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TITLE PAGE

α4β2 Nicotinic Receptors Partially Mediate Anti-Inflammatory Effects through JAK2-STAT3 but not Calcium or cAMP signaling

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α4β2 anti-inflammation and JAK2-STAT3 signaling

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ABBREVIATIONS

AD, Alzheimer's disease; BAPTA-AM, 1,2-*bis* (*o*-Aminophenoxy) ethane-N,N,N′,N′-tetraacetic Acid Tetra (acetoxymethyl) Ester; hα4β2 SHEP1, human SHEP1 neuroblastoma-derived cells stably transfected with human α4β2 nicotinic receptors; HBSS, Hank's Balanced Salt Solution; IκBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; JAK2, janus kinase 2; MS, multiple sclerosis; nAChR, nicotinic acetylcholine receptor; NFκB, nuclear factor kappa B; PBS, Phosphate Buffered Saline; PD, Parkinson's disease; PIC, pro-inflammatory cytokine; RFU, relative fluorescence units; RLU, relative luminescence units; STAT3, Signal Transducer and Activator of Transcription 3; TLR4, toll-like receptor 4.

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ABSTRACT

Despite evidence that smoking confers protection against neurological disorders, how and whether specific nicotinic receptor subtypes are involved is unknown. We previously reported that nicotine suppresses constitutive NFκB activity and thereby pro-inflammatory cytokine (PIC) production in SHEP1 cells stably transfected with α4β2 nicotinic receptors. Here we report the anti-inflammatory effects of nicotine pre-treatment in lipopolysaccharide (LPS)-stimulated SHEP1 cells. Nicotine (100-300 nM, concentrations found in smoker's blood) blocked LPS-induced NFκB translocation and production of PICs IL-1β and IL-6, but only partially blocked IκBα-phosphorylation. These effects were exclusively in cells transfected with α4β2 receptors but not in wild-types. A cell-permeable calcium chelator BAPTA-AM, the adenylate cyclase stimulant forskolin and a specific PKA inhibitor PKI 14-22 amide failed to block the effect of nicotine on LPS-induced NFκB translocation and IκBα-phosphorylation. However, the effects of nicotine on NFκB activity were significantly blocked by the highly specific JAK2 inhibitor AG-490 and the STAT3 inhibitor NSC74859. These findings reveal a calcium-and cAMP-PKA independent signaling cascade and suggest a role for JAK2-STAT3 transduction in α4β2-mediated attenuation of LPS-induced inflammation. Anti-inflammatory effects of nicotine may therefore be mediated through α4β2 receptors, the predominant high-affinity binding sites for nicotine in the CNS, in addition to the better-established α7 receptors.

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INTRODUCTION

Neurological disorders such as Alzheimer's (AD), Parkinson's disease (PD) and schizophrenia result in millions of deaths every year globally. Nicotine may be neuroprotective, as smokers are less susceptible to AD and PD, and schizophrenic smokers self-medicate with nicotine and report marked improvement in metal alertness and cognition (Shimohama, 2009). Although substantial evidence points toward a neuroprotective evidence of nicotine (Piao et al., 2009), the underlying receptor subtypes and the molecular and cellular mechanisms are still unclear. Previous studies investigating nicotine-mediated neuroprotection suggest that chronic nicotine stimulates α4β2 receptors in dopaminergic neurons and enhances neurotransmission (Penton and Lester, 2009). In agreement with this hypothesis, the protective effects of nicotine in a mouse model for PD are lost in α4* knockout animals (Ryan et al., 2001), and activation of α4* nAChRs is protective in an AD animal model (Kihara et al., 1998).

Gahring et al. (2004) report expression of α4* nAChR subtypes on non-neuronal CNS cells, emphasizing the diversity and hitherto unknown functions of cholinergic signaling. At concentrations achieved in smoker's blood, nicotine attenuates CNS inflammation as well as autoimmune responses in experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis (Shi et al., 2009). Furthermore, van der Zanden et al. find that α4/β2 nAChRs mediate cholinergic anti-inflammatory effects in certain peripheral macrophages, (van der Zanden et al., 2009). While evidence for neural involvement in CNS cytokine regulation is still emerging, it is established that the vagus nerve activity regulates cytokine production in the periphery through activation of α7 nAChRs (Tracey, 2007).

We previously found that nicotine suppresses constitutive NFκB activity and thereby pro-inflammatory cytokine (PIC) production in the SHEP1 human neuroblastoma cell line stably transfected with human α4β2 nicotinic receptors (hα4β2 SHEP1 cells) (Hosur et al., 2009). Here we investigated the mechanisms that underlie the anti-inflammatory effects of nicotine pre-treatment in LPS-stimulated hα4β2 SHEP1 cells. In the case of α7 nAChR, attenuation of inflammatory and immune responses is associated with

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JAK2-STAT3 signaling (de Jonge et al., 2005). Thus, we investigated whether α4β2 nAChRs utilize a similar signaling cascade in suppressing LPS-induced NFκB activity. Additionally, because nicotine exposure causes cAMP accumulation both in vivo and in vitro (Gueorguiev et al., 1999; Oshikawa et al., 2003), we asked whether Ca^{2+} and cAMP-PKA are involved in mediating the effects of nicotine. Our findings show that nicotine-induced α4β2 nAChR suppression of endotoxin-induced NFκB activation is $Ca^{2+}$and cAMP-PKA independent but nicotine-stimulated JAK2-STAT3 signaling blocks NFκB translocation, but only partially blocks IκBα phosphorylation.

MATERIALS AND METHODS

Cell culture and Reagents- hα4β2 SHEP1 cells were a generous gift from Ron Lukas, and grown as previously described (Hosur et al., 2009). Nicotine tartrate and endotoxin lipopolysaccharide were purchased from Sigma (St Louis, MO, USA). JAK inhibitor I, JAK2 inhibitor AG-490, NFκB peptide inhibitor SN50 (peptide: AAVALLPAVLLALLAPVQRKRQKLMP) , calcium chelator BAPTA-AM, forskolin, STAT3 inhibitor VI NSC74859 (also known as S3I-201), PKA inhibitor peptide PKI 14-22 amide (myristoylated-GRTGRRNAI-NH_2) and calcium ionophore A23187 (also known as calcimycin) were obtained from Calbiochem (San Diego, CA, USA). Primary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA) and secondary antibody was from Millipore (Billerica, MA, USA).

Real-time PCR- As described previously (Hosur et al., 2009). Toll-like receptor 4 (TLR4) primers were designed using IDT SCiTools PrimerQuestSM; Forward-TCC TGC AAT GGA TCA AGG ACC AGA; Reverse-GCC AGC AAG AAG CAT CAG GTG AAA.

Cytokine ELISA- hα4β2 cells seeded in 6-well plates were pre-treated with nicotine before stimulation with LPS for 48hrs and IL-1β and IL-6 levels were measured as previously described (Hosur et al., 2009).

