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Research Article
Mechanisms Mediating the Lipogenic Effect of Nonylphenol in Human Preadipocytes

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Abstract

Background and Objective: Metabolic syndromes may be induced or exacerbated by environmental exposure to industrial pollutants that act as endocrine disruptors, such as the widely used chemical surfactant Nonylphenol (NP). We previously examined the effects of NP on fat metabolism in mice and found that developmental exposure led to increased weight gain and adipogenesis of adult males. We further observed that NP could induce adipogenesis for human preadipocyte cells. However, the effects of NP on adipogenesis for human preadipocyte cells are not clear enough. This study examined the mechanisms of NP on lipogenesis upon human preadipocytes. **Materials and Methods:** Human preadipocytes were incubated with NP plus the cAMP-dependent Protein Kinase A (PKA) inhibitor H89 or the Extracellular Signal-regulated Kinase (ERK) inhibitor U0126 under differentiation-inducing conditions. The cells after incubation were harvested for western blotting and real-time PCR measurements of lipogenic markers. **Results:** With human preadipocytes, NP stimulated lipogenesis by increasing c/EBPα mRNA expression and both proliferator-activated receptor-γ (PPARγ mRNA and protein expression and reducing PPARα protein expression. The cells incubation with U0126 for the first day inhibited NP-induced upregulation of c/EBPα mRNA, PPARγ mRNA and PPARγ protein expressions. H89 did not affect NP-stimulated expression of FASN and PPARγ mRNA but decreased NP-induced PPARα protein expression. **Conclusion:** This study demonstrated that NP-stimulated lipogenesis by increasing c/EBPα mRNA expression and both PPARγ mRNA and protein expression and reducing PPARα protein expression. The action mechanism of NP looks mediates through PKA and ERK cascades.

Key words: Nonylphenol, lipogenesis, human preadipocytes, triacylglycerol, phosphorylation, lipogenic markers, western blotting

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Many industrial and environmental pollutants such as nonylphenol (NP), Dichlorodiphenyltrichloroethane (DDT), Diethylstilbestrol (DES) and Bisphenol A (BPA) are Endocrine Disruptors (ENDRs). The rising incidences of metabolic syndromes such as obesity, diabetes and hypertension are pandemic to public health threat[1,2]. Accumulation evidence suggests that exposure to ENDRs may contribute to disease prevalence and (or) severity[3,4]. The major routes of human exposure to ENDRs are consumption through contaminated food and water as well as inhalation of airborne particles. Human exposure to ENDRs has been found to promote the onset of obesity[5-7] by inducing excessive adipogenesis and by disturbing energy balance and lipid metabolism[8]. Those ENDRs that accelerate Triacylglycerol (TAG) accumulation and proliferation of adipocytes (hyperplasia) are defined as "obesogens"[9,10]. Such effects may arise through enhanced differentiation of adipocyte precursors, accelerated lipid synthesis and (or) reduced lipid breakdown (lipolysis). Conversely, cellular and animal models of obesity have illustrated that normalization of glucose utilization and adipokine expression can restore appropriate lipid metabolism and reduce adipogenesis[11].

Many signal transduction pathways are involved in adipogenesis. The differentiation of preadipocytes requires activation of MAPK/ERK signalling pathways[12]. Bost *et al.*[13] reported that ERK1 knockout (KO) mice have fewer adipocytes and reduced adiposity compared to Wild-Type (WT) mice and that preadipocytes extracted from ERK1 KO exhibit diminished differentiation *in vitro* compared to WT preadipocytes under identical differentiation culture conditions. Activation of MEK/MAPKs in preadipocytes induces expression of PPARγ[14]. Conversely, acceleration of lipolysis requires enhanced expression/activity of lipase to hydrolyze triglycerides into glycerol and free fatty acids[15]. The activity of lipase is dependent on serine phosphorylation by cAMP-dependent Protein Kinase A (PKA)[16].

In this study, we elucidated the mechanisms underlying the adipogenicity effect of NP upon human preadipocytes.

MATERIALS AND METHODS

Study area: This research project was implemented at the Department of Chemical and Materials Engineering, Chinese Culture University, Taipei, Taiwan from January, 2019-December, 2020.

