THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 41, pp. 29919–29926, October 12, 2007

Stress Hormones Regulate Interleukin-6 Expression by Human Ovarian Carcinoma Cells through a Src-dependent Mechanism*

Received for publication, December 18, 2006, and in revised form, June 21, 2007 Published, JBC Papers in Press, August 23, 2007, DOI 10.1074/jbc.M611539200

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Recent studies have demonstrated that chronic stress promotes tumor growth, angiogenesis, and metastasis. In ovarian cancer, levels of the pro-angiogenic cytokine, interleukin 6 (IL-6), are known to be elevated in individuals experiencing chronic stress, but the mechanism(s) by which this cytokine is regulated and its role in tumor growth remain under investigation. Here we show that stress hormones such as norepinephrine lead to increased expression of IL-6 mRNA and protein levels in ovarian carcinoma cells. Furthermore, we demonstrate that norepinephrine stimulation activates Src tyrosine kinase and this activation is required for increased IL-6 expression. These results demonstrate that stress hormones activate signaling pathways known to be critical in ovarian tumor progression.

Chronic stress, which results in increased production of catecholamines such as norepinephrine (NE)[3] and epinephrine from the adrenal medulla and sympathetic neurons, has long been believed to adversely influence health (1). While elevated levels of such stress mediators are known to be associated with consequences such as increased risk of heart disease and infection (2–4), several studies have indicated that chronic stress can also promote tumor development and progression (5). For example, chronic stress has been associated with an increased incidence of mammary tumors in female mice carrying the Bittner oncogenic virus (6), and experimentally stressed mice exhibited accelerated development of ultraviolet-induced cutaneous tumors compared with non-stressed animals (7, 8). We have recently demonstrated that chronic stress resulted in greater tumor burden and markedly increased vascularization in orthotopic models of ovarian cancer (9). These studies demonstrate that chronic stress influences tumor progression, but to date, the specific underlying mechanisms responsible for this outcome are poorly understood.

A growing number of studies have demonstrated that chronic stress and other behavioral factors have specific effects on the immune system of cancer patients (10). These effects include decreased natural killer (NK) cell cytotoxicity, weakened response of NK cells to recombinant interferon γ, and reduced T-cell response to mitogen stimulation (10, 11). However, additional factors are known to play a major role in tumor growth; therefore, we asked whether stress mediators could directly alter tumor cell production of cytokines known to contribute to tumor progression.

Angiogenesis, the formation of new blood vessels from the preexisting vasculature, is necessary for tumor expansion and ascites formation and is regulated by the equilibrium of proangiogenic and antiangiogenic molecules (12, 13). Ovarian cancer cells have been shown to express β-adrenergic receptors, and stimulation of these cells with catecholamines results in enhanced expression of an important pro-angiogenic factor, vascular endothelial growth factor (9, 14). These findings suggest that stress hormones may promote tumor angiogenesis. Interleukin-6 (IL-6) has been implicated as an important molecule in tumor progression and angiogenesis (15). Elevated levels of IL-6 are frequently detected in the serum of ovarian cancer patients and are associated with a poor prognosis and increased tumor burden (16, 17). IL-6 has been shown to be secreted by ovarian cancer cells and to facilitate tumor cell proliferation (18), migration (19), and chemotherapy resistance (20, 21). The receptor for IL-6 (IL-6R) is expressed on endothelial cells, and stimulation of ovary and mesentery-derived endothelial cells with exogenous IL-6 results in enhanced cell migration *in vitro* (15). Furthermore, IL-6 is a potent angiogenic cytokine *in vivo* (15).

Previously, IL-6 has been linked to chronic stress, in that individuals experiencing chronic stress have been shown to exhibit elevated circulating levels of IL-6 (22). Moreover,

* This work was supported in part by National Institutes of Health Grants CA109298 and CA110793, The Marcus Foundation, the University of Texas M.D. Anderson Cancer Center SPORE in Ovarian Cancer (P50 CA 083639), a Program Project development grant from the Ovarian Cancer Research Fund, Inc., and the Zarrow Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[3] The abbreviations used are: NE, norepinephrine; NK, natural killer; IL, interleukin; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; RIPA, radioimmune precipitation buffer; PBS, phosphate-buffered saline; siRNA, short interfering RNA; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine; Bt_2cAMP, dibutyryl cyclic AMP.

behavioral factors have been associated with circulating and ascites IL-6 levels in ovarian cancer patients (23). Epinephrine has been shown to increase IL-6 gene expression in adipose tissue (24), and norepinephrine infusion increases IL-6 expression in myocytes (25). However, whether tumor cell expression of IL-6 is influenced by chronic stress is unknown. In the current study, we evaluated the effect of stress-related hormones on IL-6 production by ovarian cancer cells and found that catecholamines enhance IL-6 promoter activity and protein levels. Moreover, the effect of norepinephrine on IL-6 expression is mediated through β-adrenergic receptors and requires the subsequent activation of the Src proto-oncogene product.

EXPERIMENTAL PROCEDURES

Cell Culture—SKOV3.ip1 is a variant derived from SKOV3 cells (ATCC, Manassas, VA) that was established from the ascites fluid of female nude mice following intraperitoneal injection of the parental line (26). The human ovarian cancer cell line Hey-A8 was a kind gift from Dr. Gordon B. Mills (The University of Texas M. D. Anderson Cancer Center, Houston, TX). EG cells have been described previously (14). Cell lines were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, vitamins, sodium pyruvate, L-glutamine, and nonessential amino acids (Invitrogen).

To evaluate the effect of stress hormones on IL-6 secretion, 1.0×10^5 SKOV3.ip1, Hey-A8, and EG cells were seeded into individual wells of a 24-well plate. Following a 24-h incubation, triplicate cultures (wells) were stimulated with 500 μl of media containing norepinephrine, epinephrine, or the synthetic β-adrenergic receptor agonist, isoproterenol (Sigma), at 0-, 0.1-, 1-, and 10-μM concentrations. After 1, 3, and 6 h, medium was collected, centrifuged, and tested for the presence of IL-6 by ELISA (R&D Systems, Minneapolis, MN). The doses of catecholamines used for our studies were selected to reflect physiologic conditions of these hormones at the level of the tumor. While circulating plasma levels of norepinephrine range from 10 to 1000 pM in a normal individual and may reach as high as 100 nM in conditions of stress (27), 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 μM (28, 29).

