Alpha-7-nicotinic Acetylcholine Receptor Agonist Ameliorates Nicotine plus High-Fat Diet-Induced Hepatic Steatosis in Male Mice by Inhibiting Oxidative Stress and Stimulating AMPK Signaling.
Alpha-7-nicotinic Acetylcholine Receptor Agonist Ameliorates Nicotine plus High-Fat Diet-Induced Hepatic Steatosis in Male Mice by Inhibiting Oxidative Stress and Stimulating AMPK Signaling


Endocrinology
Endocrine Society
Submitted: June 26, 2017
Accepted: December 13, 2017
First Online: December 20, 2017

Advance Articles are PDF versions of manuscripts that have been peer reviewed and accepted but not yet copyedited. The manuscripts are published online as soon as possible after acceptance and before the copyedited, typeset articles are published. They are posted "as is" (i.e., as submitted by the authors at the modification stage), and do not reflect editorial changes. No corrections/changes to the PDF manuscripts are accepted. Accordingly, there likely will be differences between the Advance Article manuscripts and the final, typeset articles. The manuscripts remain listed on the Advance Article page until the final, typeset articles are posted. At that point, the manuscripts are removed from the Advance Article page.

DISCLAIMER: These manuscripts are provided "as is" without warranty of any kind, either express or particular purpose, or non-infringement. Changes will be made to these manuscripts before publication. Review and/or use or reliance on these materials is at the discretion and risk of the reader/user. In no event shall the Endocrine Society be liable for damages of any kind arising references to, products or publications do not imply endorsement of that product or publication.
Role of α7nAChR in hepatic steatosis.

Alpha-7-nicotinic Acetylcholine Receptor Agonist Ameliorates Nicotine plus High-Fat Diet-Induced Hepatic Steatosis in Male Mice by Inhibiting Oxidative Stress and Stimulating AMPK Signaling

Mohammad Kamrul Hasan,1 Theodore C. Friedman,1,2 Carl Sims,1 Desean L. Lee,1 Jorge Espinoza-Derout,1 Adaku Ume,1 Victor Chalfant,1 Martin L. Lee,1,2 Indrani Sinha-Hikim,1,2 Kabirullah Lutfy,3 Yanjun Liu,1 Sushil K. Mahata,4,5 and Amiya P. Sinha-Hikim1,2

1Division of Endocrinology, Metabolism and Molecular Medicine, Department of Internal Medicine, Charles R. Drew University of Medicine and Science, Los Angeles, CA 90059; 2 David Geffen School of Medicine at University of California, Los Angeles, Los Angeles, CA 90095; 3 College of Pharmacy, Western University of Health Sciences, Pomona, CA 91766; 4Department of Medicine, University of California at San Diego, CA 92093; and 5VA San Diego Health Care System, San Diego, CA

Received 26 June 2017. Accepted 13 December 2017.

Alpha7-nicotinic-acetylcholine receptor (α7nAChR) agonists confer protection against a wide variety of cytotoxic insults and suppress oxidative stress and apoptosis in various cell systems, including hepatocytes. We recently demonstrated that nicotine when combined with a high-fat diet (HFD) triggers oxidative stress, activates hepatocyte apoptosis, and exacerbates HFD-induced hepatic steatosis in male mice. This study evaluates whether PNU-282987 or PNU, a specific α7nAChR agonist, is effective in preventing nicotine plus HFD-induced hepatic steatosis. Adult C57BL6 male mice were fed a normal chow diet or HFD with 60% of calories derived from fat and received twice daily IP injections of 0.75 mg/kg BW of nicotine, PNU (0.26 mg/kg BW), PNU plus nicotine, or saline for 10 weeks. PNU treatment was effective in attenuating nicotine plus HFD-induced increase in hepatic triglyceride levels, hepatocyte apoptosis, and hepatic steatosis. The preventive effects of PNU on nicotine plus HFD-induced hepatic steatosis were mediated by suppression of oxidative stress and activation of AMP-activated protein kinase (AMPK) together with inhibition of its downstream target sterol regulatory element binding protein 1c (SREBP1c), fatty acid synthase (FAS), and acetyl-coenzyme A-carboxylase (ACC). We conclude that α7nAChR agonist PNU protects nicotine plus HFD-induced hepatic steatosis in obese mice. PNU appears to work at various steps of signaling pathways involving suppression of oxidative stress, activation of AMPK, and inhibition of SREBP1c, FAS, and ACC. α7nAChR agonists may be an effective therapeutic strategy for ameliorating fatty liver disease, especially in obese smokers.

Pharmacological activation of alpha-7-nAChR by PNU-282987 through activation of AMPK and suppression of oxidative stress protects nicotine plus high-fat diet-induced hepatic steatosis in obese mice.

Introduction

Cigarette smoking is the leading preventable cause of death and disability worldwide (1). Smoking is a major risk factor for cardiovascular disease, chronic obstructive pulmonary disease and lung cancer and nonalcoholic fatty liver disease (NAFLD) (2-6). Importantly, the health risk associated with smoking is further exaggerated by obesity (7,8).

Nicotine acts on both high-affinity nicotinic acetylcholine receptors (nAChRs), such as α4/β2 receptor and the low-affinity receptor such as α7, both centrally and peripherally (9). In...
rodents, nicotine blocks high-fat diet (HFD)-induced weight gain, that was completely blocked by mecamylamine, a nonselective nAChR antagonist, but only partially blocked by the $\alpha_4\beta_2$ nAChR partial agonist/antagonist, varenicline (10). Nicotine when given with a HFD, leads to hepatic and muscle steatosis that is thought to be due to, at least in part, through increased abdominal fat lipolysis (11-13), although the nAChRs involved in this response are not characterized.

Hepatic $\alpha_3/\beta_4$ nAChR has been implicated in chronic nicotine exposure-induced improvement of glycemia and insulin sensitivity (14). Chronic exposure of nicotine, through the $\alpha_7$nAChR-mediated pathway, improves glucose homeostasis and insulin sensitivity in genetically obese and diet-induced obese mice (15). Treatment with PNU-282987, a specific $\alpha_7$nAChR agonist is also found to be protective against a wide variety of cytotoxic insults and suppresses oxidative stress and apoptosis in various cell systems, including hepatocytes (16-19). Indeed, $\alpha_7$nAChRs are present in the liver (14,16,20) and the $\alpha_7$nAChR-mediated pathway plays a major role in mitigating hepatic ischemia-reperfusion injury (21,22). TC-7020, an $\alpha_7$-nAChR agonist, reduced weight gain and improved metabolic disorders in db/db mice (23).

