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Suppression of adenosine monophosphate-activated protein kinase selectively triggers apoptosis in activated T cells and ameliorates immune diseases

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ABSTRACT

Deficient apoptosis of activated T cells can result in immunological disorders. Molecules associated with energy and metabolisms are suggested to be involved in pathogenesis of immune diseases, but remain uninvestigated. In the present study we reported that glibenclamide exerted a new pharmacological effect on inflammatory responses by selectively triggering apoptosis of activated T cells. Glibenclamide demonstrated an inhibition on activated T lymphocytes, whereas showed no toxicity in the naive cells. This effect was mainly related with its ability to facilitate apoptosis in activated T cells with an up-regulation of cleaved-caspases and cleaved-PARP. Glibenclamide enhanced Fas expression and suppressed the expression of antiapoptotic cellular FLICE-inhibitory protein. The underlying mechanism of glibenclamide was not associated with its classical inhibitory effect on ATP-sensitive potassium channels, but due to a unique suppression on the phosphorylation of 5′ adenosine monophosphate-activated protein kinase, which was augmented during T cell activation. An *in vivo* experiment further demonstrated that glibenclamide ameliorated T-cell-mediated contact hypersensitivity in mice. Altogether, these results suggest that AMPK inhibition by glibenclamide can regulate the survival and death of T lymphocytes and be beneficial for the treatment of autoimmune diseases.

1. Introduction

Insufficient cell death of activated T cells can result in immunological disorders [1]. Apoptosis is an essential mechanism to eliminate activated T cells during the shutdown process of excess immune responses [2]. However, elimination of too many T cells unselectively can lead to immunodeficiency. Therefore, selectively inducing apoptosis of activated T cells is essential for the clearance of pathogenic injurious cells and subsequent efficient resolution of inflammation without affecting quiescent T cells.

In order to mount an adequate immune response, T cells must have an adequate and rapidly available energy regulation to activate and to sustain function. Although some metabolic changes occurring during T cell activation are characterized, such as the oxidative phosphorylation for energy in Th17 effector cells [3], the energy regulations for T lymphocytes survival are largely unexplored. ATP-sensitive potassium (K_{ATP}) channels, which are present in many tissues, including pancreatic islet cells, heart, and muscles [4], is regulated by intracellular ATP/ADP radio and energy balances [5]. Many researchers including our group have reported that pharmacological activation of these channels has anti-inflammatory effects in nervous system [6–8]. Taken together, these findings remind us to investigate the potential effects of K_{ATP} channels in the survival and death of T lymphocytes. We examined protein expression for the K_{ATP} channels subunits in T cells after concanavalin A (ConA) stimulation and further examined whether administration of a K_{ATP} channel opener or blocker might affect T cell fate. To our surprise, we found that although there was no apparent difference in expression of K_{ATP} channel proteins between the activated and naïve T cells, the K_{ATP} channel blocker glibenclamide could selectively trigger apoptosis of activated T cells.

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Moreover, this novel effect of glibenclamide is due to its inhibition on the phosphorylation of another vital energy-regulatory molecule - 5′ adenosine monophosphate-activated protein kinase (AMPK).

2. Materials and methods

2.1. Mice

Animal welfare and experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006) and the related ethical regulations of our university. All the animal experiments were approved by Nanjing University Animal Care and Use Committee (NJU-ACUC) and made to minimize suffering and to reduce the number of animals used. Female BALB/c mice (Specific pathogen-free, 8–10 weeks old, 18–22 g) were obtained from the Experimental Animal Center of Yangzhou University (Yangzhou, China).

