Cholinergic agonists regulate JAK2/STAT3 signaling to suppress endothelial cell activation

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Cholinergic agonists regulate JAK2/STAT3 signaling to suppress endothelial cell activation. Am J Physiol Cell Physiol 297: C1294–C1306, 2009. First published September 9, 2009; doi:10.1152/ajpcell.00160.2009.—The cholinergic anti-inflammatory pathway is a physiological mechanism that inhibits cytokine production and minimizes tissue injury during inflammation. Previous investigations revealed that cholinergic stimulation (via cholinergic agonists and vagus nerve stimulation) suppresses endothelial cell activation and leukocyte recruitment. The purpose of this study was to investigate the mechanisms by which cholinergic agonists (e.g., nicotine and GTS-21) regulate endothelial cell activation. Specifically, we examined the effects of cholinergic agonists on IL-6-mediated endothelial cell activation through the JAK2/STAT3 signaling pathway. Treatment of macrovascular human umbilical vein endothelial cells (HUVECs) and microvascular endothelial cells (MVECs) with the cholinergic agonists nicotine and GTS-21 significantly reduced IL-6-mediated monocyte chemoattractant protein-1 (MCP-1) production and ICAM-1 expression which are regulated through the JAK2/STAT3 pathway. We found that treatment of endothelial cells with cholinergic agonists significantly reduced STAT3 activation by phosphorylation and DNA binding. The inhibition of STAT3 phosphorylation was reversed by sodium orthovanadate, an inhibitor of tyrosine phosphatases, as well as by NSC-87877 suggesting a SHP1/2-dependent mechanism. Further investigations showed that cholinergic agonists reduced the phosphorylation of JAK2, an upstream component of the JAK2/STAT3 pathway. Finally, we observed that nicotine and GTS-21 treatment decreased levels of SOCS3 (suppressor of cytokine signaling; a negative regulator of the inflammatory activity of IL-6) in activated endothelial cells. These data demonstrate that cholinergic agonists suppress IL-6-mediated endothelial cell activation through the JAK2/STAT3 pathway. Our results have significant implications for understanding the therapeutic potential of cholinergic agonists for treating IL-6-mediated inflammatory conditions.

interleukin-6; soluble interleukin-6 receptor; monocyte chemoattractant protein-1; intercellular adhesion molecule-1; suppressor of cytokine signaling; nicotine; GTS-21

The endothelium plays an important role in leukocyte trafficking during inflammation and infection. Pro-inflammatory mediators activate the endothelium to express cell-associated adhesion molecules that interact efficiently and selectively with circulating immune cells (reviewed in Refs. 32 and 41). Selectins facilitate the transient binding and rolling of leukocytes along the endothelium, and immunoglobulin superfamily members (e.g., ICAM-1, VCAM-1) mediate the firm adhesion of leukocytes via counterligands (e.g., CD11b/CD18). In addition, the activated endothelium produces numerous cytokines including monocyte chemoattractant protein-1 (MCP-1), RANTES (regulated upon activation, normal T cell expressed and secreted), macrophage inflammatory protein (MIP)-1α, and MIP-1β, which favor leukocyte infiltration during infection and inflammation (35, 47, 55).

The pro-inflammatory role of IL-6 is well described (6, 10, 36). Clinically, blockade of the IL-6/STAT3 signaling pathway via antibodies against the IL-6 receptor (IL-6R) (Atlimumab, a.k.a. Actemra or MRA) is used for treating inflammatory conditions (8, 24). Early studies by Romano and coworkers (47) revealed that IL-6−/− mice had defects in chemokine production and leukocyte trafficking suggesting a role for IL-6 in endothelial cell activation and leukocyte migration. These studies led to the discovery that endothelial cells express receptor glycoprotein 130 (gp130 or CD130) on their surface in the absence of the IL-6R and thus require IL-6 soluble receptor (IL-6sR) to respond to IL-6 (47). IL-6sR is found in normal human serum (19) and significantly elevated levels are present during inflammation (62, 70).

