Pro-inflammatory TNF and HMGB-1 Increase During Diabetic Neuropathy: The Effect of Decreasing Levels of TNF in the Brain on HMGB-1 Production

Nicole Coloney[1], Ashley Re[2], Barbara Mullan[3], Bruce Davidson[2,3], Tracey A. Ignatowski[2,6]

Depts. of [1]Biomedical Sciences, [2]Pathology & Anatomical Sciences, [3]Anesthesiology, [4]Neuroscience Program
University at Buffalo-SUNY, School of Medicine & Biomedical Sciences, Buffalo, NY 14214

Abstract

Neuropathic pain (NP) is a form of chronic pain that results in damage to the nervous system. Diabetic neuropathy is a debilitating complication of diabetes mellitus and is the most frequent form of neuropathic pain encountered in the developed world. Damaged neurons release an inflammatory protein termed high-mobility group box chromosomal protein 1 (HMGB-1). HMGB-1 is a nuclear protein; however, when released from cells it has pro-inflammatory cytokine-like activity. An increase in the pro-inflammatory cytokine tumor necrosis factor-α (TNF) underlies nerve dysfunction and death, and development of NP. HMGB-1 released from damaged neurons stimulates production of TNF. While diabetes is associated with elevated HMGB-1 levels, whether this contributes to diabetic neuropathy is unknown. TNF-silencing nanoplexes are effective for relieving diabetic NP. These nanoplexes knocked-down TNF levels in the brain and decreased pain. We hypothesize that the TNF nanoplexes will also indirectly reduce HMGB-1 levels, contributing to the reduction in diabetic NP.

Background

- Tumor necrosis factor-α (TNF), when administered i.p. to rats, increases the perception of pain [1].
- Streptozotocin (STZ)-injections induce diabetic neuropathy [2].
- TNF levels were increased in the hippocampus during diabetic neuropathy [3].
- Increased levels of TNF in the brain and enhanced TNF production by macrophages are associated with pain development [2].
- HMGB-1 levels in serum increase during diabetic neuropathy [4].
- Enzyme-linked immunosorbent assay (ELISA) detects HMGB-1 concentrations [4].
- Gold nanoparticles (GNRs) complexed with small inhibitory ribonucleic acid (siRNA) is termed nanoplexes [5].
- Nanoplexes protect siRNA from degradation [5].

Previous Methods

Male Sprague-Dawley rats, 175-250 grams, are administered a single intraperitoneal injection (45 mg/kg) of streptozotocin (STZ).

Behavioral testing for pain (mechanical allodynia) for 3 days prior to (baseline) and once per week after STZ injection for 60 days.

Rat weights are monitored every other day; blood glucose readings are performed prior to, on day 4 post-STZ, and once a week thereafter for the duration of the study.

Upon sacrifice: blood is collected, and brain regions are harvested, processed, and stored until assayed for TNF; levels of TNF are assessed via WEHI bioassay (bioactive protein).

Peritoneal macrophages are harvested by lavage and plated in multi-well Lab-Teks for short-term in vitro experiments: assess the effect of lipopolysaccharide (LPS; 30 ng/ml)-induced TNF production.

Interrelationship of Topics Under Study

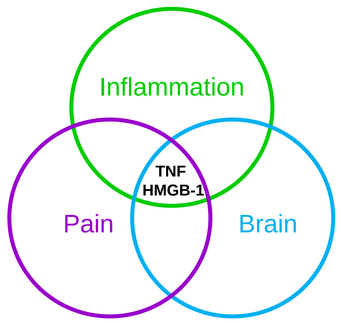
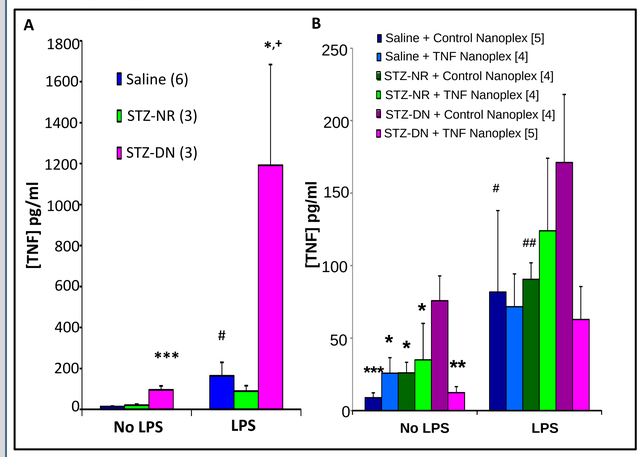


