Nicotine enhancement of lipopolysaccharide/interferon-γ-induced cytotoxicity with elevating nitric oxide production

Yen-Chou Chen a, Shing-Chuan Shen b,c,*, Hui-Yi Lin a, Shu-Huei Tsai d, Tony J.F. Lee e,f

a Graduate Institute of Pharmacognosy, School of Pharmacy, Taipei Medical University, Taipei, Taiwan
b Department of Dermatology, School of Medicine, Taipei Medical University, Taipei, Taiwan
c Department of Dermatology, Taipei Municipal Wan-Fang Hospital, Taipei, Taiwan
d Department of Orthopaedics and Traumatology, School of Medicine, Taipei Medical University Hospital, Taipei Medical University, Taipei, Taiwan
e College of Life Sciences, Neuromedical-Scientific Center, Tzu Chi Hospital, Tzu Chi University, Hualien, Taiwan
f Department of Pharmacology, Southern Illinois University School of Medicine, Springfield, IL, USA

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Abstract

Nicotine has been shown to induce relaxation via nitric oxide (NO) production with activation of endothelium nitric oxide synthase (eNOS), however the effect of nicotine on lipopolysaccharide/interferon-γ (LPS/IFN-γ)-induced NO production and inducible NOS (iNOS) gene expression is still undefined. Here, nicotine alone did not affect the NO and PGE 2 production in RAW264.7 and primary peritoneal macrophages. Interestingly, nicotine showed the dose-dependent stimulatory effect on LPS (20 ng/ml)/IFN-γ (10 ng/ml)-induced NO but not PGE 2 production in both cells. Although nicotine stimulates NO production in the presence of LPS/IFN-γ, LPS at the dose of 20 ng/ml, nicotine showed no obvious inductive effect on the expression of iNOS protein by Western blotting in both cells. However, nicotine significantly stimulates LPS (2.5, 5 ng/ml)/IFN-γ (10 ng/ml)-induced iNOS expression and NO production in RAW264.7 cells. Cytotoxicity assay showed that nicotine enhanced LPS (20 ng/ml) and IFN-γ (10 ng/ml)-induced cytotoxicity, which was inhibited by an NOS inhibitor N-nitro-L-arginine (NLA) in RAW264.7 cells. Direct and indirect NOS activity assays indicated that nicotine did not affect NOS activity. And, iNOS protein stability was not changed by nicotine after LPS/IFN-γ treatment. These data indicates that nicotine may potentiate LPS/IFN-γ-induced cytotoxic effects by enhancing NO production; enhancing iNOS gene expression induced by LPS/IFN-γ is involved. A cross-talk between inflammation and smoking was proposed in the present study.

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Keywords: Nicotine; Lipopolysaccharide/interferon-γ; Nitric oxide; Inducible nitric oxide synthase

Abbreviations: NO, nitric oxide; PGE 2 , prostaglandin E2; LPS, lipopolysaccharide; IFN-γ, interferon-γ; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase 2; DTT, dithiothreitol; NLA, N-nitro-L-arginine; NBT, nitroblue tetrazolium; BCIP, 5-bromo-4-chloro3-indolyl phosphate

* Corresponding author. Tel.: +886-2-27361661x3224; fax: +886-2-27387139.
E-mail address: scshen@tmu.edu.tw (S.-C. Shen).

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1. Introduction

Cancer was the first disease to be linked to tobacco use, and the relationship between tobacco use and cancer has been demonstrated extensively (Minna, 2003; Hecht, 2002). Several carcinogens such as N\(^{\prime}\)-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) isolated from tobacco have been identified (Balansky et al., 1994; Nair et al., 1996), and West et al. indicated that nicotine might contribute to lung tumorigenesis by Akt activation (West et al., 2003). In addition, tobacco use can affect the therapeutic effect of cancer cells to agents in lung, head, and neck cancers (Kaufmann, 1989). Previous studies demonstrated that nicotine, the most important compound in cigarette, inhibited apoptosis induced by ultraviolet light (UV), \(H_2O_2\), chemotherapeutic agents, and calcium ionophore (Sugano et al., 2001; Sugano and Ito, 2000). Moreover, some studies have shown that nicotine inhibited cell proliferation through its cytotoxicity without internucleosomal DNA cleavage in glioma cells or with apoptotic characteristics in leukemia cells (Yoshida et al., 1998; Lahmouzi et al., 2000). Therefore, both apoptosis-inductive and protective effects of nicotine have been reported.

