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Running title: Nicotine induces placental ER stress

Abstract

Nicotine exposure during pregnancy leads to placental insufficiency impairing both fetal and neonatal development. Previous studies from our laboratory have demonstrated that in rats, nicotine augmented endoplasmic reticulum (ER) stress in association with placental insufficiency, however, the underlying mechanisms remain elusive. Therefore, we sought to investigate the possible direct effect of nicotine on ER stress in Rcho-1 rat placental trophoblast giant (TG) cells during differentiation. Protein and/or mRNA expression of markers involved in ER stress (*e.g.,* phosphorylated PERK, eIF2α, CHOP, BiP/GRP78) and trophoblast giant cell differentiation and function (*e.g.,* Pl-1, Pgf, Hsd11b1, and Hsd11b2) were quantified via Western blot or Real-Time PCR. Nicotine treatment led to dose-dependent increases in the phosphorylation of PERK[Thr981] and eIF2α[Ser51], whereas pre-treatment with a nicotinic acetylcholine receptor (nAChR) antagonist (mecamylamine hydrochloride) blocked the induction of PERK phosphorylation, verifying the direct involvement of nicotine and nAChR binding. We next investigated select target genes known to play essential roles in placental TG cell differentiation and function (Pl-1, Pgf, Hsd11b1, and Hsd11b2), and found that nicotine significantly augmented the mRNA levels of Hsd11b1 in a dose-dependent manner. Furthermore, using Tauroursodeoxycholic acid (TUDCA), a safe bile acid known to improve protein chaperoning and folding, we were able to prevent nicotine-induced increases in both PERK phosphorylation and Hsd11b1 mRNA levels, revealing a potential novel therapeutic approach to reverse the deleterious effects of nicotine exposure in pregnancy. Collectively, these results implicate that nicotine, acting through its receptor, can directly augment ER stress and impair placental function.

Keywords

Nicotine, placenta, endoplasmic reticulum stress, Tauroursodeoxycholic acid, Rcho-1, trophoblast giant cell

Introduction

Nicotine exposure during pregnancy remains prevalent worldwide. Studies report that approximately 10-28% of pregnant women smoke (Cui *et al.*, 2014; Dhalwani *et al.*, 2013; Tong *et al.*, 2013), but exposure to nicotine may also occur through nicotine replacement therapies (NRTs), electronic (e)-cigarettes, and other forms of non-combustible tobacco products (Carroll Chapman and Wu, 2014; Myung *et al.*, 2012). While the risks in pregnancy associated with NRTs or e-cigarettes have been long overlooked in comparison to the known hazards of cigarette smoking, recent evidence from animal studies warrant a careful reevaluation of the safety of nicotine exposure alone on pregnancy outcomes (De Long *et al.*, 2014).

During pregnancy, the placenta sustains the health and development of the fetus through finely-balanced nutrient exchange, waste removal, immune and barrier protection, and endocrine regulation (Burton and Fowden, 2015). Interestingly, the lipid-soluble nature of nicotine allows it to rapidly traverse past the membrane barriers to enter into the placenta where it competes with endogenous acetylcholine for binding to nicotinic acetylcholine receptors (nAChRs). Many nAChR subtypes are expressed in both human and rat placentas, and nAChRs in rat placental trophoblast cells are found to be responsive to nicotine doses within the average serum concentrations (25nM-25μM) of moderate to heavy cigarette smokers and/or NRT users (Holloway *et al.*, 2014; Lips *et al.*, 2005; Machaalani *et al.*, 2014). nAChR signaling governs many functions, including cell differentiation, migration, viability, and transmitter release, thus augmented activation due to nicotine binding may elicit alternative, and potentially pathological, effects to impair placental development and function (Albuquerque *et al.*, 2009). Indeed, many animal studies reveal that nicotine exposure *in utero* can lead to placental insufficiency, as seen through structural, morphological, and functional defects *in vivo*, alongside various developmental and long-term health consequences in the offspring (Bruin *et al.*, 2010; De Long *et al.*, 2014; Genbacev *et al.*, 1995; Gruslin *et al.*, 2009; Holloway *et al.*, 2014). However, the cellular mechanisms underlying this nicotine-induced placental insufficiency have yet to be fully explored.

Endoplasmic reticulum (ER) stress, an intracellular perturbation involving the accumulation of misfolded or unfolded proteins, was recently proposed to underlie placental insufficiency associated with intrauterine growth restriction (IUGR) (Yung *et al.*, 2014; Yung *et al.*, 2008; Yung *et al.*, 2012a; Yung *et al.*, 2012b). In response to ER stress, the unfolded protein response (UPR) is activated to alleviate protein misfolding through three major signaling pathways that decrease global protein translation and increase protein folding capacity (Chambers and Marciniak, 2014). While the placenta normally exhibits low basal levels of ER stress due to high protein secretory activity, chronically unresolved ER stress leads to downstream apoptosis, which can impair placental development *in vivo* and *in vitro* (Kawakami *et al.*, 2014; Yung *et al.*, 2008; Yung *et al.*, 2007). We recently reported that maternal nicotine exposure leads to augmented ER stress in the rat placenta (Wong *et al.*, 2015), revealing a potential cellular mechanism that may underlie nicotine-induced placental insufficiency. However, due to the broad scope of indirect effects that nicotine may also elicit *in vivo* (*e.g.,* vasoconstriction leading to hypoxia and amino acid starvation (Koumenis *et al.*, 2002; Lo *et al.*, 2015; Pastrakuljic *et al.*, 1999; Wong *et al.*, 2015)), it is difficult to determine from *in vivo* studies if the ability of nicotine to cause ER stress results from a direct effect on placental cells.

To address this, mechanistic *in vitro* studies using placental trophoblast cells are necessary. The trophoblast giant (TG) cell serves as a relevant cell lineage to target due to its large population in the rat placenta, cardinal involvements in early establishment of pregnancy through uterine invasion and anastomosis of maternal blood supply, and later maintenance of healthy gestation through key endocrine roles in local and systemic physiological adaptations (Fonseca *et al.*, 2012; Hu and Cross, 2010; Soares *et al.*, 1996). Differentiated Rcho-1 TG cells *in vitro* have been very well characterized and determined to exhibit many similarities to the true placental TG cells *in vivo* in terms of cell cycle regulation, differentiation, gene transcription profile, transport processes, hormone production, and others (please refer to (Sahgal *et al.*, 2006) for full reference list on conducted studies). Therefore, utilizing differentiated Rcho-1 TG cells would provide better translatability to the mechanisms that might be occurring *in vivo*. Given that nicotine exposure has been found to negatively impact TG cell function and differentiation (Holloway *et al.*, 2014), our current study was designed to determine the direct role of nicotine on placental ER stress. Moreover, we were also interested in whether nicotine-induced ER stress could be prevented with the use of Tauroursodeoxycholic acid (TUDCA), a taurine-conjugated ursodeoxycholic bile acid endogenously produced by intestinal bacteria (Vang *et al.*, 2014). TUDCA has previously relieved ER stress and stabilized UPR activation in several cell and tissue types (Berger and Haller, 2011; Ozcan *et al.*, 2006; Xie *et al.*, 2002), however, its efficacy as a therapeutic agent in the placenta and in nicotine-induced ER stress has yet to be examined.

