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Gene regulation of α4β2 nicotinic receptors: microarray analysis of nicotine-induced receptor up-regulation and anti-inflammatory effects

Vishnu Hosur, Scott Leppanen, Adham Abutaha and Ralph H. Loring

Department of Pharmaceutical Science, Northeastern University, Boston, Massachusetts, USA

Abstract

α4β2 Nicotinic acetylcholine receptors play an important role in the reward pathways for nicotine. We investigated whether receptor up-regulation of α4β2 nicotinic acetylcholine receptors involves expression changes for non-receptor genes. In a microarray analysis, 10 μM nicotine altered expression of 41 genes at 0.25, 1, 8 and 24 h in hα4β2 SH-EP1 cells. The maximum number of gene changes occurred at 8 h, around the initial increase in ^{3}[H]-cytisine binding. Quantitative RT-PCR corroborated gene induction of endoplasmic reticulum proteins CRELD2, PDIA6, and HERPUD1, and suppression of the pro-inflammatory cytokines IL-1β and IL-6. Nicotine suppresses IL-1β and IL-6 expression at least in part by inhibiting NFκB activation. Antagonists dihydro-β-erythroidine and mecamylamine blocked these nicotine-induced changes showing that receptor activation is required. Antagonists alone or in combination with nicotine suppressed CRELD2 message while increasing α4β2 binding. Additionally, small interfering RNA knockdown of CRELD2 increased basal α4β2 receptor expression, and antagonists decreased CRELD2 expression even in the absence of α4β2 receptors. These data suggest that endoplasmic reticulum proteins such as CRELD2 can regulate α4β2 expression, and may explain antagonist actions in nicotine-induced receptor up-regulation. Further, the unexpected finding that nicotine suppresses inflammatory cytokines suggests that nicotinic α4β2 receptor activation promotes anti-inflammatory effects similar to α7 receptor activation.

Keywords: CRELD2, IL-1β, IL-6, NFκB, pro-inflammatory cytokines, receptor up-regulation.

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Nicotine is the major reinforcing component of tobacco smoke and induces up-regulation of its major high-affinity binding site in the brain, the α4β2 nicotinic acetylcholine receptor (nAChR), (Picciotto *et al.* 2008). α4β2 nAChRs are well distributed in the cortex, midbrain, hippocampus, thalamus, substantia nigra, nucleus accumbens, and brainstem (Flores *et al.* 1997) and show considerable up-regulation not only in the intact brain, but also in *Xenopus* oocytes and several mammalian cell lines (Yates *et al.* 1995; Whiteaker *et al.* 1997; Sweileh *et al.* 2000). Chronic nicotine causes variable α4β2 receptor up-regulation in different brain regions (Nguyen *et al.* 2003). For instance, nicotine induces a dose-dependent increase of α4β2 receptors in the cortex, hippocampus and striatum of rat brain, but not in the thalamus (Schwartz and Kellar 1983; Flores *et al.* 1992; Rowell and Li 1997).

Epidemiological evidence suggests that nicotine has a protective effect against neurological disorders such as Alzheimer's disease, schizophrenia and Parkinson's disease (Ripoll *et al.* 2004; Picciotto and Zoli 2008). Nonetheless, nicotine is an extremely addictive drug, and its addiction leads to several physiological and behavioral alterations, and receptor up-regulation may play a role in these processes (Buisson and Bertrand 2002). Minimizing the detrimental effects of nicotine and maximizing the possible therapeutic

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Address correspondence and reprint requests to Ralph H. Loring, Department of Pharmaceutical Science, 211 Mugar, Northeastern University, 360 Huntington Avenue, Boston, MA 02115, USA. E-mail: r.loring@neu.edu

Abbreviations used: BiP, heat shock protein 5 (immunoglobulin heavy chain-binding protein); CRELD2, cysteine-rich with EGF-like domain 2; CREM, cAMP responsive element modulator; dHβE, dihydro-β-erythroidine; ER, endoplasmic reticulum; HERPUD1, homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1; HPRT1, hypoxanthine guanine phosphoribosyl transferase 1; HSPA5, heat shock protein 5 (BiP); Mec, mecamylamine; nAChR, nicotinic acetylcholine receptor; PIC, pro-inflammatory cytokine; NFκB, nuclear kappa B; PAX5, paired box gene 5; PDIA4, protein disulfide isomerase family A, member 4; PDIA6, protein disulfide isomerase family A, member 6; PMCA4, ATPase, Ca++ transporting, plasma membrane 4; siRNA, small interfering RNA.

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uses of nicotinic agonists requires a thorough understanding of the mechanisms underlying α4β2 up-regulation.

In contrast to normal agonist-induced receptor desensitization and down-regulation, the pharmacology of α4β2 up-regulation is complicated because chronic nicotine exposure increases B_{max} without dramatically changing the affinity of the receptors measured with binding assays. Further, both competitive and non-competitive antagonists can either potentiate agonist-induced up-regulation or elicit the phenomenon themselves (Wonnacott 1990; Pauly *et al.* 1996; Darsow *et al.* 2005). Up-regulation of α4β2 receptors is a post-transcriptional event with no change in the message for α4 or β2 subunits (Marks *et al.* 1992; Pauly *et al.* 1996). However, this does not rule out gene changes of endoplasmic reticulum (ER) chaperones and cytosolic proteins that may be involved in up-regulation. Dunckley and Lukas showed that nicotine alters gene expression, including transcription factors and genes involved in the ubiquitin proteasome system, through activation of α7 and α3β4 nAChRs in SH-SY5Y cells at a concentration (1 mM) that up-regulates those receptor subtypes (Dunckley and Lukas 2003, 2006).

We used a microarray approach to investigate whether nicotine modulates gene expression while inducing up-regulation of α4β2 receptors, with special interest in ER chaperones and other proteins that promote assembly, folding, and/or receptor trafficking. We found the optimal concentration to induce up-regulation in hα4β2 SH-EP1 cells was 10 μM nicotine. Therefore, we investigated the effects of 10 μM nicotine on gene expression at 0.25 h, 1 h, 8 h and 24 h. Nicotine altered expression of 41 genes at the time points tested. Quantitative RT-PCR (qPCR) validated some of these gene expression changes. Further, we examined the effects of nicotinic antagonists on nicotine-induced gene alterations, as these agents block α4β2 activation but potentiate nicotine-induced α4β2 up-regulation. In carrying out these studies, we unexpectedly found that the expression of a set of pro-inflammatory cytokines (PICs) was significantly down-regulated by chronic nicotine exposure, which may correspond with neuroprotective effects of nicotine.

