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Stress-induced enhancement of NF-κB DNA-binding in the peripheral blood leukocyte pool: effects of lymphocyte redistribution

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Abstract

To identify signaling pathways by which the sympathetic nervous system (SNS) might alter gene expression in the immune system, we assayed activation of the inflammatory transcription factor NF-κB in peripheral blood mononuclear cells (PBMC) from 13 healthy young adults at rest and following 5 min of intense exercise. SNS activation was verified by changes in cardiovascular parameters and mobilization of NK cells into circulating blood. Electrophoretic mobility shift assays (EMSA) of nuclear protein extracts confirmed previous findings that SNS activation increased NF-κB DNA-binding activity in bulk PBMC. However, analyses of isolated leukocyte subsets failed to indicate any increase on a per-cell basis in NK cells (the major carriers of NF-κB activity in circulating PBMC), in the residual CD56− leukocyte pool, or in CD14+ monocytes. Regression analyses indicated a strong correlation between increasing NK cell prevalence and changes in NF-κB DNA-binding activity in bulk PBMC, and suggested that no change in EMSA activity would be observed in the absence of NK cell mobilization. Such results imply that SNS-induced mobilization of NK cells can rapidly (<10 min) alter NF-κB DNA-binding activity in the circulating PBMC pool without generating any true change in NF-κB activity on a per-cell basis. Implications for future efforts to analyze stress effects on leukocyte gene expression are considered.
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1. Introduction

Acute stress has several effects on circulating blood, including generalized leukocytosis and shifting composition of lymphocyte subsets (e.g., increased prevalence of NK cells and CD8+ T lymphocytes) (Benschop et al., 1996b; Herbert and Cohen, 1993). These changes occur rapidly and can be abrogated by β-adrenoreceptor antagonists, suggesting that they are mediated by catecholamine release during sympathetic nervous system (SNS) activation (Bachen et al., 1995; Benschop et al., 1996a,b). Although the physiologic significance of these effects remains unclear (Sanders and Straub, 2002), it has been suggested that rapid and selective mobilization of functionally distinct leukocyte subsets may constitute an immunologic component of the evolutionarily conserved "fight-or-flight" response (Benschop et al., 1996b). Under this hypothesis, increased circulation of neutrophils, CD8+ T lymphocytes, and NK cells may optimize the immune system's ability to respond to tissue damage sustained during stressful events.

It was recently observed that acute stress can also increase the DNA-binding activity of the proinflammatory transcription factor NF-κB in peripheral blood mononuclear cells (PBMC) (Bierhaus et al., 2003; Vider et al., 2001). Given the central role of NF-κB in controlling expression of immune system genes (Ghosh et al., 1998), these results were interpreted as showing that acute stress can rapidly alter leukocyte gene expression

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in vivo. However, the well-known redistributive effects of SNS activation suggest an alternative interpretation. If distinct leukocyte subsets maintain different levels of constitutive NF-κB activity, and the subsets showing higher levels of constitutive activity also preferentially enter circulation during acute stress, then the average level of NF-κB activity within the PBMC pool might change without any true transcriptional activation on a per-cell basis. Under this hypothesis, acute stress might not alter transcription factor activity or gene expression in the body as a whole, but it would increase the level of NF-κB activity measured in circulating mononuclear cells by changing the composition of that cell population.

In the present study, we sought to identify the mechanism by which acute SNS activity alters NF-κB DNA-binding activity in PBMC, and to define the cellular locus of that effect. We isolated bulk PBMC and specific leukocyte subsets from circulating blood of healthy individuals at rest and following a 5-min exercise task that induced pronounced SNS activation. Electrophoretic mobility shift assays (EMSAs) showed increased NF-κB DNA-binding activity in bulk PBMC, but this effect was completely attributable to the increasing prevalence of NK cells in PBMC. NK cells maintained significantly higher levels of constitutive NF-κB activity than did other leukocyte subsets, but their per-cell EMSA activity was not altered by SNS activation. No significant increase in per-cell EMSA activity occurred in the CD56− fraction of PBMC, or in the CD14+ monocyte fraction. Such results suggest that it may be necessary to re-evaluate interpretations of rapid NF-κB-mediated gene induction during acute stress in light of the changing composition of the PBMC pool. These findings also underscore an emerging theme in which stress and neuroendocrine activity regulate immune system function in large part by controlling cellular localization, rather than changing the activation of resident cells (Carlson, 2001; Cole et al., 1999; Dhabhar and McEwen, 1999; Ottaway and Husband, 1994; Sheridan et al., 1998).

