

# Tumor Necrosis Factor $\alpha$ Enhances Nicotinic Receptor Up-regulation via a p38<sup>MAPK</sup>-dependent Pathway\*

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A response by key neuronal nicotinic acetylcholine receptors (nAChRs) to sustained nicotine exposure is up-regulation. Although this unusual receptor characteristic contributes to processes ranging from aging to addiction, the normal physiological reason for this response is unknown. We find that up-regulation of [<sup>3</sup>H]epibatidine binding and function in HEK293 cells stably expressing  $\alpha 4\beta 2$ -nAChR is significantly enhanced by co-application of the proinflammatory cytokine, tumor necrosis factor  $\alpha$ . The mechanism of tumor necrosis factor  $\alpha$ -enhanced up-regulation requires transcription, new protein synthesis, and signaling through p38<sup>MAPK</sup> as demonstrated by complete inhibition using SB 202190. This finding extends the possibilities for nAChR-inflammatory interactions in normal physiological processes and offers novel insights into endogenous mechanisms that can modify up-regulation.

Neuronal nicotinic acetylcholine receptors (nAChRs)<sup>3</sup> play an important role in modulating normal neurotransmission in the central nervous system. These receptors also have a direct impact upon behavioral and physiological pathologies ranging from addiction to their early and selective loss in Alzheimer disease (1). Addiction to nicotine correlates with the curious trait of up-regulation in response to chronic exposure to receptor ligands (2–4). The majority of high affinity nicotine receptors that undergo up-regulation in the mammalian brain are composed of at least nAChR  $\alpha 4$  and  $\beta 2$  subunits as demonstrated by high affinity ligand binding and genetic studies (1, 5–7). Although multiple mechanisms contribute to up-regulation (1, 8, 9), the normal physiological reason for this response is poorly understood despite its being conserved in animals ranging from *Caenorhabditis* to mammals. Since most of these organisms have never been exposed to nicotine either acutely or at any time throughout their evolutionary history, up-regu-

lation probably reflects a normal physiological response preserved by processes of natural selection. It is also an intrinsic property of nAChRs composed of  $\alpha 4 + \beta 2$  subunits (termed  $\alpha 4\beta 2$ -nAChR; see Refs. 10 and 11 for additional nomenclature). Even when expressed in heterologous systems, such as human embryonic kidney cells, this receptor responds to nicotine with pharmacokinetics that are almost identical to those of the mammalian forebrain (1, 12).

Possible reasons for endogenous up-regulation of nAChR may involve the recent recognition of the participation by nicotinic receptors in regulating proinflammatory processes. In this context,  $\alpha 7$ -nAChR has received the greatest attention since its antagonism of the proinflammatory cytokine, TNF $\alpha$ , was documented over a decade ago (13). Subsequent investigations have supported the physiological relevance of this interaction in regulating numerous proinflammatory processes (14). However, a more complex interaction between nAChRs and inflammation is suggested by studies using both tissue culture (13, 15, 16) and animal models (14, 17). For example, proinflammatory cytokines can impact upon nAChR expression through promoting efficient receptor assembly and altering relative subunit composition of mature nAChRs when HEK293 (293) cells are co-transfected with cDNA encoding  $\alpha 4$  and  $\beta 2$  and/or  $\beta 4$ , respectively (16). In these earlier studies, we did note a small but persistent increase of [<sup>3</sup>H]epibatidine ([<sup>3</sup>H]Eb) binding in 293 cells transiently expressing  $\alpha 4\beta 2$ -nAChR that were also treated with TNF $\alpha$  (16). This has been examined further with 293 cells stably expressing nAChRs. Our findings show that the proinflammatory cytokine, TNF $\alpha$ , dramatically enhances  $\alpha 4\beta 2$ -nAChR up-regulation induced by nAChR ligands, including nicotine, cytisine, and carbachol. The pathway to enhancement of up-regulation is both actinomycin D- and cycloheximide-sensitive, and it is inhibited by SB 202190, a highly specific inhibitor of p38<sup>MAPK</sup>. These findings offer a novel insight into how proinflammatory cytokines can impact upon mechanisms of up-regulation and strongly suggest that reciprocal regulatory interactions between the nAChR and inflammatory systems are likely.

## EXPERIMENTAL PROCEDURES

**Cell Lines and Culture Conditions**—The 293 cell lines stably co-transfected with nAChR subunits  $\alpha 4 + \beta 2$ ,  $\alpha 4 + \beta 4$ ,  $\alpha 3 + \beta 2$ , or  $\alpha 3 + \beta 4$ , respectively, were generously provided by Drs. Ken Kellar and Yingxian Xiao (Department of Pharmacology, Georgetown University). These cells were maintained as described (12, 18, 19). Additional analysis of these cells by real time PCR showed expression of RNA encoding the TNFR sub-

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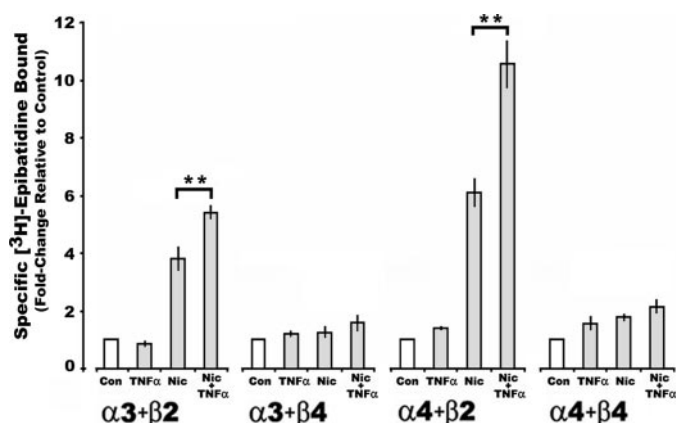
<sup>3</sup> The abbreviations used are: nAChR, neuronal nicotinic acetylcholine receptor; 293, HEK293; Eb, epibatidine; TNF $\alpha$ , tumor necrosis factor  $\alpha$ ; TNFR, tumor necrosis factor receptor; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase.