2.2. Reagents

Glibenclamide was bought from Sigma (St. Louis, MO), dissolved at a concentration of 50 mM in 100% DMSO as a stock solution, stored at −20 °C, and diluted with medium before each experiment. The final DMSO concentration did not exceed 0.1% throughout the study. Annexin V-FITC/PI kit was purchased from BD Biosciences (San Jose, CA). Purified anti-mouse CD28 and purified anti-mouse CD3 were purchased from BD PharMingen (San Diego, CA). Fetal bovine serum (FBS) and RPMI-1640 were purchased from Life Technology (Carlsbad, CA). $CD3^+$ T cells from lymph nodes were purified using the Pan T cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) via magnetic cell separation with more than 98% purity. Antibodies for phosphorylated AMPK (Thr172), AMPKα, cleaved caspase 3, cleaved caspase 9, cleaved caspase 12, PARP, cellular FLICE-inhibitory protein (c-FLIP) and Actin were from Cell Signaling Technology (Boston, MA). Antibodies for sulfonylurea receptor (SUR)1, SUR2, inward rectified potassium channels (Kir)6.1 and Kir6.2 were from Santa Cruz Biotechnology (Santa Cruz, CA). 5-(and-6)-Carboxyfluorescein diacetate, succinimidyl ester (CFSE) - mixed isomers (Vybrant CFDA-SE Cell Tracer Kit) was from Invitrogen (Carlsbad, CA). Con A, picryl chloride (PCl), 3-(4, 5-dimethyl-2-thiazyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT), Cyclosporine A (CsA), AICAR, Compound C, 5-HD, cromakalim, and other reagents were all from Sigma (St. Louis, MO).

2.3. Cell culture and cell proliferation assay

Purified T lymphocytes from mouse lymph nodes were stimulated with 5 μg/mL Con A and incubated in 96 well-plate at a density of 5×10^5/mL cells. The cell growth was measured by MTT assay as described [9].

CFSE was stored frozen as a 10 mM stock solution until used. A pellet of 2×10^6 cells was resuspended in 1 mL of CFSE labeling solution of 10 μM incubated 10 min in 37 °C in the dark. After incubation, cells were then washed three times with PBS and centrifuged at 400 *g* for 5 min at room temperature before resuspension in the culture medium. Cells stained with CFSE were analyzed after 72 h of cell culture with flow cytometry. Data were analyzed using Cell Quest software.

2.4. Flow cytometric assay

Cell apoptosis assay was performed as described before [10]. Cells were seeded in 6-well plates at a density of 1×10^6 cells/well in RPMI 1640 medium and treated with glibenclamide (2, 10 and 50 μM) or CsA (10 μM) for 48 h in the presence of 5 μg/mL Con A. Cells were harvested and washed twice with cold PBS. After incubation of cells with 2 μL PI and 2 μL Annexin V-FITC at room temperature in the dark for 20 min, flow cytometric analysis was carried out with FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA).

2.5. Western blot

Proteins were extracted in lysis buffer, separated by SDS-PAGE (10%) and electrophoretically transferred onto polyvinylidene fluoride membranes. The membranes were probed with antibodies overnight at 4 °C, and then incubated with a HRP-coupled secondary antibody. Detection was performed using a LumiGLO chemiluminescent substrate system (KPL, Guildford, UK).

2.6. PCl-induced contact hypersensitivity

Mice were divided into seven experimental groups (n = 12 per group): (I) normal group; (II) PCl control group; (III) PCl + glibenclamide (5 mg/kg, intragastrically (*i.g.*)); (IV) PCl + glibenclamide (10 mg/kg, *i.g.*); (V) PCl + glibenclamide (20 mg/kg, *i.g.*); (VI) PCl + CsA (5 mg/kg, intraperitoneally) and (VII) PCl + repaglinide (10 mg/kg, *i.g.*). On day 0, female BALB/c mice were painted on the clipped abdomen with 5% PCl (100 μl) in ethanol/acetone (3:1). Five days after sensitization (day 5), mice were challenged by painting both sides of right ear with 0.5% PCl (30 μl) in olive oil. The ear thickness of mice was measured using a digimatic micrometer (0.001 mm, Mitutoyo, Tokyo, Japan) before and 24 h after challenge. Ear swelling was calculated as (ear thickness after challenge) − (ear thickness before challenge). Drugs were given once a day from day 0 to day 6. The same volume of vehicle (PBS) was given to the normal and model control mice.

2.7. Histological analysis

Formalin-fixed, paraffin-embedded ear tissue was sectioned at 5 μm thickness, and the sections were stained with hematoxylin and eosin. The following parameters were assessed: (1) the level of leukocyte infiltration and vascular congestion; (2) the erosion and anabrosis of epidermal cells; and (3) affection of the other side of the ears. The histological scores were assessed from 1 to 4. Final data are the average scores of each animal in the same group, and the higher score means more serious inflammation.