In the current study, we used the $\alpha_7$-nAChR agonist PNU-282987 and a commonly used model of diet-induced obesity (24-26), to elucidate the role of $\alpha_7$-nAChR agonism in nicotine-induced weight loss and hepatic steatosis in mice on a HFD. Given that oxidative stress and inactivation of AMP-activated protein kinase (AMPK) coupled with hepatocellular apoptosis plays a pivotal role in pathogenesis of hepatic steatosis (27-29), we also examined the effect of $\alpha_7$nAChR agonism on these parameters.

Materials and Methods

Animals

Male C57BL/6 mice weighing 22-24 g (Taconic Farms-Germantown, NY, USA) were used in all experiments. Mice were housed under controlled temperature (22°C) and photoperiod (12-h light and 12-h dark cycle) with free access to water and food. Mice were fed either a normal chow diet (NCD) with 5% fat (8.5 KJ/g; laboratory rodent diet #5001, Lab Diet, Richmond, IN, USA) or HFD with 60% of calories derived from fat. HFD consisted of 26.2% protein, 26.3% carbohydrate, and 34.9% fat (21.9 KJ/g; D12492, Research Diets, New Brunswick, NJ, USA) for 10 weeks. Groups of 5-6 mice on either diet received twice daily IP injections of nicotine (0.75 mg/kg BW), PNU-282987 (0.26 mg/kg BW), PNU plus nicotine (PNU was given 15 min before nicotine), or saline for 10 weeks. The rationale for using twice daily IP administration of nicotine (0.75 mg/kg BW) was based on the results of our previous studies which demonstrated that this dose led to levels of nicotine that when combined with a HFD triggers greater oxidative stress, activates hepatocellular apoptosis, and amplifies HFD-induced hepatic steatosis (11,28) and causes intramyocellular lipid accumulation and intramyofibrillar mitochondrial abnormalities in the skeletal muscle (13).

The rationale for using twice daily IP injections of PNU-28987 (0.26 mg/kg BW) was based on the results of a previous study (30), which showed that chronic treatment of PNU at this daily dosage (0.52 mg/kg BW/day) enhanced insulin sensitivity, as judged by reduction in homeostasis model assessment of insulin resistance (HOMA-IR), glucose tolerance test (GTT) and insulin tolerance test (ITT), in mice fed a NCD. Additional support for using this dose of PNU was based on the results of our pilot study, which showed that long-term treatment with twice daily IP injections of PNU at this dose level was able to prevent nicotine plus HFD-induced hepatic steatosis in male mice(31).
The rationale for using male C57BL/6 mice is that these male mice when fed a HFD deriving 60% of calories from fat develop visceral adiposity, hyperglycemia, insulin and leptin resistance, as well as hepatic steatosis and are commonly used model of diet-induced obesity (22-24). Moreover, female C57BL6/J mice on a similar HFD are less sensitive and develop much milder type 2 diabetes than that in males (32).

Mice were weighed weekly. The amount of food consumed per mouse was determined daily. Food intake was measured per cage with 2-3 mice per cage to avoid the stress of individual housing (24) and calculated per mouse (10). The cumulative caloric intake was calculated in each group as described previously. Mice were fasted overnight before euthanasia with a lethal injection of sodium pentobarbital (200 mg/kg BW). Livers were removed. Portions of liver were placed in RNAlater and used for gene expression analysis by quantitative RT-PCR or snap frozen in liquid N₂ and stored frozen for subsequent measurements of triglycerides and changes in protein expression by western blotting. The remaining portions of liver were either fixed in 2.5% glutaraldehyde for high-resolution light and electron microscopy or 4% paraformaldehyde for routine histological and immunohistochemical or immunofluorescence studies. Animal handling and experimentation were in accordance with the recommendation of the American Veterinary Medical Association and were approved by the Charles R. Drew University School of Medicine and Science Institutional Animal Care and Use Committee (IACUC).

Hepatic triglyceride (TG) levels
Hepatic TG levels were measured by using Abcam’s triglyceride quantitation kit according to manufacturer’s protocol (Abcam, Cambridge, MA, USA).

Assessment of apoptosis
*In situ* detection of cells with DNA strand breaks was performed in paraformaldehyde-fixed, paraffin-embedded liver sections by the terminal deoxynucleotidyl transferase (TdT)-mediated deoxy-UTP nick end labeling (TUNEL) technique (11,12,33) using an ApopTag-peroxidase kit (Chemicon International, Inc., San Francisco, CA, USA). Negative and positive controls were carried out for all assays. For negative controls, tissue sections were processed in an identical manner, except the TdT enzyme was substituted by the same volume of distilled water. Mammary tissue sections from rat pups 3-5 days after weaning (Charles River Laboratories, Worcester, MA, USA) in which apoptosis is well characterized (34) were used as positive controls. Enumeration of TUNEL-positive nuclei was carried out in liver sections using an American Optical Microscope with an X40 objective and a pair of X10 eyepieces. Methyl green was used as a counterstain to detect non-apoptotic nuclei. A square grid fitted within one eyepiece provided a reference of 62,500 µm². The rate of hepatocellular apoptosis was expressed as the percentage of the TUNEL-positive apoptotic nuclei per total nuclei (apoptotic plus non-apoptotic) present within the reference area (11,12,33).

Liver pathology
Liver pathology was evaluated using conventional histological analysis on hematoxylin and eosin (H&E) stained sections. Further evaluation of pathology was achieved by high-resolution light microscopy using glutaraldehyde-fixed, osmium tetroxide post-fixed, epoxy-embedded, and toluidine blue-stained sections and electron microscopy (11,33) Accumulation of intracellular fat was quantified by computerized densitometry using the ImagePro Plus soft aware coupled to an Olympus BHS microscope equipped with a VCC video camera (11,33). For electron microscopic studies, thin sections from selected tissue blocks were sectioned with an LKB ultramicrotome,
stained with uranyl acetate, and examined with a Hitachi 600 electron microscope (Hitachi, Indianapolis, USA).

