

Neuroendocrine Modulation of Signal Transducer and Activator of Transcription-3 in Ovarian Cancer

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Abstract

There is growing evidence that chronic stress and other behavioral conditions are associated with cancer pathogenesis and progression, but the mechanisms involved in this association are poorly understood. We examined the effects of two mediators of stress, norepinephrine and epinephrine, on the activation of signal transducer and activator of transcription-3 (STAT3), a transcription factor that contributes to many promalignant pathways. Exposure of ovarian cancer cell lines to increasing concentrations of norepinephrine or epinephrine showed that both independently increased levels of phosphorylated STAT3 in a dose-dependent fashion. Immunolocalization and ELISA of nuclear extracts confirmed increased nuclear STAT3 in response to norepinephrine. Activation of STAT3 was inhibited by blockade of the β 1- and β 2-adrenergic receptors with propranolol, and by blocking protein kinase A with KT5720, but not with the α receptor blockers prazosin (α 1) and/or yohimbine (α 2). Catecholamine-mediated STAT3 activation was *not* inhibited by pretreatment with an anti-interleukin 6 (IL-6) antibody or with small interfering RNA (siRNA)-mediated decrease in IL-6 or gp130. Regarding the effects of STAT3 activation, exposure to norepinephrine resulted in an increase in invasion and matrix metalloproteinase (MMP-2 and MMP-9) production. These effects were completely blocked by STAT3-targeting siRNA. In mice, treatment with liposome-incorporated siRNA directed against STAT3 significantly reduced isoproterenol-stimulated tumor growth. These studies show IL-6-independent activation of STAT3 by norepinephrine and epinephrine, proceeding through the β 1/ β 2-adrenergic receptors and protein kinase A, resulting in increased matrix metalloproteinase production, invasion, and *in vivo* tumor growth, which can be ameliorated by the down-regulation of STAT3. [Cancer Res 2007;67(21):10389–96]

Introduction

There is growing evidence that stress and other behavioral conditions may be associated with cancer pathogenesis and

progression (1–3). However, the mechanisms through which stress may promote malignant progression are poorly understood. Deleterious effects on cancer may be mediated by the same neurohormones coordinating the stress response itself (1, 4–7). Stress is associated with an increase in the neuroendocrine hormones norepinephrine and epinephrine, which are released from sympathetic nervous system neurons and the adrenal medulla. Stress can also activate the production of cortisol by the hypothalamic pituitary adrenal axis. Catecholamines have been shown to increase tumor growth and angiogenesis via β -adrenergic receptors (β AR) on malignant ovarian cancer cells (1, 8). Moreover, we and others have shown that chronic stress results in a more infiltrative pattern of tumor growth (1, 9), and the increase in invasive potential is related to increased levels of matrix metalloproteinases (MMP; refs. 9, 10). Because cytokines such as interleukin 6 (IL-6) have been implicated in malignant progression through the activation of signal transducer and activator of transcription-3 (STAT3), we sought to determine whether stress hormones have a direct effect on the expression and activation of STAT3.

STAT3 is a member of a family of seven homologous transcription factors initially identified as mediators of IFN signaling, and have been noted to participate in numerous processes key to malignant progression (11). Constitutive STAT3 activation has been noted in hematologic, head and neck, brain, breast, lung, prostate, pancreas, and ovarian cancers. STAT3 has transforming properties in and of itself, and is a participant in many other steps in cancer pathogenesis. Pathways in which STAT3 may directly participate in cancer progression include inhibition of apoptosis (12, 13), cell cycle dysregulation (14), induction of angiogenesis (15, 16), and evasion of the immune response (17, 18). If constitutively increased levels of neuroendocrine hormones contribute to the activation of STAT3, this could be an important pathway and potential target of intervention in cancer pathogenesis.

The purpose of this study was to determine if the neuroendocrine hormones norepinephrine and epinephrine have the potential to activate STAT3, to identify participants in this pathway, and to examine the efficacy of targeting STAT3 in a preclinical mouse model of ovarian cancer. We have shown that these hormones can indeed activate STAT3 and induce its nuclear translocation through β ARs and protein kinase A (PKA), independent of IL-6. Neuroendocrine stimulation results in promalignant biological effects, including MMP production, invasion, and tumor growth, which can be blocked with small interfering RNA (siRNA)-based STAT3 down-regulation.

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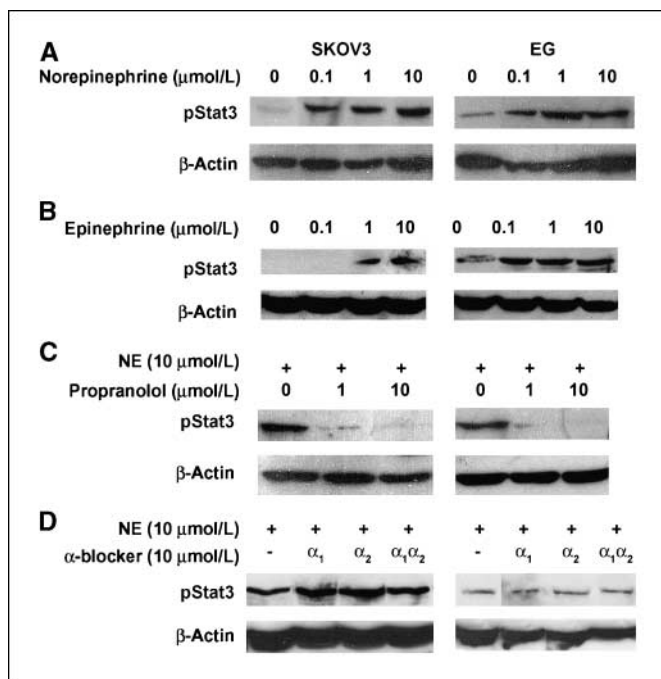