Inflammation is a complex process through activation of a series of genes including nitric oxide synthases (NOSs) and cyclooxygenases (COXs). Three types of NOSs including endothelium nitric oxide synthase (eNOS), neural NO synthase (nNOS) and inducible NO synthase (iNOS) and two types of COXs including COX-1 and COX-2 have been identified (Shen et al., 2002; Chen et al., 2001a,b). Nitric oxide (NO) derived from NOS activation as a signaling molecule involved in a variety of pathophysiological activities. The physiological role of NO from eNOS or nNOS play as a neurotransmitter in central nervous system or as a potent vasorelaxant in regulating the blood pressure though modulating muscular tone (Moncada et al., 1989). However, NO derived from iNOS has been defined as a deleterious molecule in the process of inflammation and sepsis (Southan and Szabo, 1996). Exposure to outer bacterial toxins such as lipopolysaccharide (LPS) stimulates cellular inflammatory responses, and released a lot amount of NO induced by iNOS activation to promote inflammatory responses. Similar to NO, prostaglandin \(E_2\) (PGE\(_2\)) is over-produced at inflammatory sites by activating COX-2 (not COX-1) gene expression, an inducible enzyme in the conversion of arachidonic acid to prostaglandin \(H_2\), prostacyclin and thromboxane \(A_2\). Elevation of cyclooxygenase-2 protein has been demonstrated to be associated with the occurrence of some human diseases such as colon carcinoma (Manning et al., 2002).

The relationship between nicotine and NO release has been studied extensively. Nicotine was able to induce NO release in several tissues such as arteries, vein, colitis and nerve (Lee, 2002; Si and Lee, 2001). NO from smoke may be able to increase nicotine absorption and increase post-synaptic dopamine levels (Vleeming et al., 2002). In food-deprived rats, nicotine administration decreased NOS gene expression in the hypothalamus (Jang et al., 2002). In the process of carcinogenesis, nicotine stimulates angiogenesis and promotes tumor growth and atherosclerosis through endothelial production of NO, prostacyclin and vascular endothelial growth factor (Heeschen et al., 2001). Although many biological effects of nicotine have been identified, the effect of nicotine on LPS-induced inflammatory responses is still undefined. Results of the present study show that nicotine was able to enhance LPS/IFN-\(\gamma\)-induced cytotoxicity through stimulating NO production in RAW264.7 macrophages and primary peritoneal macrophages, and iNOS gene expression is involved.

2. Materials and methods

2.1. Cells

RAW264.7, a monocyte-macrophage cell line, was obtained from the American Type Culture Collection. Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 2 mM glutamine, antibiotics (100 U/ml of penicillin A and 100 U/ml of streptomycin), and 10% heat-inactivated fetal bovine serum (Gibco/BRL) and maintained in a 37 \(^{\circ}\)C humidified incubator containing 5% \(CO_2\). Thioglycollate-elicited peritoneal macrophages are obtained from specific pathogen-free male Balb/c mice at 5–8 weeks of age by injection of 3% thioglycollate solution (Difco, Detroit, MI) for 4 days before
lavage with 10 ml of phosphate-buffered saline. The peritoneal macrophages were purified and identified as described in our previous paper (Shen et al., 2002).

