Compound C Increases Sestrin2 Expression via Mitochondria-Dependent ROS Production

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Compound C is a widely used chemical inhibitor that down-regulates AMP-activated protein kinase (AMPK) activity. However, it has been suggested that compound C exerts AMPK-independent effects in various cells. Here, we investigated whether compound C induces Sestrin2 (SESN2), an antioxidant enzyme induced by diverse stress. In addition, the mechanism responsible for SESN2 induction by compound C was determined. Our results showed that compound C increased SESN2 protein expression in HepG2 cells in a concentration- and time-dependent manner. The induction of SESN2 mRNA was also observed in cells treated with compound C. Increase of SESN2 luciferase activity confirmed transcriptional regulation by compound C and this substance also increased nuclear factor erythroid 2 (NF-E2)-related factor-2 (Nrf2) phosphorylation, which implies that Nrf2 was involved in SESN2 induction. Next, we sought to demonstrate whether production of reactive oxygen species (ROS) accompanied SESN2 expression. Compound C increased ROS production, but this effect was prevented by pretreatment with antioxidants or the mitochondrial complex I inhibitor. Moreover, cyclosporin A, an inhibitor of pore formation in the mitochondrial membrane, attenuated compound C-induced SESN2 induction. However, overexpression of a constitutively active form of AMPK was not able to abolish SESN2 induction by compound C, which implies that its action is independent of AMPK inhibition. In conclusion, this is the first study demonstrating that compound C alters mitochondrial function and induces ROS production, which ultimately leads to phosphorylation of Nrf2 and induction of SESN2.

Key words compound C; Sestrin2; reactive oxygen species (ROS); mitochondria; nuclear factor erythroid 2 (NF-E2)-related factor-2 (Nrf2)

Compound C is a cell-permeable pyrazolopyrimidine derivative that has been widely used as a chemical inhibitor of AMP-activated protein kinase (AMPK). Many reports have provided evidence that AMPK is deeply involved in the regulation of energy metabolism. AMPK is activated in response to an increase of the AMP/ATP ratio and switches on catabolic pathways including glucose uptake, glycolysis, and fatty acid oxidation. Moreover, various cellular events such as proliferation, autophagy, and apoptosis are regulated by AMPK. In this regard, the value of AMPK as a drug target is recognized. SESN2 is a stress-inducible antioxidant gene. SESN2 can also affect redox-homeostasis via the regeneration of peroxiredoxins (PRXs). Rescue of PRX from overoxidation by SESN2 plays a critical role in the control of hydrogen peroxide concentration. ROS are important stimuli necessary to induce SESN2. SESN2 can also affect redox-homeostasis via the regeneration of peroxiredoxins (PRXs). Rescue of PRX from overoxidation by SESN2 plays a critical role in the control of hydrogen peroxide concentration. ROS are important stimuli necessary to induce SESN2. SESN2 can also affect redox-homeostasis via the regeneration of peroxiredoxins (PRXs). Rescue of PRX from overoxidation by SESN2 plays a critical role in the control of hydrogen peroxide concentration.

Although compound C is still being adopted as an AMPK inhibitor, numerous reports have indicated that the mechanisms of compound C are not restricted to AMPK targeting only. Based on the previous reports that the function of ROS production is inevitable and important for cells in order for them to control a diverse of functions ranging from cell homeostasis to cell death. However, excessive ROS cause oxidative modifications of cell components, subsequently inducing detrimental effects on the cells. Hence, cells have diverse antioxidant systems to maintain redox homeostasis. The close relationship between Sestrin2 (SESN2) and the adaptive response to various types of stress are well recognized. SESN2 is a stress-inducible antioxidant gene. Initially, SESN2 was known as Hs95, which is involved in p53-dependent regulation of autophagy and cell viability. SESN2 can also affect redox-homeostasis via the regeneration of peroxiredoxins (PRXs). Rescue of PRX from overoxidation by SESN2 plays a critical role in the control of hydrogen peroxide concentration. ROS are important stimuli necessary to induce SESN2. Collectively, SESN2 can be considered as an essential regulator of the cellular adaptive response against various stresses.

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SESN2 is highly related with AMPK, and that SESN2 is an nuclear factor erythroid 2 (NF-E2)-related factor 2 (Nrf2)-mediated antioxidant gene, we investigated the effects of compound C on SESN2 regulation. The present study is the first to report that compound C could induce SESN2 and that this effect is mediated by ROS production.