Materials: NP was purchased from Fluka (Buchs, Switzerland) and dissolved in methanol as a 0.5 M stock solution and all other reagents w ere purchased from Sigma Chemical Co. (St. Louis, MO, USA). Anti-fatty acid synthase (FASN), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and anti-Vinculin antibodies were purchased from GeneTex Inc. (San Antonio, TX, USA) and both anti-proliferator-activated receptor-a (PPAR-α) and anti-proliferator-activated receptor-γ (PPAR-γ) from Cayman Chemical (Ann Arbor, MI, USA). Peroxidase-conjugated anti-mouse and anti-rabbit IgG secondary antibodies were purchased from ICN Pharmaceuticals (Aurora, OH, USA). Cell Application (San Diego, CA, USA) provided human preadipocytes. Preadipocyte medium (PAM) and preadipocyte differentiation medium (PADM) were purchased from ScienCell™ Research Laboratories (Carlsbad, CA, USA). In our previous study[17], we confirmed that NP has no significant effects on human preadipocyte viability at concentrations $\leq$20 μM. Therefore, 20 μM NP was used in all experiments described here.

Cell culture and treatment

Cell culture: Human preadipocytes were maintained in PAM at 37°C under a 5% CO_2 atmosphere. For differentiation into adipocytes, human preadipocytes were seeded onto plates or dishes at 48000 cm^{2-1} in PAM medium for 2 days and then incubated in PADM for 6 days as indicated with medium exchange every two days. In experiments studying the effect of NP on lipogenesis and the associated signalling mechanisms, human preadipocytes were cultivated in 6-well plates and differentiated for 6 days in PADM plus vehicle (control) or 20 μM NP in the presence or absence of 10 μM H89 (PKA inhibitor) or 10 μM U0126 (ERK inhibitor). Cells were then harvested for western blotting and real-time PCR.

Western blot analysis: A total of 6-12% acrylamide sodium dodecyl sulfate-polyacrylamide gels[18] were used and transferred to Immobilon-P PVDF membranes. Membranes were blotted with the indicated antibodies and target protein bands visualized by enhanced chemiluminescence (ECL) detection reagents (Amersham Pharmacia Biotech. Buckinghamshire, UK), captured by a Luminescent Image Analyzer (Las-4000, Fuji-Film, Stamford, CT, USA) and analyzed using Multi Gauge software (Fuji Film). All target protein signals were normalized against Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) or Vinculin protein signal as the gel loading control.

Table 1: Real-time PCR primers

Genes	Sequence (5'-3' forward)	Sequence (5'-3' reverse)
c/EBPα	CGGTGGACAAGAACAGCAAC	CGGAATCTCCTAGTCCTGGC
FASN	CGCGTGGCCGGCTACTCCTAC	CGGCTGCCACACGCTCCTCT
PPARα	CAGAACAAGGAGGCGGAGGTC	TTCAGGTCCAAGTTTGCGAAGC
PPARγ	AGGCGAGGGCGATCTTGACAG	GATGCGGATGGCCACCTCTTT
18s rRNA	TGCCATGTCTAAGTACGCACG	TTGATAGGGCAGACGTTCGA

RNA extraction and quantitative real-time PCR: Human adipocyte RNA was extracted using TRIzol reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) following the manufacturer's protocol. Concentration and purity were measured by the absorbance at 260 nm (A260) and by the A260/A280 ratio, respectively, using a Nanodrop spectrometer (Thermo Scientific, Waltham, MA, USA). Only samples with A260/A280 $\geq$1.9 were used for subsequent gene expression analyses. Sample RNA was reverse transcribed into cDNA using a 2XRT-Master mix cDNA synthesis kit (Bioman Scientific Co., Ltd, Taipei, Taiwan) and gene expression estimated by real-time PCR reactions using an SYBR Green PCR Master Mix kit (Applied Biosystems, Inc., Foster City, CA, USA) and the appropriate primer pairs (synthesized by Tri-I Biotech Inc., Taipei, Taiwan, sequences shown in Table 1). Briefly, 20 μL reaction mixtures including 250 nM of each primer, 50 ng cDNA and 10 μL enzyme solution containing the DNA double strand-specific SYBR Green I dye were prepared in 96-well plates. The reaction conditions included a 10-min activation step at 95°C and 40 cycles of 95°C for 15 sec and 60°C for 1 min were controlled by an ABI real-time PCR detection system (Applied Biosystems). Relative gene expression levels were estimated using the $2^{-\Delta\Delta}$Ct method with normalization to 18s rRNA expression as the internal control. Reactions including vehicle rather than cDNA were run in parallel and expression set at 1.0 to calculate relative gene expression changes. Melting curve analysis was used to verify the purity of PCR products.