Figure 1. The relationship between chronic pain and inflammation, while complex, shares similar pathologic changes, including changes in the brain. An increase in the pro-inflammatory cytokines and neural mediators TNFα and HMGB-1 are common to both disorders.

Supporting Data

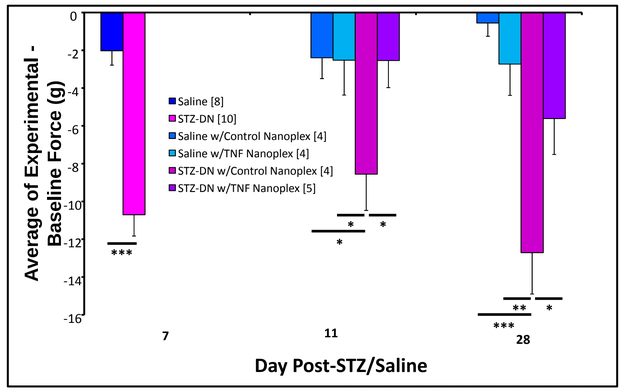
Macrophage TNF Production



A. Re, Master's Thesis, University at Buffalo, 2013

Figure 2. A) Effect of lipopolysaccharide (LPS, 30 ng/ml) stimulation of peritoneal macrophages on TNF production at day-61 post-STZ/saline injection (i.p., 45 mg/kg). Bars= mean ± S.E.M. (number of rats in parentheses.) No LPS groups: Statistical analysis was by ANOVA followed by SNK: *** p < 0.001, compared to Saline and STZ-NR No LPS groups. LPS groups: Statistical analysis was by ANOVA followed by Dunn's Method: * p < 0.05, compared to the STZ-NR LPS group. **B) Peritoneal macrophage production of TNF with and without LPS (30 ng/ml) stimulation.** Macrophage from rats on day-28 post-STZ (45 mg/kg, i.p.) or saline (vehicle control); rats also received bilateral intra-hippocampal injection of either control nanoplexes (GNR-scrambled siRNA, 0.2 nmol/3 µl) or TNF nanoplexes (GNR-TNF siRNA, 0.2 nmol/3 µl) on day-7 post-STZ/saline. Bars= mean ± S.E.M. (number of rats in brackets.) No LPS groups: Statistical analysis was by ANOVA followed by Fisher LSD: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared to 'STZ-DN + Control Nanoplex' No LPS groups. LPS groups: Statistical analysis by ANOVA, NS.

