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Toxicomics Report

PPAR gamma negatively regulates the expression of TRPM8 in normal epidermal cells but mutually regulate their expressions with TRPM8 by feed-back loop regulation in squamous carcinoma cells

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ABSTRACT — TRPM8, non-selective cation channel of the transient receptor potential (TRP) superfamily, required for the transduction of moderate cold temperatures, regulates proliferation of epidermal cells in cyclin-dependent kinase inhibitor p21/Cip1-dependent manner. Given that downregulation of TRPM8 decreases p21/Cip1 level, increasing risk for carcinogenesis, and other TRP family is regulated by nuclear receptor peroxisome proliferator-activated receptor (PPAR) gamma, we examined whether TRPM8 expression was regulated by PPAR gamma. Knockdown assay and inhibition of PPAR gamma revealed that PPAR gamma negatively regulates the expression of TRPM8 in normal epidermal cells but positively regulates that in squamous carcinoma cells. Later restoration of decreased TRPM8 level in PPAR gamma antagonist-treated squamous carcinoma cells was attributed to feed-back loop regulation between TRPM8 and PPAR gamma using TRPM8 knockdown assay. Consisting with this finding, p21/Cip1 decrease by TRPM8 blocker, N-(4-Tertiarybutylphenyl)-4-(3-chloropyridin- 2-yl) tetrahydropyrazine-1 (2H)-carbox-amide (BCTC), was restored by additional BCTC treatment in squamous carcinoma cells.

Key words: TRPM8, PPAR gamma, Feed-back loop regulation, Squamous carcinoma cells

INTRODUCTION

TRPM8 is a calcium-permeable, non-selective cation channel of the transient receptor potential (TRP) superfamily, required for the transduction of moderate cold temperatures (Peier *et al*., 2002). TRPM8 mRNA has been detected in malignant cells, and has been extensively studied in prostate cancer (Valero *et al*., 2012). TRPM8 expression is also markedly up-regulated in human pancreatic adenocarcinoma cell lines and tissues, and is important for cell proliferation. A deficiency in TRPM8 in pancreatic cancer cells leads to impaired proliferation and cell cycle progression with elevated levels of cyclin-dependent kinase (CDK) inhibitors (Hantute-Ghesquier *et al*., 2018; Yee *et al*., 2010). TRPM8 is highly expressed at both the mRNA and protein levels in the MCF-7 breast cancer cell line, and breast adenocarcinomas, and is especially correlated with estrogen receptor positive (ER+) tumors (Hantute-Ghesquier *et al*., 2018; Chen *et al*., 2014). Interestingly, while TRPM8 stimulates the proliferation of breast and pancreatic cell lines, it negatively regulates melanoma proliferation (Hantute-Ghesquier *et al*., 2018; Guo *et al*., 2012).

Recently we found that the CDK inhibitor p21/Cip1 is a key factor involved in the regulation of TRPM8-mediated proliferation of epidermal cells, which are direct-

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ly affected by cold temperatures (Fujino, 2022). p21/Cip1 causes G1 arrest resulting in decreased DNA synthesis while downregulation of p21/Cip1 is associated with carcinogenesis (Paramio *et al*., 2001; Jackson *et al*., 2002). Our study showed that p21/Cip1 levels were reduced by TRPM8 knockdown in HaCaT cells derived from normal human keratinocytes (CLS Cell Lines Service, Eppelheim, Deutschland, 300493) (Boukamp *et al*., 1988) and squamous carcinoma SAS (JCRB cell bank, Osaka, Japan: JCRB0260) (Takahashi *et al*., 1989) cells.), suggesting that TRPM8 downregulation may increase the risk of carcinogenesis attributed to a decrease in p21/Cip1 and stimulated proliferation of carcinoma cells. Therefore, we examined the regulation of TRPM8 expression in the present study. We focused on the fatty-acid-activated nuclear receptor peroxisome proliferator-activated receptor (PPAR) gamma, since the expression of other TRP family is regulated by PPAR gamma (Wolfrum *et al*., 2018).

