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α4β2 Nicotinic Receptors Partially Mediate Anti-Inflammatory Effects through Janus Kinase 2-Signal Transducer and Activator of Transcription 3 but Not Calcium or cAMP Signaling[S]

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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 reported previously that nicotine suppresses constitutive nuclear factor κB (NF-κB) activity and thereby proinflammatory cytokine (PIC) production in SHEP1 cells stably transfected with α4β2 nicotinic receptors. Here, we report the anti-inflammatory effects of nicotine pretreatment in lipopolysaccharide (LPS)-stimulated SHEP1 cells. Nicotine (100–300 nM, concentrations found in smoker's blood) blocked LPS-induced NF-κB translocation and production of PICs interleukin (IL)-1β and IL-6 but only partially blocked inhibitor of nuclear factor-κBα (IκBα) phosphorylation. These effects were exclusively in cells transfected with α4β2 receptors but not in wild types. The cell-permeable calcium chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid-acetoxymethyl ester, the adenylate cyclase stimulant forskolin, and a specific protein kinase A (PKA) inhibitor PKI 14-22-amide failed to block the effect of nicotine on LPS-induced NF-κB translocation and IκBα phosphorylation. However, the effects of nicotine on NF-κB activity were significantly blocked by the highly specific janus kinase 2 (JAK2) inhibitor α-cyano-(3,4-dihydroxy)-N-benzylcinnamide (AG-490) and the signal transducer and activator of transcription 3 (STAT3) inhibitor 2-hydroxy-4-[[[[(4-methylphenyl)sulfonyl]oxy]acetyl]amino]-benzoic acid (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 central nervous system, in addition to the better-established α7 receptors.

Introduction

Neurological disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), and schizophrenia result in millions of deaths every year globally. Nicotine may be neuroprotective, because smokers are less susceptible to AD and PD, and smokers with schizophrenia 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 long-term nicotine exposure 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

ABBREVIATIONS: AD, Alzheimer's disease; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; AM, acetoxymethyl ester; hα4β2 SHEP1, human SHEP1 neuroblastoma-derived cells stably transfected with human α4β2 nicotinic receptors; HBSS, Hanks' balanced salt solution; IκBα, inhibitor of nuclear factor of κ light polypeptide gene enhancer in B-cells, α; JAK2, Janus kinase 2; nAChR, nicotinic acetylcholine receptor; NF-κB, nuclear factor κB; PD, Parkinson's disease; PIC, proinflammatory cytokine; RFU, relative fluorescence units; STAT3, signal transducer and activator of transcription 3; TLR4, Toll-like receptor 4; AG-490, α-cyano-(3,4-dihydroxy)-N-benzylcinnamide; NSC74859, 2-hydroxy-4-[[[[(4-methylphenyl)sulfonyl]oxy]acetyl]amino]-benzoic acid; CNS, central nervous system; PKA, protein kinase A; IL, interleukin; LPS, lipopolysaccharide; RT, room temperature; TBS, Tris-buffered saline; HRP, horseradish peroxidase; TNFα, tumor necrosis factor α; ELISA, enzyme-linked immunosorbent assay; A23187, calcimycin.

are lost in $\alpha4*$ knockout animals (Ryan et al., 2001), and activation of $\alpha4*$ nAChRs is protective in an AD animal model (Kihara et al., 1998).

Gahring et al. (2004) report expression of $\alpha4*$ 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. (2009) find that $\alpha4/\beta2$ nAChRs mediate cholinergic anti-inflammatory effects in certain peripheral macrophages. Whereas 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 $\alpha7$ nAChRs (Tracey, 2007).

We found previously that nicotine suppresses constitutive NF-κB activity and thereby proinflammatory cytokine (PIC) production in the SHEP1 human neuroblastoma cell line stably transfected with human $\alpha4\beta2$ nicotinic receptors (h$\alpha4\beta2$ SHEP1 cells) (Hosur et al., 2009). Here, we investigated the mechanisms that underlie the anti-inflammatory effects of nicotine pretreatment in LPS-stimulated h$\alpha4\beta2$ SHEP1 cells. In the case of $\alpha7$ nAChR, attenuation of inflammatory and immune responses is associated with JAK2-STAT3 signaling (de Jonge et al., 2005). Thus, we investigated whether $\alpha4\beta2$ nAChRs use a similar signaling cascade in suppressing LPS-induced NF-κB activity. In addition, 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 $\alpha4\beta2$ nAChR suppression of endotoxin-induced NF-κB activation is Ca^{2+}- and cAMP-PKA-independent; nicotine-stimulated JAK2-STAT3 signaling blocks NF-κB translocation but only partially blocks IκBα phosphorylation.

