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Azoxystrobin, a mitochondrial complex III Q_o site inhibitor, exerts beneficial metabolic effects in vivo and in vitro

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ABSTRACT

Background: Several anti-diabetes drugs exert beneficial effects against metabolic syndrome by inhibiting mitochondrial function. Although much progress has been made toward understanding the role of mitochondrial function inhibitors in treating metabolic diseases, the potential effects of these inhibitors on mitochondrial respiratory chain complex III remain unclear.
Methods: We investigated the metabolic effects of azoxystrobin (AZOX), a Q_o inhibitor of complex III, in a high-fat diet-fed mouse model with insulin resistance in order to elucidate the mechanism by which AZOX improves glucose and lipid metabolism at the metabolic cellular level.
Results: Acute administration of AZOX in mice increased the respiratory exchange ratio. Chronic treatment with AZOX reduced body weight and significantly improved glucose tolerance and insulin sensitivity in high-fat diet-fed mice. AZOX treatment resulted in decreased triacylglycerol accumulation and down-regulated the expression of genes involved in liver lipogenesis. AZOX increased glucose uptake in L6 myotubes and 3T3-L1 adipocytes and inhibited de novo lipogenesis in HepG2 cells. The findings indicate that AZOX-mediated alterations to lipid and glucose metabolism may depend on AMP-activated protein kinase (AMPK) signaling.
Conclusions: AZOX, a Q_o inhibitor of mitochondrial respiratory complex III, exerts whole-body beneficial effects on the regulation of glucose and lipid homeostasis in high-fat diet-fed mice.
General significance: These findings provide evidence that a Q_o inhibitor of mitochondrial respiratory complex III could represent a novel approach for the treatment of obesity.

1. Introduction

The mitochondrion is a subcellular organelle where fatty acids and glucose are consumed to produce ATP through oxidative phosphorylation. A growing body of evidence suggests that overloading lipid oxidation in the mitochondria is a potential risk factor for the pathogenesis of insulin resistance [1]; genetic approaches have demonstrated that transgenic mouse models with enhanced capacity for fat oxidation are more susceptible to diet-induced insulin resistance [2,3]. Insulin sensitizing medicines, including metformin [4], thiazolidinediones (TZDs) [5], and berberine [6], have been shown to exert their beneficial effects in part via inhibition of mitochondrial respiratory chain complex I activity, while other natural products with blood glucose-lowering potential, such as resveratrol and quercetin [7], inhibit ATP synthase. Inhibition of the mitochondrial respiratory system thus represents an effective strategy to ameliorate insulin insensitivity in the context of modern lifestyle-related mitochondrial overload.

While much progress has been made in understanding the role of mitochondrial functional inhibitors in treating metabolic diseases, complex III inhibitors are not well studied. Mitochondrial complex III consists of 11 subunits, three of which have known electron transport activity (Rieske Fe–S protein, cytochrome b, and cytochrome c1). Electron flux from ubiquinol (QH_2) to cytochrome c occurs through the ubiquinone (Q) cycle within this complex [8]. The Q cycle reaction mechanism of complex III postulates separate quinone reduction (Q_i) and quinol oxidation (Q_o) sites. Based on the Q cycle reaction mechanism, two distinct groups of complex III inhibitors have been identified: Q_i site inhibitors (i.e. antimycin A) and Q_o site inhibitors (i.e. stigmatellin, myxothiazol and strobilurin derivatives) [9–11].

Azoxystrobin (AZOX), one of the first strobilurins to be commercialized as a new class of fungicides, binds very tightly to the Q_o site of complex III [11]. AZOX was discovered through research on *Oudemansiella*

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mucida and *Strobilurus tenacellus*, small white or brown colored mushrooms commonly found in Czech forests, and is now the leading proprietary fungicide in the world. AZOX is considerably less toxic to mice and has passed stringent toxicity scrutiny (U.S. Environmental Protection Agency, Pesticide Fact Sheet for azoxystrobin).

In this study, AZOX was used to evaluate the therapeutic potential of the mitochondria Q_o site of complex III inhibitors for treatment of overnutrition-related metabolic diseases. We demonstrate that AZOX treatment improves insulin sensitivity in a rodent model of diet-induced obesity, reduces whole-body adiposity, and shifts energy substrate preference away from the use of fatty acids, a favorable adjustment for disorders characterized by glucose intolerance. Some of the observed effects of AZOX are at least partially mediated by activating the crucial cell-intrinsic energy sensor AMP-activated protein kinase (AMPK).

2. Materials and methods

2.1. Materials

AZOX was obtained from Sino Chemtech CO., LTD (Shanghai). Antimycin A, Compound C, cytochrome c, decylubiquinone and insulin were obtained from Sigma-Aldrich (St. Louis, MO). Wesson pure corn oil was obtained from ConAgra Foods.

2.2. Cell culture

L6 myotubes and HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% FBS (Invitrogen) and penicillin–streptomycin. For the differentiation of L6 myoblasts, the concentration of FBS was kept at 2% for 6 days. The culture and differentiation of 3T3-L1 cells were conducted as described previously [12]. Rat hepatocytes were isolated using Selgen's two-step perfusion method [13] and maintained in DMEM.

2.3. Measurement of respiration in isolated mitochondria and intact cells and the complex III specific activity

Mitochondria were isolated from rat liver according to previously reported methods [14]. Respiration measurements were conducted using a Clark-type oxygen electrode as previously described [6]. To measure mitochondrial respiration, AZOX or DMSO was added in the presence of the following respiratory chain complex substrate inhibitors and ADP: complex I (malate/pyruvate), complex II (succinate/rotenone) or complex IV (ascorbate/N1,N1,N1,N1-tetramethyl-1,4-phenylene diamine (TMPD)), and the effects on the rate of oxygen consumption were recorded. For cellular respiration, AZOX or DMSO was added to a cell suspension system.

The complex III (ubiquinol–cytochrome c reductase) specific activity was measured by an improved spectrophotometric method using rat liver mitochondria, as previously described [15]. Decylubiquinol was prepared according to Fisher's method [16].

2.4. Adenine nucleotide extraction and measurement

Adenine nucleotides were extracted from cells with perchloric acid and measured by HPLC, as previously described [17].

2.5. Measurement of 2-deoxy-[^{3}H]-D-glucose uptake

2-Deoxyglucose uptake was measured as described previously [17].

2.6. Measurement of glucose and fatty acid oxidation

After 4-h treatment of L6 myotubes in serum-free medium containing [U–^{14}C]D-glucose at 37 °C, each treatment well was immediately covered with a piece of square shaped Whatman paper and fixed with tape. Reactions were stopped at 4 h by wetting the cover paper with 3 mol/l NaOH and injecting 70% perchloric acid into the medium. $^{14}CO_2$ formed from glucose oxidation was collected in the NaOH filter paper for over 1 h. The filter paper traps were counted in liquid scintillation fluid (Perkin-Elmer, MA, USA).

