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Original Article

Palmitic acid induces inflammatory cytokines and regulates tRNA-derived stress-induced RNAs in human trophoblasts

Changwon Yang[1,#], Garam An[1,#], Jisoo Song[2], Gwonhwa Song[1,*] and Whasun Lim[2,*]

[1]Institute of Animal Molecular Biotechnology and Department of Biotechnology, College of Life Sciences and Biotechnology, Korea University, Seoul 02841, Korea
[2]Department of Biological Sciences, Sungkyunkwan University, Suwon 16419, Korea

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***Correspondence**
Gwonhwa Song
E-mail: ghsong@korea.ac.kr
***Correspondence**
Whasun Lim
E-mail: wlim@skku.edu

[#]These authors contributed equally to this work.

Author's Position and Orcid no.
Yang C, Research professor,
https://orcid.org/0000-0001-5326-9124
An G, Graduate student,
https://orcid.org/0000-0002-0065-793X
Song J, Graduate student,
https://orcid.org/0000-0002-6522-1803
Song G, Professor,
https://orcid.org/0000-0003-2817-5323
Lim W, Associate professor,
https://orcid.org/0000-0002-1328-0465

ABSTRACT High levels of proinflammatory cytokines have been observed in obese pregnancies. Obesity during pregnancy may increase the risk of various pregnancy-related complications, with pathogenesis resulting from excessive inflammation. Palmitic acid (PA) is a saturated fatty acid that circulates in high levels in obese women. In our previous study, we found that PA inhibited the proliferation of trophoblasts developing into the placenta, induced apoptosis, and regulated the number of cleaved halves derived from transfer RNAs (tRNAs). However, it is not known how the expression of tRNA-derived stress-induced RNAs (tiRNAs) changes in response to PA treatment at concentrations that induce inflammation in human trophoblasts. We selected concentrations that did not affect cell viability after dose-dependent treatment of HTR8/SVneo cells, a human trophoblast cell line. PA (200 μM) did not affect the expression of apoptotic proteins in HTR8/SVneo cells. PA significantly increased the expression of inflammatory cytokines including *interleukin (IL)-1β, IL-6, IL-8*, and *tumor necrosis factor (TNF)-α*. In addition, 200 μM PA significantly increased the expression of tiRNAs compared to 800 μM PA treatment. These results suggest that PA impairs placental development during early pregnancy by inducing an inflammatory response in human trophoblasts. In addition, this study provides a basis for further research on the association between PA-induced inflammation and tiRNA generation.

Keywords: cytokine, inflammation, palmitic acid, tRNA-derived species, trophoblast

INTRODUCTION

Inflammation is a hallmark of metabolic diseases such as obesity and diabetes, and is characterized by high levels of proinflammatory cytokines (Schenk et al., 2008). Obesity during pregnancy increases the risk of complications such as preeclampsia, gestational diabetes, and intrauterine growth retardation (Simko et al., 2019). Obesity is a chronic, low-grade inflammatory condition. Inflammatory cytokines, including interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, are positively correlated with obese pregnancy (Madan et al., 2009). Obesity is associated with an increase in fatty acids circulating in the bloodstream, and inflammation may be enhanced or alleviated depending on the type and levels of fatty acids in the placental environment. Increased saturated fatty acids during pregnancy induce lipotoxicity in the placenta, which is alleviated by monounsaturated fatty acids

(Natarajan et al., 2021). Palmitic acid (PA) is the dominant saturated fatty acid in the diet and circulates in high amounts in obese women (Chen et al., 2010). Our previous studies and additional evidence have revealed that PA induces mitochondrial dysfunction and endoplasmic reticulum (ER) stress-mediated apoptosis in human trophoblasts (Colvin et al., 2017; Yang et al., 2018). Although PA has been reported to induce inflammation in a variety of cells, including macrophages and hepatocytes, its effect on human trophoblasts is unclear.

