Danthron activates AMP-activated protein kinase and regulates lipid and glucose metabolism in vitro

Rong ZHOU¹, Ling WANG¹, Xing XU², Jing CHEN², Li-hong HU², Li-li CHEN², *, Xu SHEN¹, 2, *

¹School of Pharmacy, East China University of Science and Technology, Shanghai 200237, China; ²State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China

Aim: To discover the active compound on AMP-activated protein kinase (AMPK) activation and investigate the effects of the active compound 1,8-dihydroxyanthraquinone (danthron) from the traditional Chinese medicine rhubarb on AMPK-mediated lipid and glucose metabolism in vitro.

Methods: HepG2 and C2C12 cells were used. Cell viability was determined using MTT assay. Real-time PCR was performed to measure the gene expression. Western blotting assay was applied to investigate the protein phosphorylation level. Enzymatic assay kits were used to detect the total cholesterol (TC), triglyceride (TG) and glucose contents.

Results: Danthron (0.1, 1, and 10 µmol/L) dose-dependently promoted the phosphorylation of AMPK and acetyl-CoA carboxylase (ACC) in both HepG2 and C2C12 cells. Meanwhile, danthron treatment significantly reduced the lipid synthesis related sterol regulatory element-binding protein 1c (SREBP1c) and fatty acid synthetase (FAS) gene expressions, and the TC and TG levels. In addition, danthron treatment efficiently increased glucose consumption. The actions of danthron on lipid and glucose metabolism were abolished or reversed by co-treatment with the AMPK inhibitor compound C.

Conclusion: Danthron effectively reduces intracellular lipid contents and enhanced glucose consumption in vitro via activation of AMPK signaling pathway.

Keywords: 1,8-dihydroxyanthraquinone (danthron); traditional Chinese medicine; AMPK; SREBP1c; fatty acid synthetase; cholesterol; triglyceride; glucose consumption

Introduction

Impaired glucose and lipid metabolism occur commonly in several major diseases such as obesity, type 2 diabetes, hypertension and atherogenic dyslipidemia[1, 2]. AMP-activated protein kinase (AMPK), which is expressed in the liver, brain, and skeletal muscle tissues, has been a key sensor in the regulation of fatty acid and glucose homeostasis[3] and a potential therapeutic target for the prevention and treatment of diabetes[4, 5]. AMPK becomes activated when phosphorylation takes place at threonine-172 by the AMPK kinases[6]. In the liver, AMPK activation increases fatty acid oxidation and inhibits cholesterol synthesis, triglyceride synthesis and gluconeogenesis. In muscle, AMPK promotes fatty acid oxidation and glucose uptake. In adipose tissue, activated AMPK increases fatty acid oxidation and glucose uptake while attenuating adipocyte lipolysis and lipogenesis[3–5].

Acetyl-Coenzyme A carboxylase (ACC) is a direct downstream target of AMPK, and its active dephosphorylated form can catalyze the conversion of acetyl-CoA to malonyl-CoA in the de novo lipid synthesis pathway. AMPK phosphorylates and inactivates ACC, which inhibits the conversion from acetyl-CoA to malonyl-CoA, and thus increases the transport of fatty acids into the mitochondria for oxidation[7]. Meanwhile, AMPK activation reduces the expression of sterol regulatory element-binding protein 1c (SREBP1c) and its downstream gene fatty acid synthetase (FAS) to control triglyceride synthesis[8]. Moreover, activated AMPK regulates cholesterol synthesis by phosphorylating and inactivating 3-hydroxy-3-methylglutaryl-CoA reductase[9]. In addition, in adipose and skeletal muscle tissues, AMPK promotes the translocation of glucose transporter type 4 (Glut4) to the cellular membrane to transport glucose[10, 11]. In the liver, AMPK helps regulate glucose homeostasis by reducing the gluconeogenic key genes including phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase)[12, 13]. Furthermore...
more, the AMPK activator AICAR[11] could enhance the Glut2-dependent glucose uptake in the jejunum, implying a possible new role for AMPK[13].

