Nicotine Inhibits Cardiac Apoptosis Induced by Lipopolysaccharide in Rats

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OBJECTIVES
Apoptosis develops in several heart diseases, but the therapeutic options are limited. It was hypothesized that nicotine, which inhibits apoptosis in several cells, inhibits cardiac apoptosis induced by lipopolysaccharide (LPS).

BACKGROUND
Over-the-counter nicotine produces sustained levels (10 to 25 ng/ml) that may be antiapoptotic. Low levels of LPS induce apoptosis by activating tissue renin-angiotensin to stimulate angiotensin II, type 1 (AT1) receptors in cardiac myocytes.

METHODS
Adult Sprague-Dawley rats were pretreated with nicotine (6 mg/kg/day) or saline for seven to ten days (miniosmotic pumps). The LPS (1 mg/kg) was injected intravenously. Toll-like receptor 4 (TLR4) and angiotensinogen messenger ribonucleic acid (mRNA) were measured in the heart after 0, 4, 8, 16, and 24 h. Cardiac apoptosis was measured by terminal deoxy-nucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining after 24 h. In vitro effects of LPS (10 ng/ml, 24 h) were studied in cardiac myocytes isolated from rats pretreated with nicotine for 7 to 10 days, or after pre-exposing myocytes to nicotine (15 ng/ml) for 1, 4, 16, or 24 h.

RESULTS
Neither nicotine nor LPS affected systolic blood pressure. The LPS increased cardiac apoptosis after 24 h in saline-treated, but not nicotine-treated rats, despite similar increases in cardiac TLR4 and angiotensinogen mRNA over 8 to 16 h. The LPS-induced apoptosis was blocked by pre-exposing myocytes to nicotine for 4 to 24 h (partial inhibition after 1 h). Nicotine did not inhibit apoptosis induced by angiotensin II (100 nM, 24 h).

CONCLUSIONS
Therapeutic levels of nicotine inhibit LPS-induced cardiac apoptosis. This occurs after LPS increases TLR4 and angiotensinogen mRNA, but proximal to AT1 receptor activation. Nicotine may be a novel inhibitor of cardiac apoptosis in conditions associated with circulating LPS (e.g., decompensated heart failure, acute and chronic infections). (J Am Coll Cardiol 2003;41:482–8) © 2003 by the American College of Cardiology Foundation.

Nicotine inhibits apoptosis in several cells induced by diverse stimuli (1–5). This may have adverse effects since apoptosis normally removes excessive or potentially dangerous cells (e.g., predispose to cancer in smokers). This effect of nicotine may be advantageous in the heart, where apoptosis is undesirable. Cardiac myocytes are terminally differentiated cells with a limited (but not absent [6]) capacity to regenerate. Cardiac apoptosis is increased in multiple forms of heart failure and may contribute to the progression of disease (7). The therapeutic options for inhibiting apoptosis in heart disease are limited. It is unknown if nicotine has antiapoptotic effects on cardiac cells. The therapeutic use of nicotine for inhibiting apoptosis has not been previously investigated.

It was hypothesized that therapeutic levels of nicotine inhibit cardiac apoptosis induced by lipopolysaccharide (LPS). The LPS from gram-negative bacteria induces cardiac apoptosis in sepsis (8,9). Low levels of LPS also induce cardiac apoptosis (10). We found that a single dose of LPS that causes no distress and does not affect blood pressure is sufficient to increase cardiac apoptosis for days (10). The heart is sensitive to LPS because of abundant expression of the LPS receptor, toll-like receptor 4 (TLR4), on cardiac myocytes (11). This has important implications since plasma LPS levels in pg/ml to low ng/ml range occur in several conditions (12), including decompensated heart failure (13), chronic infections (14), and periodontitis (15). Inhibiting cardiac apoptosis may minimize injury to the heart as an innocent bystander in these common conditions.