2. Methods

2.1. Participants

Thirteen healthy young adults were recruited by posted advertisements to participate in a study of "exercise and the immune system." Prospective participants were screened to exclude any individuals with chronic illness, acute infection (including upper respiratory infection), or current use of prescription drugs other than birth control pills. Age ranged from 18 to 32, and seven participants were female. All procedures were approved by the UCLA Medical Institutional Review Board, and all participants provided written informed consent.

2.2. Procedure

Peripheral blood was obtained by venipuncture after 15 min of seated rest and following 5 min of physical exercise (running down and then up 14 flights of stairs). At least 40 ml of blood were drawn at each time point into 10 ml sodium heparin Vacutainers (Becton–Dickinson, San Jose CA). Immediately prior to each blood draw, heart rate and blood pressure were assessed by digital oscultory monitoring (LifeSource UA-767, A&D Engineering, Milpitas, CA). All procedures were conducted between 10 AM and 12 noon to minimize diurnal influences, and the total time from pre- to post-exercise blood draw was 15 min. Ten minutes elapsed between the initiation of exercise the post-exercise blood draw.

2.3. Leukocyte phenotyping

The circulating prevalence of CD3+ T lymphocytes and CD3−/CD56+ NK cells was measured by flow cytometry after whole blood staining with a FITC-conjugated antibody to CD3 (clone KT3) and a PC5-conjugated antibody to CD56 (clone N901) (Beckman Coulter, Miami, FL). Data were acquired on a FACScan flow cytometer and analyzed using CellQuest software (BD Immunocytometry Systems, San Jose CA), with live lymphocytes gated on the basis of forward vs. side scatter profiles. At each time-point, 400 μL of whole blood was stained and resuspended in 200 μL of phosphate-buffered saline containing 2% paraformaldehyde. Absolute cell counts were established by acquiring samples for 20 s at a constant flow rate of 1 μl/s. Total lymphocyte counts and cell viability were verified by hemacytometry and trypan blue exclusion. Flow cytometry data are unavailable for some participants due to technical difficulties in sample preparation (e.g., RBC contamination).

2.4. Transcription factor DNA-binding activity

DNA-binding activity of NF-κB was assessed by EMSA of nuclear extracts from 10^7 Ficoll-separated PBMC or equivalent numbers of CD56+, CD56−, or CD14+ PBMC isolated by immunomagnetic positive selection (Miltenyi Biotec, Auburn, CA). Nuclear extracts were obtained by differential lysis at 4 °C (Read, 1996), and 1/25 (2 μl) of the resulting extract was incubated at room temperature for 15 min with 1.75 pmol of ^{32}P-labeled NF-κB consensus oligonucleotide (Promega, Madison, WI) in a 10 μl aqueous binding reaction containing 2 μl of 5× gel shift binding buffer (20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris–HCl, pH 7.5, and. 25 mg/ml poly(dI–dC); Promega). Bound oligonucleotides were resolved

on a 6% polyacrylamide gel (run for 90 min at 250 V following a 15 min pre-run) and quantified on a Storm 860 phosphorimager using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). All binding reactions were oligonucleotide-specific as demonstrated by competitive inhibition when protein extracts were preincubated with 100-fold excess of unlabeled target oligonucleotide, but not when extracts were preincubated with similar concentrations of an unlabeled control oligonucleotide. Equivalent loading of nuclear protein into each binding reaction was verified by Bradford assay (Bio-Rad Laboratories, Hercules, CA).