Noncoding RNAs are known to have diverse physiological and pathological functions, and play important roles in inflammatory regulation (Marques-Rocha et al., 2015). We previously determined that PA regulates the expression of microRNAs (miRNAs) that regulate stearoyl-CoA desaturase (*SCD1*) and diacylglycerol acyltransferase (*DGAT1*) genes associated with fatty acid metabolism, suggesting a role for PA in regulating the generation of noncoding RNAs (Yang et al., 2018). Recent studies on noncoding RNAs have focused on the functionality of transfer RNA (tRNA)-derived species in cells. Multiple tRNA-derived species are distinguished by different cleavage sites in mature tRNAs and enzymes such as Dicer, angiogenin, and RNase Z (Lyons et al., 2018). Among several tRNA-derived species, tRNA-derived stress-induced RNAs (tiRNAs) result from angiogenin-mediated tRNA cleavage and are generally considered to be the 5'- and 3'- halves of tRNAs (Yamasaki et al., 2009). The levels of tiRNAs produced by the action of angiogenin are increased by external stresses, including oxidative stress (Saikia et al., 2012). A recent study reported that the generation of tRNA-derived species is regulated by inflammatory cytokines, including IL-6, and implicated in the carcinogenic mechanism of pancreatic ductal adenocarcinoma resulting from chronic inflammation (Pan et al., 2021). In a previous study, we showed for the first time that PA can regulate the production of tiRNAs involved in trophoblast death (Yang et al., 2022). Knockdown of angiogenin or inhibition of tiRNA$^{GlyCCC/GCC}$ in human trophoblasts promotes PA-induced apoptosis and mitochondrial dysfunction. However, it is unknown whether PA can induce inflammation and regulate the expression of tiRNAs at low concentrations that do not induce apoptosis in human trophoblasts. Therefore, in the current study, PA was administered in a dose-dependent manner to HTR8/SVneo cells, a human trophoblast cell line. We then analyzed whether the inflammatory response was induced at a concentration that did not affect cell viability or the expression pattern of tiRNAs in HTR8/SVneo cells.

MATERIALS AND METHODS

Chemicals

PA was purchased from Sigma-Aldrich (St. Louis, MO, USA) and the working solution was prepared as previously described (Yang et al., 2018). Antibodies against P53, poly (ADP-ribose) polymerase (PARP), Bcl-2, and LC3B were purchased from Cell Signaling Technology (Danvers, MA, USA).

Cell culture

HTR8/SVneo cells were purchased from the American Type Culture Collection and cultured as previously described (Yang et al., 2018). The cells were maintained in RPMI-1640 medium with 5% fetal bovine serum (FBS) at 37℃ in a CO_2 incubator.

MTT assay

Cells treated with PA for 48 h were incubated with the MTT labeling reagent for 4 h and then incubated overnight with solubilization solution. The absorbance at 560 and 650 nm was measured using a microplate spectrophotometer.

Western blot analysis

Changes in protein expression in HTR8/SVneo cells in response to PA treatment for 24 h were measured by western blotting, as previously described (Yang et al., 2018). Bradford protein assay was performed to determine the total protein concentration in the cells.

Annexin V and propidium iodide staining

Apoptosis was analyzed using the Annexin V Apoptosis Detection Kit I (BD Biosciences, San Jose, CA, USA) as previously described (Bae et al., 2020). Briefly, HTR8/SVneo cells were treated with PA for 48 h, harvested, and stained with equal amounts of annexin V and propidium iodide (PI). The fluorescence intensity was measured using a flow cytometer.

Quantitative PCR

For the quantitative analysis of genes, quantitative PCR

(qPCR) using SYBR Green dye was performed as previously described (Yang et al., 2020; Choi et al., 2022). For quantification of tiRNA expression, qPCR Master Mix (Agilent Technologies, Santa Clara, CA, USA) was used after cDNA synthesis based on polyadenylation, as previously described (Yang et al., 2022).

Statistics

The SAS program (SAS Institute, Cary, NC, USA) was used for statistical analysis, as previously described (Yang et al., 2018). Differences with a probability value of $p < 0.05$ were considered statistically significant. Data are presented as mean ± standard error of the mean (SEM) unless otherwise stated.

RESULTS

Selection of a concentration of PA that does not affect the viability of trophoblasts

We first investigated changes in cell viability after dose-dependent treatment with PA in HTR8/SVneo cells. PA at < 200 μM did not significantly affect the viability of HTR8/SVneo cells, whereas 400 μM (23.5%, $p < 0.01$) and 800 μM (32.7%, $p < 0.001$) of PA decreased viability (Fig. 1A). These results are similar to those of our previous study on the effect of PA on trophoblasts (Yang et al., 2018). PA (200 μM) also did not affect the expression of p53, a master regulator of apoptosis, or cleaved PARP, which indicates the progression of apoptosis (Fig. 1B). Furthermore, 200 μM PA did not affect the expression of the anti-apoptotic protein Bcl-2 or the autophagy marker LC3B. These results suggested that 200 μM PA had little effect on the apoptosis-related pathways in HTR8/SVneo cells. This conjecture was clarified by performing staining of annexin V and PI on HTR8/SVneo cells. In our previous study, 800 μM PA induced significant apoptosis in HTR8/SVneo cells (Yang et al., 2022). However, 200 μM PA did not increase the proportion of cells undergoing apoptosis (Fig. 1C).