Nicotine may be a novel therapy to inhibit cardiac apoptosis. Nicotine is a safe, over-the-counter medication used in smoking cessation and to treat ulcerative colitis, Alzheimer’s disease, Parkinson’s disease, and Tourette’s syndrome (16,17). Nicotine patches and gum produce sustained blood nicotine levels of 10 to 25 ng/ml for over 24 h (17). Although nicotine has adverse cardiovascular effects when combined with cardiotoxic substances in smoking (18), nicotine can be used safely as a medication even in patients with cardiac disease (18–20).

This study examines if therapeutic levels of nicotine inhibit cardiac apoptosis using in vivo and in vitro models of LPS (10). Rats were exposed to nicotine for one week with miniosmotic pumps, or isolated cardiac myocytes were exposed to nicotine in vitro using levels comparable to those...
achieved with commercially available preparations (e.g., nicotine gum and transdermal patches) (17). The results from this study may expand the therapeutic uses of nicotine (16) to include inhibition of LPS-induced cardiac apoptosis.

**METHODS**

Experiments were performed in accordance with institutional guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Systolic blood pressure was measured by tail cuff in adult Sprague Dawley rats (250 to 400 g, either gender). Rats were anesthetized with ketamine (100 mg/kg intramuscularly) and xylazine (8 mg/kg intramuscularly) to implant Alzet miniosmotic pumps (model 2002, Alza Corp., Mountain View, California) subcutaneously through 1 to 2 cm incisions under sterile conditions. The pumps were filled with saline or nicotine dihydrochloride to infuse solution at 0.5 μl/h, with delivery of nicotine at 6 mg/kg/day (21). The rats were allowed to recover for one week to ensure full recovery from anesthesia, surgery, and to allow complete wound healing. This minimized the risk of systemic exposure to bacteria from the wound site, which may induce LPS tolerance in the heart (22). After 7 to 10 days, 1 mg/kg LPS (Escherichia coli 055, LPS no. B5, lot 2039F, List Biological Laboratories, Cambell, California) or vehicle (saline) were injected into the tail vein. This dose of LPS does not alter blood pressure (10) and in a preliminary study, heart rate and left ventricular function (measured by echocardiography) were unaffected at 24 h (23). Rats were euthanized 24 h after injections; the heart was excised and fixed in 4% formalin phosphate buffered saline for TUNEL assays with over 2,000 cells scored for each group. In protocols examining nicotine in vitro, cardiac myocytes were preincubated with nicotine (15 ng/ml) or vehicle for 1 to 24 h, prior to LPS (10 ng/ml) exposure for an additional 24 h.

Cardiac levels of TLR4 and angiotensinogen messenger ribonucleic acid (mRNA) were measured from heart sections placed in RNAlater (Qiagen, Inc., Valencia, California) to preserve ribonucleic acid (RNA) integrity and stored at −70°C. Total RNA was isolated with TRIzol reagent according to the manufacturer’s protocol (Life Technologies, Rockville, Maryland). The RNA was treated with RNase-free DNase I (Roche Molecular Biochemicals, Indianapolis, Indiana), precipitated and 10 μg used for first strand complementary deoxyribonucleic acid (cDNA) synthesis with SuperScript II reverse transcriptase (Life Technologies).

The relative levels of TLR4 and angiotensinogen mRNA were measured with a Perkin-Elmer ABI Prism 7700 and Sequence Detection System software (Foster City, California). Equal amounts of cDNA were used in duplicate and amplified with the Taqman Master Mix provided by Perkin-Elmer. The sequences used for the rat angiotensinogen were from GenBank Accession # L00090, exon 5, nucleotides 157 to 176, 213 to 235, forward and reverse primers, respectively, and 178 to 202 for the probe. The rat TLR4 sequences were from Accession #AF057025, nucleotides 2107 to 2131, 2158 to 2177, and 2133 to 2156 as above. Amplification efficiencies were validated and normalized against 18S ribosomal RNA and relative increases were calculated using the Standard Curve Method for quantitation (Ref: ABI Prism 7700 SDS User Bulletin #2 P/N 4303859 Rev. A).
Statistical analyses were performed with one- or two-way analysis of variance (ANOVA) to compare results from different rats. One- or two-way repeated measures ANOVA were used when myocytes from the same rat underwent different treatments. Posthoc comparisons were performed by Student–Newman–Keuls methods. Results are presented as mean ± SE with p < 0.05 used to indicate statistical significance.