Materials and Methods

Cell Culture and Reagents. h$\alpha4\beta2$ SHEP1 cells were a generous gift from Ron Lukas and were grown as described previously (Hosur et al., 2009). Nicotine tartrate and endotoxin lipopolysaccharide were purchased from Sigma-Aldrich (St. Louis, MO). JAK inhibitor I, JAK2 inhibitor α-cyano-(3,4-dihydroxy)-N-benzylcinnamide (AG-490), NF-κB peptide inhibitor SN50 (peptide: AAVA LLPAVLLALLAPVQRKRQKLMP), calcium chelator BAPTA-AM, forskolin, STAT3 inhibitor VI 2-hydroxy-4-[[[[(4-methylphenyl)sulfonyl]oxy]acetyl]amino]-benzoic acid (NSC74859, also known as S3I-201), PKA inhibitor peptide PKI 14-22 amide (myristoylated-GRT-GRRNAI-NH$_2$), and calcium ionophore calcimycin (A23187) were obtained from Calbiochem (San Diego, CA). Primary antibodies were purchased from Cell Signaling Technology (Danvers, MA), and secondary antibody was from Millipore Corporation (Billerica, MA).

Real-Time Polymerase Chain Reaction. As described previously (Hosur et al., 2009). Toll-like receptor 4 (TLR4) primers were designed using IDT SciTools PrimerQuest (Integrated DNA Technologies, Coralville, IA): forward, TCC TGC AAT GGA TCA AGG ACC AGA; reverse, GCC AGC AAG AAG CAT CAG GTG AAA.

Cytokine ELISA. h$\alpha4\beta2$ cells seeded in six-well plates were pretreated with nicotine before stimulation with LPS for 48 h, and IL-1β and IL-6 levels were measured as described previously (Hosur et al., 2009).

Phospho-IκBα ELISA. Phospho-IκBα levels were examined in h$\alpha4\beta2$ SHEP1 cells by Function ELISA IκBα assay (Active Motif Inc., Carlsbad, CA). Cells seeded in six-well plates and grown for 2 days were pretreated with nicotine, BAPTA-AM, or AG-490 before stimulation with or without 1 μg/ml LPS for 25 min. Thereafter, cells were washed two times with ice-cold phosphate buffered saline (PBS), and a cell-scraper was used to remove cells. Cells were centrifuged for 10 min at 1000 rpm at 4°C, and then pellets were incubated in lysis buffer containing protease inhibitor for 30 min on ice. Samples were again centrifuged for 20 min at 14,000g at 40°C. Samples were stored at -80°C until further use. Protein concentration was determined by a NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA). Cell lysate (100 μg) was added to capture antibody precoated plates and incubated for 4 h at 4°C. Plates were thoroughly washed, and detection antibody was added and allowed to incubate for 1 h at RT. After a second wash, secondary antibody was added for 1 h before quantifying the signal using a chemiluminescent substrate.

Calcium Indicator Assay. A23187-induced increases in intracellular calcium were measured using Fluo-4-AM (Invitrogen, Carlsbad, CA). Cells plated in a 96-well plate (5×10^4 cells/well in Costar 3603 plates; Corning Life Sciences, Acton, MA) 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 of Hanks' buffered salt solution (HBSS) with 0.1% dimethyl sulfoxide and 5.5 mM glucose, without phenol red]. A BioTek-HT microplate reader (BioTek, Winooski, VT) determined baseline fluorescence at excitation and emission wavelengths of 485 ± 20 and 520 ± 20 nm, respectively, by taking nine readings at 0.5-s intervals. At 4.8 s, 100 μl of the calcium ionophore A23187 or nicotine were added by automated dispenser and fluorescence measurements were read at 0.5-s intervals for a total elapsed time of 40.8 s. All readings were made within 4 h 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 six-well plates were incubated with 10 μM nicotine or 10 μM 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.

NF-κB Secreted Luciferase Reporter Assay. The NF-κB secreted luciferase reporter assay was used as described previously (Hosur et al., 2009).

Western Blotting. To investigate whether $\alpha4\beta2$-mediated anti-inflammatory effect involves the phosphorylation of IκBα, we probed using antibodies against total IκB and phospho-IκBα (phosphorylated IκBα undergoes ubiquitination and allows translocation of NF-κB to the nucleus) after treating h$\alpha4\beta2$ SHEP1 cells with nicotine/BAPTA-AM/AG-490 inhibitor/LPS. In brief, cells seeded onto six-well plates were treated with the varying concentrations of nicotine with or without inhibitors. After incubation, cells were washed three times with PBS and lysed using Mammalian Protein Extraction Reagent (Pierce Chemical, Rockford, IL). Protein concentration was determined using a NanoDrop ND-1000 UV-Vis Spectrophotometer. Equal concentrations of protein were heated at 95°C for 5 min before loading onto precast gels (Pierce Chemical) and run at ~140 V for 45 min at RT. Protein was transferred to a polyvinylidene difluoride membrane at 30 V for 2 h at 4°C. Membranes were then stained with Ponceau S reagent to visualize the efficiency of protein transfer before incubating with blocking buffer [$1\times$ TBS, 5% (w/v) nonfat dry milk, and 0.1% Tween 20] at RT for 1 h. The blots were incubated in primary antibody buffer (0.2g/ml antibody and blocking buffer) overnight at 4°C. A thorough wash (five times with TBS/Tween 20) was followed by incubation with secondary antibody (HRP-conjugate) buffer (10 ng/ml) at RT for 1 h. Membranes were washed again five times with TBS/Tween 20 before exposing the blots to a chemiluminescent substrate (SuperSignal West Pico; Thermo Fisher Scien-

tific). A charge-coupled device camera (Kodak Image Station In-Vivo FX; Carestream Health, Rochester, NY) was used to visualize luminescence.

