The Expression and Functional Role of Nicotinic Acetylcholine Receptors in Rat Adipocytes

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ABSTRACT

To clarify whether nicotine has a direct effect on the function of adipocytes, we evaluated nicotinic acetylcholine receptor (nAChR) expression in adipocytes by reverse transcriptase-polymerase chain reaction (RT-PCR) and immunocytochemistry and the direct effects of nicotine on the production of adipocytokines by enzyme-linked immunosorbent assay and Western blot analysis. Receptor binding assays were performed using [3H]nicotine. RT-PCR studies revealed that α1–7, 9, 10, β1–4, δ, and ε subunit mRNAs are expressed in adipocytes. Immunocytochemical experiments also suggested the presence of α7 and β2 subunits. The receptor binding assay revealed a binding site for nicotine (Kd = 39.2 × 10⁻⁹ M) on adipocytes. Adipocytes incubated with nicotine for 12 and 36 h released tumor necrosis factor-α (TNF-α), adiponectin, and free fatty acid (FFA) into the medium in a dose-dependent manner with increasing nicotine concentration from 6 × 10⁻⁶ to 6 × 10⁻⁴ M. However, TNF-α protein levels in adipocytes incubated for 12 and 36 h decreased in a dose-dependent manner with increasing nicotine concentration from 6 × 10⁻⁶ to 6 × 10⁻⁴ M. These results show that adipocytes have functional nAChRs and suggest that nicotine reduces TNF-α protein production in adipocytes through the activation of nAChRs. Nicotine may temporarily lower insulin sensitivity by stimulating the secretion of TNF-α and FFA, whereas long-term direct stimulation of nAChRs by nicotine in addition to autonomic nervous system stimulation may contribute to better insulin sensitivity in vivo through a modulated secretion of adipocytokines.

Nicotinic acetylcholine receptors (nAChRs) are a family of ionotropic receptor proteins formed by five homologous or identical subunits and are involved in signal transduction between neurons and muscle cells (Albuquerque et al., 1997). nAChRs are divided into muscle (α1, β1, γ, ε, and δ) and neuronal AChRs (α2–10 and β2–4), and neuronal AChRs are further subdivided into those that form homomeric receptors when expressed in heterologous systems (α7–10) and those that form heteromeric receptors (α2–6 and β2–4 in different combinations) (Gotti et al., 2000). The α8 nAChR subunit has not been studied since it is only expressed in the chicken (Rubbo et al., 1994). The muscle receptor subtypes are comprised of α1, β1, δ, and γ (embryonal) or α1, β1, δ, and ε (adult) subunits (Tassonyi et al., 2002). ACh and its receptors are among the best characterized neurotransmitter/receptor systems (Albuquerque et al., 1997). Cholinergic neurotransmission is used in a variety of neuronal systems and in a broad range of animals ranging from invertebrates to mammals (Lindstrom et al., 1995). The main function of this receptor family is to transmit signals through the neurotransmitter acetylcholine at neuromuscular junctions and in the central and peripheral nervous systems (Marubio and Changeux, 2000). In addition, non-neuronal cells may express a functional nAChR. Human bronchial epithelial cells and aortic endothelial cells express a functional α3 subtype nAChR, which modulates cell shape and affects cell-to-cell contact (Maus et al., 1998). Human skin keratinocytes express functional nAChRs of different subtypes, including α3, α9, and possibly α7 subunits (Nguyen et al., 2000). Specific receptors for nicotine are present on lymphocytes in humans (Hoss et al., 1986) and rats (Maslinski et al., 1992); however, whether functional nAChRs exist in adipocytes is unclear.

Excess adipose tissue leads to insulin resistance, thereby increasing the risk of type 2 diabetes mellitus and cardiovascular disease (Saltiel and Kahn, 2001). Adipocytes release cytokines that influence energy expenditure, insulin sensitivity, vasomotor tone, and fibrinolysis, and obesity perturbs the regulation of these cytokines. Physiologically active substances produced in adipose tissues, called adipocytokines and free fatty acid (FFA), play a role in the progression of insulin resistance in obesity (Matsuzawa et al., 1999). Adipocytes...
Adipocytes play a role in systemic energy homeostasis by producing molecules such as leptin, plasminogen activator inhibitor-1, and several cytokines including tumor necrosis factor-α (TNF-α) and interleukin-6 that influence key metabolic pathways (Mohamed-Ali et al., 1998). TNF-α is a mediator of lipid metabolism, adipocyte differentiation, and in vivo insulin sensitivity (Hotamisligil et al., 1993). TNF-α is expressed in macrophages and adipocytes and is substantially elevated in obesity in rodents (Hotamisligil et al., 1993) and humans (Hofmann et al., 1994).

