Reserpine treatment activates AMP activated protein kinase (AMPK)

Rackhyun Park1,†, Kang Il Lee1,†, Hyunjoo Kim1, Minsu Jang1, Thi Kim Quy Ha2, Won Keun Oh2, and Junsoo Park1,*

1Division of Biological Science and Technology, Yonsei University, Wonju 26493, Republic of Korea
2Korea Bioactive Natural Material Bank, College of Pharmacy, Seoul National University, Seoul 08826, Republic of Korea

Abstract – Reserpine is a well-known medicine for the treatment of hypertension, however the role of reserpine in cell signaling is not fully understood. Here, we report that reserpine treatment induces the phosphorylation of AMP activated protein kinase (AMPK) at threonine 172 (T172) in PC12 cells. Phosphorylation of AMPK T172 is regulated by upstream signaling molecules, and the increase of phospho-T172 indicates that AMPK is activated. When we examined the FOXO3a dependent transcription by using the FHRE-Luc reporter assay, reserpine treatment repressed the FHRE-Luc reporter activity in a dose dependent manner. Finally, we showed that reserpine treatment induced the phosphorylation of AMPK as well as cell death in MCF-7 cells. These results suggest that AMPK is a potential cellular target of reserpine.

Keywords – Reserpine, AMPK, FOXO3a, PC12, MCF-7

Introduction

AMP activated protein kinase (AMPK) is the key regulator of the cellular response to lowered ATP levels in essentially all eukaryotic cells.1,2 AMPK activity is induced by various stresses including oxidative damage, osmotic shock, hypoxia, and glucose deprivation.2,3 AMPK complex is composed of catalytic α subunits and regulatory β and γ subunits, and mammals genome encode multiple AMPK isofroms (α1, α2, β1, β2, γ1, γ2, γ3).4 While AMPK α1 complex is ubiquitously expressed, the expression of AMPK α2 is high in skeletal muscle, heart and liver.4 AMPKα is activated when threonine 172 of AMPK is phosphorylated by upstream kinases such as Liver kinase B1 (LKB1), calcium dependent protein kinase kinase-β (CaMKK-β) and TGFβ-activated kinase 1 (TAK1).5,6 AMPK activity is also induced by the presence of small molecules such metformin and 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR).7 Recent reports showed that metformin, an anti-diabetic drug, exerts anti-tumoral effect in vitro and in vivo.8,9

Reserpine (structure as Fig. 1.A) is a natural plant alkaloid isolated from Rauwolfia serpentine,10 and a well-known medicine for the treatment of hypertension and schizophrenia.11,12 Reserpine inhibits the vesicular monoamine transporter 2 (VMAT2), and reduces the concentration of monoamine neurotransmitters such as dopamine in synaptic vesicles by blocking uptake through VMAT2.13 Therefore, chronic administration of reserpine can induce Parkinson’s disease like symptoms.14,15 Recently, we demonstrated that reserpine treatment inhibits autophagic flux and induces autophagosome formation.16 However, the role of reserpine in cell signaling is not fully understood. Here we examined the effect of reserpine treatment in cell signaling and found that AMPK treatment induces the phosphorylation of AMPK at T172 in PC12 cells and MCF-7 cells.

Experimental

Cell culture – PC12, MCF-7 and HEK293T cells were grown in Dulbecco’s Modified Eagle’s medium (DMEM; Welgene, Korea) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA). Cell viability was measured using the [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, MCF-7 cells were seeded in a 24-well plate and treated with reserpine for 24 h and 48 h. Later the formazan was solubilized with acidified isopropanol (4 mM HCl in
absolute isopropanol) and the intensity was measured colorimetrically at 570 nm. Reserpine was obtained from the Korea Bioactive Natural Material Bank (KBNMB) and from Sigma-Aldrich (St. Louis, MO, USA).

**Cell cycle analysis** – For cell cycle analysis, cells were washed with phosphate buffered saline (PBS) and fixed with 70% ethanol. After centrifugation, cells were washed and resuspended in PBS containing 50 µg/ml propidium iodide (PI) and 10 mg/ml RNase A. Reserpine treated cells were analyzed in a FACScalibur flow cytometer (Beckton-Dickson, Mountain View, CA).

**Luciferase reporter assay** – For the reporter assay, HEK293T cells were seeded in 24 well plates in DMEM 24 h prior to transfection. Total DNA for the transfection was 0.5 µg per well, each assay was normalized with renilla luciferase activity. Transfection was performed using lipofectamine (Invitrogen, Carlsbad, CA).

**Western blotting** – For Western blotting, polypeptides in whole cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose membrane filters. Detection was conducted with a 1:5,000 dilution of phospho-AMPK or AMPK using an enhanced chemiluminescence (ECL) system. Images were acquired using a Chemidoc-it 410 imaging system (UVP, Upland, CA). The antibodies for AMPK and phospho-AMPK were purchased from Cell Signaling Technology (Beverly, MA, USA).

**Statistical methods** – The results of the luciferase assay, western blot and cell viability analysis were evaluated by a two-tailed t test using Microsoft Excel software (Seattle, WA, USA). P < 0.05 was considered significant.

