Guggulsterone (GS) inhibits smokeless tobacco and nicotine-induced NF-κB and STAT3 pathways in head and neck cancer cells

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Understanding the molecular pathways perturbed in smokeless tobacco (ST)-associated head and neck squamous cell carcinoma (HNSCC) is critical for identifying novel complementary agents for effective disease management. Activation of nuclear factor-kappaB (NF-κB) and cyclooxygenase-2 (COX-2) was reported in ST-associated HNSCC by us [Sawhney, M. et al. (2007) Expression of NF-kappaB parallels COX-2 expression in oral precancer and cancer: association with smokeless tobacco. Int. J. Cancer, 120, 2545–2556]. In search of novel agents for treatment of HNSCC, we investigated the potential of guggulsterone (GS), ((4,17(20)-pregnadiene-3,16-dione), a biosafe nutraceutical, in inhibiting ST- and nicotine-induced activation of NF-κB and signal transducer and activator of transcription (STAT) 3 pathways in HNSCC cells. GS inhibited the activation of NF-κB and STAT3 proteins in head and neck cancer cells. This inhibition of NF-κB by GS resulted from decreased phosphorylation and degradation of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha the inhibitory subunit of NF-κB. Importantly, treatment of HNSCC cells with GS abrogated both ST- and nicotine-induced nuclear activation of NF-κB and pSTAT3 proteins and their downstream targets COX-2 and vascular endothelial growth factor. Furthermore, GS treatment decreased the levels of ST- and nicotine-induced secreted interleukin-6 in culture media of HNSCC cells. In conclusion, our findings demonstrated that GS treatment abrogates the effects of ST and nicotine on activation of NF-κB and STAT3 pathways in HNSCC cells that contribute to inflammatory and angiogenic responses as well as its progression and metastasis. These findings provide a biologic rationale for further clinical investigation of GS as an effective complementary agent for inhibiting ST-induced head and neck cancer.

Abbreviations: CLSM, confocal laser scan microscopy; COX-2, cyclooxygenase-2; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis (aminoethylthelylether)-tetracetic acid; HEPES, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid; HNSCC, head and neck squamous cell carcinoma; GS, guggulsterone; IL, interleukin; IkBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; NF-κB, nuclear factor-kappaB; PBS, phosphate-buffered saline; SCC, squamous cell carcinoma; SDS, sodium dodecyl sulfate; STAT, signal transducer and activator of transcription; ST, smokeless tobacco; VEGF, vascular endothelial growth factor.

Introduction

Tobacco is used by >1.3 billion people worldwide (1); it kills >5 million people annually and the World Health Organization estimates this number to increase to 8 million by 2030 (2). Tobacco use is the single largest cause of cancer globally (3,4). The number of adult smokers has declined in the USA (3,4), but the use of smokeless tobacco (ST) products (e.g. chewing tobacco, khaini and snuff) is increasing worldwide, and these products can be a gateway for lifelong addiction (1,5). ST consumption is emerging as a major risk factor for head and neck squamous cell carcinoma (HNSCC) (6,7), the most common cancer in men in many Asian countries and the sixth most common cancer in USA (8). The causal association between smoking and HNSCC has been unequivocally established (9–12). The evidence that ST causes oral cancer was confirmed by the International Agency for Research on Cancer and ST was classified as a human carcinogen, with nitrosamines being one of the major carcinogenic constituents (13). In a recent study, 23 polycyclic aromatic hydrocarbons have been identified in ST (14). Nicotine, a major component of ST, besides causing addiction has been shown to regulate cell proliferation, angiogenesis and inhibit apoptosis induced by anticancer drugs (15). An in-depth understanding of the molecular mechanism(s) underlying ST-related head and neck carcinogenesis will lead to the development of effective strategies to prevent and treat ST-related HNSCC.

