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PAPER

The effect of nicotine and dextrose on endoplasmic reticulum stress in human coronary artery endothelial cells

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Abstract

Cigarette smoking is one of the major causes of coronary artery disease (CAD) as is diabetes. However, nicotine has been generally regarded as safe and is used in smoking cessation programs. This presumption of nicotine safety was examined in human coronary artery endothelial cells (HCAEC). Endoplasmic reticulum (ER) stress was measured using the secreted alkaline phosphatase (SAP) assay. The ER stress markers inositol-requiring enzyme 1α (IRE1α), phospho-IRE1α, double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase (PERK), phospho-PERK, activating transcription factor 6 (ATF6), and glucose-related protein 78 (GRP78) were measured by western blot. Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and crystal violet staining. Intact and cleaved caspase 3, BH3 interacting-domain death agonist (BID), and B-cell lymphoma 2 (Bcl2) were measured by western blot. In cells transfected with the SAP expression plasmid, treatment with nicotine resulted in a dose-dependent decrease in SAP expression with no noticeable toxicity. Nicotine (10 nM) also increased IRE1α and PERK phosphorylation, and ATF6 and GRP78 expression. Although nicotine at concentrations up to 10 μM did not cause cell death, treatment of HCAEC with 10 nM nicotine in the presence of 13.8 mM dextrose aggravated ER stress, increased cell death, increased cleaved caspase 3 and BID, and decreased BCL2. Nicotine at concentrations commonly achieved in nicotine-replacement therapy (NRT) significantly increased ER stress in HCAEC and aggravated dextrose-induced ER stress and cell apoptosis. People using electronic cigarettes and on NRT may be at increased risk for CAD.

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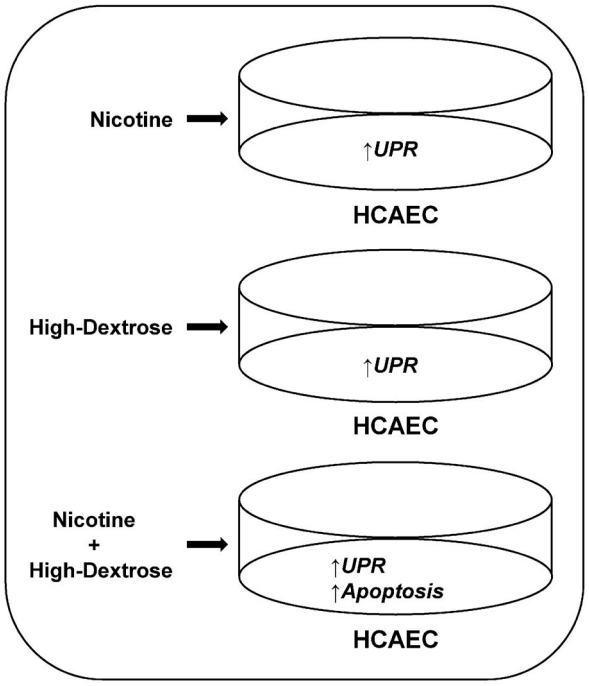
Graphical Abstract



Key words: nicotine, ER stress, apoptosis, HCAEC

Introduction

Cigarette smoking is the leading cause of preventable disease and death in the USA, accounting for more than 480 000 deaths every year [1]. Through educational efforts and the availability of various tools to overcome nicotine addiction, current smoking rates have declined from 20.9% in 2005 to 15.5% in 2016 [2]. Of the many diseases promoted by cigarette smoking, coronary heart disease continues to be the number one cause of morbidity and mortality [1]. Among men in the 45–54 age group, the mortality rate in those who smoke more than 25 cigarettes/day is almost 20-fold higher (425/100 000 men) than those who have never smoked (25/100 000 men) [3]. The American Heart Association lists tobacco smoke as the number one risk for heart disease [4]. Cigarettes contain about 2000 compounds including tar, nicotine, and carbon monoxide. The latter reduces oxygen carrying capacity of the blood while tar contains hydrocarbons and other carcinogenic substances. Nicotine causes release of epinephrine and norepinephrine that alters cardiac physiology and hemodynamics and has been considered the main culprit of the addiction to cigarette smoking.

Clinicians often use nicotine-replacement therapy (NRT) to assist people to stop smoking and consider NRT to be of minimal risk compared to the risk of continued smoking. NRT options include nicotine gum, nicotine patch, inhalers, nasal spray, lozenges, and sublingual tablets. The euphoria people get from the nicotine is related to blood concentration and the rate of increase in blood nicotine concentration achieved during NRT is generally thought to be safe even in patients with cardiovascular disease. This presumption of safety was critically examined in controlled laboratory conditions. The results indicate that nicotine at concentrations commonly achieved during NRT can cause endoplasmic reticulum (ER) stress and can aggravate dextrose-induced ER stress and apoptosis in human coronary artery endothelial cells (HCAEC).