2.5. Data analysis

Change in EMSA DNA-binding activity was quantified as the ratio of post-exercise to pre-exercise values for each individual, and analyzed in a log-transformed format by paired t test (Miller, 1986). Alterations in circulating NK cell prevalence (CD3−/CD56+ cells as a percent of total lymphocytes), heart rate, and blood pressure were expressed as change scores (post-exercise–pre-exercise), and analyzed by paired t test. Relationships between change in NF-κB DNA-binding activity and change in NK cell prevalence were analyzed using linear regression (Miller, 1986). The slope of the ordinary least-squares regression line indicated the extent to which increases in NF-κB DNA-binding activity varied with the magnitude of NK cell mobilization. In this regression equation, the intercept indicates the magnitude of NF-κB activity that would be expected in the absence of any change in NK cell prevalence.

3. Results

3.1. Acute SNS activation

To ensure that the exercise induced significant increases in SNS activity, we monitored heart rate and blood pressure immediately prior to baseline and post-exercise blood draws. Both parameters increased significantly following exercise (Fig. 1). Heart rate rose from a mean 71 beats per minute (bpm) to 128 ($t(11) = 9.27$, $p < .0001$), systolic blood pressure rose from a mean 124 mm Hg to 189 ($t(11) = 13.98$, $p < .0001$), and diastolic blood pressure rose from a mean 75 mm Hg to 86 ($t(11) = 3.93$, $p = .0028$). All participants showed an increase on each parameter.

3.2. NF-κB DNA binding in bulk PBMC

To determine whether acute SNS activation is associated with increased NF-κB DNA binding in PBMC, we performed EMSAs on nuclear extracts from Ficoll-separated leukocytes obtained at rest and following exercise. Cell numbers were equalized prior to assay, and equivalent loading of nuclear protein was confirmed by Bradford assay (all variations < 10%). Fig. 2A shows

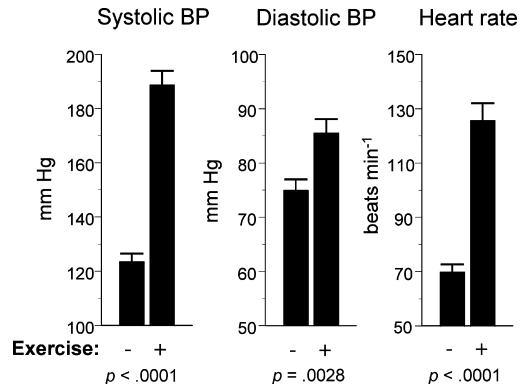

Fig. 1. Activation of the sympathetic nervous system by acute exercise. Sympathetic nervous system activity was monitored by assessing heart rate, systolic blood pressure, and diastolic blood pressure in 12 healthy young adults following 15 min of rest and after 5 min of intense exercise (running down then up 14 flights of stairs). Graphs give mean value of each parameter (±SE), and statistical significance of changes was evaluated by paired t test. All participants showed an increase on each parameter.