IL-6 mRNA Assay by Real-time Reverse Transcription PCR—Total cellular RNA was isolated (Qiagen RNeasy) and treated with RNase-free DNase (Qiagen), both according to the manufacturer's protocol. 10% of the resulting RNA was assayed using a one-step real-time reverse transcription PCR protocol (Bio-Rad iQ SYBR enzyme mix in a Bio-Rad iCycler), with 30 min of reverse transcription at 60 °C, 15 min of reverse transcriptase inactivation at 95 °C, and 40 cycles of DNA amplification involving 60 s of annealing and extension at 60 °C followed by 15 s of melting at 95 °C (15 s). Assays used commercial primers for human glyceraldehyde-3-phosphate dehydrogenase (forward, GAAGGTGAAGGTCGGAGTC; reverse, GAAGATGGTGATGGGATTTC; probe, CAAGCTTCCCGTTCTCAGCC) and IL-6 (forward, GTGTGAAGCAGCAAAGAAGC; reverse, CTGGAGGTACTCTAGGTATAC; probe, GGATTCAATGAGGAGACTTGC). SYBR Green fluorescence intensity data were analyzed using iCycler™ software, with gene induction measured as the change in glyceraldehyde-3-phosphate dehydrogenase-normalized threshold cycle numbers (Ct):-fold change $= 2^{\text{Ct experimental} - \text{Ct control}}$.

Western Blot Analysis—Cell lysate was prepared with modified radioimmune precipitation (RIPA) lysis buffer as previously reported (30). Protein concentrations were determined using a BCA protein assay reagent kit (Pierce). 50 μg of whole cell lysate protein was subjected to 15% SDS-PAGE separation and transferred to a nitrocellulose membrane via wet transfer (Bio-Rad Laboratories). Nonspecific sites were blocked with 5% nonfat milk and incubated with IL-6-specific antibody (1:500; R&D Systems, Minneapolis, MN) overnight at 4 °C. Primary antibody was detected utilizing anti-goat IgG (Roche Applied Science) and developed with a chemiluminescence detection kit (PerkinElmer). β-Actin antibody (1:3,000; Sigma) confirmed equal loading.

IL-6 Promoter Activity—SKOV3, SKOV3.ip1, and HEY-A8 cells were transfected with 1 μg of a luciferase reporter construct driving production of firefly luciferase from a 651-bp sequence upstream of the transcription start site for the human *IL6* gene. Transfections were carried out using Lipofectamine 2000 reagent (Invitrogen) in OPTI-MEM-reduced serum according to the manufacturer's protocol. 1 h after transfection, cells were treated with pharmacologic antagonists for 30 min, followed by NE exposure for 90 min. Cells were washed in phosphate-buffered saline, lysed, and assayed for firefly luciferase activity using standard substrate reagents (Promega, Madison, WI) and a Bacterial Systems BG-1 luminometer (GEM Biomedical, Sparks, NV). To determine potential regions of the IL-6 promoter responsible for mediating stress hormone-mediated transcriptional activation, we generated luciferase expression vectors driven by variants of the human IL-6 promoter bearing point mutations in the cAMP-response element (CRE) or C/EBP-β (NFIL6)-response element, as previously described (31).

Receptor Inhibitors and cAMP Agonists—To inhibit activation of β-adrenergic receptors, SKOV3.ip1 and Hey-A8 cells were seeded into 24-well plates as described above. Cells were pretreated with the β_2-adrenergic receptor inhibitor, DL-propranolol (Sigma-Aldrich) at a concentration of 1 μM. Following a 1-h incubation, medium was removed and cells were stimulated with 10% fetal bovine serum DMEM containing 10 μM NE with or without 1 μM propranolol for 3 h. Similarly, cells were incubated with the adenylyl cyclase activator forskolin (10 μM) (Sigma Aldrich) or 100 μM of an analogue of cAMP that specifically activates Epac (8-(4-chloro-phenylthio)-2′-*O*-methyladenosine-3′, 5′-cyclic monophosphate [8CPT-2Me-cAMP]; (Biolog Life Science Institute, Bremen, Germany) for 3 h. Conditioned medium was collected, centrifuged, and tested for the presence of IL-6 by ELISA.

Detection of Src Phosphorylation—SKOV3.ip1 cells (1×10^6) were seeded into 10-cm dishes in 10% fetal bovine serum/DMEM. One day later the cells were incubated in serum-free DMEM in the presence or absence of the Src inhibitor AP23846 (1 μM; Ariad Pharmaceuticals, Cambridge, MA) for 18 h. Cells were then stimulated for 5, 10, 15, or 45 min with serum-free DMEM containing 10 μM NE in the presence or absence of 1 μM

AP23846. Control cells were incubated in serum-free medium. Cells were then washed in ice-cold PBS, and cells were lysed by scraping in RIPA B buffer (20 mM sodium phosphate buffer, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate), supplemented with 1% aprotinin, 20 μM leupeptin, and sodium orthovanadate (1 mM, pH 7.4). Protein lysates were cleared by centrifugation at 15,000 $\times$ *g* for 15 min. 500 μg of sample protein in 650 μl of RIPA buffer was rotated overnight with 6 μl of antibody against total c-Src (mAb 327; Oncogene Sciences, Cambridge, MA). 50 μl of a 1:1 slurry of protein G-agarose in RIPA buffer was added to each sample, and rotation was continued for an additional hour. Bound proteins were pelleted by centrifugation, washed with RIPA buffer, and eluted by boiling in Laemmli's sample buffer. 50 μg of protein was separated on an 8% SDS-PAGE gel, transferred onto a nitrocellulose membrane, immunoblotted using phospho-Src^{Y418}-specific antibodies (1:1000; Cell Signaling Technology), and stripped and reprobed with antibodies against total Src (1:1000; Oncogene Sciences).

