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RESEARCH ARTICLE

HUHS1015 DOWNREGULATES PKM2 THROUGH AN AMPK/p38MAPK PATHWAY IN MKN45 CELLS

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ABSTRACT

In the earlier study, the newly synthesized anticancer drug HUHS1015 decreased pyruvate kinase M2 (PKM2) in MKN45 human gastric cancer cells due to autophagic degradation. The present study was conducted to gain further insight into the mechanism underlying HUHS1015-induced autophagic degradation of PKM2. HUHS1015 phosphorylated at Thr172 and activated AMP-activated protein kinase (AMPK). HUHS1015, alternatively, phosphorylated at Thr180/Tyr182 and activated p38 mitogen-activated protein kinase (p38MAPK). HUHS1015-induced decrease of PKM2 was cancelled by the AMPK inhibitor Compound C or combination of the MAP kinase kinase (MAP2K) inhibitor PD98059 and the p38MAPK inhibitor SB203580. Taken together, these results indicate that HUHS1015 activates AMPK and the effector p38MAPK, which triggers autophagic degradation, to decrease PKM2 in MKN45 cells.

INTRODUCTION

Accumulating evidence has shown that 1-[2-(2-methoxy phenylamino) ethylamino]-3-(naphthalene-1-yloxy) propan-2-ol (HUHS1015) exhibits a beneficial anticancer effect on a variety of cancer cells through diverse signaling pathways (Kaku *et al.*, 2016; Kanno *et al.*, 2013; Nishizaki *et al.*, 2014). HUHS1015 induces caspase-dependent apoptosis in MKN45 human gastric cancer cells by upregulating expression of tumor necrosis factor α (TNF α), a ligand of TNF α receptor relevant to activation of caspase-8 and the effector caspase-3 (Kaku *et al.*, 2015). HUHS1015, alternatively, induces caspase-dependent apoptosis in MKN45 cells by activating caspase-3 in association with mitochondrial damage, regardless of cytochrome c, DIABLO, apoptosis-inducing factor (AIF), or AMID (Ohyama *et al.*, 2015). Moreover, HUHS1015 promotes autophagic degradation of X-linked inhibitor of apoptosis protein (XIAP), thereby neutralizing caspase-3 inhibition due to XIAP, to activate caspase-3 and induce apoptosis in MKN45 cells (Tsuchiya *et al.*, 2015). Pyruvate kinase generates ATP from ADP without oxygen supply, allowing survival of the organs under the hypoxic conditions as seen in solid cancers (Vaupel and Harrison, 2004). Pyruvate kinase M2 (PKM2) plays a critical role in the oncogenesis, progression, and prognosis of cancers (Hsu and Hung, 2018; Li *et al.*, 2018). In the earlier study, I have found that HUHS1015 decreases PKM2 in MKN45 cells due to autophagic degradation (unpublished data). The present study aimed at understanding the mechanism underlying HUHS1015-induced autophagic degradation of PKM2.

The results show that HUHS1015 activates AMPK and the effector p38 mitogen-activated protein kinase (p38MAPK), causing decrease of PKM2 in MKN45 cells.

MATERIALS AND METHODS

Cell culture: MKN45 cells, a poorly differentiated gastric adenocarcinoma cell line, were kindly gifted from Dr. Tatematsu (Nagoya University, Japan). Cells were plated and grown in a RPMI1640 solution (Sigma, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (final concentration, 100 U/ml), and streptomycin (final concentration, 0.1 mg/ml) in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C.

Western blotting: After treatment, cells were lysed and the lysates were loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membrane. The blotting membrane was reacted with an antibody against PKM2 (Cosmo Bio, Tokyo, Japan), β -actin (Cell Signaling, Beverly, MA, USA), phospho (Thr172)-AMPK (Cell Signaling Technology, Danvers, MA, USA), AMPK (Cell Signaling Technology), phospho (Thr845)-ASK1 (Cell Signaling Technology), ASK1 (Cell Signaling Technology), phospho (Thr202/Tyr204)-ERK 1/2 (Thermo Fisher Scientific, Rockford, IL, USA), ERK1/2 (Thermo Fisher Scientific), phospho (Thr180/Tyr182)-p38 MAPK (Cell Signaling Technology), p38MAPK (Cell Signaling Technology), phospho (Thr183/Tyr185)-JNK (Cell Signaling Technology), or JNK (Cell Signaling Technology), followed by a horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody. Immunoreactivity was detected with an ECL kit

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(Invitrogen, Carlsbad, CA, USA) and visualized using a chemiluminescence detection system (GEHealthcare, Piscataway, NJ, USA). Protein concentrations for each sample were determined with a BCA protein assay kit (Thermo Fisher Scientific).