Figure 1. Effect of norepinephrine and epinephrine on pSTAT3. The ovarian cancer cell lines SKOV3 and EG were serum-starved for 15 h before the addition of increasing concentrations of norepinephrine (A) or epinephrine (B). Cell lysate was collected 3 h later, and subjected to Western blotting using anti-pSTAT3 antibody. pSTAT3 increases significantly with as low as 0.1 $\mu\text{mol/L}$ of both hormones. Total STAT3 levels were not affected (data not shown). Probing for β -actin confirms equal loading in all lanes. Treatment with propranolol, a β 1/ β 2-adrenergic receptor blocker, for 1 h prior to exposure to 10 $\mu\text{mol/L}$ of norepinephrine (NE) prevented an increase in pSTAT3 (C). Cells pretreated with prazosin (an α 1 blocker), yohimbine (an α 2 blocker), or the combination of both, did not prevent norepinephrine-induced increases in pSTAT3 (D).

Materials and Methods

Reagents and cell lines. The SKOV3 cell line was obtained from American Type Tissue Culture and maintained in minimal essential media (SRB core) with 10% fetal bovine serum. The SKOV3ip1 line was derived from SKOV3 by culture of intraperitoneal tumors from mice (19). EG cells (20) were maintained in RPMI 1640 with 15% fetal bovine serum and 0.1% MITO Plus reagent (BD Biosciences). For all experiments, 80% confluent cells were incubated for 15 h in serum-free medium prior to administration of the drug. Norepinephrine, epinephrine, and propranolol were purchased from Sigma and reconstituted and stored in 5N H_2SO_4 at -20°C . Prior to each experiment, stock hormones were diluted at least 1:1,000 in appropriate serum-free medium and exposed to cells within 5 min of preparation, to minimize spontaneous degradation. Prazosin and yohimbine (Tocris), and KT5720 (Calbiochem) were stored at -20°C . Anti-IL-6 antibody was from Biosource used at a dose of 50 $\mu\text{g/mL}$. When used, these inhibitory substances were exposed to cells for 1 h prior to exposure to norepinephrine or epinephrine, with the exception of siRNA-mediated down-regulation (see "siRNA" in Materials and Methods), which was introduced 24 h prior to the addition of neuroendocrine hormones.

Western blot. Cell lysates were prepared by washing cells with PBS and incubated for 10 min at 4°C in modified radioimmunoprecipitation assay lysis buffer (50 mmol/L Tris, 150 mmol/L NaCl, 1% triton, 0.5% deoxycholate plus the protease inhibitors leupeptin, aprotinin, EDTA, and sodium vanadate; Sigma). Cells were scraped from plates, centrifuged at 13,000 rpm for 20 min at 4°C , and the supernatant was stored at -80°C . Protein concentrations were determined using a bicinchoninic acid protein assay reagent kit (Pierce), and 50 μg of whole cell lysates were subjected to 10% SDS-PAGE separation. Samples were transferred to a nitrocellulose membrane by semidry electrophoresis (Bio-Rad Laboratories), blocked

with 5% nonfat milk for 2 h at room temperature, and incubated with 2 $\mu\text{g/mL}$ of anti-phosphorylated STAT3 or anti-STAT3 antibody (Upstate) overnight at 4°C . Primary antibody was detected with anti-mouse IgG (Amersham), and developed with an enhanced chemiluminescence detection kit (Pierce). Membranes were probed for β -actin (0.1 $\mu\text{g/mL}$ anti- β -actin antibody, Sigma) to confirm equal loading.

Immunolocalization. SKOV3 cells, at 80% confluency in a 16-well glass chamber slide (Nunc Technologies), were serum-starved overnight, and then treated for 15 min with norepinephrine at final concentrations of 0.1, 1, and 10 $\mu\text{mol/L}$. For blocking experiments, the cells were pretreated with 10 $\mu\text{mol/L}$ propranolol for 1 h before the addition of norepinephrine. Following treatment, the slide was fixed immediately with cold acetone for 10 min and stored in PBS at 4°C prior to further processing. Endogenous peroxidase was blocked with 3% H_2O_2 for 10 min, followed by a 15-min incubation in a biotin blocking system (CSA Ancillary Kit; DAKO, Ltd. UK). The cells were then exposed for 2 h to polyclonal rabbit anti-STAT3 (total) antibody (Upstate) diluted to 20 $\mu\text{g/mL}$, followed by a three-step staining procedure using biotinylated secondary antibody, streptavidin peroxidase reagents (LSAB+ kit; DAKO Ltd. UK) and 3,3'-diaminobenzidine, then counterstained with hematoxylin (Vector Laboratories).