Due to the important regulation of AMPK on glucose and lipid metabolism, novel agents to activate AMPK were of great interest. Currently, in addition to known activators such as AICAR[11], metformin (Met)[13], berberine[14], and A-769662[10], more active natural products have been increasingly discovered from the plant kingdom. In particular, many effective AMPK activators have come from traditional herbs[15–17], such as the natural anthraquinone derivative emodin, which ameliorates dyslipidemia in high-fat-diet rats by activating AMPK[15]. As with emodin, 1,8-dihydroxyanthraquinone (danthron, Figure 1A) is also a natural anthraquinone derivative extracted from the traditional Chinese medicine rhubarb. Given the structural similarity of these anthraquinones, we thus explored the potential role of danthron in regulating AMPK activation.

Previously, danthron was used as a laxative[18]. Since danthron induced DNA damage and inhibited DNA repair, it exhibited efficient anticancer[19] and antiproliferative[20] and antimetastatic[20] effects. In addition, we previously found that danthron was a retinoic X receptor antagonist and that it helped improve insulin resistance in high-fat diet-induced obesity mice[20]. Our current work shows that danthron functions in regulating glucose and lipid metabolism by activating AMPK in both HepG2 and C2C12 cells. These findings prolong the pharmacological actions of danthron and provide a new clue for anti-diabetic drug development.

Methods and materials
Reagents and plasmids
Cell culture plastic ware was purchased from Corning Inc (Corning, New York, USA). All cell culture media and sera were purchased from Invitrogen (Carlsbad, CA, USA). Danthron and guggulsterone (GS) were purchased from J&J&K Chemical Ltd (Beijing, China); Metformin (Met), compound C, TO901317 (TO90) and methylthiazolyldiphenyl-tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St Louis, MI, USA); RNAiso reagent kit, PrimeScript™RT reagent kit, and SYBR Premix Ex Taq Real-time PCR master mix were kindly donated from TakaRa (Otsu, Shiga, Japan). Anti-GAPDH antibody was from KangCheng Bio-tech (Shanghai, China). The other antibodies were from Cell Signaling Technology (Beverly, Massachusetts, USA). The SuperSignal West Dura and Pico substrates were from Thermo Fisher (San Jose, CA, USA). Lipofectamine 2000 Transfection Reagent was purchased from Invitrogen. The Dual Luciferase Reporter Assay kit was from Promega (Madison, Wisconsin, USA). Other solvents and reagents were purchased in analytical grade and used without further purifications. The pGL3-ABC1-promoter-luc, pGL3-ABCG1-promoter-luc and pRL-SV40 plasmids were kindly donated by Prof Gerd SCHMITZ (Institute for Clinical Chemistry, University of Regensburg), Prof Steven L SABOL (Laboratory of Developmental Biology NHLBI Division of Intramural Research) and Prof J Larry JAMESON (Department of Medicine, Northwestern Memorial Hospital), respectively.

Cell culture and differentiation
HepG2 cells were cultured in MEM medium, and C2C12 cells were cultured in DMEM medium. All cells were cultured at 37°C in media supplemented with 10% FBS and in a humidified atmosphere with 5% CO2. After 100% confluence, C2C12 cells were differentiated using DMEM containing 2% horse serum for 4 d to obtain mature myotubes.

Cell viability assay
Cells were treated with the indicated compounds for 24 or 48 h, and then incubated with 0.5 mg/mL MTT in culture media for another 4 h. Cell viability was evaluated using MTT assay as previously described[22].

Transient transfection and promoter activity measurement
HepG2 cells were transfected with pGL3-ABCA1 promoter-luc or pGL3-ABCG1 promoter-luc and pRL-SV40 plasmids using Lipofectamine 2000 Transfection Reagent according to the manufacturer’s protocols. At 6 h post-transfection, the cells were incubated with danthron (0–20 µmol/L), TO90 (2 µmol/L) or DMSO for 24 h. Luciferase activity was measured using the Dual Luciferase Reporter Assay kit.