Statistical analysis: All results are expressed as the Mean±standard error of the mean (SEM) Treatment group means were compared by one-way ANOVA or unpaired Student's t-test as indicated. For all tests, a $p<0.05$ (two-tailed) was significant in statistics.

RESULTS

Nonylphenol promotes lipogenesis through ERK-dependent mechanism: There are two sets of studies to incubate NP human preadipocyte cells with/without U0126. First set of studies, the cells were incubated with NP (0 or 20 μM) combined with U0126 (0 or 10 μM) in PADM medium for 6 days. For the second set of studies, the cells were incubated with NP (0 or 20 μM) combined with U0126 (10μM) in PADM medium for 0-1 day, the following days (2-6 days) the cells were incubated with NP (0 or 20 μM) in PADM medium only. The human preadipocytes incubated with U0126 for the entire 6 days exhibited unchanged c/EBPα mRNA expression (Fig. 1a) but enhanced FASN mRNA expression ($p<0.05$, Fig. 1b), decreased PPARα mRNA expression ($p<0.01$, Fig. 1c). Alternatively, preadipocyte cells incubated with U0126 for 0-1 day exhibited reduced PPARγ mRNA expression ($p<0.05$, Fig. 1d). Furthermore, the human preadipocyte cells incubated with U0126 for 0-1 day exhibited increased PPARα protein expression ($p<0.05$, Fig. 2a) and preadipocytes incubated with U0126 for the entire 6 days exhibited enhanced PPARγ protein expression(include 53, 57 kDa) ($p<0.01$, Fig. 2b). Treatment with NP alone for 6 days increased the expression levels of c/EBPα mRNA ($p<0.05$, Fig. 1a), FASN mRNA ($p<0.05$, Fig. 1b), PPARγ mRNA ($p<0.01$, Fig.1d), decreased PPARα protein expression ($p<0.05$, Fig. 2a) and increased PPARγ protein (include 53, 57 kDa) ($p<0.05$, Fig. 2b). Further, incubation with U0126 for the first day inhibited NP-induced upregulation of CCAAT Enhancer Binding Protein a (c/EBPα) mRNA ($p<0.05$, Fig. 1a), PPARγ mRNA ($p<0.01$, Fig. 1d) and PPARγ protein (include 53, 57 kDa) ($p<0.05$, Fig. 2b) expressions. If the human preadipocyte cells were incubated U0126 for all the different times, U0126 could stimulate cells lipogenesis but it could not affect NP-stimulated lipogenesis. Thus, ERK inhibition during the initial phase of NP exposure blocked the stimulation of adipogenesis. Conversely, prolonged ERK inhibition enhanced adipogenesis and did not alter NP-induced lipogenesis.

Nonylphenol promotes lipogenesis through PKA-dependent cascade: To examine the contributions of PKA, human preadipocyte cultures were incubated in PADM containing NP (20 μM) with or without the PKA blocker H89 (10 μM) for 6 days. Incubation in NP alone again increased FASN mRNA expression ($p<0.05$, Fig. 3a), reduced PPARα mRNA expression ($p<0.05$, Fig. 3b) and increased PPARγ mRNA expression ($p<0.05$, Fig. 3c). Alternatively, the addition of H89 increased the mRNA expression levels of FASN ($p<0.01$, Fig. 3a) and PPARγ ($p<0.05$, Fig. 3c), suggesting that lipogenesis can be stimulated by blocking PKA activity. However, H89 did not

