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Hyperglycemia decreases anti-cancer efficiency of adriamycin via AMPK pathway

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Abstract

Accumulating clinical evidence indicates that diabetic liver cancer patients are less sensitive to intra-arterial chemotherapy than non-diabetic cancer patients. However, the underlying mechanism remains largely uncharacterized. Here, we report that hyperglycemia inhibits AMPK pathway and subsequently reduces adriamycin (ADR)-induced DNA damage, resulting in decreased chemotherapeutic sensitivity of ADR. HepG2 and Bel-7402 cells were treated with ADR in various glucose conditions and then subjected to cell proliferation assay and apoptosis. The IC₅₀ of ADR greatly increased with the increasing concentration of glucose (15 ± 4 nM to 93 ± 39 nM in HepG2, 78 ± 8 nM to 1310 ± 155 nM in Bel-7402). Both FACs and Western blot analysis indicated that high concentration of glucose protected cells from ADR-induced apoptosis. Mouse hepatoma H22 xenografts were established both in db/db diabetic mice and STZ-induced diabetic mice. The inhibitory effect in tumor growth of ADR was significantly reduced in diabetic mice, which could be recovered by insulin therapy. Hyperglycemia greatly ameliorated AMPK activation and H2AX expression caused by ADR treatment. Pretreatment with compound C or AMPK silencing eliminated hyperglycemia reduced cytotoxicity of ADR. However, the impaired cytotoxicity in hyperglycemia was recovered by treatment with AMPK activator AICAR. This study indicates that hyperglycemia impairs the chemotherapeutic sensitivity of ADR by downregulating AMPK pathway and reducing ADR-induced DNA damage.

Key Words

- ▶ diabetes
- ▶ hepatic carcinoma
- ▶ hyperglycemia
- ▶ adriamycin
- ▶ AMPK

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Introduction

Clinical investigations show that the population of patients with both diabetes and malignancies is growing (Currie *et al.* 2009, Westley & May 2013). According to statistic data, 47% of liver cancer patients and 16% of breast cancer patients also suffer from diabetes (Davila *et al.* 2005, Wolf *et al.* 2005). Diabetes has long been considered as a high risk factor for malignancies, which increases

the incidence of diverse varieties of cancer (Currie *et al.* 2009, Gallagher & LeRoith 2011), including liver, breast (Larsson *et al.* 2007), colon (Yang *et al.* 2004), pancreatic (Everhart & Wright 1995, Rahn *et al.* 2017) and kidney cancer (Moore *et al.* 1998). Diabetes not only influences the occurrence and progression of malignancies, but also impairs the sensitivity of chemotherapy (Richardson &

Pollack 2005). In the latest retrospective review, compared with non-diabetic cancer patients, diabetic cancer patients showed an obvious increase of recurrence and death rates after chemotherapy (Bonovas *et al.* 2004, Barone *et al.* 2008, Currie *et al.* 2009, Srokowski *et al.* 2009). Diabetic rectal cancer patients are less sensitive to neo-adjuvant chemotherapy than non-diabetic cancer patients (Caudle *et al.* 2008). Compared with other cancers, diabetes mellitus is more closely associated with hepatocellular carcinoma (HCC) (Yang *et al.* 2016). Diabetes also reduces the sensitivity of liver cancer patients to intra-arterial chemotherapy (Feng *et al.* 2011a). Although the impact of diabetes mellitus on complications and outcomes of chemotherapy has gained much attention in clinical studies, whether diabetes impaired chemotherapeutic sensitivity in HCC is not clear.

Adriamycin (ADR) is widely used as a first-line anti-hepatoma drug alone or in combination (Judson *et al.* 2014). Multiple action mechanisms are involved in ADR-induced apoptosis, intercalating with DNA and RNA synthesis, inhibiting topoisomerase II leading to DNA strand breakages, causing the formation of reactive oxygen species and free radical damage on cells (Salzano *et al.* 2014, Yang *et al.* 2014a). Despite its multifactorial antitumor activity, only 20% of HCC patients respond to ADR treatment. Whether hyperglycemia is associated with decreased sensitivity to ADR remained unknown.

AMP-activated protein kinase (AMPK) is originally well known as a key sensor of fuel that regulates metabolism and energy homeostasis (Kim *et al.* 2014b). Recently, large number of evidence indicates that activation of AMPK inhibits cell proliferation and induces apoptosis via multiple pathways (Faubert *et al.* 2013). Several anti-cancer drugs have been reported to activate AMPK pathway. For example, AMPK plays an important role in the inhibitory effect of celastrol on cancer cell proliferation (Kim *et al.* 2013). Anti-diabetic drugs such as metformin and berberine, which are able to activate AMPK have shown anti-cancer activity (Dowling *et al.* 2012, Kim *et al.* 2015, Aznar *et al.* 2016). Ji and colleagues found that ROS-dependent AMPK activation contributed to the anti-tumor activity of ADR (Ji *et al.* 2010). Furthermore, exogenous cell-permeable C6-ceramide exerts synergistic anti-cancer effects with ADR via activation of AMPK. Given that AMPK activity is generally reduced in diabetic patients, whether lower chemotherapeutic sensitivity in diabetic cancer patients are owned to loss of AMPK activity worth further study.

In present study, we first used HCC cell lines to investigate the effects of hyperglycemia on the cytotoxicity of ADR. Then, mouse hepatoma H22 xenografts were

established in db/db diabetic mice or streptozotocin (STZ)-induced hyperglycemic mice to evaluate the impact of hyperglycemia or hyperinsulinemia on the anti-cancer activity of ADR *in vivo*. Then, the involvement of AMPK was confirmed in the *in vitro* model. This study not only characterized the effect of diabetes on the anti-tumor activity of ADR, but also clarified the potential mechanisms involved.

