Enhanced Nuclear Factor (NF)-κB Activation in Experienced Divers in Response to Acute Hyperbaric Stress

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Abstract

Background: Nuclear factor (NF)-κB is a master transcriptional switch in inflammation, immunity and apoptosis. Activated NF-κB migrates into the nucleus to regulate the expression of multiple target genes influencing many aspects of normal and disease physiology. The aim of this study was to investigate nuclear translocation of NF-κB within circulating leukocytes in subjects exposed to progressive levels of hyperbaria.

Materials and Methods: Naive subjects (N, *n*=9) and experienced divers (D, *n*=11) underwent 30-min hyperbaric exposures at 180 (18 msw; low stress); 300 (30 msw; moderate stress); or 450 kPa (45 msw; high stress)] separated by 1-week intervals. Peripheral blood samples were drawn pre-dive, 20 and 60-min post-dive. A whole blood flow cytometric assay was used to quantify spontaneous and *in vitro* LPS-stimulated NF-κB translocation in peripheral blood mononuclear cells (PBMC) and neutrophils (PMNs). Results are expressed as the median fluorescence intensity (MFI) of intranuclear NF-κB in each population.

Results: Resting spontaneous expression of NF-κB in PBMC and PMNs was similar between N and D. Following acute hyperbaric stress, D exhibited a significant ($p < .05$) time-dependent elevation (up to 60%) of intranuclear NF-κB expression (MFI) in both PBMC and PMNs at all depths; whereas, significant translocation occurred only after 45 m, 60-min post-dive in N. Subsequent LPS stimulation induced significantly greater increases in both pre-dive (20%) and 20-min post-dive (45%) levels of NF-κB translocation in D compared to N responses.

Conclusions: This study demonstrates enhanced nuclear translocation of NF-κB in humans following acute hyperbaric exposure that is upregulated in experienced divers following repetitive episodes of hyperbaric stress. These results suggest that modulation of NF-κB-dependent pathways may contribute to diving acclimatization and a reduced susceptibility to decompression sickness in individuals undergoing chronic hyperbaric stress.

Introduction

Cellular responses to external stimuli require rapid signal transduction, leading to activation of specific transcription factors that induce the expression of appropriate target genes. NF-κB is an ubiquitous transcription factor that is sequestered in the cytoplasm in an inactive form when bound to an inhibitory protein, IκB. Upon stimulation, NF-κB is released from its naturally occurring inhibitor and translocates into the nucleus, where it binds to DNA and ultimately initiates mRNA transcription (*Figure 1A*).

NF-κB is actually a collective term referring to a family of five transcriptional proteins of the Rel family: p65 (Rel-A), Rel-B, c-Rel, NF-κB1 (p105/p50), and NF-κB2 (p100/p52). The activated form of NF-κB is a heterodimer, which usually consists of two proteins, a 65-kD subunit (p65 or relA) and a 50-kD subunit (p50).

The "classical" NF-κB activation pathway is primarily involved in cell stress and inflammatory signaling. It is initiated by extracellular receptor binding (e.g., toll-like receptors) in response to a wide array of physiologic or pathologic stimuli, including cytokines, endotoxin, hypoxia, oxidative stress and changes in vascular shear stress. This complex signaling cascade involves, phosphorylation, proteolysis and redox changes in a variety of adaptor proteins (e.g., IRAK, TRAF, PKC), which converge on the IKK (inhibitor of NF-κB kinase) complex (*Figure 1A*).

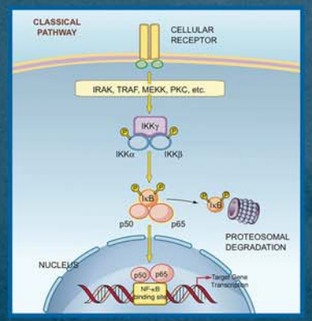

Figure 1A

Phosphorylation of IKKβ in turn induces phosphorylation of inhibitory IκB bound to NF-κB. This targets the IκB for proteasomal degradation and the dissociated p65–p50 dimer is free to translocate to the nucleus, where it binds to and activates κB motif-containing promoters. This in turn, leads to enhanced transcription of numerous NF-κB-responsive genes encoding multiple immune and inflammatory mediators (e.g., cytokines, chemokines, adhesion molecules), inducible enzymes (e.g., iNOS, COX2) and regulatory proteins for cell proliferation and survival (i.e., apoptosis vs necrosis), thereby, influencing many aspects of normal and disease physiology (*Figure 1B*).