2.8. Statistical analysis

All results were expressed as mean ± S.E.M. of three independent experiments with each experiment including triplicate sets *in vitro*, or of twelve animals per group *in vivo*. Comparisons between groups were evaluated using one-way analysis of variance (ANOVA) followed by Dunnett's test. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Glibenclamide induces apoptosis in activated T lymphocytes

The $CD3^+$ T lymphocytes were purified and stimulated with ConA. Total cell numbers were counted using the MTT assay and a dose-dependent reduction by glibenclamide was shown in Fig. 1A. Glibenclamide also demonstrated inhibitory effects on the numbers

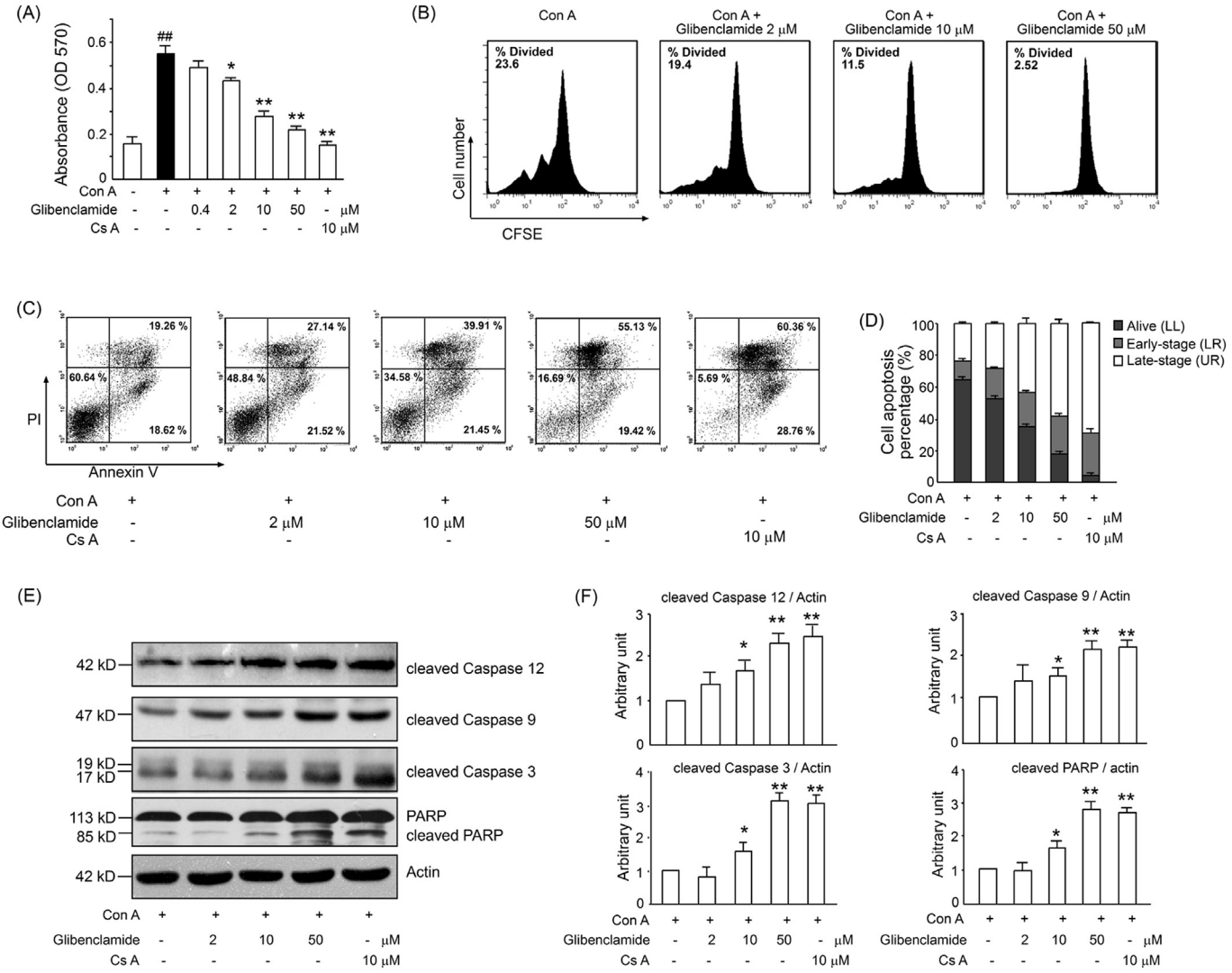

Fig. 1. Glibenclamide induced apoptosis of activated T lymphocytes. Purified mouse $CD3^+$ T cells (5×10^5/mL) were incubated at 37 °C and 5% CO_2 in the presence of Con A (5 μg/mL) as well as 0.4, 2, 10 and 50 μM glibenclamide. (A–B) T lymphocytes proliferation was determined by MTT assay and CFSE-based assay 72 h after ConA administration. (C) The apoptosis of cells was determined by Annexin V/PI staining 48 h after ConA administration. (D) Annexin V^+/PI^- and Annexin V^+/PI^+ cells of three independent experiments were shown in column statistics. (E–F) Expression of apoptotic proteins in activated T cells 36 h after ConA stimulation was shown with representative Western blot bands and data summary. Data are expressed as a histogram of mean ± SEM of three independent experiments. One-way ANOVA revealed a significant difference at $P < 0.01$. $^{\#\#}P < 0.01$, *vs.* naïve; $^{*}P < 0.05$, $^{**}P < 0.01$, *vs.* ConA control (Dunnet's test).