MATERIALS AND METHODS

Materials

siRNAs against TRPM8 and PPAR gamma were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). PPAR gamma antagonist GW9662 was obtained from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). TRPM8 blocker, N-(4-Tertiarybutylphenyl)-4-(3-chloropyridin- 2-yl) tetrahydropyrazine-1 (2H)-carboxamide (BCTC), was purchased from MedChemExpress (Tokyo, Japan).

Cell culture

HaCaT cells derived from normal human keratinocytes and squamous carcinoma SAS cells were maintained in Dulbecco's modified eagle medium containing 10% fetal calf serum, 50 units/mL penicillin G sodium salt, and 50 μg/mL streptomycin sulfate and cultured in a humidified atmosphere of 8.5% CO_2 at 37°C.

RNA interference experiments

To knockdown endogenous PPAR gamma and TRPM8, cells were seeded on 60-mm dishes at a density of 2.0×10^5 cells per dish and transfected with siRNA against PPAR gamma and TRPM8 (10 nM each) using HiPerfect Transfection Reagent (Qiagen) according to the manufacturer's instructions. After incubating for 24 hr, total RNA was extracted for real-time polymerase chain reaction (PCR). In RNA interference experiments, "Nonsilencing Control" siRNA (#1022076) from Qiagen was used as a control.

Quantification of mRNA

Quantification of mRNA was performed using real-time PCR. Briefly, 4 μg of total RNA was reverse-transcribed using a ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). The resultant cDNA was subjected to real-time PCR analysis using a TaqMan Gene Expression Assay kit (Applied Biosystems, Tokyo, Japan). mRNA levels were determined using TaqMan assay mixtures for TRPM8 (Hs00368574), PPAR gamma (Hs01115513), and β-actin (4310881E). Amplification and quantification were performed using the StepOne Real-Time PCR System (Applied Biosystems). mRNA levels were normalized to those of β-actin as an internal control.

Statistical analysis

Data are presented as the mean ± S.E.M. of three experiments performed in triplicate and were analyzed using Two-way ANOVA with Bonferroni post-hoc test.

RESULTS

PPAR gamma negatively regulates the expression of TRPM8 in normal epidermal cells but positively regulates that in squamous carcinoma cells

Based on the report showing other TRP family is regulated by PPAR gamma (Wolfrum *et al*., 2018), we examined whether PPAR gamma is a regulator of the expression of TRPM8 in the epidermal cell line HaCaT derived from normal human keratinocytes and squamous carcinoma SAS cells. As shown in Fig. 1A, knockdown of PPAR gamma (Fig. 1A-1) significantly increased the TRPM8 level in HaCaT cells (Fig. 1A-2). The stimulation of TRPM8 expression caused increase in the level of CDK inhibitor p21/Cip1 (Fig. 1A-3), consistent with our previous study (Fujino, 2022). In contrast to HaCaT cells, knockdown of PPAR gamma in squamous carcinoma SAS cells (Fig. 1A-4) significantly decreased TRPM8 and p21/Cip1 level (Fig. 1A-5 and 6). Results of PPAR gamma knockdown assay indicate that PPAR gamma negatively regulates the expression of TRPM8 in normal epidermal cells but positively regulates that in squamous carcinoma cells. Similarly with knockdown assay, 24 hr treatment with PPAR gamma antagonist significantly increased the TRPM8 and p21/Cip1 level in HaCaT cells (Fig. 1B-1 and 3) but decreased those in SAS cells (Fig. 1B-4 and 6), however, decreased TRPM8 and p21/Cip1 in SAS cells restored by additional 24 hr treatment (Fig. 1B-4 and 6). In SAS cells, PPAR gamma level was significantly increased by PPAR gamma antagonist (Fig. 1B-5), suggesting that PPAR gamma increase was involved in restoration of TRPM8 and p21/Cip1.

