UNIVERSITY OF CALIFORNIA, SAN DIEGO

Mechanisms Underlying Nicotine-induced Development of Insulin Resistance in Mice

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

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2012

The Thesis of Christine Uyen Vu is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego

2012

DEDICATION

Foremost, my thesis is dedicated to my loving parents, especially my mom. Thank you for all your tender love and support. I would not be the young woman that I am today without your guidance. I love you.

I would also like to dedicate this to my four amazing sisters. Thank you for your endless love and timeless friendship. I am forever grateful.

EPIGRAPH

If you can imagine it,
you can achieve it;
if you can dream it,
you can become it.

William Arthur Ward

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ACKNOWLEDGEMENTS

I'd like to start by thanking Dr. Mahata for his continuous support and encouragement. Thank you for always believing in me and seeing the potential that I possess. My experience in your lab would not have been the same without your guidance.

Dr. Colin Jamora and Dr. Gen-Sheng Feng thank you both for serving on my committee board.

I'd also like to thank Dr. Bandyopadhyay for all his scientific feedback throughout this project and for always being there when I needed laboratory supplies. I can always count on you for that.

Next, I'd like to thank Stefano, Agnes, Rahul, Tina, Paul, and Jawed for making lab a fun, friendly environment. Thank you for also helping me on various experiments.

Lastly, the majority of my master's thesis, in part is currently being prepared for submission for publication of the material. Jiaur R. Gayen, Christine U. Vu, Gautam K. Bandyopadhyay, Paul Wadensweiler, Jawed M. Siddiqui, Agnieszka Radziszewska, Stefano Gentile, Nilima Biswas, Nai-Wen Chi, Daniel T. O'Connor, Sushil K. Mahata. I am the co-first author of this material.

ABSTRACT OF THE THESIS

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Professor Sushil Mahata, Chair

Cigarette smokers develop insulin resistance. However, administration of nicotine, a key toxicant of tobacco leaves, to insulin resistant animal models decreases inflammation and improves glucose tolerance. Nicotine activates nicotinic acetylcholine receptor (nAChR) system that integrates signaling in central and peripheral nervous

systems and regulates glucose metabolism. Insulin sensitive animals, not suffering from inflammation, may not benefit from nicotine intake.

We hypothesize that non-inflamed insulin sensitive mice, like the smokers, would develop insulin resistance upon nicotine administration due to interference with the insulin action.

Glucose tolerance tests and plasma insulin assays demonstrated that the activation of nAChR by nicotine in mice fed normal chow diet (NCD) led to fasting hyperinsulinemia as well as hyperglycemia that depended on nAChR, catecholamine and nitric oxide (NO) suggesting development of insulin resistance. Nicotine-induced hyperinsulinemia did not stimulate, rather inhibited, Akt phosphorylation in liver or muscle cells. Treatment with nAChR antagonist chlorisondamine and inhibitors of NO synthase prevented nicotine-induced hyperglycemia and reversed nicotine-mediated inhibition of hepatic insulin signaling. Nicotine augmented NO production in liver cells *in vitro*, and caused secretion of catecholamines from the adrenal medulla, leading to activation of α- and β-adrenergic receptors in liver. Net results were increased gluconeogenesis and glycogenolysis. Although α1-7, α9-10, and β1-4 subunits of nAChR are expressed in liver and muscle, their contributions have not been determined.

The anti-inflammatory effects of nicotine did not prevent hyperglycemia in NCD-fed mice. The activation of nAChR in insulin-sensitive mice caused hyperglycemia through increased gluconeogenesis and glycogenolysis.

INTRODUCTION

1.1 Smoking, Cigarettes, and Nicotine

It has been well established that smoking is one of the leading causes for the development of cardiovascular diseases (CVD), chronic lung cancer, and type 2 diabetes mellitus (T2DM). In the U.S., 435,000 people die prematurely from smoking-related disease each year (1). Smoking causes 1 in 5 deaths (1). Studies have also demonstrated that 94,000 infant deaths are correlated to mothers that were smokers during their pregnancy. Smoking and its hazardous effects not only affect the smoker but also those that are around them, the secondhand smokers.

Cigarettes, as well as cigars, pipes, snuffs, and chewing tobacco are mainly composed of tobacco and are made up of over 200 components. One of its principal ingredients is nicotine, which is a β-pyridyl-α-N-methyl-pyrrolidine (2). Nicotine is the major addictive component in cigarettes (3). This liquid alkaloid is both lipid and water-soluble and is very poisonous. A cigarette contains 10 to 15 mg nicotine and delivers on average 1 mg nicotine to the smoker, which reaches the brain in 10 seconds after inhalation, and raises the peak plasma concentration from 10 to 50 ng/ml with an half-life of ~2 hours (4). Nicotine (>80%) is metabolized primarily by lung, liver, and kidney to cotinine, which has a half-life of ~15 hours and is used as a biomarker of nicotine exposure (4). Although it is colorless, once exposed to air it will turn brown and obtain the lucid tobacco smell. Nicotine is found in two types of tobacco plants *Nicotiana tobacum* and *Nicotiana rusticum*. Once it is burned its 4,800 distinct components exist as a gas or particle form that can be very reactive and toxic. Of the 4,800 components, 63 of

them have been linked to cancer and disease. One of tobacco's gaseous byproducts is carbon monoxide (CO), which has been thought to be the main culprit for causation of CVD.

1.2 Nicotine's mechanistic action through the Nicotinic Acetylcholine Receptors (nAChRs)

Acetylcholine is an important intracellular signaling molecule released from cholinergic nerve terminal. Upon release, in response to stress, acetylcholine acts through two different receptors: nicotinic and muscarinic receptors. The nicotinic acetylcholine receptors (nAChRs) are a part of a four transmembrane domain superfamily that consists of neurotransmitter-gated ion channels (5), which are of two types: neuronal and muscle type. The muscle type consists of two α subunits and one each of β, γ, and δ subunit. The neuronal nicotine receptor, on the other hand, consists of two α and three β subunits and is a pentamer that forms a barrel shape with a gated ion channel (5). The α subunit is the agonist binding subunit and the β subunit is the structural component. In the peripheral nervous system, the main receptor composition for the neuronal type is α3β4.

Nicotine is an acetylcholine analog that acts through nAChR as well (6). When nicotine binds to nAChR, it opens a Na^+ channel, which is believed to be the first step in nicotinic-cationic signal transduction. Entry of Na^+ inside the cell causes depolarization of the cell membrane, which in turn opens the voltage-gated Ca^{2+} channel (VGCC), resulting in increment in intracellular Ca^{2+} concentration (from 100 nM to 1μM). The increase in intracellular Ca^{2+} is a prerequisite for catecholamine/neurotransmitter secretion from stored vesicles via exocytosis. SNAREs (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors) comprising of syntaxin 1A and

SNAP-25 (t-SNAREs) and synaptobrevin1 (v-SNARE) and associated proteins such as synaptotagmin 1 (Ca^{2+} and phospholipid binding protein) and complexin 1/2 (Ca^{2+}-dependent regulator of SNARE complex assembly and disassembly) play a critical role in vesicle docking, priming, fusion and synchronization of catecholamine/neurotransmitter release. Ca^{2+} upon entry through VGCC binds to synaptotagmin 1 (7). When nicotine binds to the nAChR in adrenal chromaffin cells, it induces the release of catecholamines and co-stored proteins (chromogranins) and peptides (neuropeptide Y). Catecholamines travel to the liver to induce glycogenolysis and also goes to the heart to increase the heart rate.

1.3 Nicotine and its Adverse Effects

Nicotine, carbon monoxide, and tar are the major toxicants in tobacco smoke. Smoking is an independent risk factor for CVD and T2DM. The main mechanisms by which nicotine causes CVD is by activation of the sympathetic nervous system. Nicotine increases heart rate, blood pressure, and myocardial contractility. Smoking a cigarette typically increases blood pressure (by 5-10 mmHg) for ~30 minutes and increases heart rate (by 10-20 bpm) for ~1 hour (8). Because of the production of a hypercoagulate state, cigarette smoking may contribute to accelerated atherosclerosis eventuating in acute cardiac events (9). Blood flow in the heart is also affected as nicotine causes vasoconstriction of coronary blood vessel (9). A recent study shows that 32 weeks of exposure to cigarette smoke in mice results in high blood pressure, endothelial dysfunction, leucocyte activation with ROS (reactive oxygen species) generation, and decreases bioavailability of nitric oxide (NO) (10).

More than 90% of patients with T2DM are hypertensive or dyslipidemic or both.

Smoking decreases insulin sensitivity by affecting insulin secretion from pancreatic β-cells in the islets of Langerhans, and affects glucose and lipid metabolism. Pancreatic β-cells express α2, α3, α4, α5, α7, and β2 nAChR subunits. Nicotine is believed to act through those receptors to decrease insulin secretion (11). Thus, both acute and chronic nicotine exposure results in significant decrease in tolbutamide-induced insulin secretion from rat islets *ex vivo* and high-glucose-induced insulin secretion from pancreatic islets *ex vivo* (12). Steady state plasma glucose concentration is reported to be higher in response to glucose, insulin, and somatostatin in smokers as compared to non-smokers (13). In humans, insulin resistance is positively correlated with smoking habits and nicotine doses (14). Euglycemic-hyperinsulinemic clamp studies in humans reveal lower HDL-C and higher triglyceride levels (14). Like humans, nicotine administration in rats results in increments in serum concentrations of cholesterol, phospholipids, triglycerides and LDL-C and decrement in HDL-C (15). Lipolysis is the most prominent effect seen in human after nicotine administration. This is achieved indirectly via the release of catecholamines and their effects on β-adrenergic receptors located on adipocytes. This can also be achieved through direct action of nicotine of nAChR present on adipocytes (16). Smoking and obesity are the leading causes of morbidity and morality worldwide. The negative effects of smoking on CVD are prominent in T2DM subjects. T2DM and obesity are menacing challenges to our nation because these problems have become epidemic in both young and old people. Diabetic patients develop CVD, which worsen with nicotine intake through smoking. Insulin resistance is a primary effect underlying the development of T2DM and is the hallmark of metabolic syndrome or syndrome X (17).