To assess free fatty acid oxidation, L6 myotubes were exposed to [^{3}H]-palmitate and the production of tritiated water was measured. Myotubes were exposed to DMEM containing 0.25 mM palmitate and 1.5 μCi [9,10-^{3}H (N)]-palmitic acid with or without AZOX for 4 h. The nonmetabolized palmitate in the medium was absorbed with charcoal slurry (0.1 g/ml charcoal in 20 mM Tris–HCl pH 7.5) for 30 min and removed by centrifugation. The radioactivity of the medium was then measured.

2.7. Determination of lipid synthesis in HepG2 cells

Fatty acid synthesis was measured as described previously [18].

2.8. Sulforhodamine B (SRB) cytotoxicity assay

L6 myotubes, 3T3-L1 fibroblasts and HepG2 cells were treated with or without the indicated doses of AZOX for 24 h, followed by SRB assay, as previously described [19].

2.9. Detection of mitochondrial ROS

Mitochondrial ROS was measured using MitoSOX Red (Invitrogen, USA), which is a live-cell permeant and is rapidly and selectively targeted to mitochondria. Once in the mitochondria, MitoSOX ™ Red reagent is oxidized by superoxide and exhibits red fluorescence (with excitation at 510 nm and emission at 580 nm). After drug treatment for the indicated time periods, cells were incubated in Hank's balanced salt solution (HBSS) containing 5 μM MitoSOX Red for 30 min at 37 °C. After incubation, cells were washed twice with PBS, then trypsinized, re-suspended, and immediately submitted for flow cytometric analysis. Data based on the FL2 channel were analyzed using the Cell Quest program.

2.10. Animal experiments

All animal experiments were approved by the Animal Care and Use Committee of the Shanghai Institute of Materia Medica, where the experiments were conducted. Six-week-old male C57BL/6J mice (Shanghai SLAC Laboratory Animal Co., Shanghai, China) were housed in a temperature-controlled room (22 ± 2 °C) with a light/dark cycle of 12 h. For chronic anti-diabetic and anti-obesity studies, mice were fed high fat diets (60% calories from fat; Research Diets, New Brunswick, NJ, USA). At 14 weeks of age, mice were randomly assigned to treatment groups. High-fat diet-fed mice were orally administered either vehicle (100% corn oil) or AZOX (25 mg/kg/day) for 35 days. Body weight and food intake were monitored daily. The fasting blood glucose levels and glucose tolerance test (2 g/kg glucose i.p.) were performed in mice fasted for 6 h. The insulin tolerance test (0.75 units/kg insulin i.p.) was conducted after 4 h of fasting. At the end of the study, blood samples were collected. Tissues were dissected, weighed, immediately frozen in liquid nitrogen and stored at −80 °C. For choric study of mice on normal diets, eight-week-old C57BL/6J mice were orally administered either vehicle (100% corn oil) or AZOX (25 mg/kg/day) for 28 days. Body weight and food intake were monitored daily. The fasting blood glucose levels and glucose tolerance test (2 g/kg glucose i.p.) were performed in 6 h-fasted mice.

2.11. Indirect calorimetry

Oxygen consumption rate (VO_2) and respiratory exchange ratio (RER) were measured under a consistent environmental temperature

(22 °C) using an indirect calorimetry system (TSE PhenoMaster, TSE system, Gmbh, Bad Homburg, Germany). For mice, studies were initiated after 24 h of acclimation to the metabolic chamber using an airflow rate of 0.40 l/min. VO_2 was measured in individual mice at 17 min intervals during a 53 h period. Animals were treated with vehicle or AZOX at the dose indicated in the figure legends. During these studies, mice had ad libitum access to food and water.

2.12. Metabolite analysis

Plasma insulin and leptin levels were measured using ELISA kits (Linco Research). Plasma triacylglycerol (TG), cholesterol (TC), HDL-cholesterol (HDL-c), LDL-cholesterol (LDL-c), lactate and nonesterified fatty acids (NEFA) were assayed using kits from Shanghai Fudan-Zhangjiang and Wako Diagnostics. Hepatic and muscular TG content were measured according to Folch's method [20].

2.13. Quantitative RT-PCR

Total RNA was isolated from homogenized tissues using TRIzol reagent (Invitrogen). Two and one-half micrograms of total RNA was reverse transcribed using PrimeScript Reverse Transcriptase (TaKaRa). The resulting cDNAs were amplified using SYBR® Premix Ex Taq™ (TaKaRa) and a 7300 Fast Real-Time PCR System (Applied Biosystems). Expression was normalized to β-actin. The following primer sequences were used: Mouse *Srebp-1c*, 5′-GGAGCCATGGATTGCACATT-3′ and 5′-GGCCCGGGAAGTCACTGT-3′; mouse *Fasn*, 5′-GGGTGACTCATTCCAGAACC-3′ and 5′-ACCAGCCCTCTCCATATCCT-3′; mouse *Acox1*, 5′-GCTCAGCAGGAGAAATGGATGC-3′ and 5′-AATGAACTCTTGGGTCTTGGGG-3′; mouse *Cpt1*, 5′-ATGACGGCTATGGTGTTTCC-3′ and 5′-TGTCCATCATGGCTTGTCTC-3′; and mouse β-*actin*, 5′-AGCCATGTACGTAGCCATCC-3′ and 5′-CTCTCAGCTGTGGTGGTGAA-3′.

2.14. Immunoblotting

Total proteins from tissues or cells were prepared in RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 1 mM Na_3VO_4, 1 mM DTT, 1 mM EDTA, and 1 mM EGTA) containing complete protease inhibitors (Roche). Protein (20 μg per sample) was electrophoresed through SDS–PAGE after boiling for 5 min in SDS loading buffer. The antibodies for AMPK, phospho-AMPK (Thr172), total ACC, phospho-ACC (Ser79), AS160 and phospho-AS160 (Thr642) were purchased from Cell Signaling Technology.