Phospho-IκBα ELISA- Phospho-IκBα levels were examined in hα4β2 SHEP1 cells by FunctionELISA IκBα assay (Active motif, CA, USA). Cells seeded in 6-well plates and grown for 2 days were pre-

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treated with nicotine, BAPTA-AM or AG-490 before stimulation with or without 1μg/ml LPS for 25 min. Subsequently, cells were washed 2X with ice-cold Phosphate Buffered Saline (PBS) and a cell scraper was used to remove cells. Cells were centrifuged for 10min at 1000rpm at 4^0C and then pellets were incubated in lysis buffer containing protease inhibitor for 30 min on ice. Samples were again centrifuged for 20 min at 14,000 x g at 40C. Samples were stored at -80^0C until further use. Protein concentration was determined by a NanoDrop® ND-1000. 100μg of cell lysate was added to capture antibody pre-coated plates and incubated for 4h at 4^0C. Plates were thoroughly washed and detection antibody was added and allowed to incubate for 1h at RT. After a second wash, secondary antibody was added for 1h before quantifying the signal using a chemiluminescent substrate.

Calcium indicator assay- A23187-induced increases in intracellular calcium were measured using Fluo-4-AM (Invitrogen, CA, USA). Cells plated in a 96-well plate (50,000 cells/well in Corning Costar 3603 plates) were grown for 24 h before incubating for 30 min with a final concentration of 3 μM Fluo-4-AM in darkness at RT, and then exposed to BAPTA-AM or control media for an additional 30 min (Final volume: 100 μl Hank's Buffered Salt Solution [HBSS] with 0.1% DMSO and 5.5 mM glucose, without phenol red). A BioTek-HT microplate reader determined baseline fluorescence at excitation and emission wavelengths of 485±20 nm and 520±20 nm respectively, by taking nine readings at 0.5 sec intervals. At 4.8 sec, 100 μl of the calcium ionophore A23187 or nicotine were added by automated dispenser and fluorescence measurements were read at 0.5 sec intervals for a total elapsed time of 40.8 sec. All readings were made within 4h of adding the Fluo-4-AM solution. Baseline measurements were recorded by dispensing either HBSS (diluent for nicotine) or HBSS with an equivalent dilution of methanol (solvent for A23187).

cAMP ELISA- Cells grown to confluence in 6-well plates were incubated with 10μM nicotine or 10μM forskolin and intracellular cAMP levels were monitored using competitive ELISA kit (R & D systems, Minneapolis, MN). Activity was measured on a BioTek-HT microplate reader at 450 nm.

NFκB secreted luciferase reporter assay- As described previously (Hosur et al., 2009).

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Western blotting- To investigate whether α4β2-mediated anti-inflammatory effect involves phosphorylation of IκBα, we probed using antibodies against total IκB and phospho-IκBα (phosphorylated IκBα undergoes ubiquitination and allows translocation of NFκB to the nucleus) after treating hα4β2 SHEP1 cells with nicotine/BAPTA-AM/ AG490 inhibitor/LPS. Briefly, cells seeded onto 6-well plates were treated with the varying concentrations of nicotine with or without inhibitors. Following incubation, cells were washed 3X with PBS and lysed using Mammalian Protein Extraction Reagent (Pierce, IL, USA). Protein concentration was determined using a NanoDrop® ND-1000 UV-Vis Spectrophotometer. Equal concentrations of protein were heated at 95^0C for 5min before loaded onto pre-cast gels (Pierce, IL, USA, and run at ~140V for 45min at RT. Protein was transferred to a PVDF membrane at 30V for 2h at 4^0C. Membranes were then stained with panceau-S reagent to visualize efficiency of protein transfer before incubating with blocking buffer (1X TBS, 5% w/v nonfat dry milk, 0.1% tween) at RT for 1hr. The blots were incubated in primary antibody buffer (0.2g/ml of antibody and blocking buffer) overnight at 4^0C. A thorough wash (5X with TBS/T) was followed by incubation with secondary antibody (HRP-conjugate) buffer (10ng/ml) at RT for 1hr. Membranes were washed again 5X with TBS/T before exposing the blots to a chemiluminescent substrate (SuperSignal West Pico, Thermo scientific). A CCD camera (Kodak Image Station In-Vivo FX) was used to visualize luminescence.

Data analyses-Statistical analysis was performed by SPSS v17 and GraphPad Prism 5 was used to develop graphs. One-way analysis of variance (ANOVA) estimated differences among groups. Semi-quantitative gel density analysis was performed using ImageJ (http://rsbweb.nih.gov/ij/index.html).

RESULTS

Nicotine blocks the LPS-induced NFκB pathway. Previously, we showed that nicotine exposure inhibits constitutive NFκB activity in hα4β2 cells (Hosur et al., 2009). Nicotinic activation of α7 receptors attenuates endotoxin-induced NFκB translocation and pro-inflammatory cytokine (TNFα, IL-1β and IL-6) production (Borovikova et al., 2000; Wang et al., 2004). To test the hypothesis that α4β2

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nAChRs mediate anti-inflammatory effects similar to α7 activation, we stimulated hα4β2 SHEP1 cells with varying concentrations of the bacterial endotoxin LPS. SHEP1 cells express a message for the LPS receptor, TLR4, as determined by qPCR and DNA sequencing (not shown). Initial experiments were carried out to determine optimal concentrations of LPS to induce NFκB translocation (Fig. 1a and Supplemental Fig.1). 1μg/mL and 3μg/mL LPS caused a ~2 fold and ~ 3-4-fold increase, respectively, in NFκB activity, although, nicotine failed to attenuate LPS-induced NFκB transactivation in wild-type SHEP1 cells lacking α4, β2 or α7 nAChR subunits (Fig.1a). Conversely, pre-treatment of hα4β2 SHEP1 cells with nicotine effectively attenuated LPS-induced NFκB transactivation in a dose-dependent manner, as measured by NFκB-reporter assay (Fig. 1b), with 1μM causing approximately a 40% decrease. SN50 peptide inhibits nuclear translocation of NFκB, and acts as a positive control to block LPS-induced nuclear translocation. SN50 dose-dependently blocked LPS-induced NFκB activity (data not shown; 20μM caused a 90% decrease), with 10μM causing approximately a 50% decrease (Fig. 1b). Further, the effect of a sub-maximal dose of SN50 (10μM) was additive when applied with 300nM nicotine.

LPS exposure increases PIC production in part through the NFκB pathway. Because nicotine blocked LPS-induced NFκB activity, we tested whether nicotinic activation of α4β2 blocks LPS-induced cytokine production downstream of the NFκB pathway. hα4β2 SHEP1 cells treated with 1μg/mL LPS increased both IL-1β (2-fold) and IL-6 (1.5-fold) production (Fig. 1c, d), compared to untreated controls. However, nicotine pre-treatment reduced LPS-induced IL-1β and IL-6 production at concentrations found in smoker's blood (Henning et al., 1994; Russell et al., 1980), with 100nM nicotine blocking both cytokines down to control levels (Fig.1c, d).

JAK2 mediates α4β2-mediated anti-inflammation- α7 nAChRs activate the JAK2-STAT3 pathway to attenuate endotoxin-induced inflammation (Tracey, 2007). We investigated the involvement of JAKs in α4β2 mediated anti-inflammation using a broad spectrum JAK inhibitor I on LPS-induced NFκB translocation. Nanomolar concentrations of JAK inhibitor I robustly blocked nicotine-induced reduction

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in NFκB translocation in LPS stimulated cells (Fig.2a), suggesting a tentative evidence for JAKs' contribution in opposing LPS inflammation.