## TNF $\alpha$ Enhances Nicotinic Receptor Up-regulation

type 1 (cycle thresholds of 22) and the less abundant TNFR type 2 (cycle threshold of 35) compared with  $\beta$ -actin (cycle threshold of 17; not shown). Application of TNF $\alpha$  (human recombinant TNF $\alpha$ ; BioSource) to these cells (25 ng/ml for 2–4 h) revealed that RNA encoding the inducible form of cyclooxygenase, COX2, was elevated 3-fold, demonstrating that TNF $\alpha$  stimulates cellular responses in these cells (not shown). The same assays revealed no evidence for the expression of the acetylcholine-synthesizing enzyme, choline acetyltransferase, or other human nAChR subunits (not shown).

**Radioligand Binding**—The binding of [ $^3$ H]Eb to cell membrane preparations was done essentially as described (12, 16), with the following modifications. Cells were distributed into 100-mm culture dishes and treated (e.g. with nicotine) 48 h later. Cells were harvested 18–24 h after treatment, at which time the cultures were 50–75% confluent, into 50 mM Tris buffer (pH 7.4, 4 °C), pelleted, resuspended, and homogenized. Cellular debris and nuclei were removed by low speed centrifugation (100  $\times$  g; 5 min), and the supernatant was collected and centrifuged (20,000  $\times$  g for 10 min) to pellet remaining membranes for ligand-binding assays. For binding assays, 5  $\mu$ g of membrane was incubated with 5 nM [ $^3$ H]Eb for 2–4 h at room temperature ( $\sim$ 25 °C). Nonspecific binding was assessed by adding 500  $\mu$ M nicotine hydrogen tartrate (Sigma) for 30 min before the addition of [ $^3$ H]Eb to block specific binding. Samples were tested in triplicate. Bound ligand was separated from free ligand by vacuum filtration through Whatman GF/C filters and prepared for scintillation counting. Specific binding was defined by averaging the total binding minus the nonspecific (nicotine-blocked) binding. Data were analyzed using Prism 3 (GraphPad Software Inc., San Diego, CA) as described (12, 18, 19).

**Calcium Imaging**—Fura-2/AM calcium imaging was done essentially as before (20). Cells were grown on glass coverslips coated with poly-L-lysine and laminin and washed four times with Hanks' solution (with 1.3 mM CaCl<sub>2</sub> (Invitrogen)) over 20 min, and fresh buffer was added containing 5  $\mu$ g/ml freshly prepared Fura-2/AM (Molecular Probes) in Me<sub>2</sub>SO (Sigma). After a 30-min incubation, the cells on coverslips were again washed, and fresh Hanks' buffer was added before testing in a Zeiss Axiovert microscope equipped with the Attotfluor calcium ratio system. Upon calibrating resting calcium-bound Fura-2 (340 nm) *versus* nonbound Fura-2 (380 nm) ratios, 1  $\mu$ M nicotine was rapidly applied directly onto the cells using a fixed pipette or a picospritzer. The cell response (change in the 340/380 nm ratio) was then recorded. Nicotine was then added a second time, and the measurement was repeated before adding 1 mM acetylcholine to activate muscarinic receptors as a positive control for cell responsiveness ( $\sim$ 98% in each test group). Recordings were prepared from multiple coverslips in independent platings, which were scored for responsiveness. A responsive cell was defined as having a change in the peak intensity of the 340/380 nm ratio of 0.01 units from resting upon nicotine application and that failed or exhibited a very poor response to the second application (desensitization). Primary data were analyzed using Excel.



**FIGURE 1. The influence of nicotine and/or TNF $\alpha$  on [ $^3$ H]Eb binding site density in 293 cells stably expressing nAChRs of different subunit composition.** Cells were grown in the presence of 1  $\mu$ M nicotine hydrogen tartrate, TNF $\alpha$  (25 ng/ml), or both nicotine (Nic) and TNF $\alpha$  for 18–24 h before measuring [ $^3$ H]Eb binding (5 nM for 2–4 h at room temperature) to membrane preparations. Results are expressed as the -fold change in specific [ $^3$ H]Eb binding (average of three samples after subtracting a parallel sample blocked with 500  $\mu$ M nicotine) from the control (Con) (vehicle added), which is 1.0 in all experiments. The values shown are the mean -fold difference  $\pm$  S.E. from 3–12 independent measurements. Exposure to nicotine increased [ $^3$ H]Eb binding at all nAChR subtypes but to differing degrees, as reported (19). The dramatic enhancement of ligand binding is observed in the  $\alpha 4\beta 2$ -nAChR-expressing cells treated with nicotine + TNF $\alpha$  (almost 2-fold) and to a lesser, but still highly significant extent, for  $\alpha 3 + \beta 2$  ( $\sim$ 1.5-fold; \*\*,  $p < 0.001$ ). A lesser increase in enhanced binding relative to the nicotine-treated cells was observed for the  $\alpha 4\beta 4$ -nAChR combination. TNF $\alpha$  also increased binding sites for  $\alpha 4\beta 2$ -nAChR (small but significant change at  $p < 0.05$  and  $\alpha 4\beta 4$ -nAChR ( $p < 0.01$ )). Cells expressing  $\alpha 3\beta 4$ -nAChR failed to exhibit a significant TNF $\alpha$  enhancement of [ $^3$ H]Eb binding sites.

