

Research Article

# Oxidative stress regulates cellular bioenergetics in esophageal squamous cell carcinoma cell

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The aim of the present study was to explore the effects of oxidative stress induced by CoCl<sub>2</sub> and H<sub>2</sub>O<sub>2</sub> on the regulation of bioenergetics of esophageal squamous cell carcinoma (ESCC) cell line TE-1 and analyze its underlying mechanism. Western blot results showed that CoCl<sub>2</sub> and H<sub>2</sub>O<sub>2</sub> treatment of TE-1 cells led to significant reduction in mitochondrial respiratory chain complex subunits expression and increasing intracellular reactive oxygen species (ROS) production. We further found that TE-1 cells treated with CoCl<sub>2</sub>, a hypoxia-mimicking reagent, dramatically reduced the oxygen consumption rate (OCR) and increased the extracellular acidification rate (ECAR). However, H<sub>2</sub>O<sub>2</sub> treatment decreased both the mitochondrial respiration and aerobic glycolysis significantly. Moreover, we found that H<sub>2</sub>O<sub>2</sub> induces apoptosis in TE-1 cells through the activation of PARP, Caspase 3, and Caspase 9. Therefore, our findings indicate that CoCl<sub>2</sub> and H<sub>2</sub>O<sub>2</sub> could cause mitochondrial dysfunction by up-regulation of ROS and regulating the cellular bioenergy metabolism, thus affecting the survival of tumor cells.

## Introduction

Esophageal squamous cell carcinoma (ESCC) is the most frequent histological subtype of esophageal cancer and is the sixth most common cause of cancer-associated mortality worldwide [1]. Despite advances in early diagnosis, surgery, and chemoradiotherapy, the prognosis of ESCC is still very poor and remains a challenge [2]. Thus, our understanding of mechanisms of ESCC tumorigenesis is urgent, which will facilitate the development of diagnosis marker and novel therapeutic strategies.

Unlike normal cells, tumor cells demand a vast amount of energy (ATP) and metabolites to support their rapid proliferation. Indeed, some tumor cells are addicted to glycolysis rather than oxidative phosphorylation (OXPHOS) even in normoxic conditions and need quick reprogramming of energy metabolism. This phenomenon is often referred to as the Warburg effect [3]. Importantly, glycolysis provides cancer cells with not only energy but also crucial intermediates for biosynthesis of macromolecules, lipids as well as NADPH which is important for redox homeostasis. Metabolic reprogramming is required for cell malignant transformation, tumorigenesis, invasion, metastasis, and resistance to cancer treatments [4]. In solid tumor, hypoxia is a crucial microenvironmental stimuli that leads to significant up-regulation of hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) in majority of human cancers [5-7]. Cobalt chloride (CoCl<sub>2</sub>), a well-known hypoxia-mimicking agent *in vitro*, which blocks HIF-1 $\alpha$  degradation and thus causes HIF-1 $\alpha$  accumulation in cells, therefore leading to an intracellular hypoxia-like microenvironment and enhancing tumor malignancy.

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Because hydrogen peroxide ( $H_2O_2$ ) can generate large amounts of oxygen free radicals and cause oxidative stress in tumor cells, it is widely used as an apoptosis inducer [8]. Indeed, many anticancer drugs promote cancer cell death through enhancing the intracellular  $H_2O_2$  generation [9,10]. Thus, studying the effects of  $H_2O_2$  on cancer cells is helpful to provide novel strategies for the prevention and treatment of cancers.

$H_2O_2$  is also an important signaling molecule in tumor cells, which can regulate cell signaling pathways and transcription factors at different levels [11,12]. For example,  $H_2O_2$  can induce the activation of EGFR, which leads to the amplification of Ras signaling cascade and activation of mitogen-activated protein kinases (MAPKs) [13]. Moreover,  $H_2O_2$  can indirectly regulate PTK-EGFR-Ras signaling in cancer cells, and finally the activation of MAPK, so that ERK, JNK, p38, three subfamilies signaling pathways are regulated [13,14].

Low concentration of  $H_2O_2$  plays an essential role in regulating cell division and growth as a signaling molecule [15,16]; however, when the amount of  $H_2O_2$  exceeds a certain critical value, the cell cycle will be blocked and even lead to apoptosis [17]. Therefore, the up-regulation of  $H_2O_2$  amount in tumor cells is one of the strategies to kill tumor cells. This process can also be rescued by up-regulation of catalase expression. Overexpression of SOD inhibits tumor cell proliferation, metastasis, and other malignant phenotypes. Similarly, catalase or glutathione reductase can reverse the antitumor effect of SOD [18].

In the present study, we examined the effects of  $CoCl_2$ -induced hypoxia and  $H_2O_2$  on the regulation of cellular bioenergy metabolism in ESCC cells and sought to investigate the underlying molecular mechanism.

## Materials and methods

### Reagents and antibodies

RPMI-1640 medium and FBS (Life Technologies, Grand Island, NY); oligomycin, FCCP, rotenone, antimycin A, glucose, 2-DG, and  $CoCl_2$  were purchased from Sigma (St. Louis, MO); Annexin V-FITC/propidium iodine (PI) apoptosis detection kit was from BD Pharmingen (San Diego, CA); 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), horseradish peroxidase (HRP) conjugated anti-rabbit, anti-mouse Ig, trypsin were from Beyotime (Shanghai, China). The following antibodies were used: anti-COXI, anti-COXII, anti-COXIV, and anti-SDHA (Abcam, Cambridge, MA); anti-ND2, anti-NDUFA5, anti-NDUFA6, anti-NDUFA9, anti-HIF-1 $\alpha$ , anti-ATP5A, anti-PARP, anti-Caspase-9, anti-Caspase-3, anti-cleaved Caspase-3 (Cell Signaling, Beverly, MA); anti- $\beta$ -actin (Abmart, Shanghai, China); anti-Cyt. b (Santa Cruz Biotechnology, Santa Cruz, CA). All other chemicals used were of highest analytical grade and obtained from Sigma, unless otherwise stated.