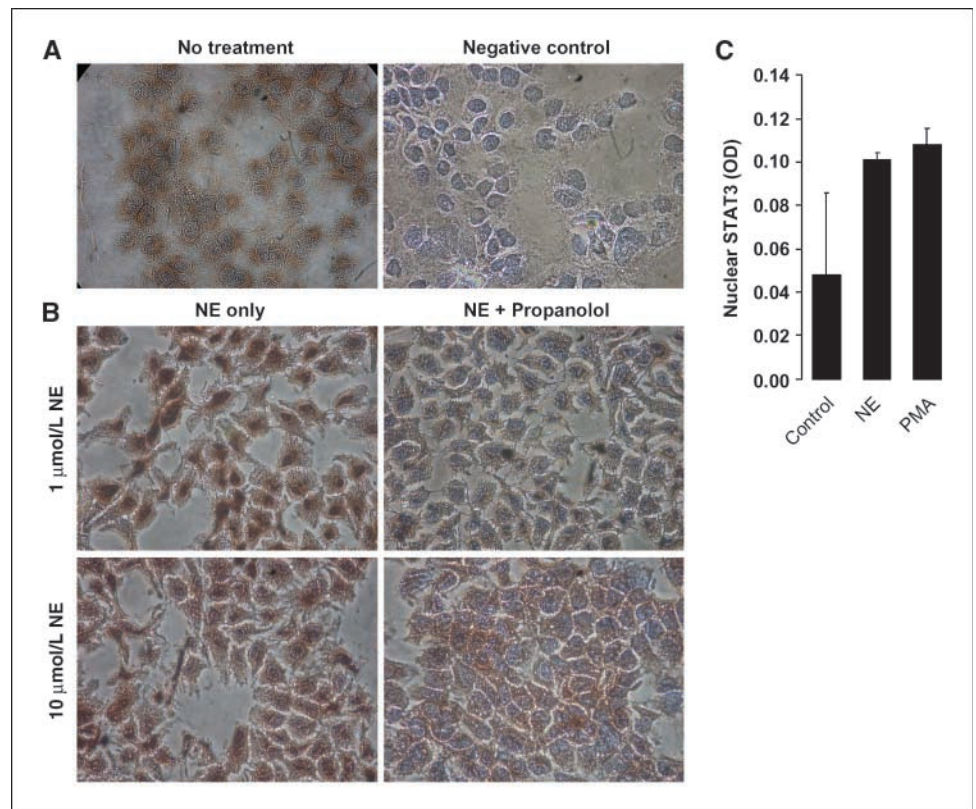
siRNA. For down-regulation of STAT3 *in vitro* and *in vivo*, siRNA was employed. STAT3-specific siRNA was purchased from Qiagen, HP validated anti-STAT3 siRNA (sequence unpublished). The IL-6-specific target sequence was 5'-CCAGGAGAAGAUUCCAAAGAUGUA-3'. The gp-130-specific target sequence was 5'-CAGAATTGTTTATGGAATCACA-3'. Control siRNA used was also from Qiagen, target sequence 5'-AATTCTCCGAACGTGTACAGT-3'. For *in vitro* studies, mRNA-specific or nonspecific (control) siRNA was incorporated into Qiagen RNAiFect transfection agent (1 μg siRNA to 6 μL RNAiFect) and exposed to cells at 70% to 80% confluence. After 36 h, the media was changed to standard siRNA-free media, and after another 12 h, an experiment was started (addition of norepinephrine) for the measurement of phosphorylated STAT3 (pSTAT3), use in the Membrane Invasion Culture System (MICS), or collection of supernatant for assessment of MMP production. For *in vivo* studies, siRNA was incorporated into the neutral liposome 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) as previously described (21). For each treatment, 3.5 μg of siRNA was reconstituted in 200 μL of PBS, and administered by i.v. injection. The dosing schedule is described in the *in vivo* tumor model below.

ELISA. To determine whether siRNA against IL-6 was effective at reducing levels of secreted IL-6, an ELISA was employed. SKOV3ip1 (1.0×10^5 cells) were seeded in triplicate into individual wells of a 24-well plate. Cells were then exposed to medium containing 0.7 $\mu\text{g}/\mu\text{L}$ of nonsilencing or IL-6-targeting siRNA with RNAiFect as described above. Following 24, 48, 72, or 96 h of incubation, medium was collected, centrifuged for 5 min at 13,000 rpm and 4°C , and tested for the presence of IL-6 by ELISA (R&D Systems) according to the manufacturer's instructions.

As an additional method to determine levels of nuclear STAT3 protein after norepinephrine exposure, nuclear extracts were assayed by ELISA (TransAM STAT3; Active Motif) 2 h after exposure of 10^6 SKOV3 cells to 0 or 10 $\mu\text{mol/L}$ of norepinephrine, or phorbol 12-myristate 13-acetate (a known activator of STAT3, as a positive control).

Invasion assay. The MICS chamber was used to measure the *in vitro* invasiveness of all cell lines used in this study. (22, 23) siRNA was exposed to cells 24 h prior to cell harvest, and 10 $\mu\text{mol/L}$ of norepinephrine was exposed for 3 h prior to harvest and testing for invasion. For the MICS assay, a polycarbonate membrane with 10- μm pores (Osmomics) was uniformly coated with a defined basement membrane matrix consisting of human laminin/type IV collagen/gelatin and used as the intervening barrier to invasion. The defined matrix was prepared (stored at 4°C) in a 10 mL stock solution as follows: laminin (50 $\mu\text{g/mL}$) 1 mL + type IV collagen (50 $\mu\text{g/mL}$) 0.2 mL + gelatin (2 mg/mL) 4 mL + 4.8 mL PBS. Using a disposable pipette, 1 mL of the matrix solution was dispensed across a long side of the membrane. An 8 mm glass rod was used to spread the matrix across the membrane, and allowed to dry for 30 min. The matrix-coated filter was placed coated side-up on the lower plate followed by carefully attaching the upper plate. Both upper and lower wells of the chamber were filled with