PA increases the expression of inflammatory cytokines in trophoblasts

We previously suggested that nuclear factor (NF)-κB activation by external factors and induction of inflammation, represented by increased inflammatory cytokines, can lead to gestational diseases, such as premature birth (Yang et al., 2019). Therefore, we investigated whether PA at concentrations that did not affect cell viability increased the mRNA levels of inflammatory cytokines in trophoblasts. PA (200 μM) significantly increased the mRNA expression levels of inflammatory pathway factors such as *NF-κB* and *IL-1 receptor-associated kinase 1 (IRAK1)*

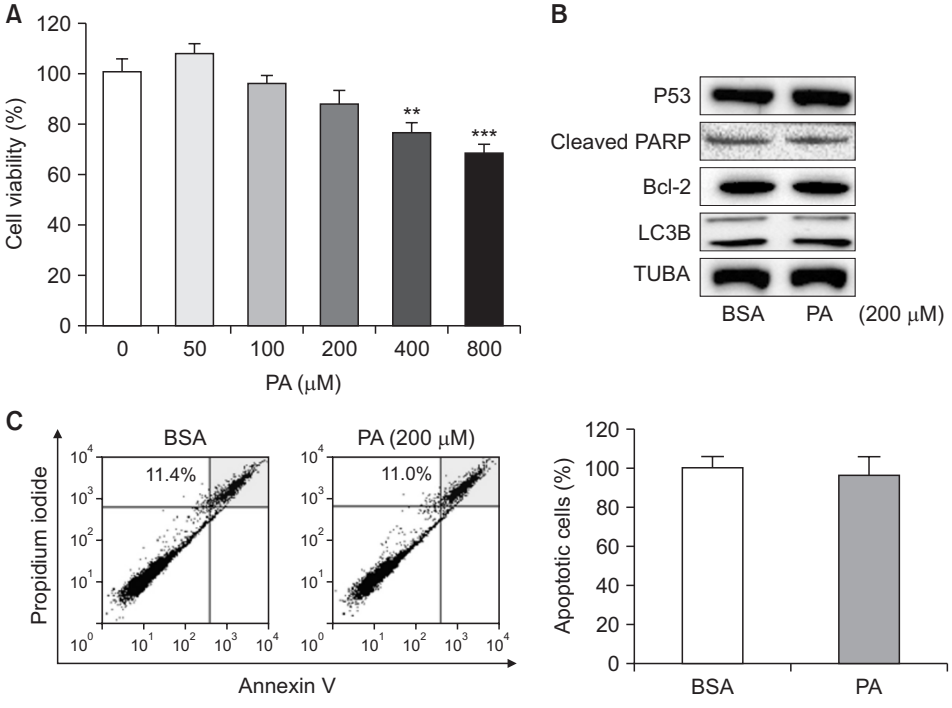

Fig. 1. Effect of palmitic acid (PA) on cell viability and apoptosis of human trophoblasts. (A) Changes in viability of HTR8/SVneo cells following dose-dependent treatment (0, 50, 100, 200, 400, and 800 μM) with PA for 48 h. (B) Expression analysis of p53, cleaved PARP, Bcl-2, and LC3B following 200 μM PA treatment for 24 h in HTR8/SVneo cells. (C) Changes in apoptosis following 200 μM PA treatment for 48 h in HTR8/SVneo cells. The proportion of cells corresponding to the upper right quadrant was quantified. Data are presented as representative results from three independent experiments. Asterisks indicate a statistically significant difference compared to controls (***$p < 0.001$; **$p < 0.01$).

(Fig. 2). In addition, mRNA expression levels of inflammatory cytokines, including *IL-1β, IL-6, IL-8*, and *TNF-α*, in trophoblasts was significantly increased in response to PA. These results suggest that even PA concentrations that do not affect cell viability can induce inflammatory responses in trophoblasts.