Materials and Methods

Materials

HCAEC (PCS-100-020) as well as vascular endothelial cell growth medium and supplements were purchased from American Type Culture Collection (Manassas, VA). Nicotine, simvastatin, and pravastatin were purchased from Cayman Chemical (Ann Arbor, MI). Antibodies for activating transcription factor 6 (ATF6) (MA1-25358), inositol-requiring enzyme 1α (IRE1α) (PA1-46027), phospho-IRE1α (PA1-16927), double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase (PERK) (PA5-38811), phospho-PERK (PA5-40294), and glucose-regulated protein 78 (GRP78) (PA5-22967) were purchased from Thermo-Fisher Scientific (Pittsburg, PA), as was HALT protease/phosphatase inhibitor cocktail and crystal violet stain. Antibodies to β-actin (sc-4967), cleaved caspase 3 (sc-271028), caspase 3 (sc-9665), B-cell lymphoma 2 (BCL2) (sc-4223S), and BH3 interacting-domain death agonist (BID) (sc-2002) were purchased from Cell Signaling Technology (Danvers, MA), while secondary antibodies conjugated to horseradish peroxidase (HRP) (goat-anti-mouse IgG, 1030-05; goat-anti-rabbit IgG, 4010-05) were purchased from Southern Biotech (Birmingham, AL). The chemiluminescent secreted alkaline phosphatase (SAP) substrate disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2′-(5′-chloro)tricyclo [3.3.1.13,7]decan}-4-yl)phenyl phosphate (CSPD) was purchased from CloneTech (Mountain View, CA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (Saint Louis, MO). Plasmid DNA preparation kits were purchased from

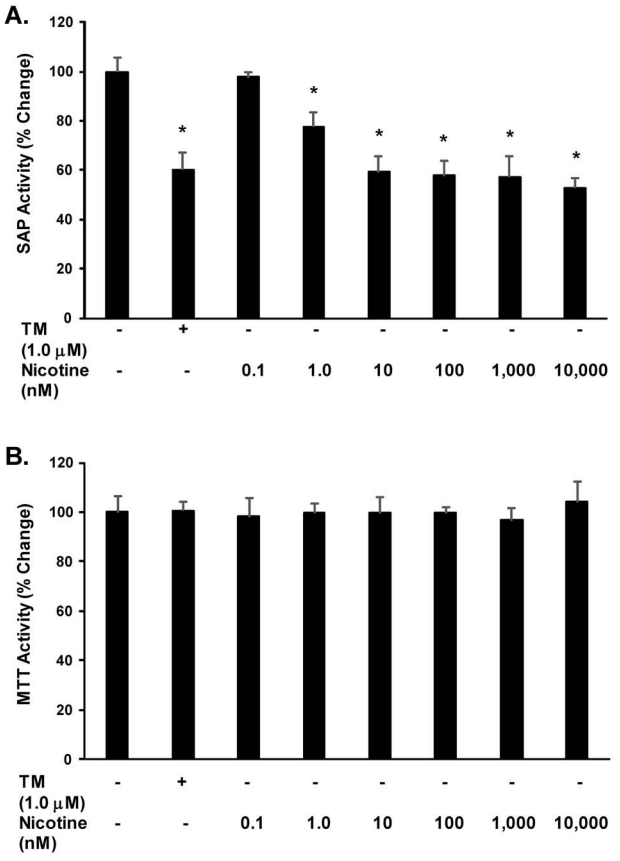


Figure 1: The effect of nicotine on SAP activity. HCAEC were transfected with the plasmid pSEAP2.control and 24-h later treated with solvent (DMSO), 1.0 μM tunicamycin (TM), or 0, 0.1, 1.0, 10, 100, 1000, and 10 000 nM nicotine. After 24-h the conditioned medium was collected and assayed for SAP activity (A) and cell viability was measured using the MTT assay (B). Treatment with nicotine resulted in a dose-dependent decrease in SAP activity with no toxicity. $N = 6$; $^{*}P < 0.05$ relative to cells treated with solvent. One-way ANOVA followed by Student's *t*-test were used to calculate statistical differences.

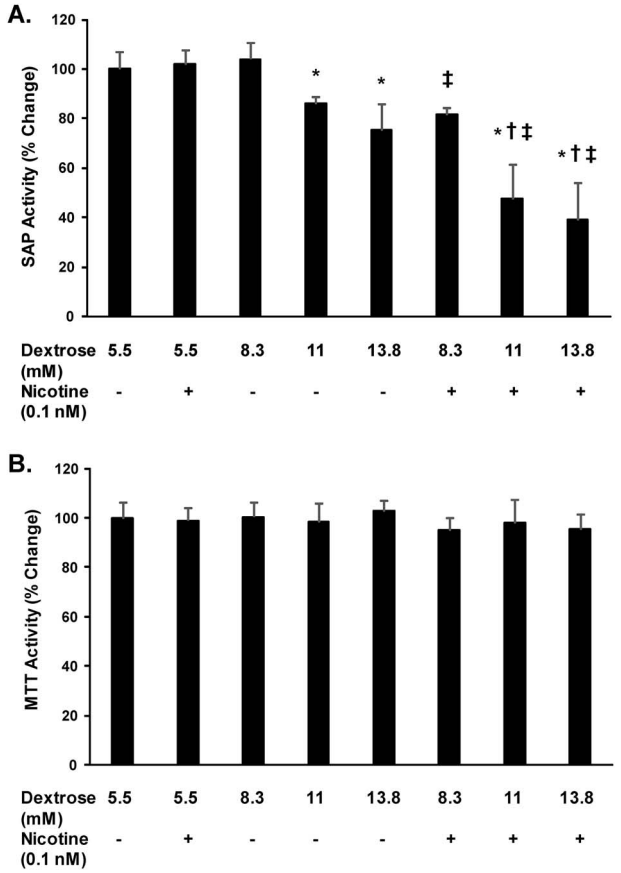


Figure 2: The effect of dextrose and low-dose nicotine on SAP activity. HCAEC were transfected with the plasmid pSEAP2.control and 24-h later treated with 5.5, 8.3, 11, or 13.8 mM dextrose in the presence or absence of 0.1 nM nicotine. After 24-h, the conditioned medium was collected from the cells and SAP activity was measured (A), while cell viability was measured using the MTT assay in the remaining cells (B). Treatment with 0.1 nM nicotine had no effect on SAP activity while treatment with 11 and 13.8 mM dextrose reduced SAP activity relative to control cells. However, addition of nicotine and dextrose produced a dose-dependent decrease in SAP activity. Cell survival was not altered by any of the treatments as indicated by the MTT assay (B). $N = 6$; $^{*}P < 0.05$ relative to cells exposed to 5.5 mM dextrose. $^{\ddagger}P < 0.05$ relative to cells treated with 0.1 nM nicotine and exposed to 5.5 mM dextrose. $^{\dagger}P < 0.05$ relative to cells treated with 0.1 nM nicotine and exposed to 8.3 mM dextrose. One-way ANOVA followed by Student's *t*-test were used to calculate statistical differences.

