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ORIGINAL RESEARCH COMMUNICATION

Stimulation of Alpha7 Nicotinic Acetylcholine Receptor by Nicotine Attenuates Inflammatory Response in Macrophages and Improves Survival in Experimental Model of Sepsis Through Heme Oxygenase-1 Induction

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Abstract

Activation of nicotinic acetylcholine receptor alpha7 subunit (α7nAChR) by nicotine leads to the improved survival rate in experimental model of sepsis. Previously, we demonstrated that heme oxygenase *(HO)-1* inducers or carbon monoxide significantly increased survival of lipopolysaccharide (LPS)-induced and cecal ligation and puncture-induced septic mice by reduction of high mobility group box 1 release, a late mediator of sepsis. However, that activation of α7nAChR by nicotine provides anti-inflammatory action through *HO-1* upregulation has not been elucidated. Here we show that *HO-1*-inducible effect by nicotine was mediated through sequential event—Ca^{2+} influx, classical protein kinase C activation, and reactive oxygen species production—which activates phosphoinositol-3-kinase/Akt/Nrf-2 pathway. In addition, *HO-1* is required for nicotine-mediated suppression of tumor necrosis factor-α, inducible nitric oxide synthase, and high mobility group box 1 expression induced by LPS in macrophages, as evidenced by the fact that nicotine failed to inhibit production of these mediators when *HO-1* was suppressed. Importantly, nicotine-induced survival rate was reduced by inhibition of *HO-1* in LPS- and cecal ligation and puncture-treated septic mice. Collectively, these data suggest that activation of α7nAChR by nicotine is critical in the regulation of anti-inflammatory process, which could be mediated through *HO-1* expression. Thus, we conclude that activation of α7nAChR by nicotine provides anti-inflammatory action through *HO-1* upregulation. *Antioxid. Redox Signal.* 14, 2057–2070.

Introduction

Sepsis, the systemic inflammatory response syndrome that occurs during severe infection, kills more than 210,000 people in the United States annually (1). Pathogenesis of sepsis is commonly explained by the overwhelming pro-inflammatory response that subsequently leads to the multiple organ failure and death (1). However, these days, this classical view of sepsis is questioned, partially due to failure of pro-inflammatory-targeted therapeutics to improve survival in clinical trials (15). Recent studies demonstrated that immunosupression, which goes after immune activation, is involved in the immunopathology of sepsis probably by viral reactivation in septic patients (27, 29). In this point of view, anti-inflammatory mediators may play a detrimental role in sepsis (25). Thus, pathobiology of sepsis is much more complex as it has been considered before, and it seems that the identification of balance between pro- and anti-inflammatory responses will help to control appropriate immune response during sepsis (15).

Recently, it has been suggested that high mobility group box 1 (HMGB1) protein is the late mediator of lethal systemic inflammation and sepsis (47). Originally described as an intracellular DNA-binding protein, HMGB1 is released into extracellular milieu by activated macrophages. After binding to DNA or bacterially derived materials, extracellular HMGB1 develops the ability to induce cytokines (28). Therefore, it is suggested that neutralization of HMGB1 release can be perspective intervention window in the treatment of sepsis.

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In 1997, Romanovsky and colleagues demonstrated that hypothermic response to lipopolysaccharide (LPS) is exaggerated in vagotomized animals, thus implying the role of cholinergic nervous system in endotoxic shock (36). Later, the importance of vagus nerve, with great attention to nicotinic acetylcholine receptor (nAChR) activation, in the regulation of inflammatory response in sepsis has been described (5, 48, 49). This concept, the so-called cholinergic anti-inflammatory pathway, has been increasingly recognized that innate immune response can be under neuronal control (32). Acetylcholine or nicotine dose-dependently decreased the production of proinflammatory mediators, including HMGB1 in macrophages stimulated with endotoxin (48, 49). Although activation of janus kinase 2 (Jak2)-signal transducer and activator of transcription 3 (STAT-3) signaling pathway by nicotine on peritoneal macrophages showed anti-inflammatory action *in vitro* and *in vivo* (8), the molecular mechanism by which it provides anti-inflammatory response has been poorly investigated. Recently, we demonstrated that heme oxygenase (HO)-1 inducers or carbon monoxide (CO) significantly increased survival of LPS-induced and cecal ligation and puncture (CLP)-induced septic mice by reduction of HMGB1 release, a late mediator of sepsis (45). Thus, here we asked whether activation of nicotinic acetylcholine receptor can upregulate anti-inflammatory genes such as HO-1 and thus provide anti-inflammatory properties. We found that nicotine, through activation of α7nAChR, significantly increased HO-1 protein level in RAW 264.7 cells, which depended on dosage and incubation time. HO-1-inducible effect was mediated by series of events: activation of α7nAChR by nicotine increases Ca^{2+} influx that activates classical protein kinase C (cPKC); the activated PKC produces reactive oxygen species (ROS) *via* NADPH oxidase; the produced ROS activates phosphoinositol-3-kinase (PI3K)/Akt/Nrf-2, which results in HO-1 induction in macrophages.

Materials and Methods

Materials

Anti-HMGB1 was purchased from Abcam, anti-inducible nitric oxide synthase (iNOS) from Transduction Laboratories, and anti-*β*-actin and anti-HO-1 from Santa Cruz Biotechnology. Enhanced chemiluminescence Western blotting detection reagent was from Amersham. SB203580, SP600125, PD98059, LY294001, wortmannin, and Gö6976 were obtained from Calbiochem. All other chemicals, including LPS (*Escherechia coli* 0111:B4), ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid, 1,2-Bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetrakis(acetoxymethyl ester), and A23187, were purchased from Sigma-Aldrich.

Cell culture

RAW 264.7 cells were obtained from the American Type Culture Collection (ATTC). The cells were grown in RPMI-1640 medium supplemented with 25 m*M* *N*-(2-hydroxyethyl)piperazine-*N*-2-ethanesulfonic acid, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum.

Cell viability

Cell viability was determined colorimetrically using the methylthiazol tetrazolium assay. Cells at the exponential

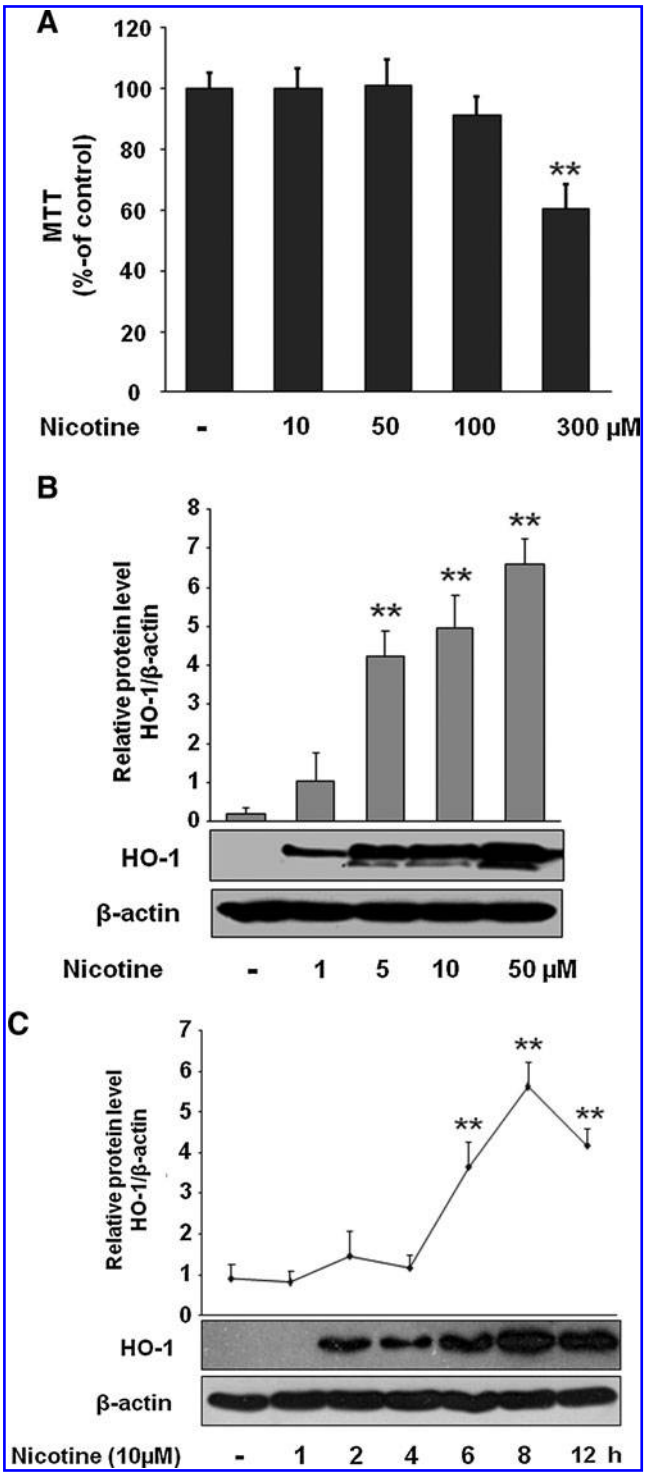


FIG. 1. Effect of nicotine on the expression of HO-1 in macrophages. (A) Cytotoxicity of nicotine was determined by methylthiazol tetrazolium assay. **(B)** Cells were treated with nicotine (1, 5, 10, and 50 μM) for 8 h, or with nicotine (10 μM) in a time-dependent fashion (1, 2, 4, 6, 8, and 12 h). **(C)** After incubation, cells were harvested and subjected to Western blotting. Data are presented as mean ± SD of three independent experiments. Significance compared to control, $^{**}p < 0.01$. HO-1, heme oxygenase-1.