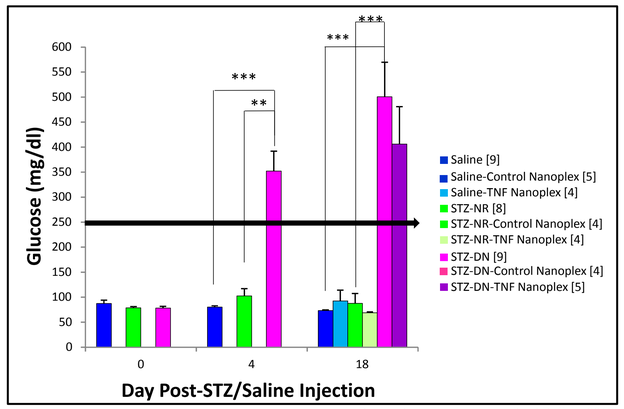
Mechanical Allodynia



A. Re, Master's Thesis, University at Buffalo, 2013

Figure 3. Alleviation of STZ-induced mechanical allodynia. Bars= mean ± S.E.M. values for the differences of baseline (average of 3 recordings taken 2 days prior to and on day of, but prior to, the injection) withdrawal force from experimental withdrawal force at the indicated days post-injection (number of rats indicated in brackets). Statistical significance was by One-way ANOVA followed by Fisher LSD Method for multiple comparisons: * p < 0.05, ** p < 0.01, *** p < 0.001.

Effect of Nanoplexes on Blood Glucose Levels



A. Re, Master's Thesis, University at Buffalo, 2013

Figure 4. Average rat blood glucose levels in rats receiving control or TNF nanoplex. Values >250 mg/dl are indicative of hyperglycemia in this model. Bars= mean ± S.E.M. of three separate experiments with the number of rats indicated in brackets. Statistical analysis of the control nanoplex administered rat groups was by One Way ANOVA followed by Tukey Test for multiple comparisons: *** p < 0.001, ** p < 0.01. STZ-NR = STZ-injected non-responder group; STZ-DN = STZ-induced diabetic neuropathy group.

Cellular Mechanism

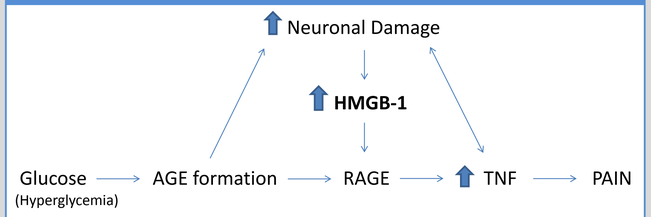


Figure 5. Schematic of cellular mechanisms involved in the development of diabetic neuropathy.

Purpose

Determine whether HMGB-1 levels change (i.e., decrease) in samples from diabetic neuropathy rats that received treatment for their neuropathy/pain.

Methods

Preparing the Standard Curve

A standard curve is run to compare known concentrations of HMGB-1 protein to samples (serum and macrophage) containing an unknown amount of HMGB-1 protein. Supernatants from cultured macrophages are compared to a PBS standard curve, whereas the serum samples are run with a spiked standard curve. Serum samples have a higher level of background noise due to the presence of other proteins and immunoglobulins. All samples are from rats; therefore rat serum is used to spike the curve. Initial optimization experiments determined that the curve is run with 1% rat serum with PBS.

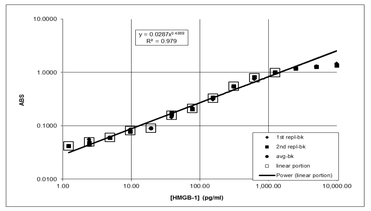
ELISA Procedure

Day 1

1. Prepare all reagents, samples and standards.
2. Add 50µL of standards to wells of a 96-well Maxisorp plate.
3. Seal plate with adhesive cover and incubate on shaker at room temperature (RT) for 3 hours.
4. Aspirate solution and wash all wells 2X using plate washer.
5. Add 150µL of Superblock blocker to all wells, seal and incubate at RT for 1 hour on shaker. Wash 2X as in step 4.
6. Add 50µL of the Primary Antibody to wells.
7. Seal plate, incubate at RT for 20 hours on shaker.

Day 2

1. Aspirate solution and wash all wells 2X using plate washer.
2. Add 50µL of HRP-conjugated Antibody Solution to all wells, seal and incubate at RT for 1 hour on shaker. Wash 3X as in step 1.
3. Add 100µL of TMB solution to each well. Do not seal plate.
4. Incubate 20-40 minutes in the dark.
5. Add 100µL of Stop Solution to each well.
6. Read absorbance at 465nm (with 590nm correction) within 30 minutes of adding the stop solution. Calculate the standard curve.



