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The expression and functional role of nicotinic acetylcholine receptors in rat adipocytes

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Non-Standard Abbreviations: nAChRs, nicotinic acetylcholine receptors; TNF-α, tumor necrosis factor alpha; FFA, free fatty acid; PAI-1, plasminogen activator inhibitor-1; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; (RT)-PCR, reverse transcriptase-PCR; ELISA, enzyme linked immunosorbent assay; PBS, phosphate-buffered saline; ECL, enhanced chemiluminescence; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

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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 RT-PCR and immunocytochemistry, and the direct effects of nicotine on the production of adipocytokines by ELISA and Western blot analysis. Receptor binding assays were performed using [^{3}H] 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 ($K_d = 39.2 \times 10^{-9}$ M) on adipocytes. Adipocytes incubated with nicotine for 12 and 36 h released TNF-α, adiponectin, and FFA into the medium in a dose-dependent manner with increasing nicotine concentration from 6×10^{-8} 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^{-8} 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.

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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, 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 (Rubboli et al., 1994). The muscle receptor subtypes are comprised of α1, β1, δ, γ (embryonal) or α1, β1, δ, 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 nAChRs. 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

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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 et al., 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, 1999). Adipocytes play a role in systemic energy homeostasis by producing molecules such as leptin, plasminogen activator inhibitor-1 (PAI-1), and several cytokines including tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-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

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secreted from adipocytes and has antidiabetic 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 (Liu et al., 2001) and reduced hepatic glucose release (Liu et al., 2003). To clarify whether nicotine has a direct effect on the function of adipocytes, we evaluated nAChR expression in adipocytes and the direct effects of nicotine on the production of adipocytokines (TNF-α and adiponectin).

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Materials and Methods

Cell culture. Adipocytes were obtained from male Wistar rat (8 weeks of age) abdominal subcutaneous fat tissue by collagenase digestion using a kit (Toyobo, Japan). Preadipocytes were seeded into 75 cm^2 flasks (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) in medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% fetal calf serum (FCS) 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% CO_2 in air. Preadipocytes were grown to confluence in preadipocyte growth medium (TAGM-250, Toyobo) for the first 5 days. The medium was changed after one day and thereafter every two days. Preadipocytes were differentiated in vitro to mature adipocytes using preadipocyte differentiation medium (TADM-250, Toyobo). 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, Japan) dye exclusion method. We used a similar procedure for plating cells onto 4-well glass slides (Nalge Nunc, Naperville, IL, USA) for immunofluorescence assays.

Conditioned media. Preadipocytes seeded at 2×10^4 cells/cm^2 into 24-well plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) were cultured to confluence. Under the culture conditions, preadipocytes differentiated into matured adipocytes. Adipocytes were incubated in medium for 12 and 36 h with nicotine (nicotine tartrate dihydrate dissolved in

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DMEM) 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 1,000 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 (ELISA) using kits obtained from BioSource International, Inc. (California, CA, USA) and Otsuka Pharmaceutical Co., Ltd (Japan), respectively. The medium FFA was measured by spectrophotometric assays using a commercially available kit (Wako, Richmond, VA).

Adipocyte number and form. Adipocytes were counted by a hemocytometer, and viable and dead cells were counted by 0.4 % trypan blue staining. Micrographs were taken at 40-200 × magnification.

RNA extraction and reverse transcriptase-PCR (RT-PCR). Total RNA was isolated from the adipocytes using a QIAamap RNA Mini kit (QIAGEN, Japan). Prior to RT-PCR experiments, total RNA was quantified by measuring the absorbance at 260 nm (HITACHI, U-1100 Spectrophotometer, Japan). The RNA samples had *A* 260 /*A* 280 ratios ranging from 1.8 to 2.0. All RNA samples were treated with amplification grade DNase I according to the manufacturer's instructions (Gibco BRL, Carlsbad, CA) to eliminate residual DNA. cDNA synthesis and PCR were performed in a single tube using gene-specific primers and total RNA by SuperScript ™One-Step RT-PCR with a Platinum® Taq Kit (Invitrogen, Japan). For the conversion of total

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RNA (0.5 μg) to cDNA, a 50 μl single-tube reaction mixture was prepared from a master-mix containing 25 μl of 2x reaction mix, 0.5 μg of template RNA, and 1 μl of RT/Platinum® *Taq* Mix. Then, 10 μM of each gene-specific primer pair was added to the tubes. Primer sequences were selected from the unique cytoplasmic domain region of each nAChR subunit (Table 1) (Witzemann et al., 1990; Liu et al., 1998; Tseng et al., 2001; LaPolla et al., 1984; Buonanno et al., 1989; Rohwedel et al., 1995). Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (TOYOBO Co., LTD. Japan) 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: Perkin-Elmer, Branchburg, NJ) 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 and (46-57°C, respectively, in Table 1) for 1 min and 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 four-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, USA) or rabbit anti-β2 antibody (1:200 dilution in PBS, Santa Cruz Biotechnology Inc., USA) at room temperature,