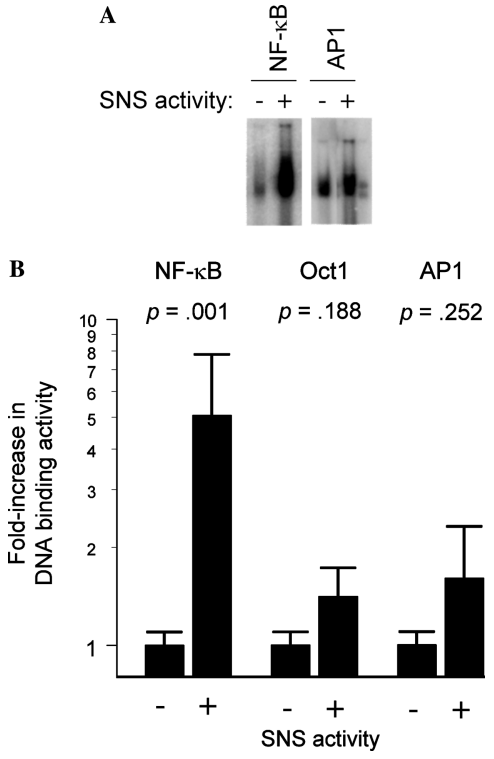

Fig. 2. Effect of SNS activity on NF-κB DNA-binding activity in bulk PBMC. Electrophoretic mobility shift assays (EMSAs) were conducted as described in Methods to assay levels of NF-κB, AP1 or Oct1 DNA-binding activity in Ficoll-separated PBMC obtained at baseline and following SNS activation. (A) Representative EMSA results for one participant, and (B) the mean change in NF-κB DNA-binding activity across all participants (±SE). Statistical significance was assessed by paired t test.

representative results for one participant in which PBMC NF-κB DNA-binding activity increased by approximately 10-fold following SNS activation. In contrast, activity of the transcription factor AP1 changed by only 2-fold. Across all participants studied, NF-κB EMSA activity increased by an average of more than 4-fold following SNS activation ($t(10) = 4.64$, $p = .0009$). EMSA activity for Oct1 and AP1 transcription factors changed by less than 50% (neither reaching statistical significance; Fig. 2B). Effects of acute SNS activation dissipated within 60 min, at which point NF-κB DNA-binding activity was statistically indistinguishable from baseline levels (data not shown).

3.3. Leukocyte dynamics

As in previous studies (Benschop et al., 1996b; Herbert and Cohen, 1993; Sanders and Straub, 2002; Vider et al., 2001), SNS activation led to a 2-fold increase in the total circulating white blood cell counts ($t(11) = 10.77$, $p < .0001$). Fig. 3 shows that the composition of the circulating leukocyte pool also changed significantly. Granulocyte numbers rose by approximately 2-fold ($t(11) = 4.17$, $p < .0042$), and lymphocyte counts increased from a mean of 1.49×10^6 cells/mm^3 at baseline to 4.60×10^6 post-SNS activation ($t(11) = 4.23$, $p = .0039$). Within the lymphocyte population, the relative prevalence of NK cells (% of total lymphocytes) increased an average of 2-fold from pre- to post-exercise ($t(9) = 7.33$, $p < .0001$) whereas the prevalence of CD3+ T cells and CD19+ B lymphocytes both declined slightly (T cells: $t(9) = -8.15$, $p < .0001$; B cells $t(9) = -2.85$, $p = .019$). The prevalence of CD14+ cells as a fraction of total PBMC was not significantly altered ($t(9) = -1.21$, $p = .267$).

3.4. NF-κB DNA-binding activity in circulating leukocyte subsets

To identify the cellular source of increased NF-κB activity in PBMC, we isolated distinct leukocyte subsets by immunomagnetic positive selection and performed EMSAs on equalized numbers of purified cell populations from a subset of study participants. Purity of isolated cell populations was confirmed by flow cy-