Data Analyses. Statistical analysis was performed by SPSS version 17 (SPSS Inc., Chicago, IL), and GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA) was used to develop graphs. One-way analysis of variance estimated differences among groups. Semiquantitative gel density analysis was performed using ImageJ (http://rsbweb.nih.gov/ij/index.html).

Results

Nicotine Blocks the LPS-Induced NF-κB Pathway. We showed previously that nicotine exposure inhibits constitutive NF-κB activity in hα4β2 cells (Hosur et al., 2009). Nicotinic activation of α7 receptors attenuates endotoxin-induced NF-κB translocation and proinflammatory cytokine (TNFα, IL-1β, and IL-6) production (Borovikova et al., 2000; Wang et al., 2004). To test the hypothesis that α4β2 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 qualitative polymerase chain reaction and DNA sequencing (data not shown). Initial experiments were carried out to determine optimal concentrations of LPS to induce NF-κB translocation (Fig. 1a and Supplemental Fig. S1). LPS (1 and 3 μg/ml) caused a ~2- and ~3- to 4-fold increase, respectively, in NF-κB activity, although nicotine failed to attenuate LPS-induced NF-κB transactivation in wild-type SHEP1 cells lacking α4, β2, or α7 nAChR subunits (Fig. 1a). On the other hand, pretreatment of hα4β2 SHEP1 cells with nicotine effectively attenuated LPS-induced NF-κB transactivation in a dose-dependent manner, as measured by NF-κB reporter assay (Fig. 1b), with 1 μM causing approximately a 40% decrease. SN50 peptide inhibits nuclear translocation of NF-κB and acts as a positive control to block LPS-induced nuclear translocation. SN50 dose-dependently blocked LPS-induced NF-κB activity (data not shown; 20 μM caused a 90% decrease), 10 μM causing a decrease of approximately 50% (Fig. 1b). Furthermore, the effect of a submaximal dose of SN50 (10 μM) was additive when applied with 300 nM nicotine.

LPS exposure increases PIC production in part through the NF-κB pathway. Because nicotine blocked LPS-induced NF-κB activity, we tested whether nicotinic activation of α4β2 blocks LPS-induced cytokine production downstream of the NF-κB pathway. hα4β2 SHEP1 cells treated with 1 μg/ml LPS increased both IL-1β (2-fold) and IL-6 (1.5-fold) production (Fig. 1, c and d) compared with untreated controls. However, nicotine pretreatment reduced LPS-induced IL-1β and IL-6 production at concentrations found in the blood of patients who smoke (Russell et al., 1980; Henning et al., 1994), with 100 nM nicotine blocking both cytokines down to control levels (Fig. 1, c and 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 NF-κB translocation. Nanomolar concentrations of JAK inhibitor I robustly blocked nicotine-induced reduction in NF-κB 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 NF-κB into the nucleus. Because nicotinic activation of α4β2 receptors attenuated LPS-induced NF-κB activation and thereby cytokine production, we asked whether α4β2 signaling interferes with IκBα phosphorylation. hα4β2 SHEP1 cells, preincubated with either PBS or 300 nM nicotine, were stimulated with 1 μg/ml LPS for 25 min at 37°C. Nicotine (300 nM) decreased IκBα phosphorylation approximately 50% compared with PBS-treated controls in Western blots (Fig. 2b).
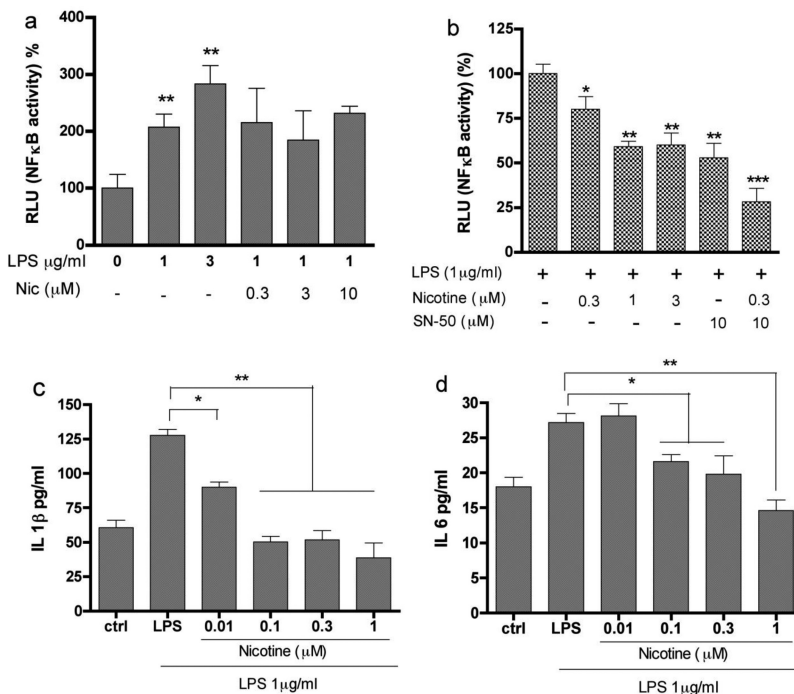