Western blotting
Cells were harvested and separated by SDS-PAGE, and then transferred into the nitrocellulose membrane. Corresponding antibodies were incubated, and the SuperSignal West Dura (and Pico) substrates were applied for signal detection. ImageQuant LAS 4000 mini (GE Health) was used for signal collection.

Real-time RT-PCR
Starved overnight, cells were incubated with the compounds for 24 h, and then total RNA was extracted and reversed using a corresponding reagent kit (TAKARA). Real-time PCR was performed using SYBR Green Real-time PCR master mix on the DNA Engine Opticon 2 System (Bio-Rad Laboratories, USA). The relative mRNA levels were normalized to β-actin or GAPDH. PCR primer pairs are listed in Table 1.

TG/TC content measurement
HepG2 and mature C2C12 cells were starved overnight and treated with indicated compounds in a FBS-free medium for 24 h. The samples’ harvest, treatment and detection were performed as described previously[25]. Cellular total cholesterol (TC) and triglycerides (TG) were measured using the kits (Beihua-kangtai Clinical Reagent Limited Company, Beijing, China).

Glucose consumption assay
HepG2 and mature C2C12 cells were planted in 96-well plates with 6 blank wells without cells as the control. After the cells reached 80% confluence, the medium was replaced by phenol red- and FBS-free DMEM adding 11.1 mmol/L glucose and
Table 1. The primer pairs sequences.

| Gene       | Forward primer (5′–3′) | Reverse primer (5′–3′) |
|------------|------------------------|------------------------|
| SREBP1c    | GGATTGCACCTTGGAGACAGT  | AGGATGCCTGATGGCACTG    |
| FAS        | TTGGACATGTGCAGGAC      | GCCATTCAACCTAGACAGGTC  |
| ABCA1b     | ACCAATGTGAAACACATG     | GAGTCGCTGGACAGGAACAG    |
| ABCG1b     | CTGTGCCGTTGTCGGTTT     | CCACTGTAGGTACGTTGGGAT  |
| GAPDHb     | ACAGCAACAGGGCTGTGGAC   | TTTGAGGGTGCAATGCGAACTT |
| β-actin    | CATGTCAGGTGCATCAGGC    | CTCCCTAATGTCAACCGAAG    |
| SREBP1c    | CCGGAGGGCTCTCTCAGG     | GTGTCAGTGGCGTTACAGG    |
| FAS        | GGCTCTAGGTTACCAAGCC    | CCAGTGTTGCTCTCAGGA     |
| ABCA1b     | GCTGGTTGCGCTTCAAGGG    | GTAGCTCAGGGCCTACAGAGAT |
| ABCG1b     | CCTTCTTCTCTTGTACCCGAG  | CCGGAGCTCCATGTAAGG     |
| GAPDHb     | ACAGCAACAGGGCTGTTGAC   | TTTGAGGGTGCAACCGAAGTT  |

*a *homo sapiens primers; *b *mus musculus primers.

0.2% BSA. Meanwhile, cells were incubated with the indicated compounds for 24 h. The glucose content in the medium was then determined by the glucose oxidase method according to the manufacturer’s protocol (Rsbio, Shanghai, China). Next, the MTT assay was performed to evaluate cell viability and adjust the glucose consumption values. Glucose consumption =glucose concentration of blank wells – glucose concentration of wells plated with cells.

Statistical analysis
Results were presented as mean±SEM for at least three separate experiments in each group. Differences between groups were examined for statistical significance using Student’s t-test or one-way ANOVA. The value of *P*<0.05 was considered to be a statistically significant difference.