Future Outlook

- Continue optimizing standard curves for macrophage and serum samples.
- Continue running macrophage and serum samples to increase n number.
- Run collected nanoplex samples to compare to TNFα data.
- Create a standard curve for homogenized tissue, and run samples to compare to current HMGB-1 data.

Results

Macrophage HMGB-1 Production

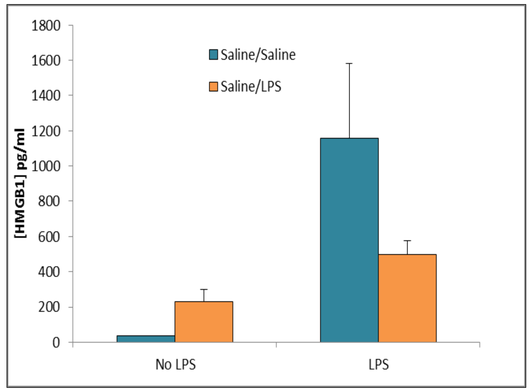


Figure 6. Effect of LPS stimulation of peritoneal macrophage on saline/saline vs saline/LPS injected rats on HMGB-1 production.

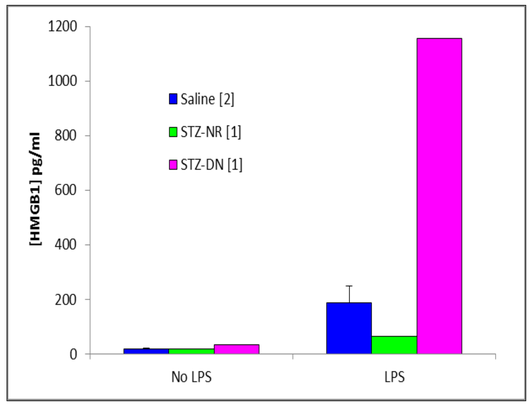


Figure 7. Effect of LPS stimulation of peritoneal macrophage on HMGB-1 production in saline and STZ injected rats.

Conclusions

- Peritoneal macrophage stimulated with LPS increases HMGB-1 production (Fig. 6).
- Peritoneal macrophages from STZ-DN rats are more pro-inflammatory than those from STZ-NR and saline control rats (Fig. 7).
- STZ-DN rats show increase HMGB-1 production when stimulated with LPS (Fig. 7).

*Due to low n numbers, statistical analysis not be performed.

Acknowledgements

- Research supported by Center for Undergraduate Research and Creative Activities.
- Special thanks to the members of the Ignatowski lab and Davidson lab.

References

[1] Watkins LR, Goehler LE, Relton J, Brewer MT, Maier SF. Mechanisms of tumor necrosis factor-alpha (TNF-alpha) hyperalgesia. Brain Res. 692: 244-250, 1995.

[2] Re A, Alnaji AR, Knight PR, Davidson B, Ignatowski TA. The pathogenesis of the chronic pain associated with STZ-induced diabetic neuropathy is associated with increased levels of tumor necrosis factor (TNF) in the brain. Upstate New York Pharmacology Society Second Annual Meeting: Frontiers in Neuropharmacology (ASPET), Buffalo, New York. The Pharmacologist 55(2): B-5 (A19), 2013.

[3] A. Re, Master's Thesis, University at Buffalo, 2013.

[4] Nin JW; Ferrerira I,Schalwijk CG; Prins MH; Chaturvedi N; Fuller JH; Stehouwer CD; EURODIAB Prospective Complications Study Group. European Journal of Endocrinology. 166 (2): 325-32, 2012 Feb.

[5] Bonoiu AC, Bergey EJ, Ding H, Hu R, Kumar R, Yong K-T, Prasad PN, Mahajan S, Picchione KE, Bhattacharjee A, Ignatowski TA. 2011. Gold nanorod-siRNA induces efficient in vivo gene silencing in the rat hippocampus. Nanomedicine 6(4):617-630.