In Vivo Tumor Model—Female C.B-17/IcrHsd-*Prkdc*^{SCID} mice were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). The mice were housed and maintained under specific pathogen-free conditions in facilities approved by the American Association for Accreditation of 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 National Institutes of Health. The mice were used according to institutional guidelines when they were 8–12 weeks of age. SKOV3.ip1 tumor cells were harvested from subconfluent cultures by a brief exposure to 0.25% trypsin and 0.02% EDTA. Trypsinization was neutralized with fetal bovine serum-containing medium. The cells were then washed once in serum-free medium and suspended in serum-free Hanks' balanced salt solution. Only single-cell suspensions with greater than 95% viability, as determined by Trypan blue exclusion, were used for the injections. To produce tumors, 2×10^6 SKOV3.ip1 cells (in 0.1 ml) were injected subcutaneously into the right flank of each mouse (5 mice/group). Three days after tumor cell injection, mice were treated with daily injections of PBS (200 μl, intraperitoneal (IP)), isoproterenol (10 mg/kg daily IP), or isoproterenol with siRNA-DOPC (control or IL-6-specific, 3.5 μg IP twice per week) for 3 weeks. All treatments were administered in a total volume of 200 μl. Three weeks after tumor cell injection, all mice were sacrificed. Tumors were measured and harvested. Tumor volume was calculated as (length/2) $\times$ (width^2).

Short Interfering RNA (siRNA) Preparation—We purchased siRNAs targeted against IL-6 (target sequence 5′-CCCAGGAG-AAGAUUCCAAAGAUGUA-3′ from Qiagen and incorporated them into a neutral liposome (1,2-dioleoyl-sn-glycero-3-phosphatidylcholine; DOPC), as previously described (32, 33).

Statistical Analysis—Changes in IL-6 levels were analyzed with an analysis of variance (both factors treated as repeated measures). A p value ≤ 0.05 was considered statistically significant.

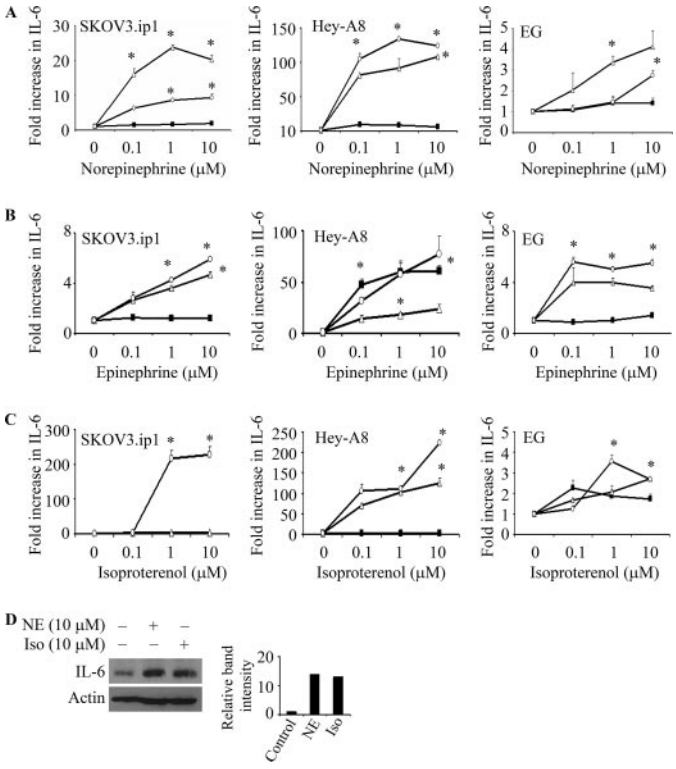

FIGURE 1. **Catecholamines enhance IL-6 secretion by tumor cells.** Human ovarian cancer cell lines (*SKOV3.ip1*, *Hey-A8*, and *EG*) were stimulated with 0.1-, 1-, or 10-μM concentrations of norepinephrine (*A*), epinephrine (*B*), or isoproterenol (*C*). Medium was collected after 1 h (*closed square*), 3 h (*open triangle*), or 6 h (*open oval*) and tested for the presence of IL-6 by ELISA. Data are graphed as mean -fold increase in IL-6 secretion relative to basal level of IL-6 secretion with *error bars* representing S.D. *, $p < 0.005$. *D*, SKOV3.ip1 cells were stimulated with norepinephrine 10 μM or isoproterenol 10 μM for 3 h, and protein was obtained from cell lysate for Western blot analysis using an IL-6 antibody. The quantification of band intensity relative to β-actin intensity is shown on the *right*.

RESULTS

Adrenergic Stimulation of Ovarian Cancer Cells Enhances IL-6 Expression—To examine the effect of stress hormones on IL-6 expression in ovarian tumor cell lines, SKOV3.ip1, Hey-A8, and EG cells were stimulated with increasing concentrations of norepinephrine, epinephrine, or isoproterenol, and culture supernatants were assayed for IL-6 by ELISA (Fig. 1, *A*–*C*). In all three human ovarian carcinoma cell lines stimulated with isoproterenol or physiological stress levels of norepinephrine or epinephrine, a significant increase in IL-6 secretion resulted. Specifically, stimulation of SKOV3.ip1 cells with norepinephrine resulted in a peak >20-fold increase in IL-6 production at the 3-h time point ($p = 0.004$). The mean basal level of IL-6 secretion by SKOV3.ip1 cells at 3 h was 6.4 + 2.25 pg/ml, and after treatment with 1 and 10 μM norepinephrine IL-6 levels increased to 152.38 + 5.12 and 129.47 + 8.70, respectively. Similarly, norepinephrine led to a 4-fold increase in IL-6 secretion by EG cells ($p = 0.02$) and a 120-fold increase in IL-6 secretion by Hey-A8 cells ($p < 0.001$). Stimulation of human ovarian cancer cell lines with epinephrine also increased IL-6 production. Specifically, Hey-A8 cells treated with 10 μM epinephrine produced 656.06 + 151.02 pg/ml of IL-6, resulting in a 77-fold increase ($p = 0.018$) in comparison to controls. Epinephrine induced an ~5-fold increase in IL-6 secretion in both