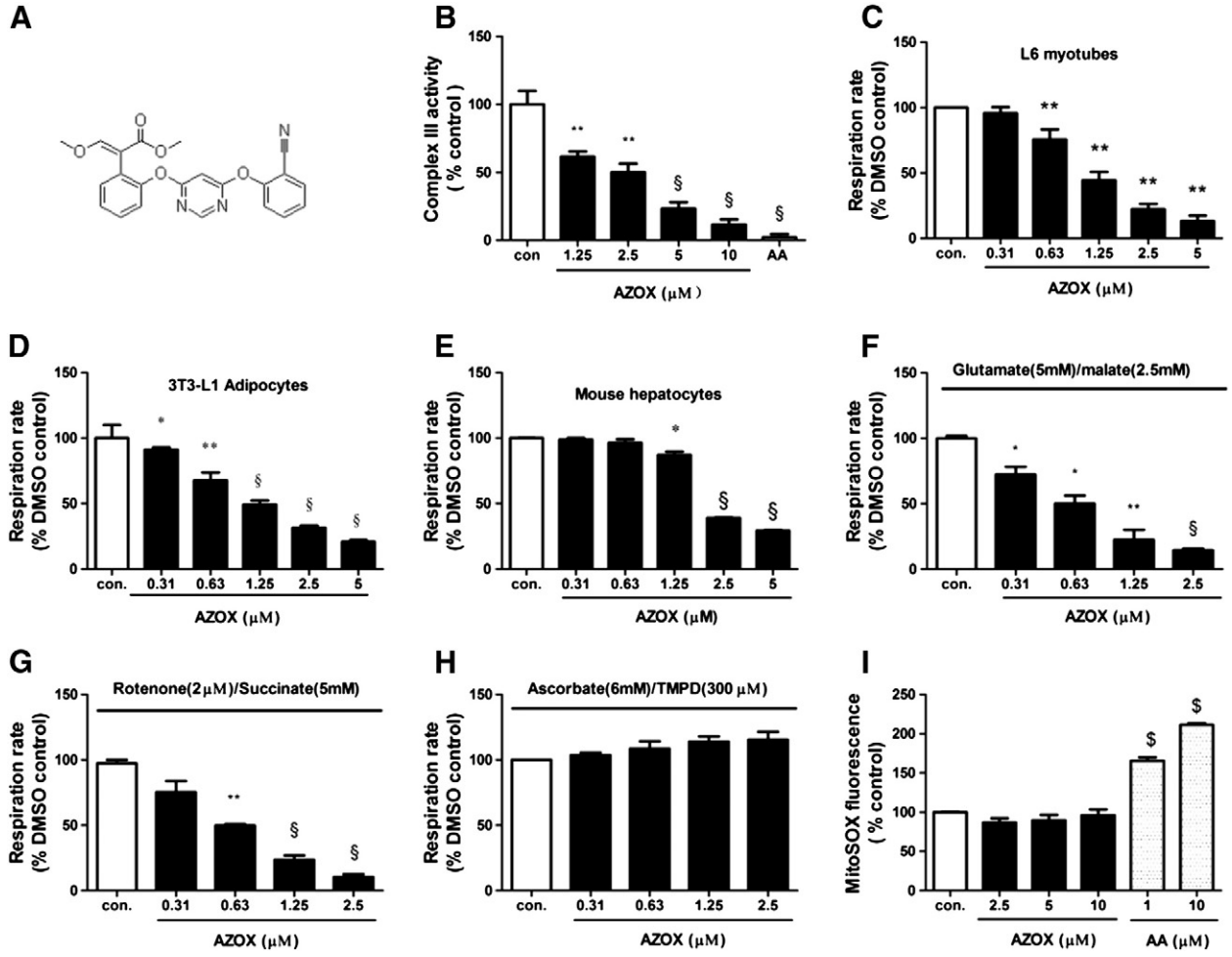

Fig. 1. AZOX affects O_2 consumption in metabolic cells via the Q_o site of the mitochondrial complex III. (A) The chemical structure of AZOX. (B) Dose-dependent inhibition of complex III specific activity in rat liver mitochondria. (C–E) AZOX inhibited the respiration of L6 myotubes, 3T3-L1 adipocytes and primary mouse hepatocytes in a dose-dependent manner. (F–H) Effects of AZOX on the respiration of mitochondria isolated from rat liver. AZOX dose-dependently inhibited mitochondrial ADP-stimulated respiration in the presence of complex I (glutamate + malate) substrates and complex II (succinate) substrates but not in the presence of complex IV (ascorbate + TMPD) substrates. (I) L6 myotubes were treated with AZOX and antimycin A (AA) for 3 h, then incubated with MitoSOX Red (5 μM) for 30 min. Fluorescence signals were measured and expressed as a percentage of the control treatment. AZOX did not increase mitochondrial superoxide, whereas antimycin A, a Q_i inhibitor, induced ROS production. *$p < 0.05$, **$p < 0.01$ and §$p < 0.001$ compared with relative the negative control groups (con.).

2.15. Statistical analysis

The results represent the mean ± SEM. All in vitro experiments were conducted at least three times. Differences between groups were examined using a two-tailed unpaired Student's *t*-test. The energy expenditure (EE) data in mice were assessed by analysis of covariance (ANCOVA), using body mass as a covariate. $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. AZOX reduces O_2 consumption in intact major metabolic cells and isolated mitochondria from rat liver by inhibiting respiratory complex III

Strobilurin fungicides inhibit mitochondrial respiration by binding to the Q_o site of cytochrome b, blocking electron transfer and disrupting the production of ATP in fungi and eukaryotes [11]. In this study, AZOX dose-dependently inhibited the activity of mitochondrial complex III (ubiquinol–cytochrome c reductase) isolated from rat liver (Fig. 1B). To investigate the effect of AZOX in intact major metabolic cells, we examined oxygen consumption in L6 myotubes, 3T3-L1 adipocytes, and mouse hepatocytes. AZOX induced a dose-dependent inhibition of respiration (Fig. 1C–E). To delineate the mechanism by which AZOX inhibited respiration in intact cells, we evaluated its effect on ADP-stimulated respiration in the presence of complex I (glutamate + malate), complex II (succinate) or complex IV (ascorbate + TMPD) substrate in isolated liver mitochondria. AZOX inhibited oxygen consumption in a dose-dependent manner in the presence of complex I and complex II substrates, but did not inhibit complex IV-linked respiration (Fig. 1F–H).

These results suggest that AZOX inhibits cellular respiration mainly via binding to mitochondrial complex III. Complex III is responsible for the major generation of cellular superoxide through the Q_o site, resulting in the release of superoxide into the intermembrane space or the matrix [21]. Incubation of L6 myotubes with AZOX did not increase mitochondrial superoxide. In contrast, the complex III inhibitor antimycin A, which binds to the Q_i site, inhibited the oxidation of ubiquinol in the electron transport chain of oxidative phosphorylation, resulting in ROS production (Fig. 1I). Thus, consistent with previous studies on strobilurin fungicides, AZOX affected the respiration of metabolic cells by targeting the Q_o site of complex III.

3.2. AZOX enhanced RER, but did not affect energy expenditure

Based on in vitro data, we examined whether AZOX could acutely affect whole-body energy homeostasis in standard diet-fed normal mice. In mice administered a single dose of 25 mg/kg AZOX, no changes in whole-body energy expenditure (EE) (Fig. 2A–C) or physical activity (Fig. 2D) were observed during the monitoring period. However, AZOX significant increased RER, indicating a relative shift away from fatty acid utilization (Fig. 2E–F). As skeletal muscle is the major tissue contributing to the basal metabolic rate, we investigated whether the increased RER can be explained by a direct effect at the cellular level by treating L6 myotubes with AZOX in the presence of either ^{14}C-palmitate or ^{14}C-glucose tracers. As shown in Fig. 2G–H, AZOX treatment inhibited glucose oxidation in a dose-dependant manner in L6 myotubes, and caused a more marked reduction in palmitate oxidation. Thus, fatty acid oxidation was more deeply depressed than glucose oxidation, suggesting the fundamental association with increased RER.