Materials and methods

Reagents

ADR was supplied by Actavis Italy S.p.A. (Viale Pasteur, Nerviano, Italy). A stock solution of ADR (2mM) was prepared with DMSO and stored at -20°C for *in vitro* testing. It was further diluted with DMSO before use. A stock solution of ADR (1 mg/mL) was dissolved with normal saline (NS) and stored at 4°C for *in vivo* experiments. It was freshly diluted with normal saline before use. Insulin glargine injection (insulin) was obtained from Sanofi-Aventis Deutschland GmbH. AICAR and streptozotocin were supplied by Sigma-Aldrich and compound C was from Merck & Co. RPMI 1640 medium (no glucose) was supplied by Gibco-BRL (Gaithersburg, MD). The primary antibodies for $\gamma\text{H2A.X}$ (Ser139), p-AMPK α (Thr172), AMPK, acetyl-CoA carboxylase (ACC) and p-ACC (Ser79), cleaved PARP and cleaved caspase-3 were obtained from Cell Signaling Technology. The primary antibody for β -actin and secondary antibodies for mouse IgG, goat IgG and rabbit IgG were purchased from Santa Cruz Biotechnology. Enhanced chemiluminescence (ECL), a Western blot detection reagent, was obtained from Pierce Chemical.

Cell lines and cell culture

Human hepatoma cell line HepG2 and Bel-7402 were purchased from the Cell Bank of the China Science Academy (Shanghai, China). Cells were maintained in RPMI-1640 or DMEM medium at 37°C in a humidified atmosphere with 5% CO_2 . All media were supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GibcoBRL, Grand Island, NY, USA) plus penicillin (100units/mL) and streptomycin (100units/mL). Cells adapted to RPMI-1640 containing 5.5 mM glucose for 24 h before experiments.

Cell proliferation assay

Cell proliferation was assessed by sulforhodamine B (SRB) colorimetric assay. 3000 Cells were seeded in 96-well

plates, incubated in RPMI-1640 containing 5.5 mM glucose for 24 h, and then the medium was replaced with various concentrations of glucose with a total volume of 200 μ L per well. After treatment with indicated concentrations of ADR for 48 h, cell proliferation was assessed by the SRB assay (Kim *et al.* 2014a). The cell survival fraction for each well was calculated as A510 treated cells/A510 control cells $\times$ 100%.

Compound C pretreatment

Cells were pre-incubated with compound C (5 μ M) for 30 min to inhibit AMPK activation, followed by further treatment with corresponding agents for the indicated time.

Gene silencing by small interfering RNA

The siRNA sequence was duplexes produced by Genepharm, Co. (Shanghai, China) and transfections were performed as previously described (Zheng *et al.* 2014). The sense sequence of the AMPK α siRNA was 5'-GCAUAUGCUGCAGGUAGAUTT-3'.

Apoptosis analysis

For apoptosis analysis, cultured cells were trypsinized and stained with Annexin V/PI after 0.5 μ M ADR treatment for 48 h according to the manufacturers' instructions, then analyzed on a FACS calibur cytometer (Becton Dickinson, San Jose, CA, USA).

Protein extraction and Western blotting

Cells were harvested after treatment with compounds for the indicated times. Whole cell lysates were prepared as previously described (Si *et al.* 2013). Then, Western blotting was performed as previously described (Zheng *et al.* 2011).

Animal experiment

All animal experiments were carried out in accordance with the ethical standards according to the Declaration of Helsinki and the national and international guidelines and has been approved by the Institutional Animal Care and Use Committee (IACUC) on the Ethics of Animal Experiments of Zhejiang University (Permit Number: Zju2009101004 and Zju2010101033).

Male C57BL/6 and C57BL/KsJ db/db (db/db) mice, 9 weeks of age, were obtained from the Model Animal Research Center of Nanjing University (Nanjing, Jiangsu,

China). Male ICR mice, 22–24 g, were obtained from the National Rodent Laboratory Animal Resource (Shanghai, China). The animals were maintained on a 12-h light/darkness cycle with free access to standard rodent chow and water.

Mouse hepatoma H22 xenografts were established by subcutaneously inoculating 1×10^6 cells into db/db or C57BL/6 mice. After inoculation, db/db and C57BL/6 mice were randomized into control and treatment groups ($n=6$ per group) respectively according to blood glucose levels and the mean of which was 6.6 mM in C57BL/6 mice or 28.88 mM in db/db mice. The control group mice received normal saline intraperitoneally (i.p.) while the treatment group mice received ADR (2 mg/kg, i.p.) every two days for 11 days. Random blood glucose levels were evaluated during the course. After treatment, plasma insulin levels were investigated and tumors were harvested and weighted for further analysis. Therapeutic treatment effects were shown in terms of inhibition ratio = (tumor weight of the control group – tumor weight of the treated group) / tumor weight of the control group $\times$ 100%. Furthermore, blood glucose levels after administration within 24 h were measured and areas under the blood glucose curve (AUC) were calculated using the trapezoidal method. Then, the correlations between blood glucose levels and the anti-cancer activity of ADR were analyzed.