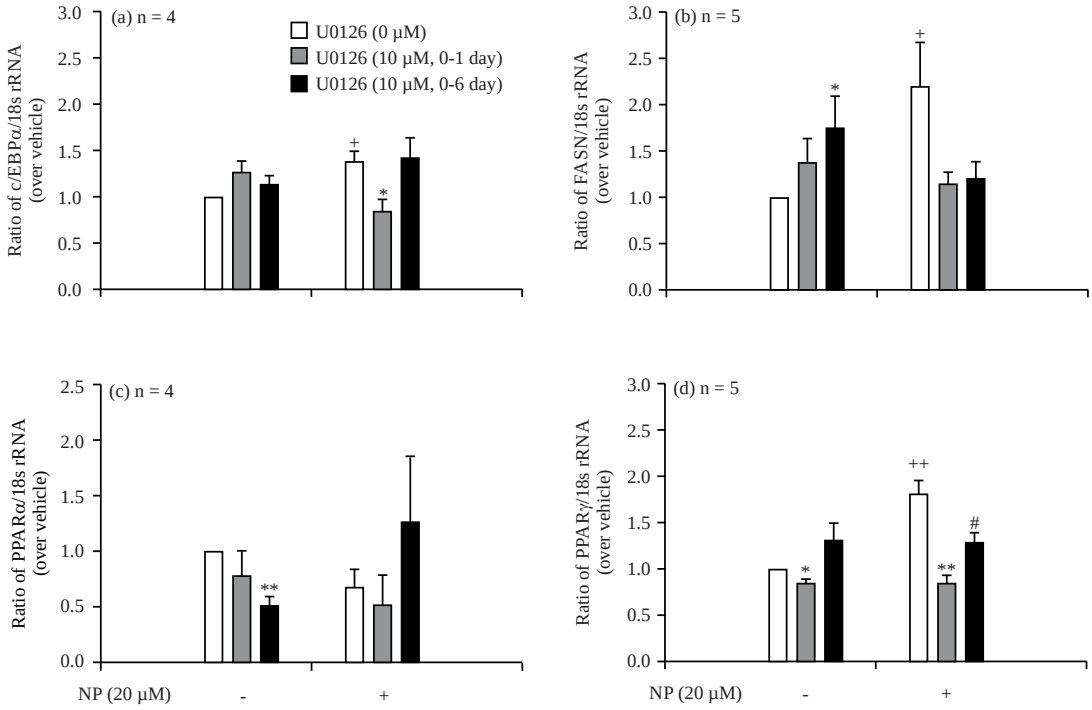

Fig. 1(a-d): Effects of ERK blockade on nonylphenol (NP)-induced expression of lipogenic mRNA marker. Human preadipocytes were incubated with the ERK blocker U0126 under two conditions. First, the cells were incubated with NP (0 or 20 μM) combined with U0126 (0 or10 μM) in a PADM medium for 6 days. Second, the cells were incubated with NP (0 or 20 μM) combined with U0126 (10 μM) in PADM medium during 0-1 day, the following days (2-6 days) the cells were incubated with NP (0 or 20 μM) in PADM medium only. After 6 days of incubation, real-time PCR analysis was used to determine (a) c/EBPα, (b) FASN, (c) PPARα and (d) PPARγ mRNA levels in the cells and the values were normalized against 18srRNA.

Means of independent cultures are shown as the Means±SEM and represent as the fold-change against control cells (cells differentiated with PADM only). $^{+, ++}$p<0.05, 0.01 for NP effect conditioned on the same concentration of U0126. *,**p<0.05, 0.01 for U0126 effect conditioned on the same concentration NP. $^{\#}$p<0.05 U0126 effect 0-6 days compare with U0126 effect 0-1 day

increase NP-stimulated expression of FASN and PPARγ mRNA, suggesting that NP may inhibit PKA activity at baseline, mitigating any effects of H89 on FASN or PPARγ gene expression. Incubation with either H89 or NP increased FASN protein expression (p<0.05, Fig. 4a), while H89 decreased NP-induced PPARα protein expression (p<0.01, Fig. 4b). Furthermore, incubation with NP increased PPARγ (include 53, 57 kDa) (p<0.01, Fig. 4c).

DISCUSSION

In this study, we explore the effects of NP on lipogenesis for human preadipocytes and quest the mechanisms of NP affect lipogenesis.

We have observed developmental exposure to environmental ENDR, i.e., NP results with overweight/obesity during an adult period by rat model[19,20]. One of the pathogenic mechanisms is the increased adipogenesis. Our studies have shown that NP induced hypoadrenalism not only on the production of adrenal corticosterone and aldosterone but also increase the cellular 11beta hydroxysteroid dehydrogenase type 1 (11b-HSD1). The cellular 11b-HSD1activity converts inactive corticosteroid to active one which created insulin resistance. The NP also activates adipogenesis and lipogenesis mechanisms, i.e., PPARγ, FASN[17]. This study is to explore whether observations in the rodent model also reflect in the human model. For human study, we use preadipocyte as a study model.