RESULTS

Miniosmotic pumps delivered nicotine (6 mg/kg/day) or saline in vivo to rats (n = 12 each) for 7 to 10 days. In pilot studies, three rats treated with nicotine-filled pumps had blood nicotine levels of 21, 30, and 52 ng/ml, while nicotine was nondetectable in three rats treated with saline-loaded pumps. Systolic blood pressure (tail cuff) did not change two days or one week after implanteing the pumps (Table 1). After one week of pretreatment, LPS (1 mg/kg) or vehicle was injected into the tail vein of rats with saline pumps (six rats in the saline-vehicle or control group, six rats in the saline-LPS group) and nicotine-filled pumps (five rats in the nicotine-vehicle group, seven rats in the nicotine-LPS group). None of the rats developed any signs of distress or change in systolic blood pressure over the ensuing 24 h (Table 1).

Figure 1 shows TUNEL staining in the left ventricle 24 h after LPS or vehicle injections. The rate of TUNEL positive stained nuclei per 10^6 nuclei was calculated based on sampling approximately 10^5 nuclei from each heart. The LPS increased positive TUNEL stained nuclei in rats pretreated with saline-filled pumps (saline-LPS compared with saline-vehicle), but not in rats pretreated with nicotine-filled pumps (nicotine-LPS compared with nicotine-vehicle) (p < 0.05, two-way ANOVA, p < 0.01 for interaction between nicotine and LPS, posthoc analysis with multiple comparison procedures by Student–Newman–Keuls method). Thus, 7 to 10 days of nicotine exposure blocked cardiac apoptosis induced by LPS in vivo.

Since LPS activates multiple cells to release a myriad of endogenous mediators in vivo, the direct cardiac effects of LPS can be discerned with isolated cardiac myocytes exposed to LPS in vitro (10). Cardiac myocytes isolated from rats after seven to ten days of nicotine (6 mg/kg/day) or saline infusions were exposed to LPS (10 ng/ml) or vehicle in vitro for 24 h. Figure 2 shows that LPS in vitro had similar effects as LPS in vivo. The LPS increased TUNEL staining in myocytes from saline-treated rats, but not in myocytes from nicotine-treated rats (p < 0.05, n = 13, two-way repeated measures ANOVA, p < 0.001 for interaction between nicotine and LPS, posthoc analysis with multiple comparison procedures by Student–Newman–Keuls method).

Cardiac myocytes are activated by LPS through the LPS receptor, TLR4 (11) and cardiac renin-angiotensin induces apoptosis through angiotensin II receptor, type 1 (AT_1) receptors (10). The time course for LPS (1 mg/kg intrave-

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**Table 1. Systolic Blood Pressure**

<table>
<thead>
<tr>
<th>Time</th>
<th>Control (Saline-vehicle)</th>
<th>LPS (Saline-LPS)</th>
<th>Nicotine (Nicotine-vehicle)</th>
<th>Nicotine-LPS (Nicotine-LPS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−1 week (before pump)</td>
<td>126 ± 5</td>
<td>122 ± 2</td>
<td>125 ± 2</td>
<td>123 ± 1</td>
</tr>
<tr>
<td>−5 days (2 days after pump)</td>
<td>127 ± 5</td>
<td>122 ± 2</td>
<td>126 ± 2</td>
<td>122 ± 2</td>
</tr>
<tr>
<td>Baseline = 0 (1 week after pump)</td>
<td>122 ± 3</td>
<td>121 ± 2</td>
<td>128 ± 0</td>
<td>124 ± 1</td>
</tr>
<tr>
<td>15 min</td>
<td>128 ± 3</td>
<td>119 ± 2</td>
<td>122 ± 2</td>
<td>124 ± 2</td>
</tr>
<tr>
<td>30 min</td>
<td>n/a</td>
<td>121 ± 1</td>
<td>n/a</td>
<td>125 ± 2</td>
</tr>
<tr>
<td>45 min</td>
<td>n/a</td>
<td>121 ± 1</td>
<td>n/a</td>
<td>124 ± 0</td>
</tr>
<tr>
<td>1 h</td>
<td>125 ± 4</td>
<td>119 ± 2</td>
<td>n/a</td>
<td>123 ± 2</td>
</tr>
<tr>
<td>2 h</td>
<td>128 ± 3</td>
<td>121 ± 1</td>
<td>122 ± 2</td>
<td>124 ± 2</td>
</tr>
<tr>
<td>4 h</td>
<td>n/a</td>
<td>119 ± 3</td>
<td>n/a</td>
<td>129 ± 3</td>
</tr>
<tr>
<td>24 h</td>
<td>124 ± 2</td>
<td>120 ± 2</td>
<td>128 ± 2</td>
<td>126 ± 2</td>
</tr>
</tbody>
</table>