2.2. Agents

Lipopolysaccharide (LPS) (Escherichia coli, serotype 055:B5), trypan blue, nitroblue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), interferon-γ, sulfanilamine, naphthylenediamine dihydrochloride, and N-nitro-l-arginine (NLA) were purchased from Sigma (St. Louis, MO). The prostaglandin E2 enzyme linked immunosorbent assay (ELISA) kits were purchased from Cayman Chemical (Ann Arbor, MI). The antibodies of anti-iNOS, anti-COX-2, anti-HO-1, and anti-α-tubulin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.3. Cell viability assay

Trypan blue was used as an indicator of cell viability as determined by the integrity of cellular membrane. RAW264.7 cells were plated at a density of 5 × 10^5 cells per well into 24-well plates for 12 h, followed by treatment with different components for a further 12 h. The cells were collected by trypsinization and the viability of cells was determined by trypan blue exclusion.

2.4. Nitrite assay

RAW264.7 cells were plated at a density of 5 × 10^5 cells/ml in 24-well plates for 12 h, followed by treatment with LPS (20 ng/ml) and interferon-γ (10 ng/ml) and different concentrations of indicated components for a further 12 h. The amount of NO production in the medium was detected with the Griess reaction. One hundred microliters of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide in 1% phosphoric acid and 0.1% naphthylenediamine dihydrochloride in water). The absorbance of the mixture at 530 nm was determined using an ELISA plate reader (Dynatech MR-7000; Dynatech Laboratories), and nitrite concentration was determined using a dilution of sodium nitrite as a standard (Chen et al., 2002). The basal level of nitrite in DMEM medium without cells has been measured by Griess reaction, and the basal amount of nitrite in DMEM medium is below 1 μM. The blank in the study has been corrected by the basal nitrite in the medium.

2.5. Western blotting

Total cellular extracts were prepared according to our previous papers (Lee et al., 2002; Ko et al., 2002), separated on 8%–12% SDS-polyacrylamide minigels, and transferred to immobilon polyvinylidenefluoride membranes (Millipore). Membranes were incubated with 1% bovine serum albumin and then incubated with anti-iNOS, anti-COX-2, anti-HO-1, and anti-α-tubulin antibodies (Santa Cruz, CA) overnight at 4 °C. Expression of protein was detected by staining with NBT and BCIP suggested by the manufacture.

2.6. Measurement of iNOS enzyme activity

Direct iNOS enzyme activity was measured as described in our previous paper (Chen et al., 2001a). Briefly, 200 μg of cell lysates from LPS/INF-γ-treated RAW264.7 cells was incubated in 20 μM Tris-HCl (pH 7.9) containing 4 μM FAD, 4 μM tetrahydrobiopterin, 3 mM dithiothreitol (DTT), and 2 mM each of l-arginine and NADPH. The reaction was carried out in duplicate for 180 min at 37 °C in 96-well plates. Residual NADPH was oxidized enzymatically and the Griess reaction was performed as above. In the indirect iNOS enzyme activity assay, cells were treated LPS/INF-γ for 12 h, followed by trypsinization and subculture to 24-well plates in the presence of indicated compounds for a further 12 h. Griess reaction was performed as above to measure the amount of NO in the medium.

2.7. Statistical analysis

Values are expressed as the mean ± S.E. The significance of the difference from the respective controls for each experimental test condition was assayed by using Student’s t-test for each paired experiment. A P value <0.05, or 0.01 was regarded as indicating a significant difference.
3. Results

3.1. Nicotine enhancement of NO, but not PGE₂ production in LPS/IFN-γ-treated RAW264.7 and peritoneal thioglycolate-elicited macrophages