Qiagen (Germantown, MD). Lipofectamine was purchased from Invitrogen (Gaithersburg, MD). All other reagents were purchased from either Thermo-Fisher Scientific or Sigma-Aldrich.

Cell culture

HCAEC were grown in vascular endothelial cell growth medium supplemented with 0.2% bovine brain extract, 5 ng/ml recombinant human epidermal growth factor, 10 mM glutamine, 1 μg/ml hydrocortisone hemisuccinate, 0.5 units/ml heparin sulfate, 50 μg/ml ascorbic acid, 2% fetal bovine serum, 10 units/ml penicillin, and 10 μg/ml streptomycin. Experiments were performed with cells between passages 2 and 5. The cells were housed in a dedicated incubator with 5% CO_2 at 37°C. All experiments performed with tunicamycin and nicotine included control cells treated with an equivalent volume and concentration of dimethylsulfoxide (DMSO).

Measurement of ER stress using the ER stress secreted alkaline phosphatase assay

ER stress was measured using the ER stress secreted alkaline phosphatase (ES-TRAP) assay as previously described [5]. HCAEC in 6-well culture dishes were transfected with 2 μg of the plasmid pSEAP2.control using lipofectamine and 24-h later treated as described in each figure. After 24-h, SAP activity was measured in the conditioned medium using the chemiluminescent substrate CSPD, and cell viability was measured using the MTT assay. CSPD luminescence was measured with a Turner Biosystems luminometer (Sunnyvale, CA) and is expressed as percent change from control.

Protein isolation and Western blot analysis

HCAEC in 6-well plates were incubated as described above and protein samples were prepared as follows. After removal of the conditioned media from the cells, the cells were washed three-times in Hank's Balanced Salt Solution. After addition of 200 μl of lysis buffer (2% sodium dodecylsulfate (SDS), 10% glycerol, 60 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-Cl) (pH 6.8), 1x HALT protease/phosphatase inhibitor cocktail),