Systolic blood pressure (mm Hg, mean ± SE) measured in four groups of rats before, 2 days and 1 week after implanting miniosmotic pumps to deliver nicotine (6 mg/kg/day) or saline. After 1 week of pretreatment (baseline time = 0), blood pressure before and after injecting lipopolysaccharide (LPS) (1 mg/kg) or vehicle into the tail vein (n/a = not applicable, not measured). There was no change in systolic blood pressure in any group at any time after any intervention (p = ns, one-way repeated measures analysis of variance).

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**Figure 1.** Terminal deoxy-nucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining in the left ventricle (mean ± SE, n = 5 to 7) of rats pretreated for 7 to 10 days with nicotine (6 mg/kg/day) versus saline (miniosmotic pump) and then injected with intravenous lipopolysaccharide (LPS) (1 mg/kg) versus vehicle. After 24 h, LPS increased TUNEL staining in saline pretreated rats (*p < 0.05, saline-LPS compared with other three groups).
nously) to activate TLR4 and angiotensinogen (precursor of angiotensin I) mRNA in the heart was measured in rats pretreated with 7 to 10 days of nicotine (6 mg/kg/day) or saline. Figure 3 shows that LPS increased cardiac TLR4 mRNA over 8 to 16 h (p < 0.05 compared with 0, 4, or 24 h, post-hoc comparisons by Student-Newman-Keuls method), with no difference in response between nicotine and saline-treated rats (p > 0.83 for nicotine vs. saline pump, two-way ANOVA). Figure 4 demonstrates increased cardiac angiotensinogen mRNA 8 h after LPS (p < 0.05 compared with 24 h, posthoc comparisons by Student-Newman-Keuls method), with no difference between nicotine and saline-treated rats (p = 0.89 for nicotine vs. saline, two-way ANOVA). Thus, nicotine does not decrease the sensitivity of cardiac myocytes to respond to LPS or events leading up to the activation of angiotensinogen mRNA.

Since LPS induces apoptosis by activating cardiac AT₁ receptors (10), the response to LPS (10 ng/ml, 24 h) and Ang II (100 nM, 24 h) were compared in cardiac myocytes isolated from nicotine and saline-exposed rats. In Figure 5, both LPS and Ang II increased TUNEL staining compared with vehicle in myocytes from saline-treated rats (p < 0.05, one-way repeated measures ANOVA, n = 10, posthoc comparisons by Student-Newman-Keuls method). In myocytes from nicotine treated rats, only Ang II and not LPS increased TUNEL staining (p < 0.01, n = 11, posthoc comparisons by Student-Newman-Keuls method). Thus, nicotine inhibited LPS-induced apoptosis proximal to the activation of AT₁ receptors in cardiac myocytes.