Figure 2. Norepinephrine increases nuclear STAT3. SKOV3 cells incubated on chambered slides were serum-starved for 15 h, and exposed to increasing concentrations of norepinephrine with or without a 1-h pretreatment of propranolol (10 $\mu\text{mol/L}$). After 3 h, cells were fixed in acetone and total STAT3 was detected with immunohistochemistry. **A**, constitutive presence of cytoplasmic STAT3 without norepinephrine stimulation compared with a negative control (no anti-STAT3 antibody). With 1 and 10 $\mu\text{mol/L}$ norepinephrine (NE) exposures (**B**), the nuclear signal increases dramatically, an effect inhibited by pretreatment with propranolol. Additionally, ELISA done on nuclear extracts showed that nuclear total STAT3 quantitatively increased with norepinephrine treatment ($P < 0.05$), and to a similar extent as phorbol 12-myristate 13-acetate (PMA), a known activator of STAT3 (**C**).



serum-free RPMI containing $1 \times \text{MITO}+$ (Collaborative Biomedical). Single cell tumor suspensions were seeded into the upper wells at a concentration of 1×10^5 cells per well. Following a 24-h incubation in a humidified incubator at 37°C with 5% CO_2 , cells that had invaded through the basement membrane were collected through the sideport by replacing the medium in the lower chamber with 2 mmol/L of EDTA/PBS (pH 7.4), for 20 min at 37°C . The cells recovered from the bottom of the filter were then loaded onto a dot blot manifold containing 3- μm pore polycarbonate filters, fixed, stained, and counted by light microscopy. Invasiveness was calculated as the percentage of cells that had successfully invaded through the matrix-coated membrane to the lower wells relative to the total number of cells seeded into the upper wells. The invasion assays were done in triplicate and conducted twice.

Determination of MMP concentration. Serum-free conditioned media from cultures of ovarian cancer cells were collected 3 h following exposure to 10 $\mu\text{mol/L}$ of norepinephrine, with or without prior exposure to siRNA for 24 h. The supernatants were microfuged to remove debris and then stored at -80°C . The samples were thawed only once for determining the MMP concentration. An identical number of cells were plated without the three-dimensional matrix for comparison. The protein concentration of total MMP-2 (pro- and active MMP-2), and total MMP-9 (92 kDa pro- and 82 kDa active forms) were determined using Quantikine immunoassays (R&D Systems) according to the protocols of the manufacturer. The concentrations of active MMP-2 and MMP-9 were determined using the Biotrak Activity Assay System (Amersham Biosciences) according to the manufacturer's protocols. The MMP experiments were done in triplicate and repeated once.

In vivo tumor model. Female nude mice were purchased from the National Cancer Institute-Frederick Cancer Research Facility. The mice were housed and maintained under specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care in accordance with current regulations and standards of the U.S. Department of Agriculture, U.S. Department of Health and Human Services, and the NIH. The mice were used according to

institutional guidelines when they were 8 to 12 weeks of age. SKOV3ip1 tumor cells were harvested from subconfluent cultures by a brief exposure to 0.25% trypsin and 0.02% EDTA. Trypsinization was stopped with medium containing 10% fetal bovine serum. The cells were then washed once in serum-free medium and resuspended in HBSS (serum-free). Only single-cell suspensions with >95% viability, as determined by trypan blue exclusion, were used for the injections. To produce tumors, 1×10^6 SKOV3ip1 cells (in 0.1 mL) were injected s.c. into the right flank of the nude mice. A total of five mice per group were used. Starting 4 days after tumor cell injection, mice were treated with daily injections of PBS (200 μL , i.p.), isoproterenol (10 mg/kg daily, i.p.), or isoproterenol (10 mg/kg) in combination with siRNA (control or STAT3-specific, 3.5 μg in DOPC every 3 days, i.p.) for 7 days (24). All treatments were administered in a total volume of 200 μL . Eight days after tumor cell injection, mice were euthanized by cervical dislocation. Tumors were measured in two dimensions, dissected and fixed in formalin. Tumor volume was calculated as $(\text{length} / 2) \times (\text{width}^2)$. Tumor samples were analyzed using H&E staining. Representative images were taken from each tumor using a light microscope at $40\times$ and $100\times$ magnification.

Statistical analysis. The χ^2 test was used to determine differences between cell counts of the MICS invasion assay using SPSS (SPSS, Inc.). For comparisons between continuous variables, the Student's *t* test was used. $P < 0.05$ was considered statistically significant.

Results

Stress hormone-mediated activation of STAT3. On ligand stimulation, STAT3 is phosphorylated at Tyr⁷⁰⁵, dimerizes, and translocates to the nucleus to transactivate target genes. We first examined the effects of norepinephrine and epinephrine on the levels of pSTAT3. After 15 h in serum-starved medium, cells were exposed to increasing concentrations of norepinephrine and epinephrine (0.1, 1, and 10 $\mu\text{mol/L}$). pSTAT3 levels increased

markedly when exposed to just 0.1 $\mu\text{mol/L}$ of norepinephrine in both cell lines (Fig. 1A), to 0.1 $\mu\text{mol/L}$ of epinephrine in EG, and to 1 $\mu\text{mol/L}$ of epinephrine in SKOV3 (Fig. 1B). There was no effect on total STAT3 levels (data not shown).

When cells were pretreated for 1 h with propranolol, a β_1/β_2 -adrenergic receptor blocker, exposure to even 10 $\mu\text{mol/L}$ of norepinephrine was not able to induce pSTAT3 expression (Fig. 1C). Propranolol also prevented pSTAT3 induction by epinephrine (data not shown). Because norepinephrine and epinephrine interact with both β - and α -adrenergic (αAR) receptors, blockade of α -receptors was also examined. Inhibition of α_1 and α_2 blockers, either individually or in combination, could not prevent pSTAT3 induction (Fig. 1D), suggesting that the effects of catecholamine on the activation of STAT3 were transmitted through βARs .