ICR mice were injected with streptozotocin (i.p.) after fasted overnight to establish a diabetic model and then the mean blood glucose level was up to 27.35 mM while the mean blood glucose level in the control group was 7.07 mM indicated that the diabetic model was established successfully. H22 xenografts model were established by subcutaneously inoculating 1×10^6 cells into diabetic mice or normal mice. After inoculation, normal mice and diabetic mice were randomized into control and treatment groups ($n=6$ per group) to receive the following treatments once a day for 10 days: NS (i.p.), ADR (2 mg/kg, i.p.), insulin (12.5 IU/kg, s.c.) and combination therapy (12.5 IU/kg insulin + 2 mg/kg ADR). Random blood glucose levels and blood glucose levels after insulin and ADR treatment were detected during the course. After treatment, plasma insulin levels and tumor weight were measured. Therapeutic treatment effects were calculated by above description.

Blood glucose assay

Blood glucose was analyzed before administration and at the indicated time points after insulin or ADR administration. Blood glucose was measured from tail

vein using a blood glucose meter (Contour TS, Bayer, Leverkusen, Germany).

Plasma insulin assay

Blood was obtained and centrifuged for plasma. Plasma insulin levels were measured using an Elisa kit according to the manufacturers' instructions (Wuhan YIAab Science Co, Ltd, Wuhan, China).

TUNEL assay

To evaluate the apoptotic response of tumor tissues, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed with one-step TUNEL Apoptosis Assay kit (Beyotime) according to the manufacturer's instructions (Zheng *et al.* 2011). Cells stained with green fluorescence were defined as apoptotic cells.

Statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology. All the experimental data are presented as means $\pm$ S.D./S.E., and Student's *t*-test was used to analyze the statistical differences between two groups by use of the SPSS 18.0 program (SPSS). A value of $P < 0.05$ was considered to be significant.

Results

Hyperglycemia attenuates the antitumor effect of ADR in HCC cell lines

As diabetic HCC patients have a worse prognosis compared with non-diabetic patients, we speculated that hyperglycemia may impair the activity of antitumor agents. To test this hypothesis, HepG2 and Bel-7402 cells were exposed to ADR for 24 h and 48 h under different glucose conditions, and cell survival was analyzed by SRB assay. At 24 h of ADR treatment, the cytotoxicity of ADR was not significantly affected by the increase of glucose concentration (Supplementary Fig. 1C and D, see section on [supplementary data](#) given at the end of this article). However, as shown in Fig. 1A, the cytotoxicity of ADR decreased with the increase of glucose concentration as indicated with elevated IC_{50} values (15 ± 4 to 93 ± 39 nM in HepG2, 78 ± 8 to 1310 ± 155 nM in Bel-7402) at 48 h of ADR treatment. Mannitol was used as an osmotic control to exclude that this phenomenon is caused by

glucose itself. Mannitol did not affect the cytotoxicity of ADR in the concentration indicated (Supplementary Fig. 1A and B). Further, the ADR-triggered cell apoptosis was evaluated by FACs. As shown in Fig. 1B, 79.7% of the HepG2 cells were found to be apoptotic following ADR treatment in 5.5 mM glucose conditions, while the percentage of apoptotic cells were decreased with the increased glucose concentrations (54.4% in 11 mM glucose and 43.7% in 25 mM glucose). Similar results were obtained from Bel-7402 cells (from 86.0% to 58.2 and 45.5%). To further confirm that high concentration of glucose impaired ADR-induced apoptosis, protein expression of PARP and Caspase 3 were analyzed. As shown in Fig. 1C, ADR caused significant expression of c-PARP and c-Caspase 3 in 5.5 mM glucose; however, no significant upregulation was observed in 25 mM glucose in both HepG2 and Bel-7402 cells. These data indicate that hyperglycemia attenuates the antitumor effect of ADR in HCC cell lines.

The antitumor effect of ADR was decreased in H22 tumor-bearing db/db mice accompanied with hyperglycemia and hyperinsulinemia

To further address the role of diabetes on the antitumor activity of ADR, we established H22 mouse HCC model in db/db mice. In accordance with previous reports, the blood glucose level in db/db mice was much higher than that in C57BL/C mice during the entire treatment ($P < 0.05$) (Fig. 2B). In C57BL/C mice, the tumor growth was markedly inhibited by ADR (2 mg/kg) treatment (inhibition ratio 65.4%, $P < 0.05$), while no significant tumor growth inhibition was observed in db/db mice (inhibition ratio 25.8%, $P > 0.05$) (Fig. 2C). And the mice body weight during the experiment was shown in Supplementary Fig. 2A. At the end of treatment, TUNEL assay was employed to detect the apoptotic cells in tumor tissues. As shown in Fig. 2D, TUNEL-positive area was much less in db/db mice than that in C57BL/C mice. These results suggest that the anti-tumor activity of ADR is reduced in db/db diabetic mice.

To determine whether the higher blood glucose level was related to the decline of antitumor efficacy in db/db mice, we analyzed blood glucose and plasma insulin after ADR administration. Blood glucose level was measured in 24 h, and AUC was calculated using the trapezoidal method. As shown in Fig. 2E, the anti-tumor responses of ADR declined accompanied with increased AUC. Meanwhile, the blood insulin levels were apparently increased in db/db mice (Fig. 2E,

Figure 1

High glucose attenuates the cytotoxicity of ADR in HCC cell lines. (A) Human hepatocellular carcinoma HepG2 and Bel-7402 cells were exposed to ADR as indicated in 5.5, 11 and 25 mM glucose respectively for 48 h. Cell survival was evaluated by SRB assay and the IC₅₀ values were calculated by Calcsyn software. Data are expressed as the mean $\pm$ s.d., $n=3$. (B) Cell apoptosis was evaluated by Annexin V/PI staining. (C) Whole cell lysates were harvested and determined by Western blotting with antibodies against c-PARP, cleaved caspase-3 and β -actin.

$P<0.05$). These results indicate that hyperglycemia and hyperinsulinemia may be associated with the decreased antitumor efficacy of ADR.