In contrast to other adipocytokines, adiponectin is proposed to play a role in the regulation of energy homeostasis and insulin sensitivity (Hu et al., 1996). Adiponectin levels are depressed in obesity and associated comorbidities such as type 2 diabetes. Decreased expression of adiponectin correlates with insulin resistance. Adiponectin is a hormone secreted from adipocytes and has anti-diabetic and antiatherogenic effects (Yamauchi et al., 2003). The mechanism of regulation of adiponectin secretion remains to be clarified.

In a previous study, we reported that oral nicotine administration reduces insulin resistance in obese diabetic rats possibly through decreased expression of TNF-α in visceral fat tissues and reduced hepatic glucose release (Liu et al., 1989; Witzemann et al., 1990; Rohwedel et al., 1995; Liu et al., 1998; Tseng et al., 2001). Glyceroldehyde-3-phosphate dehydrogenase (G3PDH) (Toyobo Engineering) was used as an internal control to verify the quality of each RNA sample and its subsequent RT-PCR analysis. The RT-PCR cycling profiles using a Thermal Cycler (GeneAmp PCR System 9600; PerkinElmer Life and Analytical Sciences, Boston, MA) were as follows: 1 cycle at 50°C for 30 min, 1 cycle at 94°C for 2 min, 35 cycles at 94°C for 1 min (46–57°C, respectively, in Table 1), 72°C for 1 min, and a final cycle at 72°C for 7 min. A 12-μl aliquot of each sample was electrophoresed on a 2.4% agarose gel containing 0.6 mg/ml ethidium bromide.

Immunofluorescence Microscopy of α7 and β2 Subunits in Cultured Adipocytes. Adipocytes grown on Labtek 4-well slides were cooled on ice and washed in ice-cold PBS. The cells were fixed with 4% paraformaldehyde in PBS for 20 min and permeabilized with 0.1% Triton X-100 in PBS for 20 min at room temperature. The slides were then blocked for 20 min with 5% normal goat serum in PBS, after which they were incubated for 1 h in mouse anti-α7 antibody (1:200 dilution in PBS, mAb 306; Sigma-Aldrich, St. Louis, MO) or rabbit anti-β2 antibody (1:200 dilution in PBS; Santa Cruz Biotechnology Inc., Santa Cruz, CA) at room temperature, respectively. The cells were then washed three times for 5 min with ice-cold PBS and incubated for 1 h at room temperature with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (1:200 dilution in PBS; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) or fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:200 dilution in PBS, Santa Cruz Biotechnology Inc., respectively). They were then washed three times with PBS for 5 min each, mounted in PermaFluor mounting medium (Thermo Shandon, Pittsburgh, PA), and observed with a Fluoview FV500 confocal laser scanning microscope (Olympus Optical Co., Ltd., Tokyo, Japan).

Materials and Methods

Cell Culture. Adipocytes were obtained from 8-week-old male Wistar rat abdominal subcutaneous fat tissue by collagenase digestion using a kit (Toyobo Engineering, Osaka, Japan). Preadipocytes were seeded into 75-cm² flasks (Falcon; BD Biosciences, Franklin Lakes, NJ) in medium consisting of Dulbecco’s modified Eagle’s medium supplemented with 1% fetal calf serum and antibiotics (penicillin 100 units/ml, streptomycin 0.1 mg/ml, and nystatin 50 units/ml). The cultures were kept at 37°C in an atmosphere of 5% CO₂ in air. Preadipocytes were grown to confluence in preadipocyte differentiation medium (TAGM-250, Toyobo Engineering) for the first 5 days. The medium was changed after 1 day and thereafter every 2 days. Preadipocytes were differentiated in vitro to mature adipocytes using preadipocyte differentiation medium (TADM-250, Toyobo Engineering). Differentiation to mature adipocytes was confirmed by the microscopic appearance of intracellular lipid droplets. A cell count was performed using a hemocytometer, and cell viability was assessed by the 0.4% trypan blue solution (Sigma-Aldrich, Tokyo, Japan) dye exclusion method. We used a similar procedure for plating cells onto 4-well glass slides (Nalge Nunc International, Naperville, IL) for immunofluorescence assays.