Interactions between membrane-bound gp130 and IL-6+IL-6sR promote the dimerization of gp130 and tyrosine phosphorylation of JAK family members (JAK1, 2, 3) and STAT3 (reviewed by Refs. 18 and 26). Phosphorylation of STAT3-Tyr705 facilitates STAT3 dimerization, nuclear translocation, and the activation of gene transcription (reviewed by Refs. 18 and 26), which is negatively regulated through members of the suppressor of cytokine signaling-3 (SOCS3) protein family (58). Recent studies by Yasukawa and coworkers (68) demonstrated that the pro-inflammatory actions of IL-6 requires the presence of suppressor of cytokine signaling-3 (SOCS3). Excessive and sustained STAT3 phosphorylation has been reported in patients with inflammatory bowel disease and rheumatoid arthritis (23). Likewise, treatment of endothelial cells with IL-6+IL-6sR promotes JAK2/STAT3 activation and an inflammatory response resulting in enhanced cell adhesion molecule expression and the production of several cytokines/chemokines, including MCP-1 and IL-8 (47, 67).

Several studies demonstrating the anti-inflammatory effects of cholinergic agonists, such as nicotine and acetylcholine, led to the discovery of the cholinergic anti-inflammatory pathway, a physiological mechanism that inhibits cytokine production and diminishes tissue injury during inflammation (reviewed by Ref. 60). Previous studies by our laboratory showed that cholinergic agonists, including nicotine, suppressed endothelial cell activation in vitro and in vivo (50). In addition, using the carrageenan air-pouch model of leukocyte trafficking previously employed to investigate the role of [IL-6+IL-6sR-gp130]-mediated endothelial cell activation and leukocyte migration in vivo (47), we demonstrated that vagus nerve stimu-
loration or treatment with cholinergic agonists suppressed leukocyte infiltration into localized sites of inflammation in vivo (50). GTS-21 is a selective agonist of the α7 nicotinic acetylcholine receptor (α7-nAChR) originally developed for the treatment of Alzheimer’s disease based on its neuroprotective and cognitive enhancing activities (reviewed by Ref. 28). Administration of GTS-21 or nicotine reduced leukocyte infiltration in rodent models of renal ischemia-reperfusion injury (69) and endotoxia (11). Therefore, we examined the effect of cholinergic agonists on IL-6-mediated ICAM-1 expression and MCP-1 production by endothelial cells via the JAK2/STAT3 signaling pathway.

METHODS AND MATERIALS

Materials. Nicotine was purchased from Sigma-Aldrich (St. Louis, MO). AG490 was purchased from Calbiochem (San Diego, CA). Stattic (CAS 19983-44-9; 6-nitro-1H-1-lamabda-6-benzo[b]thiophene-1,1-dione or 6-nitro-benzo[b]thiophene-1,1-dioxide) (56) was purchased from Ryan Scientific (Mount Pleasant, SC). GTS-21 (I3-[2,4-dimethoxybenzylidene]-anabasine) or DMXBA) was provided by Y. Al-Abed (The Feinstein Institute for Medical Research). IL-6 and IL-6sR were purchased from R&D Systems (Minneapolis, MN). Phospho-STAT3 (Tyrosine 705 and Serine 727), phospho-JAK2 (Tyrosine 1007/1008), and SOCS3 antibodies were purchased from Cell Signaling Technology (Danvers, MA). The STAT3 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The JAK2 antibody was purchased from Upstate/Millipore (Billerica, MA). Sodium orthovanadate (Na3VO4), purchased from Alfa Aesar (Ward Hill, MA), was prepared and activated according to Gordon (13). The SHP1/2 inhibitor NSC-87877 and α-bungarotoxin (α-BGT) were purchased from Tocris Cookson (Ellisville, MO).

Endothelial cell cultures. Human umbilical vein endothelial cells (HUVECs) were isolated from fresh anonymous human umbilical cords collected by the National Cord Blood Program at North Shore University Hospital (Manhasset, NY) by using collagenase to digest the subendothelial basement layer, as previously described (25). The Institutional Review Board reviewed the protocol and deemed it exempt from approval. HUVECs were grown on gelatin-coated flasks (0.3%) in Medium 199 containing 10% FCS, endothelial cell growth supplement (Upstate/Millipore), heparin, penicillin, streptomycin, gentamycin, and l-glutamine (Invitrogen, Carlsbad, CA). HUVECs were subcultured by using trypsin/EDTA Reagent Pack, according to the manufacturer’s directions (Lonza, Walkersville, MD) and incubated overnight in Medium 199 containing 10% FCS, penicillin, streptomycin, and l-glutamine before experimentation. Human dermal microvascular endothelial cells (MVECs), purchased from Lonza, were grown in EGM2-MV (Lonza) and subcultured using trypsin/EDTA Reagent Pack according to the manufacturer’s directions. Before the experimentation, MVECs were incubated in EBM2 (Lonza) basal media containing 5% FCS overnight.