Results
Danthron activated AMPK and attenuated lipid accumulation in HepG2 cells
To evaluate the effect of danthron on AMPK phosphorylation in HepG2 cells, phosphorylated AMPK (p-AMPK) and total AMPK (t-AMPK) were detected by Western blot assay. First, we identified the effect of danthron on HepG2 cell viability to determine the appropriate concentration for later experiments. We found that 10 µmol/L danthron/24 h might be safe for HepG2 cells (Figure 1C). With 80% confluence, HepG2 cells were incubated with danthron (0.1–10 µmol/L) in FBS-Free media for 8 h. Subsequently, cells were harvested for Western blot assay. As shown in Figure 1B, danthron increased the p-AMPK protein in a dose-dependent manner, and no changes in t-AMPK protein were observed. It was suggested that danthron activated AMPK in HepG2 cells. In addition, we also detected the phosphorylation of ACC, which was the downstream protein regulated by AMPK. As expected, the phosphorylated ACC, but not the total ACC, was also augmented under the treatment of danthron (Figure 1B).

Given that AMPK modulated lipid biosynthesis by controlling its downstream gene expression at the transcriptional level, such as SREBP1c and FAS, we therefore analyzed the mRNA levels of these genes. HepG2 cells were starved overnight and treated with different concentrations of danthron for another 24 h. Both SREBP1c and FAS mRNA levels were attenuated by danthron in a dose-dependent manner (Figure 1D and 1E). Meanwhile, cells were treated as described above, and TG and TC contents were measured using GS as a positive control. As expected, 40 µmol/L GS obviously lowered both TG and TC contents. Similar actions were observed in danthron-treated groups in a dose-dependent manner (Figure 1F and 1G).

In addition, considering the protein expression of ABCA1 and ABCG1 regulated by AMPK in endothelial cells, we further explored the corresponding effects of danthron by determining their promoter activities in HepG2 cells. As shown in Figure 2A and 2B, danthron increased the promoter activities of ABCA1 and ABCG1 dose-dependently. Meanwhile, we detected the effects of danthron on ABCA1 and ABCG1 endogenous expressions in HepG2 cells by RT-PCR assay. We found that the mRNA levels of the two genes were both increased with danthron treatment (Figure 2C and 2D).

All of the above-mentioned results suggested that danthron activated AMPK and regulated lipid accumulation in HepG2 cells.

Danthron increased AMPK activation and attenuated lipid accumulation in C2C12 cells
Skeletal muscle is another important tissue that responds to the activation of AMPK as well as the liver. Thus, we detected the probable effects of danthron in C2C12 cells. Mature C2C12 cells were treated with FBS-free DMEM containing danthron (0.1–10 µmol/L) for 8 h, and then Western blot assay was performed to analyze the changes in protein expression. As shown in Figure 3A, both AMPK and ACC phosphorylation levels were increased in a dose-dependent manner with the administration of danthron. In addition, SREBP1c and FAS mRNA levels were also determined. We found that danthron efficiently attenuated the gene expressions of SREBP1c and FAS in C2C12 cells (Figure 3B and 3C). Correspondingly, we observed the ABCA1 and ABCG1 mRNA levels, which were found to be modestly increased in danthron-treated cells (Figure 2E and 2F). Subsequently, we also determined the contents of intracellular TG and TC and observed their corresponding decreases in response to different concentrations of danthron (Figure 3D and 3E). These results suggested that danthron increased the activation of AMPK and further regulated lipid metabolism in C2C12 cells.

The regulation of danthron on lipid metabolism was AMPK-dependent
To investigate whether the regulation of danthron was AMPK-dependent, we next tested the corresponding effects of danthron on lipid metabolism by co-incubation with an AMPK inhibitor compound C. HepG2 cells were treated with FBS-free media containing the indicated compounds with or without compound C for 8 h. The AMPK activator metformin (Met) was used as a positive control. We found that both dan-
thron and Met could obviously enhance the phosphorylation of AMPK and ACC, and such increases were reversed totally or partially by the compound C treatment (Figure 4A). The effects of Met (Figure 4A) were in accordance with the previous results, thus indicating the effectiveness of our study. The SREBP1c and FAS gene expressions were also detected by the RT-PCR method. As shown in Figure 4B and 4C, both danthron and Met significantly reduced the mRNA levels of these genes, and the effects were more or less reversed by compound C.