Conditioned Media. Preadipocytes seeded at 2 × 10⁵ cells/cm² into 24-well plates (Falcon; BD Biosciences) were cultured to confluence. Under the culture conditions, preadipocytes differentiated into mature adipocytes. Adipocytes were incubated in medium for 12 and 36 h with nicotine (nicotine tartrate dihydrate dissolved in Dulbecco’s modified Eagle’s medium) with a concentration ranging between 6 × 10⁻⁸ and 6 × 10⁻⁴ M. The conditioned medium was then removed, centrifuged for 5 min at 4°C at 1000 rpm, and the medium and cells were separately stored at −80°C.

Assays for TNF-α, Adiponectin, and FFA. Using a specific antibody, medium TNF-α and adiponectin were measured by enzyme-linked immunosorbent assay using kits obtained from BioSource International, Inc. (Camarillo, CA) and Otsuka Pharmaceutical Co., Ltd (Tokyo, Japan), respectively. The medium FFA was measured by spectrophotometric assays using a commercially available kit (Wako Bioproducts, Richmond, VA).

Nicotinic Acetylcholine Receptors in Rat Adipocytes

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Nicotinic Acetylcholine Receptors in Rat Adipocytes
Results

Detection of nAChR Subunits Gene in Cultured Adipocytes by RT-PCR. We investigated the expression of mRNA for \( \alpha, \beta, \gamma, \delta, \) and \( \epsilon \) nAChR subunits in cultured adipocytes by RT-PCR using specific primers for the \( \alpha \) (1–7, 9, and 10), \( \beta \) (1–4), \( \gamma, \delta, \) and \( \epsilon \) nAChR subunits (Table 1). Ethidium bromide staining of the gel shows the presence of nAChR subunits (1–7, 9, 10, 1–4, \( \delta \), and \( \epsilon \)), G3PDH, and molecular weight markers (M) obtained by RT-PCR as seen in Fig. 1. The data show that rat adipocytes express \( \alpha \) (1–7, 9, and 10), \( \beta \) (1–4), \( \gamma, \delta, \) and \( \epsilon \) subunits. Amplification yielded PCR products of expected sizes: 288 bp for \( \alpha_1 \), 300 bp for \( \alpha_2 \) and \( \alpha_9 \), 209 bp for \( \alpha_10 \), 57 bp for \( \beta_3 \) and \( \beta_4 \), 55 bp for \( \delta \) and \( \epsilon \) subunits. Amplification of the G3PDH gene product (452 bp) was used as an internal control to verify the quality of each RNA sample and its subsequent RT-PCR. However, in the present study gene-specific primers for \( \delta \) and \( \epsilon \) subunits (Table 1) amplified larger products in size (35 bp; \( \epsilon \), 402 bp) than expected from previous findings in skeletal muscle (35, 235/291 bp; \( \epsilon \), 222/340 bp) (Fig. 1). The \( \gamma \) nAChR subunit was not expressed (Fig. 1).

Immunocytochemical Studies on Adipocytes Using Specific Antibodies Against \( \alpha_7 \) and \( \beta_2 \) Subunits. To further ensure the expression of nAChR subunits in adipocytes, we performed an immunocytochemical analysis using specific antibody against \( \alpha_7 \) or \( \beta_2 \) subunits. The presence of antibodies bound to the cells was revealed by the binding of fluorescein isothiocyanate-labeled goat anti-rabbit IgG antibody. A representative field is shown in Fig. 2, A and B. \( \alpha_7 \) and \( \beta_2 \) subunit immunoreactivity was observed on the mem-
branes and in the cytoplasm of the adipocytes, whereas no immunoreactivity was detected in the nuclei.

[^3H](—)Nicotine Binding to Adipocytes. Specific binding of[^3H](—)nicotine (5 × 10^-9 M) to adipocytes decreased in a dose-dependent manner with increasing (—)nicotine concentration from 0 to 1 × 10^-8 M and was significant for unlabeled (—)nicotine concentrations from 1 × 10^-4 to 1 × 10^-3 M (Fig. 3).

Saturation Analysis of[^3H](—)Nicotine. Saturation studies on adipocytes using concentrations of[^3H](—)nicotine from 1 × 10^-10 M to 1 × 10^-7 M revealed the presence of saturable binding sites. Nonlinear regression analysis of nicotine binding yielded a $K_d$ value of 39.15 ± 2.67 × 10^-9 M and a $B_{max}$ of 43,236 ± 1152 sites/cell for the affinity site by Scatchard analysis as seen by the curved appearance of the graph (Fig. 4).

