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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