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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 PA, USA) or fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:200 dilution in PBS, Santa Cruz Biotechnology Inc., USA) respectively. They were next washed three times with PBS for 5 min each, mounted in Permafluor mounting medium (ThermoShandon, PA, USA), and observed with a Fluoview FV500 confocal laser scanning microscope (OLYMPUS Optical Co., LTD. Japan).

[^{3}H] (-)-nicotine-binding assays. The analysis of binding data was performed as previously described (Didier et al., 1995). Briefly, a 0.8 ml cell suspension (50 mM NaCl, 5 mM KCl, 10 mM $CaCl_2.2H_2O$, 2 mM EDTA, 50 mM Tris base, 50 mM HEPES; 10 mM D-glucose; 1 % BSA , pH 8.0) (cells count was 4×10^7 cells/ml) was added to tubes containing unlabeled (-)-nicotine (0, 1, 10, 100 and $1,000 \times 10^{-6}$ M), and 5×10^{-9} M [^{3}H] (-)-nicotine [(-)-N-methyl- [^{3}H] nicotine (81 Ci/mmol), PerkinElmer Life Sciences, Inc., Boston, MA, USA]. A final cell suspension of 1 ml was incubated at 37°C for 30 min. The reaction was terminated by adding 2 ml of cold buffer, and the samples were then filtered through Whatman GF/C (Whatman International Ltd. England) glass filters presoaked with 0.3 % polyethylenimine solution for 5 h. The filters were washed three times with the same ice-cold buffer and the radioactivity retained on the filter was measured by liquid scintillation spectrometry. For Scatchard analysis, the particulate fractions were incubated with various

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concentrations of [^{3}H] (-)-nicotine (0 to 100×10^{-9} M) under the same conditions as described above. The dissociation constant (K_d) and maximal binding sites (B_{max}) for adipocyte [^{3}H] nicotine binding were estimated by Scatchard analysis. The GraphPad Prism® program was used for data manipulations, and all values including $B_{\max}$, and K_d values were determined using this program.

Western blot analysis. Harvested adipocytes were homogenized in ice-cold buffer (50 mM HEPES, 150 mM sodium chloride, 1 % Triton X-100 pH 7.8, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mg/ml pepstatin, and 1 mg/ml leupeptin, 2 mg/ml aprotinin), and the resulting homogenate was centrifuged for 10 min at 4°C at 12,000 rpm. Samples containing 50 μg of protein were resolved by electrophoresis in a 12 % SDS-polyacrylamide gel. Rat TNF-α (PEPRO TECH, Inc, Rocky Hill, NJ) and Rainbow markers (Amersham, Piscataway, NJ) were used as molecular markers. Proteins were transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA). Blots were incubated with anti-mouse TNF-α polyclonal antibody (Endogen, Woburn, MA) at 0.65 μg/ml in low fat milk solution overnight at 4°C. After the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated donkey anti-rabbit IgG [0.2 mg/ml (1/5,000), Amersham)] in TBST-BSA, they were incubated with enhanced chemiluminescence (ECL) reagents (Amersham) and exposed to X-ray film for 3 min. Densitometric analysis of immunoblots was performed using Adobe PhotoShop (Adobe systems, Inc.) software for Apple Macintosh computers (Apple Computer, Inc.).

Statistical analysis. The unpaired Student's t-test was used to determine significance, and values

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were represented as the means ± S.E.M of the number of experiments stated. StatView 5.0 software was used for all statistical calculations. A p value of less than 0.05 was accepted as being statistically significant.

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Results

Detection of nAChR subunits gene in cultured adipocytes by RT-PCR. We investigated the expression of mRNA for α, β, γ, δ and ε nAChR subunits in cultured adipocytes by RT-PCR using specific primers for the α (α1-7, 9, 10), β (1-4), γ, δ and ε nAChR subunits (Table 1). Ethidium bromide staining of the gel show the presence of nAChRs subunits (α1-7, 9, 10, β1-4, δ and ε), G3PDH, and molecular weight markers (M) obtained by RT-PCR as seen in Fig. 1. The data show that rat adipocytes express α (α1-7, 9, 10), β (β1-4), δ and ε subunits. Amplification yielded PCR products of expected sizes: 288 bp for α1, 300 bp for α2-7, α9, 209 bp for α10, 355 bp for β1, 507 bp for β2, and 300 bp for β3, β4. 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 rat δ and ε subunits (Table 1) amplified larger products in size (δ: 395 bp, ε: 402 bp) than expected from previous findings in skeletal muscle (δ: 235/291 bp, ε: 222/340 bp) (Fig. 1). The γ nAChR subunit was not expressed (Fig. 1).