The antiapoptotic effects of nicotine in vivo may be due to direct effects on cardiac myocytes (which are not known to possess nicotinic receptors) or secondary to nicotine activation of noncardiac cells. To address this issue, cardiac myocytes isolated from naïve animals (without miniosmotic pumps) were exposed to nicotine in vitro (15 ng/ml) for 1 h, and then treated with LPS (10 ng/ml) or vehicle for another
This study demonstrates that therapeutic levels of nicotine inhibit cardiac apoptosis induced by LPS. Nicotine has direct antiapoptotic effects on cardiac myocytes since nicotine exposure in vitro for 4 to 24 h was as effective as in vivo exposure for 7 to 10 days. The antiapoptotic effects of nicotine develop within 1 h, are complete after 4 h, and occur with nicotine levels (15 ng/ml in vitro, 20 to 50 ng/ml in vivo) comparable to those achieved with over-the-counter preparations (17). These results demonstrate a novel therapeutic use for nicotine to inhibit cardiac apoptosis.

Nicotine did not nonspecifically decrease the response of cardiac myocytes to LPS. Initial steps in LPS-induced activation, including the increase in cardiac TLR4 (LPS receptor) and angiotensinogen mRNA were unaffected by 7 to 10 days of nicotine exposure. Nicotine does not inhibit Ang II induced apoptosis, a key step in LPS-induced apoptosis (10). These data indicate that nicotine inhibits LPS activation of cardiac renin-angiotensin (distal to angiotensinogen mRNA, but proximal to the activation of AT1 receptors) in myocytes to inhibit apoptosis. Further investigation into the effects of nicotine on the signaling pathways and transcription factors of LPS activated TLR4 are required to elucidate the mechanism of nicotine inhibition during LPS induced cardiac myocyte apoptosis.

These results raise the possibility that nicotine may inhibit apoptosis in other models involving cardiac renin-angiotensin. For example, cardiac apoptosis is induced by local renin-angiotensin activation with stretch of cardiac myocytes (24) in rat models of spontaneous hypertension (25–27) and diabetic cardiomyopathy (28). Patients with essential hypertension have increased cardiac apoptosis that is attenuated by an AT1 receptor blocker (29). Future studies are needed to determine if nicotine inhibits apoptosis in these conditions.

The direct cardiac effects of nicotine are intriguing, since cardiac myocytes are not known to possess nicotinic receptors. Nicotinic receptors for acetylcholine (nAChRs) are a large family of transmembrane proteins with a pentameric structure (30). In the heart, nAChRs are localized primarily in postganglionic neurons of autonomic nerves (18). In neuronal cells, nicotine (e.g., 10 μM) inhibits apoptosis by

### Table 2. TUNEL Staining in Isolated Cardiac Myocytes

<table>
<thead>
<tr>
<th>Pretreatment Time</th>
<th>Control (Saline-vehicle)</th>
<th>LPS (Saline-LPS)</th>
<th>Nicotine (Nicotine-vehicle)</th>
<th>Nicotine-LPS (Nicotine-LPS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 h (n = 10)</td>
<td>3.3 ± 0.5%</td>
<td>5.7 ± 0.8%*</td>
<td>2.9 ± 0.4%</td>
<td>4.7 ± 0.5%†</td>
</tr>
<tr>
<td>4 h (n = 10)</td>
<td>2.6 ± 0.4%</td>
<td>4.0 ± 0.4%*</td>
<td>2.8 ± 0.3%</td>
<td>3.0 ± 0.4%</td>
</tr>
<tr>
<td>16 h (n = 6)</td>
<td>3.4 ± 0.3%</td>
<td>4.7 ± 0.5%*</td>
<td>3.7 ± 0.6%</td>
<td>3.0 ± 0.5%</td>
</tr>
<tr>
<td>24 h (n = 4)</td>
<td>3.0 ± 0.9%</td>
<td>4.8 ± 1.1%*</td>
<td>3.2 ± 0.9%</td>
<td>2.9 ± 1.0%</td>
</tr>
</tbody>
</table>