of T cells activated by PMA/ionomycin or anti-CD3/anti-CD28, whereas it exhibited no toxicity on naive lymphocytes (Supplementary Fig. 1). CFSE assay showed that the percentage of proliferated T cells was reduced by glibenclamide in a concentration-dependent manner: more than 50% at 10 μM and almost completely at 50 μM (Fig. 1B). Subsequently, we found that glibenclamide had no effects on the expression of the activation hallmarks for T cells - CD25 and CD69 (Supplementary Fig. 2), which indicated that glibenclamide might suppress the cell numbers via triggering the death of activated cells rather than suppressing cell activation.

It was shown by the Annexin V-FITC/PI staining that Con A-activated T cells underwent an apoptosis in a concentration-dependent manner when exposed to glibenclamide (2, 5, 10 μM) for 48 h (Fig. 1C and D). Then western blot was used to measure expression of apoptosis-related proteins. The cleaved-caspases 12, 9, 3, and cleaved-PARP in activated T cells were enhanced by glibenclamide (Fig. 1E and F).

3.2. Glibenclamide increased Fas expression and decreased c-FLIP expression in activated T cells induced by Con A

Recent reports show that c-FLIP is induced in resting T cells upon initial stimulation, and appears to prevent early death-receptor-mediated T-cell apoptosis [11,12]. However, T-cell activation also leads to the induction of interleukin (IL)-2, which in turn down-regulates c-FLIP expression and sensitizes T cells to cell death [13]. Then the activation of Fas initiates a signaling cascade leading to apoptosis [14]. Therefore, c-FLIP-Fas signaling may be regarded as the switch to facilitate apoptosis of activated T cells.

As shown in Fig. 2A, glibenclamide augmented Fas expression in Con A-activated $CD4^+$ T cells in a concentration-dependent manner. Glibenclamide also induced the expression of Fas in a time-related manner (Fig. 2B). In addition, T cells activated by Con A for 24 h had significantly increased expression of c-FLIP, which was down-regulated after glibenclamide treatment (Fig. 2C).