LPS-induced cytokine production in part involves IκBα-phosphorylation and subsequent translocation of NFκB into the nucleus. Since nicotinic activation of α4β2 receptors attenuated LPS-induced NFκB activation and thereby cytokine production, we asked whether α4β2 signaling interferes with IκBα-phosphorylation. hα4β2 SHEP1 cells, pre-incubated with either PBS or 300nM nicotine, were stimulated with 1μg/mL LPS for 25 min at 37^0C. 300nM nicotine decreased IκBα-phosphorylation approximately 50% compared with PBS treated controls in Western blots (Figs.2b). However, the effects of nicotine on IκBα-phosphorylation were more modest but still significant in the ELISA assay (Fig. 2d). Pre-incubation with the specific JAK2 inhibitor AG-490 (10μM) with nicotine completely restored NFκB translocation (Fig.2c) and IκBα-phosphorylation (Fig.2b, d) in LPS stimulated cells. Together, these results suggest that nicotine opposes LPS-elicited inflammation partly by interfering with IκBα-phosphorylation through a JAK2-dependent signaling cascade, but that nicotine has additional effects that cause a more profound blockade of LPS-induced NFκB translocation.

NSC74859 restores LPS-induced NFκB activation- Activated JAK induces STAT phosphorylation , which forms either homo- or hetero-dimers and translocates to the nucleus to bind promoter elements of DNA to induce gene transcription (Heinrich et al., 2003). de Jonge et al. (2005) demonstrated that nicotine dose-dependently induces STAT3 phosphorylation, and STAT3 is an essential transducer of α7-mediated anti-inflammation. We examined the role of STAT3 by assessing the ability of a specific STAT3 inhibitor NSC74859 to inhibit nicotine-induced inhibition of LPS-stimulated IκBα-phosphorylation and NFκB activity in hα4β2 SHEP1 cells. Again, the effects of nicotine were more modest on IκBα-phosphorylation measured by ELISA than on NFκB translocation, but NSC74859 at 30μM prevented the effects of nicotine on both (Fig.3a & b).

α4β2-mediated Ca^{2+} influx does not influence LPS-induced NFκB activity. We tested the role of calcium on NFκB translocation and IκBα-phosphorylation using the cell permeable calcium chelator

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BAPTA-AM. BAPTA-AM has been used extensively both in vitro and in vivo to buffer intracellular changes in calcium. The calcium ionophore A23187 (alias calcimycin) acted as a positive control to verify that the intracellular calcium buffering capacity of BAPTA pre-treatment is sufficient when measured using Fluo-4 as the intracellular calcium indicator (Fig. 4). We did not reliably see nicotine-induced calcium entry that could be blocked by mecamylamine using the Fluo-4 assay. In three separate experiments, we observed intermittent injection-related changes in fluorescence, but in 24 injections with nicotine alone (concentration range 1 – 100 μM), 10 caused upward deflections, 6 showed downward effects, and 8 had no deflection, suggesting an injection artifact. Further, we observed upward deflections with two cases of nicotine plus 33 μM mecamylamine, a nicotinic receptor channel blocker, and in one case, we saw a downward deflection when HBSS was injected (Fig 4). We conclude from these data that our Fluo-4 assay does not have sufficient resolution to reliably measure the small calcium influx due to α4β2 receptor activation, consistent with a small α4β2 calcium permeability (4-6%) compared with α7 nAChRs (Wonnacott et al., 2006). However, 3μM A23187 caused a reliable increase in calcium-dependent fluorescence compared to controls, but 30 min pre-incubation with 5μM BAPTA-AM decreased the 3μM A23187-induced calcium flux to less than 30% of A23187 alone (Fig.4). These data suggest that BAPTA pre-treatment should effectively buffer against the small increases in intracellular calcium due to nicotinic stimulation of α4β2 receptors.

Cells pre-treated with BAPTA for 30min and further incubated with nicotine showed no significant difference between nicotine alone and nicotine plus BAPTA treatments in basal NFκB translocation (Supplemental Fig.2). 300nM nicotine partially but significantly blocked LPS-induced IκBα-phosphorylation to approximately 50% of control in Western blots (Fig.5a) but only by about 10% measured by the ELISA assay (Fig. 5b). However, BAPTA failed to block the effects of 300nM nicotine in both these assays (Fig.5a, b), suggesting a calcium-independent mechanism for α4β2-mediated inhibition of LPS-induced NFκB activity and IκBα-phosphorylation.

cAMP-dependent PKA signaling- In other systems, cAMP-dependent protein kinase A (PKA) inhibits

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NFκB transcriptional activity (Minguet et al., 2005). In addition, agents that elevate intracellular cAMP inhibit IκBα-phosphorylation and subsequent NFκB activation (Sands et al., 2004; Takahashi et al., 2002). Although nicotinic agonists cause cAMP accumulation (Gueorguiev et al., 1999; Hiremagalur et al., 1993), the involvement of cAMP signaling in nAChR- mediated anti-inflammatory effects is unknown. Our results show that nicotine and forskolin (an adenylate cyclase stimulant) cause a time-dependent increase in cAMP in hα4β2 SHEP1 cells (Fig.5c), with forskolin causing a maximum increase at 1h while nicotine at 4h. Therefore, we initially examined the effect of forskolin and nicotine on LPS-induced NFκB translocation in presence or absence of a specific PKA peptide inhibitor (PKI 14-22 amide). Forskolin had no effect on NFκB activity and the PKA inhibitor failed to block the effects of nicotine (Fig.5d). These results provide no evidence for the involvement of cAMP-PKA signaling in α4β2-mediated suppression of NFκB translocation.

DISCUSSION

We previously showed that nicotine attenuates basal PIC production in hα4β2 cells but not in wild-type SHEP1 cells (Hosur et al., 2009). SHEP1 cells are a subclone of neuroblastoma cells and have low but measurable constitutive expression of cytokine proteins. We stimulated these cells with the bacterial endotoxin LPS to increase the cytokine response and asked whether nicotine was equally effective in opposing PIC production. SHEP1 cells increase cytokine expression in response to LPS stimulation (Fig. 1), and express TLR4 (PCR data not shown). Nicotine pre-treatment appreciably reduced LPS-induced cytokine production by interfering with NFκB signaling, suggesting that nicotine decreases endotoxin-induced inflammation in hα4β2 SHEP1 cells. Matsunaga et al. (2001) found that the cholinergic agonists nicotine and 1,1-dimethyl-4-phenylpiperazinium iodide suppress production of cytokines IL-6,1L-12 and TNFα in alveolar macrophages expressing α4β2 receptors and *d*-tubocurarine blocked this effect. Likewise, van der Zanden et al. (2009) found that α4β2 receptor activation in isolated mouse intestinal and peritoneal macrophages attenuates NFκB activation and PIC production. To delineate the signaling

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cascades downstream of α4β2 nAChR activation, we investigated the involvement of JAK2-STAT3 and Ca^{2+}, cAMP-PKA pathways.