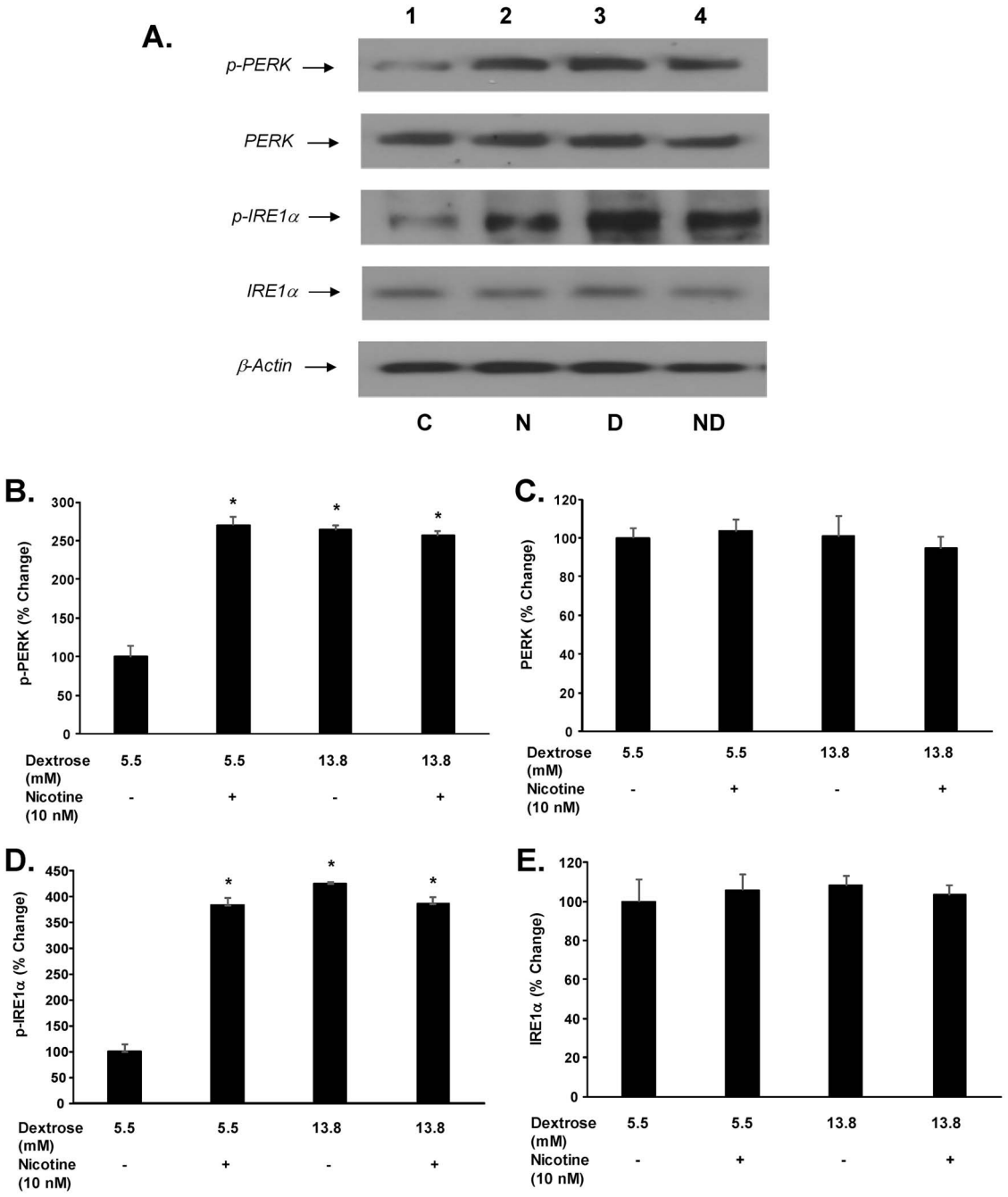


Figure 3: The effect of nicotine and dextrose on IRE1α and PERK expression and activation. HCAEC were treated with solvent (DMSO) (C), 10 nM nicotine (N), 13.8 mM dextrose (D), or nicotine + dextrose (ND) (10 and 13.8 nM, respectively) for 24-h and PERK and IRE1α levels were measured as well as their activation by phosphorylation. Representative blots are shown in (A) and quantified in (B-E). Expression levels were normalized to β-actin expression which did not change. Treatment with nicotine, dextrose, and nicotine + dextrose increased PERK (B) and IRE1α (D) phosphorylation but had no effect on their expression (C and E, respectively). $N = 3$; $^*P < 0.05$ relative to solvent treated control cells. One-way ANOVA followed by Student's *t*-test were used to calculate statistical differences.

the cells were scraped from the flask and transferred to 1.5-ml microcentrifuge tubes. The samples were then sonicated for 5-s and the supernatant fractions were collected after centrifugation at 12 000 x*g* for 10 min. Protein concentration was measured using the bicinchoninic acid (BCA) assay [6] and 50 µg of protein was fractionated on a 10% SDS-polyacrylamide gel. After transfer to Immobilon-P, the membranes were blocked in tris-buffered saline/Tween 20 (50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 0.1% Tween 20) (TBS) containing 10% newborn calf serum (NCS) (TBST/NCS) for 2-h at room temperature. Primary antibodies were diluted to the concentrations recommended by the manufacturer in TBST/NCS and incubated with the membranes overnight at 4°C. The blots were washed four times in TBST and incubated with the species-appropriate secondary antibody conjugated to horseradish peroxidase diluted 1:4000 in TBST/NCS at room temperature for 45-min. The blots were then washed four times with TBST, twice in TBS, and incubated with the enhanced chemiluminescence (ECL) HRP substrate