Materials and Methods

Cell culture and differentiation

Rcho-1, a placental trophoblast cell line derived from rat choriocarcinoma, was used as a model of placental TG cells. Rcho-1 cells can be maintained in either proliferative trophoblast stem (TS) cell or differentiated TG cell states based on the culture conditions (Faria and Soares, 1991). Rcho-1 TS cells were plated at 1.5 x 10^6 cells/mL and cultured at 37°C in 5% CO_2/95% atmospheric air (Faria and Soares, 1991). Proliferation was maintained by growing Rcho-1 TS cells in RPMI-1640 media (Gibco) supplemented with 20% fetal bovine serum (Gibco), 50µM 2-mercaptoethanol (Gibco), 1mM sodium pyruvate (Gibco), and 0.04% gentamicin (10mg/ml; Gibco). TG cell differentiation was induced as previously described (Sahgal *et al.*, 2006). Briefly, at 80-90% confluency (or after 3 days of proliferation), Rcho-1 TS cells were exposed to NCTC-135 media (Gibco) supplemented with 1% horse serum (Gibco), 50µM 2-mercaptoethanol (Gibco), 1mM sodium pyruvate (Gibco), and 0.04% gentamicin (10mg/ml; Gibco) for 10 days, with daily media changes. Removal of essential nutrients in the fetal bovine serum halted proliferation and promoted TG cell differentiation (Sahgal *et al.*, 2006). NCTC-135 media was used during these differentiation conditions as it provided better pH regulation and decreased toxicity (Faria and Soares, 1991). Rcho-1 cells between passages 26-30 were used for all experiments.

Nicotine treatments

After 10 days of differentiation, Rcho-1 TG cells were treated with vehicle or increasing doses of nicotine (0.1-100μM; Sigma-Aldrich) for 6 or 24 hours. The nicotine doses encompassed the average serum concentrations of nicotine (25nM-25μM) previously reported in pharmacokinetic studies of cigarette smoking and/or NRTs (Armitage *et al.*, 1975; DeVeaugh-Geiss *et al.*, 2010; Massadeh *et al.*, 2009; McNabb *et al.*, 1982; Oncken *et al.*, 1997; Russell *et al.*, 1980). The 6 hour time-point ensured detection of rapid protein phosphorylation events (*e.g.,* phosphorylation of PERK[Thr981] and eIF2α[Ser51]) and the 24 hour time-point allowed detection of changes in protein and mRNA expression. Activation of the UPR indicated the presence of ER stress (Schroder and Kaufman, 2005).

Mecamylamine hydrochloride (nAChR inhibitor) treatments

After 10 days of differentiation, Rcho-1 TG cells were pre-treated for 1 hour with mecamylamine hydrochloride (MH; 10μM; Santa Cruz), a non-competitive, total nAChR inhibitor, and then exposed to nicotine (10μM) for 6 hours. The dose of MH was chosen based on previously published findings demonstrating effective nAChR blocking against nicotine in neuronal cells (Collo *et al.*, 2013; Rao *et al.*, 2003; Ridley *et al.*, 2002). We selected PERK phosphorylation as a marker to assess the effect of nAChR antagonism on ER stress response induction due to the robust increase observed in response to nicotine.

TUDCA treatments

After 10 days of differentiation, Rcho-1 TG cells were pre-treated for 1 hour with TUDCA (100μM; Sigma-Aldrich), and then treated with 10μM nicotine for 6 or 24 hours. The dose of

TUDCA was chosen based on previously published findings that demonstrated effective amelioration of ER stress and UPR activation *in vitro* (Yin *et al.*, 2012; Zhu *et al.*, 2013).

RNA extraction and Real Time-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen). Chloroform (Sigma-Aldrich) was added to the solution, and then centrifuged at 12,500rpm. Supernatant was transferred to a fresh tube with an equal volume of isopropanol (Sigma-Aldrich) and centrifuged again at 12,500rpm. Total RNA was then collected from the pellet and dissolved in DEPC-treated water. Deoxyribonuclease I, Amplification Grade (Invitrogen) was added to the RNA to digest contaminating single- and double-stranded DNA. Four μg of RNA were reverse-transcribed to cDNA using random hexamers and Superscript II Reverse Transcriptase (Invitrogen). Primer sets directed against gene targets of interest were designed through National Center for Biotechnology Information's primer designing tool and generated via Invitrogen Custom DNA Oligos (**Table 1**). Quantitative analysis of mRNA expression was performed via RT-PCR using fluorescent nucleic acid dye SsoFast EvaGreen supermix (BioRad) and BioRad CFX384 Real Time System. The cycling conditions were 95 °C for 10 min, followed by 43 cycles of 95 °C for 15 sec and 60 °C for 30 sec and 72 °C for 30 sec. The cycle threshold was set so that exponential increases in amplification were approximately level between all samples. Relative fold changes were calculated using the comparative cycle times (Ct) method, normalizing all values to the geometric mean of two housekeeping genes (β-Actin and Gapdh). Suitable housekeeping genes were determined using algorithms from GeNorm (Vandesompele *et al.*, 2002), Normfinder (Andersen *et al.*, 2004), BestKeeper (Pfaffl *et al.*, 2004), and the comparative ΔCt method (Silver *et al.*, 2006) to provide an overall ranking of the most stable housekeeping genes. Given all primer sets had equal priming efficiency, the ΔCt values for each primer set were calibrated to the average of all control Ct values, and the relative abundance of each primer set compared with calibrator was determined by the formula $2^{\Delta\Delta Ct}$, in which ΔΔCt was the normalized value.