Nicotinic receptor-mediated regulation of gene transcription, neuroprotection and anti-apoptosis often involves calcium signaling (Dajas-Bailador and Wonnacott, 2004). For instance, nicotine-induced increases in tyrosine hydroxylase mRNA in PC12 cells are blocked by prior treatment with BAPTA (Gueorguiev et al., 1999). However, we found that BAPTA loading (under conditions that significantly blocked calcimycin-induced calcium influx) failed to block nicotine-induced α4β2-mediated anti-inflammatory effects. This agrees with previous findings that human leukocytes expressing α7-like nAChRs fail to elicit detectable currents in response to cholinergic agonists nicotine and acetylcholine (Villiger et al., 2002), although both agonists induce anti-inflammatory actions, suggesting a plausible role for calcium-independent signaling. Chronic smokers experience sustained exposure to low concentrations of nicotine (~100nM) and it is possible that the desensitized (non-conducting) receptors rather than activated (conducting) receptors confer neuroprotection. Furthermore, we found no evidence for the role of cAMP-PKA dependent pathways in α4β2-mediated suppression of NFκB activation. Future studies need to examine why cAMP-PKA anti-inflammatory signaling is restricted to certain receptor types, regardless of considerable cAMP accumulation.

de Jonge et al. (2005) previously noted similarities between α7 nicotinic signaling and those of the anti-inflammatory cytokine, IL-10. IL-10 receptor activates JAK2-STAT3, which inhibits LPS- and TNFα -induced PIC production by preventing IκBα-phosphorylation, NFκB activation and NFκB DNA-binding activity (Schottelius et al., 1999). We find that α4β2 activation suppresses LPS-induced effects by significantly decreasing but not completely blocking IκBα-phosphorylation, but completely blocking NFκB translocation and PIC production. This is the first indication that STAT3 inhibits NFκB activity through a non-transcriptional mechanism upon α4β2 activation, and strongly supports the previous hypotheses that cholinergic stimulation prevents NFκB activity and mimics IL-10 receptor signaling (de Jonge et al., 2005).

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Future studies need to address these important questions: 1) How is the signal transduced from the receptor to JAK2? It could be that conformational changes in the intracellular loops lead to direct JAK2 activation. JAK2 is already known to signal downstream from G-protein coupled receptors (Ahr et al., 2005) and receptor tyrosine kinases (Pelletier et al., 2006), but there seems little in common between the cytoplasmic domains of these disparate receptor families. 2) Does the open or the desensitized state of the receptor favor JAK2 binding? We previously found that both competitive and noncompetitive antagonists prevent nicotine-induced attenuation of NFκB signaling and cytokine production (Hosur et al., 2009), suggesting that an agonist-induced conformational change is required for JAK2 activation. Yet these experiments do not discriminate between "open" or "closed but high-affinity desensitized" receptors (Giniatullin et al., 2005). 3) Is the immune modulatory activity restricted to α4β2 and α7 or does it apply to other cys-loop ligand-gated ion channel receptors? Although both α7 and α4β2 receptors seem to employ JAK2-STAT3 signaling, surprisingly, there is little homology in the central portion of the major M3-M4 loop. Homology between these subunits is restricted to the first intracellular loop between M1-M2 and the beginning and the end of the intracellular loop between M3 and M4. 4) In which cell types is α4β2 receptor anti-inflammatory signaling important? Previous in vivo evidence is restricted to certain peripheral macrophages (Matsunaga et al., 2001; van der Zanden et al., 2009) but the finding of α4* receptors on both neuronal and non-neuronal CNS cells raises the possibility that α4β2 receptor mediated anti-inflammatory effects may occur in the brain (Gahring et al., 2004).

While JAK2-STAT3 is involved in nicotinic anti-inflammation, it is also implicated in mediating pro-inflammatory effects depending on the cellular context (Kox et al., 2009). de Jong et al. (2005) show phosphorylated STAT3 is involved in anti-inflammatory nicotinic α7 receptor-dependent signaling in macrophages, and many studies find that nicotinic stimulation of α7 receptors suppresses NFκB signaling (Tracey, 2007). However, a recent study finds that α7 receptors on PC12 cells stimulate anti-apoptotic effects through JAK2-STAT3–induced increases in NFκB activity (Marrero and Bencherif, 2009). Additionally, Chen et al. report that nicotine-induced STAT3 activation increases NFκB activity in

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bladder cancer cells (Chen et al., 2008). (Incidentally, the claim by Chen et al. that nicotinic effects on STAT3 in their system are mediated by α4β2 receptors is questionable, as they demonstrate that the bladder cells have both α7 and α4β2 receptors [and one bladder cell line also expresses α9 subunits]. Evidence that α4β2 receptors mediate this effect is based using lobeline as a "selective α4β2 antagonist" to distinguish nicotine's effect between these receptor subtypes at concentrations greater than 20 times lobeline's IC_{50} for human α7 receptors expressed in oocytes (Briggs and McKenna, 1998)). Very recent work suggests that in certain circumstances non-phosporylated STAT3 regulates NFκB activity and cytokine production in macrophages (Pena et al., 2010) and that the cholinergic peptide SLURP-1 modulates α7-JAK2 mediated upregulation of NFκB in keratinocytes (Chernyavsky et al., 2010). Clearly, further work is required to sort out how JAK2-STAT3 signaling can be both pro- and anti-inflammatory and both block and stimulate NFκB signaling, depending on context. However, our report demonstrates that nicotinic stimulation of α4β2 receptors in the right cellular environment can be anti-inflammatory similar to α7 receptors when activated by concentrations of nicotine found in smoker's blood.

Activation of nAChRs by cholinergic agonists attenuates PIC production (Tracey, 2007), but there may be reciprocal regulation of nAChRs by PICs. Gahring et al. report that PICs IL-1β and TNFα alter the assembly of nAChRs in HEK293 cells (Gahring et al., 2008). Nicotine exposure increased the expression of endoplasmic reticulum chaperones and down-regulated PICs while inducing up-regulation in hα4β2 SHEP1 cells (Hosur et al., 2009). Our findings point to a negative association between increases in α4β2 receptor expression and down-regulation of PICs, which could account for the following: 1. Loss of α4β2 nAChRs observed in patients with neurological disorders, manifested by excess inflammation (Ripoll et al., 2004). In this case, PICs might be altering the assembly of nAChR subunits, and 2. while positron emission tomography and post-mortem studies of chronic smoker's brains reveal an appreciable increase in high-affinity binding sites for nicotine (Wullner et al., 2008), chronic schizophrenic smokers have lower inflammatory cytokines (IL-2 and IL-6) compared with their non-smoking counterparts (Zhang et

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al., 2008). Therefore, the inverse correlation of smoking with development of neurological disorders might be due to a combination of the following: 1) Nicotine up-regulates high-affinity α4β2 receptors and their function, leading to cognitive and motor sensitization and 2) Nicotinic activation of α4β2 and α7 receptors results in attenuation of anti-inflammatory responses (van der Zanden et al., 2009).