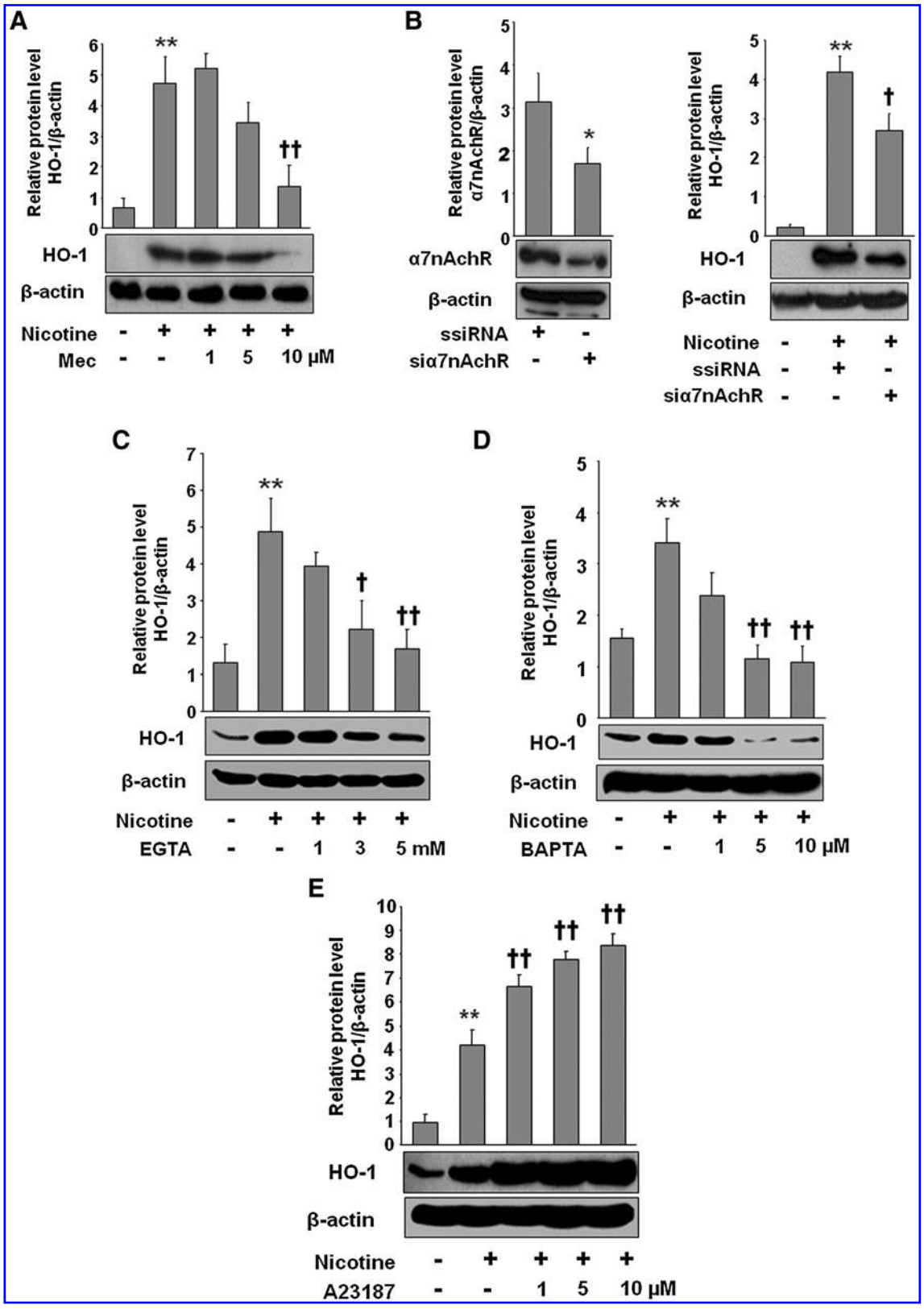
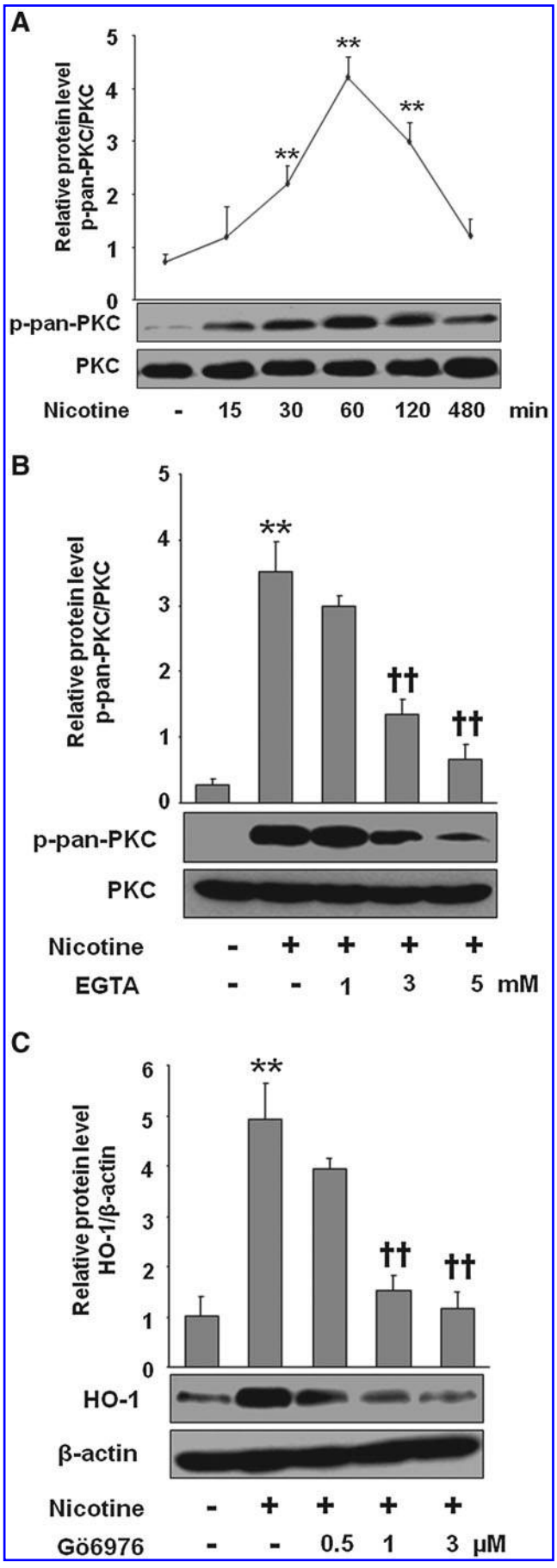


FIG. 2. Nicotine induces HO-1 expression by activation of 7α nicotinic acetylcholine receptor activation and Ca^{2+} influx in macrophages. (A) Cells were pretreated with mecamylamine or **(B)** transfected with siα7nAchR, and then cells were treated with nicotine (10 μM), and HO-1 was detected by Western blot. Cells were treated with calcium chelators such as **(C)** EGTA and **(D)** BAPTA or **(E)** calcium ionophore A23187, and then nicotine (10 μM) was applied for 8 h. After incubation, cells were harvested and subjected to Western blotting. Data are presented as mean ± SD of three independent experiments. Significance compared to control, $^{**}p < 0.01$; significance compared with nicotine or nicotine + ssiRNA alone, $^{\dagger\dagger}p < 0.01$. α7nAchR, alpha 7 nicotinic acetylcholine receptor; BAPTA, 1,2-Bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester); EGTA, ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Mec, mecamylamine.



phase were seeded at 1×10^4 cells/well in 24-well plates. After different treatments, 20 μl of 5 mg/ml methylthiazol tetrazolium solution was added to each well (0.1 mg/well) and incubated for 4 h, the supernatants were aspirated and the formazan crystals in each well were dissolved in 200 μl dimethyl sulfoxide for 30 min at 37°C, and optical density at 570 nm was read on a Microplate Reader (Bio-Rad).