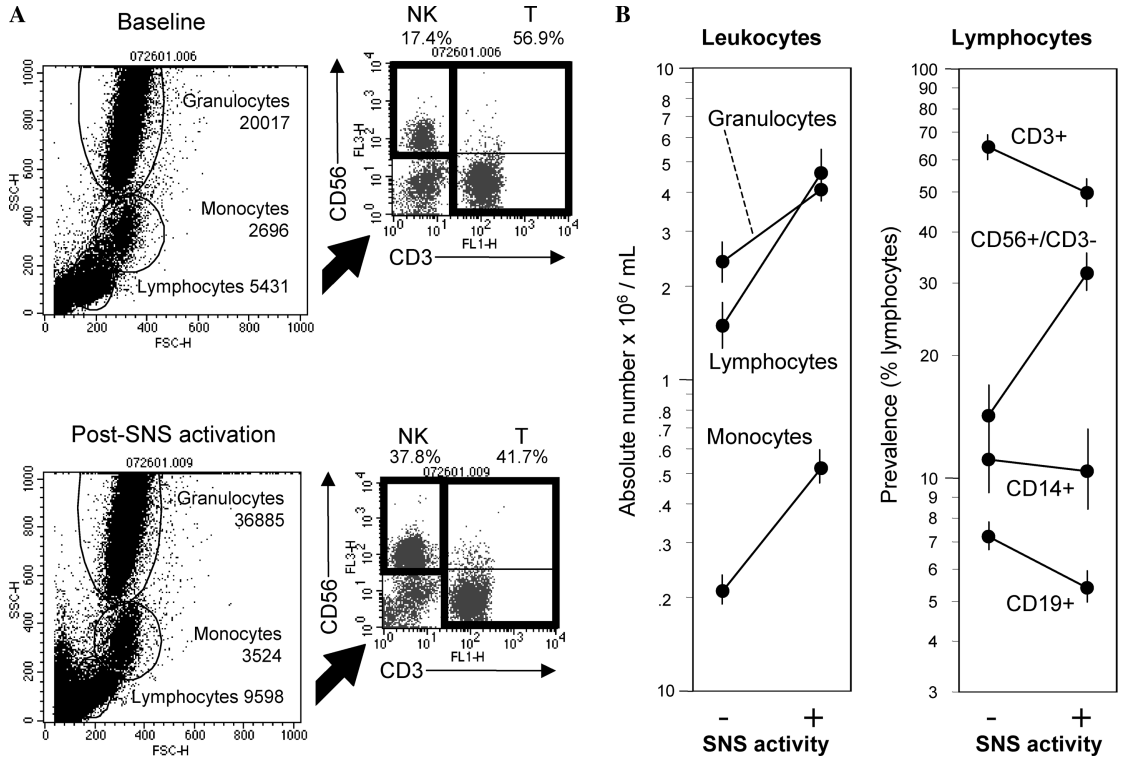

Fig. 3. Effect of SNS activity on circulating leukocytes. White blood cell counts were determined by hemacytometry and the relative prevalence of NK cells, T lymphocytes, B lymphocytes, and monocytes were assessed by flow cytometry for CD56+/CD3− lymphocytes, CD3+ lymphocytes, CD19+ lymphocytes, and CD14+ live cells, respectively (A). During flow cytometry, changes in cell counts were verified by staining equivalent volumes of blood from baseline and post-SNS activation conditions and acquiring equivalent fractions of each sample in a fixed 20 s acquisition at 1 μl/s. Values in the forward- vs. side-scatter plots of (A) reflect .5% of the total cell number/ml of original blood. Average change from pre- to post-SNS conditions was evaluated by paired t test, with means (±SEM) graphed in (B). All parameters showed statistically significant change ($p < .05$) except CD14+ cells as a fraction of total lymphocytes ($p = .267$). Individual test statistics and p values are given in the text (Results: Leukocyte dynamics).