Experimental procedures

Cell culture

SH-EP1 human neuroblastoma cells stably transfected with human α4 and β2 subunits (a generous gift from Ron Lukas) were grown at 37°C in 5% CO_2 as recommended (Pacheco *et al.* 2001). Cells grown in 75 cm^2 or 150 cm^2 flasks were passaged onto 6-, 24- or 96-well plates and grown for 2–3 days to attain confluence. Cells were then treated with (−)-nicotine or (−)-nicotine plus dihydro-β-erythroidine (dHβE) or mecamylamine (Mec) for indicated times before harvesting.

Biotinylation of surface receptors

See Appendix S1.

Immunoprecipitation of surface receptors

Cells were labeled with 1 ng/mL mAb 299 (Sigma, St Louis, MO, USA) for 2 h at 4°C with gentle shaking, washed 3 × 10 min with phosphate buffered saline, and then lysed as above. Labeled receptors were immunoprecipitated overnight at 4°C on 10 μL goat anti-rat IgG-coated beads before assaying for ^{3}[H]-cytisine binding.

Radioassay of surface α4β2 receptors

See Appendix S1.

Microarray analysis and 'retrospective' nicotinic stimulation experimental design

Total RNA was processed for analysis on Affymetrix HG-U133A human genome probe arrays following the manufacturer's protocol. Briefly, cDNA template was generated from T7/oligo dT (Affymetrix, Santa Clara, CA, USA) primed RNA. Purified cDNA template was then used to create amplified, biotin labeled, antisense cRNA through *in vitro* transcription using T7 RNA Polymerase. Labeled cRNA was quantified and added to a hybridization cocktail ready for hybridization to an Affymetrix HG-U133A. Probe arrays were hybridized and processed according to the Affymetrix Expression Analysis Manual. Data were generated using a GCS 3000 Scanner with attached Autoloader, run with GeneChip Operating System 1.1.1 (GCOS, Affymetrix). Raw image files were uploaded into Stratagene ArrayAssist software (La Jolla, CA, USA) searching for statistically significant concurrent changes within each time point and across duplicate experiments. Transcripts of interest for each time point were further interrogated using Pathway Architect (Both software packages now contained within Agilent (Santa Clara, CA, USA) GeneSpring version 10.0) for biological relatedness. In preliminary experiments, merely changing the medium caused significant changes in over 1500 gene transcripts for untreated hα4β2 SH-EP1 cells, compared to over 900 changes induced by medium change in untreated wild-type SH-EP1 cells. These non-specific gene changes threatened to mask any effects because of nicotinic stimulation. Therefore, a 'retrospective' nicotinic stimulation design minimized these non-specific effects. All cells were harvested for RNA at the same time to minimize differences in cell confluency or the number of divisions since plating. Human α4β2 SH-EP1 cells were seeded in 6-well plates in triplicate at a density of approx. 5 × 10^4 cells per well 48 h prior to harvest, and media changes brought the nicotine concentration to 10 μM at 24, 40, 47 and 47.75 h after plating (corresponding to 24, 8, 1 or 0.25 h prior to when the cells were harvested). Quadruplicate sister cultures in 24 well plates seeded at a density of approx. 1 × 10^4 cells per well were treated similarly at the indicated times and assayed for ^{3}[H]-cytisine binding to determine the amount of nicotine-induced up-regulation.

Total RNA extraction, quantitative real-time PCR and ELISA

See Appendix S1.

RNA interference

Small interfering RNA (siRNA) duplex targeting CRELD2 (cysteine-rich with EGF-like domain 2) was purchased from Sigma Aldrich (SASI_Hs01_00027506). The siRNA duplex was transfected into the cells grown to ~60% confluence, using X-tremeGene siRNA transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN, USA). The knockdown efficiency was measured by

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qPCR (Fig. S1). To determine the influence of CRELD2 on α4β2 up-regulation, cells seeded in 24-well dishes were transfected with 30 nM siRNA for 48 h and treated with or without 10 μM nicotine for 24 h. Following incubation, surface receptor expression was determined by ^{3}[H]-cytisine binding assays. Effects of Mec and dHβE on CRELD2 expression were measured using qPCR in wild-type or hα4β2 SH-EP1 cells.

NFκB secreted luciferase reporter assay

SH-EP1 hα4β2 cells were grown to confluence in a 24-well plate and transfected with pNFκB luciferase vector (Clontech, Mountain View, CA, USA), which contains the NFκB (nuclear factor kappa B) promoter element upstream of a Metridia luciferase reporter gene. Following 10 h of transfection and a media change, cells were treated with or without nicotine or nicotine plus varying concentrations of Mec or dHβE for 4 h. 50 μL of sample media was assayed immediately for secreted luciferase activity using a BioTek-HT (BioTek Instruments, Winooski, VT, USA) microplate reader.

Data analyses

SPSS v15 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA) were used for data analysis. To assess significant differences between control and drug-treated samples, Student's *t*-test was performed. One-way analysis of variance (ANOVA) was used for comparisons among groups followed by *post-hoc* tests (Tukey).

Results

Nicotine-induced up-regulation of hα4β2 receptors

Exposing SH-EP1 human neuroblastoma cells stably transfected with human α4 and β2 subunits (hα4β2 SH-EP1 cells) (Pacheco *et al.* 2001) for 24 h increased the receptors in a concentration-dependent manner with 10 μM (−)-nicotine producing the maximum up-regulation of the concentrations tested (Fig. S2). 10 μM (−)-nicotine required more than 5–6 h to induce up-regulation in hα4β2 SH-EP1 cells (Fig. 1a), whereas wild-type cells, lacking α4β2 receptors, showed no specific ^{3}[H]-cytisine binding. The magnitude of up-regulation varied from one and a half to threefold among experiments. Experiments were repeated three times, with four biological replicates. Binding assays on sister cultures confirmed α4β2 receptor up-regulation when mRNA was harvested for microarray analysis or qPCR. Treatment with 10 μM (−)-nicotine for up to 72 h did not cause significant cell toxicity (Fig. S3).