Norepinephrine induces translocation of STAT3 to the nucleus. We next sought to confirm that once STAT3 was activated, it was directed to the nucleus. Staining of SKOV3 cells in culture chambers after norepinephrine exposure showed that compared with untreated cells (Fig. 2A), increased levels of STAT3 were seen in as little as 15 min (Fig. 2B). The increase in STAT3 was predominantly in the nucleus. Pretreatment with propranolol again prevented the norepinephrine-induced effects on STAT3, suggesting that STAT3 activation is mediated via βARs . Additionally, we analyzed nuclear extracts with ELISA for total STAT3 (Fig. 2C). A significant increase with norepinephrine was observed, and to a similar extent as phorbol 12-myristate 13-acetate, a protein kinase C activator that activates STAT3 through the extracellular signal-regulated kinase pathway (25).

Norepinephrine signals to STAT3 through PKA and is independent of IL-6. We next examined the pathway connecting

norepinephrine to STAT3. Neuroendocrine hormones bind adrenergic receptors and activate the G-protein adenylyl cyclase, which activates the second messenger molecule cyclic AMP. Multiple pathways are activated by cyclic AMP, including those mediated by PKA. Furthermore, STAT3 has previously been shown to be activated through PKA-dependent pathways, such as by platelet-activating factor (26) or transforming growth factor- β_1 (27). To test if PKA was involved in STAT3 activation by norepinephrine, cells were preincubated with the PKA inhibitor KT5720 for 1 h prior to norepinephrine exposure. A concentration of 10 $\mu\text{mol/L}$ of KT5720 was able to prevent STAT3 activation with 10 $\mu\text{mol/L}$ of norepinephrine (Fig. 3A). A less specific protein kinase inhibitor, H89, achieved similar inhibition levels (data not shown). Because norepinephrine is also capable of activating IL-6 (28), one of the most potent and recognized activators of STAT3, we also examined if the norepinephrine stimulation was simply through activation of the IL-6 pathway. Cells were preincubated with anti-IL-6 antibody at 50 $\mu\text{g/mL}$, a concentration previously shown to adequately inhibit IL-6 signaling (29). Norepinephrine-induced STAT3 activation was not inhibited after treatment with the antibody (Fig. 3B), suggesting that catecholamine-mediated STAT3 activation does not occur simply by up-regulating the production of IL-6. To further test this, we used siRNA to inhibit the expression of IL-6 and gp130, a signaling component of the IL-6 receptor. First, it was confirmed that the siRNA constructs could down-regulate their targets (by ELISA of the supernatant for IL-6, and by Western blot for gp130; Fig. 3C). IL-6 and gp130 down-regulation, both alone and in combination, did not reduce norepinephrine-induced STAT3 activation (Fig. 3D). Collectively, these data show that despite the presence of low levels of constitutively activated STAT3, a further