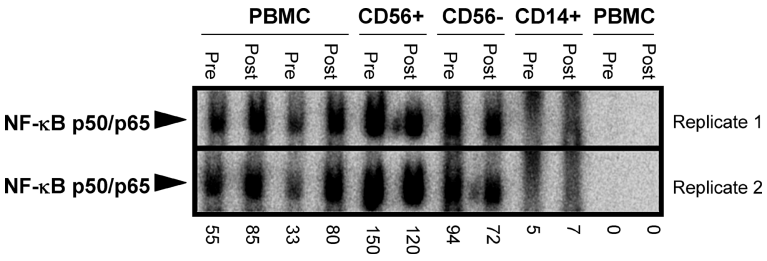

Fig. 4. Effect of SNS activity on NF-κB DNA-binding activity in distinct leukocyte subsets. Bulk PBMC were isolated by Ficoll density gradient centrifugation, and leukocyte subsets were purified into CD56+, CD56−, and CD14+ fractions by immunomagnetic positive selection for CD56 (column flow-through = CD56−) and CD14 (out of the CD56− fraction). 10^7 cells from each population were assayed by EMSA as described in Methods, and duplicate determinations of NF-κB DNA-binding activity were quantified by phosphorimagery. Mean background-corrected radioactivity intensity is given below each lane. Statistical significance of differences was analyzed by paired *t* test, with individual test statistics and *p* values reported in the text (Results: *NF-κB* DNA-binding activity in circulating leukocyte subsets). Lanes 1 and 2 show duplicate determinations on baseline (pre-SNS) and post-SNS PBMC samples from one representative participant, and analogous results are presented for a second participant in lanes 3 and 4. Lanes 5 and 6 show EMSA activity in equalized numbers of CD56+ PBMC pre- and post-SNS activation (isolated from bulk PBMC samples assayed in lanes 3 and 4), and lanes 7 and 8 give analogous results for the CD56− population of PBMC (flow-through from the positive selection column). Results for CD14+ cells are shown in lanes 9 and 10. Lanes 11 and 12 verify binding specificity of the NF-κB EMSA by showing that 100-fold excess of unlabeled NF-κB oligonucleotide can abrogate binding of PBMC nuclear extracts (assayed in lanes 1 and 2) to radio-labeled NF-κB oligonucleotides. Unlabeled target oligonucleotides for the Oct1 and AP1 transcription factors failed to inhibit nuclear protein binding to NF-κB target oligonucleotides (data not shown). All PBMC subset samples were found to be > 97% pure by flow cytometry, and equivalent loading of protein from pre- and post-SNS conditions was confirmed by Bradford assay.

tometry to be greater than 97% in all cases. Lanes 1–4 of Fig. 4 show representative magnitudes of NF-κB induction in equalized numbers of bulk PBMC from two participants at baseline and following SNS activation (determined in duplicate to verify reliability). Lanes 5–10 show comparable EMSA results for equalized numbers of CD56+ cells (lanes 5–6), CD56− cells (lanes 7–8), and CD14+ cells (lanes 9–10) isolated from the PBMC samples assayed in lanes 3 and 4. Quantitative analysis by phosphorimagery identified CD56+ cells as the primary carriers of NF-κB DNA-binding activity under both baseline and SNS activation conditions. The CD56+ population showed approximately 2-fold higher levels of EMSA activity per cell than did the remainder of PBMC ($t(6) = 3.13$, $p = .020$), and SNS activation did not significantly alter that value on a per-cell basis ($t(2) = -0.96$, $p = .437$). Among the CD56- fraction of PBMC, NF-κB DNA-binding activity actually dropped on a per-cell basis following SNS activation ($t(2) = -5.61$, $p = .030$). CD14+ monocytes accounted for comparatively little of the EMSA signal, and phosphorimagery quantified NF-κB activity in monocytes at approximately 1/4 the per-cell level observed in CD56+ cells ($t(2) = 14.61$, $p = .005$). NF-κB EMSA activity of monocytes was not significantly altered by SNS activation ($t(2) = -0.01$, $p = .934$). Thus NK cells appear to be the major source of NF-κB DNA-binding activity in human PBMC, and monocytes contribute little to the overall NF-κB activity signal either at rest or at 10 min following SNS activation. Neither CD56+ cells or CD14+ cells showed significant induction of NF-κB DNA-binding activity on a per-cell basis at 10 min after acute SNS activation.