To address whether ^{3}[H]-cytisine crosses the plasma membrane and labels internal receptors, we incubated hα4β2 SH-EP1 cells with or without 10 μM nicotine for 24 h and determined ^{3}[H]-cytisine binding to surface receptors by three methods: (i) Direct binding to receptors on intact cells, and to isolated surface receptors that had been pre-labeled on intact cells with (ii) sulfo-NHS-SS-biotin or (iii) mAb299 (rat antibody specific to the external domain of the human α4 subunit) (Whiting and Lindstrom 1988) followed by precipitation with neutravidin or goat anti-rat coated beads, respectively. Nicotine caused the same level of receptor up-regulation using each of the three assays and there was no significant difference in baseline ^{3}[H]-cytisine specific binding among the three techniques (Fig. 1b). These data are consistent with (Zhang and Steinbach 2003) showing that ^{3}[H]-cytisine does not significantly label internal receptors in intact cells, and with reports showing nicotine-induced up-regulation increases surface α4β2 receptors (Lopez-Hernandez *et al.* 2004; Kuryatov *et al.* 2005), but see (Vallejo *et al.* 2005).

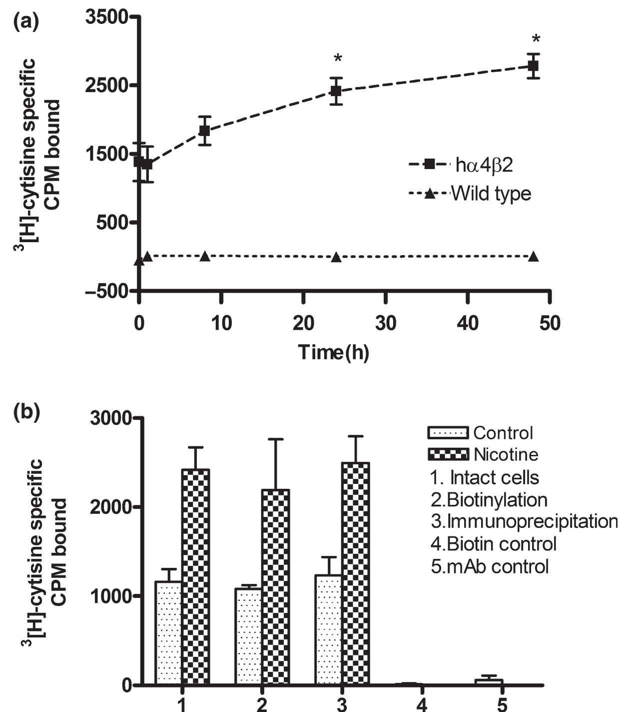

Fig. 1 The α4β2 up-regulation in hα4β2 SH-EP1 cells. (a) Time-course nicotine on hα4β2 SH-EP1 cells. Wild type and hα4β2 SH-EP1 cells seeded onto 24-well dishes were exposed to 10 μM nicotine for indicated times and specific binding was determined. 20 μM nicotine was used to estimate non-specific binding. Results represent mean ± SD of three independent experiments in quadruplicate (*$p < 0.05$). (b) Comparison between ^{3}[H]-cytisine binding to receptors on intact hα4β2 SH-EP1cells (direct binding) and to isolated surface receptors from either control cells or cells pre-treated 24 h with 10 μM nicotine. Binding is to 1 (intact cells after washing out nicotine), 2 (neutravidin precipitation of solubilized surface biotinylated receptors), 3 (immunoprecipitation of solubilized surface receptors from cells pre-treated with mAb299), 4 (neutravidin precipitation of solubilized cells without biotinylation) or 5 (immunoprecipitation of solubilized cells without mAb299 pre-treatment).

Microarray analysis of nicotine-induced α4β2 up-regulation

Nicotine (10 μM) significantly altered transcripts for 41 probe sets on Affymetrix HG-U133A arrays measured at

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0.25 h, 1 h, 8 h or 24 h exposure, with 8 h showing the maximum number of altered genes (Tables S2 and S3). However, nicotine did not change expression in the α4 probe set at the time points tested (The Affymetrix β2 probe set consists of untranslated regions, and could not measure expression changes in the β2 open reading frame used in hα4β2 SH-EP1 cells. However, qPCR confirmed that nicotine caused no change in either α4 or β2 mRNA expression – see Table 1). Pathway Architect™ (Stratagene) categorized the transcripts into two different clusters: (i) possible chaperones, transcription factors and probes with no known association (Table S2), and (ii) inflammation and immune response probes (Table S3).

Validation of transcript changes using quantitative real-time PCR

We selected the following gene transcripts (Table 1, Table S1) for validation: (i) Interleukins IL-1β, IL-11 and IL-6 and the chemokine CXCL2; (ii) Transcription factors CREM (cAMP responsive element modulator) and PAX5 (paired box gene 5); and (iii) ER resident proteins such as BiP [HSPA5, heat shock protein 5, also referred to as "immunoglobulin heavy chain-binding protein" (BiP)], PDIA4 (protein disulfide isomerase family A, member 4), PDIA6 (protein disulfide isomerase family A, member 6), CRELD2, and HERPUD1 (homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1). We considered several housekeeping genes for comparison, such as HPRT1 (hypoxanthine guanine phosphoribosyl transferase 1), PMCA4 (ATPase, Ca++ transporting, plasma membrane 4) and β-Actin, before selecting glyceraldehyde-3-phosphate dehydrogenase as the most appropriate endogenous control for qPCR (Calcagno *et al.* 2006) on the basis of its minimal Ct variation (< ± 0.15 Ct) across experimental conditions, as well as being expressed at levels similar to the transcripts under investigation. As predicted by the microarray, nicotine significantly increased IL-11 expression (8 and 24 h) but decreased the expression of the chemokine CXCL2 (1, 8 and 24 h) and the PICs IL-1β and IL-6 at both 8 h and 24 h when normalized with glyceraldehyde-3-phosphate dehydrogenase expression and compared with untreated controls (Table 1). As also predicted, nicotine increased the mRNA levels of PDIA6 (8 h only), CRELD2 (8 and 24 h), and HERPUD1 (8 h). The cAMP response element modulator (CREM) showed a significant delayed induction after 24 h nicotine exposure. In contrast, qPCR did not validate the transcript changes predicted by the microarray for BiP, PDIA4, and PAX5 (Table 1).