1.4 Objective of the study

Nicotine activates both α7 homomeric, as well as, α and β heteromeric nAChRs (such as α4β2, α3β2 in the central nervous system, and α3β4 in the peripheral nervous system). Activation of α7 nAChR by nicotine is known to suppress inflammation, decrease cytokine production, and improve insulin resistance in diet induced obese (DIO) model and in diabetic mice (18-20). Although the beneficial effects of nicotine agonist on glucose metabolism are well documented in DIO mice and diabetic models (18-22), their effects in normal, insulin sensitive models have remained elusive. In addition, the effects of nAChR activation by nicotine on insulin signaling pathway and glucose metabolism in peripheral tissues such as in liver has not yet been defined. It is, therefore, important to understand how glucose metabolism is affected by chronic nicotine exposure through regular smoking in an insulin sensitive population. We hypothesize that the metabolic effects of nicotine are modulated by nutritional states. Under normal dietary and physiological conditions, nicotine causes insulin resistance and hyperglycemia by preferential activation of heteromeric nAChRs over homomeric nAChRs. Therefore, our main objective is to determine the mechanisms whereby chronic intake of nicotine through regular smoking leads to insulin resistance by activating heteromeric nAChRs consisting of α4β2, α3β2, and α3β4.

METHODS AND MATERIALS

2.1 Animals and Diets: C57BL/6J mice were fed *ad libitum* with a normal chow diet (NCD) (LabDiet 5P00, 14% calorie content from fat).

2.2 Nicotine treatment, GTT and GSIS: Wild-type mice fed with NCD and fasted for 12 hours were injected with saline or nicotine bitartrate (0.2 μg/g BW; IP). For glucose tolerance test (GTT), glucose (1 mg/g BW) was injected IP (0 min) 15 minutes after nicotine or saline injection. Blood (10-15 μl) was collected by tail bleeding and glucose levels were monitored at -15, 0, 15, 30, 60, 90, 120, and 150 minutes after glucose injection. For chronic treatment, mice were treated daily with saline or nicotine (0.2 μg/g BW) by IP injection for 16 days. On day 16, GTT was carried out as described above. In one set of experiments, mice were injected with nicotine twice daily for 16 days. For the determination of glucose-stimulated insulin secretion (GSIS), fasted mice were treated with nicotine the same way as in GTT but blood was collected at 0 and 15 minutes after glucose injection.

2.3 Biochemical assays: Glycogen content was determined by the colorimetric method using anthrone reagent as described (23). Insulin was measured by an ELISA kit from ALPCO (Salem, NH). Nitric Oxide (NO) and cAMP concentrations were measured using commercial kits (Cayman Chemicals, Ann Arbor, MI). Catecholamine levels in plasma were measured by HPLC method as described (23).

2.4 Glucose uptake assay in L6 muscle cells: Culture and differentiation of L6 myocytes (a gift from Dr. Amira Klip, Children Hospital, Toronto, Canada) and glucose uptake assay were done as previously published (24).

2.5 HepG2 and PC12 cultures: Cells were obtained from ATCC. HepG2 was cultured following instructions from ATCC. PC12 cells were cultured as described (25).

2.6 Immunoblotting of lysates of hepatocytes and L6 myotubes: Primary hepatocytes were prepared by infusion of a collagenase called Liberase TM (Roche) through IVC for 10-15 minutes. Isolation and culture of primary hepatocytes were as described (26). Hepatocytes were cultured on collagen-coated plates in Williams E medium fortified with non-essential amino acids, glutamine, antibiotics and 10% FBS for attachment to the culture plates. After 6 hours of attachment, cultures were maintained in the same medium without serum. After 16 hours in serum-free medium with or without nicotine (20 μM), cultures (without pretreatment with nicotine) were treated with nicotine (10 μM, for 15 minutes) or insulin (10 nM, for 10 minutes) or with nicotine (15 minutes) plus insulin (10 minutes). Hepatocytes treated 16 hours with nicotine were exposed to insulin (10 nM) or vehicle for 10 minutes. At the end of incubation, cultures were homogenized in a lysis buffer containing 1% detergent (NP-40) and protease and phosphatase inhibitors as described (27). Homogenates were immunoblotted for eNOS signaling molecules and phospho- and total Akt using antisera from Cell Signaling (Danver, MA). L6 myotubes were similarly treated with nicotine and insulin and immunoblotted for phospho- and total Akt.

2.7 Glucose production in primary hepatocytes: Cultures of primary hepatocytes were washed with Krebs-Henseleit bicarbonate (KHB) buffer with 2.5 mM calcium and 1% BSA, than incubated in the same buffer containing hormones and substrates in 5% CO_2 incubator. Pyruvate (2 mM, 0.5 μCi 2-^{14}C-pyruvate) was used as a substrate. Incubations were carried out in 0.5 ml buffer in a 24-well plate seeded with 0.2 million cells per well. Hepatocytes were incubated for 30 minutes with nicotine (20 mM), glucagon (100 ng/ml) or insulin (10 nM) or combinations followed by another 3 hours incubation with 2 mM of radiolabeled pyruvate. At the end of 3 hours incubation, buffers were transferred to microfuge tubes where 0.25 ml each of 5% zinc sulfate and 0.3 N barium hydroxide were added to each tube followed by 0.5 ml of water. Tubes were vortexed and centrifuged. To quantify radiolabeled glucose released into the media, the supernatants were subjected to ion-exchange chromatography using mixed bed (cation and anion) AG-501x8 resins (BioRad, Richmond, CA). A batch treatment method was used. 150-200 mg resins were added to each tube and vortexed intermittently for 15 minutes. The tubes were centrifuged and the radioactivity of the supernatants was counted. This procedure has been adapted from several published methods (28, 29).

2.8 HepG2 cell culture and glycogenolysis: HepG2 Cells (ATCC) were cultured in alpha-MEM (InVitrogen; Carlsbad, CA) supplemented with 10% FBS, penicillin and streptomycin. At 75% confluency, cellular glycogen was labeled with U-^{14}C-glucose (1 mCi /ml, MP Biomedicals; Solon, OH) in the same culture medium, which contained 5 mM glucose and 10 nM insulin. After 24 hours of labeling, cultures were rapidly washed twice with PBS containing 10 mM Hepes, 1 mM calcium, 1 mM magnesium, 1 mM

glucose and 0.5% bovine serum albumin. Cultures were exposed to antagonists or inhibitors for 10 minutes followed by the agonists for 20 minutes, after 30 minutes of treatment, the media were collected in scintillation vials to determine the release of radiolabeled glucose as a measure of glycogenolysis. The released radioactivity was identified as glucose by passing the culture medium and a known solution of ^{14}C-glucose through an anion-exchange resin (AG-8X, formate form, 200-400 mesh, BioRad, Hercules, CA) followed by a cation-exchange resin (AG50W-8X, H^+ form, 200-400 mesh, BioRad) (30). The final eluate was counted. Compared to the known ^{14}C-glucose solution, 90% of the radioactivity of the culture medium was recovered in the final eluent. The attached cells in the culture plates were dissolved in 1M NaOH to determine protein content and radioactivity in the glycogen store. To measure the glycogen content, labeled glycogen dissolved in NaOH was precipitated with 70% ethanol. The precipitates were washed with 70% ethanol, dissolved in water and counted.

The following compounds were used: the nAChR inhibitor chlorisondamine (50 μM, Tocris, Ellisville, MO), the β-ADR inhibitor propranolol (10 μM, Tocris), the NOS inhibitors L-NIL [L-N6-(1-iminoethyl) lysine, 10 μM in culture and 15 μg/g BW *in vivo* by IP, Calbiochem, San Diego, CA) and L-NNA (N^w-Nitro-L-Arginine, 10 μM in culture and 15 μg/g BW by IP, Calbiochem), the α-ADR inhibitor prazosin (10 μM, Tocris), the nAChR agonist nicotine bitartrate (100 μM, Calbiochem), the β-ADR agonist isoproterenolol (10 μM, Tocris), the α-ADR agonist, phenylephrine (10 μM, Tocris), dibutyryl cyclic AMP (10 μM, Sigma, St Louis, MO), IBMX (100 nM, Sigma) and NO donor sodium nitroprusside (0.5 μM, Sigma). NOSI represents a combination of NOS inhibitors, L-NIL and L-NNA.

2.9 Real-Time PCR: Liver RNA was extracted using an RNA purification kit (RNeasy Plus, Qiagen, Valencia, CA) according to the manufacturer's specifications. After DNase digestion, 100 ng of RNA was transcribed into cDNA in a 20-µl reaction using a High Capacity cDNA Archive kit, then analyzed, and amplified. PCR was performed in a 25-µl reaction containing 5 µl of cDNA (5x diluted), 2× SYBR Green PCR Master Mix, and a 400 nM concentration of each primer. Differences in cycle threshold values (ΔCt) between target and the housekeeping gene *GAPDH* were used to calculate differences in expression. The primers used for qPCR are described in Table 1.