**Quantitative RT-PCR:**
Hepatic RNA from mice on a HFD treated with saline or nicotine in the absence or presence of PNU was extracted with TRIzol Reagent (Invitrogen) using a Pyrex homogenizer. Total RNA was then treated with DNase I (Ambion) for 30 minutes at 37°C and purified with RNeasy mini kit (Qiagen). The purity of RNA was determined by 260/280 ratio using NanoDrop 2000 (Thermo Fisher Scientific) and RNA with greater than 1.9 ratio was considered as highly purified RNA. RNA integrity (RIN) was measured at UCLA genomic core using 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RIN values ranged from 7.5 to 9.2. cDNA was prepared using High Quality RNA to cDNA kit (Applied Biosystems). cDNA was diluted 1:10 and 3-4 µL of the diluted cDNA were used for quantitative real time PCR. RT-q-PCR was done using Step-One plus RT-PCR system (Life Technology) with a Sybr Green PCR Master Mix (Applied Biosystems). All reactions were analyzed in triplicates and 4-5 mice from each group tested. Data were normalized to 18S rRNA transcripts using the 2^ ∆∆ Ct method for relative quantitation of gene expression. Primers sequences are:

- mSCD1-F-5’-TGCCCCTGCGGATCTT-3’ (NM_009127.4)
- mSCD1-R-5’-GCCCATTCGTACACGTATT-3’ (NM_009127.4)
- mSREBP1-F-5’-TGACCCGGCTATTCCGTGA-3’ (NM_011480)
- mSREBP1-R-5’-CTGGGCTGAGCAATACAGTTC-3’ (NM_011480)
- mFAS-F-5’- CATGACCTCGTGATGAACGTGT-3 (NM_007988.3)
- mFAS-R-5’- CGGGTGAGGACGTTTACAAAG-3’ (NM_007988.3)
- mCD36-F-5’-GGAACCTGTGGCTAATGCGT-3 (XM_006535623.3)
- mCD36-R-5’-CATGAGAATGCCTCCAAACAC-3’ (XM_006535623.3)
- mLXR-F-5’-GCCTCAATGCCTGATGTTTC-3 (XM_006499168.3)
- mLXR-R-5’-CTGCATCTTGAGTGCTTGC-3’ (XM_006499168.3)
- mSIRT1-F-5’-ATGACGCTGTGGCAGATTTT-3’ (NM_001159589.2)
- mSIRT1-R-5’-CCGCAAGGCGAGCATAGA-3’ (NM_001159589.2)
- m18SR-F-5’-GTAACCCGTTGAACCCCATT-3’ (XR_877120.2)
- m18SR-R-5’-CCATCCAATCGGTAGTGAGC-3’ (XR_877120.2)

**Western blotting**
Western blotting was performed using hepatic lysates as described previously (11,13,33). In brief, proteins (50-80 µg) were separated on a 4-12% SDS-polyacrylamide gel with 2-(N-morpholine) ethane sulfonic acid buffer purchased from Invitrogen (Carlsbad, CA) at 200V. The gel was transferred onto an Immuno-blot PVDF membrane (Bio-Rad, Hercules, CA) overnight at 4 ºC. Membranes were blocked in blocking solution (0.3% Tween 20 in Tris-buffered saline and 10% nonfat dry milk) for 1 h at room temperature then probed using a mouse monoclonal 4-hydroxynonenal (4-HNE) or rabbit polyclonal silent information regulator 1 (SIRT1), phospho-adenosine-5-monophosphate (AMP)-activated protein kinase (p-AMPK), total AMPK, phospho-acetyl-COA-carboxylase (ACC), and total-ACC antibodies (Table 1) for 1 h at room temperature or overnight at 4 ºC with constant shaking. Following 3 X 10-min washes in TBS-T buffer, membranes were then incubated in anti-mouse or anti-rabbit secondary antibody (Jackson
ImmuoResearch, Laboratories) at a 1:2000 dilution. All antibodies were diluted in blocking buffer. For immunodetection, membranes were washed three times in TBS-T wash buffer, incubated with ECL solutions per the manufacturer's specifications (Amersham Biosciences), and exposed to Hyper film ECL. Band intensities were determined using Quantity One software from Bio-Rad.

**Statistical analysis**

Statistical analyses were performed using the SigmaStat 2.0 Program (Jandel Corporation, San Rafael, CA, USA). Data are presented as mean ± SEM. We used one-way ANOVA to compare group differences. If overall ANOVA revealed significant differences, post-hoc (pairwise) comparisons were performed using Tukey’s or Tukey-Kramer-Multiple Comparison test. Differences were considered significant at P<0.05.

**Results**

**Body weight, cumulative food intake, hepatic TG levels and hepatic steatosis**

Nicotine-treated mice on a HFD had significantly lower body weight than vehicle-treated HFD-fed mice [Fig. 1(a)]. By 10 weeks of combined treatment with nicotine and HFD, mean body weight was reduced by 27% relative to mice fed with HFD alone, PNU-treated mice on a HFD also had significantly lower body weight than vehicle treated HFD fed mice [Fig. 1(a)]. The effect of PNU on body weight reduction in HFD fed mice was, however, less compared to nicotine treatment [Fig. 1(a)]. There was no difference in body weight between PNU-treated and PNU plus nicotine-treated mice on a HFD [Fig. 1(a)]. As shown in [Fig. 1 (b)], mice on a HFD consumed more calories than other experimental groups. The cumulative caloric intake over the 10-wk period in HFD fed mice was significantly reduced by nicotine treatment. [Fig. 1 (b)]. PNU-treatment alone led to a marked decrease in the cumulative caloric intake in HFD-fed mice even when compared to nicotine-treated mice on a HFD [Fig. 1 (b)]. There was no difference in cumulative caloric intake between PNU-treated and PNU plus nicotine-treated mice on a HFD [Fig. 1 (b)]. Compared to vehicle-treated, PNU-treated, or nicotine plus PNU-treated mice on a HFD, nicotine-treated mice on a HFD had significantly higher hepatic TG levels [Fig. 1 (c)].

H&E-stained liver sections revealed that compared with mice on a HFD plus vehicle, where a modest increase in lipid accumulation was detected [Fig. 1 (d)], combined treatment with HFD and nicotine led to a marked increase in lipid accumulation in the liver [Fig. 1 (e)]. PNU treatment effectively prevented HFD- [Fig. 1 (f)] or nicotine plus HFD-induced hepatic steatosis [Fig. 1 (g)]. These results were further confirmed by high-resolution light microscopy, using glutaraldehyde-fixed, osmium tetroxide post-fixed, epoxy-embedded, and toluidine blue-stained liver sections [Fig. 1 (h-k)]. Glutaraldehyde fixation followed by osmium post-fixation allows retention of fat that would have been normally washed out during tissue processing and can be easily visualized by toluidine blue staining. Nicotine plus HFD caused a striking increase in larger lipid droplets [Fig. 1(i)] compared to those from mice fed HFD and saline [Fig. 1(h)]. PNU treatment effectively prevented both HFD- [Fig. 1(j)] and nicotine plus HFD-induced hepatic steatosis [Fig. 1(k)]. In fact, PNU treatment fully attenuated HFD plus nicotine-induced fat accumulation [Fig. 1(k)] to levels seen in mice on a HFD and saline [Fig. 1 (k)].