3.5. Relationship between NK cell mobilization and NF-κB activity in PBMC

To determine whether mobilization of NK cells might account for SNS-induced increases in PBMC NF-κB DNA-binding activity, we conducted linear regression analyses quantifying the relationship between change in EMSA activity and change in NK cell prevalence. As shown in Fig. 5, there was a strong linear relationship between the mobilization of NK cells in a given participant and the extent to which that participant's PBMC showed increased NF-κB EMSA activity following SNS activation (regression slope = +.049 radioactivity units per % increase in NK cell prevalence, standard error = 0.016, $t(6) = 3.04$, $p = .023$). The regression line intercept did not differ significantly from 0 (log[1-fold] change = no change; intercept = −0.222 radioactivity units, standard error = .288, $t(6) - 0.77$, $p = .469$), implying that no significant change in NF-κB DNA-binding activity would be observed in the absence of NK cell mobilization. Put another way, changes in NK cell prevalence can account for all statistically significant SNS-induced increase in NF-κB DNA-binding activity in the bulk PBMC pool. Similar results emerged when NF-κB induction measures were adjusted to account for minor variations in protein loading (regression slope = +.024 radioactivity units per % increase in NK prevalence, standard error = 0.009, $t(6) = 2.69$, $p = .036$; intercept value = −0.080 radioactivity units, standard error = 0.157, $t(6) = -0.51$, $p = .626$). The later analysis ensures that failure to identify significant differences in per-cell NF-κB DNA-binding activity within PBMC subsets is not attributable to any SNS-induced alteration in the quantity of proteins localized

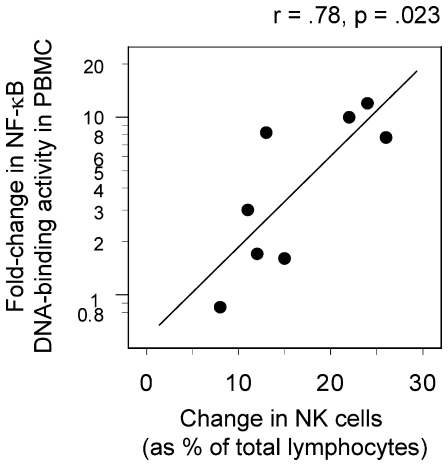

Fig. 5. Relationship between NK cell mobilization and change in NF-κB activity in PBMC. EMSA activity was quantified as described in Fig. 4, and the relative intensity of NF-κB DNA-binding activity post-SNS activation was expressed as a ratio to the baseline value determined in parallel. Change in NK cell prevalence from pre- to post-SNS activation was measured by flow cytometry for CD56+/CD3− lymphocytes (gated on forward- vs. side-scatter profiles) and quantified as the increase in NK cells as a percent of total lymphocytes. Correspondence between the magnitude of NK cell mobilization and the induction of NF-κB DNA-binding activity was quantified by linear regression. Parameter estimates for slope and intercept are given in the text along with tests of statistical significance (Results: Relationship between NK cell mobilization and NF-κB activity in PBMC).

to the nucleus. These results are consistent with Bradford assays showing that nuclear protein content of each leukocyte subset differed by less than 10% from baseline to post-SNS conditions.

4. Discussion

The present data replicate previous studies in showing increased NF-κB DNA-binding activity in PBMC following acute SNS activation (Bierhaus et al., 2003; Vider et al., 2001). However, analysis of the cellular mechanisms involved show that much of the increase in DNA-binding activity observed here can be attributed to the changing prevalence of NK cells in the circulating leukocyte pool rather than changing NF-κB activity within specific cells. CD56+ cells represent the major carriers of NF-κB DNA-binding activity in PBMC, and their mobilization by acute SNS activation may thus account for much of the increase in NF-κB DNA-binding activity observed in the total mononuclear cell pool. Quantitatively, the 2-fold elevation in basal NF-κB activity in NK cells in conjunction with their 2-fold increase in prevalence is sufficient to account for the average 4-fold increase in PBMC NF-κB activity observed following acute SNS activation. Linear regression analyses show a strong correlation between the magnitude of NK cell mobilization and increased PBMC NF-κB activity, and analysis of the regression line intercept suggests that no increase in NF-κB DNA binding would be expected in the absence of NK cell mobilization. When distinct leukocyte subsets were physically isolated and held constant in number, EMSAs confirmed that acute SNS activation did not enhance NF-κB DNA-binding activity in CD56+ cells, in the CD56− residual fraction, or in CD14+ cells. Such results suggest that the well-known redistributive effects of SNS activity could potentially account for stress-associated enhancement of NF-κB DNA binding in the total PBMC pool without entailing any true induction on a per-cell basis (Bierhaus et al., 2003; Vider et al., 2001). These results also suggest that between-subject comparisons of NF-κB DNA binding could be confounded by individual variations in circulating NK cell prevalence (Nagabhushan et al., 2001).

