Tristetraprolin Mediates Anti-inflammatory Effects of Nicotine in Lipopolysaccharide-stimulated Macrophages*

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Nicotine inhibits the release of TNF-α from macrophage through activation of STAT3. Tristetraprolin (TTP) is known to destabilize pro-inflammatory transcripts containing AU-rich elements (ARE) in 3′-untranslated region (3′-UTR). Here we show that in LPS-stimulated human macrophages the anti-inflammatory action of nicotine is mediated by TTP. Nicotine induced activation of STAT3 enhanced STAT3 binding to the TTP promoter, increased TTP promoter activity, and increased TTP expression resulting in the suppression of LPS-stimulated TNF-α production. Overexpression of a dominant negative mutant of STAT3 (R382W) or down-regulation of STAT3 by siRNA abolished nicotine-induced TTP expression and suppression of LPS-stimulated TNF-α production. Nicotine enhanced the decay of TNF-α mRNA and decreased luciferase expression of a TNF-α 3′-UTR reporter plasmid in U937 cells. However, siRNA to TTP abrogated these effects of nicotine. In this experiment, we are reporting for the first time the involvement of TTP in the cholinergic anti-inflammatory cascade consisting of nicotine-STAT3-TTP-dampening inflammation.

The cholinergic nervous system controls inflammation response (1, 2). Acetylcholine and nicotine inhibit the production of pro-inflammatory cytokines from endotoxin-stimulated macrophages through a α7 nicotinic acetylcholine receptor subunit (α7nAChR)-dependent mechanism (3–6). The anti-inflammatory effects of α7nAChR activation are mediated by the activation of the Jak2 and STAT3 (7). STAT3 is a negative regulator of the inflammatory response (8, 9). Nicotine, the prototypical α7nAChR agonist, fails to inhibit TNF production in macrophages overexpressing STAT3 with decreased DNA binding capacity (7), suggesting that DNA binding ability of STAT3 is important for its regulatory activity. Consistent with that finding, missense mutants of STAT3 that exhibit reduced DNA binding ability have been found to be associated with Hyper-IgE syndrome and showed dominant-negative effects when co-expressed with wild-type STAT3 (10, 11). However, the precise mechanism by which α7nAChR-activated STAT3 inhibits inflammatory response remains unclear.

The inflammatory response has been reported to be modulated by the post-transcriptional control (12, 13). The post-transcriptional control of inflammatory transcripts is strongly dependent on AU-rich element (ARE)-mediated mechanisms (14–16). The destabilizing function of AREs is believed to be regulated by ARE-binding proteins (17). Tristetraprolin (TTP) is an ARE-binding protein that promotes degradation of a number of inflammatory mediators including TNF-α, GM-CSF, IL-2, IL-3, IL-6, CCL2, CCL3, iNOS, and COX2 (18–24). TTP-knock-out mice develop severe inflammatory arthritis, autoimmune dysfunction, and myeloid hyperplasia, demonstrating the importance of TTP in limiting the inflammatory response (25).

Here we provide evidence that nicotine stimulates TTP production, thereby mediating the anti-inflammatory effect of nicotine in U937 cells. Further, we show that nicotine-activated STAT3 directly binds to the promoter region of TTP and enhances TTP promoter activity. However, in U937 cells transiently transfected with dominant negative STAT3 (10), nicotine does not enhance TTP expression and its anti-inflammatory effect is significantly attenuated. Nicotine increases the decay of TNF-α mRNA and decreases the expression of luciferase reporter containing TNF-α 3′-UTR. Down-regulation of TTP by siRNA treatment decreases the anti-inflammatory effect of nicotine on U937 cells. Our results show that nicotine-activated STAT3 induces the expression of TTP and, in turn, TTP plays key roles in nicotine-induced anti-inflammatory effect through destabilization of TNF-α transcripts.

**EXPERIMENTAL PROCEDURES**

**Cells**—Human monocytic U937 cells were cultured in DMEM supplemented with 10% FBS. Peripheral blood mononuclear cells were prepared by Ficoll/Hypaque (Sigma) density-gradient centrifugation and cultured in DMEM with 10% FBS. Adherent cells were allowed to differentiate for 10 days in a RPMI medium containing 2 ng/ml M-CSF (R&D Systems). Cells were preincubated with 10 nm nicotine (Sigma) for 20 min and stimulated for 4 h with LPS (1–2564, Sigma) at a concentration of 1 μg/ml. To determine the effect of α7nAChR on the expression of TTP and TNF-α, cells were preincubated with α7nAChR antagonist α-bungarotoxin (1 μg/ml, Sigma) or methyllycaconitine (1 μM, Sigma) for 30 min.