Studies carried out in our laboratory and others have shown aberrant expression of genes involved in ST-associated HNSCC (16–20). ST has been shown to increase oxidative stress (21) plays a major role in activation of nuclear factor-kappaB (NF-κB) and pSTAT3 pathways, involved in inflammation, survival and proliferation of cancer cells (21–24). Our laboratory also reported exposure of oral premalignant cultures and cancer cells (AMOS III) to ST resulted in increased cell prolifeeration and activation of NF-κB; association of ST consumption with overexpression of NF-κB and cyclooxygenase-2 (COX-2) was observed in clinical oral squamous cell carcinoma (SCC) tissue samples also (23–25). The persistent activation of NF-κB can lead to elevated expression and secretion of interleukin (IL)-6. Interestingly, blocking NF-κB diminished the accumulation of active signal transducer and activator of transcription (STAT) 3 in HNSCC cells, suggesting existence of cross talks between NF-κB and STAT3 pathways (25). Recently our group observed nuclear pSTAT3 accumulation in clinical oral squamous cell carcinoma tissue samples was significantly associated with ST consumption habits (26), indicating a plausible link between STAT3 and ST. Therefore, it is important to identify agents that can abrogate these effects of ST in head and neck cancer cells for developing therapies to prevent and treat ST-related head and neck cancer.

Guggulsterone (GS), ((4,17(20)-pregnadiene-3,16-dione), derived from the plant Commiphora mukul, is widely used to treat obesity, diabetes, hyperlipidemia, atherosclerosis and osteoarthritis (27). GS suppresses inflammation by inhibiting inducible nitric oxide synthase (28) and NF-κB induced by various carcinogens and tumor promoters (29). Our group and several other reports have shown that treatment with GS induces apoptosis and suppress proliferation of wide variety of human tumor cell types (28–33). GS inhibits invasion, angiogenesis and metastasis of tumor cells and reverses chemoresistance (31–33). GS has also been shown to inhibit both constitutive and inducible STAT3 pathways in head and neck cancer cells (34,35). Recently, Leeman-Neill et al. (35) showed antiproliferative effects of GS are partially dependent on STAT3 inactivation. They showed knocking down expression of STAT3 using small interfering RNA in head and neck cancer cells reduced GS-induced cell death in comparison with the no transfection controls. In addition, our group in collaboration with Dr Bharat Aggarwal’s laboratory showed that GS inhibits inducible and constitutive STAT3 activation through the
induction of tyrosine phosphatase, which makes it a potentially effective suppressor of tumor cell survival and proliferation (34). We further showed that activation of STAT3 by IL-6 is completely abrogated by pretreatment with GS in melphalan sensitive MM.1S cells. Similar results of GS-induced inhibition of STAT3 activation have also been reported in colon cancer cells (36). Together, these reports clearly suggest inhibition of STAT3 activation is an important mechanism of GS-induced cell death. Hence, it will be interesting to determine the effect of GS on ST- and nicotine-induced head and neck carcinogenesis.

In the current study, we determined the effect of ST and/or nicotine treatment on NF-kB and STAT3 signaling in head and neck cancer cells. Furthermore, we challenged ST- and/or nicotine-induced activation of NF-kB and STAT3 signaling by pretreatment of head and neck cancer cells (SCC4 and HSC2) with GS, to evaluate its therapeutic potential. This may help us in designing novel therapies based on such biosafe nutraceutical targets that target ST-induced inflammation, survival and proliferation in head and neck cancer cells.

Materials and methods

Antibodies and reagents

Z-GS, nicotine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and propidium iodide were purchased from Sigma–Aldrich (St Louis, MO). Z-GS was dissolved in dimethyl sulfoxide as a 100 mM stock solution and stored at −20°C. Penicillin, streptomycin, Dulbecco’s modified eagle medium/ F12 medium, fetal bovine serum, l-glutamine, sodium pyruvate, vitamins and minimum essential medium were obtained from Invitrogen (Grand Island, NY). Rabbit polyclonal antibodies against NF-κB (p65 subunit) (sc-109), NF-κB (p65 subunit) (sc-101749, Ser-276), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα) (sc-7117), plkBα (sc-101713, Ser-32), Jak1 (sc-295), pJak1 (sc-16773), Tyr 1022 and vascular endothelial growth factor (VEGF) (sc-152); goat polyclonal antibodies against COX-2 (sc-730), pSTAT3 (sc-7993, Tyr 705) and mouse monoclonal antibodies against STAT3 (sc-8019) and α-tubulin (sc-5386) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit, anti-goat, anti-mouse horseradish peroxidase conjugated proteins were purchased from DAKO Cytomations (Glostrup, Denmark), and goat anti-rabbit Alexa 594 was purchased from Molecular Probes (Eugene, OR). Bacteria-derived recombinant human IL-6 was obtained from Biosource International (Camarillo, CA). ST extract was prepared as described before by us (37).