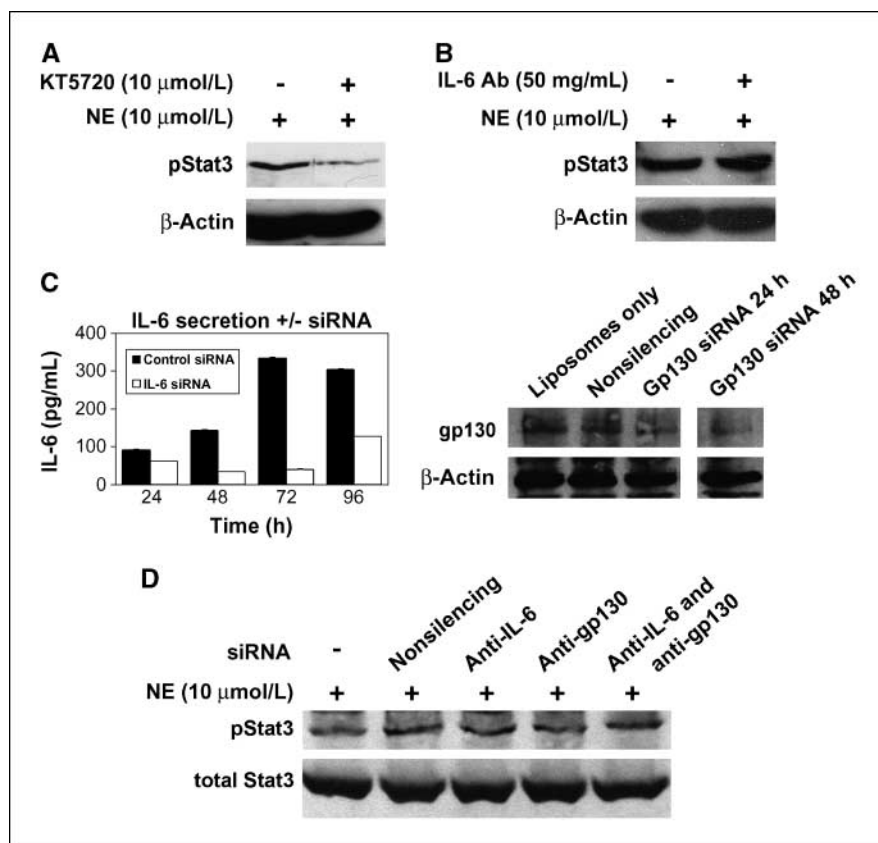
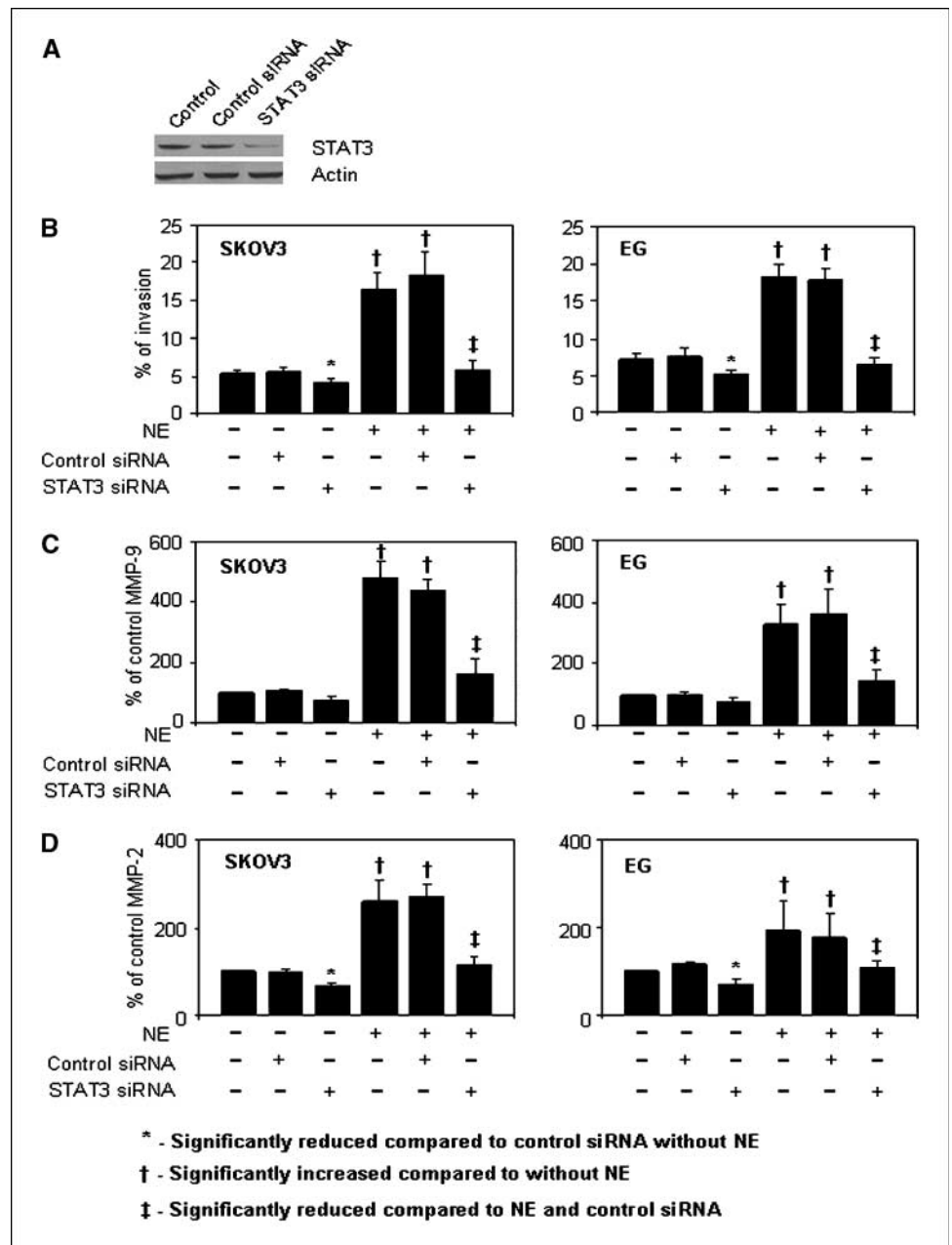


Figure 3. Blockade of PKA, but not IL-6, prevents norepinephrine-induced STAT3 activation. Pretreatment of SKOV3 cells with 1 $\mu\text{mol/L}$ of the PKA-specific inhibitor KT5720 reduces the increase in pSTAT3 seen with norepinephrine (NE), as measured by Western blot (A). However, pretreatment with 50 $\mu\text{g/mL}$ of anti-IL-6 antibody, a dose previously shown to inhibit the activating effect of IL-6 on STAT3, was not effective in reducing the norepinephrine-induced effects (B). Next, we verified IL-6 independence with siRNA against IL-6 or gp130. After confirming that siRNA down-regulated IL-6 (tested by ELISA) and gp130 (tested by Western blot; C), we found that siRNA-mediated down-regulation of IL-6, gp130, or the combination of both, did not reduce norepinephrine-induced pSTAT3 (D).

Figure 4. STAT3-mediated increases in cellular invasion and MMP expression with norepinephrine. After confirmation that an siRNA construct could reduce STAT3 expression by >80% (A), SKOV3 or EG cells were exposed to 10 $\mu\text{mol/L}$ of norepinephrine (NE) alone and with STAT3-specific siRNA or control siRNA, and were introduced into the MICS system to determine the effects on invasion (B). The percentage of cells invading through a human-defined matrix increased by 2.6- to 3.1-fold with norepinephrine, and was significantly reduced to baseline levels with STAT3-siRNA therapy in the SKOV3 and EG cell lines. Similar groups were examined for MMP production. Norepinephrine exposure resulted in a 4.8-fold increase in MMP-9 in SKOV3, and a 3.2-fold increase in EG (C). Norepinephrine lead to a 2.6- and 1.9-fold increase in MMP-2 in SKOV3 and EG, respectively (D). Treatment with STAT3-siRNA prevented norepinephrine-induced MMP up-regulation in both lines, whereas control siRNA had no effect.



increase in STAT3 is achieved with norepinephrine that is independent of IL-6 and is dependent on PKA.