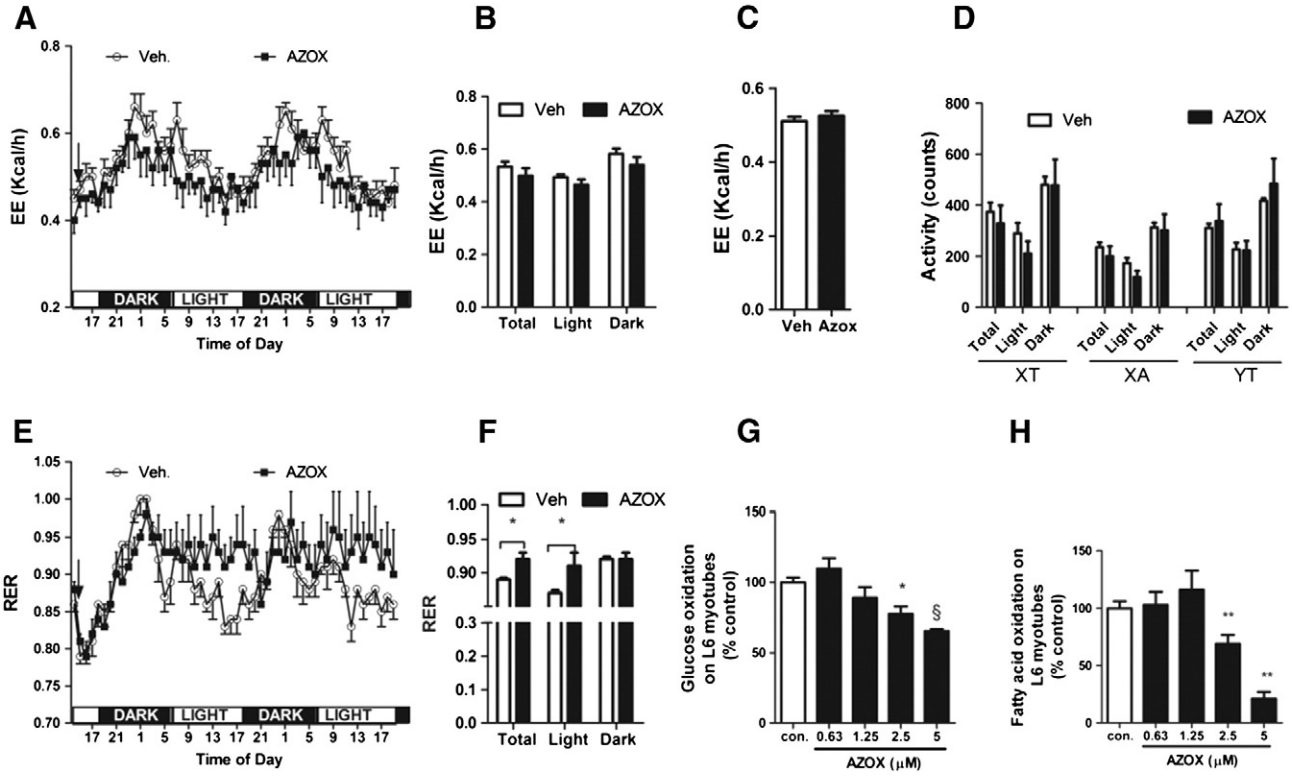

Fig. 2. Modulation of energy metabolism by AZOX in vivo. For indirect calorimetry measurement, after acclimation for 24 h in the chamber, C57BL/6J mice were orally dosed with vehicle or AZOX (25 mg/kg/day) at 14:00. (A) Changes in the hourly EE per mouse over the indicated periods. White circles, vehicle; black squares, AZOX. Arrow indicates the time of dosing. (B) The average hourly EE during the whole period of measurement, light phase and dark phase. (C) The average hourly EE adjusted for body weight by ANCOVA. (D) Physical activity was recorded by assessing *x*- and *y*-axis activity during the total, light and dark periods. XT, XA and YT denote the total activity counts along the *x*-axis, ambulatory activity along the *x*-axis, and the total activity counts along the *y*-axis, respectively. White bars, vehicle; black bars, AZOX (n = 6 per group). (E) Change in RER throughout the indicated periods of monitoring. (F) The average hourly RER during the total 53 h, light phase and dark phase. Changes in glucose (G) and fatty acid (H) oxidation were measured after 3 h treatment of L6 myotubes with AZOX or negative control. *p < 0.05 compared with the vehicle group. Exception of (G–H): *p < 0.05, **p < 0.01 and§p < 0.001 vs corresponding negative controls.

3.3. AZOX ameliorates metabolic status in high-fat diet-fed mice

To test the chronic effects of AZOX on diabetes and related metabolic disorders, we chronically administered AZOX to mice that were fed high-fat diets for 8 weeks. At the end of the 5-week treatment, obese mice treated with AZOX weighed markedly less (13.7%) than those in the vehicle obese group (Fig. 3A). While no changes in food intake were observed during AZOX treatment (Fig. 3B), AZOX treatment significantly reduced perirenal and inguinal fat, but not epididymal fat weight. The proportional weights of the pancreas and interscapular brown fat were unchanged by AZOX (Fig. 3G). Thus, body weight loss following AZOX treatment may be due to reduced white fat mass.

At the end of the 5-week treatment, administration of 25 mg/kg AZOX improved glucose tolerance (Fig. 3C–D) and insulin sensitivity, as demonstrated by the reduction in glycemia after a glucose challenge or insulin injection compared with the vehicle group. AZOX had no effect on fasting blood glucose levels (Fig. 3E–F). Investigation of the molecular mechanism underlying increased whole-body glucose disposal