Hyperglycemia attenuates the antitumor effect of ADR in STZ-induced diabetic mice bearing H22 HCC

To further investigate whether hyperglycemia and hyperinsulinemia mediate the antitumor efficacy of ADR, we established H22 mouse HCC in STZ-induced diabetic mice (DM mice) (Fig. 3A). Mice were grouped and treated as described above, and the blood glucose levels were measured on the first and tenth day. As shown in Fig. 3B, the blood glucose levels in DM mice were significantly higher than those in normal ICR mice indicating the success of STZ-induced diabetic model. And in accordance with previous reports, the blood glucose levels were downregulated after insulin treatment ($P<0.05$ – 0.001). Similar results were

observed in the AUC in 24 h after administration (Fig. 3C). As shown in Fig. 3D, the tumor growth in normal mice was significantly suppressed (inhibition ratio 70.1%) after ADR administration while the suppression was attenuated in the DM mice (inhibition ratio 27.7%). The body weight of the mice was shown in Supplementary Fig. 2B. Meanwhile, TUNEL assay showed that the ADR-induced apoptotic cells were decreased in DM mice compared to normal ICR mice (Fig. 3E). Given that the insulin secretion was destroyed in DM mice (Fig. 3F), insulin was additionally introduced to DM mice to downregulate the blood glucose levels. The anti-tumor efficacy of ADR was restored by the combined treatment of insulin, as indicated by inhibition ratio from 27.7% to 56.3% in DM mice (Fig. 3D). These results indicate that hyperglycemia plays an important role in attenuating the antitumor effects of ADR, and the antitumor efficacy of ADR declines in both db/db and DM mice regardless of insulin levels.

Figure 2

The antitumor effect of ADR was decreased in H22 tumor-bearing db/db mice accompanied with hyperglycemia and hyperinsulinemia. (A) H22 mouse hepatocellular carcinoma model was established by subcutaneously inoculating 1×10^6 cells into db/db or C57BL/C mice. After inoculation, db/db and C57BL/C mice were randomized into control and ADR treatment groups respectively according to blood glucose levels. The mice in control groups received NS (i.p.) while mice in the treatment groups received ADR (2 mg/kg, i.p.) every 2 days for 11 days. (B) Non-fasting blood glucose levels were studied on Days 1, 5 and 11 respectively during ADR treatment. (C) On Day 11, mice were killed and tumor weight was measured inhibition ratio was calculated. (D) Apoptosis cells in tumor tissues were detected by TUNEL assay. (E) Blood glucose levels after administration within 24 h were measured and areas under the blood glucose curve (AUC) were calculated using the trapezoidal method. The correlations between blood glucose levels and the anti-cancer activity of ADR were analyzed. (F) Plasma insulin levels on Day 11 were measured by ELISA kit. Data are expressed as the mean $\pm$ s.e., $n=6$. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs C57BL/C control. # $P<0.05$, ### $P<0.001$ vs C57BL/C+ADR 2 mg/kg.

AMPK activation modulates ADR-induced DNA damage in hepatoma cells

Since GLUT2 is the principal transporter for transfer of glucose, we first analyzed whether GLUT2 is involved in this process. As shown in Supplementary Fig. 3, either overexpression or silencing of GLUT2 did not affect the cytotoxicity of ADR. Considering that AMPK pathway and DNA damage play important role in

ADR-induced apoptosis, the activation of AMPK pathway and induction of γ -H2AX were detected in normal condition or hyperglycemia. As shown in Fig. 4A, when cells were treated with ADR in 5.5 mM glucose, there was a significant increase of γ -H2AX as well as p-ACC and p-AMPK. However, the induction of γ -H2AX, p-ACC and p-AMPK was declined accompanied with the increased glucose concentrations. Similar results were obtained from Bel-7402 cells (Fig. 4B), suggesting that ADR-caused

Figure 3

Hyperglycemia attenuates the antitumor effect of ADR on mouse hepatoma H22 xenograft in STZ-induced diabetic mice. (A) ICR mice were injected with streptozotocin (i.p.) after fasted overnight to establish a hyperglycemia model. H22 xenografts were established by subcutaneously inoculating cells into STZ-induced diabetic mice (DM mice) or normal mice. After inoculation, normal mice and DM mice were randomized into control and treatment groups to receive the following treatments: NS (i.p.), ADR (2 mg/kg, i.p.), insulin (12.5 IU/kg, s.c.), combination therapy (12.5 IU/kg insulin + 2 mg/kg ADR). (B and C) Non-fasting blood glucose levels and blood glucose levels during 24 h after administration were analyzed during the course. (D) At the end of the treatment on Day 10, mice were killed and the tumors were excised and weighted. (E) Apoptosis cells in tumor tissue were visualized by TUNEL assay. (F) On Day 10, blood was collected and the plasma insulin levels were measured by ELISA kit. Data are expressed as the mean $\pm$ s.e., $n=6$. $^{**}P<0.01$, $^{***}P<0.001$ vs control.

DNA damage and AMPK activation were impaired by hyperglycemia.