Immunocytochemical studies on adipocytes using specific antibodies against α7 and β2 subunits. To further ensure the expression of nAChR subunits in adipocytes, we performed an immunocytochemical analysis using specific antibody against α7 or β2 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, B). α7 and β2 subunit immunoreactivity was observed on the membrane and in the cytoplasm of the adipocytes, while no

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immunoreactivity was detected in the nuclei.

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

Saturation analysis of [^{3}H] (-)-nicotine. Saturation studies on adipocytes using concentrations of [^{3}H] (-)-nicotine from 1×10^{-10} M to 1×10^{-7} M revealed the presence of saturable binding sites. Non-linear regression analysis of nicotine binding yielded a K_d value of $39.15 \pm 2.67 \times 10^{-9}$ M and a B_{max} of 43236 ± 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

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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 dose-dependent manner with increasing (-)-nicotine concentration from 6×10^{-8} to 6×10^{-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 6×10^{-8} to 6×10^{-4} M in adipocytes (Fig. 7A, B).

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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 non-linear 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 ± 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 (Lebargy et al., 1996; Wonnacott, 1987).

[^{3}H] (-)-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

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concentrations from 1×10^{-4} to 1×10^{-3} M. Chronic administration of nicotine to animals up-regulates nAChR in the central nervous system when examined by [^{3}H] nicotine 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, adiponectin, and FFA levels in the culture cell medium with nicotine stimulation for 12 and 36 h. These results showed that TNF-α, adiponectin, and FFA was released into the medium in a dose-dependent manner with increasing nicotine concentration (Fig. 5, Fig. 6A, B). However, after stimulation with nicotine at the same concentration for 36 h, adiponectin release was significantly higher, TNF-α release was significantly lower, and FFA release did not change compared to the results for 12 h. On the other hand, TNF-α protein levels in adipocytes incubated for 12 and 36 h decreased in a dose-dependent manner with increasing nicotine concentration (Fig. 7A, B).

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

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(Yoshida et al., 1998). Nicotine inhibites the production of IL-2 and TNF-α from human mononuclear cells (Madretsma et al., 1996). Recently, Wang et al. 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. reported that TNF-α and adiponectin may be antagonists of each other or that one cytokine may control the expression of the other cytokines (Kern et al., 2003). 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 decreased TNF-α protein production. Our previous studies showed that

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nicotine reduces insulin resistance in vivo through decreased production of TNF-α protein in visceral tissues (Liu et al., 2001) and reduces hepatic glucose release (Liu et al., 2003). Together, these results suggest that adipocytes have functional nAChRs and that nicotine reduces TNF-α production in adipocytes while 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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Footnotes

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Figure legends

Fig. 1. mRNA expression of nAChR α, β, γ, δ and ε subunits in rat adipocytes. Total RNA was reverse-transcribed and the expression of each subunit mRNA was examined by PCR. The lane is labeled M for the molecular weight standards, α1-7, α9,10, β1-4, γ, δ, and ε for individual primers for rat α1-7, α9,10, β1-4, γ, δ, and ε subunits, and G3PDH for glyceraldehyde-3-phosphate dehydrogenase. Amplification of the G3PDH gene product (452 bp) was used to normalize the cDNA content in each sample and as a positive control for RT-PCR effectiveness. A sample of 12 µl of each reaction was loaded onto ethidium bromide stained 2.4% agarose gel.

Fig. 2. Immunofluorescence detection of the binding of mouse anti-α7 antibody or rabbit anti-β2 antibody specific against the rat α7 or β2 subunit to cultured rat adipocytes as indicated at left. (A): nAChR α7; (B): nAChR β2. The presence of antibodies bound to the cells is revealed by the binding of fluorescein isothiocyanate-labeled goat anti-rabbit IgG antibody. Right, extent of nonspecific labeling obtained by omitting the anti-α7 (C) or an-β2 (D) antibody. Scale bar, 20 µm.

Fig. 3. Nicotine binding assay for adipocytes. Each point represents the means ± S.E.M. obtained from four separate experiments run in triplicate. * $p < 0.05$ versus 0×10^{-6} M group. † $p < 0.01$ versus 1×10^{-6} M group.