Measurement of intracellular ROS

Production of intracellular peroxides was monitored spectrofluorometrically using dichlorofluorescein diacetate (DCFH-DA) as a fluorescent dye. Cells were trypsinized 12 h after serum deprivation, and the cell suspension was treated with DCFH-DA at a final concentration of 10 μM in the medium. Oxidation of DCFH by peroxides yields dichlorofluorescein. Fluorescence was monitored at the excitation and emission wavelengths of 485 and 530 nm, respectively, using a fluorescence plate reader (50 cycles per 20 s at 37°C) (Tecan®; Tecan US, Inc.). Data were expressed as the relative changes to the initial fluorescence.

NO assay

Nitric oxide was measured as its stable oxidative metabolite, nitrite (NOx), as described by Kang *et al.* (19). At the end of incubation, 100 μl of the culture medium was mixed with the same volume of Griess solution (0.1% naphthylethylenediamine and 1% sulfanilamide in 5% phosphoric acid). Light absorbance was measured at 550 nm, and the nitrite concentration was determined using a curve calibrated on sodium nitrite standards.

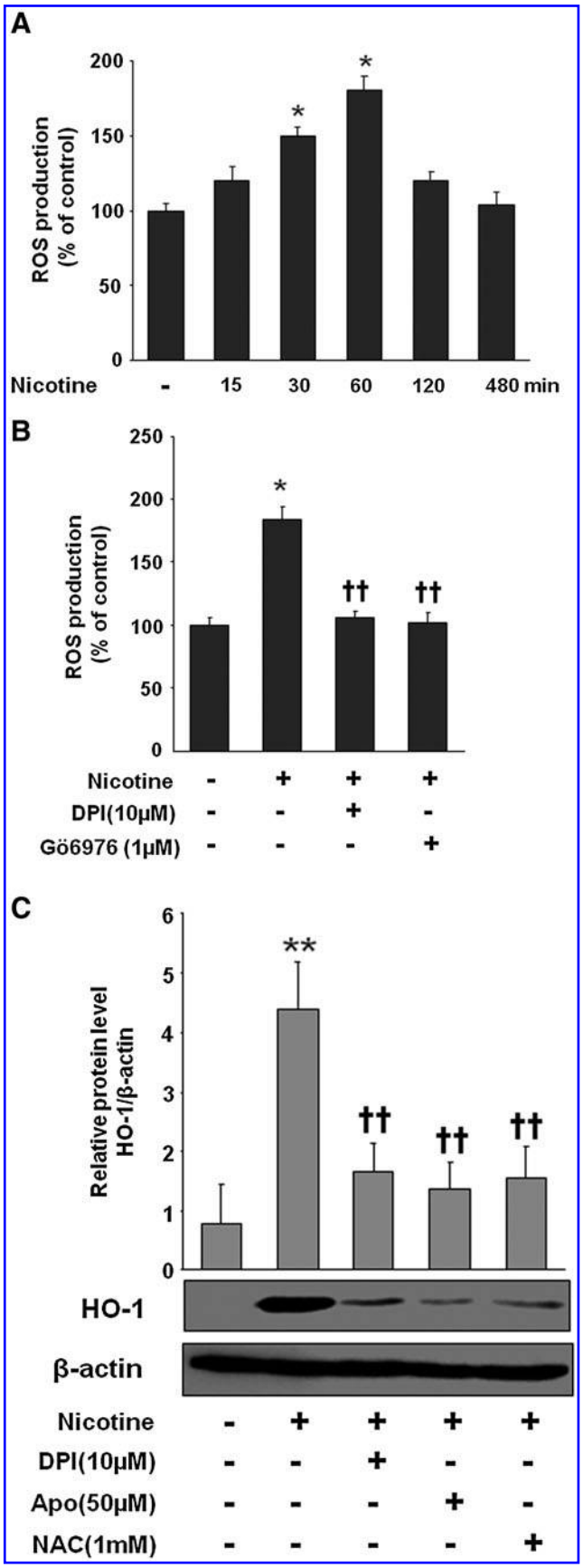
Enzyme-linked immunosorbent assay

The levels of tumor necrosis factor (TNF)-α in the culture medium were determined using commercial available enzyme-linked immunosorbent assay kits from R&D Systems according to the provided manuals.

PI3 kinase assay

RAW264.7 cells at 60%–80% confluence from six-well plates were washed in ice-cold PBS and lysed in 500 ml ice-cold lysis buffer (137 m*M* NaCl, 20 m*M* Tris-HCl [pH 7.4], 1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 0.1 m*M* sodium orthovanadate, 1% NP-40, and 1 m*M* PMSF). PI3 kinase was immunoprecipitated with 5 ml of rabbit antibody against full-length PI3 kinase (which coprecipitates the p110 catalytic subunit of PI3 kinase) and 60 ml of Protein A–Sepharose beads Santa Cruz

FIG. 3. HO-1-inducible effect by nicotine is mediated through classical PKC activation in macrophages. (A) Cells were treated with nicotine (10 μM) in a time-dependent manner (15, 30, 60, 120 and 180 min), and then cells were extracted and subjected to Western blot. Cells were treated with nicotine (10 μM) in the presence or absence of **(B)** EGTA (1, 3, and 5 m*M*) or **(C)** Gö6976 (0.5, 1, and 3 μM) for 8 h, and then cells were harvested for Western blot to detect p-pan-PKC and HO-1, respectively. Data are presented as mean ± SD of three independent experiments. Significance compared to control, $^{**}p < 0.01$; significance compared with nicotine alone, $^{\dagger\dagger}p < 0.01$. PKC, protein kinase C.



Biotechnology. PI3 kinase activity in the immunoprecipitates was analyzed with PI3 kinase enzyme-linked immunosorbent assay kits (from Echelon Biosciences) according to the manufacturer's instructions. Briefly, immunoprecipitated enzyme and PI(4,5)P_2 substrate were incubated for 1 h at room temperature in the reaction buffer. Kinase reaction was stopped by pelleting the beads by centrifugation and transferring the reaction mixture to the incubation plate and incubated overnight at 4°C with a PI(3,4,5)P_3 detector protein, and then added to the PI(3,4,5)P_3-coated microplate for 1 h for competitive binding. A peroxidase-linked secondary detection reagent and colorimetric detection (absorbance was measured at 450 nm) is used to detect PI(3,4,5)P_3 detector protein binding to the plate.