Cell culture

Head and neck cancer cells (SCC4 and HSC2) were grown in monolayer cultures in Dulbecco’s modified eagle medium (Sigma, St Louis, MO) supplemented with 10% fetal bovine serum (Sigma), 1 mM l-glutamine, 1 mM minimum essential medium, 100 μg/ml streptomycin and 100 μg/ml penicillin in a humidified incubator (5% carbon dioxide, 95% air) at 37°C as described earlier (38).

Preparation of nuclear extracts

Nuclear extracts were prepared from control, untreated and treated head and neck cancer cells (SCC4 and HSC2) as described earlier (39). Briefly head and neck cancer cells (SCC4 and HSC2) (2 × 10^6) cells were treated, harvested, washed with cold phosphate-buffered saline (PBS, pH = 7.2) and incubated on ice for 15 min in hypotonic buffer A (10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) (pH = 7.9), 10 mM KCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM ethyleneglycol-bis(ami-noethylther)-tetraacetic acid (EGTA), 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride and 0.6% NP-40). Cells were vortexed gently for 15 min, on ice for 2 h and were extracted sequentially for 10 min each in buffer B (20 mM Tris, pH 8.1, containing 1% SDS, 1% Triton X 100, 2 mM EDTA and 150 mM NaCl), buffer C (20 mM Tris, pH = 8.1, containing 500 mM NaCl, 1% SDS, 1% Triton X 100 and 2 mM EDTA), buffer D (0.5% LiCl, 1% NP-40, 1% deoxycholate 1 mM EDTA, 10 mM Tris, pH = 8.1) and 10 mM Tris, pH 8.1 and 2 mM EDTA (TE buffer). Protein–DNA complexes were eluted from the protein-A agarose beads with 1% SDS and 0.1% NaHCO3 at room temperature for 10 min with gentle agitation. The supernatant was transferred to a fresh tube, and cross-links were reversed overnight at 65°C. Samples were treated with Proteinase K (Sigma Chemical Co., St Louis, MO) at 37°C for 2 h and were extracted once with phenol and the DNA was precipitated with 2.5 vol ethanol plus 20 μg glycerogen as carrier. Precipitated DNA was pelleted, washed once with 70% ethanol, dried and resuspended in water. The purified DNA was used as a template for polymerase chain reaction amplification using primers (forward, 5'-TTGTTGCGCAAATTTCTTCC-3'; reverse, 5'-CAGACCTGCTCTACTCTC GAA-3') flanking the putative STAT-binding sites located at −848 and −630 in human VEGF promoter region. The polymerase chain reaction products were resolved on 1% agarose gel and the band intensities were quantified using Chemiimager 4400 (Alpha Innotech Corporation).

Results

Effect of GS on ST- and nicotine-induced phosphorylation and localization of p65 subunit of NF-κB

Phosphorylation of p65 subunit of NF-κB complex is required for its nuclear translocation and transcriptional activation. Therefore, we investigated the effect of GS on ST- and nicotine-induced phosphorylation of p65 in head and neck cancer cells. SCC4 and HSC2 cells were incubated with specific antibodies as per manufacturer’s recommended protocol at 4°C served as a control for protein loading in each lane. Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies, (DAKO Cytomation), diluted at an appropriate dilution in 1% bovine serum albumin, for 2 h at room temperature. After each step, blots were washed three times with Tween (0.1%)-Tris-buffer saline. Protein bands were detected by the enhanced chemiluminescence method (Santa Cruz Biotechnology) on XOMAT film. The band intensities were measured as integrated density values using AlphaEase FC Software (version 3.1) with ChemiImager IS-4400 (Alpha Innotech Corporation, San Leandro, CA).