Subsequently, we also determined the corresponding effects of danthron in C2C12 cells. Cell treatment and compounds administration were described above. As expected, we found increases in p-AMPK and p-ACC in danthron-treated cells and decreases in these proteins in compound C-treated cells (Figure 4D). Moreover, the danthron-induced increases in p-AMPK and p-ACC were obviously reversed after co-incubation with compound C (Figure 4D). Both danthron and Met reduced the mRNA levels of SREBP1c and FAS in C2C12 cells. Meanwhile, compound C reversed the effects of danthron on these gene expressions (Figure 4E and 4F).

These results suggest that danthron regulated the lipid metabolism-related genes primarily through the activation of AMPK.

**Danthron promoted glucose consumption in both HepG2 and C2C12 cells**

Because AMPK activation could modulate glucose metabolism, we investigated the potential role of danthron in glucose consumption in HepG2 and C2C12 cells. Cells were treated and measured as described in the Methods section. The effects of danthron and Met without or with compound C are shown in Figure 5A and 5B (HepG2 cells) and Figure 5C and 5D (C2C12 cells). In both cell lines, as with Met, danthron
increased glucose consumption in a dose-dependent manner (Figure 5A and 5C). Furthermore, glucose uptake was attenuated under the treatment of danthron or Met co-incubating with compound C (Figure 5B and 5D).

In addition, we also detected the effects of danthron on the key proteins involved in the insulin signaling pathway, including AKT (protein kinase B) and IR (insulin receptor). HepG2 cells were cultured overnight and incubated with different concentrations of danthron in FBS-free MEM for 8 h. Insulin was then added (16.7 nmol/L) for another 5 min, and the cells were harvested for Western blot assay. As shown in Figure 5E, HepG2 cells responded to the insulin stimulus and

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Figure 2. Increases in ABCA1 and ABCG1 promoter activities and gene expressions by danthron treatment. (A and B) The effects of danthron on ABCA1 and ABCG1 promoter activities were determined by luciferase reporter gene-based assay. T090 (T0901317, 2 µmol/L) was used as a positive control. (C and D) The gene expressions of ABCA1 and ABCG1 were detected in HepG2 cells. (E and F) The mRNA levels of ABCA1 and ABCG1 were tested in C2C12 cells. Data are presented as the mean±SEM of three independent experiments. *P<0.05, **P<0.01 vs DMSO.

Figure 3. Effects of danthron on AMPK activation and lipid accumulation in mature C2C12 cells. (A) Danthron efficiently increased the phosphorylation of AMPK and ACC. (B and C) Concentration-dependent effects of danthron on SREBP1c and FAS gene expressions were determined. (D and E) Danthron attenuated the intracellular TG and TC contents. The concentrations of danthron were indicated. Data are presented as the mean±SEM of three independent experiments. *P<0.05, **P<0.01 vs DMSO.
showed significant increases in p-AKT and p-IR. However, no increases in p-AKT or p-IR were observed in the danthron-treated cells; in fact, a downward trend was observed in the concentration of p-IR.

The above-mentioned results suggest that the promotion of glucose uptake by danthron was AMPK-dependent and p-IR/p-AKT signaling-independent.

**Discussion**

Danthron is a natural product extracted from the traditional Chinese medicine *rhubarb*. In the current work, we determined that this natural product could activate AMPK and ACC activities in both HepG2 and C2C12 cells. Further research has indicated that danthron regulates the gene expressions of lipid synthesis-related SREBP1c and FAS, and cholesterol efflux-related ABCA1 and ABCG1, reduces intracellular triglyceride and cholesterol levels, and efficiently increases glucose consumption.