To determine the effect of AMPK activation on ADR-induced DNA damage, AICAR and Compound C, the activator and the inhibitor of AMPK were introduced to our experiments. HepG2 or Bel-7402 cells were challenged with 0.5 μ M ADR, 1 mM AICAR or

combination. As shown in Fig. 4C, when the cells were treated with AICAR, AMPK was significantly activated, ADR-induced expression of γ -H2AX was enhanced by AICAR. On the contrary, the γ -H2AX expression induced by ADR was greatly impaired by Compound C (5 μ M) in both HepG2 and Bel-7402 cells (Fig. 4D). These results indicate that AMPK activation modulates ADR-induced

Figure 4

AMPK activation modulates ADR-induced DNA damage in hepatoma cells. (A and B) Human hepatocellular carcinoma HepG2 and Bel-7402 cells were seeded in six-well plates and exposed to DMSO or 0.5 μ M ADR in different glucose conditions for 2 h. Whole cell lysates were harvested and determined by Western blotting with antibodies against p-AMPK, AMPK, p-ACC, ACC, γ -H2AX and β -actin. (C) HepG2 and Bel-7402 cells were seeded in six-well plates and exposed to 0.5 μ M ADR, 1 mM AICAR or the combination of both compounds for 2 h. (D) HepG2 and Bel-7402 cells were seeded in six-well plates and exposed to 0.5 mM ADR, 5 μ M compound C or the combination of both compounds for 2 h. After incubation, whole cell lysates were harvested and determined by immunoblotting with antibodies against p-AMPK, γ -H2AX and β -actin. Western blotting bands were quantified by IPP plus 6.0. Data are expressed as the mean $\pm$ s.d., $n=3$. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs control. # $P<0.05$, ## $P<0.01$, ### $P<0.001$ vs ADR in 5.5 mM glucose conditions.

DNA damage; however, the role of AMPK in the reduced antitumor effect of ADR resulted from hyperglycemia remains to be further studied.

Hyperglycemia reduces the cytotoxicity of ADR by inhibiting AMPK pathway

Based on the results above, we next investigate whether the impaired cytotoxicity of ADR by hyperglycemia

was eliminated in the absence of AMPK. Compound C was employed to inhibit AMPK activation and Western blotting was applied to determine the inhibitory effects in HepG2 and Bel-7402 cells. The data showed that the cytotoxicity of ADR was reduced in 25 mM glucose compared to that in 5.5 mM glucose, which was eliminated when cells were pretreated with Compound C (Fig. 5A and B). Similar results were obtained when AMPK was specifically silenced in both HepG2 and

Bel-7402 cells (Fig. 5C and D). Furthermore, AICAR was employed to enhance AMPK activation and Western blotting was used to determine the activation effects. The data showed that the impaired cytotoxicity of ADR in hyperglycemia was recovered by treatment with AMPK activator AICAR (Fig. 5E and F). These results indicate that hyperglycemia decreases the cytotoxicity of ADR by inhibiting AMPK pathway.

Discussion

Clinical studies indicate that diabetic liver cancer patients are less sensitive to intra-arterial chemotherapy than non-diabetic cancer patients (Caudle *et al.* 2008). As the treatment programs for liver cancer are usually combination therapies, there is no direct evidence whether diabetes reduces therapeutic responses to individual

Figure 5

High glucose decreases the cytotoxicity of ADR by downregulating AMPK pathway. (A and B) HepG2 and Bel-7402 cells were pretreated with 5 μ M compound C for 2 h. (C and D) HepG2 and Bel-7402 cells were transfected with siRNAs specifically targeting AMPK or control siRNAs. (E and F) HepG2 and Bel-7402 cells were pretreated with 0.5 mM AICAR for 2 h. Then, cells were exposed to ADR as indicated in different glucose conditions for 48 h. Cell-proliferation inhibitory activities were determined by SRB assay. Protein expressions were detected by Western blot analysis.

anti-cancer agent. Given the extensive application of ADR in clinical practice (Wang *et al.* 2014, Gladkov *et al.* 2016), we investigated the effects of diabetes on the chemotherapeutic sensitivity of ADR. We established mouse hepatoma H22 xenografts using db/db or C57 mice and mouse hepatoma H22 xenografts using normal ICR mice or DM mice. Db/db mice and DM mice were both widely used animal models for diabetic studies (Elms *et al.* 2013, Nesca *et al.* 2013). They were subcutaneously inoculated with mouse hepatoma H22 cells to imitate diabetic cancer individuals. Blood glucose levels were monitored during the treatment to make sure that db/db mice and DM mice were all performing diabetes until the end of the experiments. Our data showed that the repression of tumor growth by ADR decreased remarkably in db/db and DM mice compared with C57 and ICR mice, respectively. This study for the first time reported that diabetes decreases the efficiency of ADR against hepatic carcinoma.

As diabetes is a complex metabolic disease with various disturbances of different factors (De Silva *et al.* 2011), the main factor that affects chemotherapeutic sensitivity of ADR are still largely unknown. Feng *et al.* reported that high insulin and glucose levels induced chemoresistance to ADR in HER18 and MCF7 breast cancer cells (Feng *et al.* 2011b). However, no *in vivo* analysis, as well as mechanical interpretation was performed in that study, leaving us a great quantity of unknown questions. Both of db/db mice and DM mice performed hyperglycemia while db/db mice displayed hyperinsulinemia but DM mice showed hypoinsulinemia. However, the anti-cancer efficacy of ADR declined regardless of insulin levels in db/db and DM mice, indicating that insulin had negligible effect on the declined anti-cancer efficacy of ADR. Our observation is contrary with the study that high insulin levels induce chemoresistance to ADR in HER18 and MCF7 breast cancer cells (Feng *et al.* 2011b). There are two possible reasons: (1) There are great differences between hepatoma cell lines and breast cancer cell lines; (2) It is inconsistent between *in vitro* and *in vivo* studies with the underlying mechanisms still yet to be known. For further investigation, insulin was used in our experiments to downregulate the blood glucose levels in DM mice. Our data showed that the anti-tumor efficacy of ADR was recovered in DM mice with the decline of blood glucose levels. These findings indicate that hyperglycemia plays an important role on the anti-cancer efficacy of ADR.