2.10 Statistics: Data are expressed as the mean ± SEM. Curve fitting was accomplished in the program Kaleidagraph (Synergy Software, Reading, PA). Multiple comparisons were made using either one-way ANOVA followed by Bonferroni's *post hoc* test. Statistical significance was concluded at $p<0.05$.

TABLE-1: RT-QPCR primers for mouse nAChR isoforms and GAPDH (5'→ 3')

Receptor Subtype	Forward Primer	Reverse Primer
α1	AGGGCGTGAAGTACATTGCAGAG	TCAGCCTCTGCTCATCCTTGTTGA
α2	AACCTGCTCTGAACTGTCCTGTGT	AATGTTCTGAAAGGCCCAATCCGC
α3	CATCGTGCTTTACAACAACGCCGA	TTAAAGATGGCCGGAGGGATCCAA
α4	GGCATGCAAGAAATCACCCTGTGT	ATCCAAACAGGGACCTCAGCTTCA
α5	ATTGTCATGGCAGAAGCATGGCAG	AAGCTCAAGTCATGTCCAGTGGGT
α6	TTCCACAGCCCTCCGTGTAGAAAT	ACAGACATAAACCGGAGTGCAGGT
α7	ACCTGCAGATGCAAGAGGCAGATA	AGGAATGAGCAGGTTGAGGCCATA
α9	TGACAGCTACTGTGCACGCTATGA	AGAAACGGTCTATGACCTTGGCGA
α10	AGAGCGTTCCACTCATCGGAAAGT	TGGATGTGCATTAGGGCCACAGTA
β1	ACCAACTCCCTGAACCACATCACT	TCACTTGGAGGCTTCCGGATGAAA
β2	ACTTCATCATTCGTCGCAAACCGC	TCTTGGAGATGAGCAGCAGGAACA
β3	TGCACGCGATGACTAGTGTCTTCA	AGGAGTACAGGCCCAACAATGACA
β4	TAGTCACCAGCACCTTGCCCATTA	TTGAAGGAGAGCAGGAGCCATTCA
δ	AGCCCACCTCTCAAATCGTGTCTT	TCTCATCTGTTTGCTCCGGCTTCT
ε	TGTGGATGCTGTGAACTTTGTGGC	TAGAACCAACGCTGAAGAGCACCA
γ	TCTGCAGTGGTTGGTGATGGGTAT	AGGCAGAGCTCTTAGGGCTTCAAA
GAPDH	TAT gTC gTg gAg TCT ACT ggT gT	gTC ATC ATA CTT ggC Agg TTT CT

RESULTS

3.1 Effects of acute and chronic nicotine treatment on GTT in mice fed with normal chow diet (NCD).

Both acute (15 minutes) and chronic (16 days) nicotine treatment (0.2 μg/g BW; IP) caused hyperglycemia in NCD-fed mice challenged with glucose 15 minutes after nicotine injection (Fig. 1A and B). The nicotine dose was chosen to recapitulate the peak blood cotinine level of cigarette smokers. Our measurement of blood levels of cotinine, a relatively stable metabolite of nicotine (half life of ~24 hours vs. 30 minutes for nicotine), 2 hours after the injection of nicotine (0.2 μg/g BW) is 93 ± 16 ng/ml. This represents the level achieved in blood of a light smoker according to Foundation for Blood Research (*www.fbr.org/publications/pamphlets/cotinine.html*). Higher nicotine dose (e.g., 1 μg/g BW) elevated plasma glucose level even further (Fig. 1C). Nicotine (0.2 μg/g BW) alone (without glucose challenge) transiently raised plasma glucose level by 10-15% (Fig. 1C). The GTT results suggest insulin resistance may have caused nicotine-induced hyperglycemia.

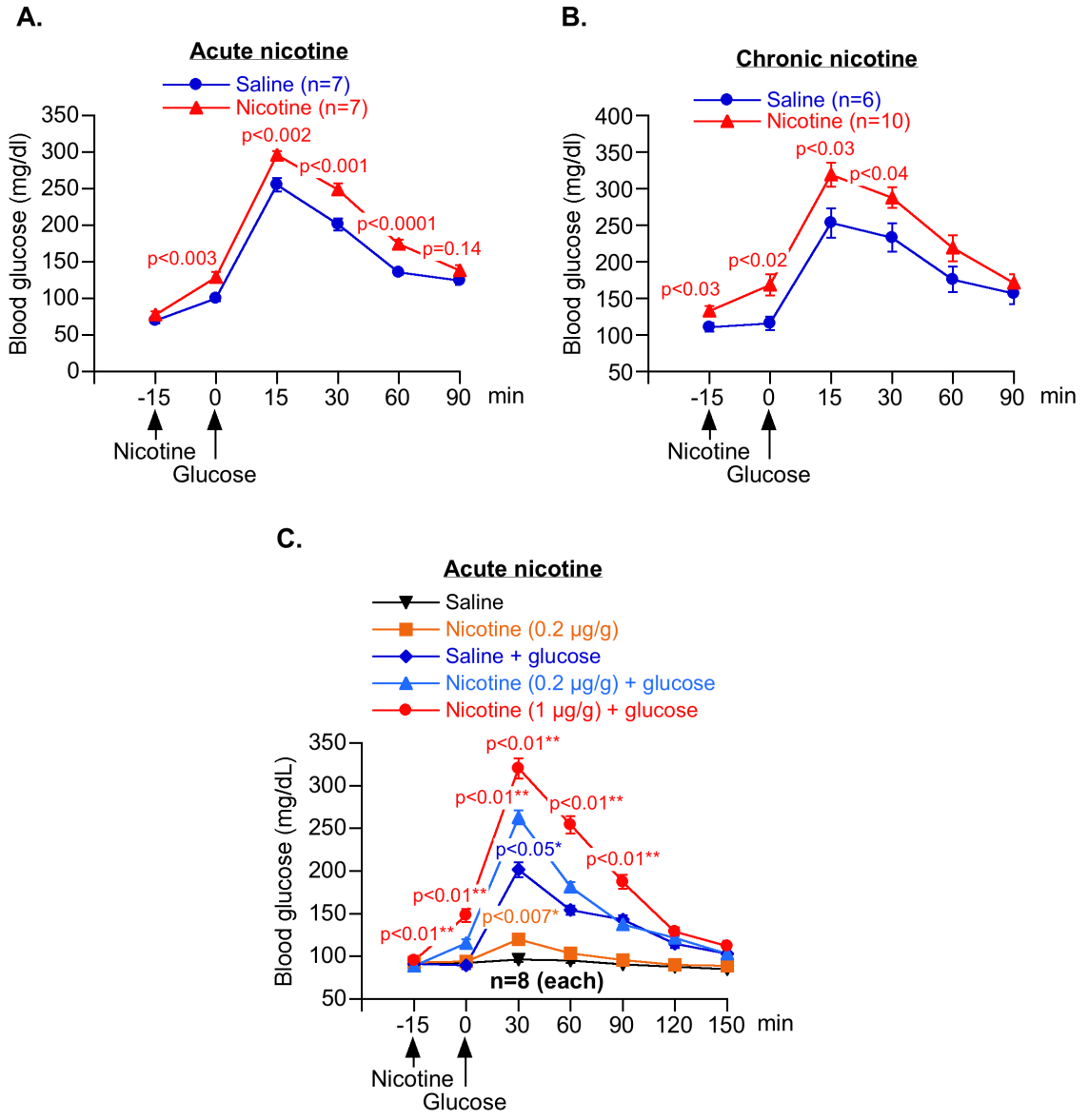

Figure 1. Nicotine's effect on glucose excursion during GTT in normal chow diet (NCD) fed mice. (A) Acute effect: Nicotine (0.2 µg/g body weight by IP) or saline was injected 15 minutes before glucose to mice (30.68±0.45 g). **(B)** Chronic effect: Nicotine (0.2 µg/g BW) or saline was injected twice to mice (31.17±0.36 g) daily for 16 days. Glucose was injected 15 minutes after the final dose of nicotine or saline**. (C)** Effects of acute treatment with saline or two doses of nicotine (0.2 and 1.0 µg/g BW) with or without subsequent glucose administration after 15 minutes. Blood glucose levels were determined at the indicated time points.

3.2. Modulation of insulin signaling through Akt by nicotine.

Nicotine treatment (for 16 hours) of cultured hepatocytes and muscle cells inhibited insulin stimulated Akt phosphorylation (Fig. 2A and 2B). These results suggest that the activation of certain subunits of nAChR may interfere with insulin signaling and disrupt glucose homeostasis, which is supported by the effects of chlorisondamine, an antagonist of various subunits of nAChR, on nicotine action. Chlorisondamine inhibits nAChR activation in both neural (31) and peripheral (32) tissues. As shown in Fig. 2C, chlorisondamine abolished the hyperglycemia induced by the chronic nicotine treatment. In line with these results, chlorisondamine treatment for 1 hour restored Akt phosphorylation in hepatocytes treated with nicotine for 16 hours (Fig. 2D). We therefore concluded that nAChR activation in insulin-sensitive, NCD-fed mice results in insulin resistance.