Fig. 1. Nicotine blocks LPS-induced NF-κB activity and cytokine production in hα4β2 but not wild-type SHEP1 cells. a, wild-type SHEP1 cells plated in 48-well plates were transfected with pNF-κB-MetLuc2-Reporter vector overnight. Cells were stimulated with varying concentrations of LPS for 4 h with or without indicated concentrations of nicotine pretreatment (2 h). Cell supernatant was collected to measure secreted luciferase activity. b, hα4β2 SHEP1cells (stably transfected with human α4β2 receptors) were pretreated with increasing concentrations of nicotine for 2 h before stimulating with 1 μg/ml LPS for 4 h. A submaximal (10 μM) dose of SN50 peptide was used as a positive control. c and d, hα4β2 SHEP1cells plated in six-well plates were stimulated with 1 μg/ml LPS for 48h with or without indicated concentrations of nicotine pretreatment. Cells were lysed and stored at −80°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 ± S.E.M. (n = 3). *, p < 0.05; **, p < 0.01; ***, p < 0.001. RLU, relative luminescence units.

However, the effects of nicotine on IκBα phosphorylation were more modest but still significant in the ELISA assay (Fig. 2d). Preincubation with the specific JAK2 inhibitor AG-490 (10 μM) with nicotine completely restored NF-κB translocation (Fig. 2c) and IκBα phosphorylation (Fig. 2, b and 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 NF-κB translocation.

NSC74859 Restores LPS-Induced NF-κB Activation. Activated JAK induces STAT phosphorylation, which forms either homo- or heterodimers 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 NF-κB activity in hα4β2 SHEP1 cells. Again, the effects of nicotine were more modest on IκBα phosphorylation measured by ELISA than on NF-κB translocation, but NSC74859 at 30 μM prevented the effects of nicotine on both (Fig. 3, a and b).

α4β2-Mediated Ca^{2+} Influx Does Not Influence LPS-Induced NF-κB Activity. We tested the role of calcium on NF-κB translocation and IκBα phosphorylation using the cell-permeable calcium chelator 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 pretreatment 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. Furthermore, 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 that was due to α4β2 receptor activation, consistent with a small α4β2 calcium permeability (4–6%) compared with α7 nAChRs (Wonnacott et al., 2006). However, 3 μM A23187 caused a reliable increase in calcium-dependent fluorescence compared with controls, but 30-min preincubation with 5 μM BAPTA-AM decreased the 3 μM A23187-induced calcium flux to less than 30% of A23187 alone (Fig. 4). These data suggest that BAPTA pretreatment should effectively buffer against the small increases in intracellular calcium that were due to nicotinic stimulation of α4β2 receptors.

Cells pretreated with BAPTA for 30 min and further incubated with nicotine showed no significant difference between nicotine alone and nicotine plus BAPTA treatments in basal NF-κB translocation (Supplemental Fig. S2). Nicotine (300 nM) partially but significantly blocked LPS-induced IκBα
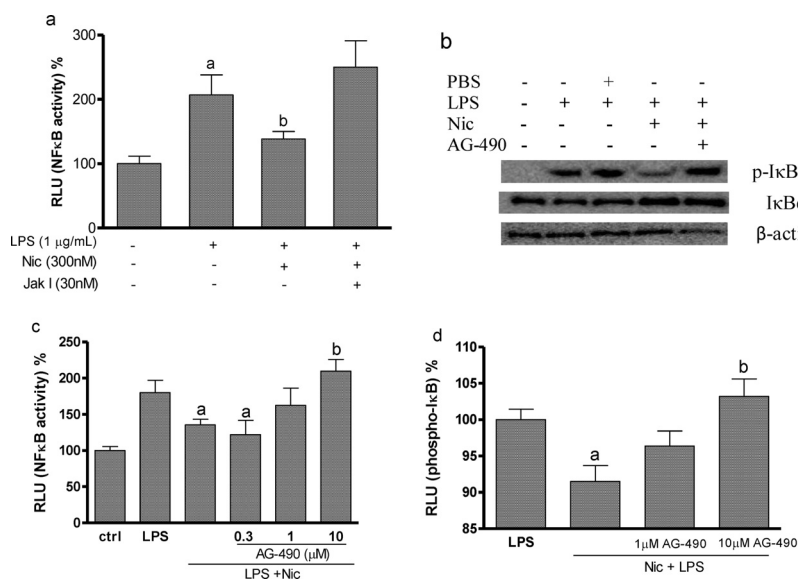