It is well known that ADR induces cell death by intercalating into base pairs as well as causing DNA damage as a result of inhibition of type 2 topoisomerase (Salzano *et al.* 2014, Yang *et al.* 2014a). Because of the high plasma

drug concentrations needed for intercalating into base pairs, ADR exerts anti-cancer effects mainly by inducing DNA damage. Recent studies showed that ROS-dependent AMPK activation is also involved in the anti-tumor activity of ADR (Ji *et al.* 2010, Gao *et al.* 2014). Several researches have revealed that radiation or chemotherapy that induces DNA damage could in turn activate AMPK pathway (Alexander & Walker 2011, Yang *et al.* 2014b). Considering that AMPK activity is generally reduced in diabetic patients, we are wondering whether the declined chemotherapeutic sensitivity of ADR in diabetic liver cancer individuals is attributed to decreased AMPK activity. Consistent with previous studies, we found that ADR-induced DNA damage as well as AMPK pathway activation. Furthermore, the DNA damage and AMPK pathway activation were suppressed by high glucose, which may lead to the declined anti-cancer efficiency of ADR in return. Further, we also found that ADR-induced DNA damage was significantly enhanced by AMPK agonist and weakened by AMPK inhibitor, indicating that AMPK also plays an important role in ADR-induced DNA damage. In addition to AMPK, Compound C also inhibits a number of other protein kinases with similar or greater potency, including ERK8, MNK1, PHK, MELK, DYRK isoforms, HIPK2, Src, Lck and Yes, FGF-R1 and Eph-A2 (Bain *et al.* 2007). Considering this limitation of Compound C, siRNA interference technology was used to further confirm that AMPK plays an important role in hyperglycemia-mediated anti-cancer efficacy of ADR. The impaired cytotoxicity of ADR by hyperglycemia was eliminated in the absence of AMPK. These findings are of certain scientific significances not only for basic research, but also for the clinical application of ADR and optimization of combined therapeutic strategies.

In summary, this study is the first time using diabetic cancer mice and hepatoma cell lines to investigate the impacts of diabetes on anti-cancer activity of ADR and the underlying mechanisms systematically. We have demonstrated that diabetes decreases the anti-cancer efficiency of ADR against hepatic carcinoma *in vitro* and *in vivo*. Hyperglycemia plays an important role while insulin has negligible effects on the declined efficacy. Hyperglycemia decreases the chemotherapeutic sensitivity of ADR by downregulating AMPK pathway, which decreases ADR-induced DNA damage in turn. These observations highlight a novel concept that diabetes affects the chemotherapeutic sensitivity of anti-cancer agents, shedding lights on overcoming the limitations to the clinical use of ADR and opening new insights into improving the efficiency of diabetic cancer treatment.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/ERC-18-0036>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