## RESULTS

The subunit composition of nAChRs determines their pharmacological and functional response (1). Therefore, we first examined how nicotine, TNF $\alpha$ , and the combination of these agents impacts upon  $\alpha 4\beta 2$ -nAChR,  $\alpha 4\beta 4$ -nAChR,  $\alpha 3\beta 2$ -nAChR, or  $\alpha 3\beta 4$ -nAChR, respectively, stably expressed by 293 cells. For these experiments, cells were treated for 18–24 h with nicotine (1  $\mu$ M), TNF $\alpha$  (25 ng/ml), or both agents, and specific binding of [ $^3$ H]Eb to 5  $\mu$ g of membrane protein was measured (see “Experimental Procedures”). Nicotine-induced up-regulation of [ $^3$ H]Eb binding to  $\alpha 3 + \beta 2$  and  $\alpha 4 + \beta 2$  equaled 4–6-fold over controls,  $\sim$ 2-fold for  $\alpha 4\beta 4$ -nAChR, and no significant change for  $\alpha 3 + \beta 4$  (Fig. 1), as previously reported (1, 12). Although not as robust as nicotine, TNF $\alpha$  induced a small but significant increase in [ $^3$ H]Eb binding by cells expressing  $\alpha 4\beta 2$ -nAChR and  $\alpha 4\beta 4$ -nAChR but not  $\alpha 3\beta 2$ -nAChR or  $\alpha 3\beta 4$ -nAChR. The most dramatic effect on ligand binding was in  $\alpha 4\beta 2$ -nAChR-expressing cells simultaneously treated with TNF $\alpha$  and nicotine. In these cells, the increase in [ $^3$ H]Eb sites exceeded 10-fold that of the control and often exceeded nicotine-treated cells by 2-fold. A smaller effect by TNF $\alpha$  on nicotine up-regulation was observed for  $\alpha 3\beta 2$ -nAChR receptors and to a lesser but significant extent  $\alpha 4\beta 4$ -nAChR. Again, cells expressing  $\alpha 3\beta 4$ -nAChR exhibited only a slight trend toward enhanced ligand binding relative to controls that was not statistically significant.

We focused the subsequent experiments toward examining the dramatic enhancement by TNF $\alpha$  on nicotine up-regulation of  $\alpha 4\beta 2$ -nAChR. To begin, dose-response assays for up-regu-

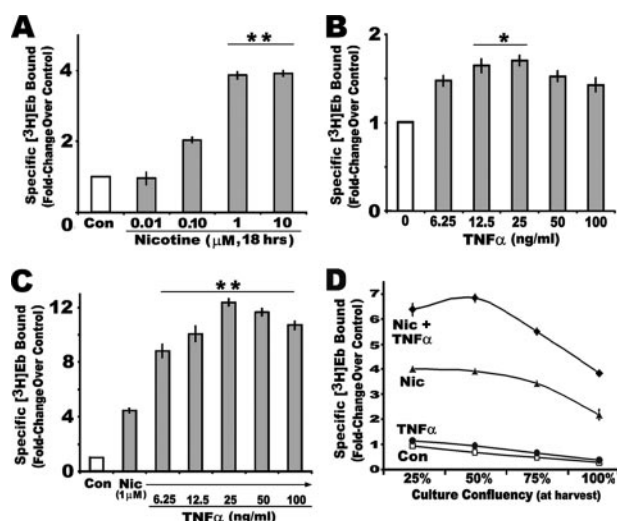


FIGURE 2. Optimal conditions for TNF $\alpha$  enhancement of nicotine-induced up-regulation of [ $^3$ H]E $\beta$  binding sites. *A*, a dose-response assay for  $\alpha 4\beta 2$ -nAChR up-regulation of [ $^3$ H]E $\beta$  binding sites at the concentrations indicated. For the 18-h period tested, 1  $\mu$ M saturated binding up-regulation did not differ statistically from 10  $\mu$ M. *B*, a dose-response assay of the effect of TNF $\alpha$  on  $\alpha 4\beta 2$ -nAChR shows that the optimal concentration inducing the small but significant up-regulation of [ $^3$ H]E $\beta$  sites was 12.5–25 ng/ml, with the 25 ng/ml amount providing the greatest consistency among all experiments. *C*, the dose response of TNF $\alpha$  when nicotine is a constant at 1  $\mu$ M. Enhancement of up-regulation [ $^3$ H]E $\beta$  sites was optimal at 1  $\mu$ M nicotine plus 25 ng/ml TNF $\alpha$ . *D*, cell density influences the magnitude of TNF $\alpha$  enhancement of up-regulation. Cells were plated to reach the confluence indicated at harvest. Basically, ligand-mediated up-regulation is not statistically different from the control at increasing confluence. However, the magnitude of TNF $\alpha$  enhancement of up-regulation decreases from 1.8-fold (50% confluence) to an average of  $\sim 1.4$ -fold as densities exceed 50% confluence. Student's *t* test was used; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ . Nic, nicotine; Con, control.

lation of [ $^3$ H]E $\beta$  binding in response to 18–24 h of exposure to nicotine, TNF $\alpha$ , or nicotine plus TNF $\alpha$  were performed. Up-regulation was observed in cells treated with as little as 100 nM nicotine ( $\sim 2$ -fold) and saturated at 1  $\mu$ M nicotine (Fig. 2*A*). TNF $\alpha$  (Fig. 2*B*) induced weak up-regulation at all doses tested (optimal dose, 12.5–25 ng/ml). The results of experiments where nicotine was held constant at 1  $\mu$ M and the concentration of TNF $\alpha$  varied are shown in Fig. 2*C*. The optimal enhancement of up-regulation (12-fold relative to 4-fold for nicotine alone in this experiment) occurred at 1  $\mu$ M nicotine and 25 ng/ml TNF $\alpha$ , which was also true in experiments where constant TNF $\alpha$  (25 ng/ml) and varied nicotine (10 nM to 10  $\mu$ M) were done (not shown). The order of addition (TNF $\alpha$  versus nicotine), including in experiments where they were added 24 h in advance of the other, had no significant influence on the results (not shown). Therefore, 1  $\mu$ M nicotine and 25 ng/ml TNF $\alpha$  were used in subsequent experiments unless otherwise noted.