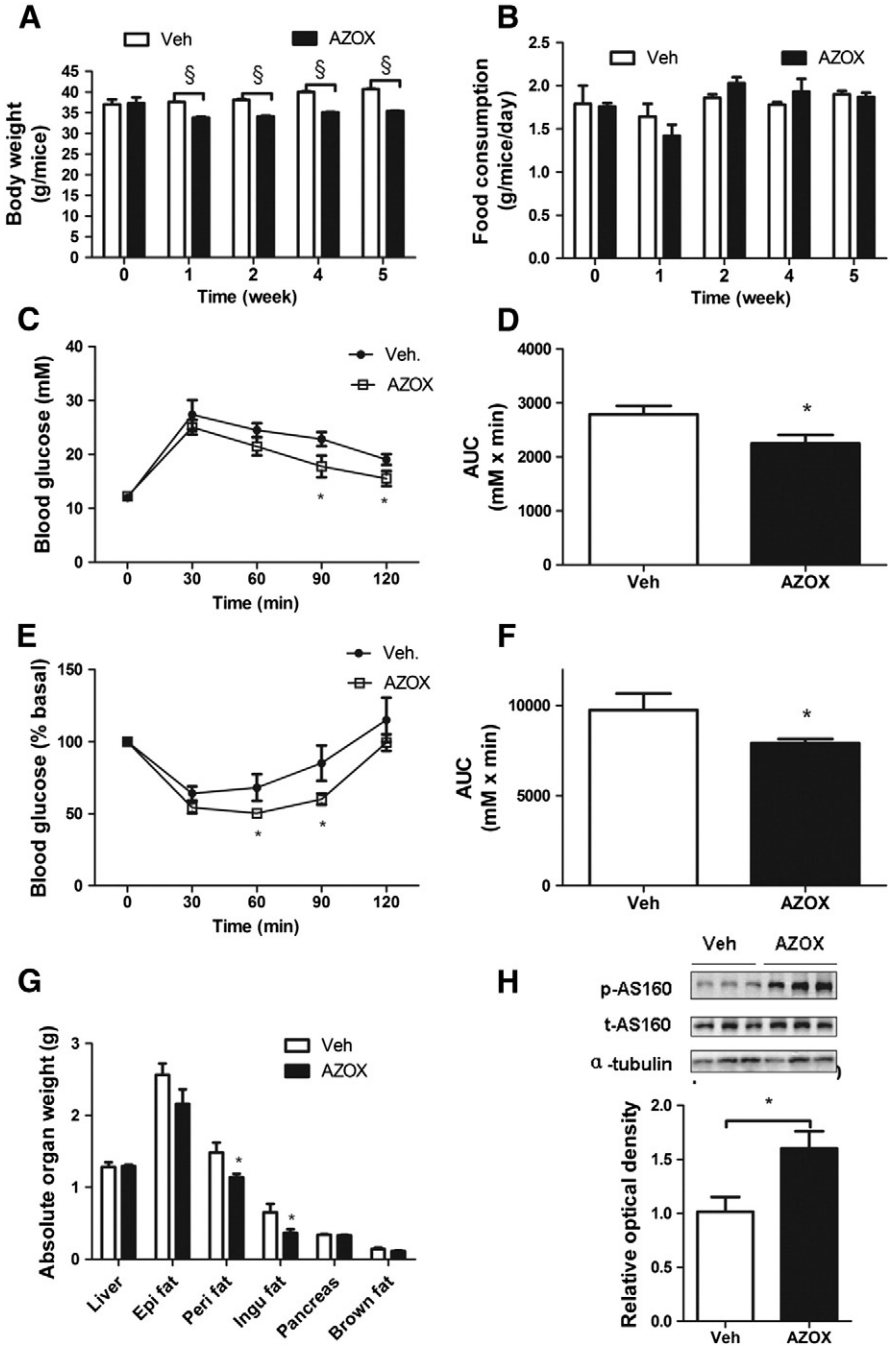

Fig. 3. Anti-obesity effect of 5-week chronic treatment with AZOX in high-fat diet-fed mice. (A) Body weight. (B) Food consumption. (C–D) Intraperitoneal glucose tolerance test was conducted at week 4 of treatment. Tail-vein blood was used for glucose assays after 6 h fasting. Blood glucose values were assessed before intraperitoneal (i.p.) injection of glucose (time 0), and at 30, 60, 90 and 120 min following the injection. The area under the glucose curve (AUC) from 0 min to 120 min was calculated. (E–F) Insulin tolerance tests were performed at week 4 of treatment. Blood glucose values were assessed before the test (time 0) and at 30, 60, 90 and 120 min afterwards. The area under the glucose curve from 0 min to 120 min was calculated. (G) Absolute weights of liver and epididymal (epi), perirenal (peri) and inguinal (ingu) fat pads, interscapular brown fat and pancreas. (H) Immunoblot of AS160 phosphorylation in the gastrocnemius muscle of HFD fed mice at the end of treatment. A representative blot is shown. The blot signal strength was quantified and presented after normalization to the respective AS160 protein level (n = 6 per group). White bars, vehicle; black bars, AZOX; black circles, vehicle; white squares, AZOX (n = 6 per group). *$p < 0.05$, §$p < 0.001$ compared with the vehicle group.

Table 1
Chronic effects of AZOX on metabolic variables in high-fat diet-fed mice.

Variable	Treatment	
	Vehicle	AZOX
Plasma insulin (ng/ml)	1.11 ± 0.25	1.11 ± 0.31
Plasma cholesterol (mg/dl)	218.96 ± 9.84	210.32 ± 13.44
Plasma triglyceride (mg/dl)	74.50 ± 2.48	62.35 ± 3.46*
Plasma LDL-c (mg/dl)	46.79 ± 2.95	44.04 ± 3.81
Plasma HDL-c (mg/dl)	38.96 ± 2.89	38.89 ± 1.92
Plasma leptin (ng/ml)	53.21 ± 6.44	28.66 ± 5.21*
Plasma NEFA (μEq/l)	1033.8 ± 48.21	895.4 ± 52.72*
Muscle triacylglycerol (μmol/g)	27.49 ± 0.82	15.39 ± 2.22*
Hepatic cholesterol (μmol/g)	8.84 ± 0.86	7.99 ± 0.54
Plasma lactic acid (mmol/l)	5.90 ± 0.27	5.60 ± 0.39
Plasma ALT (IU/L)	29.92 ± 1.47	28.78 ± 1.64
Plasma AST (IU/L)	25.28 ± 1.44	22.94 ± 1.42
Plasma UA (μmol/l)	140.37 ± 14.15	142.49 ± 5.61

Data represent the mean ± SEM of six mice. Mice were induced into obesity after 8 weeks on a high-fat diet and treated with vehicle or AZOX orally at a dose of 25 mg/kg/day. *$p < 0.05$ compared with vehicle group.

revealed that AZOX treatment significantly increased phosphorylation of AS160 in gastrocnemius muscle (Fig. 3H), which mediates insulin- and contraction-stimulated glucose uptake in mouse skeletal muscle [22].

Plasma parameter analysis indicated that AZOX treatment significantly decreased TG, NEFA and leptin levels, while plasma levels of insulin, TC, LDL-c and HDL-c were not altered after AZOX treatment (Table 1). The fact that plasma lactate levels remained unchanged after treatment with AZOX for 5 weeks suggested that the in vivo substrate utilization-shifting effect of AZOX did not cause whole-body energy starvation, which could result in a compensatory increase in anaerobic glycolysis (Table 1).

3.4. AZOX reduces hepatic lipid synthesis, possibly via the AMPK signaling pathway

Despite the potential whole-body fatty acid oxidation, triacylglycerol content in skeletal muscle decreased significantly compared with the control group after chronic AZOX treatment (Table 1). Moreover, chronic treatment of high-fat diet-fed mice with AZOX alleviated hepatic TG content (Fig. 4A), while having no substantial effect on hepatic TC content. To elucidate the mechanism underlying the beneficial effect of AZOX, we investigated fatty acid synthesis and fatty acid oxidation gene expression in liver tissue. AZOX treatment did not significantly affect genes such as acyl-CoA oxidase (*Acox1*) and carnitine palmitoyltransferase I (*Cpt1*), but it significantly decreased sterol regulatory element-binding proteins 1c (*Srebf-1c*) and fatty acid synthase (*Fasn*) mRNA levels (Fig. 4B).

The transcriptional activity of SREBP-1c, a master regulator of hepatic lipogenic gene, was previously reported to be associated with AMPK signaling. AMPK signaling has emerged as a critical regulator on lipid metabolic disorders [23,24]. Considering the depressed efficiency of energy production caused by AZOX at the cellular level, we postulated that AZOX might increase phosphorylation of AMPK. As expected, the level of phosphorylated AMPK and its direct downstream substrate acetyl coenzyme A synthase (ACC) significantly increased in the liver after chronic treatment with AZOX (Fig. 4C–D). The in vivo hepatic lipid content-lowering effect was the result of AZOX's ability to inhibit de novo lipid synthesis, as evidenced by its direct effect in HepG2 cells at the indicated non-cytotoxic concentrations (Fig. 4E, Supp Fig. 1). We observed a similar increase in the phosphorylation of AMPK and ACC after AZOX treatment at the cellular level (Fig. 4F–G). A commonly used AMPK inhibitor, Compound C, completely blocked the AZOX-meditated suppression of lipid synthesis in cultured cells (Fig. 4H), suggesting that AZOX inhibits de novo lipid synthesis predominantly via activation of AMPK.