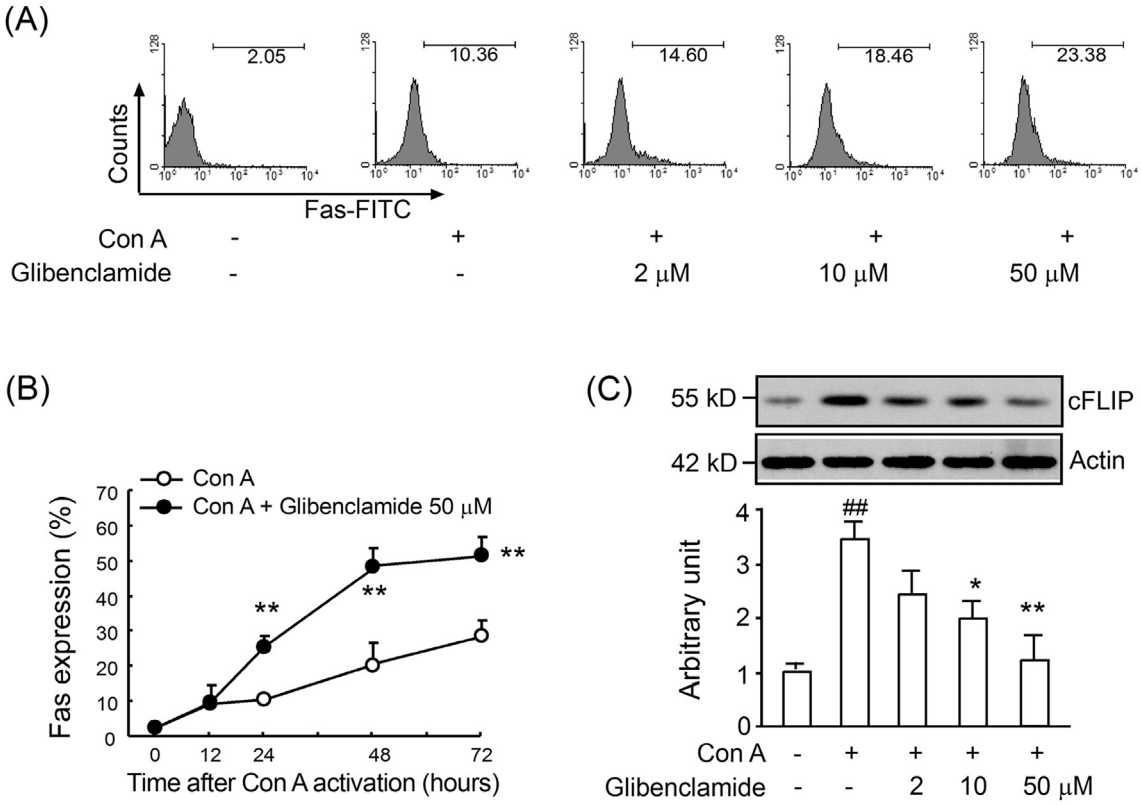

Fig. 2. Glibenclamide up-regulated Fas expression and down-regulated c-FLIP expression in activated T cells. (A) Fas expression of T cells was evaluated by flow cytometry. Cells were seeded in 6-well plate and incubated with 0, 2, 10 or 50 μM glibenclamide for 36 h in the presence of Con A (5 μg/mL). (B) Percentage of Fas^+ cells at different time points was shown in line graphs. Cells were incubated with 50 μM glibenclamide for 0–72 h in the presence of Con A (5 μg/mL). (C) Representative Western blot bands and data summary of c-FLIP were shown. T cells were activated with Con A (5 μg/mL) under the treatment of glibenclamide for 24 h. Results represent mean ± SEM of three independent experiments. One-way ANOVA revealed a significant difference at $P < 0.01$. $^{\#\#}P < 0.01$, *vs.* naive; $^{*}P < 0.05$, $^{**}P < 0.01$, *vs.* ConA control (Dunnet's test).

3.3. Inhibition of AMPK phosphorylation but not the ATP-sensitive potassium channel contributes to the apoptosis observed in glibenclamide–treated activated T cells

We further explored the mechanism underlying apoptosis induction by glibenclamide in activated T cells. Glibenclamide is reported as a K_{ATP} blocker. K_{ATP} channel is a type of potassium channel that is gated by intracellular nucleotides, ATP and ADP. It is composed of Kir6.x-type subunits and sulfonylurea receptor (SUR) subunits, along with additional components [15]. Here we showed for the first time that K_{ATP} subunits including SUR1, SUR2, Kir 6.1 and Kir 6.2 were expressed on T cells (Fig. 3A). However, all these subunits demonstrated no notable changes during the process of T cell activation. Furthermore, glibenclamide exhibited no effect on the expression of K_{ATP} subunits, either (Data not shown). Then we examined whether other K_{ATP} blocker had the same effects on activated T cells as glibenclamide. It was shown that the number of ConA-treated T cells was not altered in the presence of another K_{ATP} blocker 5-HD; and the K_{ATP} opener cromaklim did not reverse the inhibitory effect of glibenclamide on activated T cells (Fig. 3B).

Therefore, we investigated other molecules involved in intracellular energy stress. Western blot analysis showed that AMPK, a highly conserved eukaryotic enzyme (EC 2.7.11.31) that played a role in cellular energy homeostasis, was expressed in T lymphocytes. ConA treatment caused significant increases in phosphorylation of its catalytic domain at threonine-172, which started from the early phase (within 4 h) and continued to rise to even higher levels until 24 h later after activation (Fig. 3C). Glibenclamide reduced the level of phosphorylated AMPK in a concentration-related manner (Fig. 3D). Moreover, a similar efficiency on cell apoptosis was seen in the cells treated with an AMPK inhibitor compound C; whereas the effects of glibenclamide were attenuated by AICAR - an AMPK activator (Fig. 3E–F). Effects of glibenclamide on the expression of c-FLIP in activated T cells was also mimicked by compound C and reversed by AICAR (Fig. 3G). These data, taken together, indicate that glibenclamide induced the apoptosis of activated T cells via an AMPK-dependent way.