PA regulates the expression of tiRNAs in trophoblasts

We previously showed that 800 μM PA inhibited the expression of several types of tiRNAs, some of which are also associated with PA-induced trophoblast apoptosis regulation (Yang et al., 2022). However, we further analyzed the expression of tiRNAs in trophoblasts following dose-dependent treatment with PA and found that 200

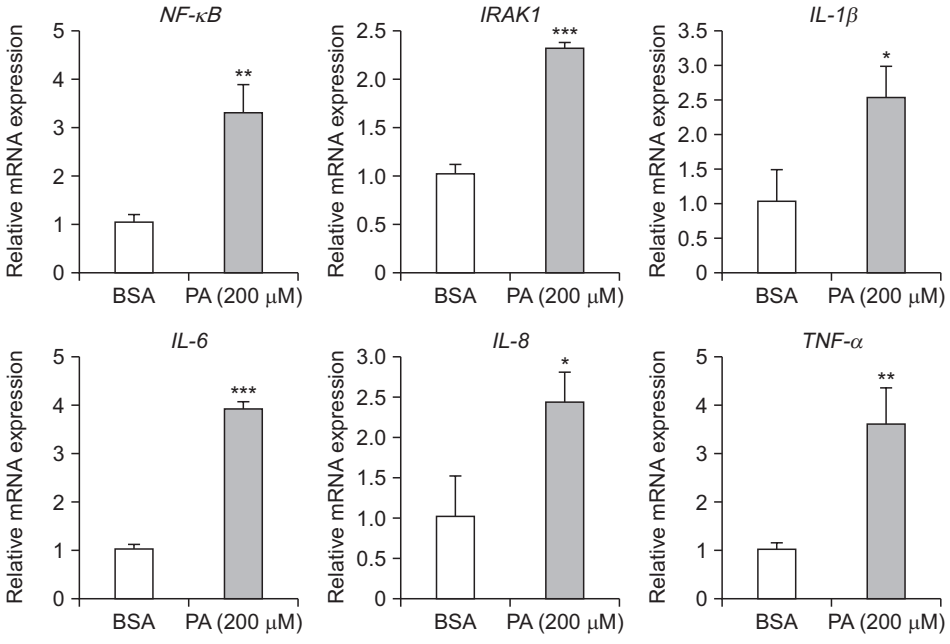

Fig. 2. PA increases gene expression in inflammatory pathways in human trophoblasts. Changes in mRNA expression levels of *NF-κB, IRAK1, IL-1β, IL-6, IL-8*, and *TNF-α* in HTR8/SVneo cells in response to 200 μM PA for 24 h were analyzed by qPCR. Data are presented as representative results from three independent experiments. Asterisks indicate a statistically significant difference compared to controls (***p < 0.001; **p < 0.01; *p < 0.05).