Fig. 4. The graph displays the specific binding of [^{3}H]-nicotine to rat adipocytes. Values represent the means ± S.E.M of 6 experiments. Scatchard plot of [^{3}H] (-)-nicotine binding to rat adipocytes. The B_{max} of the higher-affinity site was 43236 ± 1152 sites/cell with a K_d of

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$39.15 \pm 2.67 \times 10^{-9}$ M.

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.

Fig. 7A. The effect of nicotine on TNF-α expression in adipocytes. Western blot studies were performed using specific TNF-α antibody. A total of 0.1 μg of TNF-α was applied as a molecular marker in lane 1. Lanes 2-9 show samples of the rat adipocytes.

B. Relative signal intensities of TNF-α protein in the Western blot are shown in the control (white bars) or nicotine (black bars) groups. The signal intensity of the band was quantified using NIH image and Adobe PhotoShop software, and the percentage ratio to the corresponding standard TNF-α was calculated as a TNF-α protein relative intensity. Means ± S.E.M. *** $p < 0.0001$ versus the control group. # $p < 0.05$ versus the same 12 h 6×10^{-8} M nicotine group. The data display the means of 6 experiments.

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TABLE 1

Oligonucleotide PCR primers for rat nAChR subunits

nAChR	Sequence	Transcript size (bp)	Anneal temperature (°)	Reference
α1	5'-TGGAAGCACTGGGTGTTTTA-3'			
	5'-AACATGTACTTCCCGATCAGG-3'	288	53	*a*
α2	5'-TGCCCAGGTGGCTGATGATGAACC-3'			
	5'-GCTTTCTGTATTTGAGGTGACAGC-3'	300	57	*b*
α3	5'-AACCTGCTCCCCAGGGTCATGTTT-3'			
	5'-CACTTTGGATGGCTTCTTTGATTT-3'	300	57	*b*
α4	5'-GTCAAAGACAACTGCCGGAGACTT-3'			
	5'-TGATGAGCATTGGAGCCCCACTGC-3'	300	57	*b*
α5	5'-GTGGATTTAGTGAGCAGTCATGCA-3'			
	5'-TTTGGGGGGAGTTTTAAATAGTCT-3'	300	57	*b*
α6	5'-CAGGTCTTCCCCTCGATTCTGATG-3'			
	5'-CATTGTGGCTTTTCATGTTTTCTG-3'	300	57	*b*
α7	5'-AACTGGTGTGCATGGTTTCTGCGC-3'			
	5'-AGATCTTGGCCAGGTCGGGGTCCC-3'	300	57	*b*
α9	5'-ATCCTGAAGTACATGTCCAGGATC-3'			
	5'-TGGCCTTGTGGTCCTTGAGGCACT-3'	300	57	*b*
α10	5'-TAGCCAGTCTCTCCCCAAA-3'			
	5'-GCTGGAATTACCGTGCTCA-3'	209	55	*c*
β1	5'-ATAGCTCAGTAAGGCCGGCG-3'			
	5'-TAGGTGACCTGGATGCTGCA-3'	355	57	*a*
β2	5'-ACGGTGTTCCTGCTGCTCATC-3'			
	5'-CACACTCTGGTCATCATCCTC-3'	507	57	*b*

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β3	5'-GAAGATGTGGATACATCGTTTCCA-3'			
	5'-GAGCAGAGGGAGTAGTTCAGGAAC-3'	300	57	*b*
β4	5'-ATGAAGCGTCCCGGTCTTGAAGTC-3'			
	5'-GGTCATCGCTCTCCAGATGCTGGG-3'	300	57	*b*
δ	5'-CAGCCGTCTACAGTGGGATG-3'	235		
	5'-CTGCCAGTCGAAAGGGAAGTA -3'	291	55	*d*
ε	5'-ATTGAAGAGCTTAGCCTGTA-3'	222		
	5'-TACACCTGCAAAATCGTCCT -3'	340	46	*e*
γ	5'-GATGCAATGGTGCGACTATCGC-3'	360		
	5'-GCCTCCGGGTCAATGAAGATCC-3'	244	55	*f*

[a] Witzemann V et al. Eur J Biochem 1990; 194: 437-448.

[b] Liu L et al. Brain Res 1998; 809: 238-245.

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TABLE 2

Counts of viable cells in the nicotine and control groups

All values are presented as the means ± S.E.M.