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Authorship Contribution

Participated in research design: Hosur, Loring

Conducted experiments: Hosur, Loring

Contributed new reagents or analytic tools: Hosur

Performed data analysis: Hosur, Loring

Wrote or contributed to the writing of the manuscript: Hosur, Loring

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Footnotes

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FIGURE LEGENDS

Figure 1. Nicotine blocks LPS-induced NFκB activity and cytokine production in hα4β2 but not wild-type SHEP1 cells. a) Wild-type SHEP1 cells plated in 48-well plates were transfected with pNFκB-MetLuc2-Reporter vector overnight. Cells were stimulated with varying concentrations of LPS for 4h with or without indicated concentrations of nicotine pre-treatment (2 h). Cell supernatant was collected to measure secreted luciferase activity. b) hα4β2 SHEP1cells (stably transfected with human α4β2 receptors) were pre-treated with increasing concentrations of nicotine for 2h before stimulating with 1μg/mL of LPS for 4h. A sub-maximal (10μM) dose of SN50 peptide was used as a positive control. c,d) hα4β2 SHEP1cells plated in 6-well plates were stimulated with 1μg/mL LPS for 48h with or without indicated concentrations of nicotine pre-treatment. Cells were lysed and stored at -80^{0}C until further use. Protein levels of IL-1β and IL-6 were measured according to the manufacturer's instructions. Nicotine had no effect on cytokines in wild-type SHEP1 cells (data not shown). Data represent mean ±SEM (n=3). *p<0.05, **p<0.01; ***p<0.001. RLU, relative luminescence units.

Figure 2. JAK2 inhibition restores LPS-induced NFκB signaling in nicotine-treated hα4β2 SHEP1 cells. a) NFκB reporter assay: NFκB reporter transfected hα4β2 SHEP1cells were pre-treated (30min) with 30nM JAK I inhibitor (a non-selective JAK inhibitor) and 300nM nicotine and stimulated with 1μg/mL of LPS for 4h. Cell supernatant was used to monitor luciferase activity. b) Immunoblot: serum starved hα4β2 SHEP1 cells were pre-treated with 300 nM nicotine and 10μM AG-490, and stimulated with 1μg/mL LPS for 25 min at 37^{0}C. Protein samples were probed with mouse primary anti-p-IκBα or IκBα antibodies followed by a rabbit anti-HRP secondary antibody. All membranes were stripped and reprobed with a mouse anti-β-actin antibody and a rabbit anti-HRP secondary antibody. Semiquantitative Image J analysis suggests that nicotine blocked IκBα phosphorylation by approximately 50% (not shown) but AG-490 reversed nicotine's effect. c) NFκB reporter assay: NFκB-reporter transfected cells were pre-treated with nicotine and/or varying concentrations of AG-490 (30 min) and incubated with 1μg/mL of LPS for 4h. 50μL of cell supernatant was collected to assess luciferase activity. d) Phospho-IκBα ELISA:

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protein samples (same as used in figure 2b) were added to pre-coated plates and phospho-IκBα levels were determined according to the manufacturer's protocol. Data represent mean±SEM of three independent experiments. One-way ANOVA determined statistical difference among groups. a, significantly different from LPS alone; b, significantly different from LPS plus nicotine treatment (significance at 0.05 level).

Figure 3. The STAT3 inhibitor NSC74859 restores LPS-induced NFκB signaling in nicotine-treated hα4β2 SHEP1 cells. a) NFκB translocation: hα4β2 SHEP1 cells transiently transfected with NFκB reporter vector were stimulated with LPS for 4h with or without nicotine and/or increasing NSC74859 pre-treatment for 30 min. 50μl of cell supernatant was used to measure luciferase activity (n=3). b) phospho-IκBα ELISA: cells grown in 6-well plates were serum starved for 16h and then pre-incubated with 300nM nicotine and indicated concentrations of NSC74859 before stimulating with 1μg/mL of LPS for 25 min at 37^0C. 100μg of cell lysate was used to monitor phospho-IκBα levels by functional ELISA (n=4). Results represent mean ± SEM. a, significantly different from LPS treated samples ($p<0.05$); b, significantly different from nicotine plus LPS treated samples ($p<0.05$).

Figure 4. BAPTA loading blocks A23187-induced increases in intracellular calcium. hα4β2 SHEP1 cells in a 96 well black-walled plate were pre-treated with 3μM Fluo-4-AM (dissolved in 100 μl HBSS with 0.1% DMSO and 4.5 mM glucose, but without phenol red, 1 h in darkness at RT) with or without 5μM BAPTA-AM (30 min). To establish a baseline, a BioTek-HT microplate reader recorded bottom-read fluorescence at excitation 485±20 nm and emission 520±20 nm at 0.5 s intervals for a total of 4.5 s (9 recordings). At 4.8 s, the reader injected 100 μl 3μM A23187 (Calcimycin), 1 μM Nicotine or HBSS to stimulate the cells, and took fluorescence measurements at 0.5 s intervals for a total elapsed time of 40.8 s. The plot above shows single traces typical of those recorded from triplicate wells for each condition. To adjust for baseline drift, traces are normalized to the average RFU of the HBSS baseline

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recordings. The HBSS recording is an example of a trace with a downward deflection of approximately 150 RFU at the time of injection. Both upward and downward injection-related deflections were observed intermittently in this assay as discussed in the results.

Figure 5. Ca^{2+} and cAMP-PKA-dependent pathways do not regulate α4β2-mediated anti-inflammatory effects. a) Immunoblot: hα4β2 cells grown in 6-well plates were serum starved for 16h and then pre-treated (30min) with 3μM and 5μM BAPTA, and incubated with 300nM nicotine for 30min before stimulating with 1μg/mL LPS for 25min at 37^0C. Equal amounts of protein were analyzed by SDS-PAGE, followed by electrophoretic transfer to PVDF membranes, blotting and ECL detection. The membranes were stripped and reprobed with an anti-β-actin antibody. No phospho-IκBα band was detected for untreated cells (not shown). Semi-quantitative Image J analysis suggests that nicotine blocked LPS-induced IκBα phosphorylation by approximately 50% (not shown) b) phospho-IκBα ELISA: Proteins samples isolated for western blotting in Figure 3a were used according to the manufacturer's instructions. c) Time course of cAMP induction by nicotine or forskolin: cells were exposed to 10μM nicotine and 10μM forskolin for indicated times, and cell lysates were used to monitor cAMP levels by ELISA. d) NFκB reporter assay: NFκB reporter transfected cells were pre-incubated with 10μM Forskolin or 300nM nicotine and/or PKA inhibitor and stimulated with 1μg/mL of LPS for 4h. PKI inhibitor alone had no effect on LPS-induced NFκB activity. a, significantly different from untreated controls; b, significantly different from LPS-alone treatment; $p<0.05$.

Figure 6. Model for α4β2 receptor-dependent nicotinic suppression of LPS-induced PIC production through JAK2-STAT3 but not through calcium or cAMP signaling.

This figure summarizes nicotine's effects on LPS-stimulated hα4β2 SHEP1 cells and previous work on nicotine's effects on constitutive PIC expression in these cells (Hosur et al., 2009). LPS, acting in part through Toll-like Receptor 4 (TLR4) and a multistep pathway, is known to cause phosphorylation and dissociation of IκBα from NFκB. Dissociated NFκB translocates to the nucleus to promote the PIC's IL-1β and IL-6 production, but translocation is blocked by SN50. We previously showed that nicotine

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acting through α4β2 receptors blocks constitutive PIC production in these cells, but the antagonists dihydro-beta-erythroidine (dHβE) and mecamylamine (Mec) block nicotine-induced decreases in NFκB translocation and cytokine production (Hosur et al., 2009), suggesting that a receptor conformational change is required for anti-inflammatory signaling. Our present data demonstrate that specific JAK2 and STAT3 antagonists block nicotine's actions on NFκB, but nicotine only partially blocks phosphorylation of IκBα. The question marks indicate that the effects of STAT3 on NFκB translocation are not understood (see Pena et al., 2010), given the partial nicotine-induced inhibition of IκBα phosphorylation, and that either a direct or multi-step association of JAK2 with the α4β2 receptor remains to be shown. In contrast, stimulating or antagonizing the cAMP-PKA pathway or buffering agonist-elicited intracellular Ca^{2+} with BAPTA-AM has little effect on nicotine's attenuation of NFκB signaling.