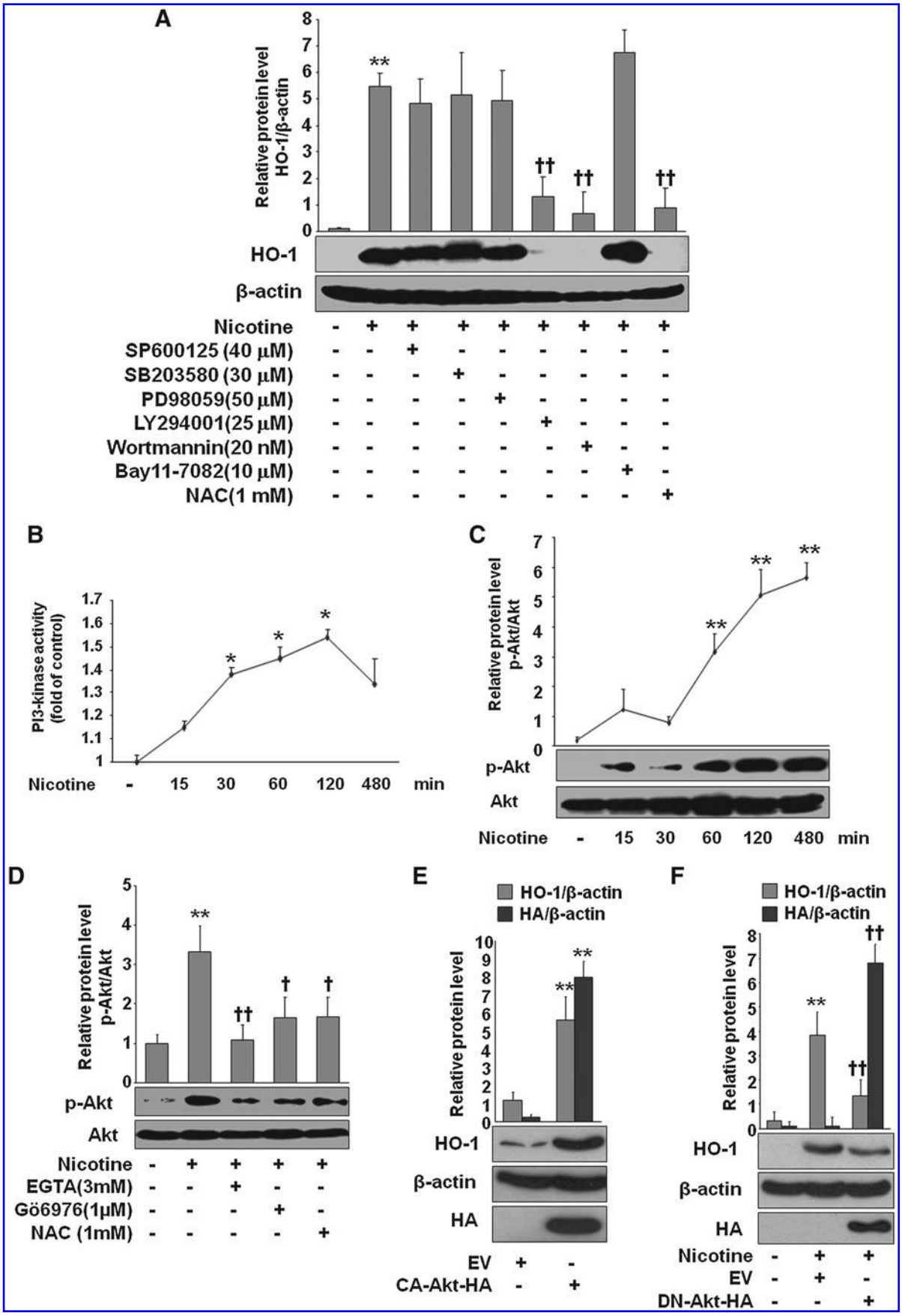
HMGB1 analysis

Culture medium or blood samples were briefly centrifuged. Same volumes of samples were then concentrated 40-fold with Amicon Ultra-4-10000 NMWL (Millipore). Centrifugation conditions were fixed angle (35 degree) and 7500 *g* for 20 min at 4°C. The concentrated samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis electrophoresis.

Western blot

Whole-cell lysate was performed using buffer containing 0.5% SDS, 1% Nonidet P-40, 1% sodium deoxycholate, 150 m*M* NaCl, 50 m*M* Tris-Cl (pH 7.5), and protease inhibitors. Concentrated supernatants, to detect HMGB1, and whole-cell lysates, to detect iNOS, *β*-actin, and HO-1, were subjected to electrophoresis in different-percentage polyacrylamide gels depending on the size of interested protein. The gels were transferred to polyvinylidene difluoride membranes by semidry electrophoretic transfer at 15 V for 60–75 min. The membranes were stained with Ponceau S solution (2 μg/ml) for 5 min to determine efficiency of transfer or/and protein loading levels per track. Then, the polyvinylidene difluoride membranes were blocked overnight at 4°C in 5% bovine serum albumin. The cells were incubated with primary antibodies diluted 1:500 in Tris/buffered saline/Tween 20 containing 5% bovine serum albumin for overnight in 4°C and then incubated with secondary antibody at room temperature for 1 h. The signals were detected by enhanced chemiluminescence.

FIG. 4. Nicotine increases ROS production through NADPH oxidase in macrophages. (A) Cells were incubated with nicotine (10 μM) for 15, 30, 60, 120, and 480 min. After incubation, cells were harvested and ROS production was measured. **(B)** Cells were treated with nicotine (10 μM) in the presence or absence of DPI (10 μM) or Gö6976 (1 μM) for 60 min. After incubation, ROS production was measured. **(C)** Cells were treated with nicotine (10 μM) for 8 h in the presence or absence of DPI (10 μM), Apo (50 μM), or NAC (1 m*M*). Then, cells were lysed and subjected to Western blotting. Blot bands are representative of three independent experiments. Data are presented as mean ± SD of three independent experiments. Significance compared to control, $^{*}p < 0.05$ or $^{**}p < 0.01$; significance compared with nicotine alone $^{\dagger\dagger}p < 0.01$. NAC, *N*-acetyl-cystein; ROS, reactive oxygen species.


A
Relative protein level HO-1/β-actin
HO-1
β-actin
Nicotine
SP600125 (40 μM)
SB203580 (30 μM)
PD98059(50 μM)
LY294001(25 μM)
Wortmannin(20 nM)
Bay11-7082(10 μM)
NAC(1 mM)
B
PI3-kinase activity (fold of control)
Nicotine - 15 30 60 120 480 min
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Relative protein level p-Akt/Akt
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Akt
Nicotine - 15 30 60 120 480 min
D
Relative protein level p-Akt/Akt
p-Akt
Akt
Nicotine
EGTA(3mM)
Gö6976(1μM)
NAC (1mM)
E
HO-1/β-actin
HA/β-actin
Relative protein level
HO-1
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CA-Akt-HA
F
HO-1/β-actin
HA/β-actin
Relative protein level
HO-1
β-actin
HA
Nicotine
EV
DN-Akt-HA

Transfection

pCMV5-HA-Akt and pCMV5-HA-DN-Akt were provided by Dr. Hye Gwang Jeong (Chonsun University, Korea) and Dr. Sung Hee Hong (Korea Institute of Radiological and Medical Sciences, Korea), respectively. The transfection of 2 μg of DNA constructs was performed using Superfect© from QIAGEN according to the manufacturer's protocol. Briefly, 1×10^5 cells were plated into 60-mm or 1×10^7 into 100-mm dishes the day before transfection and grown to about 70% confluence. Transfections were allowed to proceed for 8 h. The transfected cells were washed with 4 ml of PBS and then stimulated with nicotine (10 μM) for 8 h.

siRNA technique

siHO-1, and scramble siRNA were purchased from Invitrogen. The sequence of mouse HO-1 siRNA (5-end prime; to 3-end prime) is as follows: UUACAUGGCAUAAA UUCCCACUGCC. siα7nAchR and siNrf-2 were purchased from Santa Cruz Biotechnology. The siRNA was transfected into RAW264.7 cells according to the manufacturer's protocol using transfection reagent Superfect from Qiagen. The cells were incubated with 100 nM HO-1 or scramble siRNA for 48 h in serum- and antibiotic-free media. Then, the cells were incubated for 12 h in media containing antibiotics and fetal bovine serum, and cells were washed and pretreated with our without nicotine, after LPS stimulation.

ARE luciferase assay

RAW 264.7 cells were plated at a confluence of 50% density in 6-well plate and grown in DMEM supplemented with 10% heat-inactivated horse serum at 37°C in a humidified atmosphere of 5% CO_2/95% air. The cells were cotransfected with 2 μg of the luciferase reporter gene fusion construct (pTi-luciferase), wild-type ARE, and 0.5 μg of pCMV-β-galactosidase control vector with Superfect from Qiagen according to the manufacturer's instructions. After 24 h transfection, the cells were treated with nicotine for additional 6 h, and the cell lysis was carried out with the 1×reporter lysis buffer (Promega). After mixing the cell extract with a luciferase substrate (Promega), the luciferase activity was measured by TD-20/20 luminometer (Tuner Designs) according to the manufacturer's protocol. The β-galactosidase assay was done according to the supplier's instructions (Promega β-galactosidase Enzyme Assay System) for normalizing the luciferase activity.

Animal model of endotoxemia and sepsis

Endotoxemia was induced in BALB/c mice (male, 7–8 weeks, 20–25 g) by intraperitoneal injection of bacterial endotoxin (LPS 15 mg/kg). To induce CLP-induced sepsis, BALB/c mice were anesthetized with ketamine (30 mg/kg) and xylazine (6 mg/kg). Next, a 2-cm midline incision was performed to allow exposure of the cecum with adjoining intestine. The cecum was tightly ligated with a 3.0-silk suture at 5.0 mm from the cecal tip and punctured once with 22-gauge needle. The cecum was then gently squeezed to extrude a small amount of feces from the perforation sites and returned to the peritoneal cavity. The laparotomy site was then stitched with 4.0 silk suture. In sham control animals, the cecum was exposed but not ligated or punctured and then returned to the abdominal cavity. Mice were maintained in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication 85–23, revised 1996) and were treated ethically. The protocol was approved in advance by the Animal Research Committee of the Gyeongsang National University.

Statistical evaluation

Data are expressed as the mean ± SD of results obtained from the number of replicate treatments. Differences between data sets were assessed by one-way analysis of variance followed by Newman–Keuls tests. The Kaplan–Meier method was used to compare the differences in mortality rates between groups. $p < 0.05$ was accepted as statistically significant.