An important variable in tissue culture experiments is cell culture density. This is particularly true when variables such as cell cycle, nutrients, pH, and cell-cell contact (that may also affect accessibility by ligands to receptors) influence the experimental results. The influence of cell density on TNF $\alpha$ -enhancement of nicotine-mediated up-regulation was examined in cultures that were plated to reach confluence of 100, 75, 50, and 25%, respectively, following a total of 36 h in culture and 24 h after treatment with nicotine, TNF $\alpha$ , or both agents as above. The results show (Fig. 2*D*) that cell cultures at  $\sim 50\%$  confluence display the greatest enhancement by TNF $\alpha$  of up-

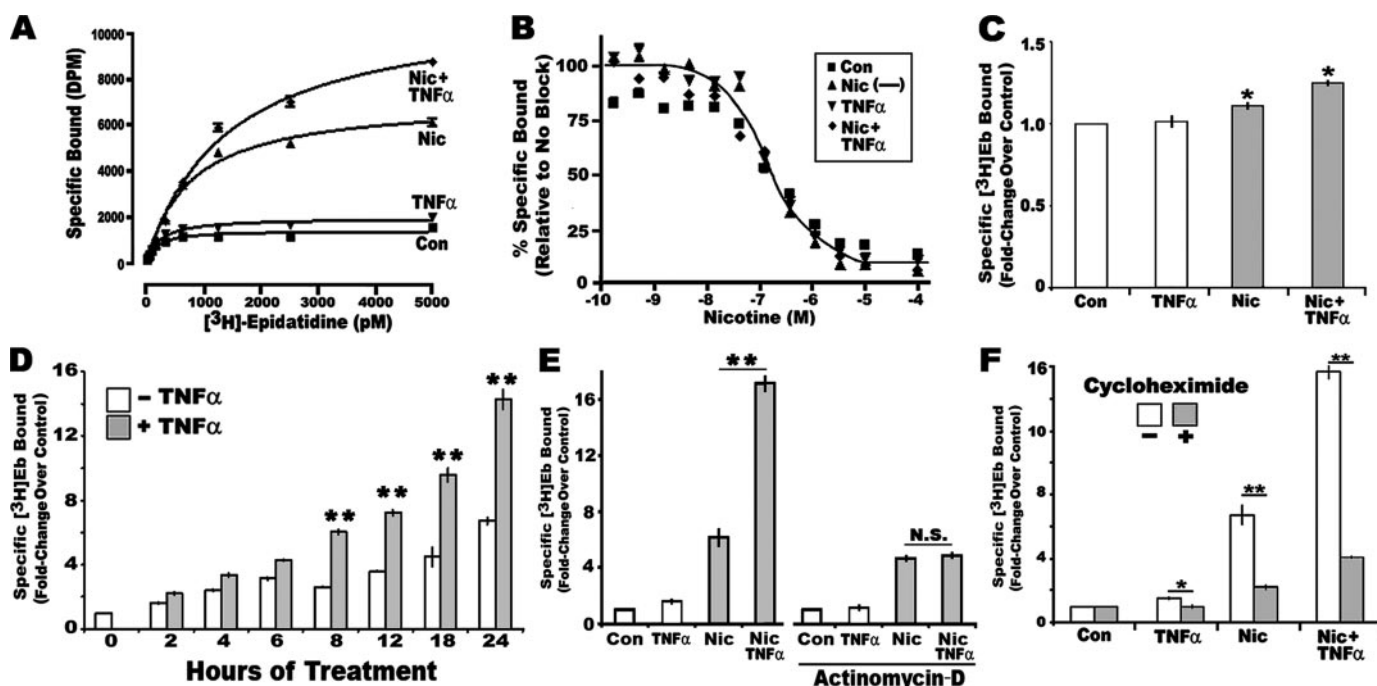
regulation (1.8-fold). There was, however, a decline in the amount of TNF $\alpha$  enhancement of up-regulation with increasing cell density ( $\sim 1.4$ -fold). This result indicates that cell culture conditions influence the ability of TNF $\alpha$  to enhance nicotine-mediated up-regulation. This finding is consistent with cell density influencing nAChR expression by transfected cultured cells. For all experiments reported, culture density was  $\sim 50\%$  confluence at the time of harvest.

**TNF $\alpha$  Enhancement of Nicotine-mediated Up-regulation Requires New RNA Transcription and Protein Synthesis**—The mechanism of nicotine plus TNF $\alpha$ -enhanced up-regulation was examined next. To begin, saturation binding of [ $^3$ H]E $\beta$  over a concentration range of 1–5000 pM for each treatment group was measured (Fig. 3*A*). For control cells, the [ $^3$ H]E $\beta$ -specific binding was  $537 \pm 140$  fmol/mg, and the saturable best fit was to a single high affinity site (control mean  $K_d$  of  $53 \pm 14$  pM) as calculated using Prism 3.0 software (12). Nicotine competition curves averaged from three independent experiments (Fig. 3*B*; nicotine binding competition against 500 pM [ $^3$ H]E $\beta$ ) show that at  $10^{-4}$  M to  $\sim 5 \times 10^{-5}$  M, nicotine competed for  $>85\%$  of the specific [ $^3$ H]E $\beta$  binding. There was no significant difference in the affinity of this compound measured in these experiments (nicotine  $IC_{50}$  of  $12 \pm 4$  nM; see Ref. 12) among the different treatment groups. Collectively, these data support a model where combining nicotine and TNF $\alpha$  up-regulation by co-exposure is related to changes in receptor number.

How TNF $\alpha$  enhances nicotine-mediated up-regulation of  $\alpha 4\beta 2$ -nAChR [ $^3$ H]E $\beta$  binding sites was examined further. Although previous reports show ligand-mediated up-regulation of nAChRs to be independent of transcription (1), how TNF $\alpha$  enhancement of up-regulation occurs is unknown. The first measurement determined if intact cells were required for TNF $\alpha$  to enhance nicotine-mediated up-regulation. For this experiment, a crude membrane fraction of untreated cells was prepared as described for binding assays. Equal aliquots of these preparations were treated with saline, 1  $\mu$ M nicotine, 25 ng TNF $\alpha$ , or both nicotine and TNF $\alpha$  and incubated at 37  $^{\circ}$ C for 24 h with gentle rocking. Membranes were then collected by centrifugation and washed, and radioligand-binding measurements were performed. The results (Fig. 3*C*) show that incubation of membranes with TNF $\alpha$  alone had no effect on [ $^3$ H]E $\beta$  binding, although a small but significant increase was measured in nicotine-treated samples and those with nicotine plus TNF $\alpha$ . This persistent increase is consistent with reports of up-regulation through mechanisms that influence receptor affinity for ligand (9). Nevertheless, the changes in ligand binding were well below the 4–6-fold change expected from whole cell preparations, and no TNF $\alpha$  enhancement of this up-regulation effect was present, indicating that TNF $\alpha$  acts on up-regulation through mechanisms requiring intact cells.