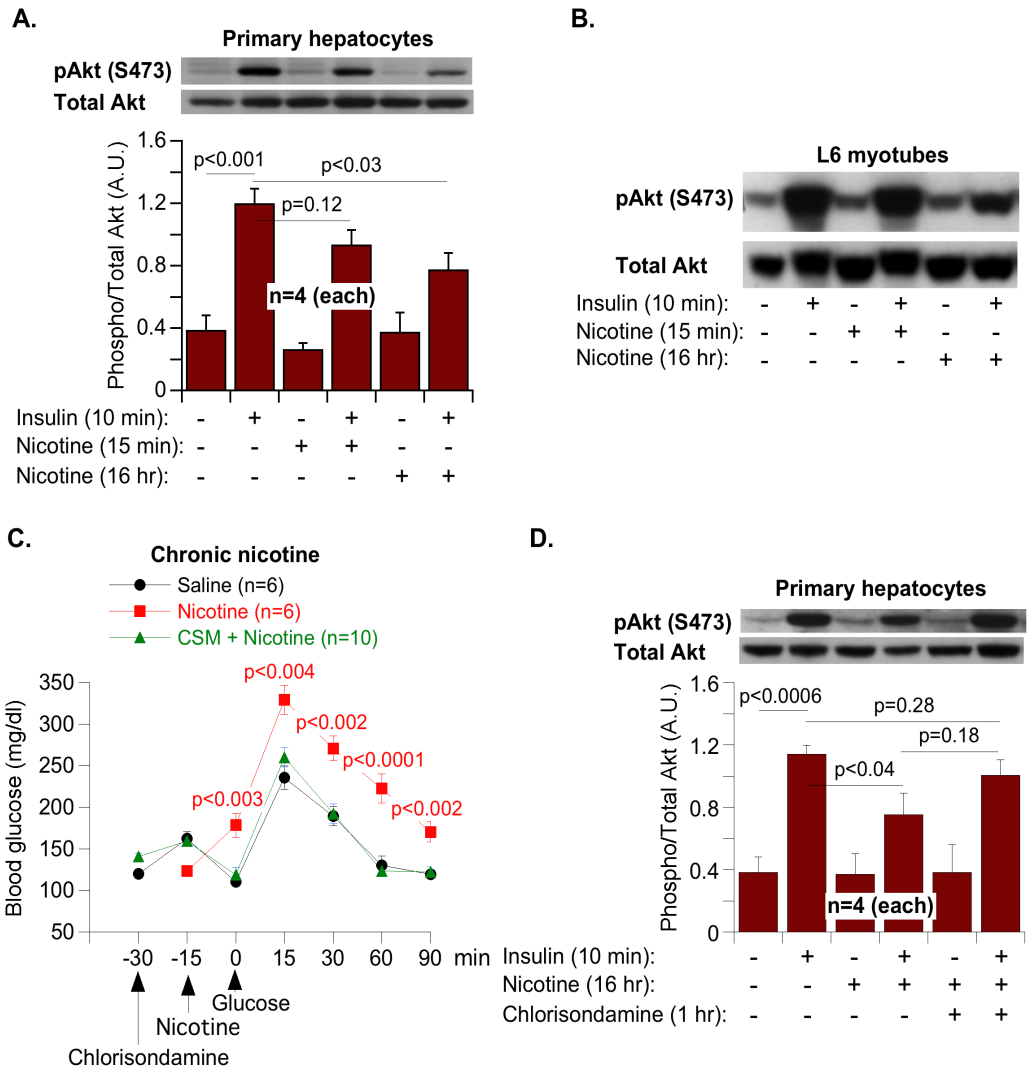

Figure 2. Modulation of insulin signaling through Akt by nicotine (A and B). Cultures of primary hepatocytes from NCD fed WT mice (A) and L6 myotubes (B) were treated with nicotine (10 µM) for 15 minutes or 16 hours before a 10-minute insulin treatment (10 nM). Cell lysates were immunoblotted for phospho- and total Akt. Results shown with hepatocytes (A) are representative of 4 experiments and with myotubes (B) are representative of duplicate experiment. **(C)** Mice were treated with nicotine (0.2 µg/g BW twice daily by IP injection) or saline for 16 days before GTT was carried out 15 minutes after the final injection. A group of nicotine-treated mice were injected with chlorisondamine (50 µM, i.p.) 30 minutes before the glucose injection. Blood glucose concentrations were monitored at the indicated time points. **(D)** Primary hepatocytes from NCD fed mice were treated with nicotine (10 µM) for 16 hours and with chlorisondamine (CSM, 50 µM) for the final 1 hour as indicated. Insulin (10 nM) was added as indicated for the final 10 minutes prior to harvesting. Lysates were immunoblotted for phospho- and total Akt.

3.3 Expression of nAChR subunits in adrenal, liver and muscle tissues.

An examination of the expression pattern of mRNA for nAChR subunits revealed that adrenal glands, liver and muscle express α1-7, α9-10, and β1-4 subunits (Fig. 3). Adrenal gland expressed most of the subunits at a higher level compared to liver and muscle except α2 and β2 subunits. α2 was more abundant in liver (Fig. 3B) whereas β2 was in muscle (Fig. 3K). α1 was barely detectable in liver and α3 was undetectable in muscle (Fig. 3A and C). Among the three tissues, liver had the lowest expression of most of the subunits (except α2). Interestingly, while chronic nicotine treatment down-regulated the expression of α3 subunit β2 expression was upregulated in adrenal gland (Fig. 3N). Our results on adrenal nAChR expressions are comparable to previous reports (33-35). Of note, in β2-knockout mice, 95% of the nicotine response in terms of secreting catecholamines is lost (36), suggesting a role of the β2 subunit in nicotine action on catecholamine secretion. We also observed increased mRNA expression of α1 and α5 in liver and α2 in muscle after chronic nicotine treatment (Fig. 3O and P).

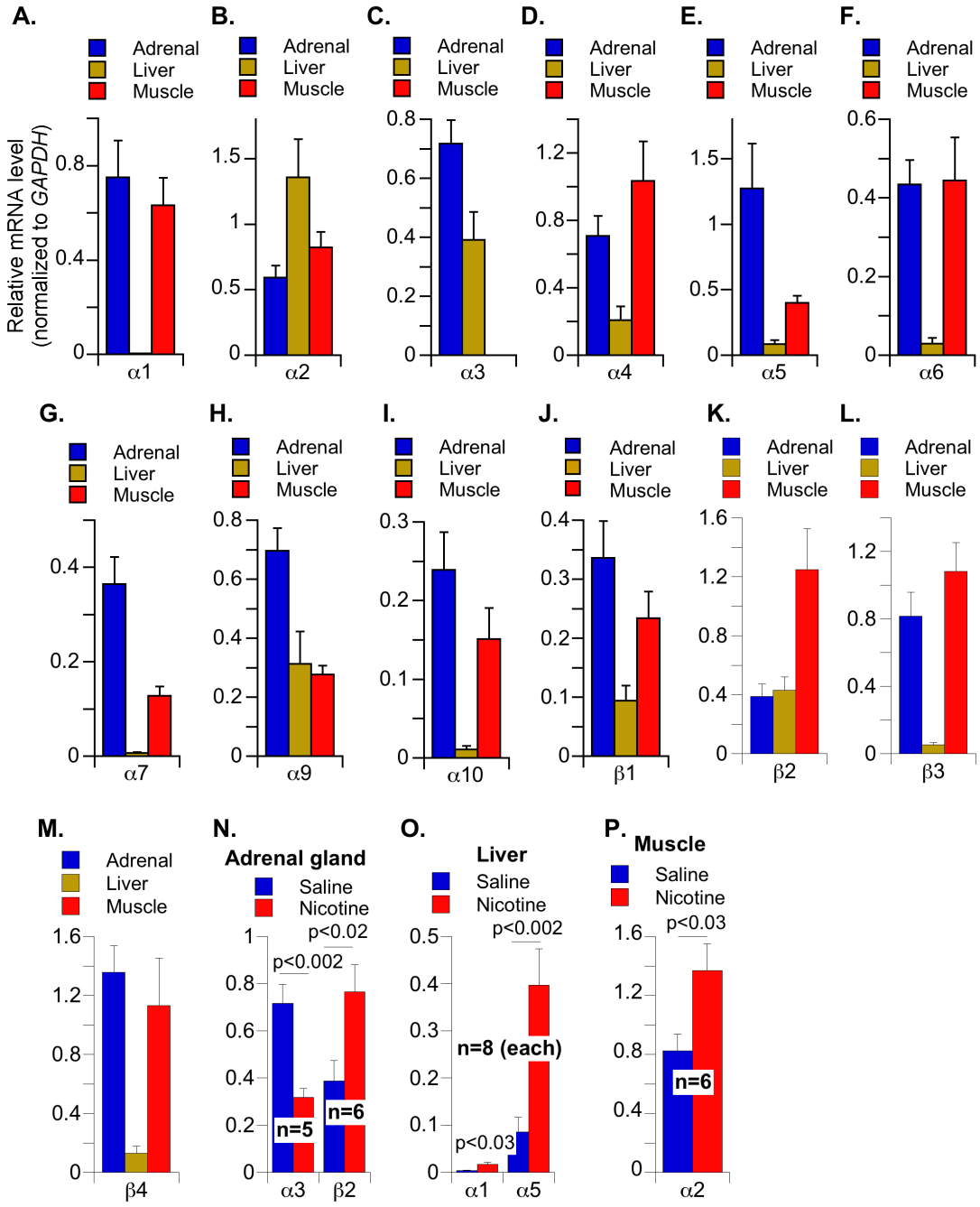

Figure 3. Qualitative expression of nAChR subunits in adrenal gland, liver and muscle. Adrenal glands, liver and gastrocnemius muscle from mice treated with nicotine (0.2 µg/g BW twice daily by i.p.) or saline for 16 days were collected in liquid nitrogen. RNA and cDNA were prepared from the tissues and subjected to quantitative real-time PCR analysis for various nAChR subunits. The expression of each subunit in three tissues is shown. Although the effects of nicotine treatment were compared to the saline treated mice, only the expression of subunits, which showed statistically significant differences in response to nicotine treatment, are shown in Fig. 3N-P.