Percent terminal deoxy-nucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) positive staining (mean ± SE) in cardiac myocytes pretreated with saline or nicotine (15 ng/ml) for 1 to 24 h, and then treated with vehicle or lipopolysaccharide (LPS) (10 ng/ml) for an additional 24 h. Lipopolysaccharide increased TUNEL staining in myocytes pretreated with saline, which was completely blocked by 4 to 24 h of nicotine pretreatment (*p < 0.05 for LPS group compared with other three groups, two-way repeated measures analysis of variance; posthoc comparisons by Student-Newman-Keuls method). One hour of nicotine pretreatment attenuated, but did not completely abolish LPS effects (†p < 0.05 for Nicotine-LPS group compared with other three groups).
activating nAChR (2,31). However, nicotine also inhibits apoptosis by nonreceptor-mediated mechanisms in several cells with an inhibitory concentration of drug with 50% of maximum effectiveness (IC_{50} = 50 to 100 μM nicotine (1)). Low μM levels of nicotine inhibit apoptosis in human (32) and cancer cell lines (5,33) and regulate several apoptotic genes in endothelial cells (34). It is unknown if low levels of nicotine (15 ng/ml = 92 nM) inhibit LPS-induced apoptosis in cardiac myocytes by a nonreceptor or nAChR-mediated mechanism.

Nicotine has non–nAChR-mediated effects on cardiac fibroblasts (35), sinoatrial node cells (36), and ventricular myocytes. Nicotine (10 nM to 100 μM) inhibits transient outward (I_{to}, IC_{50} 40 nM nicotine) and inward rectifier (I_{Kr}) potassium currents and lengths action potential duration in ventricular myocytes (37,38). This is not reversed by nAChR antagonist (mecamylamine), muscarinic acetylcholine receptor antagonist (atropine), or a beta-adrenergic receptor blocker indicating a direct effect of nicotine on ion channels (38). Thus, low levels of nicotine may have direct effects on cardiac myocytes, independently of nicotinic receptors.

Nicotine has been used therapeutically (e.g., in ulcerative colitis and smoking cessation programs) (16) with nicotine gum and transdermal patches producing sustained blood nicotine levels of 10 to 25 mg/ml for hours (17,18). Nicotine has a 2 to 3 h elimination half-life, with a 20 h terminal half-life as nicotine is released from body tissues (16). Cotinine, the major metabolite of nicotine, has a 16-h half-life. Cotinine also inhibits apoptosis (1). Since nicotine is safe even in patients with cardiac disease (18–20), over-the-counter preparations may be useful to produce nicotine levels that effectively inhibit LPS-induced cardiac apoptosis.

These results may be applicable in conditions associated with circulating LPS. Inhibiting apoptosis may attenuate myocyte losses in sepsis (8,9). In patients with decompensated heart failure, elevated plasma LPS levels (13) may induce apoptosis and contribute to further cardiac damage. The LPS is elevated in patients with cirrhosis, pancreatitis, hemodialysis (12), abdominal surgery (39), cardiopulmonary bypass surgery (40), and colonoscopy (41). Nicotine may provide a convenient therapy to protect the heart from apoptosis during transient elevations in LPS in these conditions and procedures.

Periodontitis may be an important source of systemic LPS. Elevated LPS levels occur with oral procedures (e.g., scaling) or mastication in patients with severe periodontal disease (15). This produces systemic inflammatory effects with elevated levels of C-reactive protein directly related to the severity of periodontitis (42,43). Chronic systemic effects develop, as periodontal disease has been associated with increased carotid artery intima-media wall thickness in the Atherosclerosis Risk In Communities (ARIC) study (44). Since LPS induces cardiac apoptosis, recurrent episodes of subclinical exposure to LPS may have significant cumulative effects. Nicotine may prevent cardiac apoptosis in this common clinical condition.

In summary, nicotine inhibits LPS-induced cardiac apoptosis. Nicotine has direct effects on cardiac myocytes to inhibit apoptosis within an hour (complete inhibition after 4 h) with nicotine levels comparable to those achieved with over-the-counter preparations. Nicotine inhibits LPS activation of cardiac angiotensin, which stimulates AT_1 receptors in myocytes. Nicotine may be a novel therapy to inhibit cardiac apoptosis during periods of increased susceptibility with exposure to LPS.

**REFERENCES**