The time course of TNF $\alpha$ -enhanced up-regulation was examined to determine when TNF $\alpha$ -enhanced up-regulation begins. These experiments were done as above, only cells were harvested at various times after the addition of nicotine and/or TNF $\alpha$ . As shown in Fig. 3*D*, a small enhancement by TNF $\alpha$  of nicotine up-regulation was observed within 2 h of treatment; however, significant enhancement of up-regulation was not observed until  $\sim 8$  h post-treatment. This result indicates that



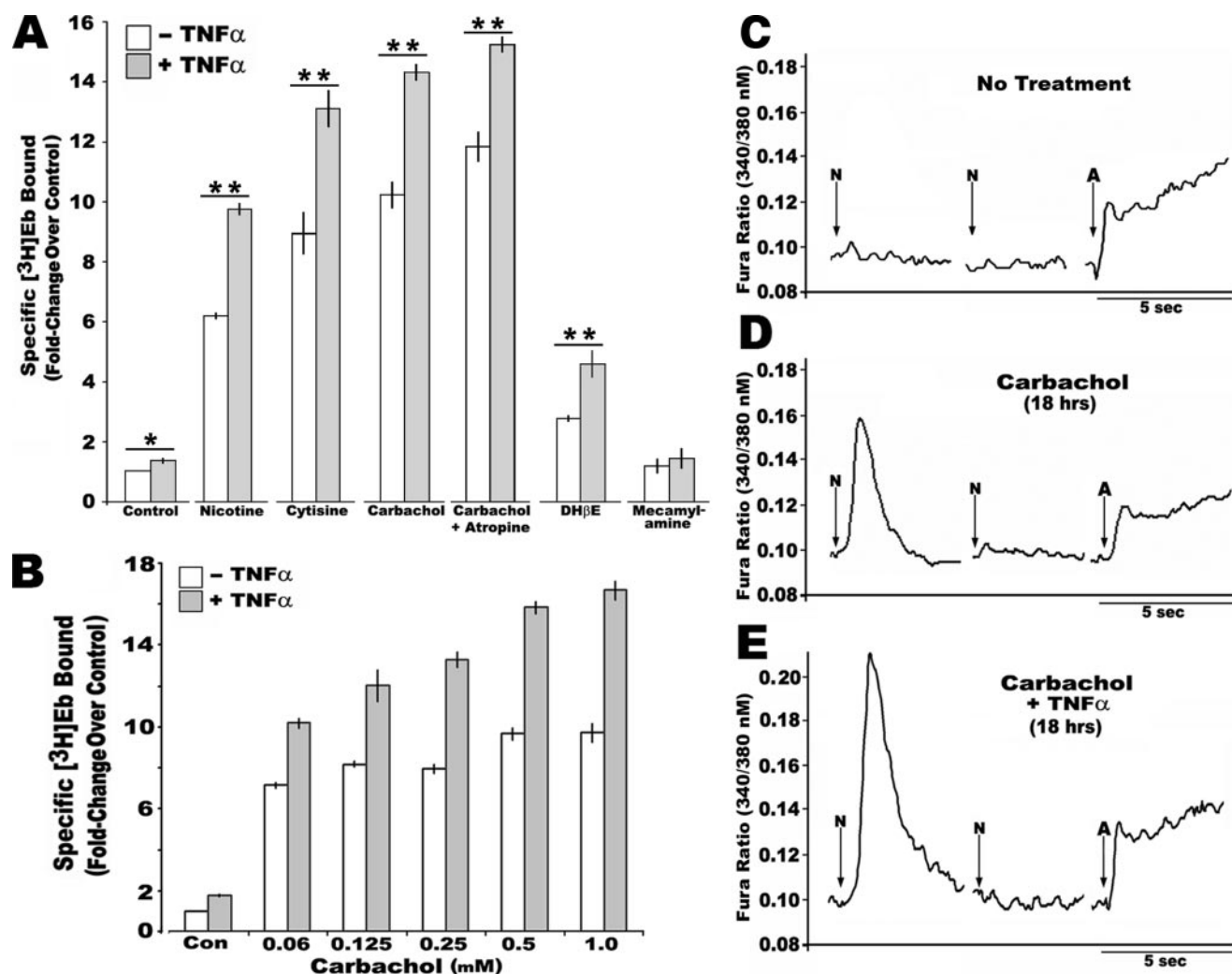


**FIGURE 3. TNF $\alpha$  enhancement of up-regulation is dependent upon increased receptor number and is sensitive to inhibitors of transcription and translation.** A, saturation binding of [<sup>3</sup>H]Epb to membrane homogenates (5 μg) prepared from α4β2-nAChR-expressing cells treated for 24 h with saline (control), 1 μM nicotine (Nic), TNF $\alpha$  (25 ng/ml), or both nicotine and TNF $\alpha$ . Saturation binding assays were carried out as described under "Experimental Procedures" for a [<sup>3</sup>H]Epb concentration range of 1–5000 pM. Binding data were analyzed by nonlinear least square regressions using Prism 3 software and the one-site saturation binding model. The data shown are from a single representative experiment of three that gave essentially equivalent results. B, ligand binding competition profiles for nicotine on membrane homogenates from cells expressing α4β2-nAChRs as in A. Competition binding assays used 500 pM [<sup>3</sup>H]Epb. The data were analyzed by the nonlinear least square regression method using Prism 3 software as reported elsewhere (12, 19), and they reflect results from one experiment that is typical. Error bars were omitted for clarity. A single curve fit (for nicotine-treated cells) is shown, but curve fits among different treatment groups were essentially identical when all experiments ( $n = 3$ ) were averaged. C, the results of [<sup>3</sup>H]Epb binding to membrane homogenates prepared from control α4β2-nAChR cells, which were then treated for 24 h with saline (control), 1 μM nicotine, TNF $\alpha$  (25 ng/ml), or both nicotine and TNF $\alpha$ . The results are shown as the ratio of specific ligand binding to the control, which is 1.0. D, cells expressing α4β2-nAChRs were treated with 1 μM nicotine (open bars) or 1 μM nicotine plus 25 ng/ml TNF $\alpha$  (gray bars) for the times indicated. The fold change in [<sup>3</sup>H]Epb binding site density over controls is shown. Highly significant ( $p < 0.01$ ) TNF $\alpha$  enhancement of up-regulation is apparent by 8 h postincubation. E, the effect of 10 μM actinomycin D on TNF $\alpha$  enhancement of [<sup>3</sup>H]Epb site up-regulation by nicotine. All values are normalized to the relevant control set (saline or saline plus actinomycin D-only treatments). F, the effect of 10 μM cycloheximide on inhibiting the up-regulation of [<sup>3</sup>H]Epb sites. All values are normalized to the control (saline, saline plus cycloheximide-only treatments). Both actinomycin D and cycloheximide inhibit TNF $\alpha$ -enhanced up-regulation without decreasing nicotine-mediated up-regulation relative to control samples. Error bars,  $\pm$ S.E.; Student's  $t$  test; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ . N.S., not significant.