3.4. Glibenclamide ameliorates PCl-induced contact hypersensitivity in mice

To determine whether glibenclimide could affects T cells *in vivo*, we used a T-cell-mediated contact hypersensitivity model and treated the mice with glibenclamide. Swelling of the right ear was monitored for changes occurring as a result of T-cell-induced inflammation. Administration of glibenclamide to the mice obviously ameliorated the ear swelling (Fig. 4A). The histopathologic changes, in the ears of mice in the control group, were mainly observed in the dermis as moderate edema, vascular congestion and severe inflammatory infiltration (Fig. 4B–C). Glibenclamide treatment significantly extenuated the inflammatory reactions. CsA, as an immunosuppressant and the positive control, also showed a strong therapeutic effect. Repaglinide, another oral blood glucose-lowering drug [15], hardly possessed a protective effect on PCl-induced contact dermatitis, which indicated that the

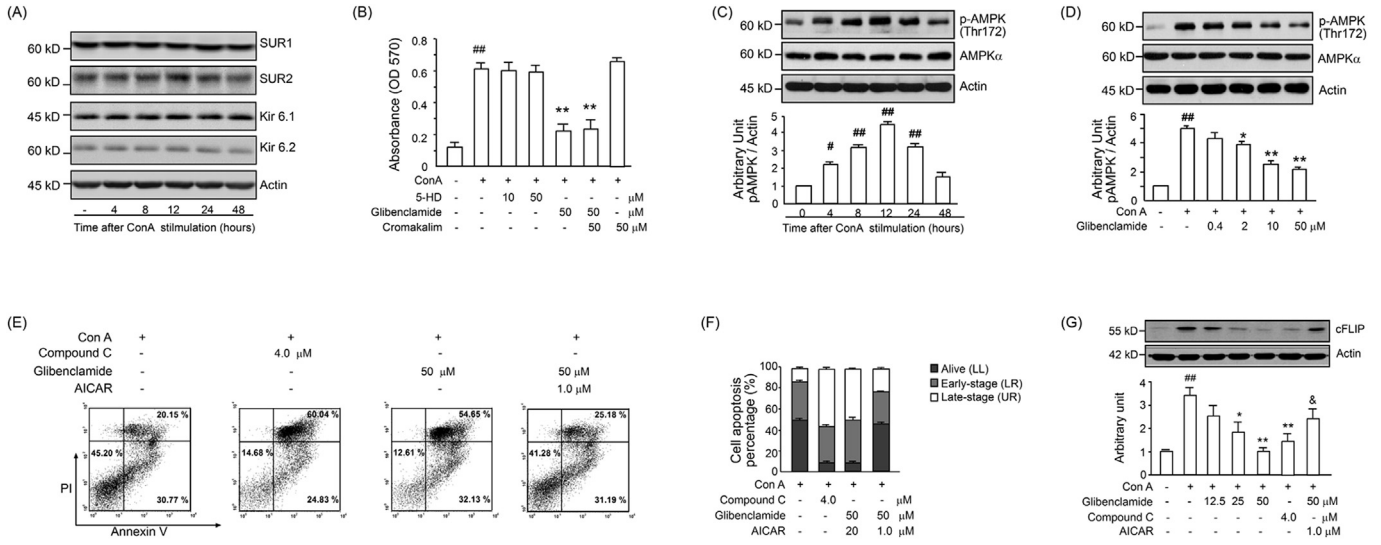

Fig. 3. Inhibition of AMPK phosphorylation contributes to glibenclamide-induced apoptosis in activated T cells. (A) The expression of KATP subunits in ConA-activated T cells. (B) Treatment with the KATP blocker 5-HD (50 μM, 72 h) did not suppressed T cell proliferation stimulated with Con A; and the KATP opener cromaklim did not reverse the suppressive effects of glibenclamide on T cell proliferation. (C) Time course of AMPK phosphorylation in T cells activated with ConA. (D) Glibenclamide inhibited AMPK phosphorylation in activated T cells. The purified T cells from lymph node were treated with glibenclamide (0.4, 2, 10, and 50 μM) for 24 h in the presence of Con A (5 μg/mL). (E–F) Treatment with the AMPK inhibitor compound C (50 μM, 48 h) induced apoptosis of T cell stimulated with Con A; whereas the AMPK activator AICAR reversed glibenclamide -induced apoptosis of the activated T cells. (G) Treatment with compound C (50 μM, 24 h) suppressed expression of c-FLIP; whereas AICAR reversed glibenclamide-suppressed expression of c-FLIP. Data represent mean ± SEM of three independent experiments. One-way ANOVA revealed a significant difference at $P < 0.01$. $^{\#\#}P < 0.01$, *vs.* naïve; $^{*}P < 0.05$, $^{**}P < 0.01$, *vs.* ConA control (Dunnet's test).

suppressive effects of glibenclamide on inflammation were not associated with regulation of blood glucose.

4. Discussion

Our study demonstrates that it is the phosphorylation of AMPK but not regulation of the K_{ATP} channels that may play vital roles in the energy metabolism in activated T cells and contribute to T lymphocytes fate, thus the AMPK inhibitor can reduce T-cell-related inflammation partly via inducing cell apoptosis. The principal findings are (1) AMPK is phosphorylated during the activation of T lymphocytes while the K_{ATP} channel subunits are normally expressed and shows no changes during cell activation; (2) administration of glibenclamide produces a dose-dependent inhibition on AMPK phosphorylation; (3) glibenclamide induced apoptosis in activated T cells with an enhancement of Fas expression and down-regulation of c-FLIP; (4) glibenclamide-induced apoptosis and suppression of c-FLIP is reversed by an AMPK activator AICAR and mimicked by an AMPK inhibitor compound C.