Effect of STAT3 blockade on the invasiveness of ovarian cancer cells and production of MMP-2 and MMP-9. We have previously shown that catecholamines promote the invasion of ovarian cancer cells and stimulate the production of MMP-2 and MMP-9 levels (9). Based on growing information regarding the role of STAT3 in the production of MMPs and cancer cell invasion (30, 31), we asked whether such a mechanism could be operative in stress hormone-mediated invasion of ovarian cancer cells. After confirming that STAT3 could be down-regulated by >80% with siRNA (Fig. 4A), the MICS chamber system was used to quantify cell invasion with norepinephrine with or without STAT3 down-regulation (Fig. 4B). Norepinephrine treatment stimulated the invasion of SKOV3 cells by 3.1-fold ($P < 0.01$). Similar increases

in invasion were observed with the EG cells (2.6-fold increase, $P \leq 0.01$). The STAT3-siRNA completely blocked the norepinephrine-induced increase in invasion, whereas the control siRNA had no effect. Because some studies have shown the effects of STAT3 activation on cell proliferation (11), we asked whether the invasion rates could be affected by changes in proliferation. The effects of norepinephrine and epinephrine on ovarian cancer cell proliferation were examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Neither of these two catecholamines affected cell proliferation despite testing multiple doses and time periods (data not shown). These data suggest that the effects on invasion are independent of proliferation.

We also used STAT3-siRNA to determine the contribution of STAT3 activation for stimulation of MMP-9 and MMP-2 production (Fig. 4). Consistent with our previous results (9), norepinephrine

induced a 3.2- to 4.8-fold increase in MMP-9 levels, and a 1.9- to 2.6-fold increase in MMP-2 levels in the SKOV3 and EG cell lines. STAT3 silencing with siRNA resulted in the blockade of catecholamine-mediated increases in both MMP-2 and MMP-9 levels.

Effect of STAT3 silencing on stress hormone-stimulated tumor growth and infiltration *in vivo*. We have previously shown that a nonspecific β -agonist, isoproterenol, stimulated the *in vivo* growth and infiltration of ovarian cancer cells (9). We have recently characterized a novel method of *in vivo* siRNA delivery using a neutral liposome (DOPC; ref. 21), and here, used this approach to test the role of STAT3 in tumor growth infiltration *in vivo*. Female nude mice were injected s.c. with SKOV3ip1 cells and treated according to the following groups ($n = 5$ per group): (a) PBS control, (b) control siRNA-DOPC, (c) STAT3 siRNA-DOPC, (d) daily isoproterenol, (e) daily isoproterenol + control siRNA-DOPC, and (f) daily isoproterenol + STAT3 siRNA-DOPC for 7 days. The tumor volume in the daily isoproterenol was significantly larger (by 846%, $P < 0.001$) and deeply infiltrating compared with the PBS controls (Fig. 5). Control siRNA-DOPC had no effect on basal or isoproterenol-stimulated tumor growth and infiltration. STAT3 siRNA-DOPC reduced tumor growth by 47% ($P < 0.01$) under basal conditions. Furthermore, STAT3 siRNA-DOPC completely blocked isoproterenol-stimulated tumor growth, reducing tumor volume by 85%.

Discussion

The key findings in this study are that the stress hormones norepinephrine and epinephrine are capable of activating STAT3, with subsequent translocation to the nucleus. The pathway is

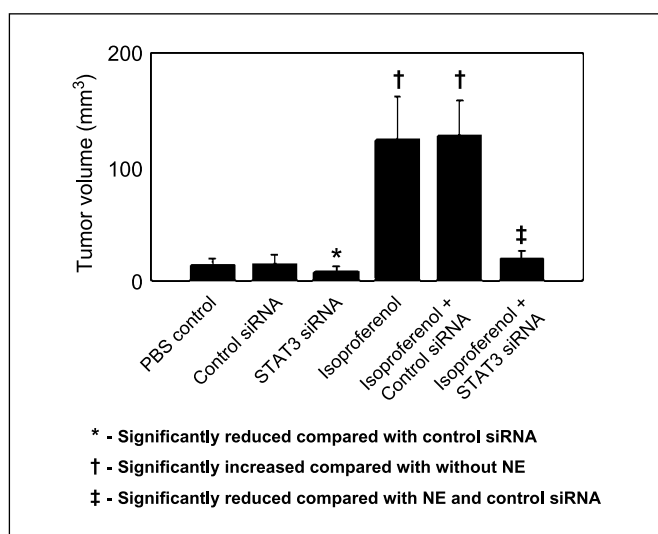


Figure 5. siRNA-directed STAT3 down-regulation prevents stress-induced tumor growth. Mice were injected s.c. with SKOV3 cells, and 24 h later, were treated via i.p. injection with PBS or the β AR agonist isoproterenol (10 mg/kg). With or without isoproterenol, mice were treated with vehicle, a control nonsilencing siRNA in the delivery liposome DOPC, or STAT3-targeting siRNA in DOPC. After 7 d of treatment, tumor volumes were measured and volume calculated as described in Materials and Methods. Tumor volume was reduced by 47% with administration of STAT3-specific siRNA in the liposomal delivery vector DOPC, in which control siRNA in DOPC did not reduce tumor volume. Isoproterenol administration led to an 8.5-fold increase in tumor volume, which was not affected by treatment with a control siRNA in DOPC, but was almost completely abrogated by STAT3-specific siRNA in DOPC.

independent of IL-6/gp130 and proceeds through β ARs and PKA in a rapid fashion. Furthermore, targeting STAT3 with siRNA abrogates stress-induced MMP production, tumor cell migration, and *in vivo* tumor growth.