MATERIALS AND METHODS

Materials Compound C and cycloheximide were provided by Calbiochem (San Diego, CA, U.S.A.). Anti-SESN2 antibody was purchased from Proteintech (Chicago, IL, U.S.A.). Phospho-acetyl-CoA carboxylase (ACC) antibody was supplied by Cell Signaling Technology (Danvers, MA, U.S.A.). Anti-Nrf2 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-beta-actin antibody, N-acetyl-L-cysteine (NAC), rotenone, trolox, and other reagents were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.).

Cell Culture The HepG2 cell lines were purchased from the American Type Culture Collection (Manassas, VA, U.S.A.). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, U.S.A.). Cells were maintained in Dulbecco’s modified Eagle’s medium containing 1% FBS for 14 h. The transfected cells were then incubated in minimum essential media containing 1% FBS for 14 h. Finally, the activity of luciferase in the lysates was detected using the dual-luciferase reporter assay system (Promega, Madison, WI, U.S.A.) as previously described.23

Infection of Recombinant Adenoviral Constitutively Active Form of AMPK (CA-AMPK) Adenovirus was diluted in DMEM containing 10% FBS, and then HepG2 cells were infected with adenovirus at a multiplicity of infection (MOI) of 50 for 36 h. Adenovirus that expresses LacZ (Ad-LacZ) was used as an infection control. Efficiency of infection was consistently >90% with this method.

Measurement of ROS Generation The level of hydrogen peroxide production was determined by measuring the increase in dichlorofluorescein fluorescence after treatment with DCFH-DA, a cell-permeable nonfluorescent probe that is cleaved by intracellular esterases and oxidized primarily by hydrogen peroxide. Cells were stained with 10 μM DCFH-DA during the final hour of incubation. The cells were then harvested by trypsinization and washed twice with PBS. The intensity of the fluorescence in the cells was measured using a fluorescence microplate reader (Gemini XPS; Molecular Device, Sunnyvale, CA, U.S.A.). ROS production was normalized to the protein concentration in each treated sample and calculated relative to the vehicle-treated control.

Mitochondrial Membrane Permeability Analysis Rhodamine 123, a membrane-permeable cationic fluorescent dye, was used to determine changes in mitochondrial membrane permeability. Cells were treated with 0.05 μM/mL rhodamine 123 for 1 h and collected by trypsinization. Fluorescence intensity was measured and calculated as described in ROS production.

Statistical Analysis For each statistically significant effect of treatment, one-way ANOVA was used for comparisons between multiple group means. The data were expressed as mean±standard error (S.E.) from at least three independent experiments. The criterion for statistical significance was set at p<0.05 or p<0.01.

RESULTS

Compound C-Induced SESN2 Expression in HepG2 Cells SESN2 is an inducible antioxidant enzyme that is regulated by the Nrf2-antioxidant response element (ARE) pathway under oxidative stress. Recently, it has been reported as an upstream regulator of AMPK in response to various stresses.2,28 Given that compound C inhibited AMPK activity and also affects Nrf2-ARE signaling,9 the effects of compound C on SESN2 expression were tested in HepG2 cells. Cells were treated with various concentrations of compound C (from 1 to 20 μM) and then the protein levels of SESN2 were observed. Immuno blot analysis showed that compound C increased the expression of SESN2 when compared with vehicle-treated cells (Fig. 1A). In accordance with protein levels, compound C treatment resulted in a marked increase in SESN2 mRNA level (Fig. 1B). A significant increase in SESN2 protein or mRNA level was detected starting from 3–6 h after compound C treatment and peaked at 24 h (Fig. 1C) or 12 h (Fig. 1D), respectively.
respectively. These results suggest that compound C positively regulates SESN2 at the mRNA and protein levels.