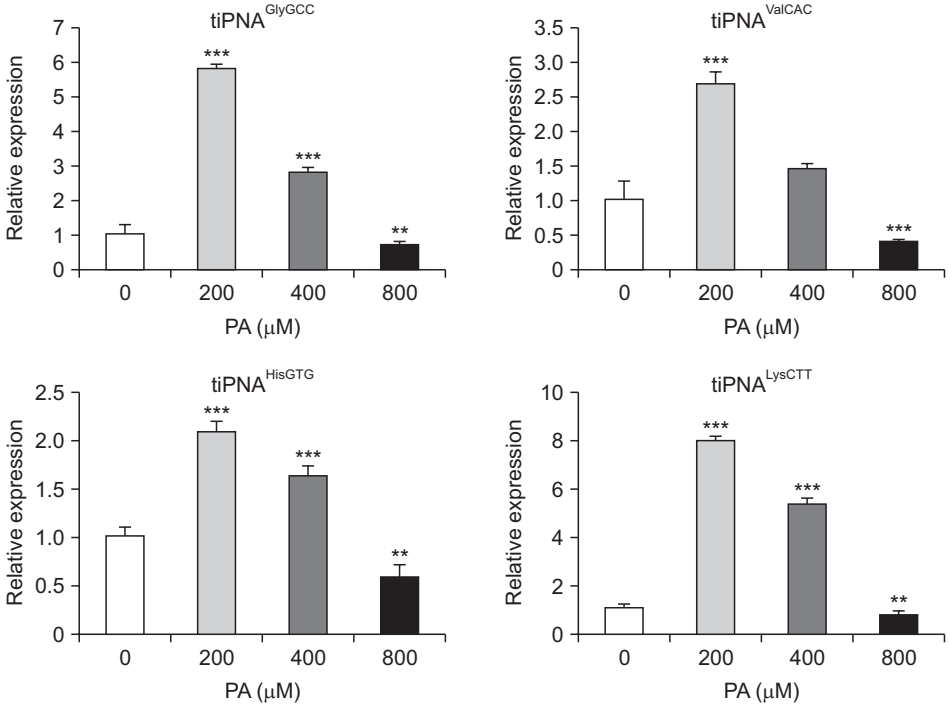

Fig. 3. PA regulates tRNA-derived stress-induced RNA (tiRNA) expression in human trophoblasts. Expression analysis of tiRNA^{GlyGCC}, tiRNA^{ValCAC}, tiRNA^{HisGTG}, and tiR^{NALysCTT} following dose-dependent treatment (0, 200, 400, and 800 μM) with PA for 24 h in HTR8/SVneo cells. Data are presented as representative results from three independent experiments. Asterisks indicate a statistically significant difference compared to controls (***p < 0.001; **p < 0.01).

μM PA significantly increased the expression of tiRNAs, including tiRNA^{GlyGCC}, tiRNA^{ValCAC}, tiRNA^{HisGTG}, and tiR^{NALysCTT} (Fig. 3). In addition, 400 μM PA induced the expression of tiRNA^{GlyGCC}, tiRNA^{ValCAC}, tiRNA^{HisGTG}, and tiR^{NALysCTT} in trophoblasts. These results suggest that the profile of tiRNAs may be different in response to a high concentration of PA, which induces apoptosis, and a low concentration of PA, which induces an inflammatory response. Considering that tiRNA production is regulated by external stress, further studies on the mechanism of tiRNA production by PA in trophoblasts are needed.

DISCUSSION

In the present study, we verified that PA increased the mRNA expression of inflammatory cytokines in human trophoblasts, even at low concentrations that did not affect their viability. Although saturated fatty acids are speculated to induce mitochondrial dysfunction and ER stress-mediated apoptosis in the placental environment, it is unclear whether PA regulates inflammatory pathway genes in human trophoblasts (Eastman et al., 2021). Several reports, including our previous study, have suggested that saturated and unsaturated fatty acids have opposing effects on the oxidative state and inflammatory response in the placenta (Yang et al., 2017; Manuel et al., 2018). High levels of saturated fatty acids in the blood have been observed in pregnant women who experience excessive weight gain during pregnancy. PA is the most abundant saturated fatty acid in the blood and promotes the production of NF-κB-dependent inflammatory cytokines (Wen et al., 2011). PA is one of the toll-like receptor (TLR) agonists and several studies have reported the inflammation-inducing effect of PA in a TLR4-dependent manner through the activation of NF-κB signaling (Korbecki and Bajdak-Rusinek, 2019). PA also had a synergistic effect when co-treated with lipopolysaccharide (LPS), an inflammation-inducing factor, as a TLR4 activator in macrophages and hepatocytes. In mice injected with PA, the NLRP3 inflammasome is activated within the placenta during pregnancy (Sano et al., 2020). In this context, it is unsurprising that even in human trophoblasts, PA regulates the expression of NF-κB and IRAK1, which play important roles in NF-κB signaling.