Confocal laser scan microscopy

For confoal laser scan microscopy (CLSM), 5 × 10⁴ SCC4 cells were plated on coverslips and grown for 24 h. Expression and localization of p65 protein was observed in treated and untreated, control SCC4 cells using CLSM as described by us (38). Briefly, treated and control SCC4 cells were, fixed in methanol for 20 min at −20°C, rinsed with PBS (pH = 7.2) and incubated with p65 antibody overnight at 4°C. After rinsing in PBS, the coverslips were incubated with biotinylated secondary antibody (LSAB plus Kit; DAKO Cytomations) for 45 min at 37°C followed by incubation with streptavidin-conjugated fluorochrome, fluorescein isothiocyanate (DAKO Cytomations). Thereafter, the coverslips were counterstained with propidium iodide (10 mg/ml; Sigma) for 30 s. Coverslips were rinsed and mounted in mounting medium. Slides were examined using a CLSM-LSM510 scanning module (Carl Zeiss Microscopy; Jena GmbH, Germany).
treated with GS (50 μM)/ST (20 μg/ml)/nicotine (10 μM) for different time intervals, and protein extracts obtained from cytosolic fraction were checked for phosphorylated p65 protein. As shown in Figure 1a, GS completely inhibited the phosphorylation of p65, whereas either ST- or nicotine-induced the p65 phosphorylation in a time-dependent manner with significant increase of phosphorylated p65 (pp65) observed after 2 h of treatment in SCC4 cells (Figure 1b and c). Interestingly, we observed that treatment with GS (50 μM) for 4 h completely abrogated the ST- or nicotine-induced phosphorylation of p65, respectively, in both the cell lines (Figure 1d and e). Thus, GS inhibited ST- or nicotine-induced NF-κB activation by inhibiting p65 phosphorylation.

GS treatment decreased nuclear accumulation of p65 as compared with the untreated control (Figure 2a), whereas ST or nicotine increased nuclear accumulation of p65 as shown by western blotting.

Fig. 1. Effect of GS on ST- and nicotine-induced phosphorylation p65. SCC4 cells were treated with (a) 50 μM GS for 0 and 30 min and 1, 2, 4 and 6 h; (b) ST (20 μg/ml) for 0, 1, 2, 4, 6 and 9 h; or (c) 10 μM nicotine for 0, 30 min, 1, 2, 3 and 4 h. SCC4/HSC2 cells were pretreated with 50 μM GS for 0, 1, 2, 4, 6 and 9 h, followed by ST for 4 h (d) or with nicotine for 1 h (e). Cytoplasmic extracts were prepared, separated on 10% SDS–PAGE. Proteins were then electrotransferred on polyvinylidene fluoride membrane followed by blocking with 5% non-fat milk overnight. Blots were incubated with specific antibody against pp65. Protein expression was determined using enhanced chemiluminescence method. The same blots were stripped and reprobed with α-tubulin antibody to show equal protein loading.
Fig. 2. (a–e) Effect of GS on ST- and nicotine-induced nuclear translocation of p65. SCC4 cells were treated with (a) 50 μM GS for 0, 30 min, 1, 2, 4 and 6 h; (b) ST (20 μg/ml) for 0, 1, 2, 4, 6 and 9 h; or (c) 10 μM nicotine for 0 and 30 min and 1, 2, 3 and 4 h. SCC4/HSC2 cells were pretreated with 50 μM GS for 0, 30 min, 1, 2, 4, and 6 h, followed by ST for 4 h (d) or with nicotine for 2 h (e). Cytoplasmic and nuclear extracts were prepared according to materials and methods and separated on 10% SDS–PAGE. Proteins were then electrotransferred on polyvinylidenedifluoride membrane followed by blocking with 5% non-fat milk overnight. Blots were incubated with specific antibody against p65. Protein expression was determined using enhanced chemiluminescence method. The same blots were stripped and reprobed with α-tubulin or laminin B antibody to show equal protein loading in cytoplasmic and nuclear fraction respectively. (f) Effect of GS on ST- and nicotine-induced nuclear translocation of p65 using immunofluorescence. SCC4 cells grown on coverslips, were kept untreated (i–iii) or treated with GS (50 μM) for 4 h (iv–vi); ST (20 μg/ml) for 6 h (vii–ix); with nicotine (10 μM) for 2 h (x–xii); pretreated with 50 μM GS for 4 h and stimulated with ST (20 μg/ml) for 6 h (xiii–xv) or with nicotine (10 μM) for 2 h (xvi–xviii). Then immunolabelled with anti-p65 antibody followed by streptavidin-conjugated fluorescein.

GS inhibits NF-κB and STAT3 activation.
using cytoplasmic and nuclear fractions obtained from treated and control SCC4 cells (Figure 2b and c). However, treatment with GS (50 μM) for 4 h completely abrogated the ST- or nicotine-induced nuclear translocation, with concomitant increase in cytoplasmic accumulation of p65 in both SCC4 and HSC2 cells (Figure 2d and e). These findings were further confirmed by CLSM of p65 subunit using control and GS-treated SCC4 cells (Figure 2f).