Antagonists block nicotine-mediated gene alterations

Previous studies demonstrate that up-regulation of α4β2 receptors requires receptor occupation, but not necessarily ion flux through the receptors (Gopalakrishnan *et al.* 1996; Darsow *et al.* 2005). In addition, rather than blocking agonist-induced up-regulation, nicotinic antagonists may either elicit up-regulation themselves or potentiate agonist-induced up-regulation (Wonnacott 1990). 24 h application of the competitive antagonist dHβE (100 μM) did not prevent about a 2-fold increase in binding when applied

Table 1 Gene transcripts selected for validation by qPCR

Gene symbol	Accession #	mRNA fold change by qPCR (10 μM nicotine exposure) 1 h	8 h	24 h
IL1B (IL-1β)	NM_000576	1.03 ± 0.02	0.77 ± 0.03*	0.68 ± 0.05*
IL6 (IL-6)	NM_000600	0.80 ± 0.02	0.70 ± 0.06*	0.66 ± 0.02*
CRELD2	NM_024324	1.28 ± 0.20	1.46 ± 0.08*	1.37 ± 0.20*
HERPUD1	NM_001010990	1.01 ± 0.12	1.36 ± 0.04*	0.86 ± 0.12
PDIA6	NM_005742	0.97 ± 0.04	1.38 ± 0.11*	1.08 ± 0.07
PDIA4[a]	NM_004911	0.98 ± 0.03	1.12 ± 0.09	1.01 ± 0.08
HSPA5 (BiP)[a]	NM_005347	1.00 ± 0.10	0.91 ± 0.01	0.98 ± 0.03
CREM	NM_001881	1.01 ± 0.05	1.28 ± 0.01	1.42 ± 0.08*
PAX5[a]	NM_016734	1.00 ± 0.07	0.94 ± 0.05	1.01 ± 0.08
CXCL2	NM_002089	0.45 ± 0.02*	0.36 ± 0.03*	0.41 ± 0.18*
SOD2	NM_000636	0.73 ± 0.01*	0.73 ± 0.05*	1.11 ± 0.20
IL8 (IL-8)[a]	NM_000584	1.00 ± 0.04	0.95 ± 0.05	0.86 ± 0.08
IL11 (IL-11)	NM_000641	0.82 ± 0.18	1.62 ± 0.26*	1.81 ± 0.25*
CHRNA4 (α4)	NM_000744	1.01 ± 0.04	0.87 ± 0.08	1.01 ± 0.04
CHRNB2 (β2)	NM_000748	1.03 ± 0.02	1.12 ± 0.15	1.13 ± 0.12
TNF (TNFα)	NM_000594	1.06 ± 0.21	0.82 ± 0.05	0.86 ± 0.23
NFKB1(NFκB)	NM_003998	1.10 ± 0.01	0.89 ± 0.08	0.89 ± 0.12

[a]Gene transcripts predicted by microarray but not validated by qPCR.
*Significant at 0.05 level (n = 3 with quadruplicate readings).

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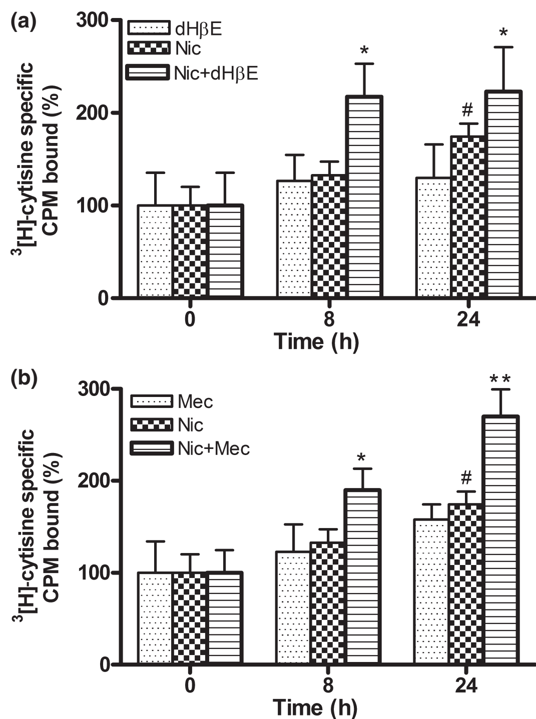

Fig. 2 Antagonists potentiate nicotine-induced α4β2 up-regulation. (a) Cells grown in 96-well plates were incubated with 100 μM dHβE alone or a combination of 10 μM nicotine plus 100 μM dHβE for indicated times before determining specific ^{3}[H]-cytisine binding. Specific basal binding was approximately 270 CPM. Co-application resulted in a ~2-fold increase in surface receptor binding. (b) Exposing cells to 10 μM Mec or 10 μM nicotine plus 10 μM Mec for indicated times resulted in a significant increase in specific ^{3}[H]-cytisine binding after 8 h and 24 h exposure. Experiments were in triplicate with four replicates. Results represent mean ± SD. Nic ($^{\#}p < 0.05$); Nic+ dHβE or Mec ($^{*}p < 0.05$, $^{**}p < 0.01$).

with 10 μM nicotine (Fig. 2a), even though that concentration of antagonist blocks virtually all ^{3}[H]-cytisine binding when applied during the binding assay (Fig. S4). The non-competitive antagonist Mec (10 μM) increased ^{3}[H]-cytisine binding by ~1.5 to ~2-fold at 24 h by itself, but co-treatment with 10 μM nicotine resulted in a ~3-fold increase (Fig. 2b). These data are consistent with previous reports that up-regulation of human α4β2 nAChRs does not require channel activity or receptor activation (Gopalakrishnan *et al.* 1996; Darsow *et al.* 2005; Nashmi and Lester 2007). As Mec and dHβE synergistically enhance up-regulation effects of nicotine, we expected that both antagonists should have a synergistic effect (either positive or negative) on genes that might be directly involved in nico2tine-induced receptor up-regulation. However, both antagonists blocked nicotine-mediated gene alterations of the ER chaperones tested (Table 2). Interestingly, we observed a strong negative correlation of CRELD2 mRNA with α4β2 receptor expression (Pearson correlation, −0.813; $p < 0.05$). In addition, CREM mRNA displayed a positive correlation (Pearson correlation, 0.990; $p < 0.05$). Collectively, these data suggest that nicotine-mediated gene alteration of ER chaperones requires activation of α4β2 nAChRs, and based on correlation analysis, manipulation of mRNA levels of CRELD2, and CREM may influence α4β2 receptor expression. As nicotine-induced gene alteration of CREM lagged the others and appeared after α4β2 up-regulation (24 h), we only assessed the influence of CRELD2 on surface expression of α4β2 receptors, using RNA interference.