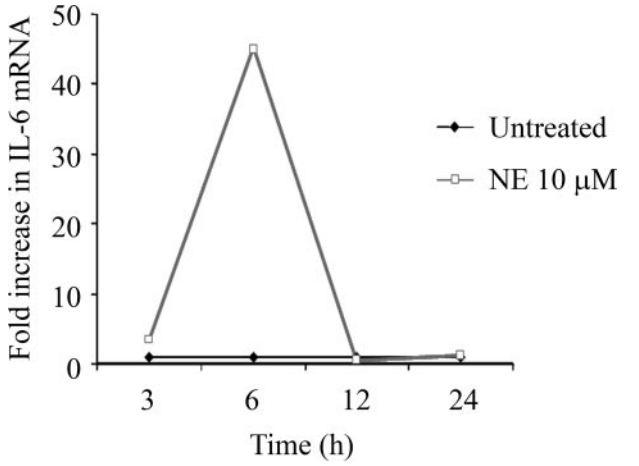

FIGURE 2. **Norepinephrine increases IL-6 RNA.** SKOV3.ip1 cells were stimulated with 10 μM norepinephrine for the indicated times. Total RNA was isolated and real-time RT-PCR was used to quantify IL-6 RNA.

SKOV3.ip1 ($p = 0.001$) and EG cells ($p < 0.001$). Moreover, the synthetic β-adrenergic receptor agonist isoproterenol also induced a significant increase in IL-6 secretion. Following treatment of Hey-A8 and SKOV3.ip1 cells, we observed an ~200-fold increase in IL-6 expression ($p < 0.004$; Fig. 1). Western blot analysis of the SKOV3.ip1 cells treated with norepinephrine or epinephrine confirmed the increases in IL-6 protein observed with ELISA (Fig. 1*D*).

Norepinephrine Stimulation Increases IL-6 RNA—To more clearly define the mechanisms by which catecholamines regulate IL-6 production by ovarian cancer cells, we examined transcriptional changes in IL-6. SKOV3.ip1 cells were stimulated with 10 μM norepinephrine, and IL-6 mRNA levels were quantified using real-time RT-PCR at multiple time points ranging from 3 to 24 h. The level of IL-6 mRNA was normalized against 18 S RNA levels. As shown in Fig. 2, norepinephrine increased IL-6 mRNA by 45-fold at 6 h, and IL-6 mRNA levels returned to base line within 12 h. Norepinephrine had a similar effect on other ovarian cancer cell lines (data not shown).

Norepinephrine Increases IL-6 Promoter Activity—To determine whether increased transcriptional activity of the *IL6* promoter was responsible for norepinephrine enhancement of *IL6* gene expression, we examined the effects of norepinephrine on a luciferase reporter construct driven by 651-bp of human DNA sequence upstream of the *IL6* transcription start site (34). When this construct was transfected into SKOV3.ip1 cells, norepinephrine enhanced luciferase production by ~10-fold within 3 h (Fig. 3), suggesting that increased IL-6 mRNA was due to increased transcription rather than increased stability. Similar effects were observed in SKOV3 and Hey-A8 cell lines (data not shown). Pharmacologic stimulation of the cellular cAMP signaling pathway with Bt_2cAMP also enhanced activity of the *IL6* promoter, suggesting that the effects of norepinephrine might be mediated by the G-protein-linked receptors that activate the adenylyl-cyclase/cAMP system. The ~10-fold induction by catecholamine was similar in magnitude to that induced by the protein kinase C activator phorbol 12-myristate 13-acetate, which functioned as a positive control in these studies.

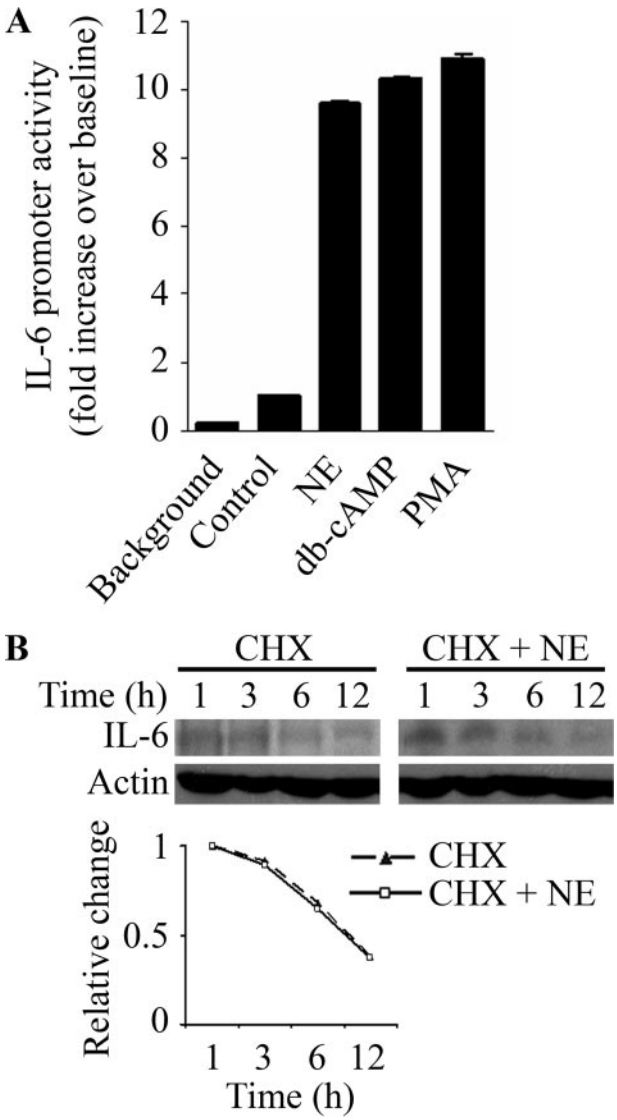

FIGURE 3. **Effects of norepinephrine on activity of the human *IL6* promoter.** *A*, SKOV3.ip1 cells were transfected with a luciferase reporter construct driving firefly luciferase expression from 651 bp of human DNA sequence upstream of the *IL6* gene transcription start site. 1 h after transfection, cells were exposed to 10 μM norepinephrine (*NE*) or an equivalent volume of PBS vehicle, and firefly luciferase activity was assayed 2 h later. Data represent the mean (±S.E.) of triplicate assay determinations, with relative light unit data normalized to values in transfected cells treated with vehicle control. Background luminance was determined in untransfected cells processed in parallel. *B*, effect of NE on IL-6 protein stability. SKOV3.ip1 cells were treated with NE (10 μM) in the presence of the translation inhibitor cycloheximide (*CHX*, 1 μM). Shown are the results of IL-6 and actin protein expression (*top*) and of densitometric scans (*bottom*).