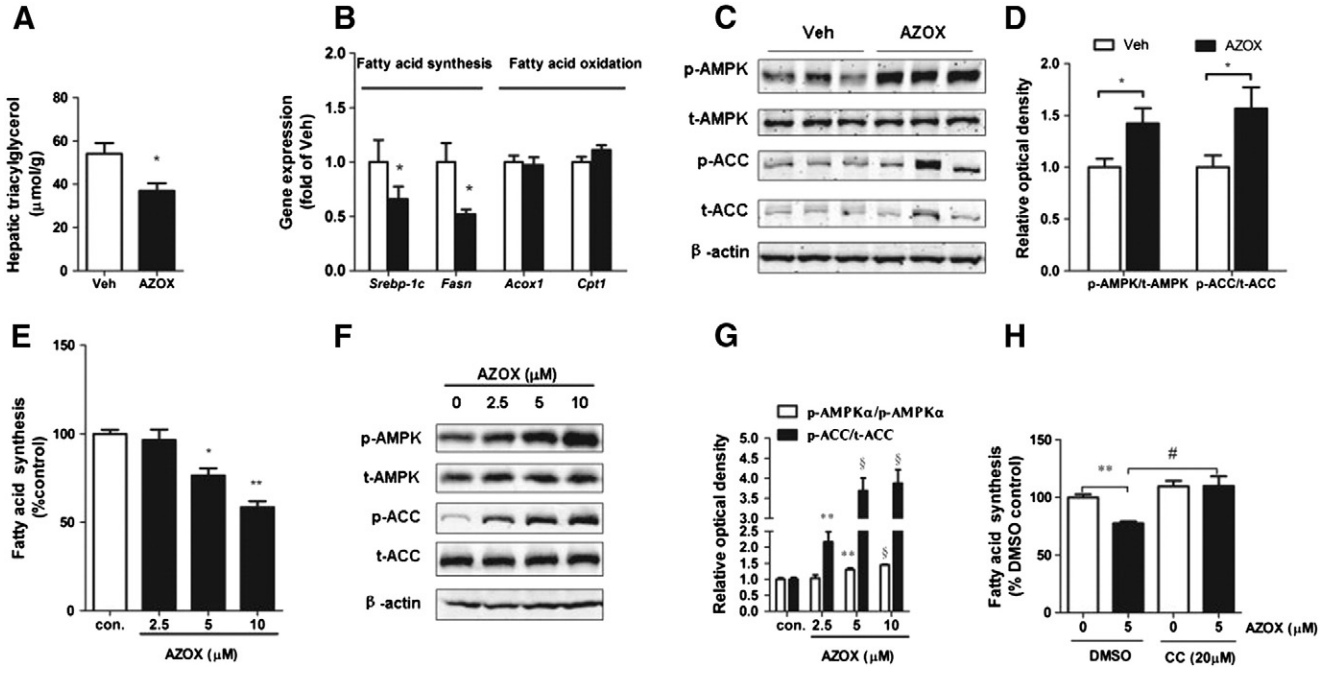

Fig. 4. Effects of AZOX on lipid accumulation in liver and HepG2 cells. (A) TG content in liver of high fat diet-fed mice. (B) RT-PCR analysis was used to measure the expression of genes related to fatty acid synthesis and oxidation in the livers (n = 6–7 per group). (C) AMPK and ACC phosphorylation in the liver of high-fat diet-fed mice. (D) The ratios of phosphorylated to total protein levels of AMPKα and ACC were determined. (E) Dose-dependent inhibition of fatty acid synthesis after 24 h exposure to AZOX at the indicated concentrations in HepG2 cells. (F) Dose-dependent increase in AMPK and ACC phosphorylation after 3 h treatment with AZOX at the indicated concentrations in HepG2 cells. (G) Ratios of the phosphorylated to total protein levels of AMPKα and ACC were determined. (H) HepG2 cells were preincubated for 30 min in the absence or presence of Compound C (20 μM) and then treated with 5 μM AZOX for 24 h, followed by the measurement of fatty acid synthesis. (A–D) White bars, vehicle; black bars, AZOX (n = 6 per group). *$p < 0.05$, §$p < 0.001$ compared with vehicle group. (E–H) White bars, control; black bars, AZOX; values represent the mean ± SEM from three independent experiments; *$p < 0.05$, **$p < 0.01$ and §$p < 0.001$ compared with corresponding negative controls. #$p < 0.05$ compared with AZOX treatment alone.

3.5. AZOX increases glucose uptake in myotubes and adipocytes

To understand the cellular basis for enhanced whole-body glucose utilization in AZOX-treated mice, we measured glucose uptake in response to AZOX treatment. AZOX stimulated glucose uptake in a dose-dependent manner in both L6 myotubes and 3T3-L1 adipocytes (Fig. 5A–B). AMPK is also involved in a signaling pathway that mediates glucose uptake [25–28]. The ADP to ATP ratio in L6 myotubes increased in a dose-dependent manner in response to AZOX treatment, accounting for the significant increase in AMPK and ACC phosphorylation (Fig. 5C–E), consistent with previous reports [29]. This enhanced AMPK activation was to some degree responsible for the stimulation of glucose uptake by AZOX, as demonstrated by the ability of Compound C to abolish the effect of AZOX on glucose uptake and AMPK activation (Fig. 5F–G).

In addition to AMPK signaling, insulin is a crucial stimulator of glucose uptake at the cellular level. As shown in Fig. 5H, treatment of 3T3-L1 adipocytes with insulin and AZOX together increased glucose uptake to a greater extent than either treatment alone. This additive effect indicates that AZOX stimulates glucose uptake in 3T3-L1 adipocytes through a pathway distinct from the insulin signaling pathway. Indeed, we also observed a significant increase in ACC phosphorylation, suggesting a role for AMPK in AZOX-mediated stimulation of glucose uptake in this cell type (Fig. 5I–J). As chronic inflammation in peripheral insulin-responsive tissues is closely related to insulin resistance [30], we examined the effect of AZOX on an inflammatory factor model with TNFα-induced insulin insensitivity in the same metabolic cell line. The impaired insulin-stimulated glucose uptake was significantly recovered after treatment with AZOX (Fig. 5K).