3.4 Effects of nicotine on glucose stimulated insulin secretion (GSIS).

Acute treatment with nicotine (15 minutes) significantly stimulated plasma insulin concentration in both the basal state (2-3 fold) and after glucose injection (Fig. 4A). After chronic nicotine treatment, basal plasma insulin concentration remained higher than saline-treated group, whereas glucose-induced increase in insulin (15 minutes) was lost (Fig. 4B). In terms of insulin secretion, beta cell functions were not impaired by chronic nicotine treatment, as plasma C-peptide levels were not different between saline and nicotine treated groups (Fig. 4C). But the lower molar ratio of C-peptide to insulin in the nicotine group suggests a decrease in the metabolic clearance of insulin. Overall, these results corroborate GTT data, suggesting that nicotine caused insulin resistance and, as a result, the increased level of plasma insulin attained by the nicotine treatment could not prevent hyperglycemia.

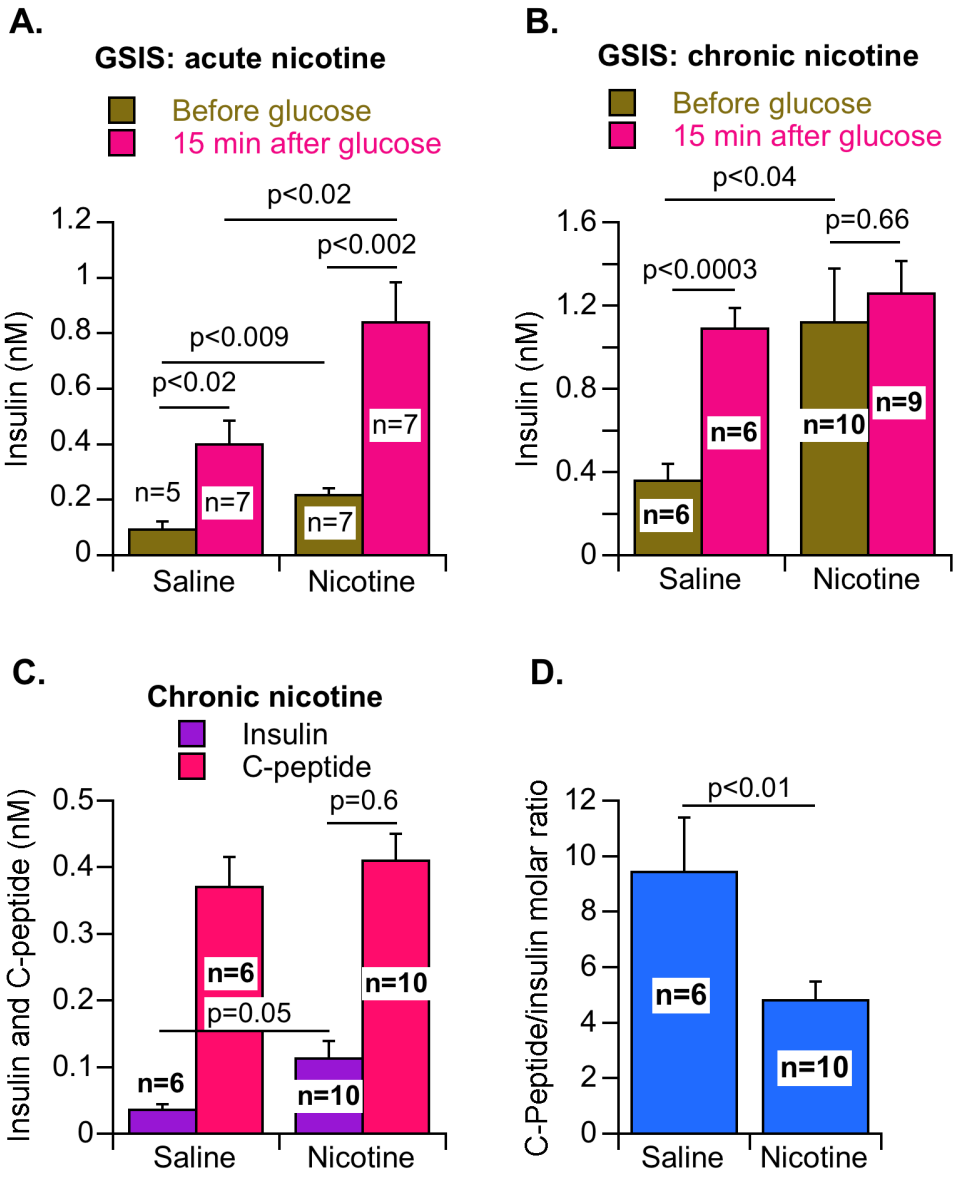

Figure 4. Nicotine regulation of basal and glucose-stimulated insulin secretion from pancreas and its clearance from liver. Mice were treated with saline or nicotine (0.2 µg/g BW by IP injection once for acute and twice daily for chronic) for 16 days. Blood samples collected at 0 and 15 minutes after glucose injection (15 minutes or 30 minutes after nicotine injection) to fasting mice were analyzed for insulin **(A-C)** and C-peptide **(C).** The C-peptide/insulin molar ratio is shown in **D**.

3.5 Effect of nicotine on glucose uptake in muscle cells.

Since chronic nicotine treatment stimulates the release of catecholamines and corticosteroids (37, 38) *in vivo*, it is important to know the effects of these hormonal changes on glucose disposal by muscle. We simulated these conditions in cultured L6 myotubes and observed that nicotine did not alter basal glucose uptake and might have somewhat suppressed insulin-stimulated glucose uptake, but nicotine effect was compensated by the presence of epinephrine or dexamethasone due to increase in basal glucose uptake (Fig. 5). Epinephrine and dexamethasone treatments either alone or in combination, increased basal glucose uptake in muscle cells but did not add to net insulin response as there was no increase in insulin minus basal counts (Fig. 5). In other words, nicotine treatment did not impair glucose disposal by muscle *in vivo* because of the compensatory effects of epinephrine and corticosteroids.

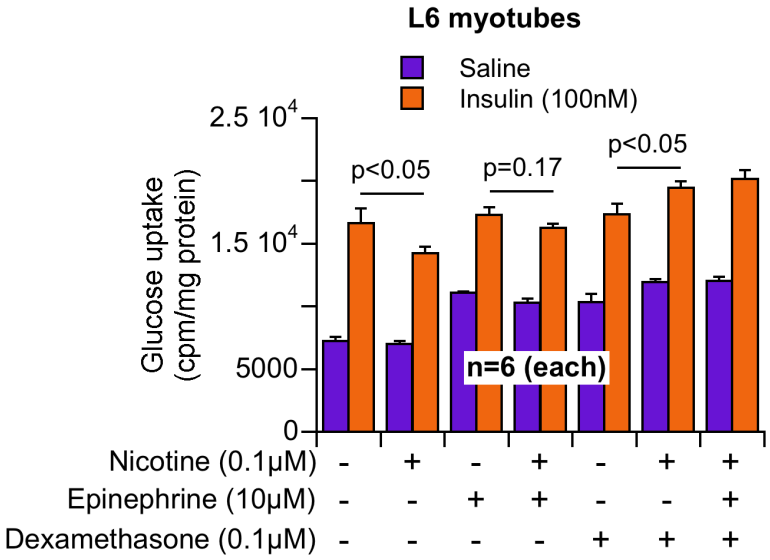

Figure 5. Nicotine regulation of glucose uptake in muscle cells. L6 myotubes were treated for 30 minutes with the indicated combination of nicotine (0.1 μM), epinephrine (10 μM), and dexamethasone (100 nM) followed by insulin (100 nM) for 20 minutes. [^{3}H]-deoxyglucose uptake was then quantified as described in Materials and Methods.

3.6 Effect of nicotine on the expression of gluconeogenic genes *Pepck* and *G6Pase* and glucose production.

Chronic nicotine treatment stimulated the mRNA expression of *Pepck* and *G6Pase*, key genes for insulin-regulated enzymes for gluconeogenesis (Fig. 6A). However, an earlier report evaluating the synthesis of ^{14}C-Glucose from [^{14}C]CO_3^- concluded that nicotine did not promote gluconeogenesis in obese, insulin-resistant rats (19). We sought to examine this issue in an insulin-sensitive model by analyzing the effects of nicotine on glucose production from ^{14}C-pyruvate in primary hepatocytes obtained from normal, insulin-sensitive mice. We pre-incubated hepatocytes with nicotine (10 μM), insulin (10 nM), glucagon (100 ng/ml), or various combinations for 30 minutes and then with the addition of ^{14}C-pyruvate for additional 3 hours (total 3.5 hours of incubation). For nicotine, there were two incubation times, 3.5 hours and 19 hours (16 hours of pre-incubation and 3 hours with pyruvate). At the end of the incubation, ^{14}C-glucose released into the media was separated from ^{14}C-pyruvate and quantified. Results shown in Fig. 6B indicate that glucagon treatment for 3.5 hours stimulated glucose production that was significantly inhibited by insulin. Similarly, 3.5 hours of nicotine treatment increased glucose production, an effect sensitive to suppression by insulin (Fig. 6B).