Currently, few studies have reported the effects of danthron on glucose and lipid metabolism. It has been reported that danthron could modestly inhibit Na⁺, K⁺-ATPase activity, while it had no effect on glucose transport in the intestine[24]. Our previous results showed that danthron as an RXRα antagonist functioned to improve insulin tolerance in diet-induced obese mice[21]. Here, we discovered a new pharmacological function of danthron as an activator of AMPK. To identify the potential effects of danthron on AMPK activation and the related regulation of glucose and lipid metabolisms, we detected the levels of AMPK and ACC phosphorylation and their downstream gene expressions in cultured hepatic and skeletal cell lines. As expected, danthron dose-dependently increased the activation of AMPK and ACC (Figures 1B and 3A) and inhibited the downstream SREBP1c and FAS gene expressions. These actions ultimately caused efficient reduction in the intracellular lipid content. The above-mentioned actions of danthron were abolished or partially reversed by AMPK inhibitor compound C. It was suggested that the effect of danthron on lipid metabolism was primarily due to its activation of AMPK. In addition, we preliminarily attempted to evaluate the effects of danthron in diet-induced obese mice, and the results demonstrated modest reduction in serum cholesterol and triglycerides in danthron-treated mice (data not shown).

Notably, an interesting finding in our study was that danthron increased the expressions of ABCA1 and ABCG1. ABCA1 and ABCG1 are known to be involved in cholesterol efflux[25] and are expressed in hepatic and skeletal muscle cells[25–27]. Moreover, studies have revealed the regulation of such gene expression by AMPK in endothelial cells[28]. Our present results (Figure 2) showed that danthron could increase the transcriptional activities and gene expressions of ABCA1 and ABCG1 dose-dependently in HepG2 and C2C12 cells. This finding explained the possible mechanism for why danthron decreased TC content. Nevertheless, danthron as
an antagonist of the retinoic X receptor α/liver X receptor α (RXRα/LXRα) heterodimer may negatively affect the expressions of ABCA1 and ABCG1\(^{[21, 29-31]}\). Thus, the effects of danthron on the ABCA1 and ABCG1 gene expressions seem to be contradictory and complicated. The details regarding the regulation of lipid metabolism by danthron are obscure and require further clarification.

Similarly, in the glucose consumption assay, the potential effects of danthron as the RXRα antagonist on insulin signaling were detected, and no beneficial changes were obtained (Figure 5E). However, the attenuated glucose uptake by compound C co-incubation with danthron revealed that danthron exerted such effects mainly through the activation of AMPK in the process. Under this condition, the increased Glut4 translocation by AMPK activation promoted the uptake of extracellular glucose in C2C12 cells\(^{[8, 32]}\). However, further studies are needed to elucidate whether the AMPK-induced Glut2-dependent glucose uptake existed and contributed to the lowered glucose content in HepG2 cells.

Considering the anti-proliferative and pro-apoptotic properties of danthron, we identified the safe concentration at the beginning of the experiments. Thus, the results we obtained exhibited the real effects of danthron rather than cytotoxic effects. In this study, we discovered a new effect of danthron on glucose and lipid metabolism in both HepG2 and C2C12 cells through the activation of AMPK. As previously reported, another anthraquinone derivative, emodin, was deemed to activate AMPK by an indirect mechanism similar to berberine\(^{[33]}\), but no explicit evidence has been provided. Considering that danthron and emodin have similar effects and chemical structures, we hypothesized that their effects on AMPK may be similar. Thus, further study is needed to identify whether danthron acts as the direct or indirect activator of AMPK, and structural modification may be necessary for drug development in the future. Briefly, our work has determined that danthron is a new, active, natural product that induces the activation of AMPK. Danthron effectively regulated lipid and glucose metabolism. Our findings are expected to prolong the new lipid-lowering function of danthron and to provide a new clue in the search for new anti-diabetic drug discovery.
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Author contribution
Prof Xu SHEN and Prof Li-li CHEN supervised the project; Rong ZHOU, Ling WANG, and Xing XU performed the experiments; Jing CHEN and Li-hong HU analyzed data; Xu SHEN, Li-li CHEN, and Rong ZHOU contributed to the writing of the manuscript.

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