**Transcriptional Induction of SESN2 by Compound C**

Next, we attempted to elucidate the detailed mechanism for SESN2 induction by compound C. To examine whether compound C transcriptionally regulates SESN2 expression, the mRNA level of SESN2 was examined after treatment with the transcriptional inhibitor actinomycin D. RT-PCR analyses showed that the compound C-induced increase in SESN2 mRNA level was completely blocked by actinomycin D treatment (Fig. 2A). Moreover, a protein synthesis inhibitor, cycloheximide, attenuated compound C-induced SESN2 expression (Fig. 2B). To confirm transcriptional induction by compound C, cells were transiently transfected with a human SESN2 promoter-driven luciferase construct and then treated with compound C. Compound C significantly increased luciferase activity (Fig. 2C). Previously, we had identified an ARE within the promoter of the SESN2 gene and reported that Nrf2 activation is involved in SESN2 expression. In addition, Liu et al. showed that compound C activates the Nrf2-ARE signaling pathway, which contributes to heme oxygenase-1 (HO-1) induction. To further examine whether the induction of SESN2 by compound C was accompanied by Nrf2 activation, the phosphorylation of Nrf2 was examined. The phosphorylated Nrf2 was increased early after compound C treatment and was maximally elevated at 1 h (Fig. 2D). However, treatment of compound C had no effect on total Nrf2 level (Fig. 2D). Collectively, these results indicate that compound C upregulates...
transcriptional induction of SESN2 via Nrf2 activation.

Role of ROS Production by Compound C in SESN2 Induction Several studies have reported that elevated ROS levels in various cells are major stimuli to induce SESN2 expression.\textsuperscript{24,29} Given that HO-1 has been shown to be increased by compound C in an ROS-dependent manner, we decided to examine whether ROS production was involved in SESN2 induction. ROS production was assessed by the increase in dichlorofluorescein fluorescence. When compared to vehicle-treated cells, compound C treated cells showed a significant increase in ROS production (Fig. 3A). In subsequent experiments, we tested whether antioxidants could affect compound C-induced SESN2 expression. Pretreatment with antioxidants, including trolox and NAC, blocked the compound C-induced
SESN2 expression (Fig. 3B). Similarly, pretreatment with rotenone, a mitochondrial complex I inhibitor which lowers ROS levels through inhibiting the mitochondrial respiratory chain,30) completely attenuated compound C-induced SESN2 induction. Moreover, pretreatment with antioxidants significantly abolished the increase of Nrf2 phosphorylation (Fig. 3C).
by compound C (Fig. 3D). Taken together, these results indicate that the SESN2 induction by compound C is associated with ROS production.

**Role of Compound C-Induced Mitochondrial Membrane Potential Transition in SESN2 Induction** In hepatocytes, mitochondria are the major site for ROS production under pathological conditions. The concept that mitochondrial dysfunction plays a critical role in the process of ROS production has been generally accepted. Therefore, in an attempt to correlate mitochondrial damage with SESN2 induction by compound C, mitochondrial membrane permeability was measured using rhodamine 123 staining. Since rhodamine 123 is a membrane-permeable, cationic fluorescent molecule that binds to the mitochondrial membranes, loss of fluorescence indicates mitochondrial dysfunction. When cells were incubated in compound C for 1–6 h, the intensity of rhodamine 123 fluorescence significantly decreased early after treatment (Fig. 4A). This result indicates that compound C exerts a deleterious effect on mitochondria. To address whether mitochondrial impairment is upstream of SESN2 induction by compound C, additional experiments were conducted with cyclosporin A. Cyclosporin A inhibits pore formation in the mitochondrial membrane, which enables it to prevent mitochondrial damage. Pretreatment with cyclosporin A was found to decrease SESN2 induction by compound C (Fig. 4B). These results strongly support the hypothesis that the mitochondrial damage caused by compound C is linked to ROS-dependent SESN2 induction.
**Effects of AMPK Inhibition by Compound C on SESN2 Induction**  
As an effort to identify involvement of AMPK inhibition by compound C, we next examined the effect of CA-AMPK on compound C-induced SESN2. Overexpression of CA-AMPK was not able to reverse SESN2 induction by compound C (Fig. 5A). Increase of ACC phosphorylation indicated overexpression of CA-AMPK (Fig. 5A). Therefore, these results suggest compound C stimulates SESN2 gene expression in HepG2 cells via ROS production (Fig. 5B), which are probably independent of AMPK inhibition.

**DISCUSSION**

In the present study, we reported that compound C, a potent inhibitor of AMPK, can induce Nrf2-dependent SESN2 expression. Compound C altered mitochondrial membrane potential and increased ROS production, which lead to SESN2 induction.