Nonylphenol acts as an environmental pollutant at least in part by interfering with endocrine signalling, thereby disrupting homeostatic control of cellular functions including lipid metabolism[21]. We previously demonstrated that NP exposure during the developmental period markedly enhanced both 11b-HSD1 activity in the liver and adipose tissues and elevated the plasma concentrations of corticosterone and aldosterone in adult rats[19,20]. The

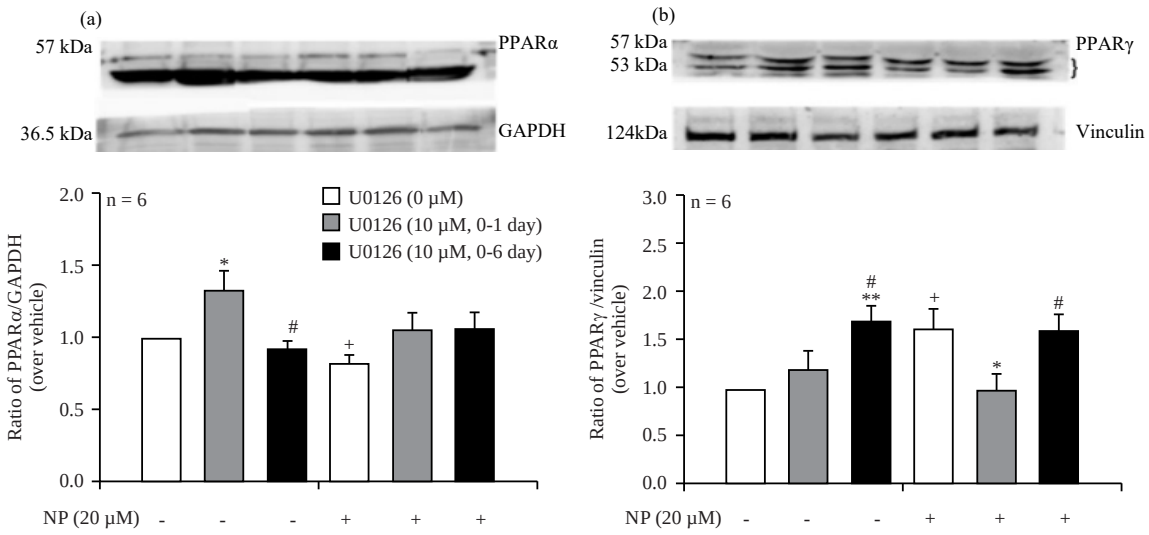

Fig. 2(a-b): Effects of ERK blockade on NP-induced expression of lipogenic protein maker. Human preadipocytes were incubated with the ERK blocker U0126 under two conditions. First, the cells were incubated with NP (0 or 20 μM) combined with U0126 (0 or10 μM) in a PADM medium for 6 days. Second, the cells were incubated with NP (0 or 20 μM) combined with U0126 (10 μM) in PADM medium during 0-1 day, the following days (2-6 days) the cells were incubated with NP (0 or 20 μM) in PADM medium only. After 6 days of incubation, western blot analysis was used to determine PPARα (57 kDa) and PPARγ (53 and 57 kDa) protein levels in the cells and the values were normalized against (a) GAPDH and (b) Vinculin

Mean of independent cultures are shown as the Mean±SEM and represent as the fold-change against control cells (cells differentiated with PADM only). $^{+}p<0.05$ for NP effect conditioned on the same concentration of U0126. *,**$p<0.05$, 0.01 for U0126 effect conditioned on the same concentration NP. $^{\#}p<0.05$ U0126 effect 0-6 days compare with U0126 effect 0-1 day

developed symptom was partially mimicking Cushing's syndrome/hyperadrenalism. These observations support Barker's hypothesis that "Fetal origins adult disease". The effect seemed mediation through epigenetic modification during in utero and the neonatal period. The epigenetic modification on the F1 generation seems to last for life long and only can be reset at F2 generation[20]. How to treat the affected F1 generation is needed. In the rodent model, we realize selective11b-HSD1 inhibitor can alleviate NP induced symptoms[17]. Does it work for humans is waiting for elucidation? This study is the first step to verify any similarity of adipogenesis mechanism in response to NP between rodent and human models.

There are regulatory systems for lipid metabolism. Adipogenesis is the process of forming new adipose cells and accumulation of lipid in those cells. Preadipose cell grows into the adipose cell through differentiation and increased lipid deposition in the cell. Cellular differentiation is a change of specific gene expressions (transcription factors). Peroxisome Proliferator-Activated Receptor γ (PPARγ) and CCAAT Enhancer-Binding Proteins (c/EBPs) are the main regulatory factors of adipogenesis. The lipid metabolism controls the balance of lipogenesis and lipolysis. Physiologically insulin is the key factor to enhance lipogenesis while counteracting glucagon, epinephrine, PPARα etc, enhance lipolysis.