On the other hand, although 19 hours of nicotine treatment significantly increased glucose production, this effect was not suppressed by insulin, suggesting the development of insulin resistance after prolonged nicotine treatment. So, we concluded that in contrast to the results obtained using obese, insulin-resistant rats (19), hepatocytes from insulin

sensitive mice produce more glucose and become insulin resistant upon exposure to chronic nicotine (Fig. 6B).

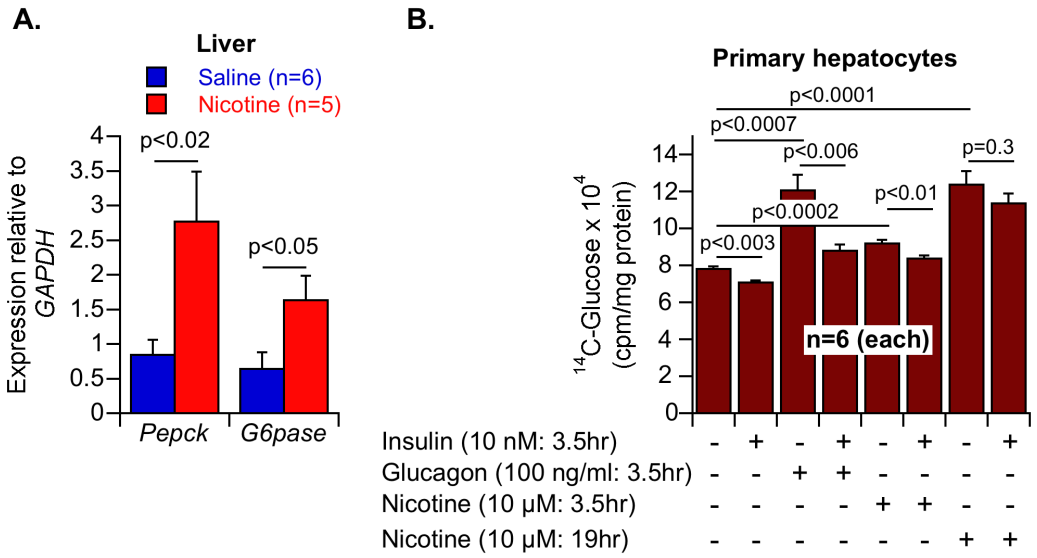

Figure 6. Nicotine regulation of expression of gluconeogenic genes in liver and glucose production in hepatocytes. (A) NCD-fed mice were treated with nicotine (0.2 µg/g BW) for 16 days and liver was harvested for mRNA extraction and processed for quantitative RT-PCR to determine the expression levels of *Pepck* and *G6Pase*. **(B)** Primary hepatocytes were treated for 30 minutes with nicotine (10 µM), glucagon (100 ng/ml), insulin (10 nM), or both glucagon and insulin. 2-[^{14}C]-pyruvate (2 mM) was then added as substrate, and cultures were incubated for an additional 3 hours. For chronic nicotine exposure, cultures were treated with nicotine (20 µM) for 16 hours followed by 3 hours incubation with radiolabeled pyruvate (2 mM) in presence or absence of insulin (10 nM). At the end of the 3-hours incubation with pyruvate, radiolabeled glucose released into the medium was measured as described in Materials and Methods.

3.7 Effects of nicotine on plasma catecholamines and hepatocyte cAMP levels.

Acute nicotine treatment for 15 minutes raised plasma catecholamine levels (Fig. 7A). While nicotine treatment did not stimulate cAMP production in hepatocytes, it did stimulate cAMP production in PC12 adrenal chromaffin cells (Fig. 7B). This was expected, as hepatocytes do not store catecholamines. In PC12 cells, cAMP production by nicotine was inhibited by the nAChR antagonist chlorisondamine, suggesting that nicotine-activated nAChR signals through β-ADR in PC12 cells but not in hepatocytes (Fig. 7B).

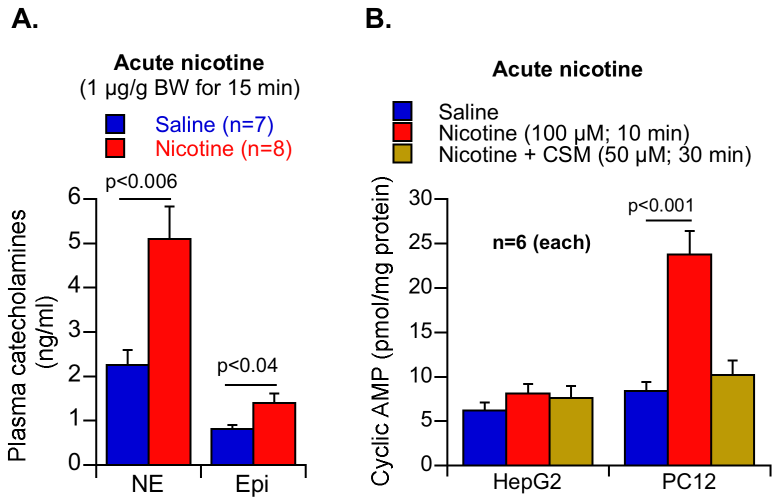

Figure 7. Nicotine regulation of catecholamine secretion and production of cAMP. Mice were treated with nicotine (1 µg/g BW) for 15 minutes **(A).** Plasma levels of norepinephrine (NE) and epinephrine (Epi) were determined by HPLC. Cyclic AMP levels in HepG2 and PC12 cells were determined after nicotine (100 µM, for last 10 minutes of incubation) treatment in the presence or absence of chlorisondamine (CSM, 50 µM for 30 minutes) **(B).**

3.8 Effects of nicotine on NO and eNOS signaling.

Nicotine also elevated plasma (Fig. 8A) as well as hepatic NO levels in NCD-fed mice (Fig. 8B). Consistent with this finding, acute nicotine treatment (30 minutes) resulted in increased eNOS phosphorylation (Fig. 8C).

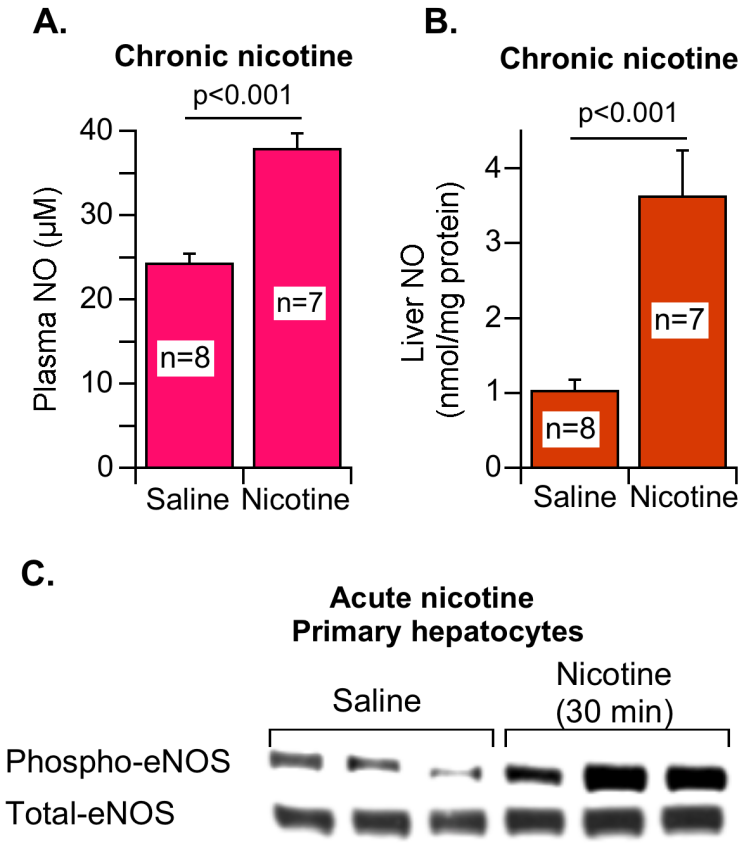

Figure 8. Nicotine regulation of NO production. NCD fed mice were treated with nicotine (0.2 µg/g BW by IP twice daily for 16 days) and sacrificed to collect plasma and liver. The effects of chronic nicotine treatment on NO concentrations in plasma **(A)** and in liver **(B)** are shown. Primary hepatocyte cultures were treated with nicotine (10 µM) for 30 minutes and immunoblotted to detect phospho- and total eNOS signals in triplicate culture samples **(C).**

3.9 Modulation of nicotine effects on catecholamine secretion and NO production by nAChR and ADR agonists and NOS inhibitors.

Hyperglycemia induced by acute nicotine treatment was normalized by NOSI (Fig. 9A). A combined treatment of L-NNA (selective for nNOS and eNOS) and L-NIL (selective for iNOS) was used to inhibit all types of NOS (39-41). Similar normalization of hyperglycemia by NOSI treatment was also observed after chronic nicotine administration (results not shown). The improved glucose tolerance by NOSI was possibly caused by preserving insulin-AKT signaling from inhibition by chronic nicotine treatment (Fig. 9B). Nicotine induced NO production in cultured HepG2 cells, an effect likely involving nAChR signaling as it was blocked by chlorisondamine (Fig. 9C). Among the ADR agonists, phenylephrine (an α agonist) was more effective than isoproterenol (a β agonist) in NO production in liver cells (Fig. 9C). Both acute nicotine and NOSI raised plasma catecholamines (Fig. 9D). NOSI-induced catecholamine secretion was significantly inhibited by nicotine (Fig. 9D).