Induction of NO and PGE₂ production by LPS (20 ng/ml)/IFN-γ (10 ng/ml) has been identified previously, however the effect of nicotine in their induction is still undefined. NO will react with ROS to form both nitrite and nitrate, and the amount of nitrite in medium can be measured by griess reaction. Our preliminary data show that reduction of nitrate to nitrite by nitrate reductase did not affect the result of NO detection in cells induced by LPS. Therefore, a direct measurement of nitrite in the medium by griess reaction as a representative of NO production was performed in the present study. Results of Fig. 1 show that both NO and PGE₂ are significantly induced in LPS/IFN-γ-treated RAW264.7 cells as analyzed by Griess reaction and ELISA kits, respectively. After 12h incubation, unstimulated macrophages produced...
background level of nitrite and PGE₂ below 5 µM and 25 pg/ml in culture medium, respectively. Nicotine alone did not exhibit any effect on NO and PGE₂ production without LPS/IFN-γ. Interestingly, in the presence of LPS/IFN-γ, nicotine showed a dose-dependent stimulation on NO production in RAW264.7 macrophages. In contrast to NO production, PGE₂ induced by LPS/IFN-γ remained at the same level as that in LPS/IFN-γ plus nicotine-treated macrophages. In order to further elucidate if nicotine possess the ability to enhance NO production induced by LPS/IFN-γ, primary macrophages were established from thioglycolate-treated Balb/c mice as described in our previous paper (Shen et al., 2002). Results of Fig. 1 C show that nicotine dose-dependently stimulated NO production in LPS (5 µg/ml)/IFN-γ (10 ng/ml)-treated primary peritoneal macrophages, however PGE₂ still did not changed by nicotine addition (data not shown). These data suggested that nicotine potentiation of NO production in the present of LPS/IFN-γ in both macrophages.

3.2. Effects of nicotine on LPS (20 ng/ml)/IFN-γ (10 ng/ml)-induced iNOS and COX-2 gene expression in protein in RAW264.7 cells

Based on the data indicating nicotine stimulation of LPS (20 ng/ml)/IFN-γ (10 ng/ml)-induced NO production, there are two possibilities for the NO stimulatory effect of nicotine. One is by inducing iNOS gene expression, and the other is by activating iNOS enzyme activity. In order to elucidate if nicotine enhanced NO production by elevating iNOS gene expression, Western blotting using specific anti-bodies were performed to examine the expression of iNOS, COX-2, and α-tubulin protein in LPS/IFN-γ or LPS/IFN-γ plus different doses of nicotine-treated RAW264.7 macrophages. Result of Fig. 2A show

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**Fig. 2.** Nicotine shows no effect on LPS (20 ng/ml)/IFN-γ (10 ng/ml)-induced iNOS and COX-2 gene expression in protein in RAW264.7 and primary macrophages. (A) RAW264.7 cells were treated with LPS/IFN-γ (LPS, 20 ng/ml; IFN-γ, 10 ng/ml) in the presence or absence of different doses of nicotine (0.2, 0.4, 0.8, 1.6 mM) for 12h, and the expression of iNOS, COX-2 and α-tubulin protein was examined by Western blotting. (B) RAW264.7 cells were treated with LPS/IFN-γ (LPS, 20 ng/ml; IFN-γ, 10 ng/ml) in the presence or absence of nicotine (1.6 mM) for different time points (4, 8, 12h), and the expression of iNOS, COX-2, and α-tubulin was examined by Western blotting. (C) Primary macrophages were treated with LPS/IFN-γ (LPS, 5 µg/ml; IFN-γ, 10 ng/ml) in the presence or absence of different doses of nicotine (0.2, 0.4, 0.8, 1.6 mM) for 12 h, and the expression of iNOS, COX-2 and HO-1 protein was examined by Western blotting.
that nicotine alone do not induce iNOS and COX-2 protein expression, and LPS/IFN-γ addition significantly induced iNOS and COX-2 protein expression. Addition of different doses of nicotine showed no obvious stimulatory effect on LPS/IFN-γ-induced iNOS and COX-2 protein expression in RAW264.7 macrophages. α-Tubulin protein was described as an internal control to verify the equal amount of protein in each lane. Similarly, a time-dependent induction pattern of iNOS and COX-2 protein in LPS/IFN-γ- and LPS/IFN-γ plus nicotine (1.6 mM)-treated RAW264.7 cells was examined, and nicotine showed no visible stimulatory effect on LPS/IFN-γ-induced iNOS and COX-2 protein expression (Fig. 2B). Similar results were obtained in peritoneal macrophages treated by LPS/IFN-γ in the presence or absence of nicotine (Fig. 2C). Furthermore, a direct and an indirect iNOS enzyme activity assay were performed to identify if nicotine affect iNOS enzyme activity. Results of Table 1 showed that nicotine showed no obvious stimulatory effect on inducible NOS enzyme activity in both direct and indirect iNOS enzyme activity assays. NLA and NAME, non-specific NOS enzyme inhibitors, decreasing NO production in the assays were described as positive controls.