Fig. 2. JAK2 inhibition restores LPS-induced NF-κB signaling in nicotine-treated hα4β2 SHEP1 cells. a, NF-κB reporter assay: NF-κB reporter-transfected hα4β2 SHEP1cells were pretreated (30 min) with 30 nM JAK I inhibitor (a nonselective JAK inhibitor) and 300 nM nicotine and stimulated with 1 μg/ml LPS for 4 h. Cell supernatant was used to monitor luciferase activity. b, immunoblot: serum-starved hα4β2 SHEP1 cells were pretreated with 300 nM nicotine and 10 μM AG-490 and then stimulated with 1 μg/ml LPS for 25 min at 37°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% (data not shown), but AG-490 reversed nicotine's effect. c, NF-κB reporter assay: NF-κB reporter-transfected cells were pretreated with nicotine and/or varying concentrations of AG-490 (30 min) and incubated with 1 μg/ml LPS for 4 h. Cell supernatant (50 μl) was collected to assess luciferase activity. d, phospho-IκBα ELISA: protein samples (same as used in b) were added to precoated plates and phospho-IκBα levels were determined according to the manufacturer's protocol. Data represent mean± S.E.M. of three independent experiments. One-way analysis of variance determined statistical difference among groups. a, significantly different from LPS alone; b, significantly different from LPS plus nicotine treatment (significance at 0.05 level).

phosphorylation by approximately 50% of control in Western blots (Fig. 5a) but only by approximately 10% measured by the ELISA assay (Fig. 5b). However, BAPTA failed to block the effects of 300 nM nicotine in both of these assays (Fig. 5, a and b), suggesting a calcium-independent mechanism for α4β2-mediated inhibition of LPS-induced NF-κB activity and IκBα phosphorylation.

cAMP-Dependent PKA Signaling. In other systems, cAMP-dependent protein kinase A (PKA) inhibits NF-κB transcriptional activity (Minguet et al., 2005). In addition, agents that elevate intracellular cAMP inhibit IκBα phosphorylation and subsequent NF-κB activation (Takahashi et al., 2002; Sands et al., 2004). Although nicotinic agonists cause cAMP accumulation (Hiremagalur et al., 1993; Gueorguiev et al., 1999), 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 1 h and nicotine at 4 h. Therefore, we initially examined the effect of forskolin and nicotine on LPS-induced NF-κB translocation in presence or absence of a specific PKA peptide inhibitor (PKI 14–22-amide). Forskolin had no effect on NF-κB 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 NF-κB translocation.

Discussion

We showed previously 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 (polymerase chain reaction data not shown). Nicotine pretreatment appreciably reduced LPS-induced cytokine production by interfering with NF-κB signaling, suggesting that nicotine
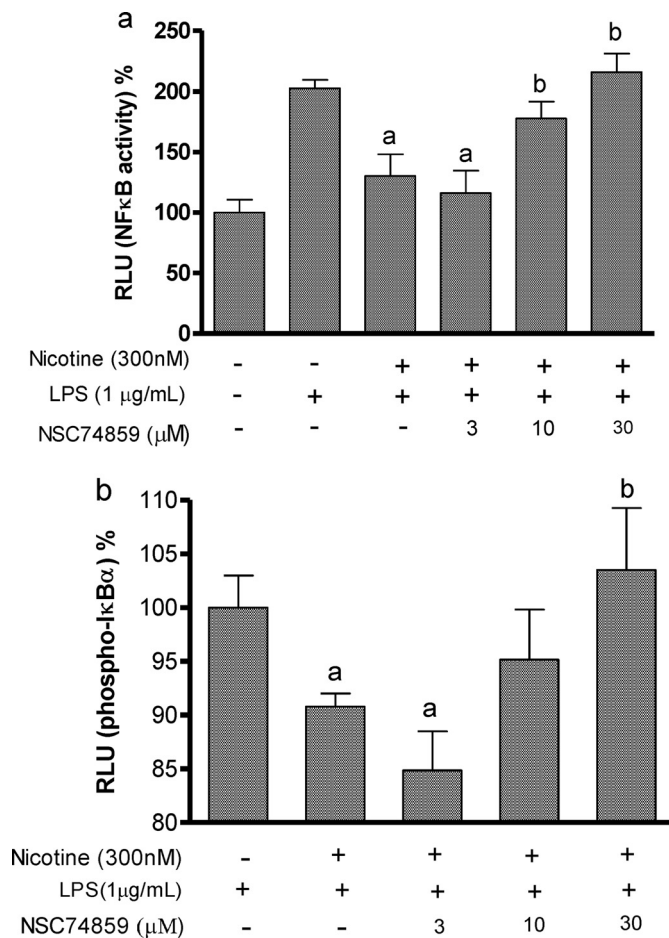