Results

Nicotine induces HO-1 expression in macrophages through nAChR and calcium influx

As shown in Figure 1A, nicotine did not induce cytotoxicity at 10 or 50 μM, whereas higher doses showed toxicity. Further, nicotine demonstrated significant upregulation of HO-1 expression in a dose- and time-dependent fashion (Fig. 1B, C). Two nAChR subtypes are involved in nicotine-mediated decrease in proinflammatory cytokine production by stimulated human and mouse macrophages: the α7 homopentamer expressed by monocyte-derived human and mouse macrophages (49), and the $\alpha4\beta2$ heteropentamer expressed by alveolar macrophages (31). Thus, to elucidate whether HO-1-inducible effect of nicotine is dependent on α7nAChR or not, we used mecamylamine (α7nAChR antagonist) (2). As shown in Figure 2A, mecamylamine dose-dependently inhibited HO-1 protein expression in nicotine-treated macrophages. However, mecamylamine is known as a nonspecific nAChR antagonist (16), so we transfected cells with siα7nAchR to attenuate α7nAchR protein expression (Fig. 2B) and to further confirm that HO-1 induction by nicotine is specific α7nAChR-mediated action. As shown in Figure 2B right pannel, HO-1 induction by nicotine was significantly reduced in cells transfected with siα7nAchR compared to positive

FIG. 5. Involvement of PI3K/Akt pathway in nicotine-induced HO-1 expression in macrophages. (A) Cells were pretreated with SP600125 (40 μM), SB203580 (10 μM), PD98059 (50 μM), LY294001 (25 μM), wortmannin (20 nM), Bay 11-7082 (10 μM), and NAC (1 mM) for 1 h, and then cells were incubated with nicotine (10 μM) for 8 h. After incubation, cells were extracted and subjected to Western blot. **(B)** Cells were treated with nicotine (10 μM) for 15, 30, 60, 120, and 480 min. Finally, cells were harvested and subjected to Western blot. **(C)** Cells were treated with nicotine (10 μM) in the presence or absence of EGTA (3 mM), Gö6976 (1 μM), and NAC (1 mM) for 1 h. Cells were then extracted and subjected to Western blot. **(D)** Cells were transfected with **(E)** constitutively active-Akt (CA-Akt-HA) and **(F)** dominant negative-Akt (DN-Akt-HA. After transfection, cells were treated with nicotine (10 μM) for 8 h, and then Western blot was performed for HO-1 detection. Significance compared to control, $^*p < 0.05$, $^{**}p < 0.01$; significance compared with nicotine alone or nicotine + empty vector, $^{\dagger}p < 0.05$ or $^{\dagger\dagger}p < 0.01$.

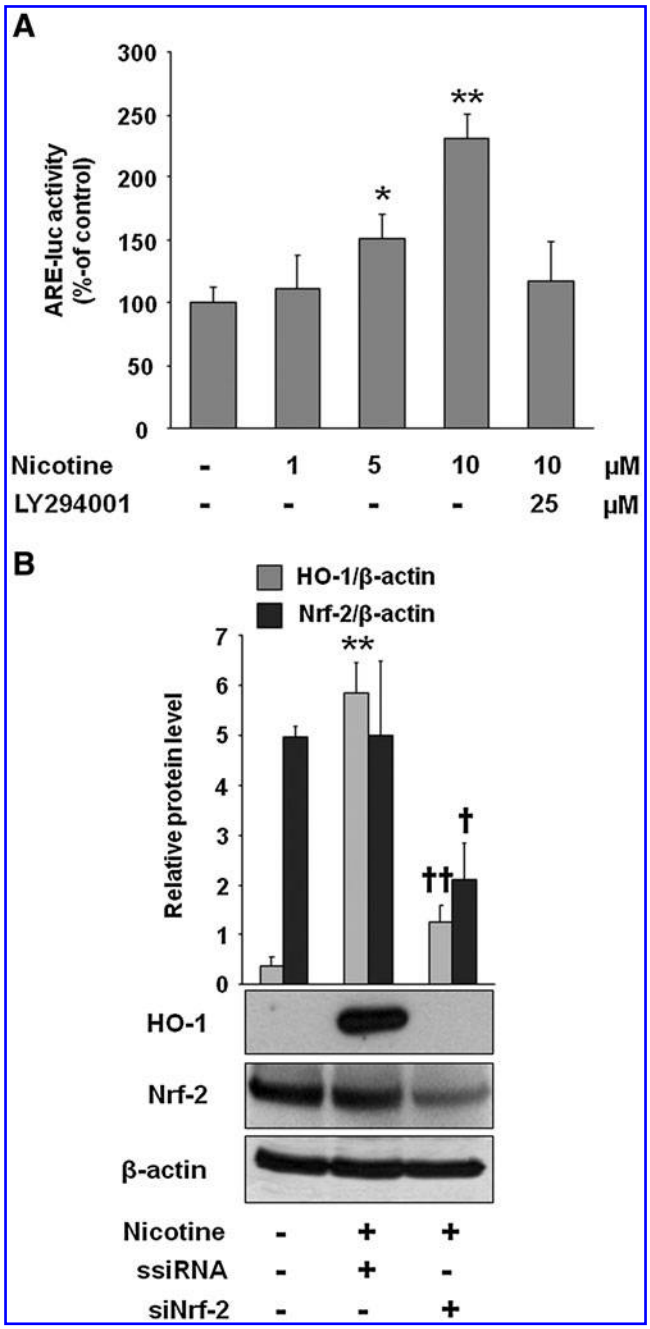


FIG. 6. Involvement of Nrf-2 in nicotine-induced HO-1 expression in macrophages. (A) Cells were transfected with ARE-luc promoter as described in Materials and Methods. After incubation, cells were treated with nicotine (1, 5, and 10 μM) with or without LY294001 (25 μM). Six hours later, cells were harvested and luciferase activity was measured as described in Meterials and Methods. **(B)** Scramble (ssiRNA) and siNrf-2-transfected cells were treated with nicotine (10 μM) for 8 h, and then cells were harvested and HO-1, Nrf-2 protein levels were evaluated by Western blot. Data are presented as mean ± SD of three independent experiments. Significance compared to control, $^{*}p < 0.05$ or $^{**}p < 0.01$; significance compared with ssiRNA $^{\dagger}p < 0.05$ or $^{\dagger\dagger}p < 0.01$.

control. The α7nAChR is highly Ca^{2+} permeable (13). Macrophages can express only α7 functionally active subunit among 16 monomer subtypes (49). Therefore, we tested whether nicotine can upregulate HO-1 gene expression by Ca^{2+} influx. Nicotine failed to induce HO-1 in the presence of calcium chelators such as ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid or 1,2-Bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester) (Fig. 2C, D). Inversely, HO-1 expression by nicotine was significantly and dose-dependently increased in the presence of calcium ionophore (A23187) (Fig. 2E).

Nicotine activates cPKC by Ca^{2+}-dependent mechanism in macrophages

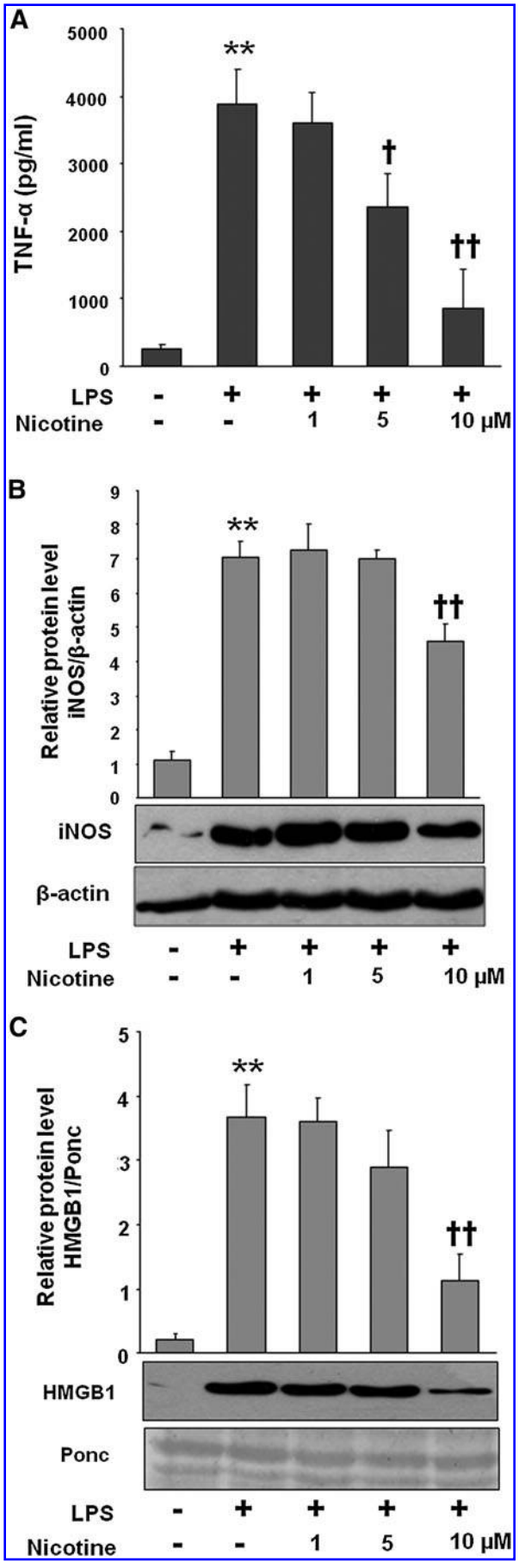
Increase of Ca^{2+} intracellularally can activate classical PKCs (18). As shown in Figure 3A and B, nicotine significantly activated PKCs, and this activation was Ca^{2+}-dependent. To further identify the role of cPKC activation in the regulation of HO-1 by nicotine, Gö6976 (specific classical PKC inhibitor) was used. Gö6976 significantly inhibited the expression of HO-1 by nicotine, suggesting that activation of cPKCs by nicotine plays an important role in HO-1 upregulation (Fig. 3C).