Fatty Acid Synthase (FASN) plays an important enzyme in lipogenesis. Lomba *et al.*[22] demonstrated that rats fed with a high-fat diet could induce weight gain, obesity and overexpression of FASN. The studies of Bost *et al.*[12,13,23] and Farmer[14] showed that activation of ERK pathway in mouse preadipocytes initiates c/EBPα, c/EBPβ, c/EBPδ and PPARγ activities to differentiate into adipocytes. In this study, we show that NP could increase c/EBPα, FASN and PPARγ mRNA expression levels (Fig. 1 and 3) and PPARγ protein expression levels (Fig. 2 and 4) for human preadipocytes. Taken together, NP could stimulate lipogenesis for human preadipocytes.

The MAPK is form ERK regulates adipogenesis *in vivo* and *in vitro*. In response to adipogenic stimuli, ERK1 is rapidly activated by MEK-catalyzed phosphorylation of specific threonine and tyrosine residues[13,24]. However, adipogenesis is subsequently downregulated by PPARγ phosphorylation[13,23]. Bost *et al.*[13] reported that ERK1 KO mice were less susceptible to insulin resistance and obesity induced by a high-fat diet and that this was associated with reduced preadipocyte

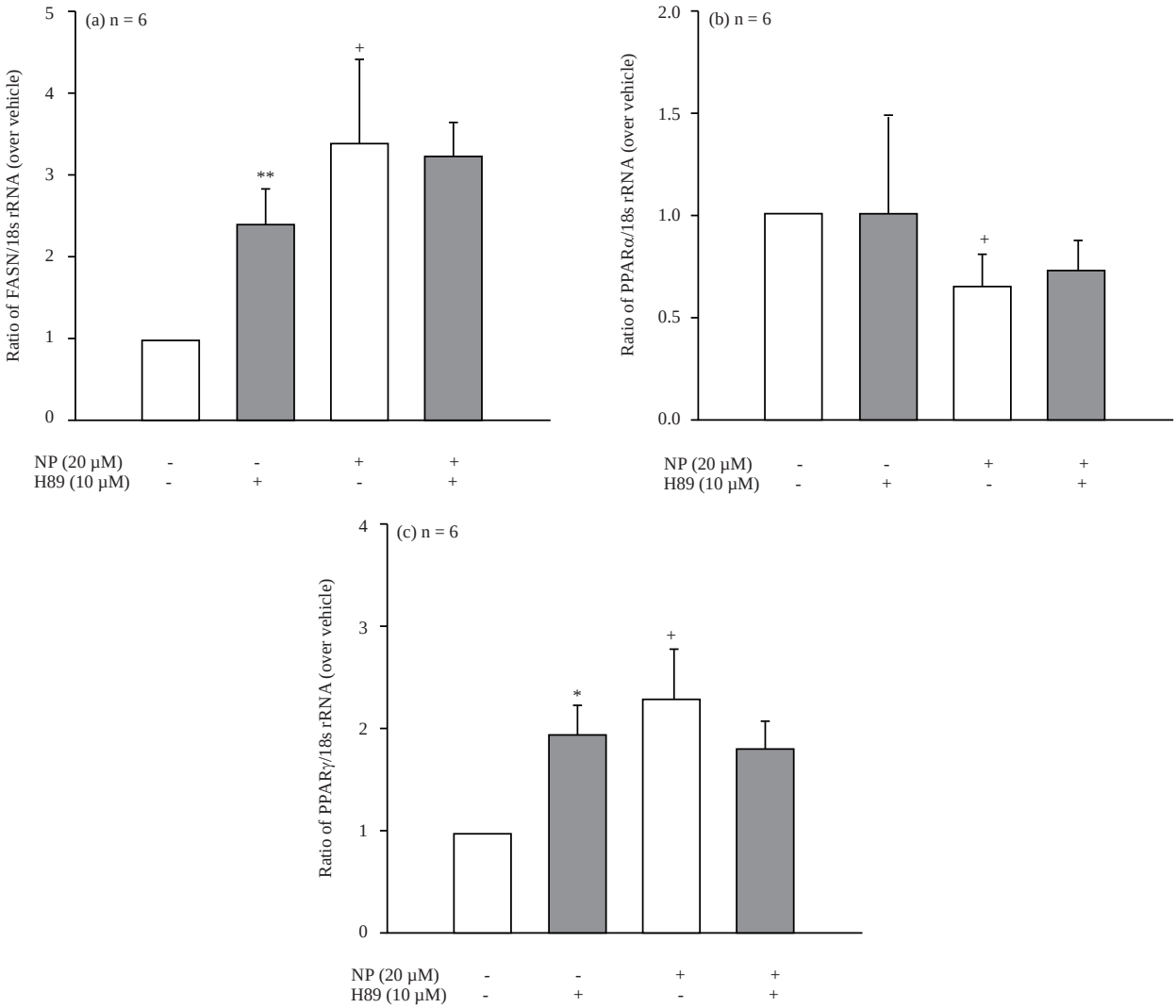

Fig. 3(a-c): Effects of PKA blockade on NP-induced lipogenic mRNA marker expression. Human preadipocytes were incubated in PADM in the presence of NP (0 or 20 μM) combined with H89 (0 or 10 μM). After 6 days of incubation, real-time PCR analysis was used to determine (a) FASN, (b) PPARα and (c) PPARγ mRNA levels in the cells and the values were normalized against 18s rRNA