Protein extraction and Western blot

Cells were homogenized in RIPA buffer (50 mM Tris-HCL, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P40, 0.25% $C_{24}H_{39}NaO_4$, supplemented with phosphatase inhibitors (20 mM NaF, 40mM Na-pyrophosphate, 40mM Na_3VO_4, 200mM β-glycerophosphate disodium salt hydrate), and a protease inhibitor cocktail (Roche)). The solution was sonicated at 30% amplitude for 5 sec total, 1 sec per pulse. It was then mixed in a rotator for 10 min at 4 °C and centrifuged at 16000g for 20 min at 4 °C. The resulting supernatant was collected as the total cellular protein extract and quantified by colorimetric DC protein assay (BioRad). Loading samples were prepared with fresh total protein extract (avoiding repeated freeze-thaw cycles), NuPAGE LDS Sample Buffer (4X) (Invitrogen), NuPAGE Reducing Agent (10X) (Invitrogen), and deionized water, and heated at 70 °C for 10 min to denature the proteins. Proteins (20μg/well) were separated by size via gel electrophoresis in gradient polyacrylamide gels (Novex), and transferred onto polyvinylidene difluoride membrane (Millipore). Membranes were blocked in 1x Tris-buffered saline-Tween 20 buffer with 5% non-fat milk (blocking solution), and then probed using primary antibodies of the protein targets of interest, diluted in the blocking solution (**Table 2**). Secondary antibodies were used to detect the species-specific portion of the primary antibody, diluted in the blocking solution (**Table 3**). Immuno-reactive bands were visualized using SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific). Relative band intensity was calculated using ImageLab software (BioRad) and normalized to the quantified total protein on each respective membrane, as determined through Amido black staining (Aldridge *et al.*, 2008).

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5 software. All results were expressed as means of normalized values ± SEM. All experiments were replicated four times (n=4). Each replicate represents an independent experiment initiated from a different frozen vial of cells. The significance of the differences ($p<0.05$) between normalized mean values were then evaluated using one-way ANOVA followed by Tukey's post-test.

Results

Determination of Rcho-1 TG cell differentiation

Rcho-1 TS cells were cultured for 10 days in NCTC-135 media + 1% horse serum to achieve large populations of actively-differentiating TG cells as previously described (Sahgal *et al.*, 2006). Successful differentiation of Rcho-1 TG cells was determined via phase contrast microscopic imaging of distinct morphological traits (*i.e.*, multiple nuclei, large cell body) (Figure 1A) and quantification of placental lactogen-I (Pl-1) mRNA levels (Figure 1B), which are uniquely expressed by TG cells (Faria and Soares, 1991).

Nicotine treatments increased PERK phosphorylation in a dose-dependent manner

Rcho-1 TG cells were treated with vehicle or increasing doses of nicotine (0.1-100µM) for 6 or 24 hours. We had previously demonstrated that this range of nicotine does not cause overt toxicity nor affect viability in Rcho-1 cells (Holloway *et al.*, 2014). Nicotine treatment led to PERK activation of the unfolded protein response in a dose-dependent manner. At 6 hours, the ratio of phosphorylated (P)-PERK[Thr981]: PERK protein was significantly increased by nicotine treatment; post-hoc testing showed a significant effect at 10 and 100µM nicotine compared to the control ($p<0.05$, $p<0.001$, respectively; Figure 2A, B). At 24 hours, the ratio of P-PERK[Thr981]: PERK protein remained significantly increased by nicotine ($p<0.01$), however this effect did not exhibit a dose-dependent relationship (Figure 2A, C).

The effect of nicotine treatments on downstream targets of the PERK pathway

Downstream of PERK in the UPR pathway, a similar response to nicotine treatments was exhibited in the phosphorylation of eIF2α. At 6 hours, the ratio of P-eIF2α[Ser51]: eIF2α was significantly increased by nicotine treatment; post-hoc testing showed a significant effect at 100µM nicotine compared to control ($p<0.01$; Figure 3A, B). However, protein levels were no longer significantly different from one another at 24 hours (Figure 3A, C). Protein levels of CHOP, a transcription factor downstream of P-eIF2α[Ser51] involved in activating ER stress-related apoptotic pathways during chronic ER stress (Matsumoto *et al.*, 1996; Oyadomari and Mori, 2004), remained unaltered at 6 hours, although it appeared to be trending towards an increase at 24 hours ($p=0.06$; Figure 3A, D, E).

To investigate other aspects of the UPR, we also measured protein levels of two chaperone proteins: BiP/GRP78, a chaperone protein up-regulated during ER stress to assist with protein re-folding (Lee, 2005), and PDI, a key enzyme and chaperone protein involved in disulfide bond formation during protein folding (Benham *et al.*, 2013; Frand and Kaiser, 1999; Wang and Tsou, 1993; Zhang *et al.*, 2014). Nicotine treatment did not significantly alter the protein expression of either marker at any time or dose tested (Figure 4A-E).

Pre-treatment with nAChR antagonist blocked nicotine-induced PERK phosphorylation

To identify whether the effect of nicotine on UPR activation occurred via nAChR signaling, Rcho-1 TG cells were pre-treated for 1 hour with mecamylamine hydrochloride (MH; 10μM), and then treated with nicotine (10μM) for 6 hours. Nicotine significantly increased PERK phosphorylation ($P<0.05$), an effect that was completely blocked with MH pre-treatment ($p<0.05$; Figure 5A, B). MH alone did not affect PERK phosphorylation.

Nicotine treatments increased 11β-hydroxysteroid dehydrogenase (Hsd11b) 1 expression

Given that nicotine can directly induce ER stress and UPR activation in Rcho-1 TG cells, we were next interested in assessing its impact on select markers of placental TG cell differentiation (*i.e.,* Pl-1 and Pgf (Faria and Soares, 1991; Vrachnis *et al.*, 2013)) and function (*i.e.,* Hsd11b1 and Hsd11b2 for placental steroid metabolism (Chapman *et al.*, 2013)). We measured the steady-state mRNA levels of Pl-1, placental growth factor (Pgf), Hsd11b1, and Hsd11b2 via RT-PCR. While nicotine treatments did not significantly affect Pl-1, Pgf, and Hsd11b2 mRNA levels after

24 hours (Figure 6A-B, D), it did significantly increase Hsd11b1 mRNA levels in a dose-dependent manner ($p<0.05$; Figure 6C).

Pre-treatment with TUDCA prevented the effects of nicotine on PERK phosphorylation and Hsd11b1 expression

Lastly, Rcho-1 TG cells were pre-treated for 1 hour with TUDCA (100μM), and then treated with nicotine (10μM) for 6 or 24 hours. Nicotine significantly increased PERK phosphorylation ($P<0.05$), and pre-treatment with TUDCA (100μM) completely abrogated this effect ($p<0.05$; Figure 7A, B). Furthermore, nicotine treatment significantly increased Hsd11b1 mRNA levels, and pre-treatment with TUDCA (100μM) also completely inhibited nicotine-induced increases in Hsd11b1 mRNA levels ($p<0.001$; Figure 7C). TUDCA pre-treatment alone did not induce any effects on PERK phosphorylation nor Hsd11b1 mRNA levels.