Activation of cPKCs by nicotine is involved in ROS increase by nicotine

The activation of cPKCs can lead to the increase of ROS in macrophages (21). In agreement with this, we also identified that nicotine can increase ROS production and this effect was mediated by cPKCs and NADPH oxidase activation (Fig. 4A, B). Next, we checked the possible involvement of ROS production in HO-1 upregulation by nicotine. As shown in Figure 4C, nicotine failed to induce HO-1 in the presence of NADPH oxidase inhibitors, including diphenyleneiodonium and apocynin (DPI, Apo) and common antioxidant *N*-acetyl-cystein.

Involvement of PI3K/Akt/Nrf-2 pathway in the nicotine-induced HO-1 expression

Since ROS production can activate plethora of signaling molecules, including mitogen-activated protein kinases (MAPKs), nuclear factor-kappa B, and PI3Ks, it was unclear what signaling molecule(s) would be involved in HO-1 induction by nicotine; thus, we performed the following experiment (Fig. 5A). We identified that administration of PI3K inhibitors, but neither MAPK nor nuclear factor-kappa B inhibitors, abrogated HO-1 induction by nicotine in macrophages. Moreover, nicotine activated Akt by its phosphorylation, which was dependent on Ca^{2+}, cPKC, and ROS formation (Fig. 5B, C). Next, nicotine failed to induce HO-1 in the presence of dominant negative-Akt, whereas constitutively active Akt induced HO-1 protein level, suggesting that nicotine induces HO-1 through Akt activation (Fig. 5D–F). It is well known that PI3K/Akt signaling can activate Nrf-2 trascription factor (14, 22). Thus, we asked whether nicotine can activate Nrf-2. As shown in Figure 6A, nicotine dose-dependently increases ARE-luc activity, but this effect is abrogated in the presence of LY294001 (PI3K inhibitor). Next, we introduced siNrf-2 and observed decreased level of HO-1 expression induced by nicotine (Fig. 6B). Thus, we suggest that nicotine-mediated HO-1 expression is dependent on PI3K/Akt/Nrf-2 signaling.



Induction of HO-1 by nicotine is critical for its anti-inflammatory activity

A recent report has suggested that nicotine attenuates pro-inflammatory response in LPS-activated macrophages (5). We also identified that nicotine dose-dependently inhibited TNF-α, iNOS, and HMGB1 release in LPS-activated macrophages (Fig. 7A–C). However, it is unclear whether anti-inflammatory effect of nicotine could be mediated through HO-1 upregulation. Therefore, we utilized siRNA technique. As shown in Figure 8A–C, transfection with scramble RNA did not interfere with inhibitory effect of nicotine on LPS-induced TNF-α, iNOS, and HMGB1 expression. In contrast, nicotine failed to inhibit the expression of these pro-inflammatory genes in siHO-1RNA-transfected cells. Thus, we concluded that HO-1 induction by nicotine is a critical step to provide anti-inflammatory activity.

Administration of ZnPPIX (HO-1 inhibitor) reverses improved survival mediated by nicotine in lethal endotoxemia and CLP- induced sepsis model

To further confirm our *in vitro* observations, we used animal models of sepsis. As shown in Figure 9A and B, administration of nicotine alone significantly improved survival in both lethal endotoxemia and CLP-induced sepsis model. However, combined treatment with nicotine and ZnPPIX (HO-1 inhibitor) reversed beneficial effect of nicotine in septic animal models (Fig. 9A, B). Figure 9C and D shows that Western blot of HMGB1 (blood) and HO-1 (heart and lung) levels 24 h after CLP-induced sepsis mice, respectively. HMGB1 in blood was significantly increased by CLP-induced sepsis; however, nicotine treatment reduced level of HMGB1, which was reversed by the presence of ZnPPIX (Fig. 9C). Next, HO-1 protein levels were checked in septic animals. As shown in Figure 9D, nicotine significantly increased HO-1 level in lung and heart tissues compared to CLP only animals. However, this effect was attenuated in the presence of ZnPPIX, which further demonstrate the dependence of therapeutic effect of nicotine on HO-1 induction.

Discussion

In the present study, we clearly showed that nicotine induced HO-1 in RAW 264.7 cells through α7nAChR and administration of nicotine increased survival of LPS- or

FIG. 7. Effect of nicotine on the expression of TNF-α, iNOS, and HMGB1 in LPS-stimulated macrophages. Cells were stimulated with LPS (1 μg/ml) in the presence or absence of nicotine (1, 5, 10 μM) for 24 h. After incubation culture medium samples were subjected to enzyme-linked immunosorbent assay for **(A)** TNF-α detection, lysates were subjected to Western blot for **(B)** iNOS and **(C)** HMGB1, respectively. Blot bands are representative of three independent experiments. Data are presented as mean ± SD of three independent experiments. Significance compared to control, $^{**}p < 0.01$; significance compared with LPS alone $^{\dagger}p < 0.05$ or $^{\dagger\dagger}p < 0.01$. HMGB1, high mobility group box 1; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; TNF, tumor necrosis factor.