To examine the effects of norepinephrine at the post-translational level, IL-6 protein levels in SKOV3.ip1 cells were evaluated in the presence of cycloheximide, 1 μg/ml. Norepinephrine had no effect on the degradation of the IL-6 protein (Fig. 3*B*). These results indicate that norepinephrine increases IL-6 levels by transcriptional activation rather than by affecting protein stability.

Induction of IL-6 Expression by Stress Hormones Requires Activation of β-Adrenergic Receptors—Human ovarian carcinoma cell lines express β-1 and β-2 adrenergic receptors that can activate the cAMP signaling pathway through G-protein-

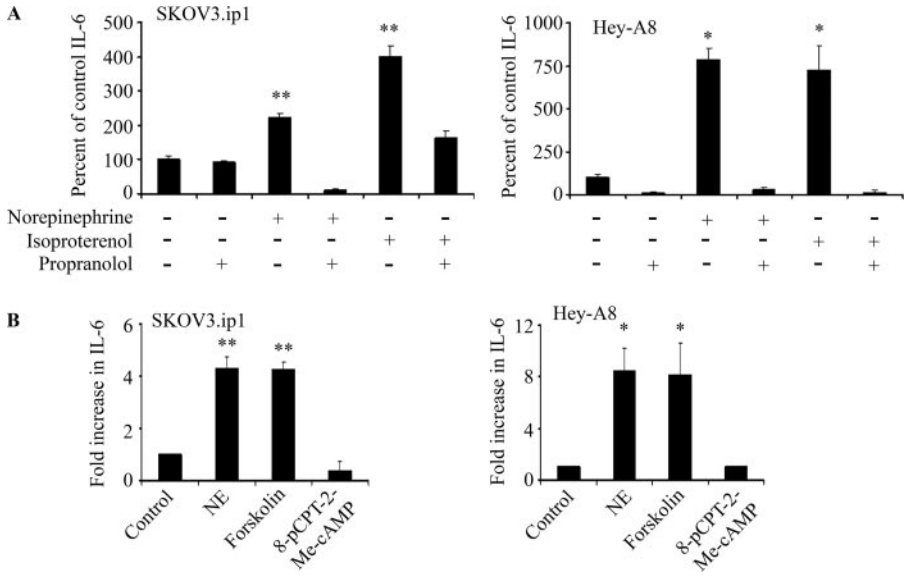

FIGURE 4. **IL-6 production is regulated through the β-adrenergic receptor and cAMP.** *A*, SKOV3.ip1 and Hey-A8 cells were pretreated with 1 μM β-adrenergic receptor inhibitor propranolol and then stimulated with 10 μM norepinephrine or isoproterenol for 3 h. Data are represented as percentage of the control (medium only), which was set to 100%. Data represent the mean of triplicate experiments with *error bars* representing S.D. *B*, SKOV3.ip1 and Hey-A8 cells were stimulated with control medium or medium containing 10 μM norepinephrine, 10 μM forskolin, or 100 μM 8-pCPT-2-Me-cAMP for 3 h. Medium was tested for the presence of IL-6 by ELISA, and IL-6 concentration is graphed as mean -fold increase in IL-6 production over control. *, $p < 0.05$; **, $p < 0.001$. *Bars*, ±S.D.

mediated stimulation of adenylyl cyclase (14). To determine whether IL-6 expression could be regulated through β-adrenergic receptors, SKOV3.ip1 and Hey-A8 cells were treated with 1 μM propranolol, a nonspecific β-adrenergic receptor antagonist, for 1 h prior to stimulation with 10 μM norepinephrine or isoproterenol. As shown in Fig. 4*A*, induction of IL-6 by norepinephrine and isoproterenol was blocked by propranolol. These effects were specific to β-adrenergic receptors, as the adrenergic antagonist prazosin (5 μM) had no effect on the ability of stress hormones to induce IL-6 production (data not shown). Because β-adrenergic receptors are known to signal via cAMP, we stimulated SKOV3.ip1 and Hey-A8 cells with the adenylyl cyclase activator forskolin and found an increase in IL-6 production similar to that induced by norepinephrine (Fig. 4*B*). Stimulation of SKOV3.ip1 cells with forskolin resulted in a 4-fold increase in IL-6 expression ($p < 0.001$), and forskolin induced an ~8-fold increase in Hey-A8 IL-6 production ($p = 0.034$). Because stimulation of the β-adrenergic receptors has been shown previously to also lead to activation of Epac (exchange protein directly activated by cAMP) (35, 36), we sought to determine whether activation of Epac could contribute to IL-6 expression in human ovarian carcinoma cells. The SKOV3.ip1 and Hey-A8 cells were treated with 100 μM 8-CPT-2Me-cAMP, a cAMP analog that specifically targets Epac. In both cell lines, 8-CPT-2Me-cAMP did not increase secretion of IL-6 (Fig. 4*B*).