Influence of CRELD2 on α4β2 receptor up-regulation

Ortiz *et al.* (Ortiz *et al.* 2005) demonstrated with yeast two-hybrid screening that the ER-localized human protein CRELD2 (Cysteine-rich EGF-like domain protein 2) interacts with both α4 and β2 nAChR subunits. Co-expression of CRELD2 with α4 and β2 subunits in Xenopus oocytes markedly impaired functional expression of α4β2 nAChRs. After noting the negative correlation between nicotine-induced α4β2 receptor up-regulation and CRELD2 expression, we measured surface receptor expression after silencing CRELD2 using siRNA designed to block α and β isoforms (Maslen *et al.* 2006). qPCR indicated an 80% reduction in

Table 2 Effect of antagonists dHβE and Mec on nicotine-induced gene alterations

	mRNA (fold change)							
	CRELD2 (8 h)	IL1B (8 h)	IL6 (8 h)	CREM (24 h)	HERP (8 h)	PDIA6 (8 h)	BiP (8 h)	PDIA4 (8 h)
Control	1.02 ± 0.02	1.03 ± 0.02	1.08 ± 0.02	1.01 ± 0.20	1.00 ± 0.02	1.02 ± 0.02	1.01 ± 0.12	1.01 ± 0.03
Nicotine	1.26 ± 0.08*	0.77 ± 0.03*	0.70 ± 0.06*	1.36 ± 0.06*	1.35 ± 0.04*	1.38 ± 0.11*	0.81 ± 0.09	0.89 ± 0.10
Nic + dHβE	0.71 ± 0.11*	0.88 ± 0.18	0.93 ± 0.11	1.90 ± 0.15*	0.95 ± 0.07	0.89 ± 0.04	0.80 ± 0.06	0.78 ± 0.07
Nic + Mec	0.47 ± 0.05*	1.16 ± 0.20	0.99 ± 0.17	2.40 ± 0.17*	0.90 ± 0.02	0.98 ± 0.07	0.86 ± 0.11	0.70 ± 0.15*

Mec and dHβE, when applied alone, significantly down-regulated CRELD2 (8 h) message both in wild-type and hα4β2 SH-EP1 cells (Fig. 3b), but had no effect on rest of the gene transcripts indicated above.
*Significant at 0.05 level (two-tailed).

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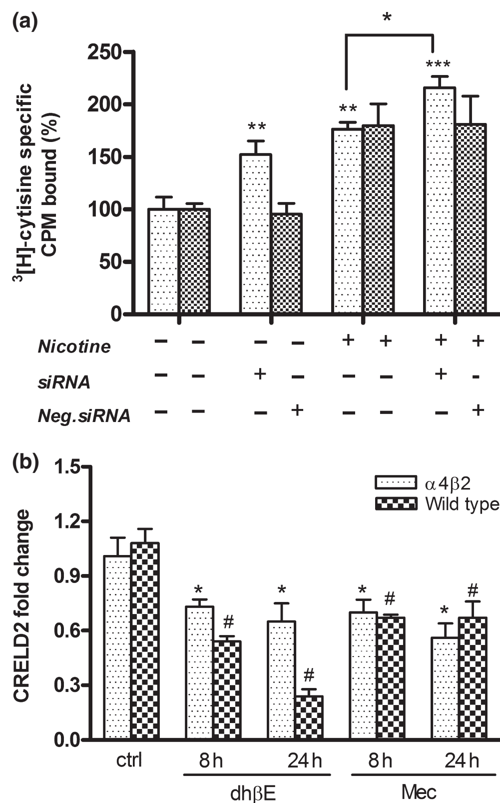

Fig. 3 CRELD2 depletion induces α4β2 up-regulation. (a) hα4β2 SH-EP1 cells in 24-well plates were transfected with 30 nM siRNA, and control wells received transfection agent alone (X-tremeGene). After 48 h, cells were exposed to 10 μM nicotine for 24 h, and surface expressing receptors were determined by ^{3}[H]-cytisine binding. Specific basal binding was approximately 1100 cpm. Silencing CRELD2 significantly ($p < 0.01$) increased the basal levels and additively enhanced nicotine-induced α4β2 up-regulation ($p < 0.05$). Similar treatment with scrambled negative-control siRNA did not affect the surface receptor expression. Data represent mean ± SD of three independent experiments. One-way ANOVA determined significant differences among the groups followed by a Tukey *post-hoc* test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (b) Treatment of stably transfected hα4β2 cells and wild-type SH-EP1 cells with dHβE (100 μM) or Mec (10 μM) for either 8 or 24 h decreased the expression of CRELD2 mRNA as measured by qPCR. α4β2 ($^*p < 0.05$); wild type ($\#p < 0.05$).