Discussion

We recently demonstrated that maternal nicotine exposure augments ER stress in the rat placenta *in vivo* (Wong *et al.*, 2015). However, chronic maternal exposure to nicotine in this study also led to increased placental hypoxia (*e.g.* HIF-1α), which is an indirect trigger for ER stress (Koritzinsky *et al.*, 2013; Romero-Ramirez *et al.*, 2004; Wong *et al.*, 2015). Thus, the direct effects of nicotine on placental ER stress could not be ascertained. In the current study, we now demonstrate that nicotine can directly augment ER stress and UPR activation in differentiating Rcho-1 TG cells, as indicated through the dose-dependent increases in PERK and eIF2α phosphorylation. The involvement of nAChR activation was further verified by the inhibition of nicotine-induced PERK phosphorylation using MH, a total nAChR antagonist. However, dissimilar to our previous *in vivo* results, the effects of nicotine on CHOP and PDI were far less prominent amidst strong PERK and eIF2α activation (Wong *et al.*, 2015). It is likely that longer exposures and/or stronger doses of nicotine may have been required to alter these downstream targets *in vitro* (*e.g.,* 10mM nicotine was used to increase CHOP expression in periodontal ligament cells *in vitro* (Lee *et al.*, 2012)). We further revealed that nicotine treatments can augment the expression of Hsd11b1 in a dose-dependent manner, in association with augmented ER stress, suggesting possible alterations in placental steroid metabolism. Subsequently, pre-treatment of Rcho-1 TG cells with TUDCA ameliorated the effects of nicotine on ER stress in placental TG cells, and consequentially, on Hsd11b1 expression, suggesting nicotine-induced ER stress may mediate its augmented expression.

Consistent with our findings, Repo *et al.* (2014) provided preliminary evidence that nicotine (15μM) can increase the expression of two ER stress markers, IRE1 and BiP, in BeWo choriocarcinoma cells. To comprehensively build upon these results, we utilized an actively differentiating placental TG cell model, a broad range of nicotine doses, along with inhibitors of nAChR activation and ER stress in order to provide a more robust, mechanistic assessment of the direct effects of nicotine on ER stress in the placenta. Rcho-1 cells are remarkable in their versatile ability to actively differentiate into TG cells, providing a more physiologically-representative cellular model of the placenta (Faria and Soares, 1991). Rcho-1 cells have also been extensively validated and exhibit many similarities to true placental TG cells in cell cycle regulation, differentiation, gene transcription, transport processes, hormone production, and others (please refer to (Sahgal *et al.*, 2006) for reference list on characterization studies).

Furthermore, the use of rat placental trophoblast cells complement what we previously reported in the rat placenta *in vivo*, allowing us to further elucidate the underlying mechanisms involved in nicotine-induced placental dysfunction (Wong *et al.*, 2015).

Considering that nicotine directly increased placental ER stress, we were next interested in investigating the influence of nicotine and ER stress on select placental target genes. The increases seen in Hsd11b1 mRNA levels in Rcho-1 TG cells are important to note as the 11β-HSD family of enzymes are essential for steroid metabolism in the placenta (Chapman *et al.*, 2013). While 11β-HSD2 (the protein product of Hsd11b2 transcript) is known for inactivating maternal glucocorticoids entering the placenta, 11β-HSD1 (the protein product of Hsd11b1 transcript), increases bioactive glucocorticoid production (Patel *et al.*, 1999; Yang *et al.*, 1994). Prenatal nicotine exposure has been associated with elevated glucocorticoid levels, low birth weight, and IUGR in rats (Chen *et al.*, 2007; Feng *et al.*, 2014), and Chen *et al.* (2007) further demonstrated that this is associated with decreased placental Hsd11b2 mRNA levels in rats. The lack of change seen in Hsd11b2 mRNA levels in our experiments may perhaps be attributed to the acute duration of our nicotine treatments (6, 24 hours) in comparison to their chronic nicotine exposures (7 days); however, Benediktsson *et al.* (1997) also reported that nicotine exposure does not alter 11β-HSD2 activity in LLC-PK1 kidney-derived cells after 24 hours, thus there may be discrepancies between the *in vivo* and *in vitro* effects. On the other hand, our finding that nicotine increased Hsd11b1 mRNA levels was consistent with a past study demonstrating that prenatal nicotine exposure led to increased Hsd11b1 mRNA levels in fetal rat hippocampus, liver, and gastrocnemius muscle *in vivo* (Xu *et al.*, 2012). It is noteworthy that nicotine increased 11β-HSD1 expression, given that bioactive glucocorticoids in the placenta have been demonstrated to act in an autocrine manner to reduce prostaglandin breakdown and promote premature parturition (Chapman *et al.*, 2013). This reveals an important area for research considering that smoking and/or nicotine exposure in pregnancy is associated with increased risk of preterm birth or intrauterine growth restriction in humans and rodents, respectively (Fantuzzi *et al.*, 2007; Feng *et al.*, 2014; Jaddoe *et al.*, 2008). Yet, the involvement of placental 11β-HSD1 has yet to be examined in these studies, thus future studies are required to investigate the changes in placental glucocorticoid metabolism upon exposure to nicotine and/or increased ER stress to further supplement these results. The specific involvement of various nAChRs, as activated by nicotine, would also be of great interest in future studies. Moreover, there are no interventions currently available to target the deleterious impact of nicotine on placental glucocorticoid metabolism. Our findings revealing a dose-dependent association of nicotine with ER stress contribute insight into potential therapeutic options.

Therefore, we next investigated the use of TUDCA in ameliorating nicotine-induced placental ER stress, and the downstream effects on Hsd11b1 expression. Findings from our study are the first to reveal that TUDCA can prevent nicotine-induced PERK activation in placental TG cells. The ability of TUDCA to prevent nicotine-induced increases in Hsd11b1 mRNA levels also suggests that ER stress may play a role in altering placental glucocorticoid metabolism, though more studies would need to be conducted to confirm this mechanism, as TUDCA is not specific to only ER stress. TUDCA is suggested to ameliorate ER stress either through direct assistance with protein folding or by increasing the expression of endogenous molecular chaperones (Berger and Haller, 2011). Exogenous supplementation of TUDCA has provided beneficial effects in treating animal models of protein misfolding disorders such as obesity, type

2 diabetes, and Alzheimer's disease (Ozcan *et al.*, 2006; Sola *et al.*, 2003). Recent developmental studies further demonstrated that postnatal TUDCA injections can reverse prenatal ethanol-induced ER stress damage in liver and skeletal muscle of rat offspring (Yao *et al.*, 2013; Yao *et al.*, 2014). Alongside the favourable effects against ER stress, TUDCA may also inhibit ROS production and protect against mitochondrial-mediated and caspase-mediated apoptosis (Miller *et al.*, 2007; Rodrigues *et al.*, 2003; Sokol *et al.*, 2005), perhaps collectively enriching global cellular health. These properties of TUDCA may be especially desirable during pregnancy, as the placenta is particularly susceptible to augmented oxidative and ER stress due to its high protein folding and secretory activity (Yung *et al.*, 2012b). Despite the fact that TUDCA, and UDCA (the non-taurine-conjugated counterpart of TUDCA), are approved by the FDA for treating primary biliary cirrhosis (Engin and Hotamisligil, 2010; Larghi *et al.*, 1997), the safety of maternal usage during pregnancy has yet to be explored *in vivo*. It will be of great interest to examine the potential benefits of TUDCA on nicotine-induced ER stress in the placenta *in vivo*, and ultimately, as a potential novel therapeutic agent to remedy the consequences of maternal nicotine exposure in the clinical setting (Please refer to Cortez and Sim (2014) for a useful review on the speculated therapeutic potential of TUDCA).