Fig. 3. The STAT3 inhibitor NSC74859 restores LPS-induced NF-κB signaling in nicotine-treated hα4β2 SHEP1 cells. a, NF-κB translocation: hα4β2 SHEP1 cells transiently transfected with NF-κB reporter vector were stimulated with LPS for 4 h with or without nicotine and/or increasing NSC74859 pretreatment for 30 min. Cell supernatant (50 μl) was used to measure luciferase activity (*n* = 3). b, phospho-IκBα ELISA: cells grown in six-well plates were serum-starved for 16 h and then preincubated with 300 nM nicotine and indicated concentrations of NSC74859 before stimulating with 1 μg/ml LPS for 25 min at 37°C. Cell lysate (100 μg) was used to monitor phospho-IκBα levels by functional ELISA (*n* = 4). Results represent mean ± S.E.M. a, significantly different from LPS-treated samples (*p* < 0.05); b, significantly different from nicotine plus LPS-treated samples (*p* < 0.05).
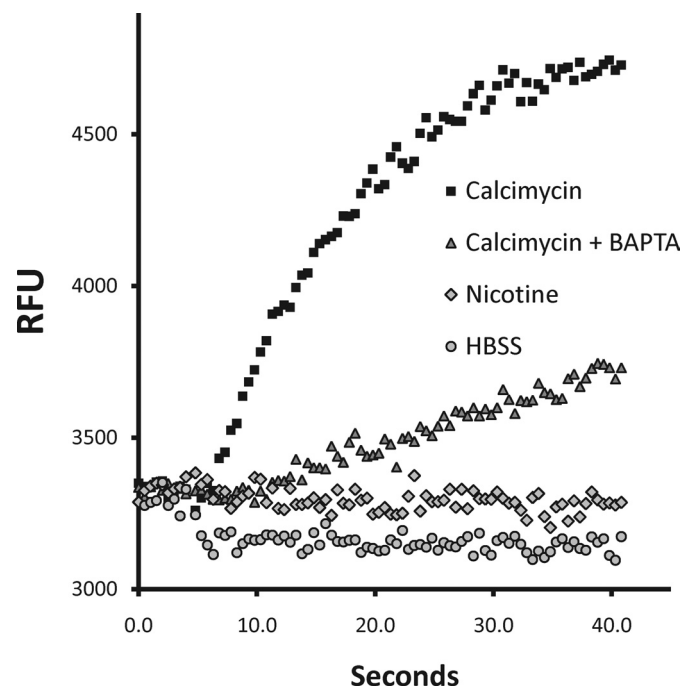

Fig. 4. BAPTA loading blocks A23187-induced increases in intracellular calcium. hα4β2 SHEP1 cells in a 96-well black-walled plate were pretreated with 3 μM Fluo-4-AM (dissolved in 100 μl of HBSS with 0.1% dimethyl sulfoxide and 4.5 mM glucose, but without phenol red, for 1 h in darkness at RT) with or without 5 μM 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 (nine recordings). At 4.8 s, the reader injected 100 μl of 3 μM A23187 (calcimycin), 1 μM nicotine, or HBSS to stimulate the cells, and fluorescence measurements were taken 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 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 under *Results*.

decreases endotoxin-induced inflammation in hα4β2 SHEP1 cells. Matsunaga et al. (2001) found that the cholinergic agonists nicotine and 1,1-dimethyl-4-phenylpiperazinium io-dide suppress production of cytokines IL-6, IL-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 NF-κB activation and PIC production. To delineate the signaling cascades downstream of α4β2 nAChR activation, we investigated the involvement of JAK2-STAT3 and Ca^{2+}, cAMP-PKA pathways as summarized in Fig. 6.