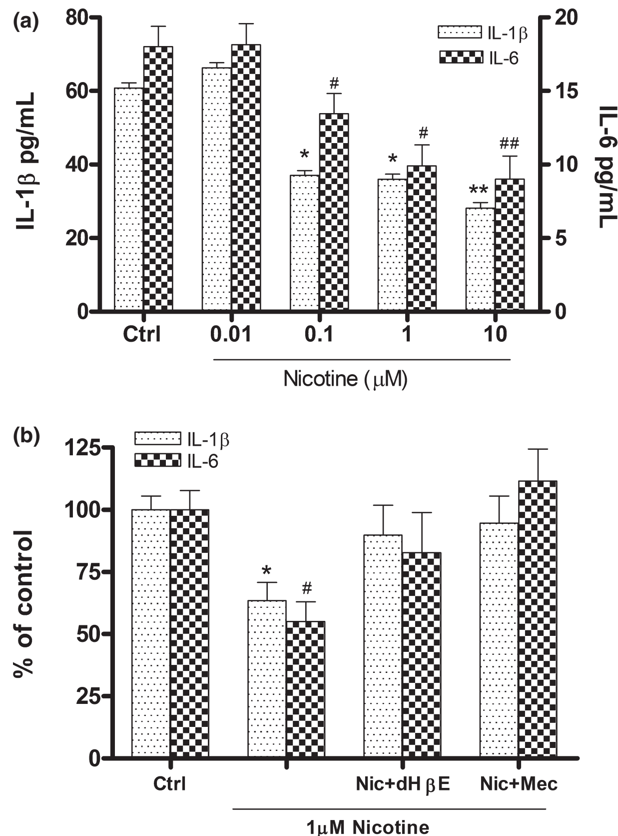

Fig. 4 Antagonists block nicotine-induced suppression of IL-1β and IL-6. Cells seeded onto 6-well dishes were exposed to nicotine or nicotine plus 100 μM dHβE or 10 μM Mec. (a) Protein levels of IL-1β and IL-6 estimated after 48 h exposure to varying concentrations of nicotine, as determined by ELISA. $n = 3$ experiments. (b) Protein levels of IL-1β ($n = 4$) and IL-6 ($n = 3$) significantly dropped after 48 h exposure to 1 μM nicotine but not when treated with 1 μM nicotine plus 100 μM dHβE or 10 μM Mec. Data represent mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by a Tukey *post-hoc* test. IL-1β ($^*p < 0.05$, $^{**}p < 0.01$); IL-6 ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$).

CRELD2 mRNA expression in cells transfected with 30 nM CRELD2 siRNA (Fig. S1). Transfection of hα4β2 SH-EP1 cells with 30 nM siRNA for 48 h significantly increased basal α4β2 receptor expression compared to control cells (Fig. 3a), while transfection with a scrambled negative-control siRNA had no effect (Fig. 3a). Further, application of 10 μM nicotine to siCRELD2-transfected cells for 24 h additively increased specific ^{3}[H]-cytisine binding compared with nicotine-alone treatment (Fig. 3a), while treatment with the scrambled negative-control siRNA had no effect on nicotine-induced up-regulation. These results suggest that CRELD2 negatively regulates α4β2 receptor expression. Finally, incubation of either hα4β2 or wild-type SH-EP1 cells in dHβE (100 μM) or Mec (10 μM) for either 8 or 24 h decreased the expression of CRELD2 mRNA as measured by qPCR (Fig. 3b).

α4β2 nAChRs-mediated down-regulation of PICs

The microarray analysis indicated that chronic exposure to 10 μM nicotine suppresses pro-inflammatory genes. We limited further evaluation to nicotine-induced suppression of the PICs IL-1β and IL-6 as examples of these effects. Addition of 100 μM dHβE or 10 μM Mec for 8 h prevented nicotine-induced suppression of mRNA for IL-1β and IL-6 (Table 2). 48 h exposure to nicotine significantly suppressed IL-1β and IL-6 protein levels, measured by ELISA, in a

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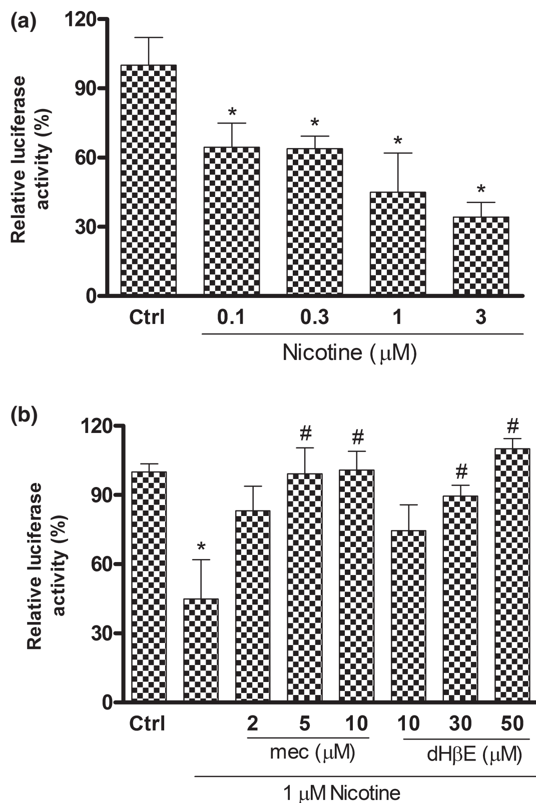

Fig. 5 Nicotine inhibits translocation of NFκB. (a) Human α4β2 SH-EP1 cells were grown to confluence and transfected with pNFκB-MetLuc2-Reporter vector for 10 h and then treated with varying concentrations of nicotine for 4 h. Sample supernatant was collected to estimate luciferase activity. (b) After 10 h of reporter vector transfection, cells were co-incubated with control media, or 1 μM nicotine with or without indicated concentrations of Mec or dHβE before determining luciferase activity. Data represent mean ± SEM of three independent experiments with triplicate samples. ANOVA determined the statistical difference among groups. *$p < 0.05$ compared to control, #$p < 0.05$ compared to 1 μM nicotine in Fig. 5(b).

dose-dependent manner (Fig. 4a), but co-incubation of 1 μM nicotine with dHβE (100 μM) or Mec (10 μM) blocked nicotine-induced suppression (Fig. 4b). Further, application of antagonists alone had no effect on either IL-1β or IL-6 message or protein levels (data not shown).

Nicotine inhibits translocation of NFκB in hα4β2 SH-EP1 cells

Nicotine (10 μM) had no effect on mRNA levels for NFκB (Table 1). Translocation of the transcription factor NFκB from the cytoplasm to the nucleus is an important canonical step in the expression of PIC's such as IL-1β and IL-6 (Perkins 2007). Varying concentrations of nicotine inhibited constitutive nuclear translocation of NFκB as measured using a secreted luciferase reporter vector (Fig. 5a). A 4-h treatment with 3 μM nicotine gave a maximum decrease of approximately a 65% relative luciferase activity, with 100 nM nicotine blocking approximately 35%. Antagonists Mec and dHβE blocked this effect (Fig. 5b), with 5 μM Mec and 50 μM dHβE completely blocking the effects of 1 μM nicotine applied for 4 h.

Discussion

We designed this study to determine whether α4β2 receptor up-regulation can involve gene expression changes other than the receptor subunits genes themselves. The α4β2 up-regulation is a post-transcriptional event, with no significant change in α4 or β2 mRNA (Marks *et al.* 1992), as we confirm here. In general, nicotine effects on gene expression in hα4β2 SH-EP1 cells are subtle to the point of being near the limit of detectable changes using qPCR. This is in keeping with the phenomenon of receptor up-regulation, which seldom involves more than a 2- to 3-fold increase in receptors. However, the observed down-stream effects of nicotine-altered expression in a selected group of these genes are not subtle changes.