Endothelial cell activation assays. Confluent monolayers of HUVECs (passages 3–8) plated in 96-well plates in Medium 199 containing 10% FCS, penicillin, streptomycin, and l-glutamine were used for experiments. Some experiments were performed with confluent monolayers of MVECs (passages 4–8) plated in 96-well plates in basal EBM-2 media containing 5% FCS. HUVECs and MVECs were treated with nicotine, GTS-21, or pathway modifying agents before overnight treatment with IL-6+IL-6sR (HUVECs: 5 + 150 ng/ml; MVECs: 5 + 500 ng/ml, unless indicated). These concentrations and time points (based on the results of pilot experiments) were found to reproducibly induce MCP-1 production and ICAM-1 expression in the linear range. Cell-free culture supernatants were collected and analyzed for MCP-1 by ELISA (R&D Systems). For some experiments, cellular ICAM-1 expression was determined using cell-based ELISA assays, as previously described (50). Control wells were treated with vehicle in media. Data from three independent experiments are shown as means (percent control) ± SE.

Assessment of STAT3 DNA binding. HUVECs were grown in T75 flasks and treated with vehicle or cholinergic agonists (nicotine or GTS-21) and then stimulated with IL-6+IL-6sR (10 + 150 ng/ml). After 40 min, nuclear extracts were isolated and assessed for STAT3-specific DNA binding with 20 μg of nuclear extracts using the TransFactor STAT3 Specific Chemiluminescent kit (Clontech, Mountain View, CA), according to the manufacturer’s directions. This assay is a quantitative ELISA-based method with a STAT3-DNA consensus binding site immobilized to the plate followed by activated STAT3 detection using specific antibodies. In addition, STAT3 phosphorylation was determined by Western blot analysis methods (see Western blot analysis).

Western blot analysis. HUVECs and MVECs were cultured as described above on 12-well plates. HUVECs or MVECs were treated with vehicle, nicotine, or GTS-21 (or Stattic or forskolin) before stimulation with IL-6+IL-6sR (2.5 + 25 ng/ml) (unless indicated). These concentrations and time points (based on the results of pilot experiments with HUVECs and MVECs) were found to reproducibly affect STAT3 and JAK2 phosphorylation or SOCS3 protein levels within the linear range. In one study, HUVECs were pretreated with activated Na3VO4 or the SHP1/2 inhibitor NSC-87877 before the addition of nicotine, GTS-21, or forskolin and then stimulated with IL-6+IL-6sR (2.5 + 25 ng/ml). Cells were lysed by the addition of lysis buffer containing Triton X-100 and deoxycholate, with a phosphatase and protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA) followed by repeated freeze-thawing procedures. After a brief centrifugation (14,000 g), lysate protein concentrations were determined using the Micro-BCA assay (Pierce, Rockland, IL). Equal amounts of lysates (20–30 μg protein/lane) were electrophoresed using NuPAGE gels (Invitrogen). Separated proteins were transferred to PVDF membranes and blotted with specific antibodies [e.g., phospho-STAT3, STAT3 (total), phospho-JAK2, JAK2 (total), and SOCS3]. For Fig. 1C, bound antibodies were probed using horseradish peroxidase-conjugated secondary antibodies followed by ECL development, and immunoblots were scanned for specific protein band intensities using NIH Image 1.62f software. All other immunoblots were probed using infrared-labeled secondary antibodies (LI-COR, Lincoln, NE), followed by imaging with the LI-COR Odyssey system (which allows quantification of band intensities). All experiments were repeated at least twice and representative blots are shown. In addition, average band intensities (corrected for loading) (± SE) from three independent experiments are shown.

Transfection studies. To selectively suppress STAT3 and JAK2 expression, HUVECs were transfected with ON-TARGET-plus SMARTpool small interfering RNAs (siRNAs) targeting STAT3 (cat. no. L-003544-00; accession numbers: NM_003150, NM_139276, NM_213662) or JAK2 (cat. no. L-003146-00; accession number: NM_004972) (Dharmacon, Lafayette, CO). Cells were transfected with siRNA (100 nM) using the DharmaFECT1 siRNA transfection reagent (Dharmacon) according to the manufacturer’s instructions for transfecting HUVECs. Control transfections were performed using ON-TARGET-plus SMARTpool siRNAs targeting GAPDH (cat. no. D-001830-10; accession number NM_002046) or DharmaFECT1 alone. The selective silencing of STAT3, JAK2, and GAPDH was determined by Western blot analysis after 48 h. The effect of silencing STAT3 and JAK2 on IL-6-mediated MCP-1 production by HUVECs (48 h posttransfection) was assessed by ELISA.