Nicotinic receptor-mediated regulation of gene transcription, neuroprotection, and antiapoptosis 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. Long-term smokers experience sustained exposure to low concentrations of nicotine ($\sim$100 nM), and it is possible that the desensitized (nonconducting) receptors rather than activated (conducting) receptors confer neuroprotection. Further-
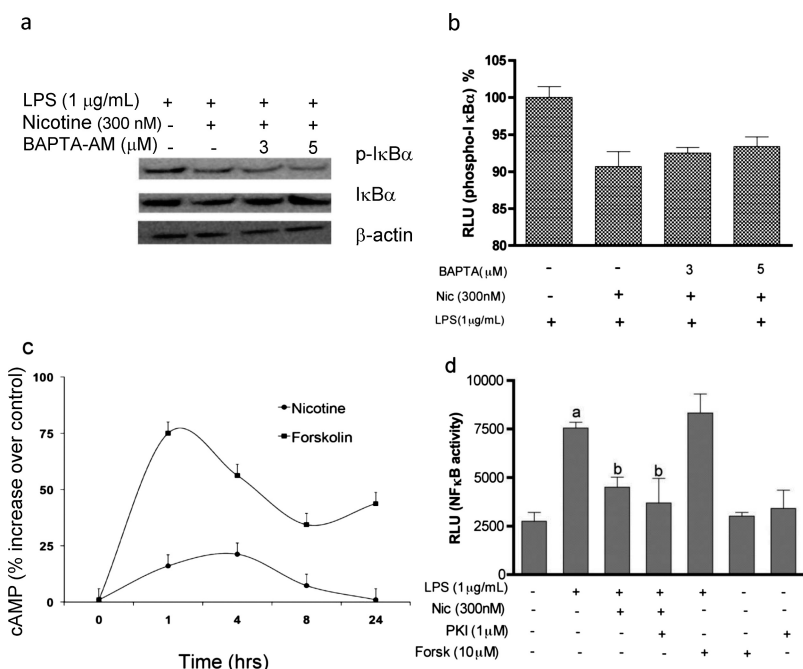

a

LPS (1 μg/mL)   +   +   +   +
Nicotine (300 nM)   -   +   +   +
BAPTA-AM (μM)   -   -   3   5

p-IκBα
IκBα
β-actin

b

RLU (phospho- IκBα) %

BAPTA(μM)   -   -   3   5
Nic (300nM)   -   +   +   +
LPS(1μg/mL) +   +   +   +

c

cAMP (% increase over control)

Nicotine
Forskolin

Time (hrs)

d

RLU (NFκB activity)

LPS (1 μg/mL)   -   +   +   +   +   -   -
Nic (300nM)   -   -   +   +   -   -   -
PKI (1μM)   -   -   -   +   -   -   +
Forsk (10μM)   -   -   -   -   +   +   -

Fig. 5. Ca^{2+} and cAMP-PKA-dependent pathways do not regulate α4β2-mediated anti-inflammatory effects. a, immunoblot: hα4β2 cells grown in six-well plates were serum-starved for 16 h and then pretreated (30 min) with 3 and 5 μM BAPTA and incubated with 300 nM nicotine for 30 min before stimulating with 1 μg/ml LPS for 25 min at 37°C. Equal amounts of protein were analyzed by SDS-polyacrylamide gel electrophoresis, followed by electrophoretic transfer to polyvinylidene difluoride membranes, blotting, and enhanced chemiluminescence detection. The membranes were stripped and reprobed with an anti-β-actin antibody. No phospho-IκBα band was detected for untreated cells (data not shown). Semiquantitative ImageJ analysis suggests that nicotine blocked LPS-induced IκBα phosphorylation by approximately 50% (data not shown). b, phospho-IκBα ELISA: protein samples isolated for Western blotting in a were used according to the manufacturer's instructions. c, time course of cAMP induction by nicotine or forskolin: cells were exposed to 10 μM nicotine and 10 μM forskolin for indicated times, and cell lysates were used to monitor cAMP levels by ELISA. d, NF-κB reporter assay: NF-κB reporter-transfected cells were preincubated with 10 μM forskolin or 300 nM nicotine and/or PKA inhibitor and stimulated with 1 μg/ml LPS for 4 h. PKI inhibitor alone had no effect on LPS-induced NF-κB activity. a, significantly different from untreated controls; b, significantly different from LPS-alone treatment; $p < 0.05$.
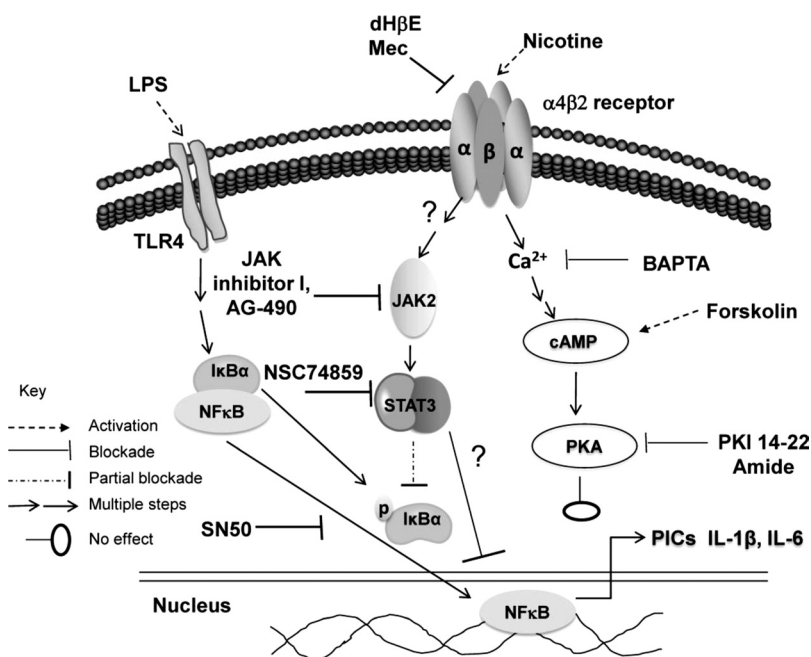
Fig. 6. Model for α4β2 receptor-dependent nicotinic suppression of LPS-induced PIC production through JAK2-STAT3 but not through calcium or cAMP signaling. This figure summarizes nicotine's effects on LPS-stimulated hα4β2 SHEP1 cells and previous work on nicotine's effects on constitutive PIC expression in these cells (Hosur et al., 2009). LPS, acting in part through TLR4 and a multistep pathway, is known to cause phosphorylation and dissociation of IκBα from NF-κB. Dissociated NF-κB translocates to the nucleus to promote the PIC's IL-1β and IL-6 production, but translocation is blocked by SN50. We showed previously that nicotine acting through α4β2 receptors blocks constitutive PIC production in these cells, but the antagonists dihydro-β-erythroidine (dHβE) and mecamylamine (Mec) block nicotine-induced decreases in NF-κB 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 NF-κB, but nicotine only partially blocks phosphorylation of IκBα. The question marks indicate that the effects of STAT3 on NF-κB translocation are not understood (Peña et al., 2010), given the partial nicotine-induced inhibition of IκBα phosphorylation, and that either a direct or multistep 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 NF-κB signaling.