3.3. Nicotine stimulates LPS (2.5, 5 ng/ml)/IFN-γ (10 ng/ml)-induced iNOS protein expression in RAW264.7 cells, but no effect on the stability of iNOS protein

Although nicotine enhancement of NO production in the presence of LPS (20 ng/ml)/IFN-γ (10 ng/ml), both iNOS protein and iNOS enzyme activity were not affected by nicotine at this condition. One possibility is a large amount of NO production induces cytotoxicity in cells and causes no significant iNOS induction was observed. Therefore, lower doses of LPS (2.5, 5, 10 ng/ml) and IFN-γ (10 ng/ml) were used to study the effect of nicotine on iNOS gene expression. Results of Fig. 3A show that nicotine shows stimulatory effect on lower doses of LPS (2.5, 5, 10 ng/ml) plus IFN-γ (10 ng/ml)-treated RAW264.7 cells. Elevation of iNOS protein expression by nicotine about 1.5-fold, compared with LPS (2.5, 5, 10 ng/ml) plus IFN-γ (10 ng/ml)-treated group, was observed (Fig. 3B). However, nicotine showed no obvious stimulation on iNOS protein expression in LPS (10 ng/ml) plus IFN-γ (10 ng/ml)-treated group. Additionally, to explore the possible action of nicotine on the stability of induced iNOS protein, cell containing an induced iNOS after LPS stimulation were washed with fresh medium to remove LPS and the decline of iNOS protein in both control and nicotine-treated group was detected. As densitometry analysis, the turn over half-life of iNOS was not significantly altered by nicotine (1.6 mM) (Fig. 3C).

3.4. Nicotine enhanced the cytotoxic effect of LPS/IFN-γ through NO production

The cytotoxic effect induced by NO has been found and involved in the pathophysiology of inflammation, therefore identifying if nicotine was able to potentiate the cytotoxic effect of LPS (20 ng/ml)/IFN-γ (10 ng/ml) in RAW 264.7 cells was performed. In the absence of LPS/IFN-γ, NLA showed no significant reduction on endogenous NO production and cellular viability in RAW264.7

Table 1

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Indirect iNOS enzyme activity assay (µM x 10^3/cell)</th>
<th>Direct iNOS enzyme activity assay (µM/200 µg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>18.6 ± 1.1</td>
<td>10.6 ± 1.3</td>
</tr>
<tr>
<td>Nicotine</td>
<td>0.4 19.4 ± 1.4</td>
<td>12.4 ± 2.1</td>
</tr>
<tr>
<td>0.8</td>
<td>17.8 ± 1.8</td>
<td>11.8 ± 0.8</td>
</tr>
<tr>
<td>1.6</td>
<td>17.6 ± 1.5</td>
<td>12.6 ± 1.8</td>
</tr>
<tr>
<td>3.2</td>
<td>19.8 ± 2.5</td>
<td>10.3 ± 2.5</td>
</tr>
<tr>
<td>NLA (100 µM)</td>
<td>1.5 ± 0.7**</td>
<td>3.1 ± 0.4**</td>
</tr>
<tr>
<td>NAME (100 µg)</td>
<td>5.6 ± 1.1**</td>
<td>2.6 ± 1.2</td>
</tr>
</tbody>
</table>

**P < 0.01, significantly different from DMSO-treated group, analyzed by Student’s t-test.

1 RAW264.7 macrophages were stimulated with LPS/IFN-γ (LPS, 20 ng/ml; IFN-γ, 10 ng/ml) for 12h, and cells were washed twice with PBS to remove LPS. RAW264.7 cells were then trypsinized and placed in a 24-well plate and indicated compounds were added and incubated at 37°C incubator for a further 12h. The amount of NO accumulated in the medium was measured as described in Section 2.