Quantitative image analysis further revealed a significant increase in intracellular lipid content after combined treatment with HFD and nicotine (215 ± 25 µm²) compared to mice on a HFD and saline (32 ± 3.2 µm²). Treatment with PNU significantly attenuated HFD plus nicotine-induced intracellular fat accumulation (35 ± 3.3 µm²) to levels almost identical to that seen in
mice on a HFD and saline (32 ± 3.2 µm²). Treatment with PNU also significantly attenuated intracellular fat accumulation triggered by HFD alone (18 ± 2.3 µm²).

We performed transmission electron microscopy (TEM) to further substantiate the light microscopic findings [Fig. 2 (a-h)]. Hepatocytes from HFD fed mice plus saline exhibited a modest lipid accumulation [Fig. 2 (a)]. Addition of nicotine to HFD led to a striking increase in lipid accumulation of varying sizes in hepatocytes along with a decrease in the amount of cellular organelle [Fig. 2 (b)]. PNU treatment effectively prevented such HFD- [Fig. 2 (c)] or nicotine plus HFD-induced hepatic steatosis [Fig. 2 (d)]. A distinct increase in the amount of hepatocyte apoptosis, characterized by nuclear condensation and fragmentation, was also noted after combined treatment with HFD and nicotine [Fig. 2 (f)] compared to mice on a HFD [Fig. 2 (e)]. PNU treatment effectively prevented such nicotine plus HFD-induced hepatocyte apoptosis [Fig. 2 (h)].

We next analyzed the occurrence of hepatocellular apoptosis by TUNEL. As demonstrated in Fig. 3 (a-f) and Fig. 3 (g), HFD alone led to a modest increase in the incidence of apoptosis. Combined with nicotine, HFD induced a further significant increase in the incidence of hepatocellular apoptosis. The incidence of hepatocellular apoptosis was similar between HFD and HFD plus PNU groups. Notably, treatment with PNU significantly attenuated HFD plus nicotine-induced hepatocellular apoptosis to levels similar to that seen in mice on a HFD and saline. There was no staining in liver section from nicotine plus HFD group [Fig. 3 (e)], when TdT enzyme was substituted by the same volume of distilled water (negative control). In the mammary tissue sections from rat pups 3-5 days after weaning in which apoptosis is well characterized (positive control), an intense immunoreactivity is detected in cells undergoing apoptosis [Fig. 3 (f)]. Nicotine treatment in the absence or presence of PNU did not alter the incidence of hepatocellular apoptosis in NCD-fed mice (data not shown).

We also used immunoblot analysis to detect α7nAChR expression in the liver [Fig. 3 (h) and Fig. 3 (i)]. Hepatic α7nAChR expression was higher in the nicotine and HFD group. PNU treatment effectively prevented nicotine plus HFD-induced increase in α7nAChR expression to levels seen in mice on a HFD alone. Hepatic expression of α7nAChR was similar in vehicle-treated, PNU-treated, or nicotine plus PNU-treated mice on a HFD.

**PNU prevents nicotine plus HFD-induced hepatic steatosis through stimulation of SIRT1 and AMPK signaling together with suppression of oxidative stress**

AMPK, a central regulator of cellular energy homeostasis, plays an important role in fatty acid metabolism through its ability to regulate key fatty acid biosynthetic pathway (35,36). To investigate whether mitigation of nicotine plus HFD-induced hepatic steatosis by PNU was associated with activation of AMPK, we carried out western blot analysis of phospho-and total-AMPK in hepatic lysates [Fig. 4 (a)]. As expected from our previous studies (11), HFD, in the absence or presence of nicotine, led to a striking decrease in hepatic phospho-AMPK levels [Fig. 4 (a)]. Consistent with the finding of inactivation of AMPK in HFD and HFD plus nicotine-treated groups, little or no phospho-ACC was detected in hepatic lysates in these groups [Fig. 4 (a)]. Treatment with PNU significantly attenuated both HFD- and HFD plus nicotine-induced decrease in phospho-AMPK levels [Fig. 4 (a)]. PNU alone failed to significantly attenuate HFD-induced decrease in phospho-ACC levels but restored phospho-ACC levels in the combined treatment group. [Fig. 4 (a)]. There were no significant differences in total-AMPK or total-ACC levels among various groups.

Given that SIRT1, a nicotinamide adenine dinucleotide-dependent histone deacetylase, plays an important role in protection against hepatic steatosis under various insults (37-39), we next...
examined the contribution SIRT1 in PNU-mediated protection of nicotine plus HFD-induced hepatic steatosis. Western blot analysis revealed that mice fed with HFD in the absence or presence of nicotine exhibited little or no hepatic SIRT1 expression [Fig. 4(a)]. While PNU treatment did not alter HFD-induced decrease in SIRT1 levels, it significantly attenuated nicotine plus HFD-induced decrease in hepatic SIRT1 expression [Fig. 4(a)]. Next, we investigated whether amelioration of nicotine plus HFD-induced hepatic steatosis was associated with suppression of oxidative stress. We performed western blot analysis of 4-HNE [Fig. 4(a)], a biomarker of oxidative stress (40,41). Nicotine treated mice on a HFD had significantly higher levels of hepatic 4-HNE relative to mice fed HFD alone. Treatment with PNU significantly attenuated both HFD- and HFD plus nicotine-induced increase in hepatic 4-HNE levels. These findings were further corroborated by densitometric analysis of band intensities [Fig.4 b)]. No significant changes in phospho-AMPK, SIRT1, and 4-HNE levels were noted among various treatment groups in mice on NCD (data not shown).