Nicotinic receptor activation induces expression of immediate early genes such as c-*fos* and junB in systems with other nicotinic receptor subtypes (Greenberg *et al.* 1986; Pich *et al.* 1997), but not in our assays. We also did not observe the same nicotine-induced gene changes reported in SH-SY5Y cells expressing α3 and α7 receptors. Dunckley and Lukas found that nicotine exposure alters the expression of contactin 1, UBE2C, UBE2S, and Perkin in SH-SY5Y cells (Dunckley and Lukas 2003, 2006) with a heavy emphasis on the ubiquitin proteosomal degradation pathway. Kane *et al.* (2004) also reported changes in the proteosomal-degradation pathway using microarray analysis of nicotine-treated rat brain, but the contributing receptor subtypes in this study are not clear.

CREM and PAX5 were the only altered transcription factors identified by our microarray analysis. However, qPCR did not validate the PAX5 message changes. In contrast, CREM, isoforms of which can be negative modulators of cAMP-response element binding protein (Don and Stelzer 2002), showed a significant but delayed induction at 24 h, suggesting that CREM changes may occur too late to account for α4β2 receptor up-regulation. Nonetheless, recent evidence implicates cAMP-dependent phosphorylation of α4 and β2 subunits in modulating expression of human α4β2 receptors (Pollock *et al.* 2009). This delayed increase in CREM mRNA in response to nicotine suggests a plausible negative feedback on α4β2-mediated signaling or up-regulation involving cAMP-response element binding protein signaling, but this requires further investigation. CREM did show a positive correlation with the increased receptor up-regulation observed when antagonists were added to nicotine (Table 2).

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ER resident proteins

The heteromeric α4β2 nAChRs exist in two stoichiometries: (α4)2(β2)3 and (α4)3(β2)2(34) and the stoichiometry may play a role in receptor up-regulation (Lopez-Hernandez *et al.* 2004). Folding, assembly, and glycosylation of these receptors occurs in the ER, and properly folded receptors are trafficked to the cell surface after passage through the Golgi. Nicotine readily crosses the cell membrane and may act as a pharmacological chaperone by interacting at the interface between α and β subunits, thereby enhancing the stability and maturation of these receptors (Kuryatov *et al.* 2005; Sallette *et al.* 2005). In a different receptor family, recent work suggests that agonist occupancy of the receptor is necessary for α-amino-3-hydroxy-5-methylisoxazole-4-propionate receptor export from the ER (Coleman *et al.* 2009).

Several ER chaperones co-immunoprecipitate with nAChRs suggesting that more than one ER chaperone modulates nAChR maturation (Ortiz *et al.* 2005; Wanamaker and Green 2007). Particularly, ER proteins BiP, CN, ERp57, UBXD4 and Ric-3 have been shown to regulate maturation and expression of nAChRs subtypes in *Xenopus* oocytes, neurons and mammalian cultured cells (Halevi *et al.* 2003; Lansdell *et al.* 2005; Wanamaker and Green 2007; Rezvani *et al.* 2009). BiP plays an important role in the unfolded protein response to ER stress (Schroder and Kaufman 2005), and although it appeared in the microarray results, qPCR indicated no significant change in BiP expression.

An 8-h exposure of hα4β2 SH-EP1 cells to nicotine enhanced expression of several possible ER chaperones (PDIA6, HERPUD1, and CRELD2) detected by the microarray, and qPCR confirmed these changes suggesting that these proteins might contribute to α4β2 up-regulation. qPCR did not confirm microarray predicted changes in PDIA4 mRNA. PDIA6, a different member of the PDI family, has two conserved thioredoxin domains (CXXC) that catalyze the formation, breakdown, and isomerization of disulfide bond between two cysteines (Kemmink *et al.* 1996; Freedman *et al.* 2002). HERPUD1 is an ER-localized protein and consists of an ubiquitin-like domain on the N-terminus, and the promoter region has c/EBP-ATF and ERSE sites that are regulated by both ER stress and cellular stress pathways (Kokame *et al.* 2000). HERPUD1 also promotes endoplasmic reticulum associated degradation by recruiting ubiquilins (Kim *et al.* 2008) that negatively regulate up-regulation of neuronal nAChRs (Ficklin *et al.* 2005). However, treatment with α4β2 antagonists blocked nicotine-induced increases of HERPUD1 and PDIA6 transcripts (Table 2) while simultaneously enhancing receptor up-regulation, suggesting that any connection of these proteins to α4β2 receptor up-regulation may be indirect, but further work is required.

CRELD2 interacts with both α4 and β2 subunits (Ortiz *et al.* 2005) and is an ER-resident protein with multiple splice variants (Maslen *et al.* 2006). Ortiz *et al.* (2005) found CRELD2 with a yeast-two-hybrid assay using the α4 cytoplasmic loop as bait. CRELD2 over-expression decreases functional α4β2 expression in frog oocytes. Our microarray data independently identified CRELD2 as a gene with nicotine-enhanced expression in hα4β2 SH-EP1 cells, and qPCR confirmed this at 8 and 24 h. Together, these data suggest that CRELD2 might play a negative-feedback role in limiting the extent of α4β2 up-regulation. Consistent with this hypothesis, siRNA knockdown of CRELD2 expression caused a significant increase in the basal expression of surface receptors, and this effect was additive with nicotine. However, the antagonists Mec and dHβE in the presence or absence of nicotine significantly decreased CRELD2 mRNA below control levels (even in wild type SH-EP1 cells), suggesting that the antagonists work to suppress CRELD2 expression even in the absence of nicotinic receptors. We propose that this antagonist-induced decrease in CRELD2 mRNA may explain both synergistic effects of nicotine with receptor antagonists (Note the combination of nicotine and antagonists at 8 h compared to either treatment alone in Fig. 2) and α4β2 receptor up-regulation observed with antagonists such as Mec and dHβE in the absence of nicotine or other agonists. One puzzling aspect of nicotine-induced up-regulation is that antagonists such as Mec or dHβE can either induce up-regulation or enhance the effects of nicotine (Peng *et al.* 1994; Pauly *et al.* 1996; Darsow *et al.* 2005), and a non-receptor mediated effect of Mec or dHβE on CRELD2 expression could explain these effects (Note that we find no evidence by PCR of mRNA for α4, β2, or α7 receptor subunits in wild-type SH-EP1 cells, although the presence of other receptor subunits cannot be ruled out).

