury causes significant increases in the expression of all genes except OXR1, which remained unchanged, and nephrilin treatment significantly reduces transcript elevations for all remaining genes except NOX4.

Several additional genes induced by burn injury and known to play key roles in mitochondrial biogenesis, function, mitophagy, fission and fusion were not affected by nephrilin treatment (Figure 1c): STEAP4,\textsuperscript{38} SHC1,\textsuperscript{39} GLUL,\textsuperscript{40} SLC23A2,\textsuperscript{41} PINK1\textsuperscript{42} and MFN2.\textsuperscript{43} Expression of ACMSD,\textsuperscript{44} PPARGC1A\textsuperscript{45} and FIS1\textsuperscript{46} genes was not changed by burn injury or nephrilin treatment.

**PLSCR1 and BACE1**

PLSCR1 and BACE1 are physically and functionally associated products in the CNS. BACE-1 is associated with elevated oxidative-stress and CNS pathologies such as Alzheimer’s.\textsuperscript{37,48} Figure 1G shows that PLSCR1 and BACE1 are similarly elevated by burn injury. Nephrilin treatment does not seem to affect these levels.

Another case of possible downstream regulations was examined by measuring transcripts for potassium/chloride channel carriers SLC12A5 and SLC12A2. Both gene products are coordinately upregulated by OSR1 kinase, which responds to oxidative stress.\textsuperscript{49} The results in Figure 1g show that burn injury causes significant increase in the expression of these two genes, but nephrilin treatment does not have a significant effect on the levels of these elevated transcripts.

**Discussion**

In rodent models of stress, nephrilin peptide has been shown to reverse elevations in neuroimmune and oxidative stress consequent to thermal, metabolic and xenobiotic insult.\textsuperscript{1–4,7} In particular, nephrilin’s efficacy in reversing the systemic effects of sepsis and burn trauma, including inflammation, catabolism and loss of kidney function\textsuperscript{5,4} suggests a mechanistic link to the body’s immunological response to stress challenge.

In this study, the focus was on the effects of burn injury on gene expression in the CNS, with an emphasis on the inter-related phenomena of astrogliosis, oxidative stress, immunosuppression and mitochondrial homeostasis. In each category, a number of gene transcripts were carefully quantified using qRT-PCR. In general, in each category, nephrilin peptide reverses burn-induced effects of oxidative stress.
Figure 1. Relative gene expression in rat CNS 14 days after scald injury was determined by qRT-PCR as described in the ‘Material and methods’ section. Sham values (white bars) are set to $= 1$. Black bars are scald + vehicle, grey bars are scald + nephrilin. *$P < 0.05$, **$P < 0.01$ versus scald + vehicle group. CNS, central nervous system; qRT-PCR, quantitative reverse transcription polymerase chain reaction.
transcript elevation for some key genes but not others. Additional studies will be needed to uncover the underlying mechanisms that may explain this observed dichotomy.

Based on these data, it does seem clear that nephrilin treatment might be expected to ameliorate some of the long-term consequences of burn trauma on the CNS. Whether the pathways affected by nephrilin are instrumental in changing quality-of-life outcomes, especially those relating to depression and dementia, remains to be established.

Some genes whose transcripts were elevated by burn injury in this study have been shown to cluster into well-characterised regulons. The TLR4/MyD88 regulon is known to regulate production of ROS, immunosuppression and chronic inflammation in response to lipopolysaccharide challenge via NOX4, ARG1 and CCL2,39–52 three genes whose burn trauma-induced elevations were not reversed by nephrilin in the current study. One possible line of investigation suggested by this result is co-treatment of thermal insult with both nephrilin and a modifier of TLR4 signalling such as fenofibric acid.53 Co-treatment concepts may also include the use of valproic acid, which not only downregulates TLR4-mediated inflammation54 but also downregulates gliosis55 and the immunosuppressive function of MDSCs.54 The use of anti-epileptic agents such as valproic acid as co-treatments with nephrilin makes additional sense, given the known link between trauma and seizure.57

In conclusion, these results show the efficacy of nephrilin treatment in mitigating the transcriptional effects of burn trauma in the CNS, but also raise intriguing questions for future study. Is it possible to modify the treatment regimen to include additional agents that address the areas that nephrilin cannot? Can this peptide be coupled to other moieties to further improve effective treatment of burn trauma? We intend to address these questions in future experiments.

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