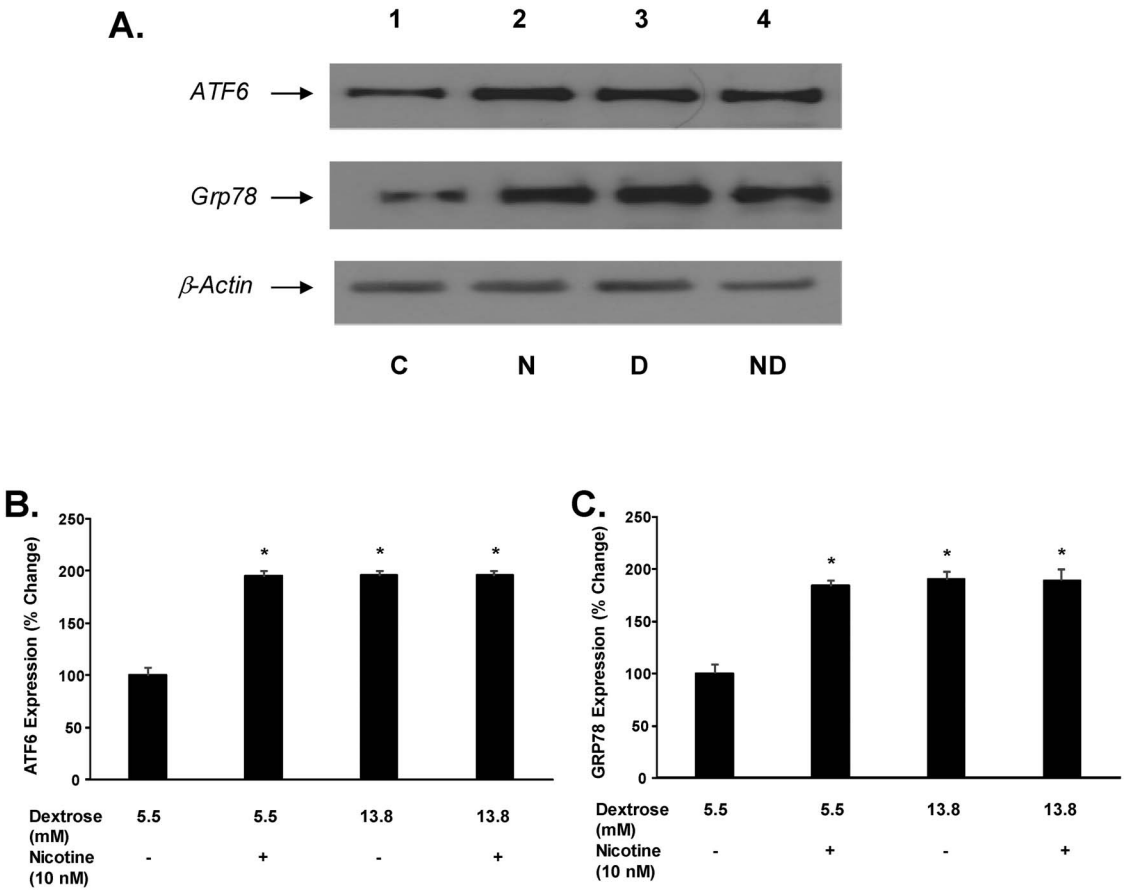


Figure 4: The effect of nicotine and dextrose on ATF6 and GRP78 expression. HCAEC were treated with the solvent (DMSO) (C), 10 nM nicotine (N), 13.8 mM dextrose (D), or nicotine + dextrose (ND) (10 nM and 13.8 mM, respectively) for 24-h and ATF6, GRP78, and β-actin levels were measured by western blot. Representative blots are shown in (A) and ATF6 and GRP78 levels, normalized to β-actin expression are quantified in (B and C). Treatment with nicotine, dextrose, and nicotine + dextrose induced ATF6 and GRP78 levels significantly. There was no effect of any of the treatments on β-actin expression. $N = 3$; $^*P < 0.05$ relative to control cells. One-way ANOVA followed by Student's *t*-test were used to calculate statistical differences.

for 1-min. The blots were exposed to film, stripped following the manufacturers recommendations, and incubated with an antibody to β-actin as a loading control. The films were scanned and quantified using ImageJ (NIH).

Measurement of cell viability

Cell viability was measured using either the MTT assay [7] or crystal violet staining [8]. For the MTT assay, cells were treated as described and half of the conditioned medium was removed for SAP measurement. To the remaining medium, 100 µl of MTT dissolved in phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) (5 mg/ml) was added and the cells were returned to the incubator until a visual inspection of the cultures indicated enough time had elapsed for formazan crystal formation. The medium was aspirated from the cells and 300 µl of stop buffer (0.1 M HCl, 10% Triton X-100 in isopropanol) was added. The plates were gently rocked until the crystals were dissolved, the liquid was transferred to a 96-well plate, and absorbance was measured at 570 and 690 nm (for background correction) on a BioTek ELx800 microplate spectrophotometer (Winooski, VT).

Crystal violet staining was performed by aspirating the culture medium from the treated cells and washing twice with tap water prior to addition of 750 µl of 0.5% crystal violet stain. After 20-min, the cells were washed four-times with tap water, and allowed to dry for 2-h. The stained cells were then dissolved in 200 µl of methanol and their absorbance at 570 nm was measured on a BioTek ELx800 microplate spectrophotometer.

Statistics

The results are presented as the mean ± standard deviation. Analysis of variance (ANOVA) was performed using Statistica (Palo Alto, CA), and subgroup analysis was carried using the Student's *t*-test for independent variables. Significant differences are reported as a two-tailed $P < 0.05$.

Results

The effect of nicotine on ER stress in HCAEC

Addition of nicotine at concentrations 1.0 nM and above significantly suppressed SAP activity with a maximal response observed at 10 nM (Fig. 1A). As anticipated, treatment with pharmacologic ER stress inducer tunicamycin (1 µM), suppressed SAP activity. The latter was included in the assay as an internal positive control. The reduction in SAP activity observed in tunicamycin or nicotine-treated cells was not due to cytotoxicity since MTT activity was equivalent in all the conditions examined (Fig. 1B). These results suggest that nicotine induces ER stress in a dose-dependent manner.

The potentiating effect of nicotine on dextrose-induced ER stress

Treatment of HCAEC with 11 and 13.8 mM dextrose inhibited SAP activity relative to cells maintained in culture media with 5.5 mM dextrose (Fig. 2A). When 0.1 nM nicotine was added to cells exposed to 8.3, 11, and 13.8 mM dextrose, a further reduction in SAP activity was observed relative to cells exposed to 8.3, 11, and 13.8 mM dextrose alone (Fig. 2A). The cell viability was not altered in these experiments as evidenced by lack of a change in MTT activity (Fig. 2B).

The effect of nicotine and dextrose on ER stress biomarkers

Representative western blots are shown in Figures 3A and 4A and the quantified values are presented in Figures 3B-E and 4B and C. Treatment with 10 nM nicotine, 13.8 mM dextrose, and the combination of the nicotine and dextrose induced PERK activity as indicated by its increased phosphorylation state (Fig. 3B) but had no effect on PERK expression itself (Fig. 3C). Likewise, IRE1α activity (as indicated by its increased phosphorylation state) was increased (Fig. 3D) but total IRE1α levels were not altered (Fig. 3E). In addition, treatment with 10 nM nicotine, 13.8 mM dextrose, and the combination of the nicotine and dextrose induced ATF6 expression (Fig. 4B) and the expression of its target gene GRP78 (Fig. 4C). All western blots were normalized to β-actin levels, which did not change with these experimental conditions. These results indicate that nicotine, like dextrose, induces the PERK, IRE1α, and ATF6 branches of the unfolded protein response (UPR).

The potentiating effect of nicotine on dextrose induced cytotoxicity

Treatment with 10 nM nicotine and 13.8 mM dextrose suppressed SAP activity relative to control cells, while treatment with the combination of nicotine and dextrose suppressed SAP activity to a greater extent than either agent alone (Fig. 5A). However, when cell viability was assessed, HCAEC treated with combination of nicotine and dextrose showed a reduction in MTT activity (Fig. 5B) as well as crystal violet staining (Fig. 5C) indicating that the combination of 10 nM nicotine and 13.8 mM dextrose induces significant cell death.

The effect of nicotine and dextrose on expression of apoptotic regulatory protein expression

Representative western blots are shown in Figure 6A. Cleaved caspase 3, total caspase 3, BID, and BCL2 levels were normalized to β-actin content and are quantified in panels B, C, D, and E, respectively. Treatment with 13.8 mM dextrose alone or treatment with the combination of 10 nM nicotine and 13.8 mM dextrose increased the amount of cleaved caspase 3 (panel B) and BID levels (panel D) and decreased BCL2 levels decreased significantly (panel E). These results suggest that dextrose-related cell death occurs through apoptosis.