In conclusion, our current *in vitro* study provides strong mechanistic insight on the direct effects of nicotine exposure on placental ER stress at the cellular level. Our findings further demonstrate that TUDCA supplementation may indeed be a promising therapeutic option to consider for treating the negative outcomes of maternal nicotine exposure, although more studies are warranted to assess its safety and efficacy in pregnancy. With the high rates of maternal nicotine exposure that continue to occur worldwide, research providing intervention strategies are urgently required.

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Figure Legends

Figure 1. Different methods used to detect the presence of differentiated Rcho-1 TG cells. (A) Phase contrast microscopic images (10X). White triangles identify several representative differentiated TG cells. (B) Steady-state mRNA levels of Pl-1 as measured through RT-PCR.

Figure 2. The effect of nicotine exposure (0.1-100µM) on phosphorylation of PERK after 6 and 24 hours in Rcho-1 TG cells. (A) Specific targeted protein bands as detected by respective antibodies via Western blot. (B) Protein levels of the ratio of P-PERK[Thr981]: PERK at 6 hours and (C) 24 hours of nicotine exposure. All arbitrary values were expressed as means normalized to Amido Black ± SEM. All experiments were performed in quadruplicates (n=4). Significant differences between treatment groups as determined by one-way ANOVA (1WA) indicated by ** ($p<0.01$) or *** ($p<0.001$). Different letters represent means that are significantly different from one another according to Tukey's post-test ($p<0.05$). Non-significant differences ($p>0.05$) indicated by n.s.

Figure 3. The effect of nicotine exposure (0.1-100µM) on downstream targets of the PERK pathway after 6 and 24 hours in Rcho-1 TG cells. (A) Specific targeted protein bands as detected by respective antibodies via Western blot. (B) Protein levels of the ratio of P-eIF2α[Ser51]: eIF2α at 6 hours and (C) 24 hours of nicotine exposure. (D) Protein levels of CHOP at 6 hours and (E) 24 hours of nicotine exposure. All arbitrary values were expressed as means normalized to Amido Black ± SEM. All experiments were performed in quadruplicates (n=4). Significant differences between treatment groups as determined by one-way ANOVA

(1WA) indicated by ** ($p<0.01$) or *** ($p<0.001$). Different letters represent means that are significantly different from one another according to Tukey's post-test ($p<0.05$). Non-significant differences ($p>0.05$) indicated by n.s.

Figure 4. The effect of nicotine exposure (0.1-100µM) on BiP and PDI after 6 and 24 hours in Rcho-1 TG cells. (A) Specific targeted protein bands as detected by respective antibodies via Western blot. (B) Protein levels of BiP at 6 hours and (C) 24 hours of nicotine exposure. (D) Protein levels of PDI at 6 hours and (E) 24 hours of nicotine exposure. All arbitrary values were expressed as means normalized to Amido Black ± SEM. All experiments were performed in quadruplicates (n=4). Non-significant differences ($p>0.05$) indicated by n.s.

Figure 5. Pre-treatment with MH (10µM) blocked nicotine-induced PERK phosphorylation after 6 hours in Rcho-1 TG cells. (A) Specific targeted protein bands as detected by respective antibodies via Western blot. (B) Protein levels of the ratio of P-PERK[Thr981]: PERK at 6 hours. All arbitrary values were expressed as means normalized to Amido Black ± SEM. All experiments were performed in quadruplicates (n=4). Significant differences between treatment groups as determined by one-way ANOVA (1WA) indicated by * ($p<0.05$). Different letters represent means that are significantly different from one another according to Tukey's post-test ($p<0.05$).

Figure 6. The effect of nicotine exposure (1-10µM) on markers of placental TG cell differentiation and function after 24 hours in Rcho-1 TG cells. mRNA levels of (A) Pl-1, (B)

Pgf, (C) Hsd11b1, and (D) Hsd11b2. All arbitrary values were expressed as means normalized to the geometric mean of β-Actin and Gapdh ± SEM. All experiments were performed in quadruplicates (n=4). All experiments were performed in quadruplicates (n=4). Significant differences between treatment groups as determined by one-way ANOVA (1WA) indicated by * ($p<0.05$). Different letters represent means that are significantly different from one another according to Tukey's post-test ($p<0.05$). Non-significant differences ($p>0.05$) indicated by n.s.

Figure 7. Pre-treatment with TUDCA (TUD; 100µM) prevented the effects of nicotine on PERK phosphorylation and Hsd11b1 expression after 6 hours in Rcho-1 TG cells. (A) Specific targeted protein bands as detected by respective antibodies via Western blot. (B) Protein levels of the ratio of P-PERK[Thr981]: PERK at 6 hours. All arbitrary values were expressed as means normalized to Amido Black ± SEM. All experiments were performed in quadruplicates (n=4). Significant differences between treatment groups determined by one-way ANOVA (1WA) indicated by * ($p<0.05$) or *** ($p<0.001$). Different letters represent means that are significantly different from one another according to Tukey's post-test ($p<0.05$).

Tables

Table 1. Forward and reverse sequences for the primers used for RT-PCR.

Gene	*Forward*	*Reverse*	*GenBank/ Reference*
Pl-1	TGACTTTGACTCTTTCGGGCT	GCTCTGAATACACCGAGAGCG	(Dai *et al.*, 1996)
Pgf	GTGAGTATGCTGAGCCTAAGGG	AGACCTTACAAGACATGGATTCCC	NM_053595.2
Hsd11b1	GTCTCGGTAGGAGATGCTCAGG	GTAAGAGGCAACTTCCAGATGGC	NM_017080.2
Hsd11b2	TCGGCATCAGCAGTAGAGG	ACAACCCAGGACCCAAAC	(Xu *et al.*, 2012)
β-Actin	CACAGCTGAGAGGGAAAT	TCAGCAATGCCTGGGTAC	NM_031144
Gapdh	GGATACTGAGAGCAAGAGAGAGG	TCCTGTTGTTATGGGGTCTGG	NM_017008.4

Table 2. Western Blot primary antibodies, dilutions used in experiments, and company and catalogue information.