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4 The abbreviations used are: ARE, AU-rich element; TTP, tristetraprolin; LPS, lipopolysaccharide; CHX, cycloheximide; αBgt, α-bungarotoxin; MLA, methyllycaconitine.
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Plasmids and siRNAs—pcDNA6/V5-TTP construct has been described previously (26). The full-length cDNA of human STAT3 was PCR amplified from the cDNA of U937 cells using Taq polymerase (SunGeneticts, Daejeon, Korea) and primer set (supplemental Table S1). A plasmid construct containing C-terminal Flag-tagged wild-type full-length cDNA of human STAT3, pCMV-Flag/wSTAT3, was generated by inserting the PCR product into HindIII and Xhol sites of the pCMV-T4-Flag. A pCMV-Flag/dnSTAT3(R382W) construct containing the dominant negative STAT3 mutant (R382W) with reduced DNA binding ability was generated by a two-step polymerase chain reaction procedure changing the CGG codon for arginine, at position 382, to TGG encoding tryptophan. We also generated a pCMV-Flag/dnSTAT3(Y705F) construct containing the dominant negative STAT3 mutant with a substitution of phenylalanine for the tyrosine at amino acid residue 705. The constructions were verified by DNA sequencing. The sequence of the human TTP genomic locus at 19q13.1 (GenBank™ accession number NT_011109) was used to engineer PCR cloning primers. A 1411 base pair (bp) genomic fragment containing the 5'-flanking region of the TTP gene was isolated by PCR amplification from human genomic DNA. Construct pGL3/TTP p-1345 contains the −1345bp promoter region of the human TTP gene up to nucleotide +66 base pair (i.e. downstream from the TTP mRNA cap site) inserted into the SacI and XhoI sites of the pGL3 basic vector (Promega). The pGL3/TTP p-1345 construct was used as a template to synthesize substitution mutant constructs of the STAT3 binding sites within the TTP promoter. PCR primer pairs used for mutant constructs are listed in supplemental Table S1. A pRL-SV40 Renilla luciferase construct was purchased from Promega. The human TNF-α 3’-UTR (nt 872–1669 of TNF-α mRNA, GenBank™ accession number NM_000594.2) was PCR amplified from the cDNA of U937 cells using Taq polymerase (SunGenetics, Daejeon, Korea) and primer set as follows: CTCGAGGGAGGACGAACATC-CAACCTTCC, GCGGCCGCTTCTTTTCTAAGCAAACTT-CAAGAAAACTC-3 

Cytokine Assays—U937 cells were transfected with various kinds of plasmid constructs and siRNAs—psiCHECK2/TNF-α (sc-24736) and control siRNA (scRNA) (sc-37007) were purchased from Promega. The human STAT3 was PCR amplified from the cDNA of U937 cells using Taq polymerase (SunGenetics, Daejeon, Korea) and primer set as follows: CTCGAGGGAGGACGAACATC-CAACCTTCC, GCGGCCGCTTCTTTTCTAAGCAAACTT-CAAGAAAACTC-3 

Cycloheximide-based Protein Chase Experiment—U937 cells were preincubated with 10 nM nicotine (Sigma) for 20 min and stimulated for 4 h with 1 μg/ml LPS (Sigma). Cells were incubated with 10 μg/ml cycloheximide (CHX, Calbiochem) to stop protein synthesis. Cell supernatants and cell lysates were collected at 0, 1, 3, and 5 h after addition of CHX and analyzed for TNF-α by ELISA.

SDS-PAGE Analysis and Immunoblotting—Proteins were resolved by SDS-PAGE, transferred onto Hybond-P membranes (GE Healthcare Bio-Sciences Corp. Piscataway, NJ), and probed with appropriate dilutions of anti-TTP (sc-8458, Santa Cruz Biotechnology), anti-STAT3 (sc-482, Santa Cruz Biotechnology), anti-phospho-STAT3 (Cat No 9131, Cell Signaling), or anti-actin (sc-1616, Santa Cruz Biotechnology). Immunoreactivity was detected using the ECL detection system (GE Healthcare Bio-Sciences Corp.).