The effect of simvastatin and pravastatin on ER stress and cell toxicity induced by nicotine and dextrose

Treatment with 10 nM nicotine, 13.8 mM dextrose, and the combination of 10 nM nicotine with 13.8 mM dextrose significantly reduced SAP activity relative to control cells (Fig. 7A). However, 10 µM simvastatin or 10 µM pravastatin treatment reversed these effects entirely (Fig. 7A). Likewise, treatment with the

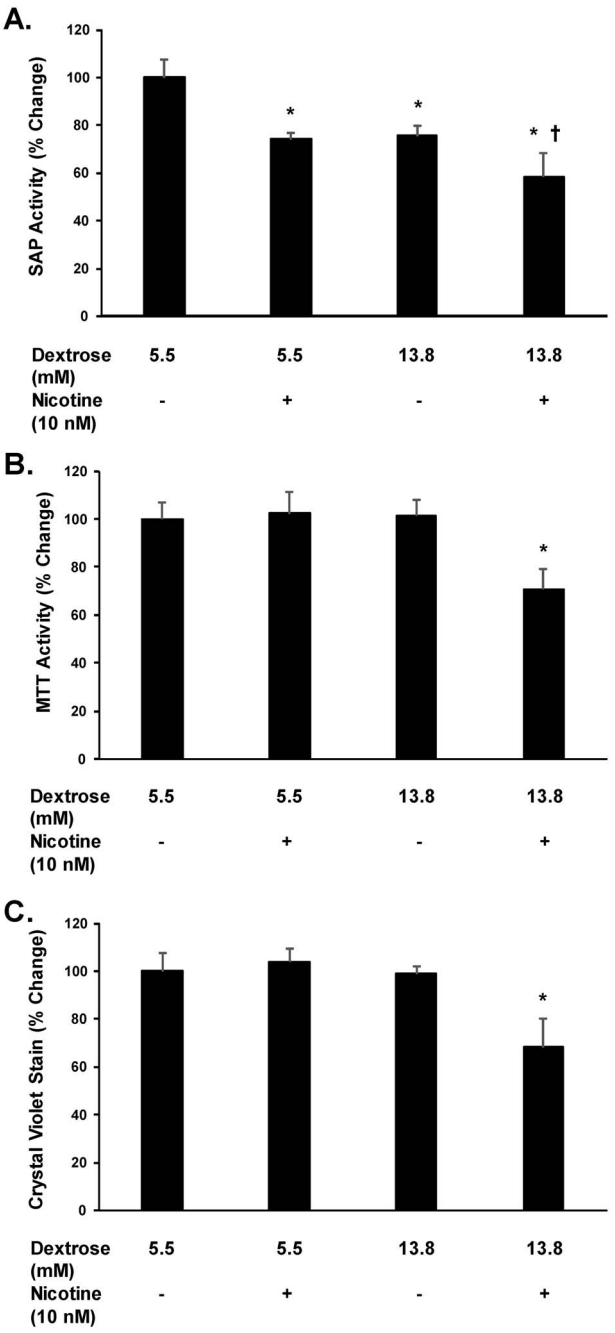


Figure 5: The effect of nicotine and dextrose on SAP activity and toxicity. HCAEC were transfected with the plasmid pSEAP2.control and 24-h later treated with the solvent DMSO, 10 nM nicotine, 13.8 mM dextrose, or 10 nM nicotine + 13.8 mM dextrose. Twenty four hours later, the conditioned medium was collected and assayed for SAP activity (A) while cell viability was measured using the MTT assay (B) or crystal violet staining (C). Treatment with nicotine or dextrose decreased SAP activity, while treatment with both nicotine and dextrose decreased SAP activity even more. While treatment with nicotine and dextrose had no effect on cell viability alone (B and C), treatment with nicotine + dextrose reduced cell viability as indicated with both the MTT assay and by crystal violet staining. $N = 6$; $^{*}P < 0.05$ relative to control cells. $^{\dagger}P < 0.05$ relative to cells treated with nicotine or dextrose alone. One-way ANOVA followed by Student's *t*-test were used to calculate statistical differences.