Effect of GS on ST- and nicotine-induced IκBα phosphorylation

The degradation of IκBα and subsequent release of NF-κB (p65 and p50) requires prior phosphorylation at Ser-32 and Ser-36 residues. Therefore, to investigate whether the inhibitory effect of GS is mediated through the alteration of IκBα phosphorylation, SCC4 cells were treated with GS (50 μM)/ST (20 μg/ml)/nicotine (10 μM) for different time intervals and western blotting was performed for phospho-IκBα.

Fig. 2. Continued.

Isothiocyanate (FITC)-labeled tertiary antibody (green fluorescence) and nuclei were counterstained with propidium iodide (PI) (red fluorescence). Panels (i), (iv), (vii), (x), (xiii) and (xvi) represent nuclei showing red fluorescence of PI; panels (ii), (v), (viii), (xi), (xiv) and (xvii) shows green fluorescence for p65 protein in cytoplasm and nuclei; panels (iii), (vi), (ix), (xii), (xv) and (xviii) shows increased green fluorescence for p65 protein in cytoplasm of SCC4 cells. (original magnification ×100 for all photomicrographs).
Effect of GS on ST- and nicotine-induced IκBα phosphorylation and IκBα degradation. SCC4/HSC2 cells were treated with GS (50 μM) for 0, 30 min, 1, 2, 4 and 6 h; ST (20 μg/ml) for 0, 1, 2, 4, 6 and 9 h; or 10 μM nicotine for 0, 30 min, 1, 2, 3 and 4 h. SCC4 cells were pretreated with 50 μM GS for 0, 1, 2, 4 and 6 h, followed by ST for 4 h or with nicotine for 2 h. Whole-cell extracts were prepared, separated on 10% SDS–PAGE. Proteins were then electrotransferred on polyvinylidenedifluoride membrane followed by blocking with 5% non-fat milk overnight. Blots were incubated with specific antibody against pIκBα (a–e) and IκBα (f–j). Protein expression was determined using enhanced chemiluminescence method. The same blots were stripped and reprobed with α-tubulin antibody to show equal protein loading.
As shown in Figure 3a, GS treatment decreased constitutively expressed phosphorylated \(\text{I}\text{kB}_\alpha\) (Ser-32/36) as revealed by western blotting. However, either ST or nicotine increased the expression of p\(\text{I}\text{kB}_\alpha\) with a significant increase in expression observed after 4 and 1 h on ST and nicotine treatment, respectively (Figure 3b and c). Interestingly, treatment with GS (50 \(\mu\)M) completely abrogated ST/nicotine-induced phosphorylation of \(\text{I}\text{kB}_\alpha\) in SCC4 and HSC2 cells (Figure 3d and e).

We determined whether the GS inhibition by either ST- or nicotine-induced NF-\(\kappa\)B activation was due to inhibition of \(\text{I}\text{kB}_\alpha\) degradation. GS induced the expression of \(\text{I}\text{kB}_\alpha\) in a time-dependent manner (Figure 3f), with maximum expression observed at 4 h; however, ST- or nicotine-induced \(\text{I}\text{kB}_\alpha\) degradation in a time-dependent manner (Figure 3g and h). ST induced the degradation of \(\text{I}\text{kB}_\alpha\) was observed within 4 h and complete loss of \(\text{I}\text{kB}_\alpha\) expression was found within 6 h of ST treatment. Upon nicotine treatment, degradation started as early as in 2 h with no detectable expression observed within 4 h. Notably, pretreatment with GS abrogated the ST-induced \(\text{I}\text{kB}_\alpha\) degradation in both cell lines (Figure 3i). Similarly we observed nicotine-induced \(\text{I}\text{kB}_\alpha\) degradation was abrogated in GS pretreated SCC4 and HSC2 cells.

Fig. 3. Continued.
Thus, GS pretreatment abolished ST- and nicotine-induced NF-κB activation by inhibiting the phosphorylation of IκBα.

GS inhibits upregulation of COX-2 in response to ST and nicotine treatment

NF-κB activation results in nuclear translocation p65-subunit followed by increased expression of its target genes, such as COX-2. Therefore, we investigated the effect of GS on COX-2 expression.