Statistical analyses. All experiments were performed at least twice, and data are expressed as means ± SD or SE, as indicated. For data with multiple comparisons, data were analyzed by one-way ANOVA followed by the Dunnett’s test (to compare each treatment to control value). Student’s t-tests were used for single comparisons between treatment and control. P values <0.05 were considered significant.
IL-6 + IL-6sR induces MCP-1 production and ICAM-1 expression by endothelial cells via the JAK2/STAT3 pathway. In the absence of IL-6sR, IL-6 does not induce MCP-1 production or ICAM-1 expression by HUVECs demonstrating their lack of IL-6R expression (Fig. 1, A and B, top, respectively). As predicted (47), treatment of HUVECs with IL-6 in the presence...
of IL-6sR-induced MCP-1 production and ICAM-1 expression following IL-6sR stimulation (18 or 30 min post-IL-6 stimulation). However, a higher concentration of IL-6sR was required (in the presence of IL-6, 5 ng/ml) to stimulate MCP-1 production and ICAM-1 expression by MVECs compared with HUVECs. Consistent with the results observed using HUVECs, inhibition of STAT3 phosphorylation and JAK2 phosphorylation by Stattic and AG490, respectively, significantly reduced MCP-1 production and ICAM-1 expression by MVECs following IL-6+IL-6sR stimulation (Fig. 3, A and B, respectively). In addition, IL-6+IL-6sR treatment promoted STAT3 phosphorylation in MVECs, and treatment with Stattic completely blocked IL-6+IL-6sR-induced STAT3 phosphorylation (Fig. 3C). Together, these data demonstrate that IL-6+IL-6sR-induced MCP-1 production and ICAM-1 expression by HUVECs and MVECs are mediated through the JAK2/STAT3 pathway.

Cholinergic agonists reduce IL-6+IL-6sR-induced endothelial cell activation with concomitant inhibition of STAT3 phosphorylation and DNA binding. Previous studies by our laboratory demonstrated the inhibitory effect of cholinergic agonists (e.g., nicotine) on tumor necrosis factor (TNF)-stimulated microvascular endothelial cell activation in vitro and leukocyte trafficking in vivo (50). Based on the role of IL-6+IL-6sR on endothelial cell activation and leukocyte trafficking, we examined the effect of cholinergic agents nicotine or GTS-21 (a selective α7-nAChR agonist) on IL-6-mediated endothelial cell activation. We found that cholinergic agonists significantly blocked IL-6+IL-6sR-induced MCP-1 production and ICAM-1 expression by HUVECs in a dose-dependent manner (Fig. 4, A and B, respectively). Likewise, treatment of MVECs with cholinergic agonists nicotine or GTS-21 significantly reduced MCP-1 production and ICAM-1 expression following IL-6+IL-6sR treatment (Fig. 4, C and D, respectively). Time-course experiments revealed that nicotine (10^{-4} M) and GTS-21 (10^{-5} M) significantly reduced MCP-1 production by HUVECs even when added either simultaneously with IL-6+IL-6sR (22 ± 3.5% and 30 ± 1.3% inhibition, respectively) or 30 min post-IL-6+ILsR stimulation (18 ± 4.7% and 24+4.6% inhibition, respectively). The inclusion of α-bunga-
Components of the JAK2/STAT3 signaling pathway are activated and/or deactivated through phosphorylation and/or dephosphorylation events. For example, the protein tyrosine phosphatase Src homology region 2 domain-containing phosphatase 2 (SHP2) dephosphorylates mediators of this pathway (18, 57). Therefore, we next examined the role of tyrosine phosphatases in mediating the inhibitory effects of cholinergic agonists on STAT3 phosphorylation. We found that treatment of endothelial cells with Na3VO4, a cell-permeable phosphatase inhibitor, partially blocked the inhibitory effects of cholinergic agonists on IL-6+IL-6sR-mediated STAT3-Tyr705 phosphorylation (Fig. 6A). We used forskolin, an inducer of cAMP and also a known activator of SHP2 (45) that dephosphorylates STAT3 as a positive control. As expected, forskolin treatment of endothelial cells suppressed STAT3 phosphorylation by endothelial cells (53) (Fig. 6A), and this inhibition was partially reversed by the phosphatase inhibitor sodium orthovanadate (Fig. 6A). These data suggest that like forskolin, the inhibitory effects of the cholinergic agonists on STAT3-Tyr705 phosphorylation are mediated, in part, through tyrosine phosphatases. Further studies using the specific SHP1/2 inhibitor NSC-87877 revealed that the inhibitory effects of both nicotine and GTS-21 on STAT3 phosphorylation were dependent, in part, on SHP1/2 (Fig. 6B and C, respectively).