To gain additional insights into the potential mechanisms of PNU-mediated mitigation of nicotine plus HFD-induced hepatic steatosis, we measured the expression of key genes related to hepatic lipid metabolism by qRT-PCR. Consisting with the finding of inactivation of AMPK in HFD plus nicotine-treated groups, we found a significant upregulation of SREBP1c and FAS [Fig. 5], indicating increased hepatic lipogenesis. Treatment with PNU significantly attenuated nicotine plus HFD-induced upregulation of SREBP1c and FAS to levels seen in HFD plus saline or HFD plus PNU groups [Fig. 5]. Consistent with our western blot data, PNU treatment also upregulated hepatic SIRT1 gene expression in nicotine-treated mice on a HFD. Intriguingly, nicotine treatment resulted in a striking downregulation of CD36 expression in mice on a HFD. PNU treatment in the absence of presence of nicotine had no effect on nicotine plus HFD-induced changes in CD36 expression. No significant changes in the hepatic expression of SCD1 and LXR expression were seen between HFD and HFD plus nicotine-treated groups. Treatment with PNU significantly attenuated both HFD- and HFD-plus nicotine-induced upregulation of SCD and LXR gene expression [Fig. 5].

Discussion

In recent studies using the model of diet-induced obesity in C57BL6J mice, we demonstrated that nicotine when combined with a HFD triggered greater oxidative stress, activated hepatocellular apoptosis, and exacerbated HFD-induced hepatic steatosis (11,12). In the current study, we evaluated the protective role of α7nAChR agonist, PNU on nicotine plus HFD-induced hepatic steatosis. We are intrigued by the observation that indeed PNU significantly blocks HFD-induced weight gain and protects nicotine plus HFD-induced hepatic steatosis in obese mice. The preventive effects of PNU on nicotine plus HFD-induced hepatic steatosis are mediated by suppression of oxidative stress, activation of SIRT1 and AMPK signaling together with inhibition of its downstream targets, including SREBP1c, ACC, and FAS.

The results of the present study confirm and extend our previous studies by demonstrating that nicotine blocks the HFD-induced weight gain in mice (10,11,13). This study is, however, unique in showing that PNU alone can reduce HFD-induced weight gain, albeit less pronounced when compared with nicotine, suggesting an important role of the α7nAChR in the weight-reducing effect of nicotine. Indeed, the α7nAChR plays an important role in both central and peripheral mechanisms regulating food intake (9). Consistent with a role of α7nAChR in food intake, we found that PNU treatment alone led to a marked decrease in the cumulative food intake in HFD-fed mice. This notion is supported by another line of evidence showing that TC-
7020, an α7nAChR agonist, reduced weight gain and food intake in a mouse model of diabetes (23). Thus, a potential mechanism by which PNU can reduce body weight in HFD-fed mice is through appetite suppression, although this needs to be demonstrated by either showing intracerebroventricular injection of PNU suppresses food intake or that a peripheral-acting α7nAChR antagonist such as hexamethonium (42), does not block the effect of α7nAChR agonist on food intake.

Oxidative stress coupled with hepatocyte apoptosis plays a pivotal role in the pathogenesis of NAFLD (27,29,43). Our study showed that nicotine when combined with a HFD generated greater oxidative stress, as evidenced by an increase in hepatic 4-HNE levels, than HFD alone. Oxidative stress has also been implicated in apoptotic signaling in various cell types, including hepatocytes (27,29,44). Thus, it is likely that generation of severe oxidative stress could trigger hepatocyte apoptosis and aggravate hepatic steatosis in the combined treatment group through the formation of reactive and biologically-active lipid peroxidation products such as 4-HNE (27,29,43,45). This is consistent with our previous data showing that both HFD and nicotine are capable of generating oxidative stress in the liver and heart ventricle, but only when combined can cause greater oxidative stress and triggers hepatocyte and cardiomyocyte apoptosis (11,28). It is noteworthy that in spite of a significant increase in oxidative stress, there was little or no change in the incidence of hepatocyte apoptosis in mice on a HFD alone, suggesting that the oxidative stress generated by HFD alone is not severe enough to trigger cellular apoptosis. This is consistent with earlier reports indicating that cellular responses to oxidative stress varies depending on the cell type, the levels of stress achieved and the duration of exposure (46). However, we cannot rule out the possibility that in addition to high levels of oxidative stress, other factors may have also contributed to increased hepatocyte apoptosis triggered by the combined treatment with nicotine and HFD.

Notably, PNU treatment was able to suppress nicotine plus HFD-induced increase in hepatic oxidative stress and hepatocellular apoptosis. Indeed, we detected α7nAChR expression in the liver and found that the highest amounts of the receptor were in the nicotine plus HFD group. This is consistent with the previous reports showing that α7nAChRs are present in the liver (14,16,20). PNU treatment significantly prevented nicotine plus HFD-induced increase in α7nAChR expression to levels seen in mice on a HFD alone. The mechanisms by which PNU prevents nicotine plus HFD-induced increase in α7nAChR expression remain unknown but could be mediated through desensitization of α7nAChR after chronic exposure of the agonist (47).

Evidence also exists that α7nAChR-mediated pathway plays a major role in mitigating hepatic ischemia-reperfusion injury (21,22). Furthermore, the α7nAChR agonist PNU is protective against a wide variety of cytotoxic insults and suppresses oxidative stress and apoptosis in various cell systems, including hepatocytes (16-19,21,48,49). The underlying mechanisms of the protective effects of PNU in mitigating nicotine plus HFD-induced oxidative stress and hepatocellular apoptosis could include induction of heme oxygenase 1 via nuclear factor erythroid-2-related factor 2 (18,22,33). It remains also possible that PNU can attenuate hepatic steatosis by reducing circulating FFA via reduction of abdominal lipolysis (11), and improving glucose homeostasis and insulin sensitivity through activation of α7nAchR-mediated cholinergic anti-inflammatory pathway (15). Other studies have found that chronic treatment of PNU at the same dose levels used by us significantly enhanced insulin sensitivity, as judged by reduction in the HOMA-IR, GTT, and ITT in mice fed a NCD (30). Available evidence also suggests that PNU administration attenuates the central insulin action-mediated hepatic
interleukin-6 and signal transducer and activator of transcription factors 3 in the liver of mice on a HFD, suggesting that cholinergic pathway can play an important role in central insulin action mediated hepatic response in diet-induced obese mice (50).