To evaluate the role of AMPK in various diseases, many reports have frequently used compound C in *in vivo* and *in vitro* experimental models.11,13 Initially, Zhou et al. identified and characterized compound C as a potent AMPK inhibitor.11 However, like any chemical inhibitor, it has shown other effects independent of its initially reported actions. Compound C induces cell cycle arrest and autophagy via the Akt/mTOR signaling pathway in glioma cells, but its inhibition of AMPK is not involved.15,35 Moreover, it represses intercellular adhesion molecule-1 and vascular cell adhesion molecule expression via nuclear factor-kappa B (NF-kB) inhibition independent of AMPK inhibition.30 In addition, a previous study by Liu et al. reported that HO-1 was induced by exposure to compound C and that ROS production accompanied this process.30 We also found that overexpression of the CA-AMPK could not reverse compound C-induced SESN2 expression (Fig. 5A). Taken together, these reports imply that compound C can exert distinct actions aside from AMPK inhibition in cells.

It is well recognized that ROS induce antioxidant genes including HO-1 and SESN2 by transcriptional modulation of Nrf2 and ARE activation.9,22,23 In the present study, we found that compound C induced Nrf2 phosphorylation (Fig. 2D). Since phosphorylation of Nrf2 enables it to release Nrf2 from an inhibitor regulatory of Nrf2, Kelch-like ECH-associated protein 1,37 an increase of Nrf2 phosphorylation means Nrf2 activation. We also observed that SESN2 luciferase activity containing an ARE was also increased by compound C (Fig. 2C). Pretreatment with antioxidants blocked compound C-induced Nrf2 phosphorylation and SESN2 induction (Fig. 3). Collectively, compound C activates ROS-dependent Nrf2 signaling, which leads to SESN2 induction.

Interestingly, Emerling et al. reported that compound C negatively regulates hypoxic activation of hypoxia-inducible factor-1 (HIF-1) by inhibition of mitochondrial ROS production.30 Since HIF-1 is also a transcription factor that induces SESN2, these results were controversial. However, under hypoxic conditions, inhibition of mitochondria may contribute to repression of ROS production.30 Therefore, inhibition of mitochondrial function by compound C can repress ROS production and HIF-1 mediated hypoxic adaptation. In normoxic conditions, the disturbed functioning of mitochondria can cause ROS production.30 Therefore, under normoxia, compound C may chiefly increase ROS production in mitochondria. Previous papers also gave evidence that compound C plays a role as a pro-oxidant.9,11 Moreover, a previous report showed that compound C inhibition of fas-associated protein with death domain-like apoptosis regulator and myeloid cell leukemia-1 in renal cancer cells was mediated by ROS, which indicates that distinct actions of compound C might originate via ROS production.23

Hepatic parenchymal cells including HepG2 produce ROS mostly from mitochondria.30 Therefore, we examined whether compound C altered mitochondrial function. Compound C caused a significant decrease in mitochondrial membrane potential after treatment (Fig. 4A). Membrane potential of mitochondria is important to generate ATP, therefore its loss directly indicates collapse of mitochondrial homeostasis and subsequently ROS can leak out into the cytosol.30 The recovery of mitochondrial function by cyclosporin A antagonized compound C-induced SESN2 expression (Fig. 4B). These results suggest the possibility of association between compound C and mitochondrial membrane pore. In other words, compound C exerts a direct effect on mitochondria and that its actions might promote ROS production. However, further detailed study is needed to understand the mechanisms underlying the effects on mitochondria and ROS production by compound C.

In general, pharmacological inhibitors can acquire its usefulness when selectivity and potency of inhibitors for target molecule are validated. To inhibit activity of AMPK in various cells, between 5 and 20 μM of compound C is generally used.35,38 In addition, 20–40 μM of compound C can significantly antagonize ACC inactivation by 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) or metformin in cultured hepatocytes.31 As shown Fig. 1A, 5 μM or more of compound C significantly induced SESN2 expression. Hence, the present study indicates that possible targets of compound C are not only AMPK and pharmacologically relevant concentrations of compound C show pleiotropic actions. Therefore, we suggest that selectivity of compound C as a pharmacological inhibitor of AMPK should be reconsidered when the role of AMPK is accessed in various experiments.

In conclusion, compound C induces ROS production and activates Nrf2, thereby increasing SESN2 expression. Although the specific mechanisms that trigger ROS production by compound C are still unclear, it is obvious that pharmacologically relevant concentrations of compound C affect redox sensitive pathways. These findings show the potential of compound C as a pharmacological modulator irrespective of its inhibition of AMPK activity.

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**Conflict of Interest**  
The authors declare no conflict of interest.

**REFERENCES**


2) Hardie DG, Ross FA, Hawley SA. AMPK: a nutrient and energy