- Alexander A & Walker CL 2011 The role of LKB1 and AMPK in cellular responses to stress and damage. *FEBS Letters* **585** 952–957. (<https://doi.org/10.1016/j.febslet.2011.03.010>)
- Aznar N, Patel A, Rohena CC, Dunkel Y, Joosen LP, Taupin V, Kufareva I, Farquhar MG & Ghosh P 2016 AMP-activated protein kinase fortifies epithelial tight junctions during energetic stress via its effector GIV/Girdin. *eLife* **5** 1–33. (<https://doi.org/10.7554/eLife.20795>)
- Bain J, Plater L, Elliott M, Shpiro N, Hastie CJ, McLauchlan H, Klevernic I, Arthur JS, Alessi DR & Cohen P 2007 The selectivity of protein kinase inhibitors: a further update. *Biochemical Journal* **408** 297–315. (<https://doi.org/10.1042/BJ20070797>)
- Barone BB, Yeh HC, Snyder CF, Peairs KS, Stein KB, Derr RL, Wolff AC & Brancati FL 2008 Long-term all-cause mortality in cancer patients with preexisting diabetes mellitus: a systematic review and meta-analysis. *JAMA* **300** 2754–2764. (<https://doi.org/10.1001/jama.2008.824>)
- Bonovas S, Filioussi K & Tsantes A 2004 Diabetes mellitus and risk of prostate cancer: a meta-analysis. *Diabetologia* **47** 1071–1078. (<https://doi.org/10.1007/s00125-004-1415-6>)
- Caudle AS, Kim HJ, Tepper JE, O'Neil BH, Lange LA, Goldberg RM, Bernard SA, Calvo BF & Meyers MO 2008 Diabetes mellitus affects response to neoadjuvant chemoradiotherapy in the management of rectal cancer. *Annals of Surgical Oncology* **15** 1931–1936. (<https://doi.org/10.1245/s10434-008-9873-6>)
- Currie CJ, Poole CD & Gale EA 2009 The influence of glucose-lowering therapies on cancer risk in type 2 diabetes. *Diabetologia* **52** 1766–1777. (<https://doi.org/10.1007/s00125-009-1440-6>)
- Davila JA, Morgan RO, Shaib Y, McGlynn KA & El-Serag HB 2005 Diabetes increases the risk of hepatocellular carcinoma in the United States: a population based case control study. *Gut* **54** 533–539. (<https://doi.org/10.1136/gut.2004.052167>)
- De Silva NM, Freathy RM, Palmer TM, Donnelly LA, Luan J, Gaunt T, Langenberg C, Weedon MN, Shields B, Knight BA, *et al.* 2011 Mendelian randomization studies do not support a role for raised circulating triglyceride levels influencing type 2 diabetes, glucose levels, or insulin resistance. *Diabetes* **60** 1008–1018. (<https://doi.org/10.2337/db10-1317>)
- Dowling RJ, Niraula S, Stambolic V & Goodwin PJ 2012 Metformin in cancer: translational challenges. *Journal of Molecular Endocrinology* **48** R31–R43. (<https://doi.org/10.1530/JME-12-0007>)
- Elms SC, Toque HA, Rojas M, Xu Z, Caldwell RW & Caldwell RB 2013 The role of arginase I in diabetes-induced retinal vascular dysfunction in mouse and rat models of diabetes. *Diabetologia* **56** 654–662. (<https://doi.org/10.1007/s00125-012-2789-5>)
- Everhart J & Wright D 1995 Diabetes mellitus as a risk factor for pancreatic cancer. A meta-analysis. *JAMA* **273** 1605–1609. (<https://doi.org/10.1001/jama.1995.03520440059037>)
- Faubert B, Boily G, Izreig S, Griss T, Samborska B, Dong Z, Dupuy F, Chambers C, Fuerth BJ, Viollet B, *et al.* 2013 AMPK is a negative regulator of the Warburg effect and suppresses tumor growth in vivo. *Cell Metabolism* **17** 113–124. (<https://doi.org/10.1016/j.cmet.2012.12.001>)
- Feng YH, Lin CY, Huang WT, Wu CL, Fang JL & Tsao CJ 2011a Diabetes mellitus impairs the response to intra-arterial chemotherapy in hepatocellular carcinoma. *Medical Oncology* **28** 1080–1088. (<https://doi.org/10.1007/s12032-010-9650-9>)
- Feng YH, Velazquez-Torres G, Gully C, Chen J, Lee MH & Yeung SC 2011b The impact of type 2 diabetes and antidiabetic drugs on cancer cell growth. *Journal of Cellular and Molecular Medicine* **15** 825–836. (<https://doi.org/10.1111/j.1582-4934.2010.01083.x>)
- Gallagher EJ & LeRoith D 2011 Diabetes, cancer, and metformin: connections of metabolism and cell proliferation. *Annals of the New York Academy of Sciences* **1243** 54–68. (<https://doi.org/10.1111/j.1749-6632.2011.06285.x>)
- Gao K, Chi Y, Sun W, Takeda M & Yao J 2014 5'-AMP-activated protein kinase attenuates adriamycin-induced oxidative podocyte injury through thioredoxin-mediated suppression of the apoptosis signal-regulating kinase 1-P38 signaling pathway. *Molecular Pharmacology* **85** 460–471. (<https://doi.org/10.1124/mol.113.089458>)
- Gladkov O, Moiseyenko V, Bondarenko IN, Shparyk Y, Barash S, Adar L & Avisar N 2016 A phase III study of balugrastim versus pegfilgrastim in breast cancer patients receiving chemotherapy with doxorubicin and docetaxel. *Oncologist* **21** 7–15. (<https://doi.org/10.1634/theoncologist.2015-0152>)
- Ji C, Yang B, Yang YL, He SH, Miao DS, He L & Bi ZG 2010 Exogenous cell-permeable C6 ceramide sensitizes multiple cancer cell lines to Doxorubicin-induced apoptosis by promoting AMPK activation and mTORC1 inhibition. *Oncogene* **29** 6557–6568. (<https://doi.org/10.1038/nc.2010.379>)
- Judson I, Verweij J, Gelderblom H, Hartmann JT, Schoffski P, Blay JY, Kerst JM, Sufliarsky J, Whelan J, Hohenberger P, *et al.* 2014 Doxorubicin alone versus intensified doxorubicin plus ifosfamide for first-line treatment of advanced or metastatic soft-tissue sarcoma: a randomised controlled phase 3 trial. *Lancet Oncology* **15** 415–423. ([https://doi.org/10.1016/S1470-2045\(14\)70063-4](https://doi.org/10.1016/S1470-2045(14)70063-4))
- Kim JH, Lee JO, Lee SK, Kim N, You GY, Moon JW, Sha J, Kim SJ, Park SH & Kim HS 2013 Celastrol suppresses breast cancer MCF-7 cell viability via the AMP-activated protein kinase (AMPK)-induced p53-polo like kinase 2 (PLK-2) pathway. *Cell Signaling* **25** 805–813. (<https://doi.org/10.1016/j.cellsig.2012.12.005>)
- Kim KH, Moon E, Kim SY, Choi SU, Lee JH & Lee KR 2014a 4-Methylthio-butanyl derivatives from the seeds of *Raphanus sativus* and their biological evaluation on anti-inflammatory and antitumor activities. *Journal of Ethnopharmacology* **151** 503–508. (<https://doi.org/10.1016/j.jep.2013.11.003>)
- Kim M, Hunter RW, Garcia-Menendez L, Gong G, Yang YY, Kolwicz SC Jr, Xu J, Sakamoto K, Wang W & Tian R 2014b Mutation in the gamma2-subunit of AMP-activated protein kinase stimulates cardiomyocyte proliferation and hypertrophy independent of glycogen storage. *Circulation Research* **114** 966–975. (<https://doi.org/10.1161/CIRCRESAHA.114.302364>)
- Kim HS, Lim J, Lee DY, Ryu JH & Lim JS 2015 Kazinol C from *Broussonetia kazinoki* activates AMP-activated protein kinase to induce antitumorigenic effects in HT-29 colon cancer cells. *Oncology Reports* **33** 223–229. (<https://doi.org/10.3892/or.2014.3601>)
- Larsson SC, Mantzoros CS & Wolk A 2007 Diabetes mellitus and risk of breast cancer: a meta-analysis. *International Journal of Cancer* **121** 856–862. (<https://doi.org/10.1002/ijc.22717>)