Statistical analysis: Statistical analysis was carried out using analysis of variance (ANOVA) followed by a Bonferroni correction.

RESULTS

HUHS1015 decreases PKM2: Like the earlier study, HUHS1015 (100 μ M) decreased PKM2 in a treatment time (1-10 min)-dependent manner in MKN45 cells (Figure 1).

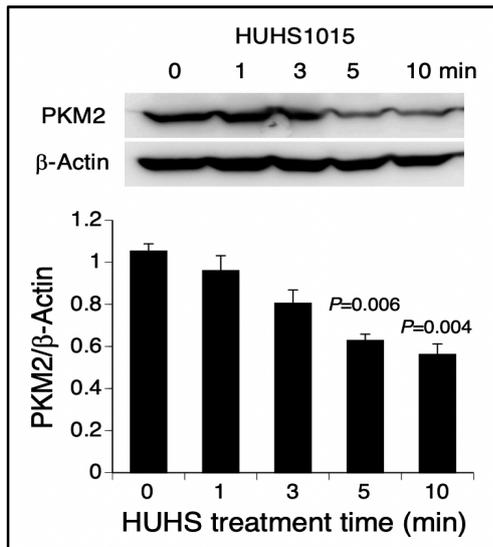


Figure 1. HUHS1015 decreases PKM2. MKN45 cells were treated with HUHS1015 (100 μ M) for periods of time as indicated, followed by Western blotting. In the graph, each column represents the mean (\pm SEM) signal intensity for PKM2 relative to the intensity for β -actin ($n=4$ independent experiments). P values, ANOVA followed by a Bonferroni correction.

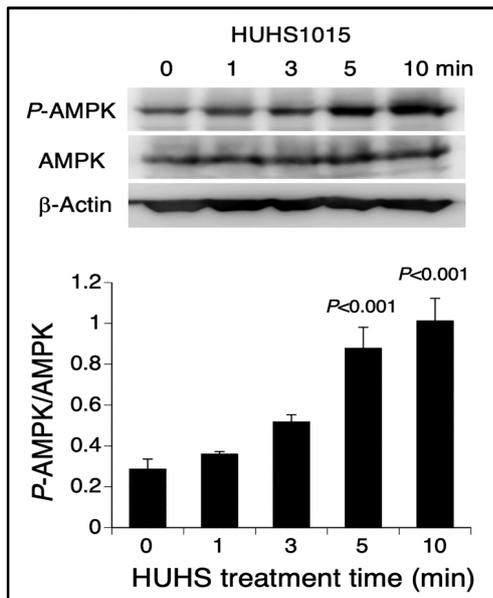


Figure 2. HUHS1015 activates AMPK. MKN45 cells were treated with HUHS1015 (100 μ M) for periods of time as indicated, followed by Western blotting. In the graph, each column represents the mean (\pm SEM) signal intensity for phospho (Thr172)-AMPK (P-AMPK) relative to the intensity for total AMPK ($n=4$ independent experiments). P values, ANOVA followed by a Bonferroni correction.

HUHS1015 activates AMPK, to downregulate PKM2: AMPK is activated by being phosphorylated at Thr172 (Russo *et al.*, 2013). HUHS1015 (100 μ M) phosphorylates AMPK at Thr172 in a treatment time (1-10 min)-dependent manner in MKN45 cells (Figure 2), indicating HUHS1015-driven AMPK activation. HUHS1015 (50 μ M)-induced decrease of PKM2 was cancelled by the AMPK inhibitor Compound C (Figure 3). Taken together, these results indicate that HUHS1015 activates AMPK, to down regulate PKM2 in MKN45.