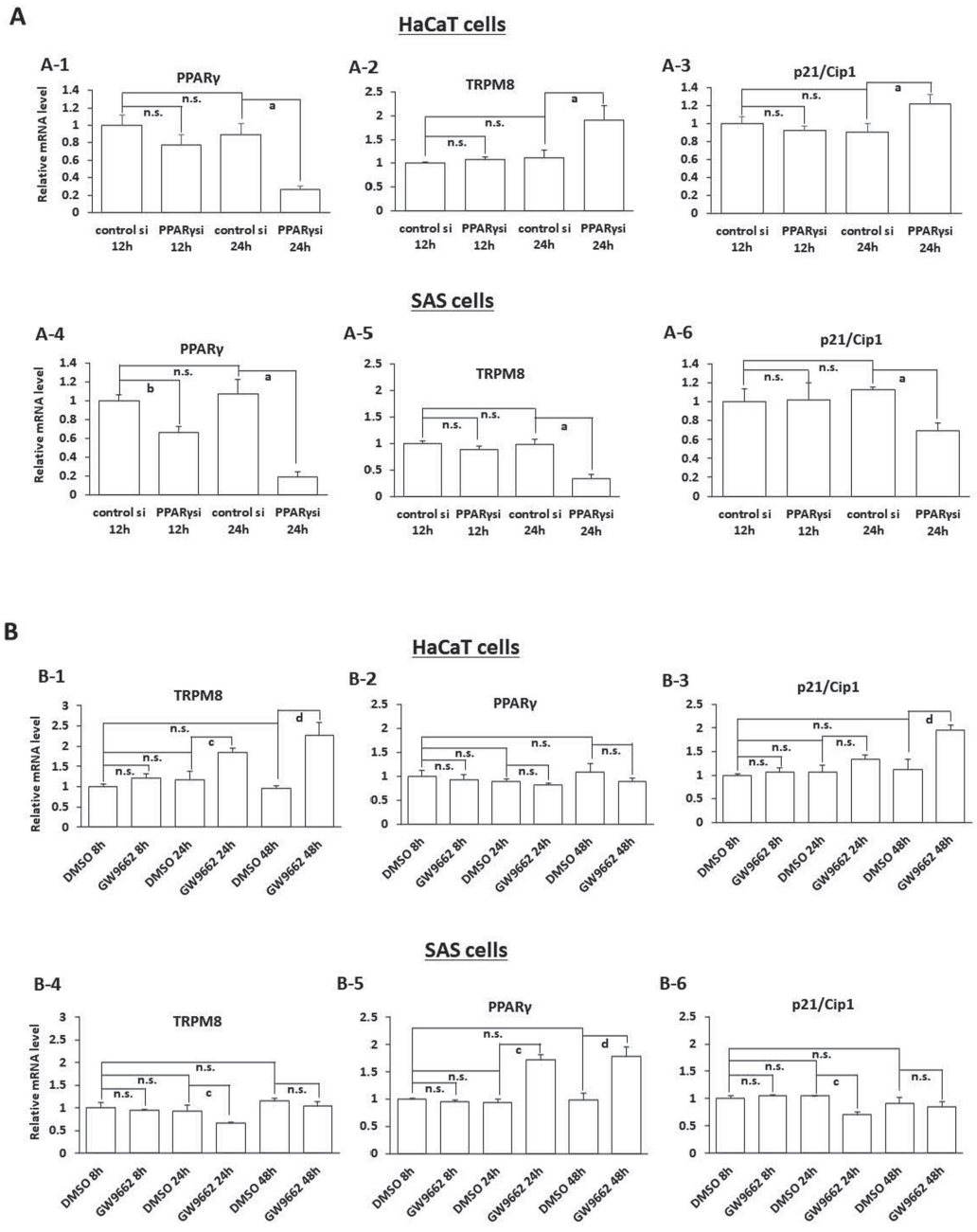

Fig. 1. Effect of knockdown of PPAR gamma and PPAR gamma antagonist on TRPM8 and p21/Cip1 expressions in epidermal cells. A: HaCaT and SAS cells seeded at 2.0×10^5 cells/60-mm dish were transfected with siRNA against PPAR gamma, or control siRNA. After 12 and 24 hrs, total RNA was quantified to determine PPAR gamma (A-1 and 4), TRPM8 (A-2 and 5), and p21/Cip1 (A-3 and 6) mRNA levels as described in the **Materials and Methods**. B: HaCaT and SAS cells seeded at 2.0×10^5 cells/60-mm dish were transfected with 1 μM of PPAR gamma antagonist GW9662, or DMSO as control. After 12 and 24 hrs, total RNA was quantified to determine TRPM8 (B-1 and 4), PPAR gamma (B-2 and 5), and p21/Cip1 (B-3 and 6) mRNA levels as described in the **Materials and Methods**. Data were analyzed using Two-way ANOVA with Bonferroni post-hoc test and are presented as the mean ± S.E.M. of three experiments performed in triplicate. n.s.: not significant. a: significant compared to "control si 24 hr"; $P < 0.05$. b: significant compared to "control si 12 hr"; $P < 0.05$. c: significant compared to "DMDO 24 hr"; $P < 0.05$. d: significant compared to "DMDO 48 hr"; $P < 0.05$.

TRPM8 and PPAR gamma mutually regulate their expression by feed-back loop regulation in squamous carcinoma cells

Given that results of PPAR gamma antagonist assay suggested that decrease in TRPM8 was restored by upregulation of PPAR gamma expression in SAS cells (Fig. 1B-4, 5, and 6), we examined whether TRPM8 regulates PPAR gamma in epidermal cells. As shown in Fig. 2A-5, knockdown of TRPM8 (Fig. 2A-4) significantly increased PPAR gamma level in SAS