the impact of TNF $\alpha$  on up-regulation is not likely to be through modifications of preexisting protein pools. To test this, we next examined the influence of the transcriptional inhibitor, actinomycin D, on TNF $\alpha$ -enhanced up-regulation. In these experiments, [<sup>3</sup>H]Epb binding of membranes isolated from cells treated with nicotine, TNF $\alpha$ , nicotine plus TNF $\alpha$ , or saline for 18 h, either in the presence or absence of 10 μM actinomycin D (a dose tolerated by the cells; not shown), was measured. Upon normalizing the results to the control cells as in Fig. 3E, it was found that consistent with the previous reports noted above, there was no effect by transcription inhibition by actinomycin D on the up-regulation of [<sup>3</sup>H]Epb binding sites treated with nicotine relative to control values. However, TNF $\alpha$  enhancement of the nicotine-induced up-regulation process was abolished. In a similar experiment, inhibition of new protein synthesis with 10 μM cycloheximide was done as described above for the actinomycin D experiments. The results of these experiments, shown in Fig. 3F, demonstrate that cycloheximide abolishes essentially all up-regulation as well as TNF $\alpha$  enhancement of up-regulation relative to control cells. Notably, a small amount of up-regulation in cells treated with nicotine did persist, similar to that seen in membrane incubation experiments

(Fig. 3C). However, we did not explicitly rule out the possibility that some residual protein synthesis could have occurred. What is important is the substantial reduction of up-regulation and enhancement by TNF $\alpha$  (>80% relative to controls). Together with the results of actinomycin D studies and the timing of the onset of enhanced up-regulation, these findings argue that the mechanism(s) of TNF $\alpha$  enhancement of [<sup>3</sup>H]Epb binding sites requires both new transcription and protein synthesis.

**Up-regulation Produced by Ligands Other than Nicotine Is Enhanced by TNF $\alpha$** —Most agonists that bind nAChRs (e.g. nicotine) and some antagonists (e.g. dihydro- $\beta$ -erythroidine) produce up-regulation (21). In this context, to examine the specificity of TNF $\alpha$  enhancement of up-regulation, the change in [<sup>3</sup>H]Epb binding sites by α4β2-nAChR-expressing cells in the presence or absence of TNF $\alpha$  for 24 h and either 1 mM carbachol, 10 μM dihydro- $\beta$ -erythroidine, 10 μM cytosine (a competitive partial agonist), or mecamylamine a noncompetitive antagonist not generally reported to produce up-regulation). All agents, except mecamylamine, consistently produce significant up-regulation of α4β2-nAChR [<sup>3</sup>H]Epb binding (Fig. 4). TNF $\alpha$  enhanced up-regulation induced by these agents proportionately to that seen with nicotine except for mecamylamine,



**FIGURE 4. TNF $\alpha$  enhances up-regulation by ligands other than nicotine.** *A*, cells were grown in the presence of the drug indicated for 18 h with and without TNF $\alpha$  (25 ng/ml) before measuring [ $^3$ H]Etb binding sites. Results are expressed as the mean  $\pm$  S.E. relative to the saline control (1.0) for at least four independent measurements. Exposure to these drugs, except mecamylamine (a noncompetitive antagonist) produced up-regulation of [ $^3$ H]Etb binding sites that was enhanced by TNF $\alpha$  (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; Student's  $t$  test). *B*, a response curve for carbachol in the presence of a fixed amount of TNF $\alpha$  (25 ng). Up-regulation and enhancement is observed at 60  $\mu$ M and saturates at 500  $\mu$ M carbachol. All treatments in *A* and *B*, except mecamylamine, produced significant up-regulation relative to controls ( $p < 0.01$ ). *C*, calcium imaging reveals that TNF $\alpha$  enhancement of carbachol up-regulation corresponds with an increased functional response to nicotine. For these experiments, cells grown on coverslips under the conditions indicated were loaded with Fura-2/AM (see "Experimental Procedures") and subjected to two repeated rapid applications of 1  $\mu$ M nicotine (*N*) followed by 1 mM acetylcholine to ensure that a cell response by this method (muscarinic activation) could be measured. Control cells show a very small (if any) response to nicotine. *D*, after carbachol (*Carb*) treatment (24 h), a robust response to nicotine application is observed in  $\sim 60\%$  of the cells tested. This response is desensitized, as indicated by the small or absent response to the second nicotine application. *E*, in cells treated with carbachol and TNF $\alpha$  (*Carb* + TNF $\alpha$ ), the magnitude of the response is consistently greater (1.6–2-fold) than in carbachol-treated cells. As in carbachol-treated cells,  $\sim 60\%$  of the cells respond.

where again no significant effect was observed. The greatest up-regulation was observed with carbachol, although enhancement of this effect by TNF $\alpha$  was proportional to that seen with other receptor ligands. Because carbachol is also an agonist of muscarinic receptors, the same experiment (Fig. 4*A*) was done in the presence of atropine (which by itself had no effect on [ $^3$ H]Etb binding; not shown). Atropine had no measurable effect on carbachol up-regulation of [ $^3$ H]Etb binding or TNF $\alpha$  enhancement. Also, because the concentration of carbachol used was relatively high, a dose-response assay was done (Fig. 4*B*). At concentrations to 60  $\mu$ M, carbachol produced significant  $\alpha 4\beta 2$ -nAChR up-regulation of [ $^3$ H]Etb, and TNF $\alpha$  (25 ng) enhanced this up-regulation. As reported (8), the up-regulation of  $\alpha 4\beta 2$ -nAChR by the competitive antagonist dihydro- $\beta$ -erythroidine was approximately one-third that of nicotine, yet

TNF $\alpha$  also enhanced [ $^3$ H]Etb binding proportionately. Collectively, these results indicate that in all cases tested where ligand binding by the nAChR initiates the up-regulation mechanism, proportional enhancement by TNF $\alpha$  occurs.