One potential limitation of our study is that at present, we are unable to answer whether PNU would have detrimental effects if used at 1.5 mg/kg BW/day dose or would nicotine have beneficial effects if used at 0.52 mg/kg/BW/day. Indeed, the low dosages (1.5 mg/kg BW/day) of nicotine used by us induces adipose tissue lipolysis and hepatic steatosis in mice (11). In contrast, other studies showed that high dosages of nicotine (4 mg/kg BW/day) improves obesity, hepatic steatosis, and endoplasmic reticulum stress in diet-induced obese male rats (51). To address the discrepancy between mice and rats, Wu and colleagues (20) further studied the metabolic effects of both low dosages (0.8 mg/kg BW/day equal to 1.5 mg/Kg BW/day in mice) and high dosages of nicotine (4 mg/kg BW/day) in rats. Intriguingly, they found that low dosage of nicotine, resulting in a serum concentrations of nicotine that is similar to the clinically relevant concentrations found in habitual smokers, induced insulin resistance, mild loss of body weight, and hepatic steatosis. In striking contrast, the high dosage of nicotine, resulting in much higher serum nicotine concentrations than found in habitual smokers, induced severe loss of body weight, improved insulin sensitivity and prevents hepatic steatosis in male rats (20). Although these observations suggest that the effects of nicotine could vary depending on dosages, it remains unresolved whether lower doses of nicotine (i.e. lower than 1.5mg/kg BW/day) will have detrimental effects on health. Likewise one could also argue that the beneficial effects of PNU that are so obvious at lower daily dosages (0.52 mg/kg/BW) ameliorating nicotine plus HFD-induced hepatic steatosis might not be so obvious if used at higher (1.5 mg/kg BW) daily dosages. In this context it is pertinent to note here that pretreatment of PNU at a dosage of 1 mg/kg BW was indeed able to protect the liver following ischemia reperfusion injury in mice (19). Daily dosages of PNU (0.3 mg/kg and 1.0 mg/kg BW) further inhibited muscular degeneration in mdx dystrophic mice with a greater efficacy at higher dosages (52). PNU at a slightly higher daily dosage (2.4 mg/kg BW) significantly promoted wound healing in streptozotcin-induced diabetic mouse model (53). Available evidence also suggests that administration of PNU at a much higher dosage (12 mg/kg BW) did confer neuroprotection in a mouse model of intracerebral hemorrhage (48). Thus, given that PNU exerts its beneficial effects at various doses levels in protecting organ damage under diverse experimental conditions, it is unlikely that PNU would have detrimental effects if used at higher daily dosages such as 1.5 mg/kg BW. However, this clearly merits further investigation.

Together, these results demonstrate a key role of α7nAchR agonist in amelioration of nicotine plus HFD-induced hepatic steatosis. Future studies are needed to understand which nAchR(s) is involved in nicotine plus HFD-induced hepatic steatosis and to what extent it is mediated by increased adipose tissue lipolysis, resulting in excess FFA delivery, and a direct effect of nicotine in the liver.

AMPK is a central regulator of lipid homeostasis and mediates suppression of lipogenic gene expression such as ACC and FAS through inhibition of SREBP1c (36,54). ACC is the rate determining enzyme for the synthesis malonyl-CoA, both a critical substrate for fatty acid biosynthesis and a potent inhibitor of fatty acid oxidation (36). AMPK can also phosphorylate and inactivate ACC leading to inhibition of de novo fatty acid and cholesterol synthesis (36). Consistent with a pivotal role for AMPK in lipid homeostasis, we herein show that the additive effects of nicotine and HFD on the severity of hepatic steatosis were associated with complete inhibition of AMPK. The net effect of AMPK inactivation is decreased phosphorylation and
activation of ACC, leading to increased lipogenesis in the liver. RT-PCR data also showed a significant upregulation of SREBP1c and FAS, indicating increased hepatic lipogenesis, in the nicotine plus HFD combined treatment group. These results are consistent with our earlier reports (11,55) as well others (20,56) linking inhibition of AMPK with NAFLD. Treatment with PNU significantly restored nicotine plus HFD-induced decrease in hepatic phospho-AMPK and phospho-ACC levels and prevented upregulation of SREBP1c and FAS. Furthermore, mice fed HFD for long term (16 weeks) also develop hepatic steatosis in association with inhibition of AMPK coupled with activation of ACC that was attenuated by betaine, a naturally occurring metabolite of choline and an essential biochemical component of the methionine-homocysteine cycle (57). Taken together, these results indicate that PNU reduced nicotine plus HFD-induced hepatic steatosis by improving AMPK signaling and, in turn, by inactivating ACC and suppressing lipogenic gene expression such as SREBP1c and FAS. At present, we are unable to determine the possible mechanisms by which PNU restores hepatic AMPK activity in nicotine treated mice on a HFD and this clearly merits further investigation.

Of further interest, activation of AMPK can also suppress oxidative stress and inhibit apoptosis in various cell systems (58-60). Thus, it is possible that the protective effect of PNU on nicotine plus HFD-induced hepatocyte apoptosis could also be mediated by activation of AMPK. Given the critical role of AMPK in modulating an ever-expanding array of biological pathways, this study further underscores that potential ability of α7nAChR agonist to mediate a variety of signal transduction pathways in regulating cell fate.

SIRT1 plays an important role in protection against hepatic steatosis under various insults (37-39). Consistent with a pivotal role of SIRT1 pathway in attenuating hepatic steatosis, here we also found that PNU treatment stimulated SIRT1 in the combined treatment group. This notion is further supported by another line of evidence showing that hepatic overexpression of SIRT1 attenuates hepatic steatosis in mice fed a HFD or after fasting (39) whereas deletion of SIRT1 exacerbates HFD-induced hepatic steatosis (38).

We found that PNU treatment suppressed HFD plus nicotine-induced upregulation of SCD1 and LXR gene expression in the liver, suggesting that both SCD1 and LXR plays an important role in attenuating nicotine plus HFD-induced hepatic steatosis. This is consistent with previous data showing SCD1-deficiency prevented hepatic steatosis observed in a number of mouse models, including mice fed high-carbohydrate and HF diets and ob/ob mice (61,62). Likewise, downregulation of LXR is involved with suppression of high-cholesterol diet-induced hepatic steatosis though suppression of lipogenic genes such as SREBP1c, SCD1, and FAS (63). Indeed, we found PNU-mediated amelioration of nicotine plus HFD-induced hepatic steatosis is associated with down-regulation of these lipogenic genes.