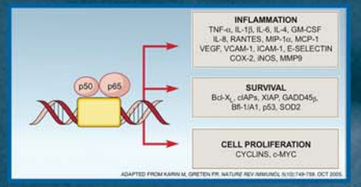

Figure 1B

There are, however, no published reports regarding circulating NF-κB status and hyperbaric stress or the potential role of NF-κB dysregulation in the development of decompression sickness (DCS).

Acknowledgements

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Purpose

We sought to determine the effect of hyperbaric stress on nuclear translocation of NF-κB within circulating leukocyte subsets, in healthy subjects exposed to progressive levels of hyperbaria.

Materials and Methods

Subjects

Following IRB approval, subjects were medically screened with a 12-lead ECG, chest x-ray and physical exam and a full explanation of procedures, discomforts and risks was given prior to obtaining written informed consent. Twenty males volunteered to participate in the study. Respective mean values and SD for age, height and weight were 38.9 ± 8.4 y, 1.76 ± 0.07 m and 83.0 ± 13.2 kg. Subjects were recruited and classified as being either naive (N, *n*=9) having never experienced hyperbaric stress or experienced (D, *n*=11) having been exposed at least once per month during the previous 6 months to hyperbaric stress. Nine of the subjects in this group were professional military divers, who were classified as explosive ordinance disposal divers and posted to DRDC Toronto while the others were recreational divers. There were no differences between groups in the descriptive characteristics listed above.

Experimental Design

For all hyperbaric exposures, subjects breathed chamber air and wore a Durrette® coverall over personal underwear and static control chamber shoes over personal socks. A minimum of 14 days prior to the first experimental session subjects completed a familiarization exposure to 90 kPa for 15 min experiencing all of the instrumentation and testing described below. The 3 experimental sessions involved 30 min of exposure to 180 (18 msw), 300 (30 msw), or 450 kPa (45 msw), which were defined as low, moderate or high level of hyperbaric stress, respectively. Pressurization rates were 18 kPa/min and decompression rates were in accordance with established dive tables and represented one, 15 and 55 min for the low, moderate and high levels of hyperbaric stress. Experimental sessions were separated by at least 6–7 days with the order of presentation standard for all subjects from the lowest through the highest level of stress.

Blood Collection

Venous whole blood was collected immediately pre-dive, 20- and 60-min post-dive using an indwelling venous catheter. Blood samples were drawn into syringes and immediately transferred into 3mL vacutainers containing sodium heparin (Becton Dickinson Biosciences, BD).

Flow Cytometric Analysis of Intranuclear NF-κB

A flow cytometric assay was used to quantify intranuclear NF-κB translocation in peripheral blood mononuclear cells (PBMC) and polymorphonuclear neutrophils (PMN). For each of the sampling time points, fresh whole blood was stained immediately for spontaneous NF-κB expression or following *in vitro* LPS (100 ng/mL) stimulation in a water bath (37°C, 30 min). Briefly, 50μL aliquots of blood were incubated for 30 min with 1mL BD Lysing Solution and then processed using BD CycleTest Plus DNA reagent kit. After washing in citrate buffer, cells were treated with 125μL Solution A (trypsin with spermine tetrahydrochloride detergent buffer) and 100μL Solution B (trypsin inhibitor and RNase buffer with spermine tetrahydrochloride). Each solution step was incubated 10 min at room temp. Without removing solution A and B, isolated nuclei were stained with either 10μL mouse IgG1 (non-specific binding) isotype control or NF-κB/p65 FITC-conjugated antibodies and incubated at room temperature for 10 min in the dark. The mouse NF-κB antibody recognizes an epitope overlapping the nuclear location signal of NF-κB-p65 and therefore selectively recognizes the activated form of NF-κB. Propidium Iodide (PI) was added to bind to clean isolated nuclei. Stained cell suspensions were acquired within 3 h of staining on a dual laser FACSCalibur flow cytometer using CellQuest software (BD), with a live gate set using a bivariate dotplot of Forward Scatter (FSC) vs Side Scatter (SSC) to distinguish the leukocyte populations; sample acquisition was stopped after 10,000 PBMC events were collected. Subsequent data analysis was performed using FlowJo software (*Figures 2A–B*). Results are expressed as the linear median fluorescence intensity (MFI) of intranuclear NF-κB in each population in arbitrary units (a.u.).