Glibenclamide is a hypolycemic agent, which is commonly and safely applied in patients with type 2 diabetes mellitus for decades [16]. The drug works by binding to and inhibiting the K_{ATP} regulatory subunit SUR1 [17] in pancreatic beta cells. In the present study, it was shown for the first time that all the four subunits of K_{ATP} channel are expressed on $CD3^{+}$ helper T cells, but neither opening nor inhibiting K_{ATP} channels affects T cell survival and death (Fig. 3A–B), which suggested that glibenclamide had a new mechanism for its effects on T cells. When the *in vivo* effects of glibenclamide were investigated, another blood glucose-lowering drug repaglinide was shown to have no therapeutic effect on contact dermatitis (Fig. 4). Repaglinide was also a K_{ATP} blocker [15], further indicating that the effect of glibenclamide on T-cell-related inflammation might not be associated with blockage of K_{ATP} channels.

Interestingly, we found that AMPK, a crucial molecule in energy homeostasis, was phosphorylated during T cell activation in a time-dependent manner (Fig. 3C). The role of AMPK in cell death is now controversial. It has been reported that AMPK activation is required for both apoptotic and autophagic death in HepG2 cells or adipocytes [18,19] and AMPK knockout protects beta cells from IL-1β-mediated apoptosis [20]. However, it seemed to be different in lymphoid cells. AMPK inhibition enhances apoptosis in MLL-rearranged pediatric B-acute lymphoblastic leukemia cells [21], and AMPK deficiency leads to leukemic T cell death [22]. In the present study, we reported for the first time that inhibition on phosphorylated AMPK by glibenclamide could induce apoptosis in activated T cells by decreasing the expression of anti-apoptotic protein c-FLIP (Fig. 2). Former studies on transgenic mice revealed that c-FLIP-Fas signaling induced T cell apoptosis [23]. Our data indicated AMPK as an important upstream signaling of c-FLIP and cell death.

Since the resistance to apoptosis in activated T cells results in the occurrence of autoimmune diseases [24–26], specific induction of pathogenic T-cell apoptosis is beneficial in depressing excess immune responses and maintaining immune homeostasis. It should be noted that AMPK was robustly phosphorylated in activated T cells, and AMPK inhibition by glibenclamide could only induce apoptosis in activated T lymphocytes while showed no effects on naïve cells (Fig. 1, Supplementary Figs. 1–2). This unique mechanism, along with its long usage history in the clinic, suggested that glibenclamide could be a safe drug used in the treatment of autoimmune diseases by selectively removing over-activated lymphocytes.

In summary, our results indicate that activation-induced phosphorylation of AMPK may regulate the survival and death of T lymphocytes, thus the AMPK-inhibiting drug, such as glibenclamide, which is currently used for treating diabetes, may be used to reduce T-cell-mediated immune reactions in certain patients clinically.