Cholinergic agonists block JAK2 phosphorylation by endothelial cells. An early signaling event leading to IL-6+IL-6sR-induced STAT3 activation is the phosphorylation of JAK2 by gp130 following IL-6+IL-6sR ligation (reviewed by Refs. 18 and 26). JAK2 inhibition by AG490 significantly reduced IL-6-mediated MCP-1 production and ICAM-1 expression by endothelial cells (Fig. 1A and B, [HUVECs], respectively, and Fig. 3, A and B, [MVECs], respectively) and JAK2 silencing significantly decreased IL-6+IL-6sR-mediated MCP-1 production by HUVECs (Fig. 2B). We found that treatment of...
HUVECs with cholinergic agonists significantly suppressed both constitutive and IL-6+IL-6sR-mediated JAK2 phosphorylation (Fig. 7, A and B). Cholinergic agonists decrease SOCS3 protein levels. The JAK2/STAT3 pathway is regulated by the SOCS family of proteins (reviewed in Ref. 18), which inhibit JAK phosphorylation or JAK stability or both and hence, STAT3 phosphorylation. In particular, SOCS3 has been reported to be induced by IL-1, IL-6/IL-6sR, TNF, and LPS, which are known to promote STAT3 activation (4, 5, 18). To address whether the effects of

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Reduced endothelial cell activation by cholinergic agonists is associated with inhibition of STAT3 phosphorylation and DNA binding. A and B: HUVECs were untreated or treated with either Nic or vehicle (A) or GTS-21 or vehicle (B) for 0.5 h before IL-6+IL-6sR stimulation. After 25 min, cells were harvested and lysates were assessed for phosphorylated-STAT3-Tyr705 (p-STAT3) or total STAT3 expression by Western blotting. This experiment was repeated twice and a representative blot is shown. The mean p-STAT3:STAT3 ratio (based on band densities) ± SE for 3 independent blots is shown in the graph below. C and D: MVECs were either untreated or treated with either Nic or vehicle (C) or GTS-21 or vehicle (D) for 0.5 h before IL-6+IL-6sR stimulation. After 25 min, cells were processed as described for HUVECs. E: HUVECs were untreated or treated with either Nic (10 μM) or GTS-21 (20 μM) before stimulation with IL-6+IL-6sR. After 40 min, nuclear extracts were isolated, and specific STAT3 DNA binding was determined using chemiluminescent detection. Data are expressed as mean relative light units (RLU) ± SD for 2 independent assays. *P < 0.05 and **P < 0.01 comparing constitutive expression vs. IL-6+IL-6sR-stimulated expression and IL-6+IL-6sR-stimulated expression in the absence vs. presence of cholinergic agonists.
cholinergic agonists might be inhibited, at least in part, by suppressing IL-6-induced SOCS3, we examined the effect of nicotine and GTS-21 on SOCS3 protein levels induced by IL-6+IL-6sR-stimulated endothelial cells. Treatment with either nicotine or GTS-21 significantly blunted IL-6+IL-6sR-induced SOCS3 protein levels in both HUVEC and MVEC (Fig. 8) while concomitantly suppressing STAT3 phosphorylation and DNA binding (Fig. 5). Cholinergic agonists had no effect on constitutive SOCS3 levels (data not shown).

**DISCUSSION**

Our previous studies showed that cholinergic agonists (including nicotine) suppressed endothelial cell activation (in vitro and in vivo) and leukocyte trafficking (in vivo) (50). IL-6 bound to IL-6sR (found at basal levels in blood and at increased concentrations in the circulation during inflammation) induces both MCP-1 production and ICAM-1 expression by endothelial cells that are regulated, in part, through the JAK2/STAT3 signaling pathway (47). Systemic IL-6 administration leads to STAT3 phosphorylation and MCP-1 expression by brain endothelial cells (49). We confirmed the effects of IL-6+IL-6sR on HUVEC and MVEC activation (Figs. 1 and 3), and we report that these effects are mediated through the JAK2/STAT3 pathway. We investigated the effects of cholinergic agonists on chemokine and adhesion molecule expression, as well as signaling pathways using IL-6+IL-6sR-stimulated endothelial cells. We found that cholinergic agonists inhibit IL-6-mediated MCP-1 production and ICAM-1 expression via the JAK2/STAT3 pathway in a SHP1/2 phosphatase-dependent manner. The inhibitory (or anti-inflammatory) effects of cholinergic