Nicotine treatment resulted in a striking downregulation of CD36, the fatty acid translocase, in mice on a HFD. PNU treatment in the absence of presence of nicotine had no effect on nicotine plus HFD-induced decrease in CD36 expression. It has been shown that the upregulation of CD36 in the liver is associated with increased steatosis in NAFLD patients (64,65) and CD36−/− mice are resistant to alcohol- and high carbohydrate-induced hepatic steatosis (66). These observations raise the question as to why mice on a HFD with significantly increased CD36 levels do not develop hepatic steatosis. Alternatively, in spite of a significant downregulation of CD36 in the combined treatment, why these mice develop hepatic steatosis. One possibility is that decreased CD36 expression in the nicotine plus HFD group could reflect a compensatory mechanism aimed to decrease fatty acid uptake to compensate HFD-induced increased hepatic lipogenesis. Evidence also exits that hepatic overexpression CD36 attenuates
HFD-induced hepatic steatosis (67). Thus, one could speculate that upregulation of CD36 in the mice on a HFD might be beneficial in protecting liver under lipid overload and metabolic stress. Thus, the regulation of hepatic lipid homeostasis by CD36 is far more complex and could vary depending on nature and severity of stress.

In summary (see Fig. 6), we have provided insights into the molecular mechanisms by which PNU attenuates nicotine plus a HFD-induced hepatic steatosis and hepatocellular apoptosis. The preventive effects of PNU on nicotine plus HFD-induced hepatic steatosis appears to be mediated by suppression of oxidative stress, activation of SIRT1 and AMPK signaling together with inhibition of its downstream targets, including SREBP1c, ACC, and FAS. The clinical implication of this study is that α7nAChR agonists may be an effective therapeutic strategy for the amelioration of NAFLD and adverse metabolic sequelae, especially in obese smokers.

Acknowledgments
This work was supported by the Diversity-Promoting Institution Drug Abuse Research Program (DIDARP) grant (R24DA017298) and the Accelerating Excellence in Translational Science (AXIS) grant (2U54MD007598) from the National Institutes of Health (NIH). We also thank the Technology Core of the AXIS grant (2U54MD007598) from the NIH for tissue preparation and hematoxylin and eosin staining.

Diversity-Promoting Institution Drug Abuse Research Program (DIDARP), NIH, R24DA017298, Theodore C Friedman; AXIS (Accelerating Excellence in Translational Science-NIH/NIMHD), 5U54MD007598, Amiya P Sinha-Hikim

Address for Correspondence: Mohammad Kamrul Hasan Ph.D. or Amiya P. Sinha-Hikim, Ph.D., Department of Internal Medicine, Charles R. Drew University of Medicine and Science, 1731 E. 120th Street, Los Angeles, CA 90059. Tel: 323-563-5974, Fax: 323-563-9352, E-mail: kamrulhasan@cdrewu.edu and amiyasinhahikim@cdrewu.edu.

Disclosure statement: The authors have nothing to disclose.

References
9. McFadden KL, Cornier MA, Tregellas JR. The role of alpha-7 nicotinic receptors in food intake behaviors. Front Psychol 2014; 5:553
21. Li F, Chen Z, Pan Q, Fu S, Lin F, Ren H, Han H, Billiar TR, Sun F, Li Q. The protective effect of PNU-282987, a selective alpha7 nicotinic acetylcholine receptor agonist, on the hepatic ischemia-reperfusion injury is associated with the inhibition of high-mobility group box 1 protein expression and nuclear factor kappaB activation in mice. Shock 2013; 39:197-203
37. Shen Z, Liang X, Rogers CQ, Rideout D, You M. Involvement of adiponectin-SIRT1-AMPK signaling in the protective action of rosiglitazone against alcoholic fatty liver in mice. Am J Physiol Gastrointest Liver Physiol 2010; 298:G364-374


46. Videla LA. Cytoprotective and suicidal signaling in oxidative stress. Biological research 2010; 43:363-369

47. Kalappa BI, Uteshev VV. The dual effect of PNU-120596 on alpha7 nicotinic acetylcholine receptor channels. Eur J Pharmacol 2013; 718:226-234


Figure 1. (a) Body weight measured over 10 weeks in mice on a HFD and treated with nicotine in the absence or presence of PNU. Both nicotine and PNU (albeit less when compared with nicotine) significantly reduced HFD-induced weight gain. (b) Average food intake in HFD mice treated with nicotine in the absence or presence of PNU. Compared to nicotine, PNU treatment alone leads to a marked decrease in cumulative food intake in HFD-fed mice. (c) Hepatic TG levels in various treatment groups. Values are given as mean ± SE of 5-6 mice per group. Means with unlike superscript are significantly (P<0.05) different. (d-g) Representative H&E -stained liver sections shows that compared with a mouse on a HFD, where a modest increase in lipid accumulation (arrow) is detected (d), combined treatment with nicotine and HFD caused a marked increase in lipid accumulation in the liver (e). PNU treatment effectively suppressed nicotine plus HFD-induced hepatic steatosis (g). (h-k) Representative light microscopic images of glutaraldehyde-fixed, osmium tetroxide post-fixed, epoxy-embedded, and toluidine-blue stained liver sections from different treatment groups show that nicotine plus a HFD causes a striking increase in lipid accumulation of varying sizes (arrow) in hepatocytes (i) compared to those from mice on a HFD alone (h). PNU treatment effectively prevented HFD plus nicotine-induced exacerbation of hepatic steatosis (k). Scale bar=25 µm (D-K). Data are representative of 5 mice in each group.

Figure 2. (a-d) Compared with mice on a HFD alone (a), nicotine plus HFD caused a striking increase in lipid accumulation (asterisk) of varying sizes in hepatocytes (b). Treatment with PNU decreased nicotine plus HFD-induced hepatic steatosis (d) to that of HFD alone (a). (e-h) Compared with mice on a HFD (e), a distinct increase in the amount of hepatocyte apoptosis, characterized by nuclear condensation and fragmentation, is noted after combined treatment with HFD and nicotine (f) and that can be effectively prevented by PNU treatment (h). Scale bar=1 µm (a-h). Data are representative of 4 mice in each group.