**Result and Discussion**

**Reserpine treatment activates AMPK in PC12 cells** – Recently, we demonstrated that reserpine is involved in autophagosome formation by blocking autophagic flux, and we attempted to identify the cell signaling, which is modulated by reserpine treatment. We used the rat PC12 pheochromocytoma cell line, and PC12 cells were incubated with various concentrations of reserpine (0, 1, 2.5, 5 and 10 µM) for 24 h. We examined the phosphorylation level of signaling proteins by Western blot and found that reserpine treatment increased the phosphorylation level of AMPK in PC12 cells (Fig. 1A and B). We used the antibody specific for AMPKα threonine 172 residue (T172) for Western blot and T172 residue of AMPKα is phosphorylated when AMPK is activated by upstream kinases such as LKB1 and TAK1. Therefore the phosphorylation level of AMPK T172 reflects the activation level of AMPK. Reserpine treatment increases the phosphorylation of AMPK, and the phosphorylation of AMPKα T172 is markedly increased at 10 µM of reserpine treatment (Fig. 1B). We also treated Compound C, an AMPK inhibitor, with reserpine and found that Compound C efficiently inhibited the activation of AMPK, which was induced by reserpine (Fig. 1C). These results indicate that reserpine treatment activates AMPK.

**Reserpine treatments reduce the cell viability** – We first examined the effect of reserpine on cancer cell viability by treating PC12 cells with various concentrations of the compound (0, 1, 2.5, 5 and 10 µM) for 24 h and 48 h followed by an MTT assay. Reserpine showed an inhibitory effect on the growths of PC12 cells at 10 µM
Low concentrations of reserpine (0, 1, 2.5, 5 μM) did not show the inhibitory effect for the short treatment (24 h). We further examined whether the inhibition of cell viability was caused by the induction of apoptosis. Since apoptosis increases the sub-G1 population, we analyzed the percentage of sub-G1 cells using flow cytometry (Fig. 2B). Compared with the sub-G1 population in control cells, reserpine treatment increased the sub-G1 population. These results indicate that reserpine reduces cell viability and induces apoptosis.

Reserpine treatment represses the FHRE-Luc activity – AMPK is involved in the FOXO3a signaling. Because reserpine treatment activates AMPK, we examined whether reserpine treatment affects the FOXO3a dependent transcription. For this purpose, we used FHRE-Luc reporter plasmid, which measures a FOXO3a dependent transcription. HEK293T cells were transfected with FHRE-Luc reporter plasmid. Twenty four h after transfection, cells were treated with various concentrations of reserpine (0, 1, 2.5, 5 and 10 μM) for 24 h and luciferase activity was measured. The luciferase activity was normalized to renilla luciferase activity and the experiment was performed in quadruplicate. The graph shows the average and the standard deviation (SD). Control vs. reserpine treatment, *P < 0.005, **P < 0.001.

Reserpine treatment induces MCF-7 cell death – Since AMPK was activated by the reserpine treatment in PC12 cells, we also examined the activation level of AMPK in the human breast cancer MCF-7 cells. MCF-7 cells were incubated with various concentrations of reserpine (0, 1, 2.5, 5 and 10 μM) and we examined the phosphorylation level of AMPK (T172). Reserpine treatment induced the phosphorylation level of AMPK (T172) in MCF-7 cells (Fig. 4A). Like PC12 cells, the phosphorylation level of AMPK is markedly increased at 10 μM of reserpine treatment, and these results suggest that 10 μM of reserpine is required for the efficient activation of AMPK. Many studies support that the activation of AMPK induces the tumor cell death. Because reserpine induced AMPK activation, we examined whether reserpine induces MCF-7 cell death. We treated various concentration of reserpine...
to MCF-7 cells for 24 h and 48 h, and measured the cell viability using MTT assay. While the viability of MCF-7 cells was slightly decreased by reserpine treatment for 24 h, the viability of MCF-7 cells was markedly decreased by 10 μM of reserpine treatment for 48 h (Fig. 4B). These results indicate that reserpine treatment induces MCF-7 cell death. In addition, we performed the cell cycle analysis of reserpine treated MCF-7 cells, and we found that reserpine treatment increased the sub-G1 population indicating that apoptosis was induced by reserpine treatment. Both PC12 cells and MCF7 cells were less sensitive to the low concentration of reserpine (< 5 μM) for the short treatment (24 h). Recently, we showed that reserpine treatment inhibited the autophagic flux. Because the activation of AMPK contributes to autophagy induction, reserpine may have multiple roles in the regulation of autophagy. Reserpine may activate autophagosome formation by activating AMPK and blocked autophagic flux. These dual regulatory roles in autophagy will induce the accumulation of autophagosomes in the cytoplasm and may contribute to the cytotoxicity of reserpine. Therefore, reserpine is potentially important to study autophagy as well as AMPK activation.

Here, we demonstrated that reserpine treatment induces the AMPK activation and decreases the viability of MCF-7 cells. We showed that both AMPK activation and MCF-7 cell death were markedly increased at 10 μM of reserpine treatment. However, it is not determined yet whether AMPK activation is mainly responsible for the MCF-7 cell death. Further study will be required to elucidate the role of AMPK activation by reserpine in tumor cell death.

Acknowledgements

This study was supported by a grant from the Leading Space Core Technology Development Program through the National Research Foundation funded by the Ministry of Science, ICT & Future Planning (2013M1A3A302042433).

References

2003, 546, 113-120.

Received January 18, 2017
Revised May 4, 2017
Accepted May 4, 2017