Mean of independent cultures are shown as the Mean±SEM and represent as the fold-change against control cells (cells differentiated with PADM only). $^{+}p<0.05$ for NP effect conditioned on the same concentration of H89. *,**$p<0.05$, 0.01 for H89 effect conditioned on the same concentration NP

differentiation. However, other studies[25,26] have found that MEK/ERK activation inhibits adipogenesis. This discrepancy may be explained by differences in measurement time following stimulation as the MEK/ERK pathway at the initial stage only induces adipocyte differentiation transiently before further differentiation is suppressed by PPARγ phosphorylation[27,28] but at late differentiation stage would inhibit adipogenesis[29,30]. In this study, human preadipocytes incubation with U0126 during early differentiation exhibited reduced PPARγ mRNA expression but increased PPARα protein expression (Fig. 1 and 2). These results indicated human preadipocytes would stimulate lipogenesis through the ERK pathway at the initial differentiation stage. Further, incubation with U0126 during early NP exposure inhibited NP-stimulated c/EBPα mRNA expression, PPARγ mRNA and PPARγ protein expression (Fig. 1 and 2). Taken together, these findings suggest that NP stimulates lipogenesis transiently and that early U0126 treatment inhibits this initial NP-stimulated lipogenic response. Alternatively, prolonged U0126 application can also upregulate lipogenesis but cannot alter this early NP-stimulated lipogenic phase.