cells, while that in HaCaT cells was unchanged (Fig. 2A-2) by TRPM8 knockdown (Fig. 2A-1). These results indicate that TRPM8 negatively regulates PPAR gamma expression while PPAR gamma positively regulates TRPM8 expression in SAS cells. Under treatment with PPAR gamma antagonist, inhibited PPAR gamma activity causes downregulation of TRPM8 expression. In squamous carcinoma SAS cells, the TRPM8 decrease stimulates PPAR gamma expression, resulting in restoration of TRPM8. In normal epidermal HaCaT cells, TRPM8 does not regulate PPAR gamma expression, then PPAR gamma antagonist-caused increase in TRPM8 sustains.

In previous study, we showed that 24 hr treatment with TRPM8 blocker BCTC downregulated p21/Cip1 expression (Fujino, 2022) in HaCaT and SAS cells. From Fig. 1A and 2A, we have revealed the feed-back loop regulation between TRPM8 and PPAR gamma expressions in squamous carcinoma cells SAS. In fact, PPAR gamma antagonist-induced decrease in TRPM8 and p21/Cip1 restored in later stage of treatment. Based on these findings, we examined whether p21/Cip1 decrease by BCTC restored by additional 24 hr treatment in SAS cells. As shown in Fig. 2B-4, decreased p21/Cip1 level by 24 hr treatment with BCTC restored by additional 24 hr treatment. Consisting with that, TRPM8 and PPAR gamma levels were increased by BCTC (Fig. 2B-5 and 6).

DISCUSSION

In the present study, we revealed that PPAR gamma negatively regulates the expression of TRPM8 in normal epidermal cells but positively regulates that in squamous carcinoma cells. TRPM8 and PPAR gamma mutually regulate their expression by feed-back loop regulation in squamous carcinoma cells (Fig. 2C).