Real-time and Semi-quantitative RT-PCR—Real-time PCR was performed using SYBR Green PCR Master Mix (Qiagen) on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). Semi-quantitative RT-PCR was performed using Taq polymerase (Solgent, Daejeon, Korea). PCR primer pairs were as follows: TTP: 5'-CGCTACAAGACTGAGCTTAT-3', 5'-GAGGTGAATCTGTGACACAAG-3'; STAT3: 5'-GGAGTACGTG-CAGAAAACCTC-3', 5'-TCTTAAACAGCTCCAGTATT-3'; and TNF-α: 5'-TCCAAAGGACCTCCAAAACG-3', 5'-AGCAA-GCTGTGCACTTTCC-3'. GAPDH: 5'-ACATCAAGAG-GTGGTGAAG-3', 5'-CTGTGTGCTGTAGCCAAATT-3'.

Electrophoretic Mobility Shift Assay (EMSA)—The biotinylated double-stranded oligonucleotides were synthesized by Amersham Pharm. Co., LTD (Seoul, Korea): TTP-pSTAT-A wild-type (WT), 5'-CAGATTTCTCCGGTAAGTCTT-3'; TTP-pSTAT-A mutant (MUT), 5'-CAG ATT TCC CCA CGT AAG TGC TT-3'. Preparation of nuclear extracts and binding reactions were carried out using NE-PER nuclear and cytoplasmic extraction reagents and LightShift™ Chemiluminescent EMSA kit, respectively, according to the manufacturer’s manual (Thermo Fisher Biotechnology). For supershift assay, 2 μl of anti-STAT3 (sc-482x, Santa Cruz Biotechnology, Inc.), 2 μl of anti-STAT1 (sc-345x, Santa Cruz Biotechnology, Inc.) or 1 μg of isotype control (Sigma) was added to the binding reaction and incubated overnight at 4 °C before adding the labeled probes. The DNA-nuclear protein complexes were resolved on 5% non-denaturing polyacrylamide gel and transferred onto a nylon membrane (Hybond™ N+), GE Healthcare Bio-Science Corp.). The DNAs on the membrane were detected using horseradish peroxidase-conjugated streptavidin according to the manufacturer’s instructions (Lightshift™ Chemiluminescent EMSA kit).

Chromatin Immunoprecipitation (ChIP) Assays—ChIP assays were performed using the EZ-Magna ChIP™ G Kit (Millipore) according to the manufacturer’s instructions. The nuclei were isolated from cells. After sonication, chromatin was immunoprecipitated with the same anti-STAT3 antibody, anti-STAT1 antibody, or isotype control that was used for the supershift assays. The complexes were collected on protein-G-magnetic beads and subsequently extracted from the beads. Bound DNA was purified and amplified by PCR. The TTP primers were as follows: 5'-AAGGCAAGCAGTTCTCAGAG-3' and 5'-CTGTTGTGCTGTAGCCAAATT-3'.
5’-AGATCAGCCTGGGCAACAGT-3’, which amplified the 194-bp region (−1026 to −833) of the TTP promoter containing target STAT3 motifs; 5’-CCTGTCCTCGAGC-TCTCTC-3’ and 5’-GCTGATAGGAGTGGCCCTTC-3’, which amplified the 160-bp region (ORF 621–780) of the TTP ORF.

Computational Analysis—For prediction of putative binding sites for transcription factors we used MatInspector software (Genomatix) as described by Quandt et al. (27).

Statistical Analysis—For statistical comparisons, p values were determined using Student’s t test.