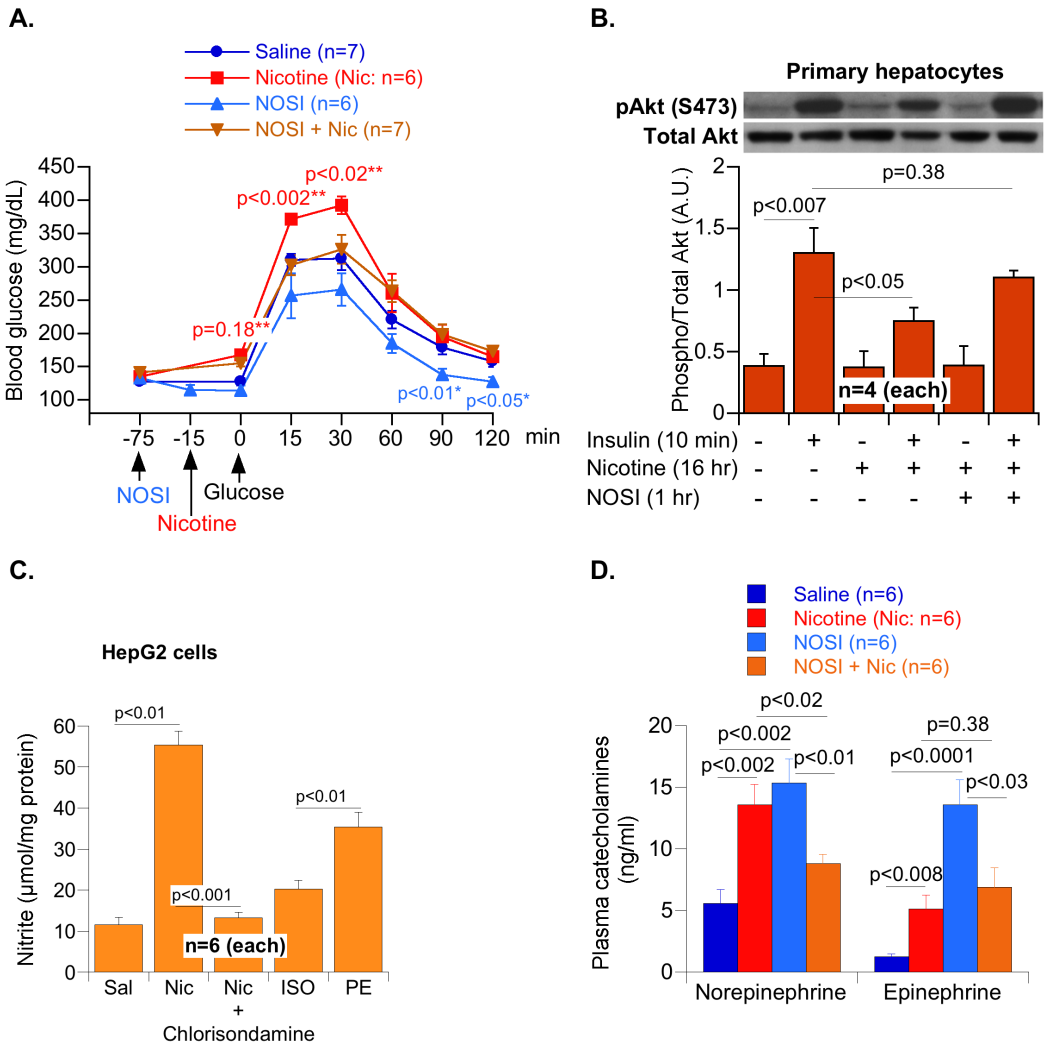

Figure 9. Modulation of nicotine effects on glucose metabolism, NO production and catecholamine secretion by NOSI. NCD fed fasted male mice were injected IP with a solution of NOS inhibitors (a combination of L-NIL and L-NNA, 15 µg/g BW each) 60 minutes before nicotine (0.2 µg/g BW) and 75 minutes before glucose injection. Blood glucose levels were determined at the indicated times after glucose injection **(A)** and plasma catecholamine levels was determined 30 minutes after nicotine injection **(D)**. Primary hepatocyte cultures pre-incubated with nicotine (20 µM) or vehicle for 16 hours were treated with the combination of NOS inhibitors (10 µM each) for the last 1 hour in presence or absence of insulin (10 nM) for the last 10 minutes incubation and phospho-Akt and total-Akt signals were determined **(B).** HepG2 hepatocyte cultures were treated with nicotine (Nic, 10 µM for 30 minutes), chlorisondamine (50 µM for 60 minutes), isoproterenolol (ISO, 10 µM for 30 minutes), phenylephrine (PE, 10 µM, for 30 minutes) and chlorisondamine (50 µM) plus nicotine (10 µM) where nicotine was added 30 minutes after chlorisondamine addition, and NO concentrations in those treated cultures were determined **(C).**

3.10 Modulation of Glycogenolysis by nAChR, ADR, cAMP and NO in HepG2 cells.

Hepatic glycogen content in fasting mice was diminished after chronic nicotine treatment (Fig. 10A). *In vivo*, activation of nAChR by nicotine leads to catecholamine release (Fig. 7A) and ADR activation (Fig. 7B), which, in turn, could activate glycogen phosphorylase via cAMP/PKA pathway and enhance glycogenolysis (42, 43). These pathways might not exist in cultured hepatocytes due to the lack of catecholamines. As a result, there was no cAMP production by nicotine treatment in cultured HepG2 cells whereas the same treatment led to cAMP production in PC12 neuroendocrine cells (Fig. 7B). In other words, activated nAChR does not communicate with β-ADR or the adenylate cyclase (AC) system in hepatocytes as it does in neuroendocrine cells. In order to dissect the pathway(s) leading to glycogenolysis, we preloaded HepG2 cells with ^{14}C-glucose to radiolabel glycogen and observed that nicotine stimulated the release of ^{14}C-glucose from these cells. This nicotine effect was completely abolished by the inhibitors of NOS as well as by chlorisondamine (Fig. 10B), suggesting that nAChR communicates with NOS to modulate glycogenolysis. The two ADR antagonists: propranolol (a β antagonist) and prazosin (an α antagonist) only partially inhibited the nicotine-induced glycogenolysis (Fig. 10B). Cyclic AMP as well as the α-ADR agonist phenylephrine stimulated glucose release from hepatocytes, whereas the β-ADR agonist isoproterenolol had no significant effects (Fig. 10C). Of note, nicotine and various ADR agonists stimulated NO production in HepG2 cells. Phenylephrine was found to be more potent than isoproterenolol (Fig. 10C). NO also stimulated glycogenolysis from the preloaded hepatocytes. The NO donor sodium nitroprusside (SNP) had a biphasic effect on glycogenolysis: low mM concentrations stimulated glycogenolysis whereas high

concentrations inhibited it (Fig. 10D). NO showed some additive effects with cAMP on glucose release from HepG2 cells (Fig. 10C). cAMP effect was partially inhibited and the stimulatory effects of phenylephrine was completely blocked by NOS inhibitors (Fig. 10C). Therefore, NO might be the crucial mediator of the chronic effects of nicotine on glycogenolysis. It is possible that the basal ADR activity also contributes to NO production and that ADR activation (by catecholamine *in vivo*) signals through both cAMP and NO production. *In vivo*, epinephrine and norepinephrine activate both α- and β-ADR and, as shown by Bugajski J et al., the α-ADR antagonist phentolamine can wipe out all catecholamine effects on hyperglycemia and glycogenolysis (42). Previously, Hutson NJ et al. demonstrated that the effects of catecholamines on glucose metabolism were mediated predominantly by α-ADR, not β-ADR (43). In summary, nicotine-induced glycogenolysis in hepatocytes primarily involved nAChR, NO, and α-ADR but not β-ADR.