2 Lysozyme preparation and iNOS activity assay were described in Section 2. Each compound was added into lysates (200 µg) from LPS-stimulated RAW264.7 macrophages and iNOS activity was measured by NO analysis using Greiss reaction.
Nicotine stimulates LPS, at the dose of 2.5 and 5 ng/ml, plus IFN-γ (10 ng/ml)-induced iNOS gene expression and NO production. (A) RAW264.7 cells were treated with LPS, at the doses of 2.5, 5 and 10 ng/ml, plus IFN-γ (10 ng/ml) in the presence or absence of nicotine (NIC; 1.6 mM) for 12 h. The amount of NO production in the medium was quantitatively assessed using NaNO₂ as a standard. Data were obtained from three independent experiments and are expressed as the mean ± S.E. **P< 0.01 indicates a significant difference from the LPS-treated group, as analyzed by Student’s t-test. (B) RAW264.7 cells were treated with LPS, at the doses of 2.5, 5 and 10 ng/ml, plus IFN-γ (10 ng/ml) in the presence or absence of nicotine (NIC; 1.6 mM) for 12 h. The expression of iNOS protein was detected by Western blotting, followed by quantification with densitometry. (C) Nicotine did not affect iNOS protein stability. After LPS (20 ng/ml)/IFN-γ (10 ng/ml)-treatment for 12 h, LPS was washed out and nicotine (1.6 mM) was added or not for 2, 4, 6, 8, 12 h. Detection of iNOS expression at indicated time points was performed by Western blotting followed by quantification with densitometry.

cells (data not shown). Results of trypan blue exclusion assay and griess reaction showed that LPS (20 ng/ml)/IFN-γ (10 ng/ml) significantly reduced the number of viable cells with elevating NO production, and nicotine addition potentiates the cytotoxic effect, accompanied by enhancing NO production, induced by LPS/IFN-γ(Fig. 4A and B). NOS inhibitor NLA attenuates both LPS (20 ng/ml)/IFN-γ (10 ng/ml)- or LPS (20 ng/ml)/IFN-γ (10 ng/ml) plus nicotine (1.6 mM)-induced decreases in the viable cell numbers by trypan blue exclusion in association with decreasing NO production. These data provide evidences to demonstrate the cytotoxic effect induced by LPS (20 ng/ml)/IFN-γ (10 ng/ml) was enhanced by nicotine addition in RAW264.7 macrophages.

4. Discussion

Data of the present study provide scientific evidences to link nicotine and inflammatory responses. Briefly, nicotine enhances LPS/IFN-γ-induced NO production with enhancing iNOS protein expression without affecting iNOS enzyme activity and iNOS
null
was able to enhance LPS/IFN-γ-induced apoptosis in macrophages by elevating NO production.

In the present study, nicotine enhances NO production in LPS-treated cells (at the dose of 20 ng/ml), however the expression of iNOS protein was not obviously elevated in the same condition. This experiment has been repeated at least 6 times, and similar results were obtained. In contrast, at lower doses of LPS (2.5, 5 ng/ml), nicotine showed significant stimulation on iNOS protein expression induced by LPS/IFN-γ. Results of cytotoxicity assay showed that LPS at the dose of 20 ng/ml exhibited cytotoxic effect in cells, and nicotine potentiated the cytotoxicity. Based on these data, we proposed that no significant stimulation of nicotine on iNOS protein expression at higher doses (10 ng/ml or 20 ng/ml) of LPS-treated cells might be due to the cytotoxic effect on cells. Therefore, enhancement of iNOS protein expression was involved in the stimulatory effects of nicotine on LPS/IFN-γ-induced NO production and cytotoxicity.

In summary, we identified that nicotine enhances LPS/IFN-γ-induced cytotoxicity by stimulation of NO production. The stimulatory mechanism of nicotine mediated by enhancing iNOS gene expression, but not related to iNOS enzyme activity and iNOS protein stability. It is suggested that nicotine from smoke may play as a deleterious factor in inflammatory process through potentiating cytotoxicity and NO production.

Acknowledgements

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References