4. Discussion

Currently, it remains debatable whether changes in mitochondrial respiration capacity are cause or effect in the development of diet-induced type 2 diabetes. Mounting evidence suggests that type 2 diabetic individuals have mitochondrial dysfunction and intrinsic deficiencies in oxidative metabolism that impair insulin signaling by diverting fatty acids away from oxidation and toward production of lipid metabolites [31–35]. Conversely, several genetically-engineered models with targeted disruptions in master transcriptional regulators of mitochondrial biogenesis (e.g., PGC-1α, TFAM) display increased insulin sensitivity relative to wild-type mice, even when fed a high-fat diet [3,36]. Modulation of mitochondrial function through pharmacological approaches, including the use of anti-diabetes drugs such as metformin

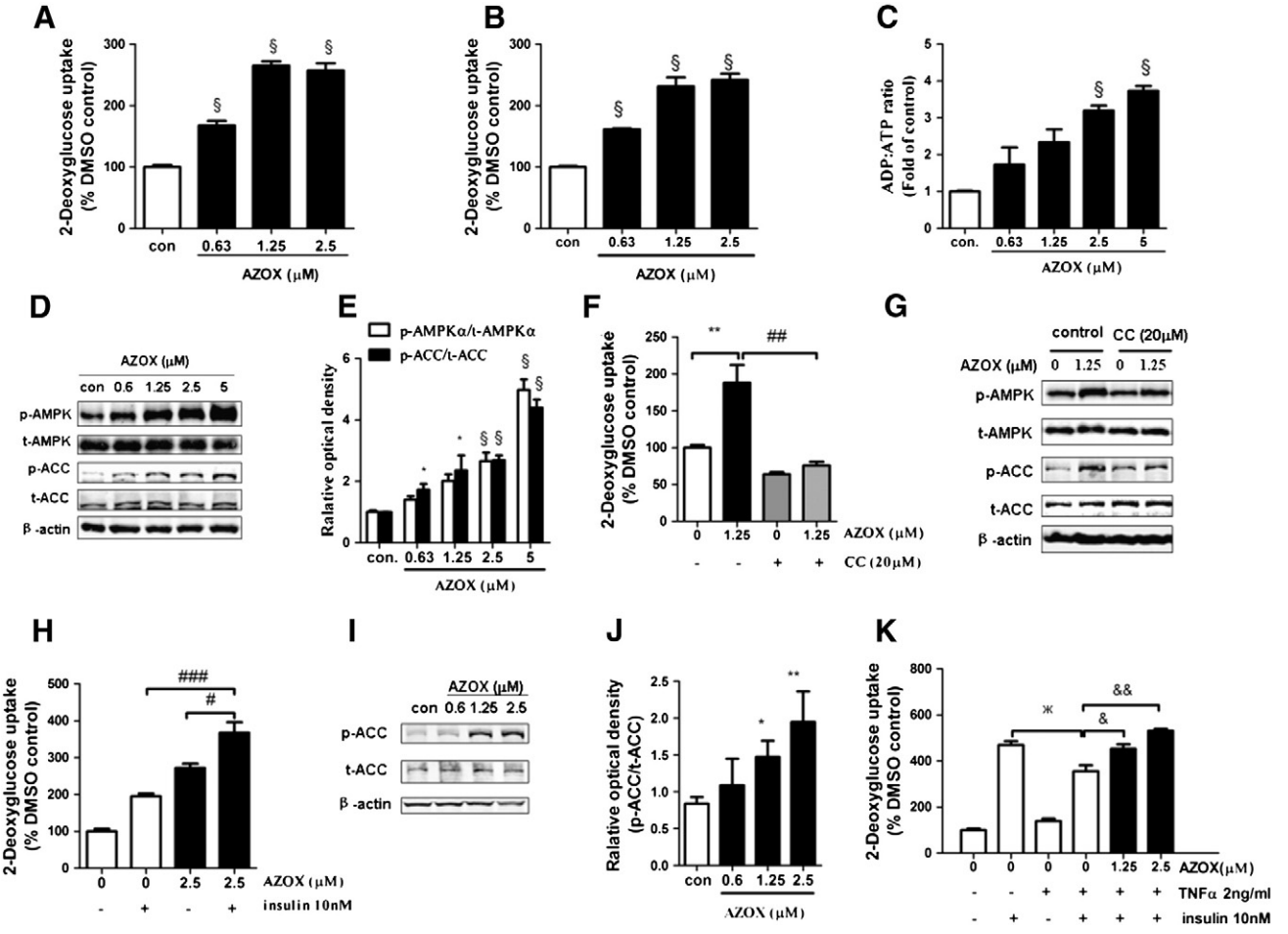

Fig. 5. AZOX increases glucose uptake in myocytes and adipocytes. Effect of AZOX on glucose uptake in L6 myotubes (A) or 3T3-L1 adipocytes (B) after 3 h treatment. (C) Dose-dependent increase in the ADP/ATP ratio in L6 myotubes exposed to AZOX at the indicated concentrations for 3 h. (D–E) Dose-dependent increase in AMPK and ACC phosphorylation in L6 myotubes after 3 h treatment with AZOX at the indicated concentrations, quantified as relative optical density. (F) L6 myotubes were preincubated for 30 min in the absence or presence of Compound C (20 μM) and then treated with 1.25 μM AZOX for 3 h, followed by measurement of glucose uptake. (G) Compound C (20 μM) treatment inhibited AZOX-induced AMPK and ACC phosphorylation in L6 myotubes. (H) 3T3-L1 adipocytes were incubated with DMSO or the indicated concentration of AZOX in the presence of insulin (10 nM). (I–J) Dose-dependent increase in ACC phosphorylation in 3T3-L1 adipocytes after 3 h treatment with AZOX at the indicated concentrations, quantified as relative optical density. (K) 3T3-L1 adipocytes become insulin resistant within the first 72 h of treatment with 2 ng/ml TNFα. Adipocytes were incubated with AZOX or control (DMSO) for the final 3 h after 72 h TNFα incubation before acute insulin (10 nM) stimulation. Insulin sensitivity was assessed by glucose uptake assay. The results represent the mean ± SEM from three independent experiments; *$p < 0.05$, **$p < 0.01$ and §$p < 0.001$ compared with corresponding negative controls (as indicated). Exception for (F, H, K): #$p < 0.05$, ## $p < 0.01$ compared with AZOX treatment alone and ###$p < 0.001$ compared with insulin treatment alone. ж$p < 0.05$ compared with insulin treatment alone, &$p < 0.01$ and &&$p < 0.01$ compared with insulin resistance control.

[4,37], thiazolidinediones (TZDs) [5,38], and other natural products with antiobesity and antidiabetic properties (i.e. berberine [6] and resveratrol [39,40]), have proven beneficial; these drugs exert their metabolic effects, at least in part, via direct reduction of the activity of respiratory complex I or ATP synthase. More interestingly, CPT-1 inhibitors such as etomoxir and oxfenicine, which decrease fatty acid oxidation, have been found to consistently elevate glucose oxidation and ameliorate hyperglycemia and hyperinsulinemia [41]. These findings raise the prospect of developing novel therapeutics for obesity-related type 2 diabetes by targeting other mitochondrial respiratory complexes.