An association between stress and malignant progression has long been suspected through retrospective analysis of incidence as well as prospective interventional studies (3, 32, 33). However, there is a paucity of data delineating the biological mechanisms involved. To date, most studies examining the mechanisms connecting stress and cancer progression have found indirect effects, such as those mediated through the immune system (33, 34). We sought to identify specific proteins and pathways that may link stress directly to malignant cell behavior. The likely candidates are those that mediate the stress response: norepinephrine, epinephrine, and cortisol. Higher levels of social support are associated with lower levels of these hormones (35), and interventions that decrease stress correspondingly decrease stress hormones (36). We have previously shown that patients with increased stress have higher circulating vascular endothelial growth factor (VEGF) levels (37), and that neuroendocrine hormones can induce VEGF production by ovarian cancer cells (8). VEGF has long been recognized as an important mediator of angiogenesis and cancer progression. Interestingly, VEGF expression has recently been associated with STAT3 activation at a tissue level (38). Collectively, these findings suggest that neuroendocrine hormones may activate both VEGF and STAT3 pathways separately, explaining this association.

The doses of norepinephrine used for our studies were selected to reflect the physiologic conditions of this hormone at the level of the tumor. Although circulating plasma levels of norepinephrine are only ~ 10 to 1,000 pmol/L in a normal individual, and may reach as high as 100 nmol/L in conditions of stress (39), catecholamine levels in the ovary are at least 100 times higher. Studies suggest that within the parenchyma of the ovary, and thus, the tumor microenvironment, concentrations may reach as high as 10 μ mol/L (40). This underlies the importance of studying STAT3 activation at all ranges of norepinephrine to which tumor cells might be exposed *in vivo*. Although measuring these hormones accurately in humans is challenging, and subject to some level of error with the standard assays used, the broad range we have examined and the uniform results seen at higher concentrations leads us to believe that they are appropriate for studying the relationship of neuroendocrine hormones and STAT3.

IL-6 is a potent activator of STAT3. Thus, we examined whether neuroendocrine hormones activated STAT3 independently, or simply via the stimulation of IL-6 production. Both incubation with IL-6 antibody at a dose known to inhibit IL-6-mediated activation (29), and inhibition of IL-6 and/or gp130 (a component of the IL-6 receptor) with siRNA were not effective in preventing norepinephrine-induced STAT3 activation. This is certainly not to suggest that IL-6-mediated STAT3 activation is less important to malignant progression, but rather these findings show that STAT3 can be activated by alternate pathways.

Neuroendocrine signaling generally proceeds through β ARs, cyclic AMP, and PKA. Related studies have suggested a similar relationship, but with important differences. Activation of STAT3 through cyclic AMP was shown in the normal mouse heart (41), and in differentiation by glioma cells (29), but in both of these instances, signaling was mediated by IL-6 activation. In normal thyroid cells and thyroid receptor-expressing Chinese hamster ovary cells, thyroid-stimulating hormones lead to STAT3 activation through cyclic AMP, but through protein kinase C and independent

of PKA (42). These data suggest that in ovarian cancer cells, the machinery exists whereby STAT3 could be activated directly, instead of through intermediaries such as protein kinase C and IL-6. Part of the answer may lie in the activity of the p300/cyclic AMP-responsive element-binding protein, a protein whose function may be altered in cancer (43).

The mechanism by which PKA may lead to activation of STAT3 is an interesting question, given that PKA activates through serine phosphorylation, and STAT3 is activated by tyrosine kinase phosphorylation. STAT3 activation through a PKA-dependent pathway has been previously shown with platelet-activating factor (26) and transforming growth factor- β 1 (27), so the fact that such signaling could go through PKA is not a new finding. The mechanism through which this may occur, however, has not been clearly shown. Src, which is a known activator of STAT3, can be activated by PKA (44). The serine site phosphorylated by PKA can then participate in autophosphorylation of the src tyrosine site. Further delineation of this pathway will be necessary, but suggests a possibly important role for src in transferring signals from serine kinases to downstream tyrosine kinases.

MMPs play a significant role in tumor cell invasion and migration, and previous reports have shown that norepinephrine could induce MMP production in both ovarian and head and neck cancers (9, 10). There have been prior reports suggesting a relationship between STAT3 and MMP production. STAT3 has been shown to be a transcriptional regulator of MMP-2 in human melanoma cells (45, 46). STAT3 is required for the maximal induction of MMP-1 by epidermal growth factor receptor in bladder cancer cells (47) and by fibroblast growth factor-1 in

prostate cancer cells (48). STAT3 activation is strongly correlated with MMP expression in breast cancer (49), and the relationship is implicated in carcinogenesis. In our stress model of ovarian cancer, isoproterenol injections as well as physiologic stressors significantly increase tumor growth, metastasis, and invasion. Given the results of these data, stress-mediated malignant progression may be primarily mediated through the up-regulation of STAT3 and activation of its multiple carcinogenic downstream effector pathways such as MMPs.

The relationship between neuroendocrine hormones and STAT3 activation warrants further study, both from the standpoint of identifying possible alternate signaling pathways through adrenergic receptors on cancer cells, and from the clinical perspective of inhibiting neuroendocrine signaling with available β -adrenergic blockers, or STAT3 with *in vivo* siRNA methods. The multiple emerging pathways relating conditions of chronic stress to carcinogenesis represent new and exciting areas of study for understanding and treating cancer in affected patients.

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