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**Fig. 6.** Protein tyrosine phosphatase inhibitors reverse the inhibitory effect of cholinergic agonists on STAT3 phosphorylation. A: HUVECs were either pretreated with vehicle, Nic (N, 10^{-4} M), GTS-21 (G, 10^{-5} M), or forskolin (F, 10^{-5} M) in the absence (solid bars) or presence (shaded bars) of Na_{3}VO_{4} (50 μM) for 0.5 h before stimulation with IL-6+IL-6sR. HUVECs were harvested 25 min later, lysed, separated by electrophoresis, and Western blotted for phosphorylated-STAT3-Tyr^{705} (p-STAT3) or STAT3 (total) expression. B and C: HUVECs were prepared as described above, except they were pretreated with the SHP1/2 inhibitor NSC-87877 (NSC, 10 μM) for 0.5 h before addition of Nic (B) or GTS-21 (C) followed by IL-6+IL-6sR, as described in A. Experiments were performed twice and representative blots are shown. The mean ratios of p-STAT3:STAT3 ± SD (based on band densities) for each condition in 2 independent blots is shown in the graph below. A: *P < 0.05 comparing constitutive expression vs. IL-6+IL-6sR-stimulated expression and IL-6+IL-6sR-stimulated expression ± cholinergic agonists (or forskolin) in the absence (solid bars) and presence (shaded bars) of Na_{3}VO_{4}; B and C: **P < 0.01 comparing constitutive expression vs. IL-6+IL-6sR stimulated expression and IL-6+IL-6sR-stimulated expression ± cholinergic agonists in the presence and absence of SHP1/2 inhibitor (NSC).
agonists were associated with reduced JAK2 and STAT3 phosphorylation and STAT3-specific DNA binding, as well as decreased levels of SOCS3, a regulator of the JAK2/STAT3 pathway (Fig. 9). Similar results were observed using human endothelial cells isolated from large veins (HUVECs) and microvascular vessels (MVECs). This is the first study to demonstrate the inhibitory effects of cholinergic agonists on IL-6-mediated signaling components of the JAK2/STAT3/SOCS3 pathway in endothelial cells, suggesting that the cholinergic anti-inflammatory pathway regulates endothelial cell activation through mediators of the JAK2/STAT3/SOCS3 signaling pathway.

Studies using IL-6 knockout mice revealed that the pro-inflammatory mediator IL-6 significantly impacts disease progression in many experimental models (26, 48). In addition, the inflammatory activity of the IL-6-STAT3 pathway is linked to inflammatory bowel disease and cancer (6). The most convincing evidence for the pro-inflammatory activity of IL-6 is the efficacious use of the anti-IL-6 receptor antibody (Atizumab, a.k.a. Actemra or MRA) for the treatment of inflammatory diseases in humans, including rheumatoid arthritis and inflammatory bowel disease (8, 24). Similar to previous studies (47) we found that IL-6/IL-6sR promotes endothelial cell activation and STAT3 phosphorylation, whereas inhibition of STAT3 phosphorylation blocked endothelial cell activation by Stattic (Figs. 1 and 3). The steep slope of inhibition by Stattic is most likely due to the “irreversible activity” of Stattic (56) and because we preincubated the cells with Stattic for 1 h.

Fig. 7. Cholinergic agonists suppress both basal and IL-6/IL-6sR-stimulated JAK2 phosphorylation. A: HUVECs were treated with vehicle (V) or cholinergic agonists [Nic (10^{-4} M) and GTS-21 (10^{-5} M)] for 0.5 h. JAK2-Tyr^{1007/1008} phosphorylation (p-JAK2) was assessed by Western blotting. B: HUVECs were treated with vehicle (V) or cholinergic agonists [Nic (10^{-4} M) and GTS-21 (10^{-5} M)] for 0.5 h followed by IL-6/IL-6sR. After 25 min, JAK2-Tyr^{1007/1008} (p-JAK2) expression was assessed by Western blotting. To correct for loading, cell lysates were blotted with JAK2 (total). This experiment was repeated twice, and the mean ratios of p-JAK2/JAK2 ± SE (based on band densities) for each condition in 3 independent experiments are shown in the graph below. **P < 0.01 comparing expression in the absence vs. presence of cholinergic agonists.