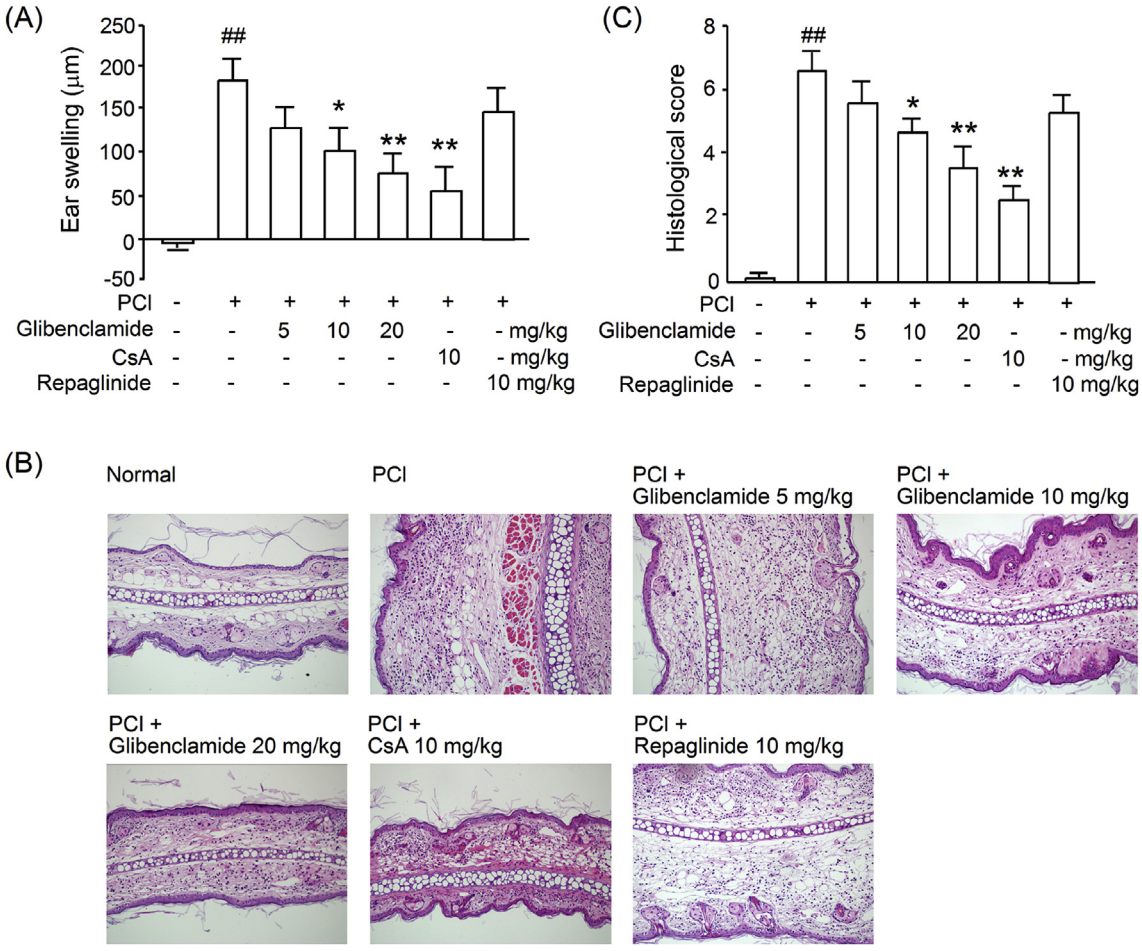

Fig. 4. Effects of glibenclamide on picryl chloride (PCl)-induced contact hypersensitivity in mice. Glibenclamide, repaglinide (intragastrically) or CsA (intraperitoneally) were administered once a day for 6 days after sensitization. Each figure indicates the mean ± SEM of 12 mice. (A) The detection of ear swelling. (B) Hematoxylin and eosin stain (original magnification 200 ×). (C) Histopathological scores. One-way ANOVA revealed a significant difference at $P < 0.01$. $^{\#\#}P < 0.01$, *vs.* normal; $^{*}P < 0.05$, $^{**}P < 0.01$, *vs.* PCl control (Dunnet's test).

Disclosure statement

The authors have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2017.04.038.

Transparency document

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