Figure 3. In situ detection of hepatocellular apoptosis by TUNEL (a-f). While HFD alone led to a modest increase in the incidence of apoptosis (a), HFD plus nicotine induced a further significant increase in the incidence of hepatocellular apoptosis (b). The incidence of hepatocellular apoptosis was similar between HFD (a) and HFD plus PNU groups (c). Notably, treatment with PNU significantly attenuated HFD plus nicotine-induced hepatocellular apoptosis (d) to levels almost identical to that seen in mice on a HFD and saline (a). A liver section from nicotine plus HFD group incubated without TdT enzyme (negative control) shows no immunostaining (e), whereas a mammary tissue section from a rat pup (positive control), shows specific immunoreactivity (f). Scale bar=25 µm (a-f). Data are representative of 5 mice in each group. (g) Quantitation of hepatocellular apoptosis. Apoptotic rate was expressed as the percentage of TUNEL-positive nuclei per total nuclei (apoptotic plus nonapoptotic nuclei) counted in a unit reference area. Values are means ± SEM (n=5). Means with unlike superscripts are significantly different. Note the marked increase in the incidence of apoptosis in the HFD plus nicotine group. PNU treatment fully attenuates nicotine plus HFD-induced hepatocyte
apoptosis. (h) Western blot analysis shows higher expression of hepatic α7nAChR expression in the combined treatment group with nicotine and HFD than in the HFD alone group. PNU treatment effectively attenuates nicotine plus HFD-induced increase in α7nAChR expression to levels seen in mice on a HFD alone. Data are representative of 6 mice in each group. (i) Quantitation of band intensities. Data are normalized to GAPDH. Values are means ± SEM. Means with unlike superscripts are significantly (P<0.05) different.

**Figure 4.** (a) Western blot analysis shows that mice fed HFD in the presence or absence of nicotine have low hepatic SIRT1, phospho-AMPK (p-AMPK) and phospho-ACC (p-ACC) levels, while nicotine-treated mice on a HFD have increased hepatic 4-HNE levels. Treatment with PNU significantly restored HFD plus nicotine-induced decrease in hepatic SIRT1, p-AMPK and p-ACC levels and prevents increase in 4-HNE levels. Data are representative of 6 mice in each group. (b) Quantitation of band intensities. Data are normalized to GAPDH. Values are means ± SEM. Means with unlike superscripts are significantly (P<0.05) different.

**Figure 5.** Quantitative RT-PCR shows upregulation of SREBP1c and FAS in the HFD plus nicotine group compared to HFD alone. Treatment with PNU fully prevented nicotine plus HFD-induced upregulation of SREBP1c and FAS. PNU treatment also upregulated hepatic SIRT1 gene expression in nicotine-treated mice on a HFD. No significant changes in the hepatic expression of SCD1 and LXR were noted between HFD and HFD plus nicotine-treated groups. Treatment with PNU, however, significantly attenuated both HFD- and HFD-plus nicotine-induced upregulation of SCD and LXR gene expression. Nicotine treatment also significantly attenuated HFD-induced upregulation of CD36. CD36 expression is similar among HFD plus nicotine, HFD plus PNU, and HFD plus nicotine plus PNU groups. Values are means ± SEM of 4-5 animals per group. Means with unlike superscripts are significantly (P<0.05) different.

**Figure 6.** Model illustrating how PNU attenuates nicotine plus HFD-induced hepatic steatosis. Nicotine plus a HFD promotes abdominal lipolysis, resulting in free fatty acid (FFA) release from adipose tissue into the circulation, thereby contributing to the buildup of lipids as triglyceride (TG) in the liver (11). In addition, nicotine plus a HFD could promote hepatic lipogenesis through inactivation of AMP-activated protein kinase (AMPK) and activation of its downstream target acetyl-coenzyme A-carboxylase (ACC), leading to the development of hepatic steatosis. Inactivation of AMPK can also stimulate lipogenesis through upregulation of key genes in the lipogenic pathway such as fatty acid synthase (FAS) and ACC by activating the transcription factor sterol regulatory element binding protein 1c (SREBP-1c). Intrahepatic lipid accumulation can also trigger hepatocellular apoptosis through generation of oxidative stress. AMPK inactivation could further sensitize liver cells to nicotine plus HFD-induced apoptosis. PNU seems to protect nicotine plus HFD-induced hepatic steatosis through suppression of oxidative stress and activation of AMPK signaling together with inhibition of its downstream target, including SREBP1c, ACC, and FAS.
<table>
<thead>
<tr>
<th>Target protein</th>
<th>Name of Antibody</th>
<th>Manufacturer /cat#/ RRID</th>
<th>Species raised in: poly or monoclonal</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospho-AMP activated Kinase (p-AMPK)</td>
<td>p-AMPK α1/2(Thr-172)</td>
<td>Santa Cruz Biotechnology No-Sc-33524</td>
<td>Rabbit, polyclonal</td>
<td>1:1500</td>
</tr>
<tr>
<td>Total AMPK</td>
<td>AMPKα</td>
<td>AB_2169714</td>
<td>Rabbit monoclonal</td>
<td>1:2000</td>
</tr>
<tr>
<td>Phospho-Acetyl CoA carboxylase (p-ACC)</td>
<td>p-ACC (S79)</td>
<td>Cell Signaling Technology. No-2532S</td>
<td>Rabbit monoclonal</td>
<td>1:2000</td>
</tr>
<tr>
<td>Total ACC</td>
<td>ACC</td>
<td>AB_330331</td>
<td>Rabbit monoclonal</td>
<td>1:2500</td>
</tr>
<tr>
<td>Sirt1</td>
<td>Anti-Sirt1</td>
<td>Cell Signaling Technology. No-3664S</td>
<td>Rabbit, polyclonal</td>
<td>1:2000</td>
</tr>
<tr>
<td>4-HNE</td>
<td>Anti-4-HNE</td>
<td>AB_330337</td>
<td>Mouse monoclonal</td>
<td>1:200</td>
</tr>
<tr>
<td>GAPDH</td>
<td>MSX GAPDH</td>
<td>Cell Signaling Technology. No-3662S</td>
<td>Mouse monoclonal</td>
<td>1:8000</td>
</tr>
<tr>
<td>AChRa7</td>
<td>AChRa7</td>
<td>AB_2219400</td>
<td>Rabbit, polyclonal</td>
<td>1:500</td>
</tr>
</tbody>
</table>

|                         |                  |                                   |                                       |          |
|                         |                  | Millipore.                        |                                       |          |
|                         |                  | No-07-131                         |                                       |          |
|                         |                  | AB_10067921                       |                                       |          |
|                         |                  | OXIS international Inc.           |                                       |          |
|                         |                  | No-24325                          |                                       |          |
|                         |                  | AB_2716829                        |                                       |          |
|                         |                  | Millipore.                        |                                       |          |
|                         |                  | No-MAB374                         |                                       |          |
|                         |                  | AB_2107445                        |                                       |          |
|                         |                  | Santa Cruz Biotechnology          |                                       |          |
|                         |                  | No-SC-554                         |                                       |          |
|                         |                  | AB_2229517                        |                                       |          |