Nicotine concentration (M)	12 h ($\times 10^5$ cells/cm^2)	36 h ($\times 10^5$ cells/cm^2)
0	3.20 ± 0.25	3.55 ± 0.22
6×10^{-8}	3.25 ± 0.17	3.70 ± 0.31
6×10^{-6}	3.35 ± 0.25	3.10 ± 0.48
6×10^{-4}	3.15 ± 0.21	2.85 ± 0.26

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Figure 1

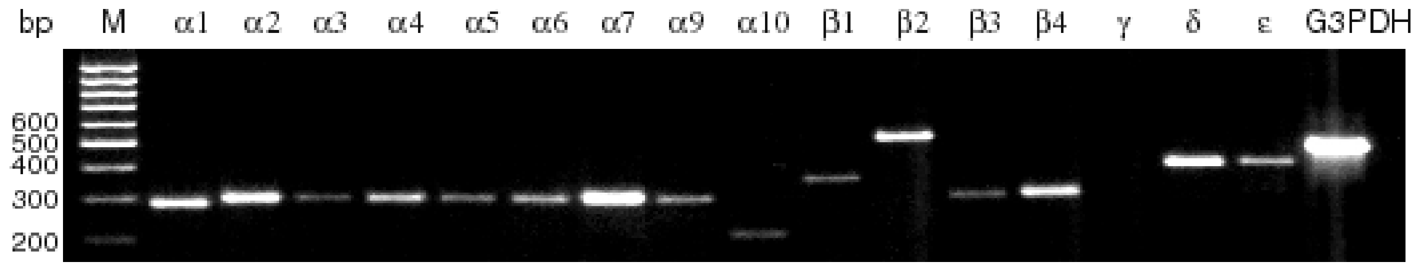

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Figure 2

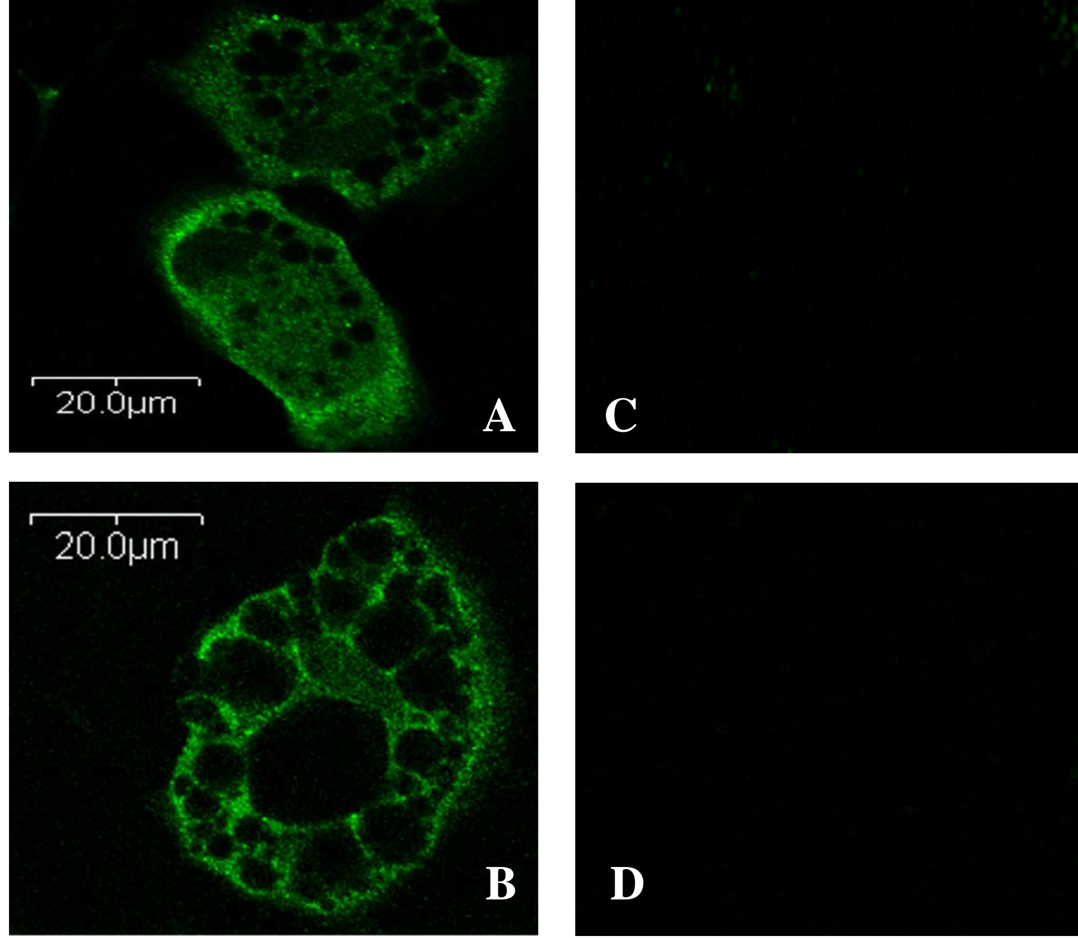

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Figure 3

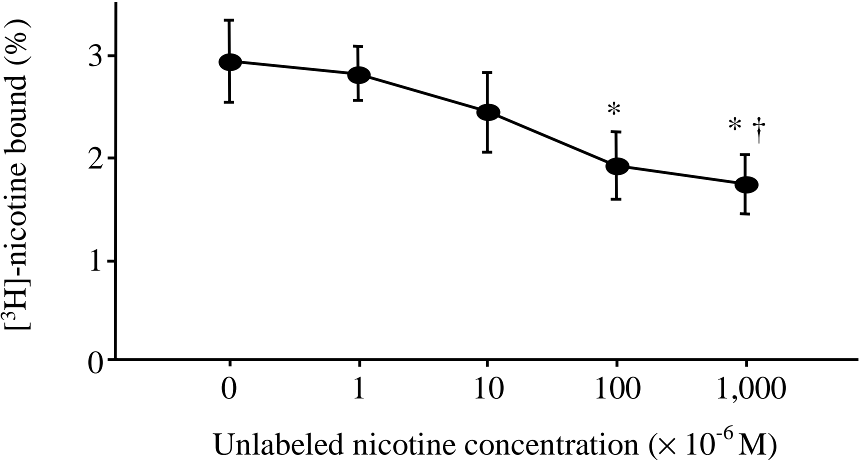