Carbachol-mediated up-regulation is particularly useful, since this method permits conditions for Fura-2 calcium imaging measurements. This is because nicotine up-regulation results in receptors that are also deeply desensitized, and the extensive amounts of washing required to restore function are often impractical. In contrast, nAChR function is restored in carbachol-treated cells within 20–30 min of washing (not shown). Following 18–24 h of 500  $\mu$ M carbachol, rapid application of 1  $\mu$ M nicotine produces an easily measurable change in the Fura-2 340/380 ratio in  $\sim 60\%$  of the cells (193 of 315 cells for the experiment shown; Fig. 4*D*), which is in contrast to con-

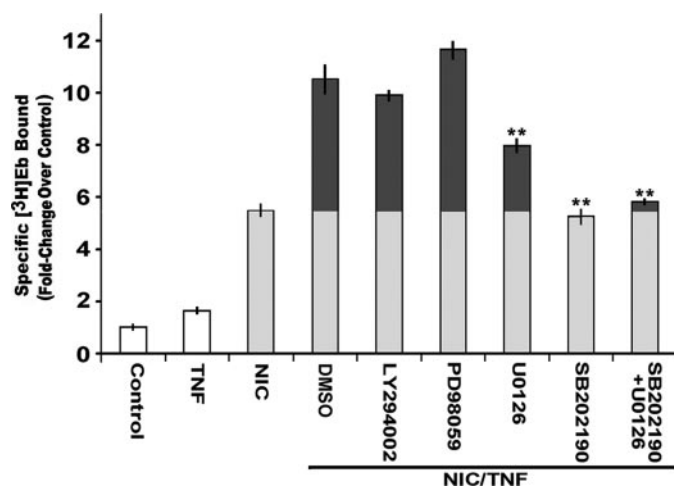


FIGURE 5. Enhancement of nicotine-induced up-regulation by TNF $\alpha$  (blackened portion of bar) is blocked by inhibitors of the mitogen-activated protein kinase, p38. Inhibitors of PI3K (LY294002; 100  $\mu$ M) and MEK1 activation (PD98059; 30  $\mu$ M) had no effect on TNF $\alpha$  enhancement of up-regulation (or on nicotine up-regulation alone (not shown)). In contrast, the MEK1 and MEK2 inhibitor, U0126, consistently interfered with enhanced up-regulation by ~50%. The most potent inhibitor of TNF $\alpha$ -enhanced up-regulation was SB 202190, a selective inhibitor of p38<sup>MAPK</sup>. Combining U0126 with SB 202190 had no additional effect. Student's *t* test was used; \*, *p* < 0.05; \*\*, *p* < 0.01. NIC, nicotine.

control cells that are rarely able to produce a convincing response (Fig. 4C). In cells treated with carbachol + TNF $\alpha$ , the peak response measured was on average 30% greater than the response measured (Fig. 4E) in carbachol only-treated cells, although there was only a small increase in the percentage of cells responding that was not seen in all experiments (236 of 350 cells). This result indicates that TNF $\alpha$ , in addition to increasing the number of [<sup>3</sup>H]Etb sites, also increases the number of functional receptors (or their duration of opening) but does not necessarily alter the number of cells able to produce a measurable functional response.

**Mechanism of TNF $\alpha$  Enhancement of  $\alpha$ 4 $\beta$ 2-nAChR Up-regulation**—TNF $\alpha$  signaling through its receptors impacts on numerous intracellular pathways (22, 23). Of interest to us has been the activation of p38 mitogen-activated protein kinase (p38<sup>MAPK</sup>) pathways. We have examined the possible contribution of this intracellular pathway to enhancement of up-regulation using various inhibitors, including those affecting phosphatidylinositol 3-kinase/protein kinase B, extracellular signal-regulated kinase, and p38<sup>MAPK</sup>. For these experiments, all treatments with saline, nicotine, TNF $\alpha$ , or both agents were done as described above. Me<sub>2</sub>SO, which is used as the carrier for inhibitor stock solutions, has no effect on ligand binding. Representative results for these inhibitors on the TNF $\alpha$  enhancement of nicotine-mediated up-regulation are shown in Fig. 5. Two inhibitors affected the TNF $\alpha$  enhancement of nicotine-induced up-regulation. The MEK1/2 inhibitor, U0126, inhibits ~50% of TNF $\alpha$ -enhanced up-regulation, but the MEK1-preferring inhibitor (24), PD98059, has no effect, suggesting that signaling through at least the MEK2 pathway is important. However, effectively all enhanced up-regulation was blocked by the p38<sup>MAPK</sup> inhibitor, SB 202190 (Fig. 5). None of these agents increased or inhibited nicotine-induced up-regulation (not shown).