FIGURE 1

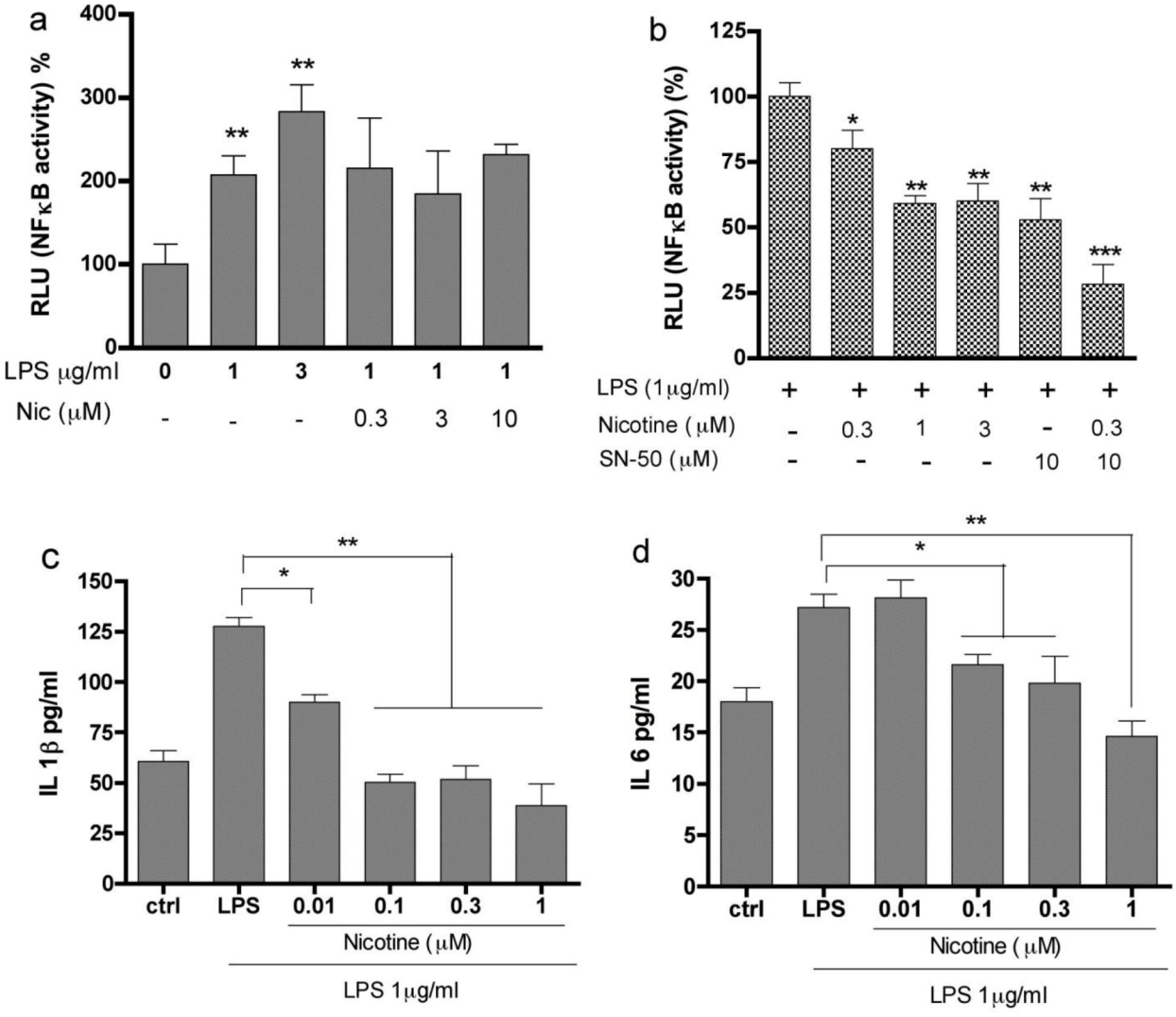

a
RLU (NFκB activity) %
400
300
200
100
0
**
**
LPS μg/ml 0 1 3 1 1 1
Nic (μM) - - - 0.3 3 10
b
RLU (NFκB activity) (%)
125
100
75
50
25
0
*
**
**
**
***
LPS (1μg/ml) + + + + + +
Nicotine (μM) - 0.3 1 3 - 0.3
SN-50 (μM) - - - - 10 10
c
IL 1β pg/ml
150
125
100
75
50
25
0
**
*
ctrl
LPS
0.01
0.1
0.3
1
Nicotine (μM)
LPS 1μg/ml
d
IL 6 pg/ml
30
25
20
15
10
5
0
**
*
ctrl
LPS
0.01
0.1
0.3
1
Nicotine (μM)
LPS 1μg/ml

FIGURE 2

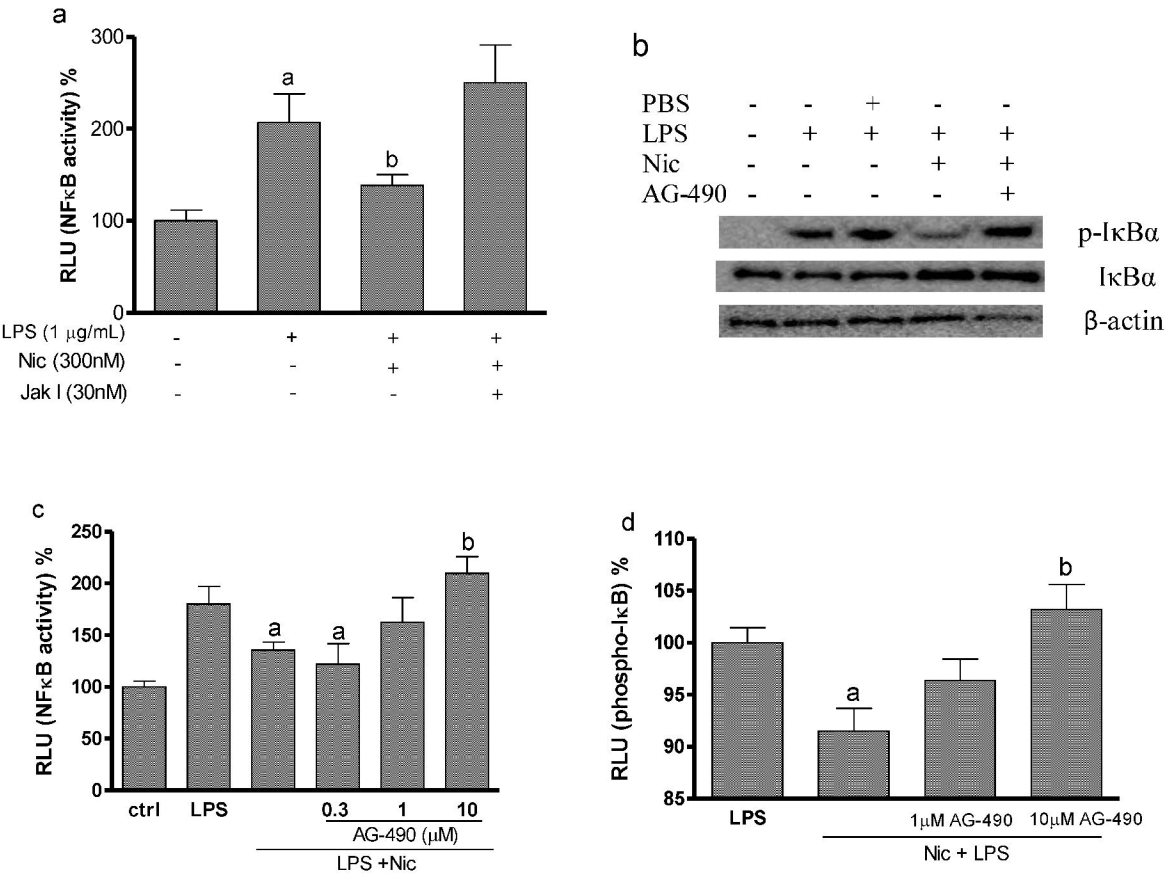
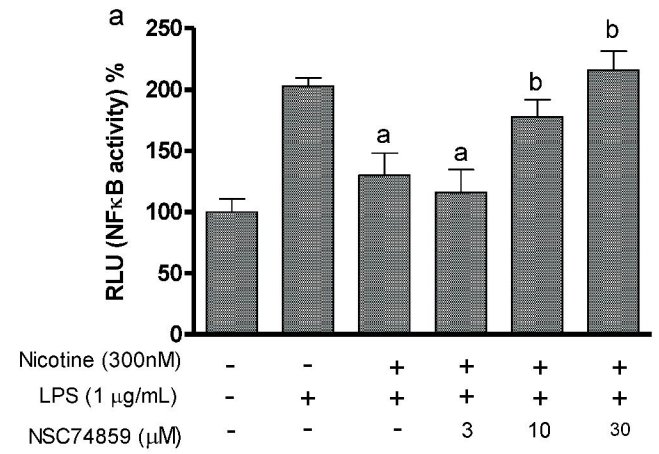