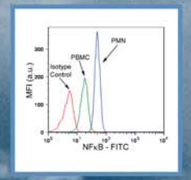


Figure 2A

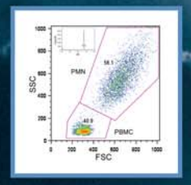


Figure 2B

Statistical Analyses

Data are shown as mean ± SE. Differences in NF-κB expression by circulating leukocyte subsets over time and between groups were analyzed by two-way repeated measures ANOVA. Statistical significance was set at $p < .05$.

Results

Figure 3. Spontaneous intranuclear NF-κB expression (MFI, a.u.) by circulating peripheral blood mononuclear cells (A; PBMC) and polymorphonuclear neutrophils (B; PMNs), sampled pre-dive, 20- and 60-min post-dive in healthy naive subjects (*N*, *n*=9) and experienced divers (*D*, *n*=11) who underwent 30-min hyperbaric exposures at 18, 30 and 45 msw. Resting spontaneous expression of NF-κB in PBMC and PMNs was similar between *N* and *D*. Following acute hyperbaric stress, *D* exhibited a significant time-dependent elevation (up to 60%) of intranuclear NF-κB expression in PBMC and PMNs at all depths; whereas, significant translocation occurred only after 45 m, 60-min post-dive in *N*. * p <0.05 post-dive vs pre-dive values.

Figure 4. Lipopolysaccharide (LPS)-stimulated (100 ng/mL, 37°C, 30 min) intranuclear NF-κB expression (MFI, a.u.) by PBMC and PMNs in naive subjects (*N*, *n*=9) and experienced divers (*D*, *n*=11). Compared to spontaneous levels, LPS stimulation induced significant increases in NF-κB expression in both groups compared; however, NF-κB translocation was greatest (up to 45%) in *D* compared to *N* responses. * p <0.05 *D* vs *N*.

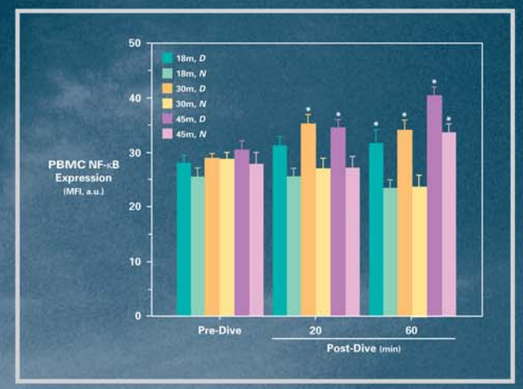


Figure 3A

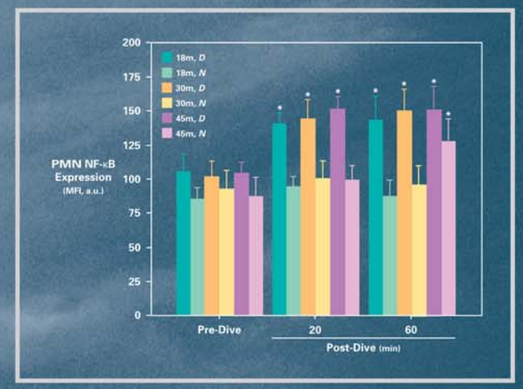
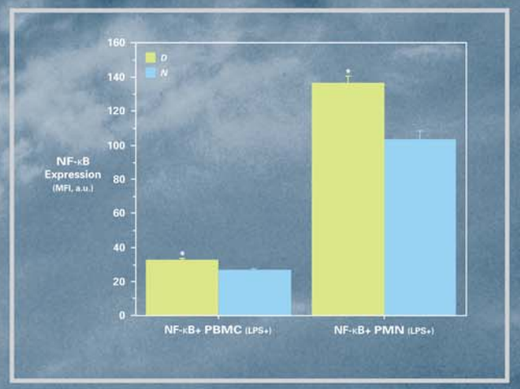


Figure 3B

Figure 4



Summary/Conclusions

- This study demonstrates enhanced nuclear translocation of NF-κB in both PBMC and PMNs from humans exposed to acute hyperbaric exposure that is greatest in experienced divers following repetitive episodes of hyperbaric stress.
- These results suggest that modulation of NF-κB-dependent inflammatory gene expression pathways may contribute to diving acclimatization and susceptibility to decompression sickness in individuals undergoing chronic hyperbaric stress.
- The complex regulatory mechanisms governing NF-κB activation/deactivation by acute/chronic hyperbaric stress/decompression are currently under investigation.

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