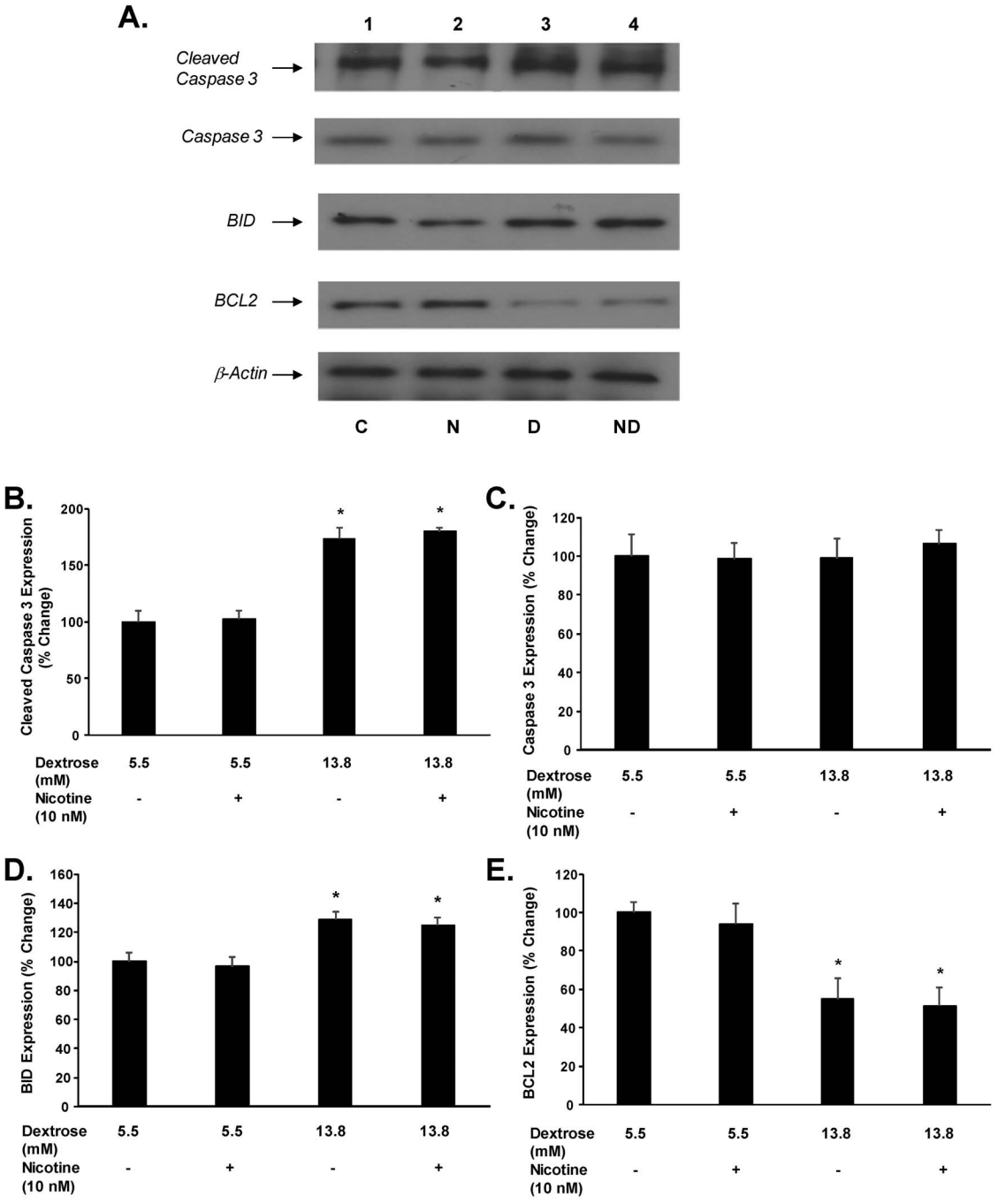


Figure 6: The effect of nicotine and dextrose on caspase 3 expression and activation, BID, and BCL2 expression. HCAEC were treated with solvent (DMSO) (C), 10 nM nicotine (N), 13.8 mM dextrose (D), or nicotine + dextrose (ND) (10 and 13.8 nM, respectively) for 24-h and caspase 3 expression and activation, BID, and BCL2 levels were measured by western blot, while β-actin levels were measured as a control. Representative blots are shown in (A). Expression levels were quantified and normalized to β-actin expression and are shown in (B) (cleaved caspase 3), (C) (total caspase 3), (D) (BID), and (E) (BCL2). Treatment with dextrose and nicotine + dextrose increased caspase 3 cleavage and increased BID expression, while treatment with dextrose and nicotine + dextrose decreased BCL2 expression. None of the treatments had any effect on β-actin expression. $N = 3$; $^*P < 0.05$ relative to control cells. One-way ANOVA followed by Student's *t*-test were used to calculate statistical differences.

combination of nicotine and dextrose reduced cell survival as measured by MTT assay (Fig. 7B), while treatment with either 10 μM simvastatin or pravastatin reversed these effects.

Discussion

NRT has been an important tool in the armamentarium of cigarette smoking cessation programs. This is based on the widely accepted assumption that nicotine replacement is safe even in current smokers who already have established cardiovascular disease. Peak plasma nicotine concentrations for a "single dose" of 4 mg nicotine gum, 2 mg nicotine gum nicotine inhaler/nasal spray and nicotine patch are 36, 25, 18, and 9.2 nM while the peak plasma nicotine concentration for cigarettes is 74 nM [9]. Compared to cigarettes, NRT products provide slower and less variable plasma nicotine concentrations