RESULTS

Nicotine Induces TTP mRNA and Protein in Human Macrophages—Nicotine, the α7nAChR agonist, suppresses production of inflammatory cytokines from macrophages (28, 29) through activation of STAT3 (7). However, the molecular basis by which nicotine-activated STAT3 shows such anti-inflammatory functions is unknown. STAT3 is a negative regulator of the inflammatory response (8, 9). It has been reported that STAT3 activated by IL-10 induces the TTP expression whereby TTP mediates the anti-inflammatory effects of IL-10 through enhancing the decay of pro-inflammatory transcripts (30). Thus, it is possible that TTP may be a target gene of nicotine-activated STAT3 that mediates the anti-inflammatory functions of nicotine. To verify this we evaluated the effect of nicotine on TTP expression in human macrophages. We first analyzed the effect of nicotine on LPS-induced TNF-α production in human macrophages. Consistent with the previous report (7), nicotine reduced the LPS-induced release of TNF-α from human macrophages in a dose-dependent manner with a half-maximal concentration (EC_{50}) of 8.7 nM (supplemental Fig. S1). Next, we determined the effect of nicotine on the expression levels of TTP in macrophages. LPS treatment alone induced slight but not significant increase of TTP. However, we observed significantly increased induction of TTP protein (Fig. 1A) and mRNA (Fig. 1B) with nicotine at concentration 10 nM. The increase of TTP mRNA level determined by real-time PCR (Fig. 1B) revealed that nicotine-mediated induction of TTP was caused by increased transcription. Similar results were obtained in U937 human monocytes cells (supplemental Fig. S2), suggesting that nicotine-mediated induction of TTP is common to macrophages and U937 cells. Exposure of macrophages to the α7nAChR antagonist α-bungarotoxin (αBgt) or methyllycaconitine (MLA) abrogated the inhibitory effects of nicotine on TNF-α production and also the nicotine induction of TTP (Fig. 1C). These results suggest that the nicotine effect on TTP expression requires binding of nicotine to its receptor, α7nAChR.

Nicotine and STAT3 Induce TTP Promoter Activity—The experiments above indicated that nicotine was able to induce TTP transcription. The nicotine exerts its anti-inflammatory effect on macrophage via STAT3 activation (7). Consistent with previous reports, we found that nicotine induced STAT3 phosphorylation (supplemental Fig. S3). LPS treatment did not increased phosphorylation of STAT3 (supplemental Fig. S3). Down-regulation of STAT3 by siRNA (Fig. 2A) abrogated the anti-inflammatory effect of nicotine (Fig. 2B), indicating the requirement for STAT3 in nicotine-mediated anti-inflammatory response in human macrophage. A search for transcription factor binding sites using online software (MatInspector) revealed the presence of three putative STAT binding sites to the regulation of the TTP promoter, we inhibited STAT3 (Fig. 2C). These data indicate that the human TTP promoter is under positive regulation by nicotine and STAT3 in U937 cells.

STAT-A Is Responsible for Nicotine-inducible Promoter Activity—To evaluate the contribution of three STAT3 binding sites to the regulation of the TTP promoter by nicotine, we made point mutations of them in the context of the reporter

FIGURE 1. Nicotine induces TTP mRNA and protein in human macrophages. Human macrophages were preincubated with 10 nM nicotine for 20 min and stimulated for 4 h with 1 μg/ml of LPS. A, TTP protein in cell lysates of macrophages was determined by immunoblot. β-Actin was used as an internal control. B, TTP mRNA was determined by quantitative real-time PCR. The expression levels obtained from no treated macrophages were set to 1. Data shown were mean ± S.E. of three independent experiments (**, <0.01). C, U937 cells were incubated with α-bungarotoxin (αBgt, 1 μg/ml) or methyllycaconitine (MLA, 1 μM) for 30 min, followed by stimulation with 1 μg/ml of LPS in the presence of nicotine. The mRNA expression of TTP and TNF-α was determined by semi-quantitative RT-PCR. GAPDH was used as internal controls.
Nicotine Enhances the Binding of STAT3 to the TTP Promoter—To determine the nature of the interaction of STAT3 with the putative binding site of the TTP promoter, STAT-A, we performed an EMSA using a 23-bp oligonucleotide bearing the STAT-A (−960 to −938) binding site as a probe. LPS treatment did not lead to increase in the intensity of the DNA-protein complexes (Fig. 3A, lane 2) in U937 cells. However, nicotine treatment significantly increased the intensity of the DNA-protein complexes (Fig. 3A, lane 3) and combination of LPS and nicotine resulted in marked increase of DNA-protein complexes (Fig. 3A, lane 4). The DNA-protein complexes were dramatically reduced by preincubation of the reaction mixture with an anti-STAT3 antibody (Fig. 3A, lane 5). However, preincubation of the reaction mixture with an anti-STAT1 antibody did not reduce the complexes, indicating that STAT1 is not involved in the DNA-protein complex (Fig. 3A, lane 6). These results indicate that STAT3 can specifically interact with the probe (−960/−938), and nicotine increases this interaction.