Induction of IL-6 Expression by Norepinephrine Is Src-dependent—Recent reports indicate a role for the protein tyrosine kinase Src in β-adrenergic receptor signal transduction (37). Based on suggested IL-6 regulation by Src (38, 39), we asked whether the catecholamine-mediated IL-6 production was Src-dependent. Following serum starvation for 18 h, the SKOV3.ip1 cells were incubated with 10 μM norepinephrine in serum-free medium for 1–45 min. Western blot analysis revealed that by 10 min after NE stimulation, Src phosphorylation increased, and the maximum increase in Src phosphorylation at Tyr-418, indicative of increased Src activity, occurred after 45 min (Fig. 5*A*). Additionally, the Src-selective inhibitor AP23846 (40) completely inhibited norepinephrine-induced Src phosphorylation. To determine whether Src phosphorylation is required for the stimulation of IL-6 production by norepinephrine, SKOV3.ip1 cells were pretreated in control medium or medium containing AP23846 for 18 h and then stimulated for 3 h with medium containing 10 μM norepinephrine in the presence or absence of AP23846. ELISA analysis of IL-6 in conditioned medium was then performed. The results demonstrated that inhibition of Src activity by AP23846 blocked the stimulatory effect of norepinephrine on IL-6 secretion (Fig. 5*B*). Furthermore, AP23846 inhibited forskolin-induced IL-6 expression in SKOV3.ip1 cells. Similar results were obtained using another Src-selective inhibitor, PP2 (Fig. 5*C*), and Src-targeted siRNA (Fig. 5*D*).

Role of Src in NE-mediated Activation of the IL6 Promoter—To determine whether norepinephrine-mediated activation of the *IL6* promoter required Src activity, SKOV3 or Hey-A8 cells were treated with AP23846 or vehicle for 30 min prior to luciferase reporter assay. As shown in Fig. 6, pharmacologic inhibition of Src completely abrogated norepinephrine-mediated induction of *IL6* promoter activity.

Analysis of *IL6* promoter constructs bearing point mutations in transcription factor binding sites showed that NE activation did not require a functional CRE (Fig. 6*B*). However, mutation of the C/EBP-β (NFIL6) motif completely abrogated NE-mediated induction of the *IL6* promoter. This effect was specific to NE-mediated signaling, as the C/EBP-β mutation did not impair *IL6* promoter response to the positive control inducer phorbol 12-myristate 13-acetate. These data suggest that β-adrenergic activation of the *IL6* promoter via the Src signaling pathway is not mediated by classical CREB response to cAMP/protein kinase A signaling but instead involves Src-dependent activation of C/EBP-β.

Role of IL-6 in Isoproterenol-induced in Vivo Tumor Growth—To determine the role of IL-6 in mediating catecholamine-induced tumor growth, we used a neutral liposomal approach (DOPC) for *in vivo* siRNA delivery that we have previously optimized for *in vivo* applications (32, 33). We first identified an IL-6-targeted siRNA sequence that decreased IL-6 levels in the SKOV3.ip1 cells (data not shown). Next, female SCID mice were injected subcutaneously with SKOV3.ip1 cells and treated

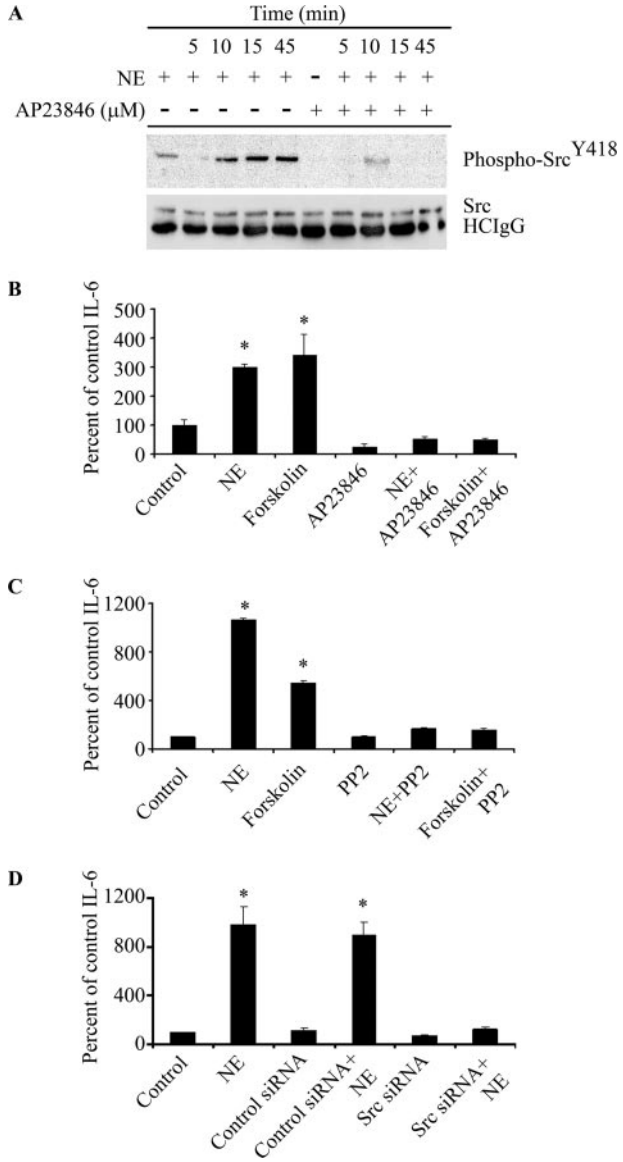

FIGURE 5. **Norepinephrine-induced IL-6 expression is Src-dependent.** *A*, SKOV3.ip1 cells were serum-starved for 18 h with control medium or medium containing the Src inhibitor AP23846. Cells were stimulated with 10 μM norepinephrine for the indicated times. Protein was lysed from cells, and total c-Src was immunoprecipitated from the samples. Samples were separated by SDS-PAGE and immunoblotted with antibodies against phospho-Src^{Y418} and total Src. *B* and *C*, SKOV3.ip1 cells were serum-starved for 18 h with control medium or medium containing the Src inhibitor AP23846 (*B*) or PP2 (*C*). Cells were then stimulated with 10 μM norepinephrine or forskolin alone or in the presence of the Src inhibitor. After 3 h, medium was collected, and the concentration of IL-6 was determined by ELISA. *D*, following transfection of SKOV3.ip1 cells with Src or control siRNA, the cells were stimulated with 10 μM norepinephrine. After 3 h, medium was collected and the concentration of IL-6 was determined by ELISA. Data are graphed as the mean percent of control (medium only), which was set to 100%. Experiments were performed in triplicate. *, $p < 0.05$. *Bars*, ±S.D.