dHβE
Mec

Nicotine

α4β2 receptor

LPS

α β α

TLR4

JAK
inhibitor I,
AG-490

JAK2

Ca^{2+} BAPTA

Forskolin

cAMP

NSC74859

IκBα
NFκB

STAT3

PKA

PKI 14-22
Amide

Key

Activation
Blockade
Partial blockade
Multiple steps
No effect

SN50

p IκBα

PICs IL-1β, IL-6

Nucleus

NFκB

more, we found no evidence for the role of cAMP-PKA-dependent pathways in α4β2-mediated suppression of NF-κB 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, NF-κB activation, and NF-κB 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 NF-κB translocation and PIC production. This is the first indication that STAT3 inhibits NF-κB activity through a nontranscriptional mechanism upon α4β2 activation and strongly supports the previous hypotheses that cholinergic stimulation prevents NF-κB activity and mimics IL-10 receptor signaling (de Jonge et al., 2005).

Future studies need to address some important questions. First, 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. Second, does the open state or the desensitized state of the receptor favor JAK2 binding? We found previously that both competitive and noncompetitive antagonists prevent nicotine-induced attenuation of NF-κB 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). Third, is the immune modulatory activity restricted to α4β2 and α7, or does it apply to other cysteine-loop ligand-gated ion channel receptors? Although both α7 and α4β2 receptors seem to use 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. Finally, 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).

Although JAK2-STAT3 is involved in nicotinic anti-inflammation, it is also implicated in mediating proinflammatory effects, depending on the cellular context (Kox et al., 2009). de Jonge et al. (2005) show that 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 NF-κB signaling (Tracey, 2007). However, a recent study finds that α7 receptors on PC12 cells stimulate antiapoptotic effects through JAK2-STAT3-induced increases in NF-κB activity (Marrero and Bencherif, 2009). In addition, Chen et al. (2008)

report that nicotine-induced STAT3 activation increases NF-κB activity in bladder cancer cells. (Incidentally, the claim by Chen et al. (2008) that nicotinic effects on STAT3 in their system are mediated by α4β2 receptors is questionable, because 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 on 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, nonphosphorylated STAT3 regulates NF-κB activity and cytokine production in macrophages (Peña et al., 2010) and that the cholinergic peptide SLURP-1 modulates α7-JAK2-mediated up-regulation of NF-κB in keratinocytes (Chernyavsky et al., 2010). Further work is clearly required to sort out how JAK2-STAT3 signaling can be both pro- and anti-inflammatory and both block and stimulate NF-κB 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. (2008) report that PICs IL-1β and TNFα alter the assembly of nAChRs in human embryonic kidney 293 cells. Nicotine exposure increased the expression of endoplasmic reticulum chaperones, down-regulated PICs, and induced 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) although positron emission tomography and postmortem studies of the brains of long-term smokers reveal an appreciable increase in high-affinity binding sites for nicotine (Wüllner et al., 2008), long-term smokers with schizophrenia have lower inflammatory cytokines (IL-2 and IL-6) compared with their nonsmoking counterparts (Zhang et 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).

Authorship Contribution

Participated in research design: Hosur and Loring.
Conducted experiments: Hosur and Loring.
Contributed new reagents or analytic tools: Hosur.
Performed data analysis: Hosur and Loring.

Wrote or contributed to the writing of the manuscript: Hosur and Loring.

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