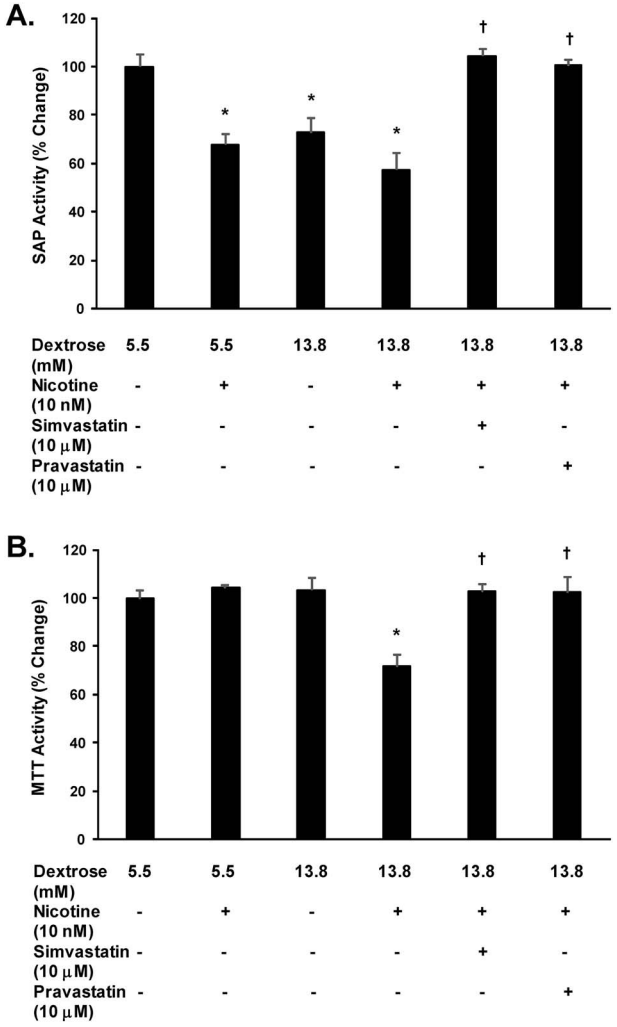


Figure 7: The effect of simvastatin and pravastatin on nicotine- and dextrose-induced ER stress and cell viability. HCAEC were transfected with the plasmid pSEAP2.control and 24-h later treated with the solvent DMSO, 10 nM nicotine, 13.8 mM dextrose, or nicotine + dextrose (10 nM and 13.8 mM, respectively). Other cells were treated with 10 nM nicotine + 13.8 mM dextrose and either 10 μM simvastatin or 10 μM pravastatin. After 24-h, SAP activity was measured in the conditioned medium (A) and MTT activity was measured in the cultures (B). As observed previously, treatment with nicotine and dextrose decreased SAP activity, as did the combination of nicotine + dextrose. However, co-treatment with either statin reversed the effect of nicotine + dextrose on SAP activity. Likewise, treatment with nicotine + dextrose decreased cell viability, however, co-treatment with either statin reversed the effects of nicotine + dextrose on cell viability. $N = 6$; $^{*}P < 0.05$ relative to control cells. $^{\dagger}P < 0.05$ relative to cells treated with nicotine + dextrose. One-way ANOVA followed by Student's *t*-test were used to calculate statistical differences.

which may minimize the addictive potential of these products [9].

The data presented in this manuscript suggest that nicotine may have hitherto unknown dire effects on endothelial cell health. The finding that nicotine at concentrations commonly encountered during NRT (10 nM) can cause ER stress and aggravate dextrose-related cytotoxicity in HCAEC, raise the specter that nicotine may promote coronary atherosclerosis especially in people with diabetes and uncontrolled hyperglycemia. It is noteworthy that nicotine at doses of as high as 10 μM did not cause cytotoxicity, while ER stress was observed with nicotine concentrations as low as 1 nM (Fig. 1). Nicotine at very small concentrations (0.1 nM) aggravated 8.3, 11, and 13.8 mM dextrose induced ER stress (Fig. 2A). The combination of modestly high dextrose concentrations and relatively low nicotine concentration was sufficient to elicit the UPR through IRE1α and PERK signaling as well as increased ATF6 expression. The cell viability was not altered in these experiments as evidenced by the lack of a change in MTT activity (Fig. 2B). However, significant cytotoxicity was observed in HCAEC treated with both nicotine (10 nM) and high-dextrose (13.8 mM), which by themselves had no associated cytotoxicity (Fig. 5B and C). In parallel, treatment with the combination of nicotine and dextrose suppressed SAP activity (i.e. increased ER stress) to a greater extent than either agent alone (Fig. 5A). Thus, ER stress assays are more sensitive than cytotoxicity assays in so far as detecting cell dysfunction. Measurements of cleaved caspase 3, BID and BCL2 in HCAEC suggest that cell death observed following treatment with the combination of 10 nM nicotine 13.8 mM dextrose was the result of apoptosis. Our data suggest that nicotine plus dextrose treatment does not have additive or synergistic effect on cell death with regard to cleaved caspase 3 levels, BID, or BCL2, which were chosen for our study because they have been clearly implicated in ER stress-induced cell death [10–12]. In addition, statin drugs that are known to have ER stress ameliorating activity [13] were also capable to reverse ER stress and cell death caused by the nicotine and high dextrose combination.

Srinivasan *et al.* [14] recently demonstrated that nicotine exposure (200 ng/ml for 2 weeks) prevented tunicamycin-induced ER stress in dopaminergic neurons isolated from the ventral midbrain of embryonic day 14 (E14) mouse embryos. Treatment with low-dose tunicamycin (50 nM) for 2 weeks induced phosphorylated eukaryotic initiation factor 2α (eIF2α), increased nuclear ATF6 and X-box binding protein 1 (XBP1) levels, and increased C/EBP homologous protein (CHOP) expression. However, when the cells were treated with 200 nM nicotine and tunicamycin, eIF2α phosphorylation, nuclear ATF6 and XBP1 levels decreased, and CHOP levels decreased. This was accompanied by an increased in the expression and activity of several neuronal acetylcholine (nAChR) receptor subunits, including α4, α6, and β3 nAChR. The authors proposed that nicotine may be suppressing ER stress in this context either by changing calcium levels in the ER or by acting directly as a molecular chaperone, stabilizing the pentameric nAChR as it is being synthesized in the ER. In contrast, Wong *et al.* [15] demonstrated that nicotine enhanced ER stress by impairing disulfide bond formation in the rat placenta. Maternal nicotine exposure induced GRP78, activating transcription factor 4 (ATF4), and CHOP expression, as well as eIF2α phosphorylation. These studies suggest that nicotine may have unique cell- or tissue-specific effects on ER homeostasis.

These results taken together suggest that nicotine can induce ER stress and aggravate dextrose-related apoptosis in HCAEC. Although the immediate clinical implications of these observations in cell culture are not known, it would be prudent to limit the dose and duration of NRT especially in people with diabetes or established coronary artery disease.

Acknowledgments

None.

Conflict of Interest

None declared.

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