Based on the present results, it does not appear that acute SNS activation necessarily produces any significant increase in cellular NF-κB activity within 10 min, and it may in fact suppress that activity in certain cell subsets (e.g., the CD56− fraction of PBMC, which constitutes primarily T cells, and to a lesser extent B cells and monocytes). The present studies document substantial differences across leukocyte subsets in basal levels of NF-κB DNA-binding activity, and they identify dynamic changes in subset prevalence as a major mechanism by which acute SNS activity can alter NF-κB EMSA activity in circulating PBMC within a short period of time. Such findings question previous interpretations of acute stress-induced changes in PBMC EMSA activity as necessarily implying alterations in gene expression. However, they do not rule out a possible additional effect of stress on per-cell NF-κB activity above and beyond the confounding effects of lymphocyte redistribution. SNS activation via physical exercise failed to increase per-cell NF-κB DNA binding within 10 min, but per-cell changes could conceivably occur under other stimulus conditions (e.g., stressors of different form, duration, or intensity) or at other time points following stress. Exercise is known to preferentially enhance norepinephrine levels, whereas psychological stressors elicit a more pronounced change in epinephrine (Dimsdale and Moss, 1980; Saitoh et al., 1995). The exercise procedure utilized here should have maximized opportunities to observe the norepinephrine-mediated increase in NF-κB DNA binding previously identified in vitro in cultured monocytic cell lines (Bierhaus et al., 2003). However, the present data do not reveal any change in NF-κB DNA-binding activity in primary monocytes following in vivo SNS activation, at least within the observed 10-min time frame. Given the minimal NF-κB activity levels observed in monocytes under both basal and SNS-activated conditions, it does not appear that this cell type mediates effects of acute stress on NF-κB DNA-binding activity in the human

PBMC pool under physiologic conditions. However, NK cell redistribution dynamics do appear to be sufficient to account for the observed SNS-induced increase in NF-κB DNA-binding activity within bulk PBMC.

The present data clearly show that stress-induced remodeling of the PBMC pool needs to be addressed as a potential confounder in efforts to analyze transcription factor activity in vivo. In addition to defining cellular mechanisms that may alter PBMC EMSA activity, these studies demonstrate a direct approach to overcoming the confounding effects of lymphocyte redistribution by physically isolating distinct cell phenotypes (e.g., by immunomagnetic positive selection or by fluorescence activated cell sorting), or by statistically controlling for alterations in PBMC composition (e.g., by analyses of covariance adjusting for NK cell prevalence). The effects of shifting PBMC composition evoke Simpson's paradox, in which dynamic population structures produce illusory correlations between two variables that actually show no association within a static population (e.g., SNS activity and NF-κB DNA binding) (Bickel et al., 1975; Wagner, 1982). Nevertheless, it is clear that stressful conditions and SNS activity can alter leukocyte gene expression in biologically important ways under other circumstances (Ader et al., 1995; Sanders and Straub, 2002). Delineation of the signaling pathways involved and the specific gene expression programs activated is critical to understanding both basic immunoregulation and its dysregulation under stress. The present data underscore the need to account for changes in the cellular composition of analyzed tissues, and they re-emphasize the profound effects that neuroendocrine activity can have on the trafficking and localization of differentiated leukocyte subsets (Carlson, 2001; Cole et al., 1999; Dhabhar and McEwen, 1999; Ottaway and Husband, 1994; Sheridan et al., 1998).

Acknowledgments

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