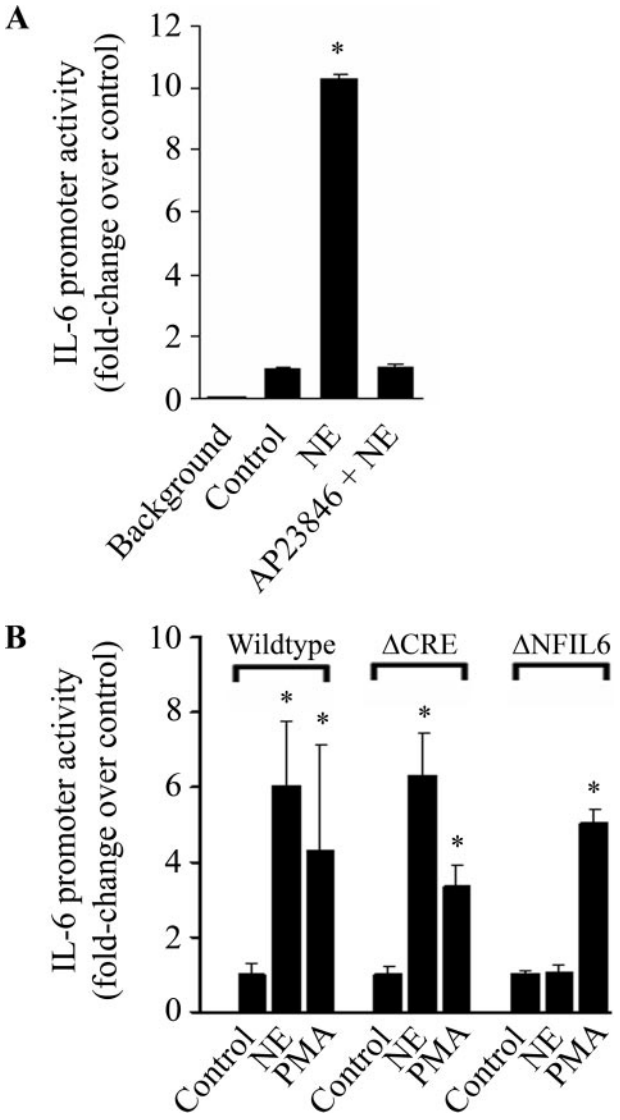

FIGURE 6. *A*, effect of Src inhibition on norepinephrine activation of the *IL6* promoter. SKOV3.ip1 cells were transfected with a luciferase reporter construct under control of the human *IL6* promoter as described in Fig. 3. 1 h following transfection, cells were treated with the Src inhibitor AP23846 or an equivalent volume of vehicle. After 30 min of antagonist exposure, cells were treated with 10 μM norepinephrine or an equivalent volume of PBS vehicle, and luciferase activity was assayed 90 min later. Data represent the mean (± S.E.) of triplicate luciferase determinations, with relative light unit data normalized to vehicle-treated control cells. Background luminance was determined in untransfected cells processed in parallel. *B*, mutational analysis of *IL6* promoter response to NE. SKOV.ip1 cells were transfected with luciferase expression vectors driven by the human *IL6* promoter or variants bearing point mutations in the cAMP-response element (*CRE*) or C/EBP-β (*NFIL6*)-response element. Following transfection, cells were cultured for 4 h in 10 μM NE, 0.2 ng/ml phorbol 12-myristate 13-acetate (positive control), or an equivalent volume of vehicle. Firefly luciferase activity was normalized to co-transfected control *Renilla* luciferase driven by an SV40 promoter and expressed as a -fold change relative to untreated controls. *, $p < 0.05$. *Bars*, ±S.D.

according to the following groups ($n = 5$/group): (*a*) control siRNA-DOPC twice weekly + PBS intraperitoneal daily; (*b*) IL-6 siRNA-DOPC twice weekly + PBS intraperitoneal daily; (*c*) control siRNA-DOPC twice weekly + isoproterenol intraperitoneal daily; (*d*) IL-6 siRNA-DOPC twice weekly + isoproterenol intraperitoneal daily. Isoproterenol treatment with control siRNA increased tumor volume by >500% ($p < 0.01$; Fig. 7). IL-6 siRNA-DOPC reduced tumor growth by 47% in the PBS control group ($p = 0.14$) and blocked the isoproterenol-induced tumor growth (Fig. 7).

DISCUSSION

This study provides direct evidence that chronic stress hormones affect expression of IL-6, a pro-angiogenic cytokine in ovarian carcinoma cells, and do so through a β-adrenergic receptor/Src tyrosine kinase signaling axis. For these studies,

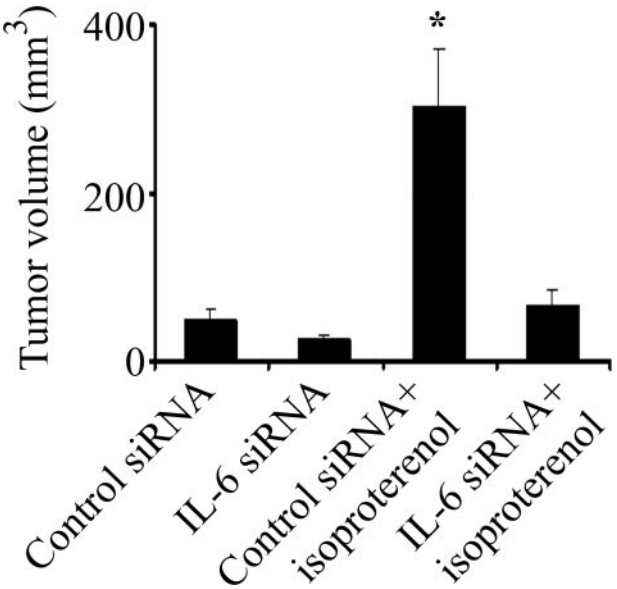

FIGURE 7. **IL-6 gene silencing inhibits catecholamine-induced tumor growth.** Mice ($n = 5$/group) were injected subcutaneously with SKOV3.ip1 cells and 3 days later randomized into four groups: 1) control siRNA-DOPC twice weekly + PBS intraperitoneal daily; 2) IL-6 siRNA-DOPC twice weekly + PBS daily; 3) control siRNA-DOPC twice weekly + isoproterenol intraperitoneal daily; 4) IL-6 siRNA-DOPC twice weekly + isoproterenol intraperitoneal daily. After 3 weeks of treatment, mice were sacrificed and the tumor volume was graphed. *, $p < 0.01$. *Bars*, ±S.D.