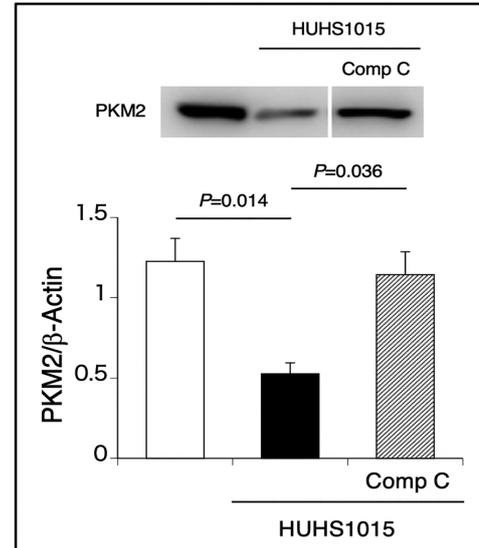


Figure 3. AMPK is required for HUHS1015-induced decrease of PKM2. MKN45 cells were treated with HUHS1015 (50 μ M) for 30 min in the presence and absence of Compound C (Comp C) (10 μ M), followed by Western blotting. In the graph, each column represents the mean (\pm SEM) signal intensity for PKM2 relative to the intensity for β -actin ($n=4$ independent experiments). P values, ANOVA followed by a Bonferroni correction.

HUHS1015 activates p38MAPK in an ASK1-independent manner, to downregulate PKM2: ASK1, a MAP kinase kinase (MAP3K) family member, is activated through its auto-phosphorylation at Thr845 (Tobiume *et al.*, 2002). HUHS1015 (100 μ M) had no effect on Thr845 phosphorylation of ASK1 in MKN45 cells (Figure 4A), indicating no implication of HUHS1015 in the activation of ASK1. ERK1/2, a MAPK family member, is activated by being phosphorylated at Thr202/Tyr204 (Roskoski, 2012). HUHS1015 (100 μ M) phosphorylated ERK1/2 at Thr202/Tyr204 in a bell-shaped treatment time (1-10 min)-dependent manner, with the maximum at 5 min, in MKN45 cells (Figure 4B), indicating HUHS1015-driven ERK1/2 activation. p38MAPK, a MAPK family member, is a downstream target of ASK1 (Hattori *et al.*, 2009). p38 MAPK is activated by being phosphorylated at Thr180/Tyr182 (Raingeaud *et al.*, 1995). HUHS1015 (100 μ M) phosphorylated p38MAPK in a treatment time (1-10 min)-dependent manner in MKN45 cells (Figure 4C), indicating HUHS1015-driven ASK1-independent activation of p38MAPK. JNK, a MAPK family member, is activated through its auto-phosphorylation at Thr183/Tyr185 (Pimental *et al.*, 2007). HUHS1015 (100 μ M) had no effect on Thr183/Tyr185 phosphorylation of JNK in MKN45 cells (Figure 4D), indicating no implication of HUHS1015 in the activation of JNK. HUHS1015 (50 μ M)-induced decrease of PKM2 was apparently suppressed in the co-presence of the MAP kinase kinase (MAP2K) inhibitor PD98059 and the p38MAPK inhibitor SB203580 (Figure 5). Overall, these results indicate that HUHS1015 activates p38MAPK, regardless of ASK1, to downregulate PKM2.