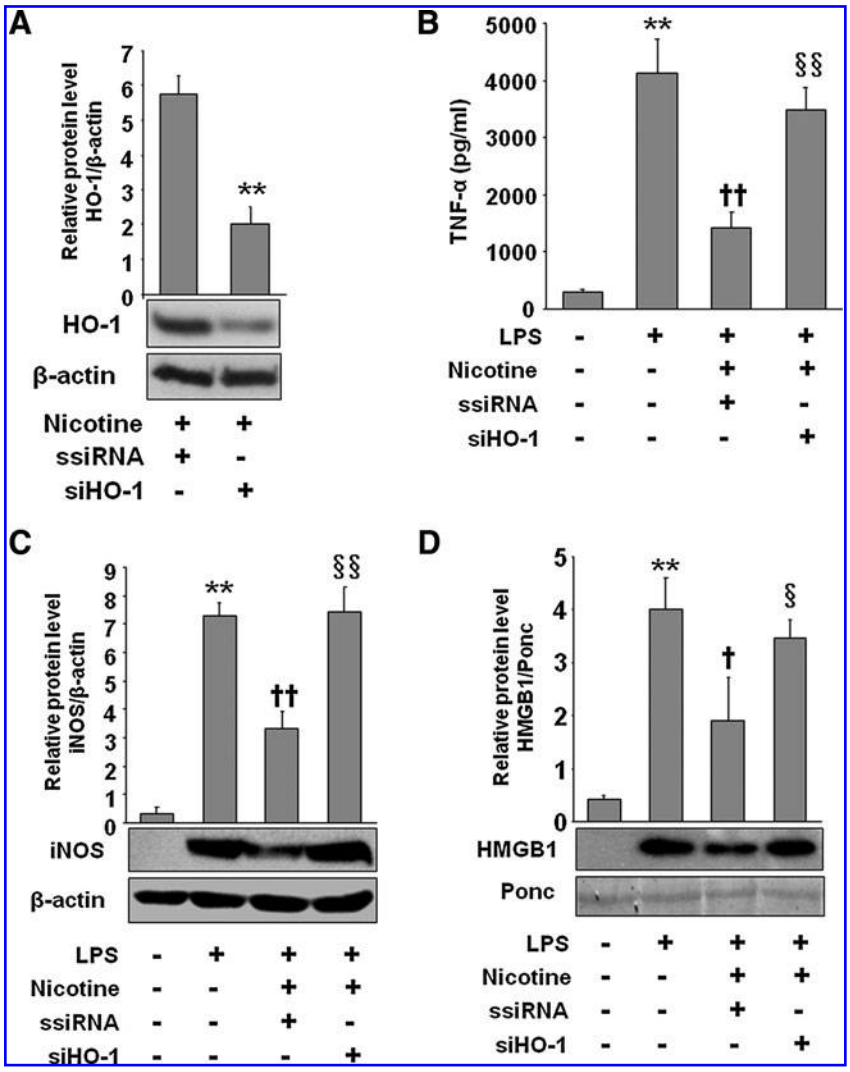


FIG. 8. Anti-inflammatory effect of nicotine is mediated *via* HO-1 induction in LPS-stimulated macrophages. (A) Scramble siRNA (ssiRNA) and siHO-1-transfected cells were treated with nicotine (10 μM) for 8 h, and then cells were harvested for immunobloting. ssiRNA and siHO-1-transfected cells were stimulated with LPS (1 μg/ml) with or without nicotine (10 μM) for 24 h. After incubation, culture medium samples were subjected to enzyme-linked immunosorbent assay for **(B)** TNF-α, and lysates were Western blot for **(C)** iNOS and **(D)** HMGB1, respectively. Blot bands are representative of three independent experiments. Data are presented as mean ± SD of three independent experiments. Significance compared to control, $^{**}p < 0.01$; significance compared with LPS alone $^{\dagger}p < 0.05$ or $^{\dagger\dagger}p < 0.01$; significance compared with ssiRNA $^{\S}p < 0.05$ or $^{\S\S}p < 0.01$.

CLP-induced septic mice in a ZnPPIX-dependent manner. Up to date, the intensive research is ongoing to elucidate how α7nAchR stimulation provides anti-inflammatory properties (8, 16, 32, 37, 42). Although it has been reported that HO-1 can be induced by nicotine in different cell lines such as chromaffin cells, keratinocytes, and gingival fibroblasts (6, 11, 23), anti-inflammatory action of nicotine *via* induction of HO-1 in inflammatory disorders such as sepsis has not yet been investigated. Here, we provide evidence that α7nAchR activation by nicotine in macrophages increases HO-1 expression as described in Figure 10, which may be responsible for increase of survival rate in LPS- and CLP-induced sepsis. Therefore, we propose that one of possible target molecules for eliciting cholinergic anti-inflammatory action by nicotine (through α7nAchR) can be HO-1 protein. This idea was based on the results that (i) nicotine increased HO-1 expression in a dose- and time-dependent manner, (ii) importantly, HO-1 induction by nicotine was significantly inhibited by silencing of α7nAchR using specific siRNA, and (iii) both Ca^{2+} and PKC affected the induction of HO-1 by nicotine in RAW 264.7 cells. In general, oxidative stress occurs when the flux of ROS or free radical generation exceeds available antioxidant defenses (12). Nicotine-induced ROS production of the present experiment may come from activation of NADPH oxidase enzymes (20), although we did not measure NADPH oxidase activity. Ample evidence suggests that activation of NADPH oxidase produces ROS in many cells, including macrophages (3, 30). In addition, PKC activation has been shown to be involved in cellular functions in response to a variety of stimuli, at least in part, by participating in the activation of NADPH oxidase (17, 39). Indeed, treatment of Gö6976, a classical PKC inhibitor, significantly reduced the ROS level, suggesting that activation of PKC by nicotine resulted in the production of ROS possibly *via* NADPH oxidase in RAW 264.7 cells. Further, two inhibitors of NADPH oxidase (DPI and apocyanine) and ROS scavenger (*N*-acetyl-cystein) significantly reduced the expression of HO-1 by nicotine, which provides additional evidence that ROS production *via* NADPH oxidase is necessary for induction of HO-1 expression by nicotine in RAW 264.7 cells. HO-1 is a rate-limiting enzyme in the oxidative degradation of heme to biliverdin, free iron, and CO. In fact, ROS are important mediators for the induction of HO-1 in many cells, including macrophages. Then, what is the signal mechanism(s) involved in HO-1 induction by nicotine? Mammalian HO-1 promoter undergoes complex regulation with the involvement of several response elements in the