The strobilurins are well-established inhibitors of complex III, where ROS are generated from the Q_o site [11]. Strobilurins inhibit electron transfer in the cytochrome bc1 complex, preventing cytochrome b reduction and thereby attenuating ROS production [42,43]. We demonstrated that AZOX potently inhibits mitochondrial respiration in metabolic cells, including myocytes, adipocytes and hepatocytes, in the presence of respective complex I and complex II substrates; it does not affect mitochondrial respiration in the presence of complex IV substrates. Furthermore, AZOX directly inhibits complex III activity in rat liver mitochondria in a dose dependent manner. Unlike Q_i site inhibitors, AZOX does not increase the level of mitochondrial superoxide. These data suggest that AZOX has the potential to alter whole-body energy metabolism by targeting the Q_o site of mitochondrial complex III.

The effect of AZOX on oxygen consumption at the cellular level can be attributed predominantly to its pronounced inhibitory effect on fatty acid oxidation relative to glucose oxidation. This is demonstrated by changes in the respective RER during light and dark periods: AZOX treatment had a more potent effect during the light period, when rodents prefer fatty acid utilization. The in vivo shift from lipid to carbohydrate substrate oxidation is consistent with the in vitro specific effect of AZOX. Mitochondrial overload and incomplete fatty acid oxidation are known to contribute to skeletal muscle insulin resistance [2]; similar abnormalities in acylcarnitine accumulation and incomplete β-oxidation have been identified in humans with obesity or type 2 diabetes [31,32]. These data suggest that AZOX improves insulin sensitivity in skeletal muscle by blocking β-oxidation and thus relieving mitochondrial overload. Further research is needed to determine whether the preferential inhibition of fatty acid oxidation is the result of a specific effect of AZOX or a common effect of any factor or compound targeting the Q_o site of respiratory complex III.

It remains unknown whether the AZOX-induced reduction in complex III activity at the cellular level could affect whole-body EE or change the activity of other mitochondrial respiratory complexes in a compensatory manner; no changes in whole-body EE were observed in this study. Previous studies have reported that upregulation of mitochondrial fatty acid oxidation does not necessarily change whole-body EE [44]. Several lines of evidence currently suggest that vascular endothelial growth factor, which is sensitive to hypoxic stress, induces brown adipose tissue with upregulation of the key thermogenesis factor uncoupling protein 1, causing a local increase in EE [45]. In addition, berberine inhibits mitochondrial respiration, but also augments energy expenditure and increases the expression of uncoupling protein 2, which is associated with energy expenditure [46]. Thus, while it is uncertain whether AZOX treatment enhances uncoupling effects, it is reasonable to conclude that AZOX-induced alteration in the whole-body energy homeostasis is not consistent with its effects at the metabolic cellular level.

Following chronic AZOX treatment, high-fat diet-fed mice displayed improved glucose tolerance; this can be attributed in part to increased glucose uptake by both myocytes and adipocytes. AMPK activation has been implicated as an insulin-independent mechanism for the stimulation of glucose uptake [47]. Reduced mitochondrial energy regeneration increases the AMP-to-ATP or ADP-to-ATP ratio, which is a major regulatory factor of AMPK signaling [48]. Considering the characteristic effect of AZOX on the respiratory system in metabolic cells, it makes sense that AZOX robustly stimulates the AMPK pathway in three types of metabolic cells. Accordingly, we demonstrated that the effect of AZOX on glucose uptake in L6 myotubes was dependent on AMPK, as an AMPK inhibitor abolished this effect. Moreover, AZOX further increased glucose uptake in adipocytes under insulin-stimulated conditions. Thus, AZOX-stimulated glucose uptake in both myocytes and adipocytes was likely mediated by AMPK activation, a pathway distinct from insulin signaling. Despite the effect of AZOX on glucose uptake, we cannot overstate the fact that any inhibitor of mitochondrial respiratory function must increase the metabolic process. Indeed, antimycin A, a Q_i site inhibitor of complex III, cannot increase glucose uptake even at low concentrations that are not toxic to cells (Supp Fig. 2).

In addition to increased glucose uptake under basal conditions, glucose tolerance was ameliorated by an improvement in compromised insulin sensitivity developed from a chronic high fat diet. Insulin resistance, a state in which peripheral tissues are rendered unresponsive to insulin's glucose-lowering and anabolic properties, is a hallmark of type 2 diabetes. Excess lipid accumulation in insulin-sensitive organs, such as skeletal muscle and the liver, is strongly associated with insulin resistance in humans and rodents [35,49]. The liver is the major site for whole-body de novo fatty acid synthesis; activation of AMPK in the liver can inhibit lipogenesis via inhibition of SREBP transcriptional activity and the production of key target lipogenic enzymes [23]. After chronic treatment with AZOX in high-fat diet-fed mice, reduced lipid accumulation in the liver and muscle was observed, accompanied by a significant decrease in serum triglyceride levels. The effect of reduced lipid accumulation in the liver was consistent with previous reports using metformin or berberine [50,51]. ACC inhibits the rate-limiting step of lipogenesis [52]; as a downstream target of AMPK activation, hepatic ACC activity should be significantly suppressed by AZOX treatment, as demonstrated by its phosphorylation. At the cellular level, the inhibitory effect on lipogenesis in HepG2 was fully abrogated by treatment with Compound C, demonstrating that AMPK activation was required for AZOX to exert this effect.

AZOX has undergone stringent scrutiny for toxicity. In a chronic toxicity study of AZOX in male mice, an oral dose of up to 37.5 mg/kg/day for 2-year Chronic Testing did not produce any toxic side effects (U.S. Environmental Protection Agency, Pesticide Fact Sheet for Azoxystrobin). Accordingly, we determined that AZOX could be tolerated in animal models at 25 mg/kg/day doses, lower than the NOAEL (No Observed Adverse Effect Level). Although high-fat diet-fed mice treated with AZOX weighed markedly less than the vehicle group at the end of 5 weeks of treatment, the fact that they displayed unchanged food intake and plasma insulin suggests no toxicity to appetite control or pancreatic β cells. After chronic administration of the same dose of AZOX in normal mice on standard diets, no alteration of glucose tolerance, body weight or food intake was observed (Supp Fig. 3). Furthermore, there was no obvious change in plasma markers of liver and kidney toxicity (Table 1). Therefore, our data demonstrate that the improved glucose tolerance and response to insulin in mice receiving AZOX is due to a direct effect on peripheral lipid and glucose metabolism rather than a secondary effect of toxicity. AZOX treatment did, however, decrease carbohydrate and fatty acid oxidation and increase lactate production of L6 myotubes (Supp Fig. 4). The potential for lactic acidosis caused by AZOX in vivo with a higher dosage still needs to be explored.

In conclusion, our study demonstrates the beneficial effects of AZOX on the regulation of whole-body glucose and lipid homeostasis in the development of obesity-related type 2 diabetes. AZOX inhibits mitochondrial respiratory complex III and subsequently provides beneficial metabolic outcomes, in part by activating AMPK. The combined beneficial effects on lipid accumulation in liver, muscle, adiposity mass and lipidemia contribute to improved insulin sensitivity in mice receiving AZOX. In addition, AZOX treatment enhances whole-body glucose tolerance and insulin sensitivity even under the condition of relatively reduced fatty acid utilization. Our findings provide evidence that a Q_o inhibitor of mitochondrial respiratory complex III can represent a novel approach for the treatment of obesity and type 2 diabetes.

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