Evidence suggests that PA increases the expression of inflammatory cytokines in several cells, similar to the trophoblasts in the current study. The expression and activity of inflammatory cytokines must be tightly regulated in the placental environment during early pregnancy. Inflammatory cytokines produced by the placenta in obese women are associated with maternal macrophage infiltration into the placenta (Challier et al., 2008). In trophoblasts, inflammatory stimuli induce the secretion of inflammatory cytokines such as TNF-α and IL-6, which contributes to the development of inflammatory diseases during pregnancy due to trophoblast damage and placental dysfunction. IL-1β is secreted by NLRP3 inflammasome activation and is a potential proinflammatory cytokine that induces placental inflammatory responses (Abrahams, 2011). Moreover, placental IL-1β may affect fetal growth by activating NF-kB and JNK, which are associated with insulin resistance and functional impairment of insulin signaling. IL-8 levels in the blood of pregnant women remains high during the first trimester (Mor, 2008). Increased IL-8 levels are associated with the pathogenesis of metabolic diseases, including obesity and diabetes, and inflammatory diseases, such as hepatitis. PA increases *IL-8* gene expression and secretion in hepatocytes via NF-κB and JNK activation (Joshi-Barve et al., 2007). PA also inhibits the phosphorylation of AKT and ERK and induces the production of IL-6 and TNF-α in human neuroblastoma cells, thereby promoting insulin resistance and inflammatory responses (Amine et al., 2021). We previously showed that LPS activates NF-κB signaling in trophoblasts and promotes the production of inflammatory cytokines; however, the regulation of inflammation in the placental environment by PA is unclear (Yang et al., 2019). In addition, we previously revealed the apoptotic effects of PA in trophoblasts, influenced by the regulation of SCD1, an enzyme involved in the unsaturation of saturated fatty acids, and DGAT1, which plays a key role in the conversion of DAG to TAG (Yang et al., 2018). We attempted to determine whether inflammation is regulated in trophoblasts by treatment with a low concentration of PA which does not affect apoptosis. The observation of inflammatory cytokines whose expression was increased by PA in the present study suggests that PA may contribute to the pathogenesis of inflammatory gestational disease by inducing an inflammatory response in the placenta, regardless of inhibiting cell viability.

The current study and other evidence suggest that noncoding RNAs, including miRNAs, can regulate inflam-

mation in trophoblasts. For instance, miR-138 increases TNF-α and IL-6 levels, which are associated with preeclampsia pathogenesis (Yin et al., 2021). We verified that the regulation of miR-146a-5p and miR-548e-5p can alleviate LPS-induced inflammation. Activation of the NF-κB inflammatory pathway following miRNA inhibition is speculated to contribute to the pathogenesis of preterm birth (Yang et al., 2019). However, little is known about whether noncoding RNAs cleaved from tRNAs can regulate inflammation in cells. Complement C3, known to contribute to the pathogenesis of alcoholic fatty liver disease, promotes the production of tRF^{Gly}, presenting tRNA-derived species as potential therapeutic targets for hepatosteatosis (Zhong et al., 2019). Recently, Su et al., (2020) found abundant expression of 5' halves derived from $tRNA^{Gly}$, $tRNA^{Glu}$, $tRNA^{Val}$, and $tRNA^{Lys}$ in the placenta and decidua of mice (Su et al., 2020). In addition, the authors verified that the 5' halves derived from tRNAs, including $tRNA^{Asp}$, $tRNA^{Gly}$, $tRNA^{Glu}$, and $tRNA^{Val}$, decreased in response to maternal immune activation at the maternal-fetal interface, although the expression of the 5' half derived from $tRNA^{His}$ was not affected. In a previous study, we found that the expression of several tiRNAs in human trophoblasts was reduced by apoptosis-inducing concentrations of PA (Yang et al., 2022). In the present study, we analyzed the expression of tiRNAs after treating trophoblasts with PA at a low concentration that induced inflammation (but did not affect apoptosis) and a high concentration that previously confirmed the effect on apoptosis. Interestingly, 200 μM PA significantly increased tiRNA expression in trophoblasts. Differences in the expression of tiRNAs depending on the concentration of PA require further study; however, a recent study on angiogenin-mediated inflammation-induced metabolic disease suggests a new link between tiRNA generation and inflammation (Park et al., 2021; Zhang et al., 2021). Angiogenin expression is increased in the mouse caput epididymis by LPS, and inflammation is inhibited in mice lacking angiogenin, accompanied by a change in the tRNA-derived species profile. Therefore, it can be inferred that the expression of tiRNAs increased by PA in human trophoblasts in the current study was also associated with the induction of inflammatory responses. However, the expression and function of tiRNAs may be altered in response to the concentrations of PA that lead to apoptosis.

CONCLUSION

In conclusion, we analyzed the inflammatory response pattern induced by PA in human trophoblasts, independent of previous studies that have focused on the inhibition of proliferation and induction of apoptosis by PA. PA increased the mRNA levels of *NF-κB*, *IRAK1*, and various inflammatory cytokines. Furthermore, we revealed that PA can regulate the amount of cleaved tRNA halves in a concentration-dependent manner in human trophoblasts. These results may serve as a basis for future studies on the link between PA exposure-induced inflammatory responses and the functional aspects of tiRNAs in placental development during early pregnancy.

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