Since TRPM8 downregulation in HaCaT cells causes p21/Cip1 decrease, increasing risk of carcinogenesis (Fujino, 2022), circumstances which activate PPAR gamma, such as environmental pollutants including phthalates (Hurst and Waxman *et al*., 2003), probably increase the risk of carcinogenesis by downregulating TRPM8. On the other hand, in squamous carcinoma cells, TRPM8 downregulation stimulates PPAR gamma expression. Since increased PPAR gamma causes restoration of TRPM8 and p21/Cip1 level, proliferation of squamous carcinoma cells is probably repressed.

We don't exclude the possibility that added PPAR gamma antagonist and TRPM8 blocker BCTC destructed during incubation, resulting in restoration of TRPM8 and p21/Cip1 levels in SAS cells (Fig. 1B-4 and 6, Fig. 2B-4). However, in HaCaT cells, increase in TRPM8 and p21/Cip1 level by PPAR gamma antagonist and decrease in p21/Cip1 by BCTC was sustained even by additional 24 hr treatment (Fig. 1B-1 and 3, Fig. 2B-1). Thus, it is more certain that increased PPAR gamma (Fig. 1B-5) and TRPM8 (Fig. 2B-5) counteracts the effect of PPAR gamma antagonist and BCTC, resulting in restored level of p21/Cip1 (Fig. 1B-6 and Fig. 2B-4).

We previously reported that expression of several transcriptional factors associated with cell proliferation and differentiation are subjected to feed-back regulation in carcinoma cells (Fujino *et al*., 2017, 2020). Briefly, in hepatocellular carcinoma and renal adenocarcinoma cells, natural product AU-1- induced increase in p21/Cip1 is restored by later stage of treatment, since p21/Cip1 suppresses its expression by miR-34-dependent manner (Fujino *et al*., 2017). In renal adenocarcinoma cells, decrease in Oct3/4, cell differentiation factor, causes downregulation of ELAVL2, transcriptional factor which negatively regulates Oct3/4 expression, resulting in restoration of Oct3/4 level (Fujino *et al*., 2020). In the present study, we have revealed novel regulation for restoring the expression of TRPM8, a factor which regulates p21/Cip1 expression in squamous carcinoma cells. Given that those feed-back regulation of factors involved in cell proliferation and differentiation is limited to carcinoma cells (Fujino *et al*., 2017, 2020), detail examination, for example, whether common factor is involved in those regulation, is useful for elucidation of the mechanism of carcinogenesis.