Adipocyte Number and Histological Examination of Culture Adipocytes. There was no significant difference in the counts of viable cells between the nicotine and control groups from 6 × 10^-8 to 6 × 10^-4 M for 12 and 36 h (Table 2). The histological forms and size of adipocytes were not significantly different in both groups from 6 × 10^-8 to 6 × 10^-4 M for 12 and 36 h (not shown).

Adiponectin Concentration in the Medium. Adipocytes incubated with (—)nicotine for 12 and 36 h released more adiponectin than the control into the culture medium. The release of adiponectin was augmented in a dose-dependent manner with increasing (—)nicotine concentration from 6 × 10^-8 to 6 × 10^-4 M. Moreover, nicotine-stimulated adiponectin secretion for 36 h was significantly higher than that for 12 h at the same nicotine-stimulated concentration (Fig. 5).

TNF-α and FFA Concentration in the Medium. Adipocytes stimulated with (—)nicotine released more TNF-α and FFA than the control for 12 and 36 h into the culture medium. The release of TNF-α and FFA was augmented in a
TABLE 2
Counts of viable cells in the nicotine and control groups
All values are presented as the means ± S.E.M.

<table>
<thead>
<tr>
<th>Nicotine Concentration</th>
<th>12 h</th>
<th>36 h</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>$10^6$ cells/cm²</td>
<td>$10^6$ cells/cm²</td>
</tr>
<tr>
<td>0</td>
<td>3.20 ± 0.25</td>
<td>3.55 ± 0.22</td>
</tr>
<tr>
<td>$6 \times 10^{-8}$</td>
<td>3.25 ± 0.17</td>
<td>3.70 ± 0.31</td>
</tr>
<tr>
<td>$6 \times 10^{-4}$</td>
<td>3.35 ± 0.25</td>
<td>3.10 ± 0.48</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>3.15 ± 0.21</td>
<td>2.85 ± 0.26</td>
</tr>
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</table>

Fig. 5. The effect of nicotine on the adiponectin concentration in rat adipocytes culture medium. Means ± S.E.M. *p < 0.05; **p < 0.01; ***p < 0.001 versus control group. †p < 0.01; ††p < 0.001 versus the same nicotine concentration.

Fig. 6. The effect of nicotine on the TNF-α (A) and FFA (B) concentration in rat adipocyte culture medium. Means ± S.E.M. *p < 0.05; **p < 0.01; ***p < 0.001 versus control group. †p < 0.05; ††p < 0.01; †††p < 0.001 versus the same nicotine concentration.

dose-dependent manner with increasing (−)nicotine concentration from $6 \times 10^{-8}$ to $6 \times 10^{-4}$ M. Nicotine-stimulated TNF-α release for 36 h was significantly lower than that for 12 h at the same nicotine concentration (Fig. 6A); however, there was no significant difference in FFA release between 12 and 36 h at the same nicotine concentration (Fig. 6B).

Expression of TNF-α Protein. The results of Western blot studies performed using anti-mouse TNF-α polyclonal antibody are shown in Fig. 7A. The percentage ratio to the corresponding standard TNF was calculated as a TNF-α protein relative intensity and is shown in Fig. 7B. Western blot analysis revealed that TNF-α protein in adipocytes in the nicotine group was significantly lower than in the control group for 12 and 36 h (p < 0.001). Moreover, the TNF-α protein levels decreased in a dose-dependent manner with increasing (−)nicotine concentration from $6 \times 10^{-8}$ to $6 \times 10^{-4}$ M in adipocytes (Fig. 7, A and B).

Discussion
In the present study, RT-PCR studies revealed that α1–7, 9, 10, β1–4, δ, and ε subunit mRNAs are expressed in adipocytes. Immunocytochemical experiments also suggested the presence of α7 and β2 subunits. Therefore, the subunits that characterize neuronal and muscle (α1 and β1) nicotinic receptors (Witzemann et al., 1990; Liu et al., 1998; Tseng et al., 2001) are present in rat adipocytes. PCR could not amplify the γ subunit transcript because the γ subunit was replaced by an ε subunit to become the adult-type receptor (Saito et al., 2002). However, the present findings of the size of δ and ε mRNA expression in the adipocytes differ from that expressed in muscle (LaPolla et al., 1984; Buonanno et al., 1989). The differences in the size of the δ and ε subunit mRNAs between muscle and fat tissues can be explained by tissue-specific splicing. Further studies are needed to clarify this.