Antibody name	*Source*	*Dilution*	*Company (#Catalogue)*
P-PERK[Thr981]	Rabbit polyclonal	1:800	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA (#sc-32577)
PERK (D11A8)	Rabbit monoclonal	1:500	Cell Signaling Technology Inc., Danvers, MA, USA (#5683)
P-eIF2α[Ser51] (119A11)	Rabbit monoclonal	1:1000	Cell Signaling Technology Inc., Danvers, MA, USA (#3597)
eIF2α	Rabbit monoclonal	1:1000	Cell Signaling Technology Inc., Danvers, MA, USA (#9722)
CHOP (D46F1)	Rabbit monoclonal	1:500	Cell Signaling Technology Inc., Danvers, MA, USA (#5554)
BiP (GRP78)	Rabbit polyclonal	1:1000	Cell Signaling Technology Inc., Danvers, MA, USA (#3183)
PDI (C81H6)	Rabbit monoclonal	1:1000	Cell Signaling Technology Inc., Danvers, MA, USA (#3501)

Table Error! No text of specified style in document.**.3. Western Blot secondary antibodies, dilutions used in experiments, and company and catalogue information.**

Antibody name	*Dilution*	*Company (#Catalogue)*
Donkey Anti-Rabbit IgG (H+L)	1:10000	Jackson ImmunoResearch Laboratories, West Grove, PA, USA (#711-001-003)

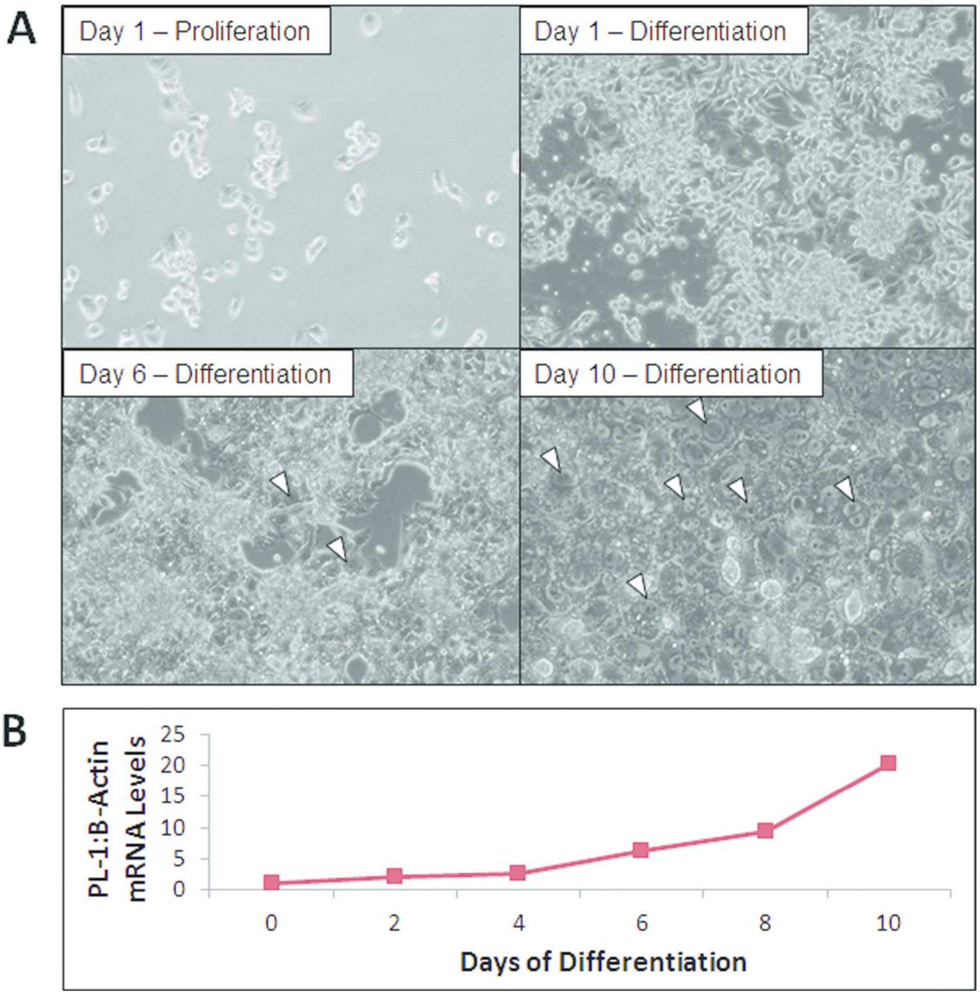

Figure 1
124x126mm (300 x 300 DPI)

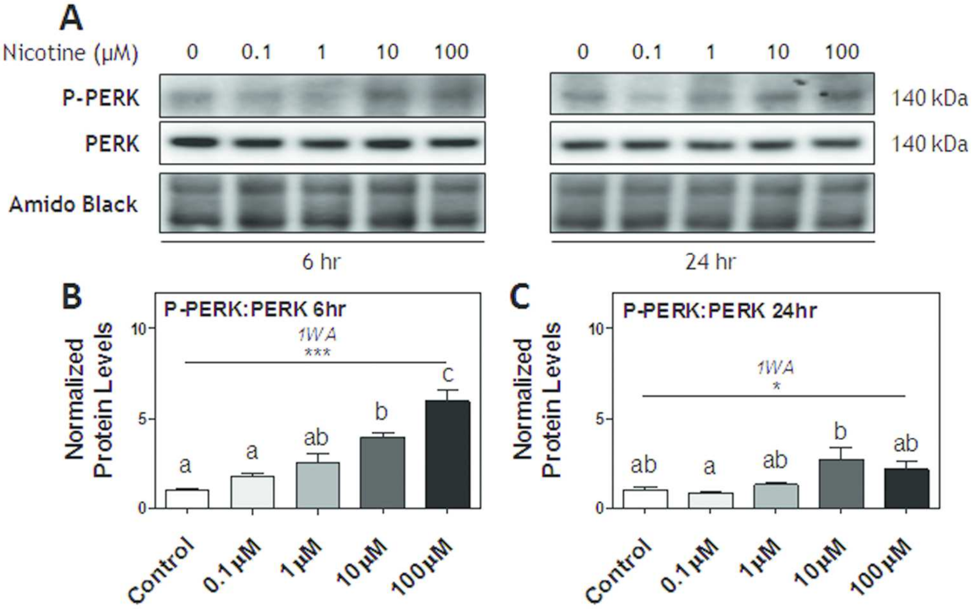

Figure 2
124x80mm (300 x 300 DPI)