To confirm the direct binding of STAT3 to the TTP promoter in vivo, we performed ChIP assay in U937 cells treated with 1 μg/ml LPS and 10 nM nicotine. Chromatin was sonicated into fragments and precipitated using anti-STAT1 or anti-STAT3 antibody. The precipitated DNA was subjected to PCR using primers designed to amplify a 194-bp fragment of the TTP promoter flanking the STAT-A site. As a negative control, a region located in the ORF was also examined. As shown in Fig.
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**FIGURE 3.** Nicotine enhances the binding of STAT3 to the TTP promoter and the DNA-binding domain of STAT3 is essential for nicotine induction of TTP promoter activity. A and B, EMSA and Chip assay. U937 cells were preincubated with 10 nM nicotine for 20 min and stimulated for 4 h with 1 μg/ml of LPS. A, an EMSA was performed as described under “Experimental Procedures.” Biotinylated oligonucleotide containing wild-type STAT-A binding site (wt) or mutant STAT-A (mut) was used as a probe. To identify STAT1 or STAT3 immunoreactivity in the DNA-protein complexes, nuclear extracts were incubated with anti-STAT1 antibody, anti-STAT3 antibody, or nonspecific mouse IgG before the addition of the biotinylated probe. An arrow indicates the position of the DNA-protein complexes containing STAT3 (STAT3). B, Chip analysis was performed to confirm the interaction of STAT1 or STAT3 with the TTP promoter in vivo in U937 cells. Formaldehyde cross-linked chromatin from U937 cells was incubated with anti-STAT1 antibody or anti-STAT3 antibody. As negative controls, the chromatin was incubated with nonspecific IgG. Total input DNA at a 1:10 dilution was used as positive control for the PCR reaction. Immunoprecipitated DNA was analyzed by PCR with primers specific for the TTP promoter or ORF. C, DNA-binding domain of STAT3 is required for nicotine-induced down-regulation of TTP. U937 cells were transfected with mock, pCMV-Flag/wtSTAT3, or pCMV-Flag/dnSTAT3 (R382W). After 24 h, cells were preincubated with 10 nM nicotine for 20 min and stimulated for 4 h with 1 μg/ml of LPS. TNF-α in the supernatant of cells was determined by ELSA. The expression levels obtained from LPS-treated U937 cells were determined by qPCR. D, DNA-binding domain of STAT3 is required for nicotine induction of TTP promoter activity. U937 cells were cotransfected with pGL3/TTP P-1345 and pCMV-Flag/wtSTAT3 or pCMV-Flag/dnSTAT3 (R382W) and analyzed the luciferase activity after treatment with nicotine. The levels of firefly luciferase activity were normalized to the luciferase activity of the vector alone. Each bar represents the mean ± S.D. of three independent experiments (***, p < 0.01). ***, p < 0.01).

3B, while LPS treatment slightly increased STAT1 binding to the TTP promoter, it did not affect the STAT3 binding to the TTP promoter. On the contrary, nicotine treatment decreased the STAT1 binding but significantly increased STAT3 binding to the TTP promoter (Fig. 3B). This result indicates that nicotine treatment increases recruitment of STAT3 to TTP promoter in living cells.

DNA Binding Domain of STAT3 Is Required for Nicotine Induction of TTP Promoter Activity and Down-regulation of TNF-α Production. The DNA binding ability of STAT3 has been reported to be responsible for the anti-inflammatory effect of nicotine (7). To determine whether the DNA binding activity of STAT3 is required for nicotine induction of TTP promoter activity, we generated a plasmid construct (pCMV-Flag/dnSTAT3(R382W)) containing dominant negative STAT3 mutant (dnSTAT3(R382W)) with reduced DNA binding ability based on previous reports (10, 11). Consistent with a previous report (7), nicotine failed to reduce LPS-induced TNF-α release in U937 cells transfected with dnSTAT3(R382W) but not those transfected with wtSTAT3 (Fig. 3C). We next cotransfected U937 cells with reporter construct containing TTP promoter (pGL3/TTP p-1345) and pCMV-Flag/wtSTAT3 or pCMV-Flag/dnSTAT3 (R382W) and analyzed the luciferase activity after treatment with nicotine. Another dominant negative mutant dnSTAT3(Y705F) that blocks STAT3 dimerization (31) was included in this experiment. As shown in Fig. 3D, nicotine did not increase TTP promoter activity in U937 cells transfected with either dnSTAT3(R382W) or dnSTAT3(Y705F). These results suggest that, in addition to the dimerization of STAT3, the DNA binding activity of STAT3 is essential for nicotine induction of TTP promoter activity.