The saturation curve was analyzed by a nonlinear regression model, and the $K_d$ and $B_{max}$ values were subsequently determined. The curved appearance of the Scatchard plot supports this interpretation. The $K_d$ values for (−)nicotine binding to the high-affinity sites is $39.15 \pm 2.67 \times 10^{-9}$ M. This is similar to that for nicotine binding sites found in brain and peripheral blood cells, which have $K_d$ for nicotine between 2 and $43 \times 10^{-9}$ M (Wonnacott, 1987; Lebarsy et al., 1996).

[3H]−nicotine binding to adipocytes decreased in a dose-dependent manner with increasing (−)nicotine concentration from 0 to $1 \times 10^{-3}$ M and was significant for unlabeled (−)nicotine concentrations from $1 \times 10^{-4}$ to $1 \times 10^{-3}$ M. Chronic administration of nicotine to animals up-regulates nAChR in the central nervous system when examined by [3H]nicotinic radiolabeled ligand for nAChRs (Ke et al., 1998). In addition, several investigations reported that the up-regulation of the receptors is due to increased numbers of α4, α2, and α7 nACHRs subtypes in neurons and non-neuronal cells (Bencherif et al., 1995). Although the nAChRs subtypes up-regulated in rat adipocytes used in this study remain to be clarified, the present findings suggest that rat adipocytes express functional nAChRs.

To investigate the direct effect of nicotine exposure on cytokine secretion from adipocytes, we examined TNF-α, adi-
Several agents such as TNF-α cytokine may control the expression of the other cytokines. Adiponectin may be antagonists of each other or that one.

Kern et al. (2003) reported that TNF-α in wild-type mice but fails to inhibit TNF synthesis in adipocytes (Ukkola and Santaniemi, 2002). TNF-α was also significantly reduced by nicotine. The mechanism responsible for this remains to be clarified.

Several studies have been carried out on nicotine inhibition of cytokine synthesis. Nicotine exerts immunosuppressive activity through T cell-dependent and -independent mechanisms (Mabley et al., 2002). It also modulates the production of various cytokines (Yoshida et al., 1998). Nicotine inhibits the production of IL-2 and TNF-α from human mononuclear cells (Madretsma et al., 1996). Recently, Wang et al. (2003) reported that the nAChR α7 subunit is required for acetylcholine inhibition of macrophage TNF release.

Electrical stimulation of the vagus nerve inhibits TNF synthesis in wild-type mice but fails to inhibit TNF synthesis in α7-deficient mice (Wang et al., 2003). The present study revealed that the amounts of TNF-α protein in adipocytes is also significantly reduced by nicotine. The mechanism responsible for this remains to be clarified.

TNF-α negatively regulates adiponectin production (Fasshauer et al., 2002). Kern et al. (2003) reported that TNF-α and adiponectin may be antagonists of each other or that one cytokine may control the expression of the other cytokines. Several agents such as TNF-α mediate their effects on insulin metabolism by modulating adiponectin secretion from adipocytes (Ukkola and Santaniemi, 2002). TNF-α expression was higher in adiponectin knockout mice, and the administration of adiponectin in these mice resulted in an improvement in insulin resistance along with a decrease in TNF expression (Maeda et al., 2002). We showed that short-term exposure to nicotine stimulates the secretion of TNF-α, adiponectin, and FFA into the culture medium. Also, long-term exposure reduces the expression of TNF-α protein in adipocytes but increases the secretion of adiponectin, possibly in part, through increased TNF-α protein production. Our previous studies showed that nicotine reduces insulin resistance in vivo through decreased production of TNF-α protein in visceral tissues and reduces hepatic glucose release (Liu et al., 2001, 2003). Together, these results suggest that adipocytes have functional nAChRs and that nicotine reduces TNF-α production in adipocytes although continuing to increase the secretion of adiponectin through the activation of nAChRs. Adipose tissues are under sympathetic and parasympathetic control (Kreier et al., 2002). The present study suggests that nicotine temporarily reduces insulin sensitivity by stimulating the secretion of TNF-α and FFA, whereas the long-term direct stimulation of nAChRs by nicotine, in addition to autonomic nervous stimulation, contributes to better insulin sensitivity in vivo through the modulatory secretion of adipocytokines.

Although nicotine administration by smoking is unlikely to be a preventative therapy for diabetes due to deleterious effects on other body systems, the discovery of the presence of nAChRs in adipocytes may lead to the development of a specific agonist for adipocytes. This may prove to be an effective therapy for increasing insulin sensitivity as described in our previous paper (Liu et al., 2001).

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