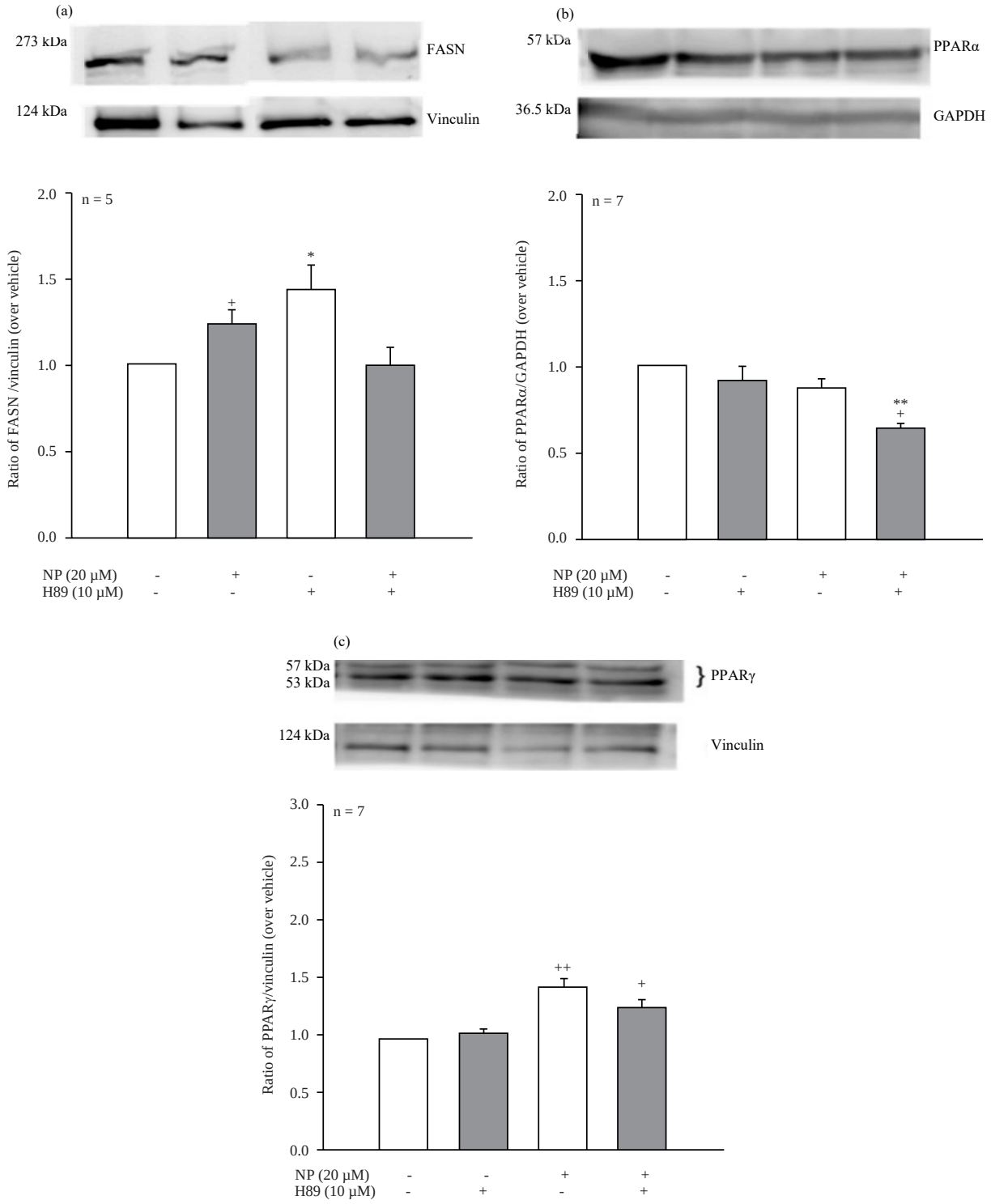

Fig. 4(a-c): Effects of PKA blockade on NP -induced expression of lipogenic protein markers. Human preadipocytes were incubated in PADM in the presence of NP (0 or 20 µM) combined with H89 (0 or 10 µM). After 6 days of incubation, western blot analysis was used to determine (a) FASN (273 kDa), (b) PPARα (57 kDa) and (c) PPARγ (53 and 57 kDa) protein levels in the cells and the values were normalized against GAPDH or Vinculin

Mean of independent cultures are shown as the Mean±SEM and represent as the fold-change against control cells (cells differentiated with PADM only). $^{+,++}$p<0.05, 0.01 for NP effect conditioned on the same concentration of H89. *,**p<0.05, 0.01 for H89 effect conditioned on the same concentration NP

Lipolysis is an important homeostatic mechanism for reducing body fat. To reduce and mobilize storage fat, triglycerides are hydrolyzed to glycerol and free fatty acids by lipase. Stimulation of b-adrenergic receptors induces lipolysis by stimulating cAMP production by adenylate cyclase, which subsequently activates PKA. PARPα, a ligand-activated nuclear transcription factor, enhances fatty acid through beta-oxidation, thereby reducing circulating triglyceride levels, liver and muscle steatosis and adiposity, which in turn raises insulin sensitivity[31-33]. In this study, NP could decrease PPARα protein (Fig. 2) and mRNA expressions (Fig. 3) from human preadipocytes. It indicated that NP might inhibit lipolysis for human preadipocytes. Human preadipocytes incubation with H89 increased the mRNA expression levels of FASN and PPARγ (Fig. 3), suggesting that blocking of PKA lipolytic activity can lead to lipogenesis in humans preadipocytes. Further, H89 inhibited NP-induced PPARα protein expression (Fig. 4b), therefore NP induces lipogenesis likely through inhibition of PKA-dependent lipolysis.

Taken together, NP transiently stimulates lipogenesis via activation of ERK during the early phase of treatment and NP induces lipogenesis likely through inhibition of PKA-dependent lipolysis.

CONCLUSION

In summary, the results show that NP transiently stimulates lipogenesis via activation of ERK as evidenced by U0126-mediated blockade during the early phase of treatment. Surprisingly, prolonged ERK blockade alone stimulates lipogenesis but cannot reduce NP-stimulated lipogenesis, consistent with the transient nature of the NP-induced response. Alternatively, blockade of PKA augments NP-induced lipogenesis by decreasing PPARα protein expression. All evidence indicates NP can enhance adipogenesis. These phenomena are similar between rat and human models.

SIGNIFICANCE STATEMENT

Many industrial or environmental chemicals are endocrine disruptors such as NP. This study discovers the NP can stimulate lipogenesis for human preadipocytes that can be used as a useful message to industries to remind paying attention to the potential negative side effects of these chemicals against human health.

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