TTP Is Required for Nicotine-induced Down-regulation of TNF-α in U937 Cells. We examined the effect of TTP on nicotine-induced down-regulation of TNF-α production in macrophages. As shown in Fig. 4A, siRNA against TTP (siTTP) significantly inhibited the expression of TTP mRNA in U937 cells. Interestingly, siTTP but not scRNA attenuated the inhibitory effect of nicotine on LPS-induced TNF-α production (Fig. 4B). These results suggest that TTP is required for nicotine-induced down-regulation of TNF-α production from U937 cells.

We determined whether nicotine affects the stability of intracellular and secreted TNF-α protein. U937 cells were treated with 1 μM nicotine in the presence or absence of 10 nM nicotine. After incubation with 10 μg/ml CHX to block protein synthesis, cell supernatants and cell lysates were collected and analyzed for TNF-α by ELISA. Intracellular TNF-α was stable until 3 h after CHX treatment and its level was not affected by nicotine. After 3 h of CHX treatment, there was a decrease of intracellular TNF-α and decreased possibly due to release into the media (supplemental Fig. S5A). The secreted TNF-α was stable until 5 h after CHX treatment, and nicotine did not affect the stability of TNF-α (supplemental Fig. S5B). This result indicates that nicotine does not affect the stability of both intracellular and secreted TNF-α.

Our next goal was to determine whether nicotine could decrease the stability of TNF-α mRNA. In LPS-treated cells, TNF-α mRNA was stable until 45 min after actinomycin D treatment. However, in U937 cells treated with nicotine, the half-life of TNF-α mRNA was less than 30 min (Fig. 4C), indicating that the inhibitory effect of nicotine on the TNF-α production from U937 cells results from enhancing the decay of TNF-α mRNA. To determine whether TTP is important for the nicotine-induced destabilization of TNF-α in U937 cells, we transfected U937 cells with siTTP to down-regulate the TTP expression. Interestingly siTTP treatment significantly increased the half-life of the TNF-α mRNA from less than 30 min to more than 45 min in nicotine-treated U937 cells (Fig. 4C), suggesting that TTP is responsible for the nicotine-induced destabilization of TNF-α mRNA. TTP protein has been reported to regulate the stability of TNF-α mRNA through
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FIGURE 4. TTP is required for nicotine-induced down-regulation of TNF-α. U937 cells were transfected with mock, siRNA against TTP (siTTP) or scRNA. After 24 h, cells were preincubated with 10 nm nicotine for 20 min and stimulated for 4 h with 1 μg/ml of LPS. A, down-regulation of TTP expression by siRNA was confirmed by real-time PCR. GAPDH was used as internal controls. The expression levels obtained from mock-treated cells were set to 1. Data shown were mean ± S.E. of three independent experiments (**, p < 0.001). B, TNF-α in the supernatant of cells was determined by ELISA. The expression levels obtained from no treated cells were set to 1. Data shown were mean ± S.E. of three independent experiments (**, p < 0.001). C, expression of TNF-α mRNA in U937 cells was determined by quantitative real-time PCR at indicated times after the addition of 5 μg/ml actinomycin D. Results shown on the graph represent means ± S.E. of three independent experiments (**, p < 0.001). D, U937 cells were cotransfected with psiCHECK/TNF-α 3′-UTR and siTTP or scRNA. After 24 h, cells were preincubated with 10 nm nicotine for 20 min, stimulated for 4 h with 1 μg/ml of LPS and luciferase activity was determined. After normalizing luciferase activity, the relative luciferase activities are presented as a fold increase over no-treated cells. Each bar represents the mean ± S.D. of three independent experiments (**, p < 0.001).

binding to the AREs within mRNA 3′-UTR (18). To determine whether the TNF-α 3′-UTR is involved in nicotine-induced down-regulation of TNF-α production in U937 cells, cells were cotransfected with psiCHECK/TNF-α 3′-UTR and siTTP or scRNA and the luciferase activity was assayed after treatment of cells with LPS and nicotine. As shown in Fig. 4D, while nicotine treatment significantly inhibited the luciferase activity in scRNA-treated cells, the inhibitory effect of nicotine was attenuated in siTTP-transfected cells (Fig. 4D). These results demonstrate that the 3′-UTR of TNF-α mRNA is important for the down-regulation of TNF-α expression by nicotine, and TTP is required for the effect of nicotine.