A time-dependent decrease in COX-2 expression was observed, with complete absence of COX-2 protein at 6 and 12 h of GS treatment (Figure 4a). Treatment with ST and nicotine increased the cytoplasmic expression of COX-2 (Figure 4b). Treatment with tumor necrosis factor-α [TNF-α (0.1 nM, 1 h)] was used as a positive control for inducing NF-κB-mediated COX-2 upregulation. Notably, GS treatment inhibited the ST- and nicotine-induced expression of COX-2 in a time-dependent manner (Figure 4b).
Effect of GS, ST and nicotine on the expression of secretory IL-6

The persistent activation of NF-κB can lead to elevated expression and secretion of IL-6 in HNSCC and may contribute to inflammatory and angiogenic responses as well as its progression and metastasis. We determined secreted IL-6 levels in GS, ST- or nicotine-treated SCC4 cells using enzyme-linked immunosorbent assay. SCC4 cells were treated with GS (50 μM), ST (20 μg/ml) and nicotine (10 μM) for different time intervals, and cell-free supernatants were collected and filtered through a 0.22 μm low-protein-binding polyethyrsulfonate membrane filter. Aliquots of 100 μl were used for IL-6 assay using an

Fig. 5. GS inhibits IL-6 inducible STAT3 phosphorylation in SCC4 cells. (a) SCC4 cells (2–3 × 10^6 cells) were treated with IL-6 (10 ng/ml) for the indicated times, and whole-cell extracts were prepared. (b) SCC4 cells (2–3 × 10^6 cells) were pretreated with 50 μM GS for the indicated durations and stimulated with IL-6 (10 ng/ml) for 2 h and whole-cell extracts were prepared. (c) GS-induced inhibition of STAT3 phosphorylation is reversible. SCC4 (2–3 × 10^6 cells) were treated with 50 μM GS for the indicated durations and stimulated with IL-6 (10 ng/ml) for 2 h and whole-cell extracts were prepared. Sixty micrograms of whole-cell extracts were resolved on 7.5% SDS–PAGE, electrotransferred to a polyvinylidenedifluoride membrane followed by blocking with 5% non-fat milk overnight. Protein expression was determined using enhanced chemiluminescence method and probed for the phosphorylated STAT3 (upper panel) and stripped and reprobed for STAT3 (lower panel). Western blot for GAPDH was done to show equal loading of protein.
enzyme-linked immunosorbent assay kit. We observed that GS decreased IL-6 levels in a time-dependent manner with complete loss of IL-6 within 6 h of GS treatment (Figure 4c). However, both ST and nicotine increased IL-6 secretion in a time-dependent manner (Figure 4c). These results were in concordance with the expression levels of NF-κB in SCC4 cells.

GS inhibits IL-6 inducible STAT3 phosphorylation in SCC4 cells

IL-6-induced signals are mediated through STAT3 phosphorylation; therefore, we determined the effect of GS on IL-6-induced STAT3 phosphorylation. SCC4 cells were treated with IL-6 (10 ng/ml) for different time intervals and whole-cell lysates were prepared. Western blot analysis showed that IL-6 induced phosphorylation of STAT3 in a time-dependent manner with phosphorylation induction as early as in 10 min and maximum phosphorylation at 2 h (Figure 5a). SCC4 cells were then preincubated with GS for different time intervals, followed by IL-6 treatment for 2 h and effect on STAT3 phosphorylation was examined. Figure 5b shows that IL-6-induced STAT3 phosphorylation was blocked by GS in a time-dependent manner. Exposure of cells to GS for 4 h was sufficient to completely suppress IL-6-induced STAT3 phosphorylation. However, GS treatment alone at these time points had no effect on STAT3 levels in these cells (Figure 5a).

GS induced inhibition of STAT3 phosphorylation is reversible in SCC4 cells

We next determined whether GS-induced inhibition of STAT3 phosphorylation was reversible. SCC4 cells were first treated with GS for 4 h, washed twice with PBS and the cells were then cultured in fresh medium without GS for different time intervals, and the levels of phosphorylated STAT3 were measured. GS treatment induced the suppression of STAT3 phosphorylation (Figure 5c), but once GS was removed, the levels of phosphorylated STAT3 increased in a time-bound manner (Figure 5c). The reversal of phosphorylation was partial at 18 h, with complete reversal at 36 h, without any changes in the STAT3 levels.