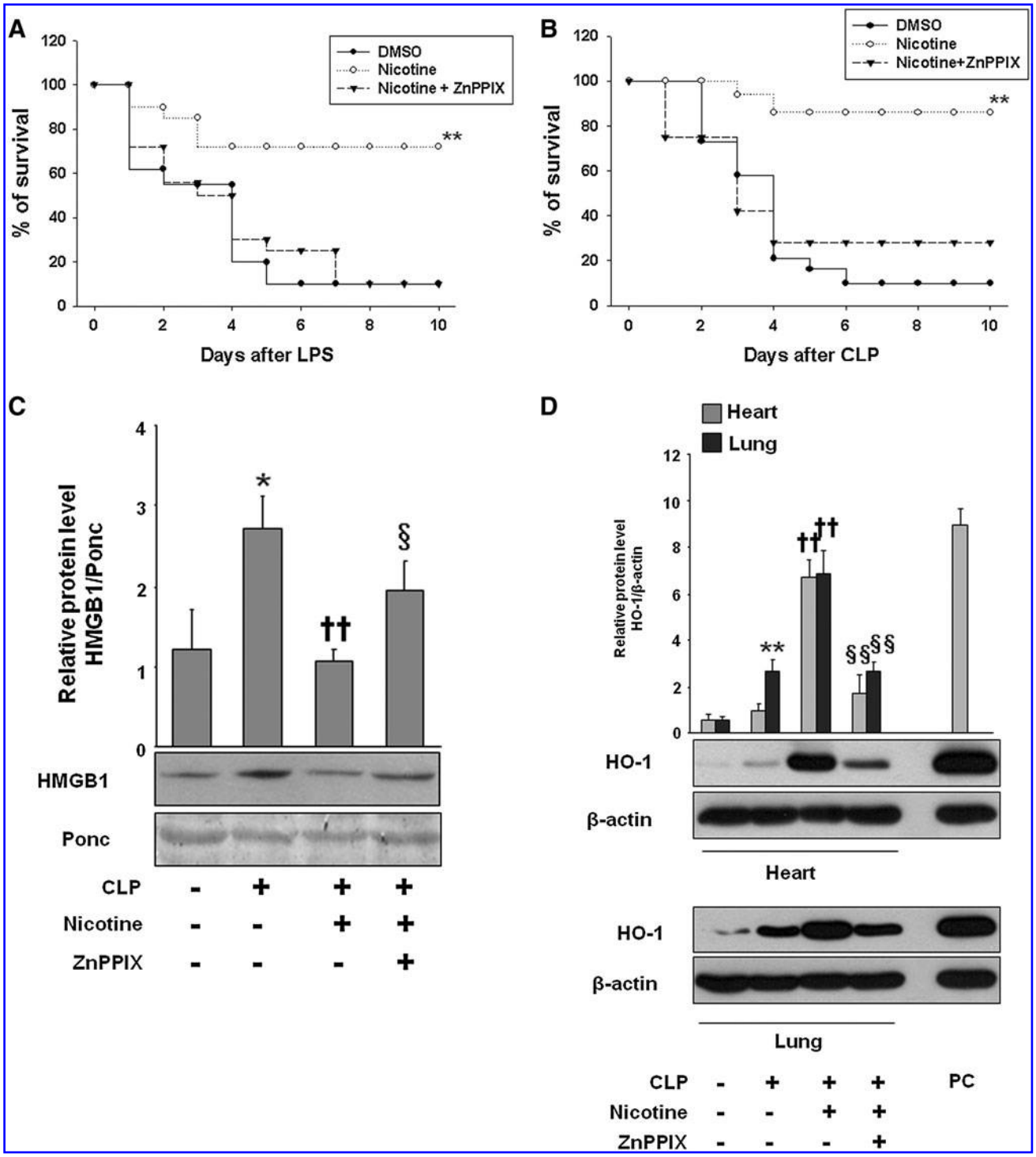


FIG. 9. Improved survival and decrease in circulating HMGB1 levels during experimental sepsis by nicotine is mediated *via* HO-1. (A, B) BALB/c mice were treated with nicotine (400 μg/kg i.p., $n = 15$) or nicotine + ZnPPIX (400 μg/kg and 5 mg/kg, respectively, i.p., $n = 15$) or DMSO treated (i.p., $n = 15$). At 0, +24, +48, +72, and +96 h after onset of endotoxemia or sepsis, animals were treated with nicotine (400 μg/kg), nicotine + ZnPPIX (400 μg/kg and 5 mg/kg, respectively), or DMSO. Survival was monitored daily for up to 2 weeks. **(C, D)** BALB/c mice ($n = 20$) were randomly divided into four groups: sham ($n = 5$), DMSO (i.p., $n = 5$), nicotine (400 μg/kg i.p., $n = 5$), and nicotine + ZnPPIX (400 μg/kg and 5 mg/kg, respectively i.p., $n = 5$). Mice were treated at 0 and 12 h after onset of sepsis (CLP). Twenty-four hours later, blood was collected by cardiac puncture and subjected to HMGB1 analysis **(C)**; hearts and lungs were extracted and homogenized and subjected to Western blotting for HO-1 detection **(D)**. Positive control (PC) (whole-cell lysate from RAW264.7 cells treated with hemin for 8 h) was used to verify HO-1 blot bands. Data are presented as mean ± SD of three independent experiments. Significance compared to control, $^{*}p < 0.05$; significance compared with CLP alone, $^{\dagger\dagger}p < 0.01$; significance compared with CLP + nicotine, $^{\S}p < 0.05$ or $^{\S\S}p < 0.01$. The Kaplan–Meier program was utilized to compare the differences in mortality rates between groups. Significance compared to DMSO, $^{**}p < 0.01$. CLP, cecal ligation and puncture; DMSO, dimethyl sulfoxide; i.p., intraperitoneal.

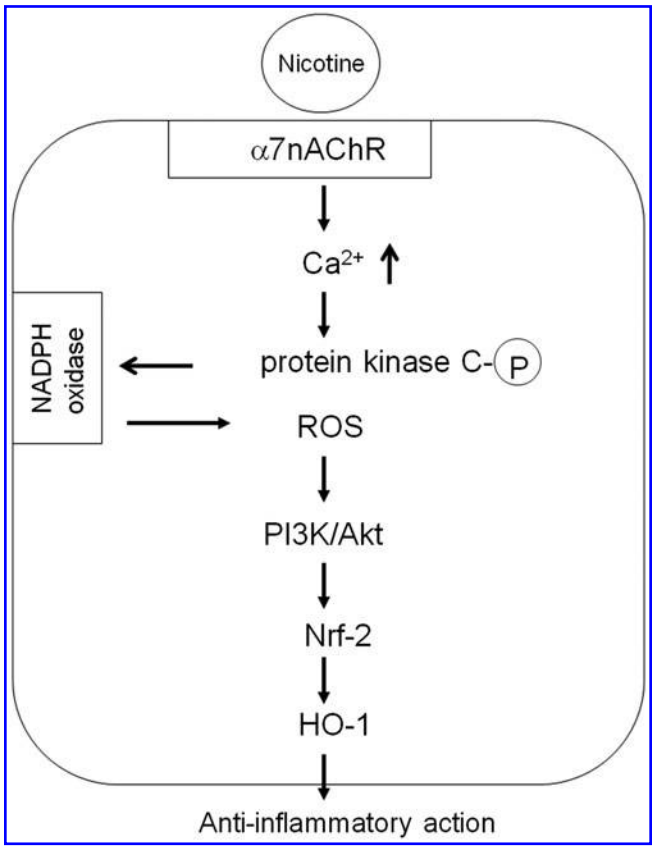


FIG. 10. Possible mechanism by which nicotine upregulates HO-1 expression in macrophages. Nicotine activates α7nAChR and subsequently increases Ca^{2+} influx. Increased Ca^{2+} level leads to the activation of classical PKCs, which in turn stimulate ROS production *via* NADPH oxidase. Increased ROS level activates PI3K/Akt/Nrf2 pathway, culminating in HO-1 induction in macrophages, which resulted in anti-inflammatory action.

promoter regions spanning −10 to −15 kb (38). This regulatory region seems to be the convergence point for a broad spectrum of agents acting through various transcription factors, including Jun, Fos, Ets, cAMP-response element, Maf, and Cap'n'collar (CNC) family of transcription factors (38). Among these, Nrf2, one of CNC family of bZIP transcription factors, plays a critical role (33). We found that nicotine-induced HO-1 in RAW 264.7 cells was significantly inhibited by transfected cells with either siNrf2 or DN-Akt, which indicates of involvement of PI3K/Akt/Nrf2 signaling. Further, we found that PI3K inhibitors (LY294002 and wortmanin) but not other MAPK inhibitors significantly inhibited HO-1 induction, reinforcing that PI3K/Akt/Nrf2 pathway is one of the important signals for induction of HO-1 by nicotine. Although the mechanism by which Akt translocates Nrf2 into nucleus is not clearly understood, Akt is not likely to translocate Nrf2 by direct phosphorylation because of lack of consensus phosphorylation sequence. Rather, Akt could stabilize Nrf2 by decreasing its proteosomal degradation by inhibition of ubiquitine ligases (33). We and others previously reported that PI3K/Akt/Nrf2 signal pathways are associated with HO-1 induction in various cells by many chemicals that provide protection against oxidative stress (24, 26, 34).

Recently, we have suggested that HO-1, particularly CO, can be beneficial in the treatment of sepsis (45). HO-1/CO system strongly inhibited HMGB1 release both *in vitro* and *in vivo* as well as improved survival in experimental model of sepsis (43, 45). Nicotine significantly reduced the expression of iNOS and HMGB1 in LPS-activated macrophages, which was inhibited by siHO-1RNA-transfected macrophages. Thus, it seems plausible that HO-1-inducible effect by nicotine is, at least, an important step for cholinergic anti-inflammatory pathway. We confirmed that activation of α7nAChR by nicotine reduced expression of HMGB1 in LPS-activated macrophages and increased survival rate in septic rodent (48). In LPS or CLP-induced septic animals, nicotine increased survival, and administration of ZnPPIX, a HO-1 inhibitor, significantly reversed the effect of nicotine. This result stressed out that increased HO-1 activity by nicotine *in vivo* reflects for increased survival in septic animals. In contrast, several reports suggested that nicotine is not efficient in the treatment of sepsis (4, 41, 46). Authors claimed that immunosuppressive effect of nicotine impairs bacterial clearance and thus does not improve survival in septic models. Now, we cannot appropriately explain this difference, but possibly this discrepancy lies in different experimental condition, treatment schedules, and different animals (4, 10, 32, 41). However, CO released from CO-releasing molecule-3 showed antibacterial activity when assayed by growth rates or viability counts or live/dead staining using bacteria (7, 9). Regarding molecular mechanism of anti-inflammatory effect of nicotine, importance of Jak2-STAT3 signaling pathway has been reported (8). Activation of Jak2-STAT3 signal also plays a crucial role for HO-1 expression in various cells, including human macrophages (35, 40, 44). Therefore, whether the activation of Jak2-STAT3 by nicotine leads to HO-1 induction in macrophages needs further investigation. Given that HMGB1 is a target molecule for HO-1/CO in the treatment of sepsis (32, 36), it would be interesting to elucidate what exact metabolites of HO-1 (*e.g.*, CO, biliverdin, or iron ion) are involved in nicotine anti-inflammatory action.

In summary, we addressed the hypothesis that nicotine stimulates HO-1 expression in RAW264.7 cells through α7nAChR, which provides anti-inflammatory action. We clearly showed that inducible effect of nicotine on HO-1 was mediated by α7-nAChR→Ca^{2+} influx → cPKC → ROS → PI3K/Akt/Nrf-2 signaling cascade, as described in Figure 10. Further, we found that upregulation of HO-1 by nicotine is a critical step in anti-inflammatory action of nicotine *in vitro* and *in vivo* septic models. Thus, we claim that induction of HO-1 through activation of α7nAChR by nicotine in macrophages can be one of possible targets for cholinergic anti-inflammatory pathway.

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Author Disclosure Statement

There is no conflict of interest.

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Abbreviations Used

α7nAchR = alpha 7 nicotinic acetylcholine receptor
BAPTA = 1,2-Bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester)
CLP = cecal ligation and puncture
CO = carbon monoxide
cPKC = classical protein kinase C
DCFH-DA = dichlorofluorescein diacetate
DMSO = dimethyl sulfoxide
EGTA = Ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid
HMGB1 = high mobility group box 1
HO-1 = heme oxygenase-1
iNOS = inducible nitric oxide synthase
i.p. = intraperitoneal
Jak2 = janus kinase 2
LPS = lipopolysaccharide
MAPK = mitogen-activated protein kinase
NAC = *N*-acetyl-cystein
ROS = reactive oxygen species
STAT3 = signal transducer and activator of transcription 3
TNF = tumor necrosis factor

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