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Figure 4

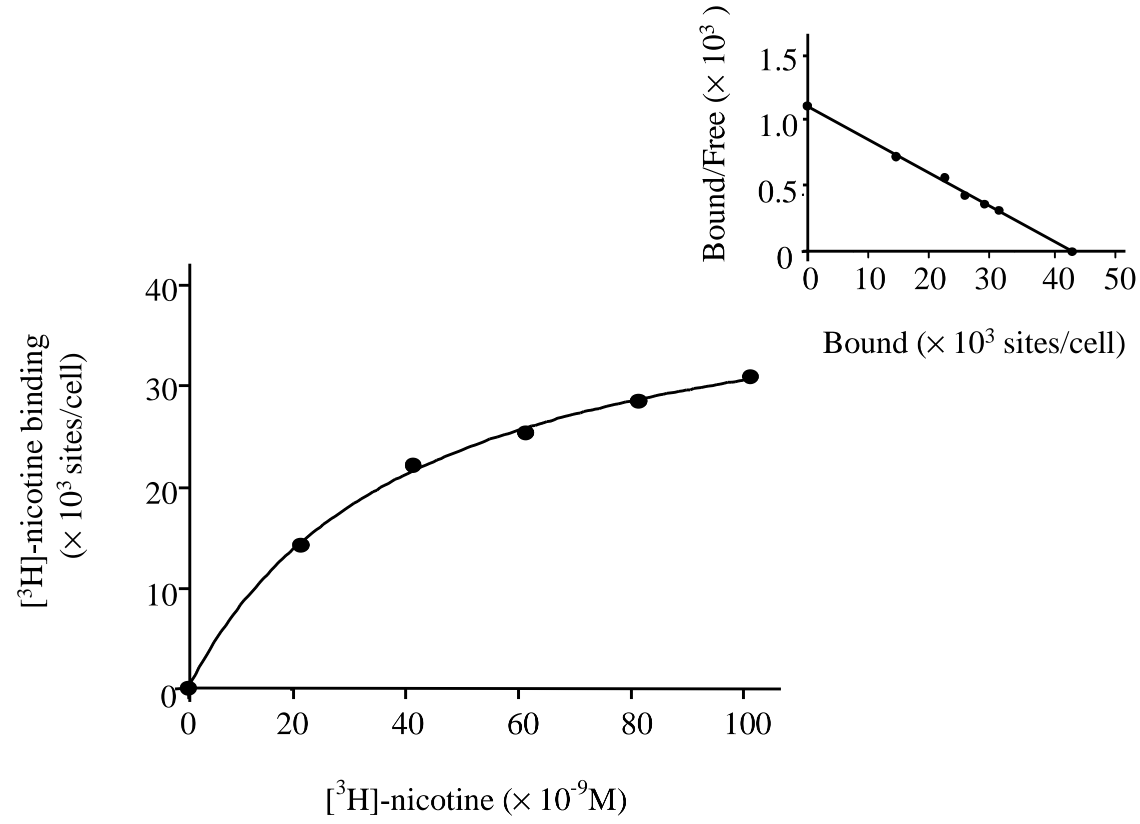

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Figure 5

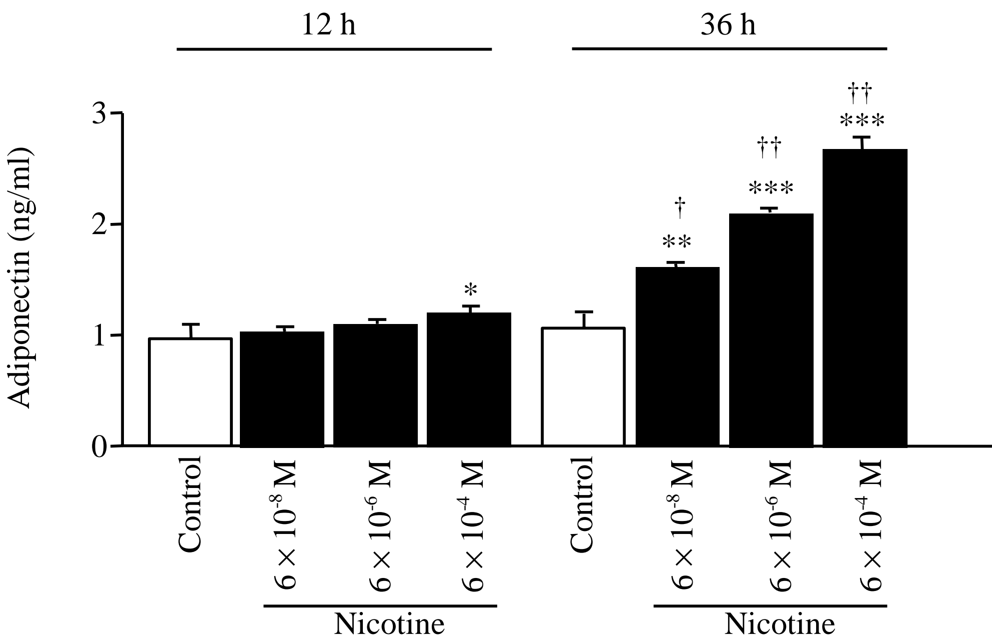

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Figure 6A

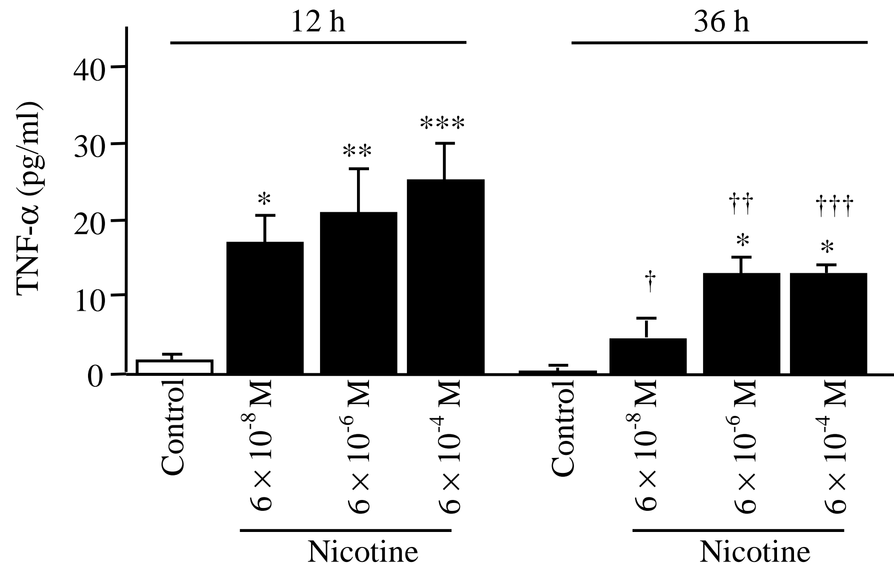