a
RLU (NFκB activity) %
300
200
100
0
a
b
LPS (1 μg/mL) - + + +
Nic (300nM) - - + +
Jak I (30nM) - - - +
b
PBS - - + - -
LPS - + + + +
Nic - - - + +
AG-490 - - - - +
p-IκBα
IκBα
β-actin
c
RLU (NFκB activity) %
250
200
150
100
50
0
a
a
b
ctrl
LPS
0.3
1
10
AG-490 (μM)
LPS +Nic
d
RLU (phospho-IκB) %
110
105
100
95
90
85
a
b
LPS
1μM AG-490
10μM AG-490
Nic + LPS

FIGURE 3


a
RLU (NFκB activity) %
250
200
150
100
50
0
a
a
b
b
Nicotine (300nM) - - + + + +
LPS (1 μg/mL) - + + + + +
NSC74859 (μM) - - - 3 10 30

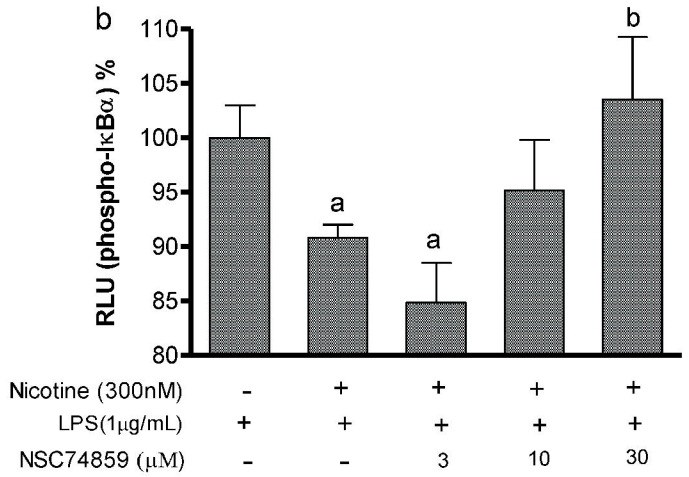

b
RLU (phospho-IκBα) %
110
105
100
95
90
85
80
a
a
b
Nicotine (300nM) - + + + +
LPS(1μg/mL) + + + + +
NSC74859 (μM) - - 3 10 30

FIGURE 4

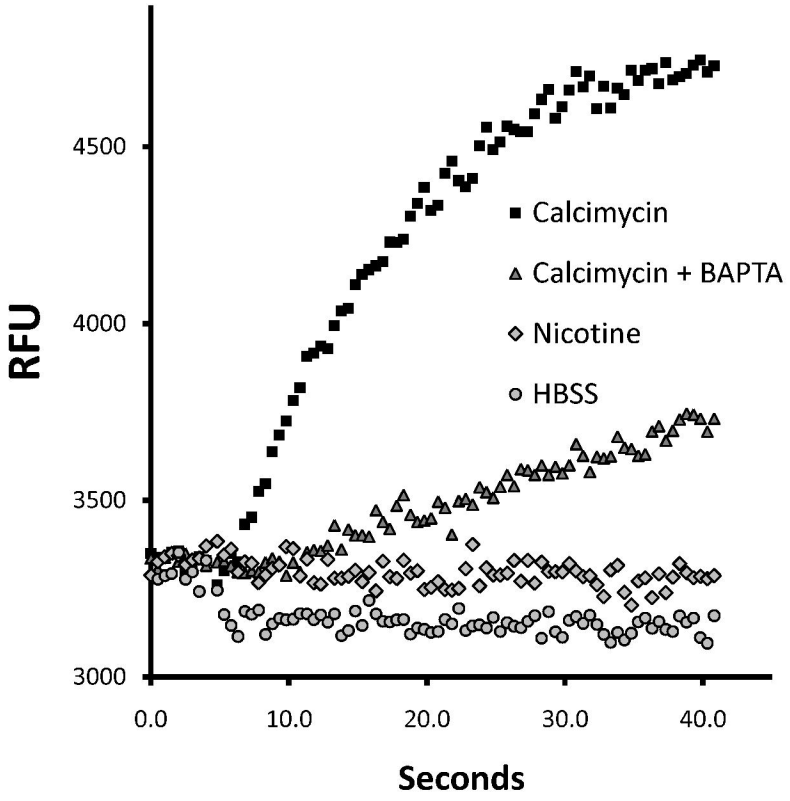

RFU
4500
4000
3500
3000
0.0
10.0
20.0
30.0
40.0
Seconds
Calcimycin
Calcimycin + BAPTA
Nicotine
HBSS