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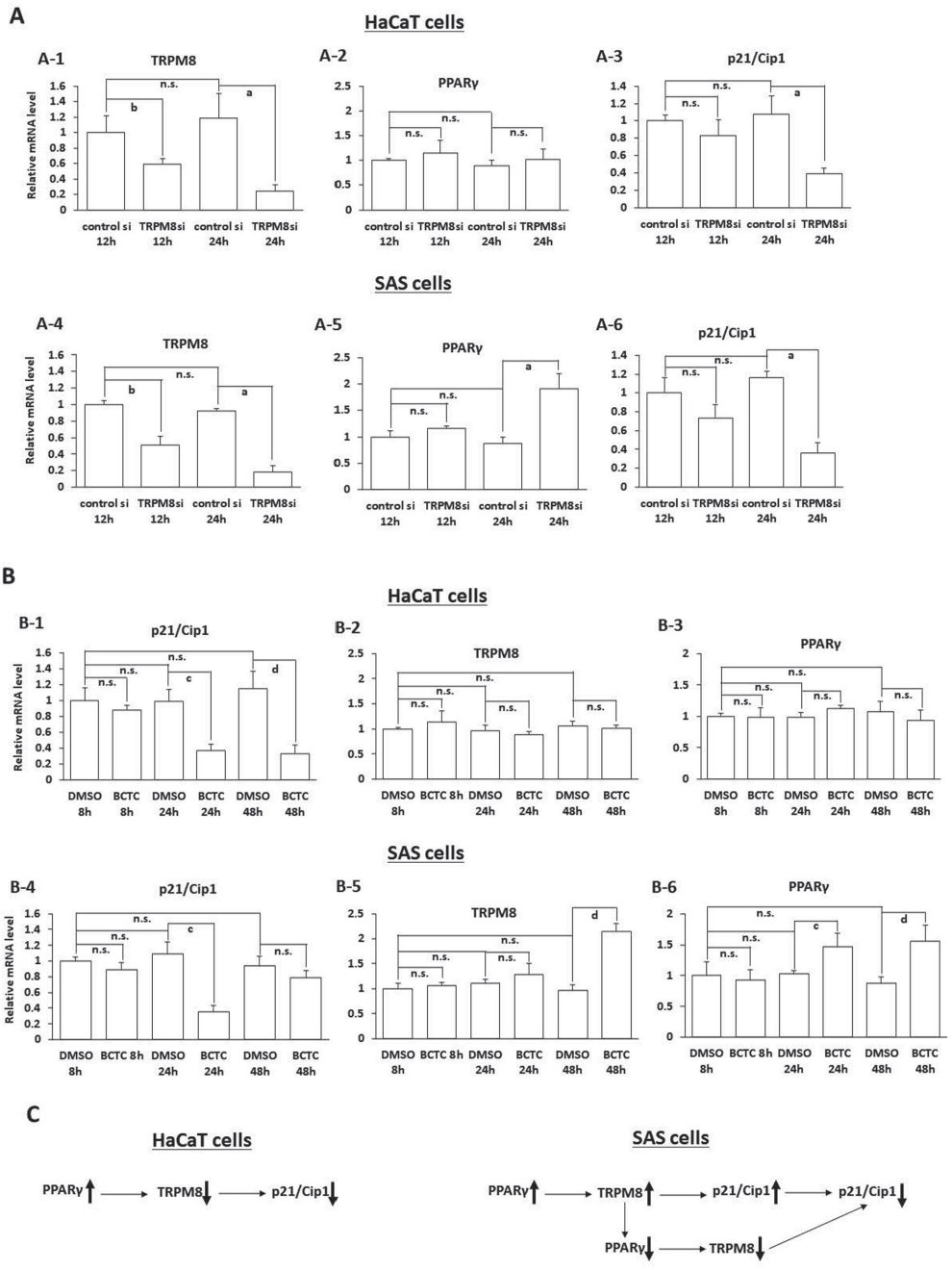

Fig. 2. Effect of knockdown of TRPM8 and TRPM8 blocker on PPAR gamma and p21/Cip1 expressions in epidermal cells. A: HaCaT and SAS cells seeded at 2.0×10^5 cells/60-mm dish were transfected with siRNA against TRPM8, or control siRNA. After 12 and 24 hrs, total RNA was quantified to determine TRPM8 (A-1 and 4), PPAR gamma (A-2 and 5), and p21/Cip1 (A-3 and 6) mRNA levels as described in the **Materials and Methods**. B: HaCaT and SAS cells seeded at 2.0×10^5 cells/60-mm dish were transfected with 10 μM of TRPM8 blocker BCTC, or DMSO as control. After 12 and 24 hrs, total RNA was quantified to determine p21/Cip1 (B-1 and 4), TRPM8 (B-2 and 5), and PPAR gamma (B-3 and 6) mRNA levels as described in the **Materials and Methods**. Data were analyzed using Two-way ANOVA with Bonferroni post-hoc test and are presented as the mean ± S.E.M. of three experiments performed in triplicate. n.s.: not significant. a: significant compared to "control si 24 hr"; $P < 0.01$. b: significant compared to "control si 12 hr"; $P < 0.05$. c: significant compared to "DMDO 24 hr"; $P < 0.05$. d: significant compared to "DMDO 48 hr"; $P < 0.01$. C: Regulation of TRPM8 expression by PPAR gamma in normal epidermal cell line HaCaT and squamous carcinoma cell line SAS.

Conflict of interest---- The authors declare that there is no conflict of interest.

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