Fig. 8. Cholinergic agonists blunt IL-6/IL-6sR-induced SOCS3 protein levels. HUVECs (A) or MVECs (B) were either untreated or treated with Nic or GTS-21 for 0.5 h before the addition of vehicle or IL-6/IL-6sR. After 25 min cells were harvested and lysates were assessed for SOCS3 protein levels by Western blotting. To correct for loading, cell lysates were blotted with STAT3 (total) and the ratio of SOCS3/STAT3 (based on band densities) for each condition is shown below the blots. This experiment was performed three times (for HUVECs, A) and twice (for MVECs, B). The data are shown as the mean ratios of SOCS3:STAT3 ± SEM (for HUVECs, A) and the mean ratios of SOCS3:STAT3 ± SD (for MVECs, B) for each condition in the graphs below the blots. *P < 0.05, **P < 0.01 IL-6/IL-6sR-induced expression in the absence vs. presence of cholinergic agonists.
signalng pathway leading to JAK2 phosphorylation and STAT3 activation. Because cholinergic agonists reduced STAT3 phosphorylation, we examined their effects on JAK2 phosphorylation, an upstream mediator. Treatment of endothelial cells with cholinergic agonists significantly reduced both basal (constitutive) and IL-6-mediated JAK2 phosphorylation (Fig. 7). This is the first report to reveal an effect of cholinergic agonists on JAK2 phosphorylation. The inhibitory effect of forskolin on STAT3 phosphorylation is reversed by orthovanadate, (50 μM), a cell-permeable inhibitor of phosphatases (12, 61) (Fig. 6A). Cholinergic agonists act similar to forskolin-cAMP to inhibit JAK2/STAT3 phosphorylation through modulating phosphatase activity (45) because the inclusion of Na3VO4, a cell-permeable tyrosine phosphatase inhibitor (12, 61), partially reversed the inhibitory effect of cholinergic agonists on JAK2/STAT3 phosphorylation (Fig. 6A). Based on previous reports revealing that 1) SHP2 phosphorylation is impaired by SOCS3 expression; 2) SOCS3 requires SHP2 recruitment site in gp130 to exert its effect on acute phase proteins, and 3) SHP2 appears to exert part of its effect on the JAK/STAT3 pathway through the recruitment of SOCS3 to the receptor complex (54), we examined the effect of NSF-87877, an inhibitor of SHP1/2 on the attenuation of IL-6-induced STAT3 phosphorylation by nicotine and GTS-21. We observed that the JAK2 inhibitor NSF-87877 significantly reversed the inhibitory effects of both nicotine and GTS-21 on STAT3 phosphorylation (Fig. 6, B and C). These data suggest that cholinergic agonists regulate signaling through the JAK2/STAT3 pathway, in part, through effects on SHP1/2 tyrosine phosphatases.

SOCS proteins are induced by and inhibit cytokine-related signaling pathways in both JAK/STAT-dependent (e.g., IL-6+IL-6sR and JAK/STAT-independent mechanisms [e.g., via cAMP (53)]. SOCS3 proteins are short lived and highly vulnerable to degradation via the proteasome pathway. We found that SOCS3 protein levels by endothelial cells were enhanced by IL-6+IL-6sR, and SOCS3 levels were significantly reduced in the presence of cholinergic agonists (Fig. 8). It is important to note that IL-6 exerts both pro- and anti-inflammatory effects (26), and SOCS3 appears to be the “regulator of this divergent activity” of IL-6 (68). In the presence of SOCS3, IL-6 exerts pro-inflammatory effects (and prevents STAT3 phosphorylation by IL-6), whereas in the absence of SOCS3, IL-6 mediates anti-inflammatory effects (68). Because the inhibitory effect of cholinergic agonists on SOCS3 levels is observed only in the presence of IL-6+IL-6sR stimulation and the effect of cholinergic agonists on SOCS3 protein levels occurs rapidly, we postulate that cholinergic agonists regulate SOCS3 degradation through the proteasome pathway. Hyperactivation of JAK2/STAT3-mediated induction of SOCS3 during inflammation and ischemia has been shown to be detrimental and therapeutic regulation of this pathway is being investigated (31).