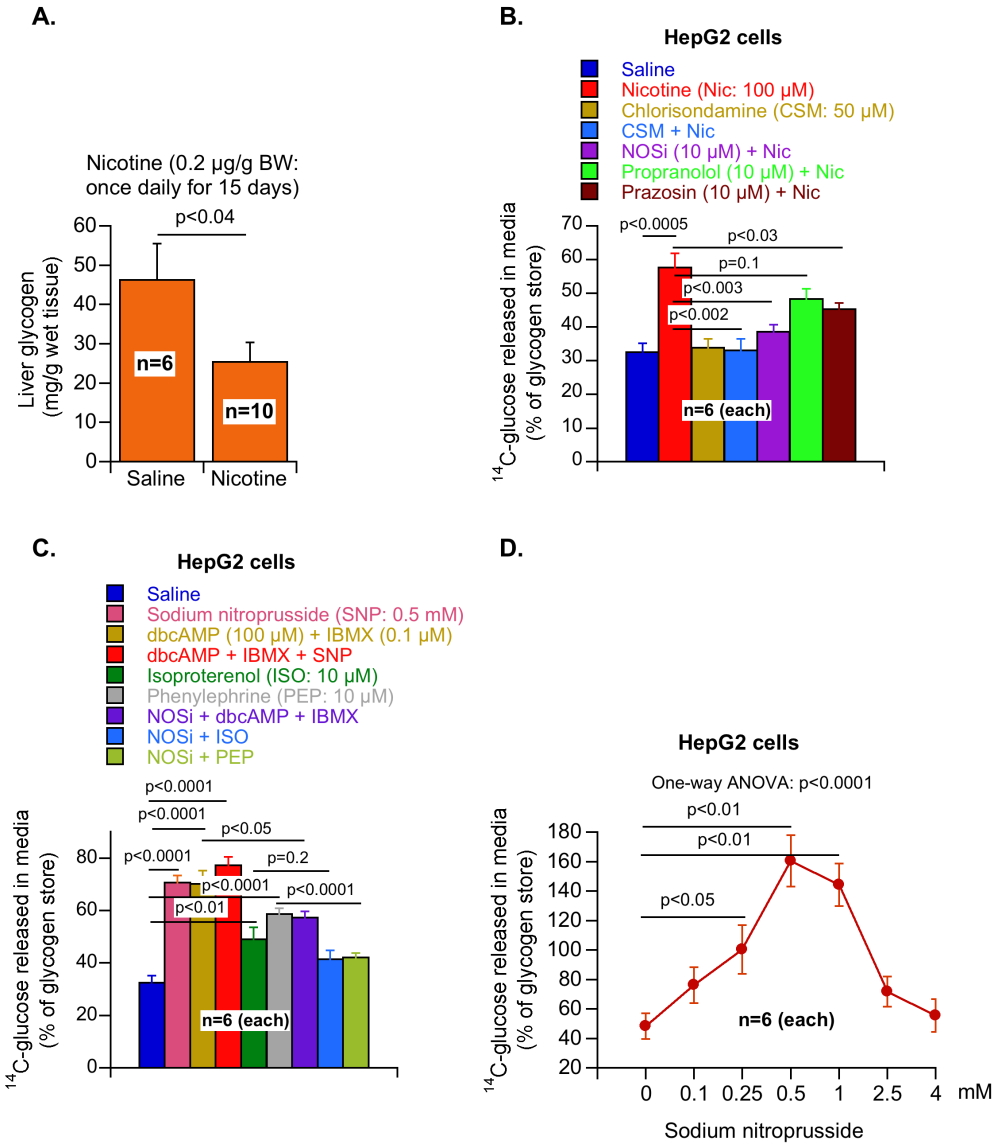

Figure 10. Regulation of glycogenolysis by nicotine and its modulation by nAChR, ADR and NO antagonists and agonists. **(A)** Liver glycogen concentrations after chronic nicotine treatment. **(B)** [^{14}C]-glycogen pre-loaded HepG2 cells were exposed to chlorisondamine (50 µM) or NOSI (10 µM), propranolol, or prazosin for 10 minutes followed by nicotine (100 µM) for 20 minutes. Conditioned media were collected to determine glycogen breakdown based on the release of [^{14}C]-glucose during the 30 minutes of the above treatments. **(C)** Another set of glycogen-preloaded HepG2 cultures were treated with NO donor sodium nitroprusside (SNP) (0.5 µM for 60 minutes) with or without dibutyryl cAMP (100 µM for last 30 minutes) and isoproterenolol (10 µM), phenylephrine (10 µM), dibutyryl cAMP (100 µM)+ IBMX (100 nM) in presence or absence of NOSI (10 µM each of L-NIL and L-NNA for 60 minutes). The agonists and cAMP were incubated for 30 minutes and added to the cultures 30 minutes after the addition of NOSI. **(D)** The dose-dependent effect of SNP (0-4 µM) was determined on the [^{14}C]-glucose release from [^{14}C]-glycogen preloaded HepG2 cell.

DISCUSSION

Although nAChR activation has a well-documented anti-inflammatory effect, it did not seem to help maintain glucose homeostasis in insulin-sensitive mice. Both acute and chronic nAChR activation by nicotine caused fasting hyperglycemia and hyperinsulinemia in NCD-fed mice. Glucose-induced insulin secretion was abolished by chronic nicotine treatment but unaffected by acute nicotine treatment. It is likely that nicotine-induced increases in plasma catecholamines triggered insulin secretion. Such a role for catecholamine has been reported (44, 45). Although chronic nicotine treatment did not affect plasma C-peptide levels, it decreases the C-peptide to insulin ratio, consistent with a decrease in insulin clearance. It is possible that chronic hyperinsulinemia downregulated insulin receptors in peripheral tissues and impaired insulin clearance through endocytosis. This could be an important issue because defective insulin clearance has been reported to cause glucose intolerance (46, 47).

Both the mRNA expression of *Pepck* and *G6Pase* and glucose production in hepatocytes increased after chronic nicotine treatment. Nicotine induces catecholamine and corticosteroid secretion *in vivo*. Simulation of such a hormonal milieu in myotube cultures by adding nicotine, catecholamine and dexamethasone to the culture medium demonstrated increased basal glucose uptake by muscle cells but no improvement of insulin-stimulated uptake. Overall, it appears that basal glucose disposal *in vivo* is not impaired by nicotine administration. Therefore, impaired glucose disposal may not be the cause for nicotine-induced hyperglycemia. It appears that chronic nicotine treatment stimulates both gluconeogenesis and glycogenolysis, whereas acute treatment act

primarily through glycogenolysis. Nicotine induces secretion of catecholamine and NO and depletes liver glycogen stores in fasting mice. Both catecholamine and NO play regulatory roles in glycogenolysis. The contribution of NO to nicotine-induced hyperglycemia is underscored by the observation that NOS inhibitors prevented nicotine effect. In hepatocyte cultures, acute nicotine treatment produced NO. Glycogen breakdown stimulated by nicotine treatment was suppressed by both chlorisondamine and NOSI, suggesting that a role of NO in glycogenolysis induced by nAChR signaling. Since NO has been reported to affect glucose metabolism (48, 49) and its production is stimulated by ADR agonists in hepatocytes (50), and by catecholamines in macrophages (51), NO could be a significant player in mediating nicotine effects in liver cells.

Moreover, nicotine treatment of hepatocytes and muscle cells for 16 hours inhibited insulin-stimulated Akt phosphorylation, signifying a resistance to insulin action on glucose metabolism. Treating hepatocytes with chlorisondamine and NOSI reversed the suppression of Akt phosphorylation by nicotine. These results provide a potential mechanism for chlorisondamine and NOSI to improve glucose tolerance in nicotine-treated mice. Nicotine did not increase cAMP levels in hepatocytes, suggesting that NO production, not cAMP-PKA signaling, was mainly responsible for nicotine-induced glycogenolysis stimulated by activated nAChR in liver. However, *in vivo*, catecholamines released from neuroendocrine tissues upon nAChR activation caused cAMP production in liver leading to glycogenolysis via activation of PKA pathway.

Therefore, hepatic glycogenolysis *in vivo* is caused by the combined actions of NO produced by nAChR activation and cAMP produced by ADR activation in response to catecholamine released from neuroendocrine tissues upon nAChR activation. It is

possible that NO-mediated nitrosylation of enzymes such as glycogen phosphorylase a/b contributed to nicotine-induced glycogenolysis. Tyrosine-nitration of IRS-1 by NO causes insulin resistance (52). It should be noted that persistently high levels of NO might contribute to oxidative damage and metabolic disorder through the formation of peroxynitrite radicals ($ONOO^-$) (53, 54).

Activation of nAChR by nicotine for 12 to 36 hours in cultured adipocytes was shown to stimulate the production of adiponectin, TNF-α and free fatty acids (FFA) (55). It is possible that nicotine initially causes insulin resistance by enhancing TNF-α and FFA production. However, GTT showed that the hyperglycemic effect of nicotine was established within 15-30 minutes, much faster than the kinetics of hyperglycemia induced by TNF-α or FFA (56).

The fact that chlorisondamine, an inhibitor of nAChR activation (31, 32), can prevent the hyperglycemic effects of nicotine highlights the role nAChR in nicotine-induced hyperglycemia. Nicotine activates both α7 homomeric (abundantly present in hippocampus) as well as α/β heteromeric nAChR such as α4β2 and α3β2 in the CNS and α3β4 in the peripheral nervous system. Liver and muscle expressed mRNA of various α and β subunits of nAChR but the level of α7 subunits were the lowest. Chronic treatment with nicotine induced mRNA expressions of α3 and β2 in adrenal gland, α1 and α5 in liver and α2 in muscle. However, protein expressions of these subunits have not been analyzed. While the α7 subunit has a strong anti-inflammatory role (57-59), deletion of β2 gene has been shown to eliminate nicotine-induced catecholamine release (36). Although selective α7-nAChR activation has been shown to improve glucose tolerance in

insulin-resistant models presumably due to its anti-inflammatory effects (20, 22), the role of other heteromeric nAChR remained unexplored. In insulin-sensitive mice, nicotine also activates α7-nAChR but produces the opposite effects, suggesting that β2 or other nAChR subunits may mediate nicotine effects. In this context, it would be important to find out whether β2-knockout mice, lacking the ability to provoke catecholamine release, are resistant to nicotine-induced hyperglycemia. In other words, heteromeric nAChR subunits, present in liver and muscle and adipose tissue, may also play important roles in glucose homeostasis.

In conclusion, the activation of nAChR exerts dual effects on glucose metabolism. In insulin-sensitive mice, it increases glycogen breakdown in an NO- and catecholamine-dependent manner and, after prolonged exposure to nicotine, enhances glucose production. The consequence of nAChR activation in insulin-sensitive, non-inflamed NCD fed mice is therefore hyperglycemia. On the other hand, the reported beneficial effects of α7-nAChR activation in insulin-resistant models with high levels of inflammation may be caused by the heightened anti-inflammatory effects and dampening of the pathway of glycogen breakdown leading to improved insulin sensitivity. In liver, direct activation of nAChR by nicotine evokes predominantly a NO-linked pathway. Nicotine does not directly evoke cAMP/PKA pathway in liver. However, *in vivo*, nicotine stimulates catecholamine production from neuroendocrine tissues that, in turn, activates α- and β-ADR pathways in liver. Attenuation of nicotine-induced NO production and activation of ADR functions by inhibiting nAChR action will prevent nicotine-induced glucose intolerance in smokers.

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