α4β2 mediated anti-inflammatory effects

Surprisingly, genes associated with inflammatory or immune response pathways comprised approximately half of the observed gene changes (19/41) in our microarray assay. Nicotine is anti-inflammatory, as stimulation of the vagus or nicotine administration suppresses several PICs through activation of α7 nAChRs (Tracey 2002; Wang *et al.* 2003). These anti-inflammatory effects are mediated via inhibition of NFκB and/or activation of janus kinase 2 – signal transducer and activator of transcription 3 signaling (Tracey 2002; de Jonge *et al.* 2005), and this response can be blocked by selective α7 antagonists (Borovikova *et al.* 2000; Tracey 2002; Wang *et al.* 2003). Besides α7, the transcripts for other nAChR subtypes such as α3, α4, α5 and β2 have been detected in different cell types including alveolar macrophages (Matsunaga *et al.* 2001; Dehkordi *et al.* 2008; Gu *et al.* 2008; Osborne-Hereford *et al.* 2008). Thus, an important question is whether α4β2 nAChRs, the major subtype for high affinity binding in the CNS, can also mediate anti-inflammatory effects of nicotine. Matsunaga *et al.* demonstrated that nicotine down-regulates production of PICs in MH-S cells expressing a message for α4 and β2 subunits (Matsunaga *et al.* 2001), and the effects were blocked by

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d-tubocurarine but not α-bungarotoxin, suggesting a possible role of α4β2 in modulating cytokine production. Zhang *et al.* found nicotine-induced alterations in gene expression in vascular endothelium, including changes in NFκB, but the nAChR subtypes involved were not identified (Zhang *et al.* 2001). A very recent paper shows that nicotine blocks cytokine production in mouse macrophages that lack α7 but express α4β2 receptors (van der Zanden *et al.* 2009). We found that nicotine suppresses PICs IL-1β and IL-6 in hα4β2 SH-EP1 cells in a time- and concentration-dependent manner. Further, selective antagonists dHβE and Mec reversed nicotine-induced suppression of PICs. A similar set of experiments in wild-type SH-EP1 cells did not detect any nicotine-induced changes to these PIC genes (data not shown), indicating that α4β2 nAChRs are required for this effect.

Nicotine signaling through α7 receptors regulates tumor necrosis factor alpha and NFκB at a post-transcriptional level (de Jonge *et al.* 2005). Nicotine did not alter expression of either gene measured by qPCR in our system (Table 1), but nicotine-mediated suppression of PICs through α4β2 receptors caused NFκB activation as shown with the secreted luciferase reporter assay (Fig. 5). Moreover, α4β2 nAChR antagonists reversed nicotine-induced suppression of PICs and NFκB transactivation, suggesting that receptor activation and signaling is required to inhibit activation of the NFκB pathway and in turn attenuate cytokine synthesis. Interestingly, the only cytokine tested that is enhanced by nicotine is IL-11, which acts to block NFκB translocation (Schwertschlag *et al.* 1999). We are presently investigating the signaling pathways downstream of α4β2 activation involved in these effects.

Nicotine-mediated neuroprotection

Postmortem analyses report elevated levels of PICs and chemokines in the pathogenesis of neurodegenerative disorders. Consistent epidemiological data suggest that chronic smokers are at lower risk for neurodegenerative diseases such as Parkinson's or Alzheimers in which neuroinflammation is a prominent feature (Quik 2004; Zhang and Kaufman 2008). In addition, schizophrenic smokers have lower IL-2 and IL-6 levels compared with non-smokers (Zhang *et al.* 2008).

Strong evidence suggests that nicotine attenuates production of several inflammatory cytokines (Tracey 2002) and improves survival in sepsis via α7 receptors (Wang *et al.* 2004). Animal studies demonstrate that nicotine attenuates immune responses for several weeks after exposure (Kalra *et al.* 2004). Our findings demonstrate that continuous exposure to nicotine suppresses the expression of PICs and chemokines (Table 1) via α4β2 receptors in hα4β2 SH-EP1 cells. The concentration of nicotine achieved in a smoker's brain is about 50–300 nM (Cao and Peng 1998; Paradiso and Steinbach 2003), which is sufficient to activate α4β2 receptors. Therefore, the putative neuroprotection seen in smokers may be due in part to an increase in high affinity α4β2 receptors and suppression of PICs via α4β2 nAChRs. Studies are underway to determine whether nicotine alters inflammatory cytokine expression in brains of wild-type, heterozygote and α4 knockout mice.

In summary, microarray analysis shows that chronic nicotine subtly alters gene expression of cytokines, chemokines, transcription factors, possible ER chaperones and cytosolic proteins in hα4β2 SH-EP1 cells. Induction of ER chaperones and cytosolic proteins after initial increase in ^{3}[H]-cytisine binding suggests a plausible negative feedback on α4β2 up-regulation triggered by nicotine, although further investigation is required to elucidate distinct functions of individual genes contributing to α4β2 receptor expression. By use of correlation analysis and RNA interference we provide early evidence that ER proteins such as CRELD2 can regulate α4β2 receptor expression. An unexpected finding of the present study is NFκB-mediated down-regulation of PICs through nicotinic stimulation of α4β2 nAChRs.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Supplementary Materials and methods.

Appendix S2. References for Supplementary Materials and methods.

Figure S1. Transfection with varying concentrations siRNA targeting CRELD2 for 48 h achieved maximum silencing at 30 nM siRNA, with no greater silencing at 100 nM, as quantified by qPCR.

Figure S2. Dose-response of nicotine-induced up-regulation of α4β2 nicotinic receptors.

Figure S3. Sulforhodamine B cytotoxicity assay using the method of Vichai and Kirtikara, (2006).

Figure S4. Radioligand displacement curves.

Table S1. qPCR primers.

Table S2. ER chaperones, transcription factors, cytosolic and other unrelated gene transcripts altered by 10 μM nicotine in hα4β2 SH-EP1 cells in microarrays compared with untreated controls [Categorized by Pathway Architect™ (Stratagene)].

Table S3. Inflammation and immune response gene transcripts altered by 10 μM nicotine in hα4β2 SH-EP1 cells in microarrays compared with untreated controls [Categorized by Pathway Architect™ (Stratagene)].

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