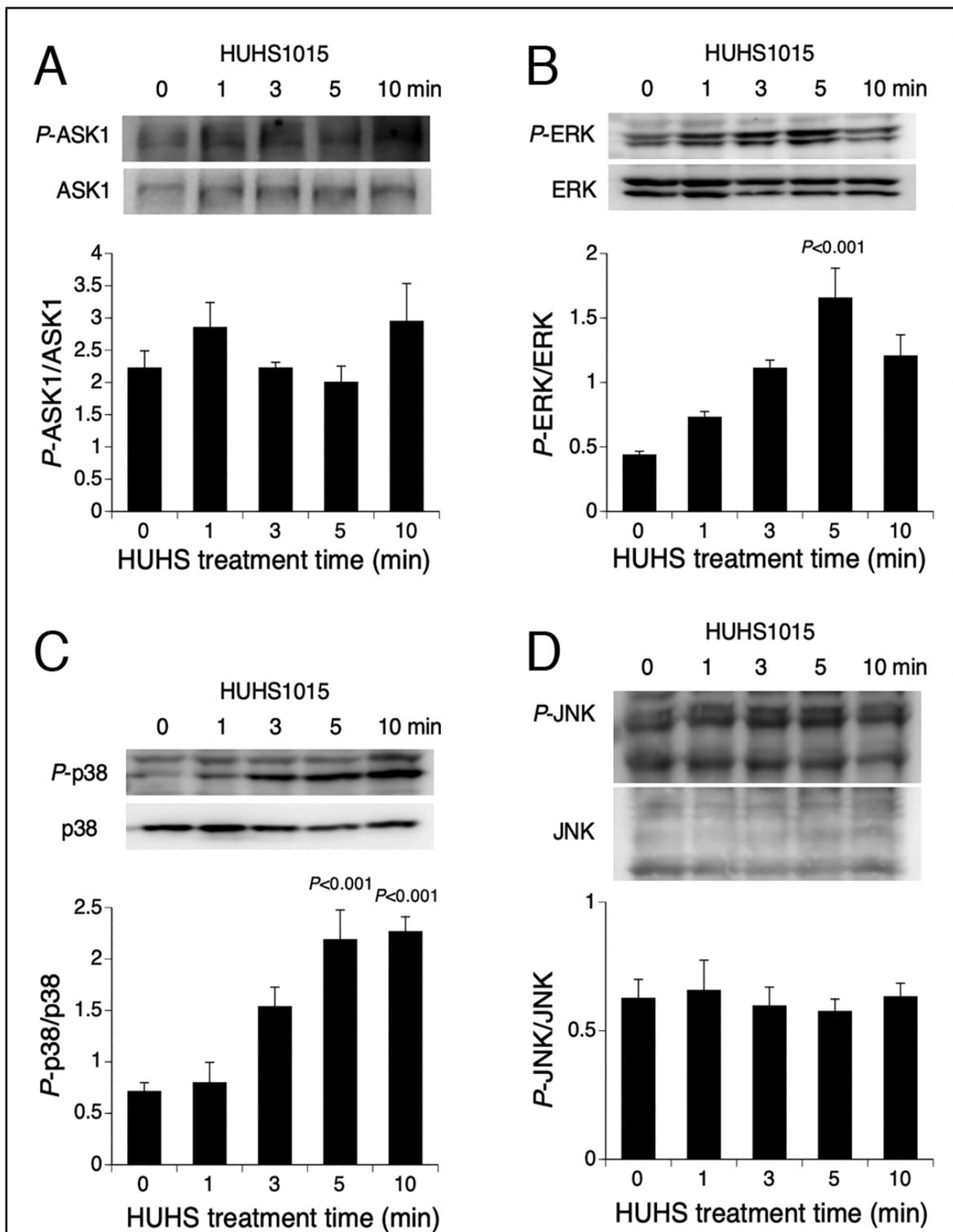


Figure 4. HUHS1015 activates p38MAPK. MKN45 cells were treated with HUHS1015 (100 μ M) for periods of time as indicated, followed by Western blotting. In the graphs, each column represents the mean (\pm SEM) signal intensity for phospho (Thr845)-ASK1 (P-ASK1) (A), phospho (Thr202/Tyr204)-ERK1/2 (P-ERK) (B), phospho (Thr180/Tyr182)-p38MAPK (P-p38) (C), or phospho (Thr183/Tyr185)-JNK (P-JNK) (D) relative to the intensity for total ASK1, ERK1/2, p38MAPK, or JNK, respectively (n=4 independent experiments). P values. ANOVA followed by a Bonferroni correction.

DISCUSSION

In the present study, HUHS1015 actually activated AMPK and p38MAPK in MKN45 cells. It is shown that isoeugenol (4-propenyl-2-methoxyphenol) increases p38MAPK phosphorylation, which is blocked by the AMPK inhibitor compound C and that isoeugenol-induced glucose uptake is suppressed by the p38MAPK inhibitor SB203580 (Kim *et al.*, 2016). This indicates that p38MAPK is a downstream target of AMPK.