GS inhibited ST/nicotine-induced STAT3 phosphorylation in SCC4 cells

The aberrant function of transcription factor NF-κB can lead to stimulation of STAT3 by an autocrine/paracrine mechanism involving the release of IL-6, prompting us to determine the effect of GS on the molecular cross talk between these two pathways. We investigated whether GS inhibits the constitutive STAT3 phosphorylation in SCC4 cells in accordance with its effect on NF-κB expression. SCC4 cells were incubated either with GS (50 μM) for indicated time intervals and compared with the untreated control cells. Western blot analysis showed that GS inhibited the constitutively active STAT3 in a time-dependent manner (Figure 5d). GS-induced inhibition was observed as early as 1 h with complete inhibition of STAT3 phosphorylation observed at 4 h; however, GS treatment did not alter the overall STAT3 protein expression (Figure 5d). We also observed similar effect of GS treatment on the expression of activated upstream STAT3 kinase, pJAK1 (Figure 5d). However, no effect was observed on the total JAK1 expression at these time points of GS treatment. These results suggested that GS-induced inhibition of STAT3 phosphorylation was mediated by inhibiting the upstream kinases.

Furthermore, we investigated the effect of GS on ST- and nicotine-induced STAT3 activation in SCC4 cells by western blotting. Both ST- and nicotine-induced STAT3 phosphorylation (Figure 5e and f). Increased STAT3 phosphorylation was observed in a time-dependent manner with maximum phosphorylation at 6 h by ST and within 4 h by nicotine, respectively. SCC4 cells were then incubated with GS for different time intervals and examined for ST and nicotine inducible STAT3 phosphorylation. Both ST- and nicotine-induced STAT3 phosphorylation was blocked by GS in a time-dependent manner (Figure 5g and h). Exposure of cells to GS for 4 h completely suppressed ST- and nicotine-induced STAT3 phosphorylation. GS, ST or nicotine, alone or in combination, had no effect on total STAT3 expression levels in these cells. These results clearly demonstrate that GS abrogated ST- and nicotine-mediated activation of pSTAT3 in SCC4 cells.

GS inhibited ST- and nicotine-induced expression of VEGF and recruitment of STAT3 to the VEGF promoter

STAT3 is a transcriptional activator of VEGF and STAT3 inhibition effectively blocks VEGF expression. STAT3 is being activated by both ST and nicotine, therefore, we demonstrated the effect of these compounds on VEGF expression and effect of GS pretreatment on ST- or nicotine-induced VEGF expression using western blotting. SCC4 cells were treated with either GS (50 μM) for different time intervals, or with ST (20 μg/ml)/nicotine (10 μM) for 6 and 12 h. As shown in Figure 6a, GS downregulated VEGF expression in a time-dependent manner, whereas both ST- and nicotine-induced VEGF expression (Figure 6b). Notably, pretreatment of cells with GS for 6 h followed by either ST or nicotine for 12 h, inhibited both ST- and nicotine-induced VEGF expression (Figure 6b).

To demonstrate the effect of GS on ST- or nicotine-induced interaction between STAT3 protein and VEGF promoter in vivo, we carried out chromatin immunoprecipitation assays using an anti-STAT3 antibody. The occupancy of the promoter was analyzed using specific pairs of primers spanning the STAT3 binding motif of the VEGF promoter. SCC4 cells were treated with either GS (50 μM) for 6 h; ST (20 μg/ml)/nicotine (10 μM) 12 h or pretreated with GS for 6 h.
followed by ST and nicotine for 12 h. Significantly greater binding of STAT3 to the VEGF promoter was observed in ST- or nicotine-treated cells (Figure 6c). GS treatment significantly inhibited both ST- and nicotine-induced recruitment of STAT3 to the VEGF promoter (Figure 6c).