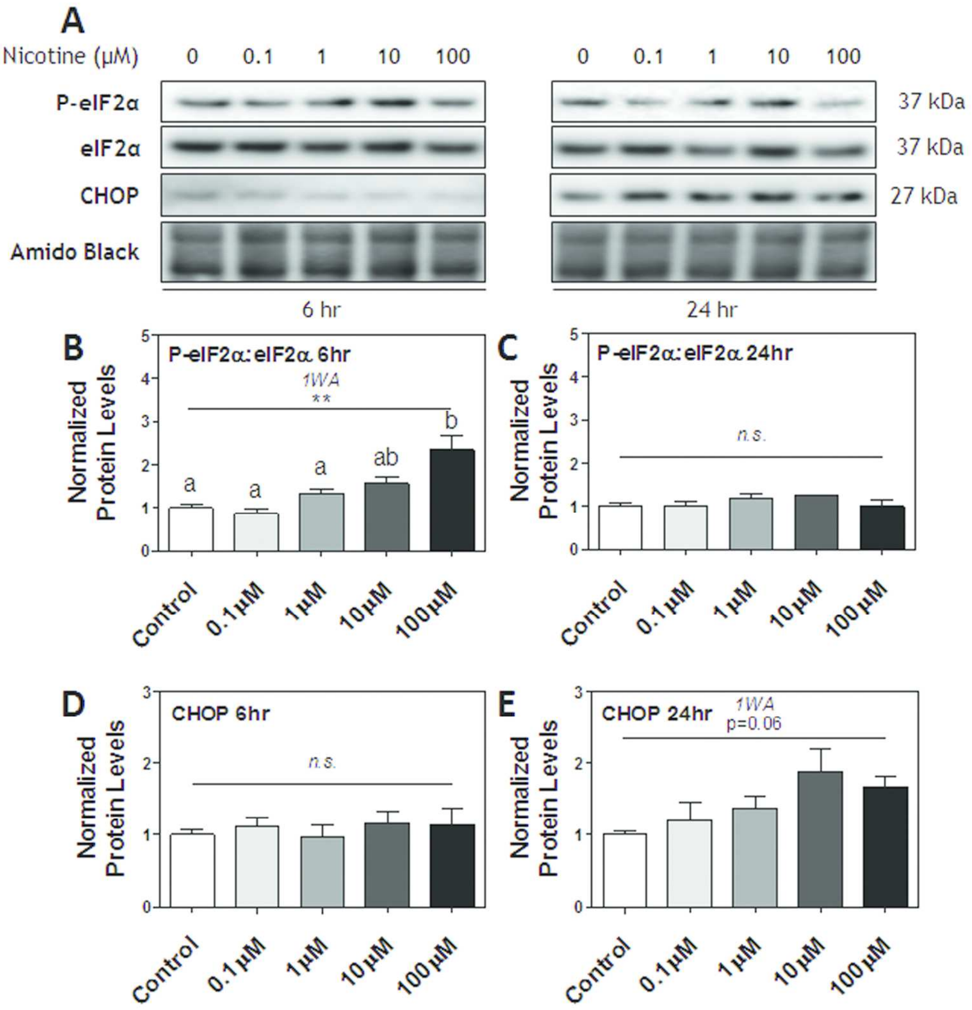

Figure 3
124x127mm (300 x 300 DPI)

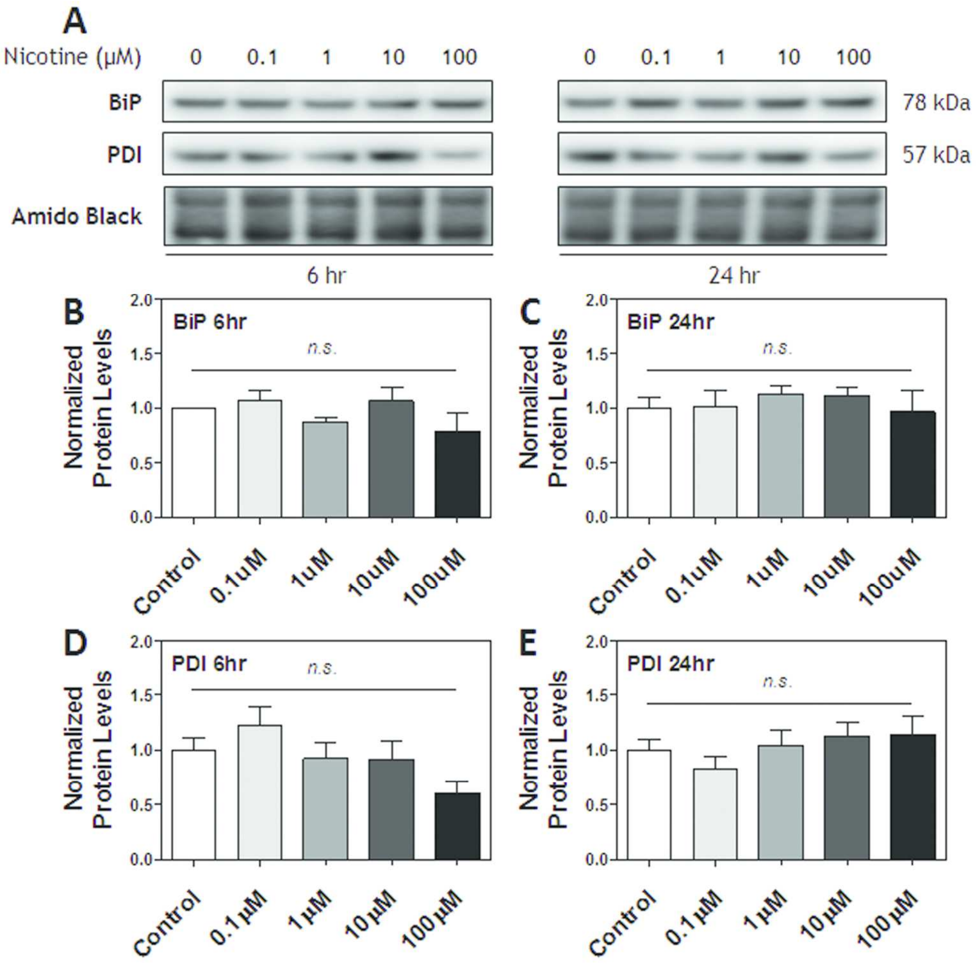

Figure 4
124x121mm (300 x 300 DPI)