- Moore MA, Park CB & Tsuda H 1998 Implications of the hyperinsulinaemia-diabetes-cancer link for preventive efforts. *European Journal of Cancer Prevention* **7** 89–107. (<https://doi.org/10.1097/00008469-199802001-00076>)
- Nesca V, Guay C, Jacovetti C, Menoud V, Peyot ML, Laybutt DR, Prentki M & Regazzi R 2013 Identification of particular groups of microRNAs that positively or negatively impact on beta cell function in obese models of type 2 diabetes. *Diabetologia* **56** 2203–2212. (<https://doi.org/10.1007/s00125-013-2993-y>)
- Rahn S, Zimmermann V, Viol F, Knaack H, Stemmer K, Peters L, Lenk L, Ungefroren H, Saur D, Schafer H, *et al.* 2017 Diabetes as risk factor for pancreatic cancer: hyperglycemia promotes epithelial-mesenchymal-transition and stem cell properties in pancreatic ductal epithelial cells. *Cancer Letters* **415** 129–150. (<https://doi.org/10.1016/j.canlet.2017.12.004>)
- Richardson LC & Pollack LA 2005 Therapy insight: influence of type 2 diabetes on the development, treatment and outcomes of cancer. *Nature Clinical Practice Oncology* **2** 48–53. (<https://doi.org/10.1038/npcnc0062>)
- Salzano M, Vazquez-Cedeira M, Sanz-Garcia M, Valbuena A, Blanco S, Fernandez IF & Lazo PA 2014 Vaccinia-related kinase 1 (VRK1) confers resistance to DNA-damaging agents in human breast cancer by affecting DNA damage response. *Oncotarget* **5** 1770–1778. (<https://doi.org/10.18632/oncotarget.1678>)
- Si M, Yan Y, Tang L, Wu H, Yang B, He Q & Wu H 2013 A novel indole derivative compound GY3 improves glucose and lipid metabolism via activation of AMP-activated protein kinase pathway. *European Journal of Pharmacology* **698** 480–488. (<https://doi.org/10.1016/j.ejphar.2012.10.006>)
- Srokowski TP, Fang S, Hortobagyi GN & Giordano SH 2009 Impact of diabetes mellitus on complications and outcomes of adjuvant chemotherapy in older patients with breast cancer. *Journal of Clinical Oncology* **27** 2170–2176. (<https://doi.org/10.1200/JCO.2008.17.5935>)
- Wang L, Wang WD, Xia ZJ, Zhang YJ, Xiang J & Lu Y 2014 Combination of gemcitabine, L-asparaginase, and oxaliplatin (GELOX) is superior to EPOCH or CHOP in the treatment of patients with stage IE/IIe extranodal natural killer/T cell lymphoma: a retrospective study in a cohort of 227 patients with long-term follow-up. *Medical Oncology* **31** 860. (<https://doi.org/10.1007/s12032-014-0860-4>)
- Westley RL & May FE 2013 A twenty-first century cancer epidemic caused by obesity: the involvement of insulin, diabetes, and insulin-like growth factors. *International Journal of Endocrinology* **2013** 632461. (<https://doi.org/10.1155/2013/632461>)
- Wolf I, Sadetzki S, Catane R, Karasik A & Kaufman B 2005 Diabetes mellitus and breast cancer. *Lancet Oncology* **6** 103–111. ([https://doi.org/10.1016/S1470-2045\(05\)01736-5](https://doi.org/10.1016/S1470-2045(05)01736-5))
- Yang YX, Hennessy S & Lewis JD 2004 Insulin therapy and colorectal cancer risk among type 2 diabetes mellitus patients. *Gastroenterology* **127** 1044–1050. (<https://doi.org/10.1053/j.gastro.2004.07.011>)
- Yang F, Teves SS, Kemp CJ & Henikoff S 2014a Doxorubicin, DNA torsion, and chromatin dynamics. *Biochimica et Biophysica Acta* **1845** 84–89. (<https://doi.org/10.1016/j.bbcan.2013.12.002>)
- Yang W, Park IJ, Yun H, Im DU, Ock S, Kim J, Seo SM, Shin HY, Viollet B, Kang I, *et al.* 2014b AMP-activated protein kinase alpha2 and E2F1 transcription factor mediate doxorubicin-induced cytotoxicity by forming a positive signal loop in mouse embryonic fibroblasts and non-carcinoma cells. *Journal of Biological Chemistry* **289** 4839–4852. (<https://doi.org/10.1074/jbc.M113.496315>)
- Yang JD, Mohamed HA, Cvinar JL, Gores GJ, Roberts LR & Kim WR 2016 Diabetes mellitus heightens the risk of hepatocellular carcinoma except in patients with hepatitis C cirrhosis. *American Journal of Gastroenterology* **111** 1573–1580. (<https://doi.org/10.1038/ajg.2016.330>)
- Zheng L, Yang W, Zhang C, Ding WJ, Zhu H, Lin NM, Wu HH, He QJ & Yang B 2011 GDC-0941 sensitizes breast cancer to ABT-737 in vitro and in vivo through promoting the degradation of Mcl-1. *Cancer Letters* **309** 27–36. (<https://doi.org/10.1016/j.canlet.2011.05.011>)
- Zheng L, Fu Y, Zhuang L, Gai R, Ma J, Lou J, Zhu H, He Q & Yang B 2014 Simultaneous NF-kappaB inhibition and E-cadherin upregulation mediate mutually synergistic anticancer activity of celastrol and SAHA in vitro and in vivo. *International Journal of Cancer* **135** 1721–1732. (<https://doi.org/10.1002/ijc.28810>)

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