Discussion

Knowledge of molecular pathways implicated in ST-induced head and neck cancers is critical for development of novel agents that are better targeted to ST-induced molecular changes and to ST-related HNSCC for effective disease management. Herein, we demonstrated that ST or nicotine activates NF-κB in HNSCC cells (SCC4 and HSC2) and this activation was mediated through phosphorylation and degradation of IκBα. ST as well as nicotine-induced IL-6 secretion, which in turn activated the STAT3 pathway. Therefore, agents that can suppress NF-κB and STAT3 activation might have potential to suppress ST/
activation of NF-κB can also stimulate STAT3 by an autocrine/paracrine mechanism involving the release of IL-6, which confers both proliferative and survival potential in HNSCC (25,41), prompting us to determine the effect of GS on molecular cross talk between these two pathways. We observed GS decreased IL-6 secretion in SCC4 cells, whereas both ST and nicotine increased the secretory IL-6 levels, and these changes in IL-6 levels were paralleled by changes in p65 subunit of NF-κB. We also observed that GS abrogated both constitutive and ST/nicotine-induced STAT3 phosphorylation in cancer cells, which may be one of the mechanisms for reducing the proliferative potential of HNSCC cells.

Interestingly, Arredondo et al. (42) showed multifold increase in STAT-3 in oral keratinocytes stimulated with aged and diluted side-stream cigarette smoke or nicotine, both at the messenger RNA and protein levels. In addition, the downregulation of NF-κB, constitutive and inducible STAT3 by GS in other HNSCC cell lines and xenografts have been reported (28,31,34,35,43,44). Importantly, these observations are in accord with clinical studies from our group and others, showing significant association of pSTAT3 activation with ST consumption habits (26,45). Previously, we have also shown that GS downregulates the expression of cyclin D1, Bcl2 and Bcl-xL in SCC4 cells (46). These genes are known to be regulated by both STAT3 and NF-κB (47,48). Notably, we found that suppression of STAT3 phosphorylation by GS was reversible, returning to nearly control values within 36 h of GS withdrawal.

Activation of NF-κB and COX-2 by ST was previously reported by our group and others using different HNSCC cell lines, namely AMOS III and SCC38 (22,24,48). Herein, we showed that ST- and nicotine-induced NF-κB activation was mediated by the phosphorylation and degradation of IκBα. Notably, other forms of ST, such as snuff extracts, have been shown to stimulate nuclear localization of p50 and p65 subunits of NF-κB in RAW264.7 cells. Normal human oral keratinocytes exposed to environmental tobacco smoke or nicotine also showed a multifold increase in NF-κB at the messenger RNA and protein levels. Importantly, GS inhibited constitutive COX-2 expression and treatment with GS abrogated the expression of both ST- and nicotine-induced COX-2 expression. COX-2 has been implicated in carcinogenic processes and its overexpression in malignant cells has been shown to enhance cellular invasion, induce angiogenesis, regulate anti-apoptotic cellular defenses and augment immunologic resistance through production of prostaglandin E2 (22,48–50). Our results suggested that nicotine-induced NF-κB activation resulting in increased expression of COX-2 protein head and neck cancer cells (SCC4), which can be inhibited by treatment with GS.

Our study also showed GS-inhibited ST/nicotine-induced STAT3 activation is required for VEGF receptor signaling and neovascularization in human oral cancer (36). In accordance with the STAT3 activation, we observed an increase in VEGF expression in SCC4 cells upon treatment with ST or nicotine and GS downregulated the levels of VEGF protein. The similar time kinetics of STAT3 and VEGF expression prompted us to determine if STAT3 regulated VEGF expression by activation of the VEGF promoter. Using co-immunoprecipitation and chromatin immunoprecipitation assays, we identified an increased interaction of STAT3 on the VEGF promoter upon ST and nicotine treatment, whereas GS treatment inhibited this interaction, suggesting that STAT3 might serve as a transcription regulator of VEGF promoter. These observations are consistent with an earlier report demonstrating GS-inhibited STAT3 binding on VEGF promoter (36). Thus, ST-induced STAT3 inhibition by GS and the subsequent reduction in VEGF expression could represent a potential clinical therapeutic approach for patients with ST-associated HNSCC.

In conclusion, our studies demonstrated that ST and nicotine activate both NF-κB and STAT3 pathways in HNSCC cells and their downstream targets—COX-2 and VEGF. Importantly, GS not only inhibits the constitutively active NF-κB and STAT3 pathways but also abrogates ST- and nicotine-induced activation of these pathways underscoring its putative chemopreventive potential. Hence, our findings provide a biologic rationale for investigating the chemopreventive and complementary therapeutic potential of GS in ST-associated head and neck cancers.

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References


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