In further support of this notion, p38MAPK functions downstream of AMPK in the anti-apoptotic effect of adiponectin (Wanget *al.*, 2017.) or AMPK-induced mitochondrial biogenesis is mediated by p38MAPK-dependent regulation of peroxisome proliferator-activated receptor γ -coactivator 1- α (PGC-1 α) (Chau *beet al.*, 2015). HUHS1015 decreased PKM2 in MKN45 cells, and the effect was restrained by the AMPK inhibitor Compound C or combination of MAP2K inhibitor PD98059 and the p38 MAPK inhibitor

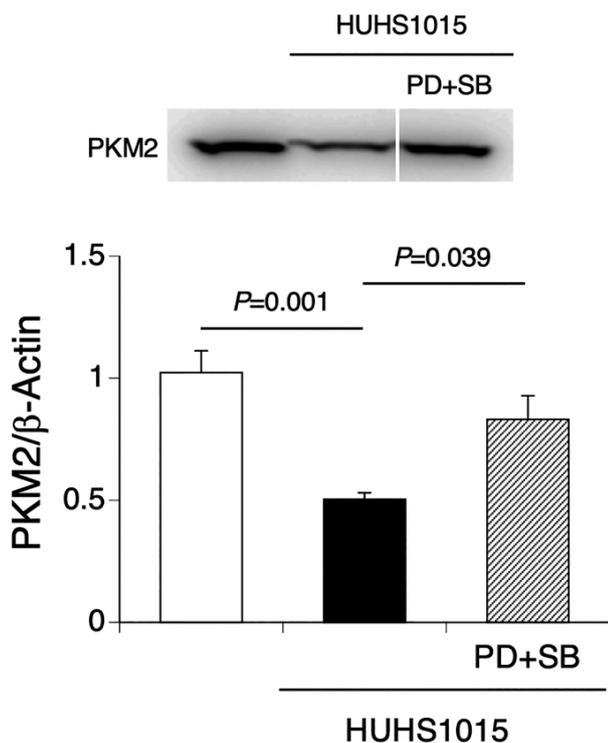


Figure 5. p38 MAPK is required for HUHS1015-induced decrease of PKM2. MKN45 cells were treated with HUHS1015 (50 μ M) for 30 min in the presence and absence of PD98059 (PD) (50 nM) plus SB203580 (SB) (10 μ M), followed by Western blotting. In the graph, each column represents the mean (\pm SEM) signal intensity for PKM2 relative to the intensity for β -actin ($n=4$ independent experiments). *P* values, ANOVA followed by a Bonferroni correction.

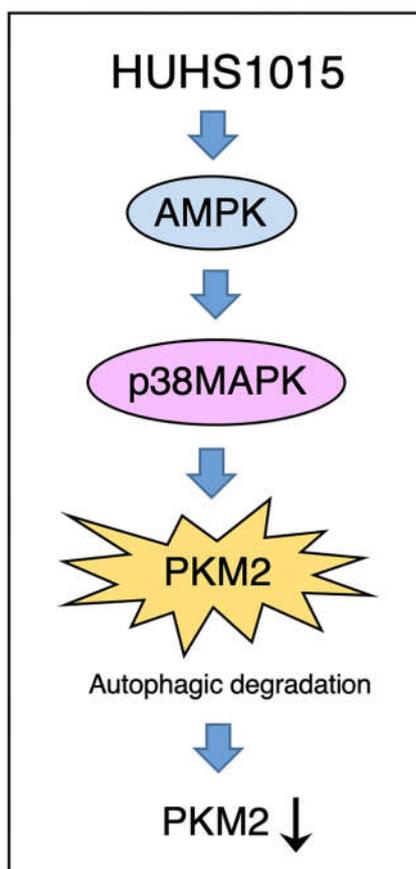


Figure 6. The mechanism underlying HUHS1015-induced decrease of PKM2.

SB203580. Taken together, these results lead to a conclusion that HUHS1015 activates AMPK followed by the effector p38MAPK, thereby downregulating PKM2 in MKN45 cells. In the earlier study, HUHS1015 decreased PKM2 in not only MKN45 cells but MKN28 human gastric cancer cells and CW2 and Caco-2 human colonic cancer cells due to autophagic degradation (unpublished data). Of particular interest is that AMPK promotes autophagic degradation through specific phosphorylation of autophagy-relevant proteins or by indirectly impacting in the activity of additional autophagy regulators (Tamargo-Gómez and Mariño, 2018). This raises the possibility that HUHS1015 activates AMPK, which triggers autophagic degradation, to decrease PKM2 in MKN45 cells. Moreover, the present results suggest that p38MAPK as a downstream effector of AMPK also participates in the regulation of autophagy.

Conclusion

The results of the present study indicate that HUHS1015 activates AMPK and the effector p38MAPK, which triggers autophagic degradation, causing decrease of PKM2 in MKN45 cells (Figure 6). This may provide further insight into the anticancer actions of HUHS1015.

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