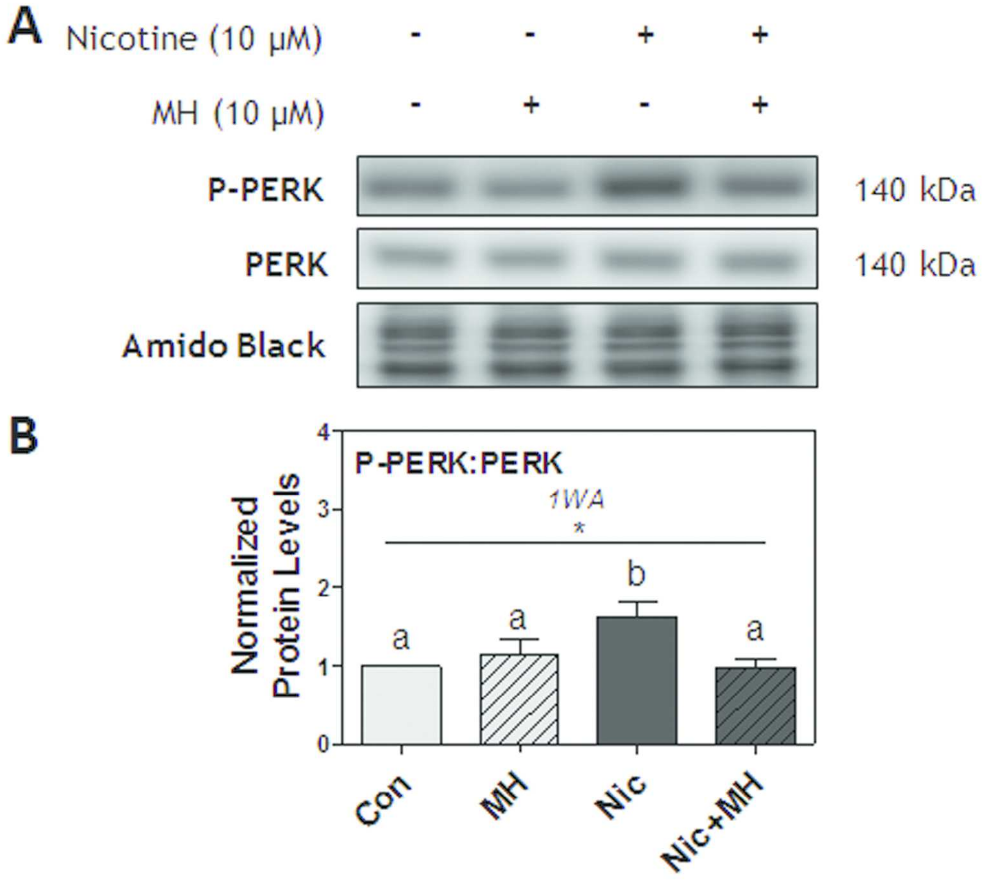

Figure 5
124x112mm (300 x 300 DPI)

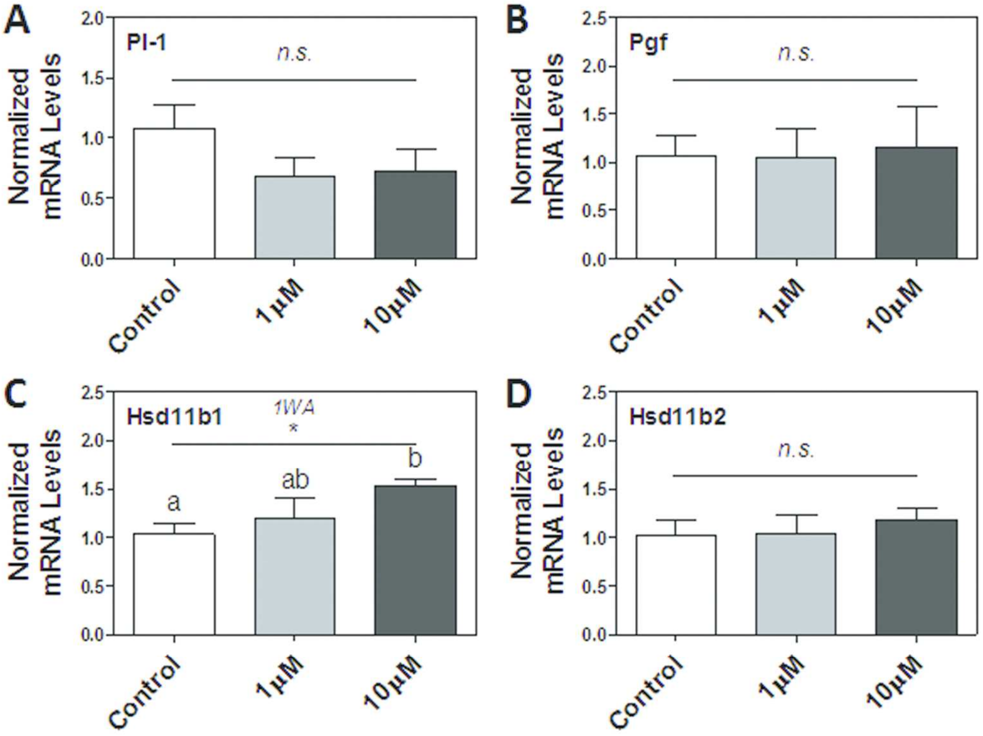

Figure 6
124x94mm (300 x 300 DPI)

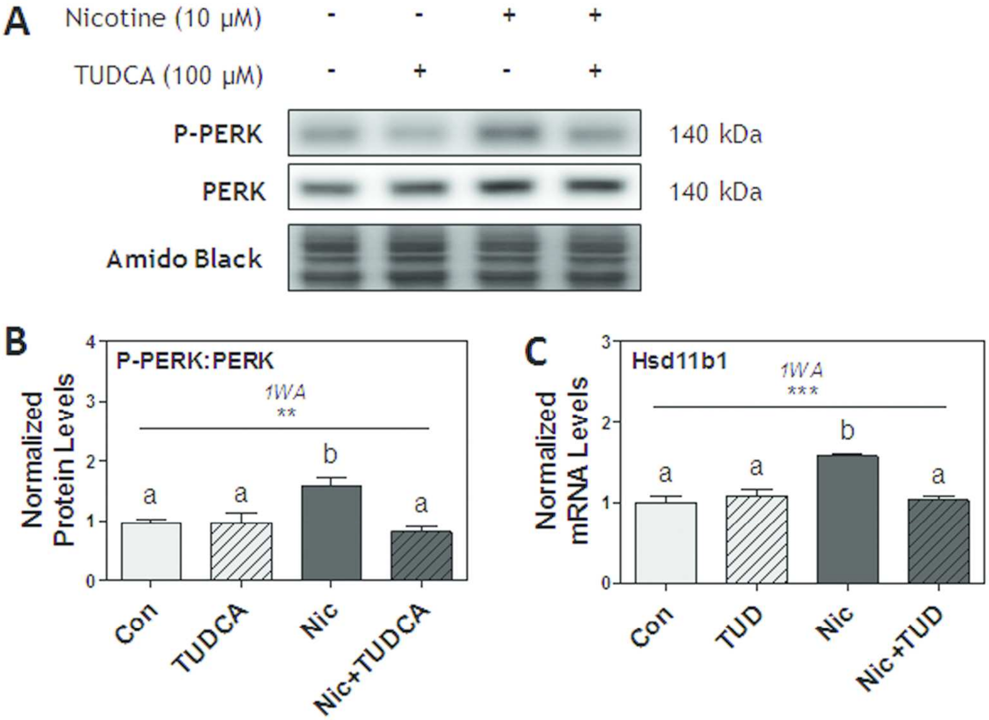

Figure 7
124x91mm (300 x 300 DPI)