The JAK2/STAT3 pathway has been the focus of investigation for numerous inflammatory conditions. For example, IL-6, STAT3, and SOCS3 have been implicated in inflammatory bowel disease (1, 17, 39, 46) and soluble gp130 has been shown to suppress inflammatory bowel disease (38). Inflammatory bowel disease is a condition that can be treated with the cholinergic agonist nicotine experimentally (59) and clinically (14, 16, 44, 52), as well as with anti-IL-6R antibody (24). Previous studies have shown the anti-inflammatory effect of nicotine on mucosal cytokine production (59) and our findings

Interestingly, previous studies by de Jonge and coworkers demonstrated that nicotine induced STAT3 activation in macrophages following LPS treatment (9, 37). Like IL-6, STAT3 activation exerts both pro- and anti-inflammatory effects (reviewed by Refs. 6, 21, 42, 63, 65). One significant difference between this study and that of deJonge and coworkers (9) is that we employed IL-6+IL-6sR as the stimulating agent and they used the bacterial product LPS. Our studies demonstrate that using siRNA approaches with endothelial cells IL-6+IL-6sR is a much more specific activator of the JAK2/STAT3 pathway than LPS (data not shown). Another difference is that we used endothelial cells and they used macrophages. Contrary to its pro-inflammatory function in endothelial cells, STAT3 phosphorylation in LPS-treated macrophages suppresses their activation (20, 42). Therefore, it is not surprising that we found that nicotine/GTS-21 reduced JAK2 and STAT3 phosphorylation and they found that nicotine induced JAK2 and STAT3 phosphorylation. Despite the opposing effects in macrophages and endothelial cells, nicotine and GTS-21 exert anti-inflammatory effects in both cell types.

The IL-6R does not contain an intracytoplasmic signaling domain (reviewed by Refs. 18 and 26). Therefore, ligation of gp130 by IL-6+IL-6sR complexes initiates an intracytoplasmic
support the role of nicotine as a regulator of the JAK2/STAT3/ SOCS3 signaling pathway in inflammatory bowel disease (38).

Our results revealing the effects of cholinergic agonists on the JAK2/STAT3 signaling pathway in endothelial cells (described in Fig. 9) have important implications for improving our understanding of the therapeutic potential of the cholinergic anti-inflammatory pathway. The cholinergic anti-inflammatory pathway is a brain-to-immune mechanism that regulates inflammatory responses through the α7-nAChR (reviewed in Ref. 60). Previous studies have shown that vagus nerve stimulation, which releases acetylcholine, and administration of cholinergic agonists, including nicotine and GTS-21, reduce cytokine production in preclinical models of acute, systemic inflammation, including endotoxemia, hemorrhagic shock, ischemia-reperfusion injury, and polymicrobial sepsis (3, 11, 15, 43). Endothelial cells, which express α7-nAChRs (33, 40, 50) and respond to cholinergic agonists to suppress chemokine/cytokine production and adhesion molecule expression, play a critical role in host responses to inflammation by serving as targets and sources of inflammatory mediators, including MCP-1, an agent that attracts and activates monocytes/macrophages. Our results demonstrate that cholinergic regulation of the IL-6-mediated JAK2/STAT3 signaling cascade attenuates inflammatory responses by endothelial cells that mediate leukocyte trafficking during inflammation and supports the role of the α7-nAChR in mediating the effects of cholinergic agonists on endothelial cell activation. Whereas our previous studies focused on the regulation of acute inflammatory mediator production and rapid neutrophil recruitment by cholinergic stimulation (via vagus nerve stimulation and cholinergic agonists) (43, 50, 64), results of these studies support examining the role of cholinergic agonists in the transition between acute-to-chronic inflammation, which is regulated, in part, by IL-6+IL-6sR (1, 22, 27). Unlike acute inflammation, this transition to chronic inflammation is characterized by sustained infiltration of monocytes, macrophages, and lymphocytes (with fewer neutrophils), as well as fibrosis and tissue destruction associated with the development of autoimmune disease. Thus future studies will focus on examining the regulation of JAK2/STAT3/SOCS3 signaling components in vivo by cholinergic agonists, as well as exploring the role of the cholinergic anti-inflammatory pathway in modifying the transition from acute-to-chronic inflammation.

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REFERENCES

anti interleukin-6 receptor monoclonal antibody in active Crohn’s disease.


48. Wang M, Zhang W, Crisostomo P, Markel T, Meldrum KK, Fu XY, Meldrum DR. Endothelial STAT3 plays a critical role in generalized...