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Figure 6B

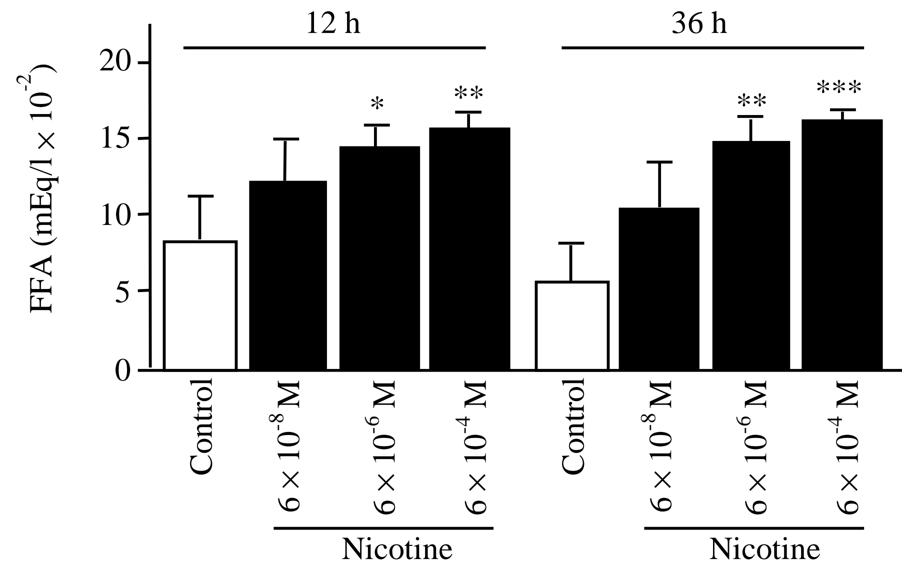

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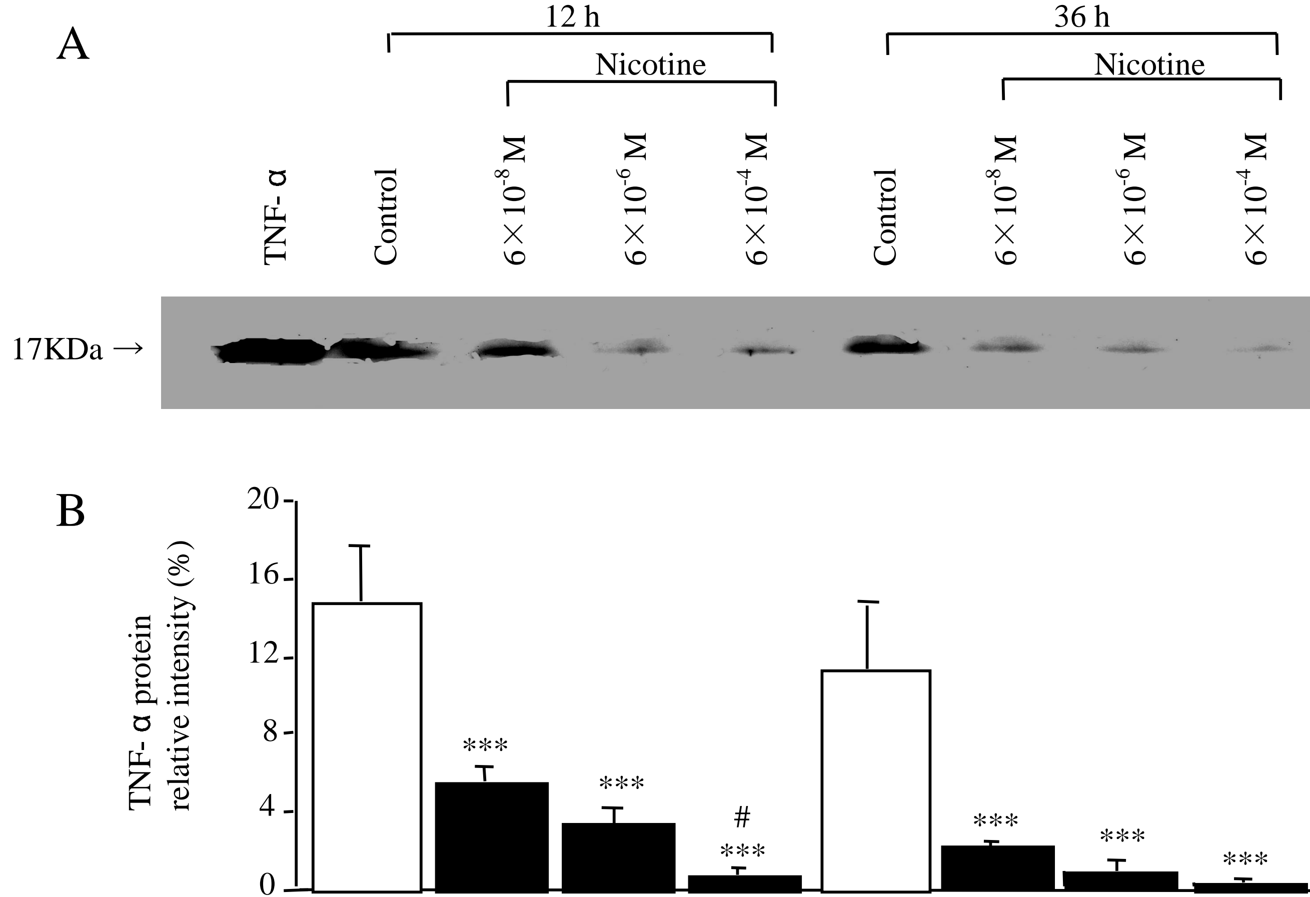
Figure 7



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