DISCUSSION

Nicotine has been found to inhibit TNF-α production from LPS-stimulated macrophages (4) through the α7nAChR-mediated STAT3 activation (7). In this study, we provide evidence that nicotine exerts its inhibitory effects through STAT3-dependent TTP expression: nicotine-TNF-α signaling down-regulates TNF-α mRNA stability in an TNF-α 3′-UTR-dependent manner; nicotine increases the STAT3 binding to the TTP promoter, the TTP promoter activity and the expression of TTP; down-regulation of STAT3 by siRNA or dnSTAT3 mutants with reduced DNA-binding activity abolishes the nicotine induction of TTP expression and inhibitory effect; siRNAs to TTP significantly restore the nicotine-mediated inhibition in TNF-α production and TNF-α-UTR Luc activity. These results indicate that the binding of nicotine-activated STAT3 to the TTP promoter and induction of TTP expression is important for nicotine-induced down-regulation of TNF-α production in U937 cells. These results are consistent with those of previous reports suggesting that the DNA binding activity of STAT3 is required for the anti-inflammatory effect of nicotine on macrophages (7). In addition, mutation within the DNA-binding domain of STAT3 resulting in the reduced DNA-binding activity has been found to be associated with the hyper-IgE syndrome, a disorder of immunity in human (10, 11). Until now, nicotine-activated STAT3 has been reported to suppress the expression of inflammatory mediators through inhibition of transcriptional activity of NF-κB (9, 32, 33). However, our results indicate that, just like IL-10-activated STAT3 (30), nicotine-activated STAT3 also exerts its anti-inflammatory function by promoting the transcription of TTP. Even though we did not directly compare the two pathways, we hypothesize that the nicotine-STAT3 signaling pathway may mimics IL-10-STAT3 pathways, or vice versa, to suppress macrophage activity.

It has been reported that stimulation with LPS results in a strong and rapid induction of TTP mRNA in mouse macrophages (34, 35). The stability of TTP mRNA is regulated by TTP itself and TTP expression peaks at early time after LPS stimulation and then decreases through autoregulation (35). Consistent with previous study, we found that LPS alone increased
TTP expression level at 4 h post-treatment in human macrophages, but its effect was mild. Even though we analyzed the TTP expression at early time (1, 2, and 3 h) after LPS stimulation, we could not detect strong induction of TTP expression (data not shown). Even though LPS increases the expression level of TTP, it also activates p38 MAPK, which results in inactivation of TTP and allows the stabilization of ARE-containing TNF-α mRNA (36, 37). IL-10 has been reported to reduce p38 MAPK activity (38) and inhibit TNF-α production from LPS-stimulated macrophages through induction of a STAT3-dependent increase of TTP expression and decrease of p38 MAPK activity (30). In this study, we found that nicotine decreased the TNF-α production from LPS-stimulated human macrophages via STAT3-dependent induction of TTP expression. Interestingly, nicotine is known to increase the phosphorylation of p38 MAPK via STAT3-dependent induction of TTP expression. Collectively, our results suggest the possibility that, even though the nicotine-STAT3 signaling pathway resembles IL-10-STAT3 pathways in induction of TTP expression, they seem to differ in regulation of p38 MAPK activity. Further studies are needed to determine the underlying mechanisms by which nicotine-STAT3 signaling pathway supports TTP to destabilize the TNF-α mRNA in the presence of p38 MAPK activity in LPS-stimulated macrophages.

We found a discrepancy between levels of the TNF-α transcripts and protein in nicotine-treated U937 cells: Whereas nicotine induced strong destabilization of TNF-α mRNA and strong down-regulation of TNF-α mRNA level, it induced mild decrease in TNF-α level. Our expectation was that there would be good correlation between the expression levels of TNF-α mRNA and protein. It has been reported that TNF-α is highly stable at neutral pH and at 37 °C (40, 41). Consistent with the previous reports, we found that TNF-α in the supernatant is stable in the presence of nicotine, which may be a possible explanation for the discrepancy.

In conclusion, we have shown that nicotine-STAT3 signaling induces the expression of TTP in macrophages and thus, down-regulates TNF-α production by destabilizing its transcripts. Because an excess of TNF-α is involved in many inflammatory diseases, the inhibition of TNF-α production through the modulation of nicotine-STAT3-TTP signaling pathway may have wide-ranging clinical implications.

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