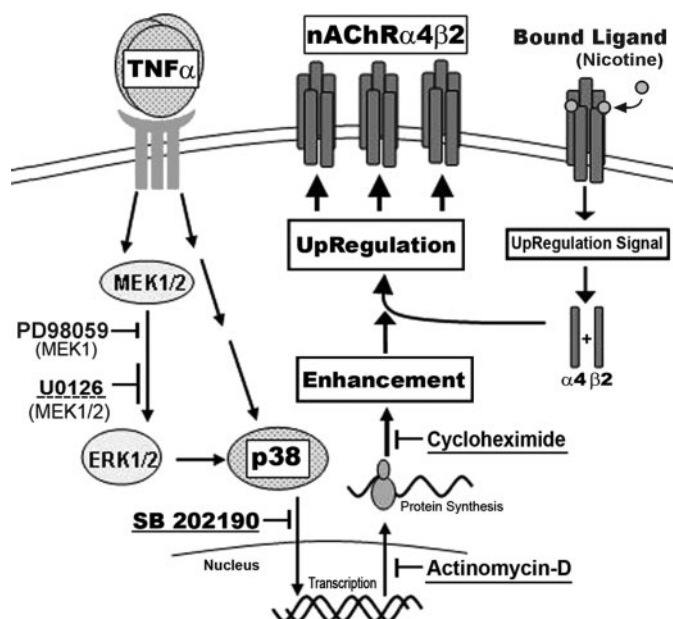


FIGURE 6. Proposed model for TNF $\alpha$  enhancement of ligand-mediated  $\alpha$ 4 $\beta$ 2-nAChR up-regulation. Up-regulation of  $\alpha$ 4 $\beta$ 2-nAChR begins when nicotine (or other ligands) bind the receptor and initiates signals leading to increased receptor numbers. TNF $\alpha$  signaling through p38<sup>MAPK</sup> (p38) and the MEK1/2 MAPK pathways enhance this up-regulation mechanism. In this scheme, at least two pathways lead from TNF $\alpha$  to p38 activation. The first is through activation of p38 via the MEK1/2 and extracellular signal-regulated kinase 1/2 cascades and is partially sensitive (~50%) to inhibition of both MEK1/2 by U0126 (broken underline) but not affected by the MEK1-preferring inhibitor, PD98059. In contrast, SB 202190, a highly specific inhibitor of p38, completely blocked (as marked by solid underlines) TNF $\alpha$  enhancement, revealing the participation by a second pathway to activate p38. In addition to blocking p38, inhibition of transcription (actinomycin D), and protein synthesis (cycloheximide) indicate that new gene transcription and translation are required to promote enhanced up-regulation of  $\alpha$ 4 $\beta$ 2-nAChRs.

## DISCUSSION

Nicotine has long been associated with its ability to up-regulate the expression of  $\alpha$ 4 $\beta$ 2-nAChRs, and this correlates with phenotypes related to addiction (1). More recently, this compound has also been found to directly impact upon inflammatory and immunologic responses (25). In a reciprocal manner, inflammatory mediators can affect the expression of nicotinic receptors (16). In this report, using stably transfected cell lines, we find that TNF $\alpha$  strongly enhances  $\alpha$ 4 $\beta$ 2-nAChR up-regulation, independent of other inflammatory conditions or nAChR subtypes, through an actinomycin D and cycloheximide-sensitive p38<sup>MAPK</sup> intracellular signaling pathway. A diagram reflecting these pathways is shown in Fig. 6. The p38<sup>MAPK</sup> cascade has a central role in regulating many immunologic and inflammatory responses as well as physiological functions related to cell growth, cell differentiation, and apoptosis. Partial inhibition of TNF $\alpha$ -enhanced up-regulation of  $\alpha$ 4 $\beta$ 2-nAChR by the MEK1/2 inhibitor U0126, but not the MEK1-preferring PD98059 (24), implies participation of an additional pathway that leads to p38<sup>MAPK</sup> activation. Collectively, the delay in the onset of TNF $\alpha$ -mediated enhancement of up-regulation, the selective diminishment of this effect by both transcription and translation inhibitors, and the general role of p38<sup>MAPK</sup> in activating stress-related genes combine to suggest that TNF $\alpha$ -mediated enhancement of up-regulation occurs through induction of a protein that favors nicotinic receptor expression by a



post-translational mechanism(s). Particularly attractive candidates are chaperone-like proteins, since these would promote more efficient folding, assembly, and/or transport from the endoplasmic reticulum to the cell surface. Also, the impact of these signals appears to be based upon subunit composition. In particular, enhancement of ligand-mediated up-regulation is most notable in receptors harboring the  $\beta 2$  subunit. However, we observed a small but significant and direct influence by TNF $\alpha$  on promoting increased ligand-binding sites for  $\alpha 4\beta 2$ -nAChR and  $\alpha 4\beta 4$ -nAChR but not for  $\alpha 3$ -containing receptors. This indicates that the  $\alpha 4$  subunit is also likely to harbor structures that are targeted directly by the TNF $\alpha$  enhancement up-regulation signal.

This study and others (13–16) also indicate that an interaction between inflammation and various nAChR subtypes occurs through multiple mechanisms. For example,  $\alpha 7$ -nAChR has been demonstrated to reduce the release of TNF $\alpha$  (14), which would dampen inflammation (and inflammatory cytokines), and indirectly regulate the amount of  $\alpha 4\beta 2$ -nAChR up-regulation. However, the relationship between  $\alpha 7$ -nAChR, TNF $\alpha$ , and enhanced up-regulation of  $\alpha 4\beta 2$ -nAChR may not be straightforward, since TNF $\alpha$  can originate from multiple sources that may or may not be regulated by  $\alpha 7$ -nAChR.

A final point is to speculate regarding the translational relevance of this observation in the animal. One place where this is possible is related to the observation that when individuals are ill, they often experience a diminished desire to continue nicotine administration. In fact this has been used as an indicator of nicotine dependence (26). Because illness is often associated with elevated TNF $\alpha$ , in the presence of chronic nicotine administration, enhanced up-regulation might actually exceed the comfort level and possibly even become toxic. A second point involves changes in nicotinic cholinergic receptor expression and inflammatory dysfunction that is characteristic of aging animals. There is a dramatic and selective loss of  $\alpha 4\beta 2$ -nAChR expression in the aged brain of both rodents (27–30) and humans (especially those with Alzheimer's disease; e.g. see Refs. 31 and 32). Coincident with the onset of old age and pathology is also an unregulated increase in TNF $\alpha$  and other proinflammatory cytokines. At present, the correlation between the loss of  $\alpha 4\beta 2$ -nAChR expression and onset (or progression) of pathology is unknown. However, the possibility of a mechanistic interaction contributing to this coincidental dysregulation in both systems is intriguing. These results and other possible interactions between inflammatory cytokines and nAChR expression extend our understanding of the physiologic relevance of up-regulation as a normal contributor to local and conditional regulation of nAChR function.

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**Tumor Necrosis Factor  $\alpha$  Enhances Nicotinic Receptor Up-regulation via a p38  
MAPK-dependent Pathway**

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