FIGURE 5

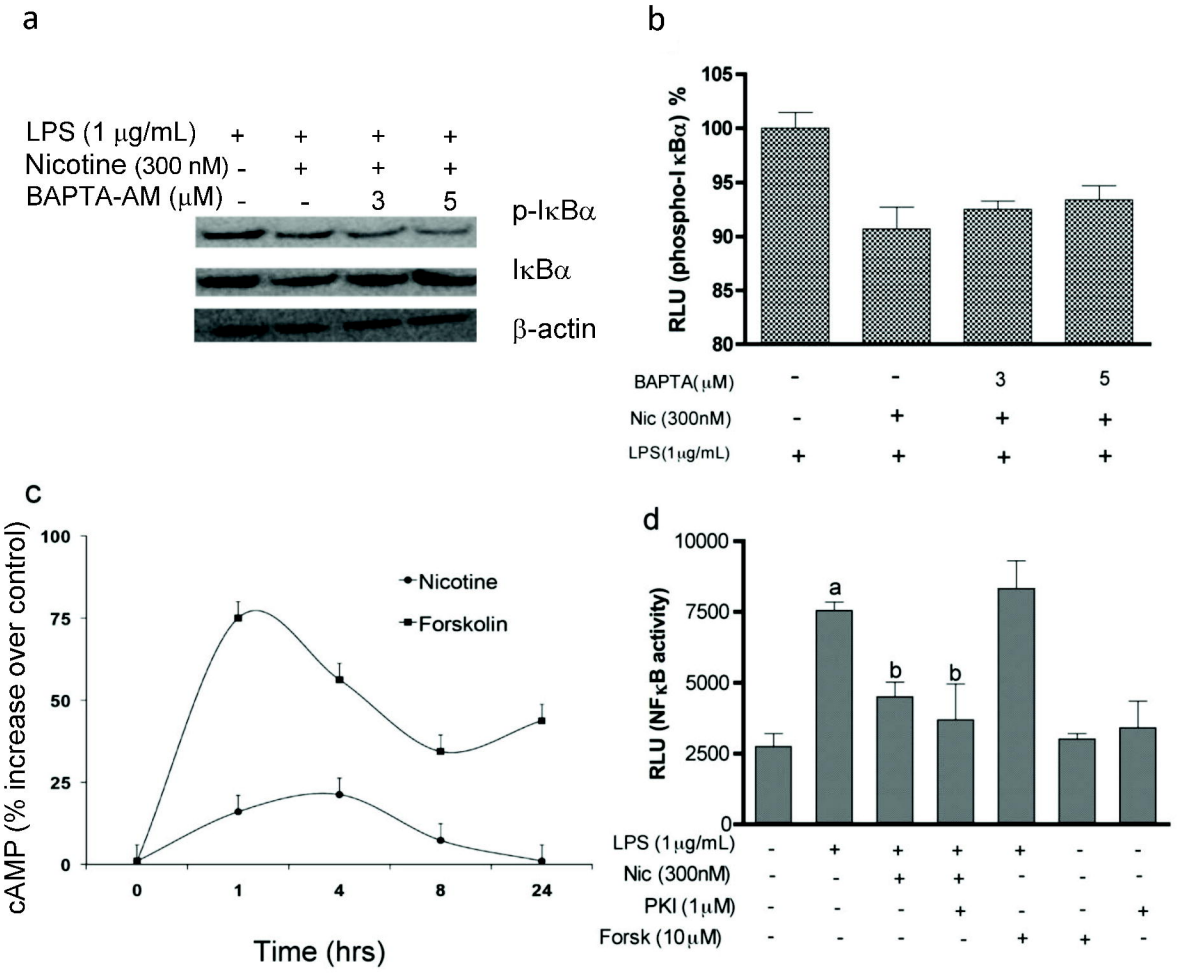
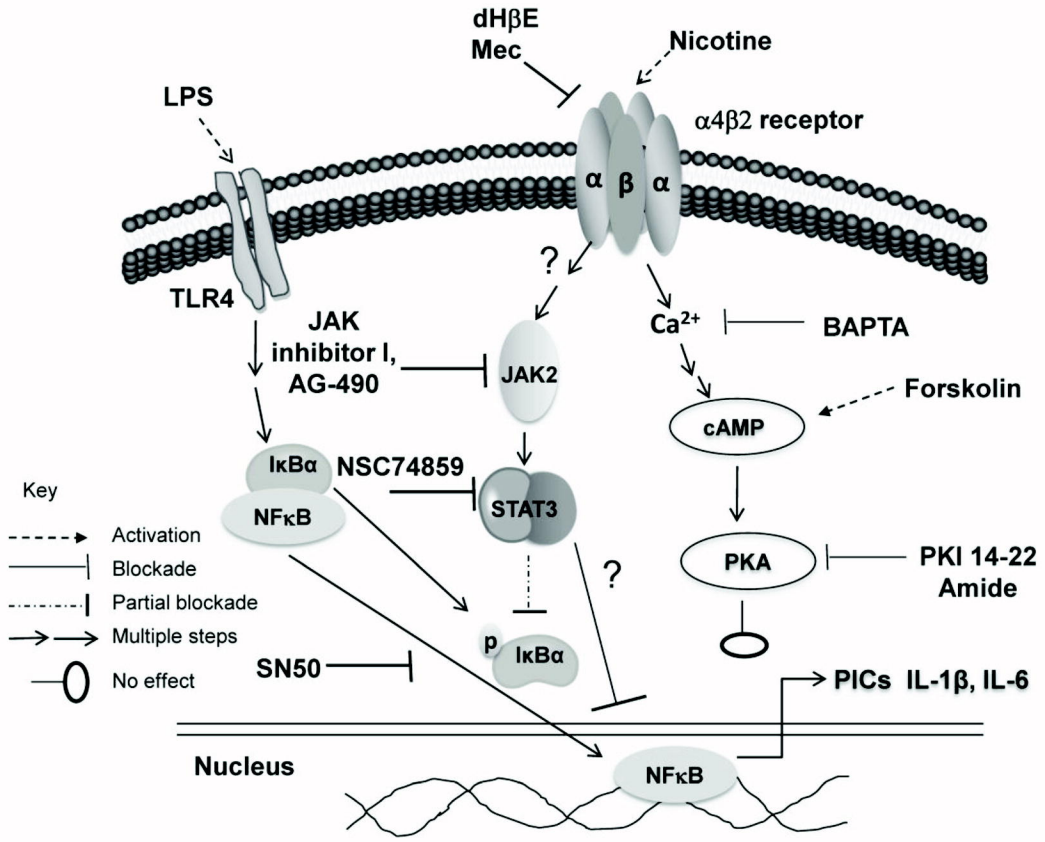

a
LPS (1 μg/mL) + + + +
Nicotine (300 nM) - + + +
BAPTA-AM (μM) - - 3 5
p-IκBα
IκBα
β-actin
b
RLU (phospho-IκBα) %
105
100
95
90
85
80
BAPTA(μM) - - 3 5
Nic (300nM) - + + +
LPS(1μg/mL) + + + +
c
cAMP (% increase over control)
100
75
50
25
0
Nicotine
Forskolin
0 1 4 8 24
Time (hrs)
d
RLU (NFκB activity)
10000
7500
5000
2500
0
a
b
b
LPS (1μg/mL) - + + + + - -
Nic (300nM) - - + + - - -
PKI (1μM) - - - + - - +
Forsk (10μM) - - - - + + -

FIGURE 6


dHβE
Mec
Nicotine
LPS
α4β2 receptor
α
β
α
?
TLR4
JAK
inhibitor I,
AG-490
JAK2
Ca2+
BAPTA
Forskolin
cAMP
IκBα
NFκB
NSC74859
STAT3
PKA
PKI 14-22
Amide
Key
Activation
Blockade
Partial blockade
Multiple steps
No effect
p
IκBα
SN50
PICs IL-1β, IL-6
Nucleus
NFκB