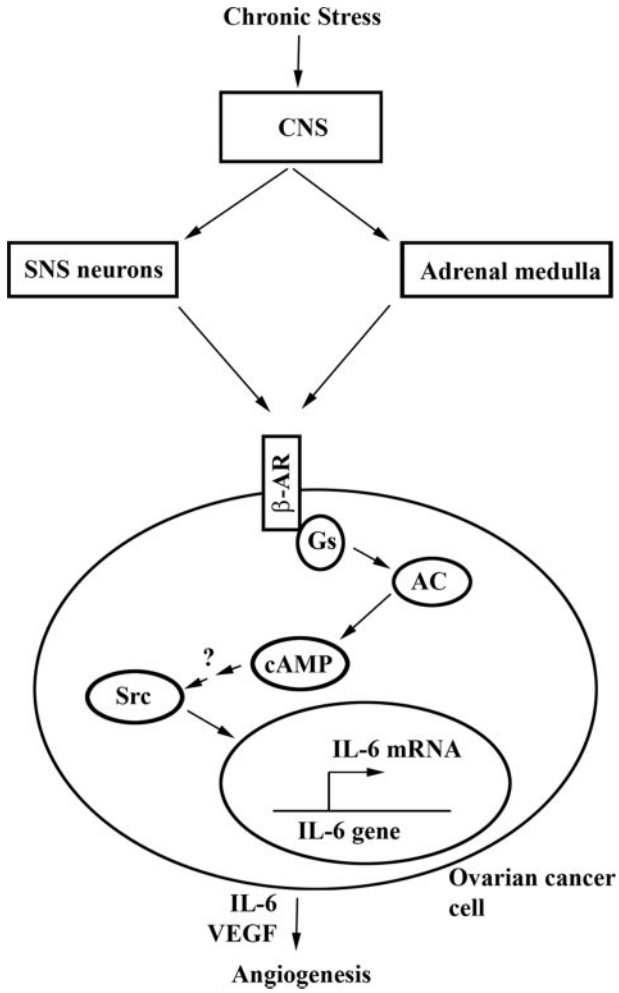

FIGURE 8. **Model of mechanism by which chronic stress enhances tumor cell secretion of proangiogenic molecules.** In response to chronic stress, catecholamines such as epinephrine and norepinephrine are released from the sympathetic nervous system. Stress-related hormones activate β-adrenergic receptors on tumor cells and enhance expression of IL-6 and vascular endothelial growth factor.

we stimulated cultured ovarian cancer cells with physiologic catecholamine concentrations and found that for all three of the cell lines tested (SKOV3.ip1, Hey-A8, and EG) norepinephrine and epinephrine significantly increased IL-6 production. Moreover, we have demonstrated that incubation of SKOV3.ip1 cells with norepinephrine results in substantial increases in IL-6 RNA and promoter activity, indicating that stress mediators regulate expression of the *IL6* gene at the transcriptional level.

The biological effects of catecholamines are mediated through binding to β-adrenergic receptors on the surface of target cells. This results in activation of adenylyl cyclase, which catalyzes the formation of the second messenger cAMP from ATP. Cyclic AMP binds to and activates protein kinase A, leading to the phosphorylation of downstream molecules (41). In our studies the effect of catecholamines on IL-6 expression was blocked by the addition of the β-adrenergic receptor antagonist propranolol but not by the α-adrenergic receptor inhibitor prazosin. Additionally, by utilizing forskolin as a direct activator of adenylyl cyclase, Bt_2cAMP as a generalized activator of cAMP effector pathways, and 8CPT-2Me-cAMP as a specific activator of the EPAC pathway, we determined that cAMP induced IL-6 expression through an EPAC-independent pathway. Recent reports have demonstrated that activation of β-adrenergic receptors results in enhanced Src activity (42, 43). Therefore, we evaluated the role of Src in norepinephrine-induced IL-6 expression and observed that norepinephrine stimulation led to an increase in Src phosphorylation. Moreover, the Src inhibitors AP23846 and PP2 blocked the induction of IL-6 expression and activation of the human *IL6* promoter by norepinephrine. These data indicate that stress-related hormones activate β-adrenergic receptors on ovarian cancer cells by increasing the kinase activity of Src, which results in enhanced transcription of the *IL6* promoter and subsequent production of IL-6 protein. Studies evaluating human ovarian cancer clinical specimens by immunohistochemistry have demonstrated that activated Src is overexpressed in the majority of ovarian carcinomas (44) and contributes to angiogenesis in these tumors; however, the mechanism(s) by which Src is activated are unclear. The current study provides a mechanism by which Src may be activated in these tumors, *i.e.* by response to stress hormones through β-adrenergic receptor. Because activation of Src has been shown to enhance expression of vascular endothelial growth factor (38) and IL-8 (45), catecholamines may increase tumor cell production of these proangiogenic molecules through a similar pathway.

Studies showing that norepinephrine levels increase in preovulatory follicles indicate that catecholamines play a role in the physiology of the normal ovary (28). Tumor cells may usurp these mechanisms to promote disease progression. Epinephrine and norepinephrine have been demonstrated to regulate IL-6 expression in adipose cells and myocytes (24, 25). Our

findings are consistent with these reports and reveal a novel pathway by which stress hormones enhance tumor production of IL-6. In other studies, catecholamines have been shown to augment ovarian cancer cell expression of vascular endothelial growth factor (14). Collectively, these data indicate that stress-induced activation of β-adrenergic signal transduction pathways on ovarian tumor cells can enhance expression of multiple proangiogenic molecules critical to ovarian cancer progression (Fig. 8).

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