### Cell lines and culture conditions

The human ESCC cell line TE-1 was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). TE-1 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and antibiotics (100 units/ml penicillin and 100  $\mu$ g/ml streptomycin) at 37°C with 5%  $CO_2$ .

### Determination of alterations in cell morphology and cell number

TE-1 cells were plated in 60-mm dishes at a density of  $0.3 \times 10^6$  and incubated with series concentrations of  $H_2O_2$  for 24 h at 37°C with 5%  $CO_2$ . Inverted microscopy (Nikon, Tokyo, Japan) was used to examine the alterations in cell morphology and cell number.

### FACS analysis for intracellular ROS

Cells were harvested by trypsin, washed with PBS, and incubated with DCFH-DA at a final concentration of 10  $\mu$ M in RPMI-1640 medium for 30 min at 37°C, then cells were washed three times with cold PBS. Intracellular reactive oxygen species (ROS) levels were measured as described previously by using the fluorescence probe DCFH-DA according to the manufacturer's protocol (Beyotime, Shanghai, China) [19]. The excitation wavelength for DCF fluorescence is 488 nm and the emission wavelength for DCF fluorescence is 525 nm, respectively.

### FACS analysis for apoptosis

Harvested cells were washed with cold PBS twice, and incubated with  $1 \times$  binding buffer at a concentration of  $1 \times 10^6$  cells/ml. Annexin V-FITC/PI (BD, San Jose, CA) was added, followed by incubation in the dark at room temperature for 20 min. Four hundred microliters of  $1X$  binding buffer was added to each tube. The samples were analyzed by flow cytometry within 1 h.

## Immunoblotting

Cells were washed with cold PBS and lysed in RIPA lysis buffer supplemented with protease and phosphatase inhibitors on ice for 20 min, followed by centrifugation at 13000 rpm for 20 min at 4°C, and the supernatants were collected. Protein concentrations of whole cell extracts were determined using the Pierce BCA protein assay kit. Cell extracts equivalent to 20 µg total protein were resolved in 10% SDS/PAGE gels followed by electrophoretic transfer on to nitrocellulose membrane (Bio-Rad, Hercules, CA) in Tris-glycine buffer. Blots were blocked at room temperature for 2 h in 3% non-fat milk in TBS-Tween (TBS-T) on a shaker, and then incubated with the primary antibodies overnight at 4°C. The membrane was washed in TBS-T for at least 3× for 10 min and then incubated with HRP conjugated anti-rabbit or anti-mouse IgG at room temperature for 1 h with gentle shaking. Signal was detected using ECL according to the manufacturer's protocol (Thermo Scientific, Rockford, IL) and exposed to X-ray films. β-actin was used as control for equal loading and the optical density was measured using National Institute of Health ImageJ software. Each experiment was repeated at least three times.

## RNA preparation and quantitative real-time PCR analysis

Total RNAs were extracted from cells using TRIzol reagent (Life Technologies, Carlsbad, CA) following manufacturer's instructions. cDNA was synthesized from 2 µg purified total RNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Dalian, China). Quantitative real-time PCR (qRT-PCR) was performed as described previously [19] using gene-specific primers. The results were normalized to β-actin in the same samples. Each sample was analyzed in triplicate and repeated three times. The primer sequences used were as follows: *COXI* 5'-TCCGCTACCATAATCATCGCT-3' (forward), 5'-CCGTGGAGTGTGGCGAGT-3' (reverse); *COXII* 5'-CGACTACGGCGGACTAATCT-3' (forward), 5'-TCGATTGTCAACGTCAAGGA-3' (reverse); *COXIV* 5'-GCAGTGGCGGCAGAATG-3' (forward), 5'-AGTCTTCGCTCTTCACAACA-3' (reverse); *ATPase6* 5'-CTGTTTCGCTTCATTGC-3' (forward), 5'-AGTCATTGTTGGGTGGTGAT T-3' (reverse); *ATPase8* 5'-AACTACCACCTACCTCCCTCAC-3' (forward), 5'-GCAATGAATGAAGC ATTCATTGC-3' (reverse); *ND3* 5'-GCGGCTTCGACCCTATATC-3' (forward), 5'-TGGCAGGTTAGTT GTTTGTAGG-3' (reverse); *ND4* 5'-TGAACGCAGGCACATACTTC-3' (forward), 5'-TCTTGGGCAGT GAGATGAG-3' (reverse); *ND5* 5'-CATTGTGCGCATCCACCTTTA-3' (forward), 5'-CTGGGTGTTTGG GTTGTG-3' (reverse); *ND6* 5'-GGTGTGTGGGTGAAAGAGT-3' (forward), 5'-CTCCCGAATCAA CCCTGAC-3' (reverse); β-actin 5'-GCCAGTGGACTCCACGAC-3' (forward), 5'-CAACTACATGGTTTA CATGTTC-3' (reverse).

## OXPHOS and glycolysis assay

The intact cellular oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of TE-1 cells were measured using a Seahorse XF-96 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA) as previously described [19]. Results were obtained by performing three independent experiments with eight replicates. After the assay was completed, a BCA Protein Assay Kit was used according to the manufacturer's instructions to determine the protein concentration to normalize OCR and ECAR.

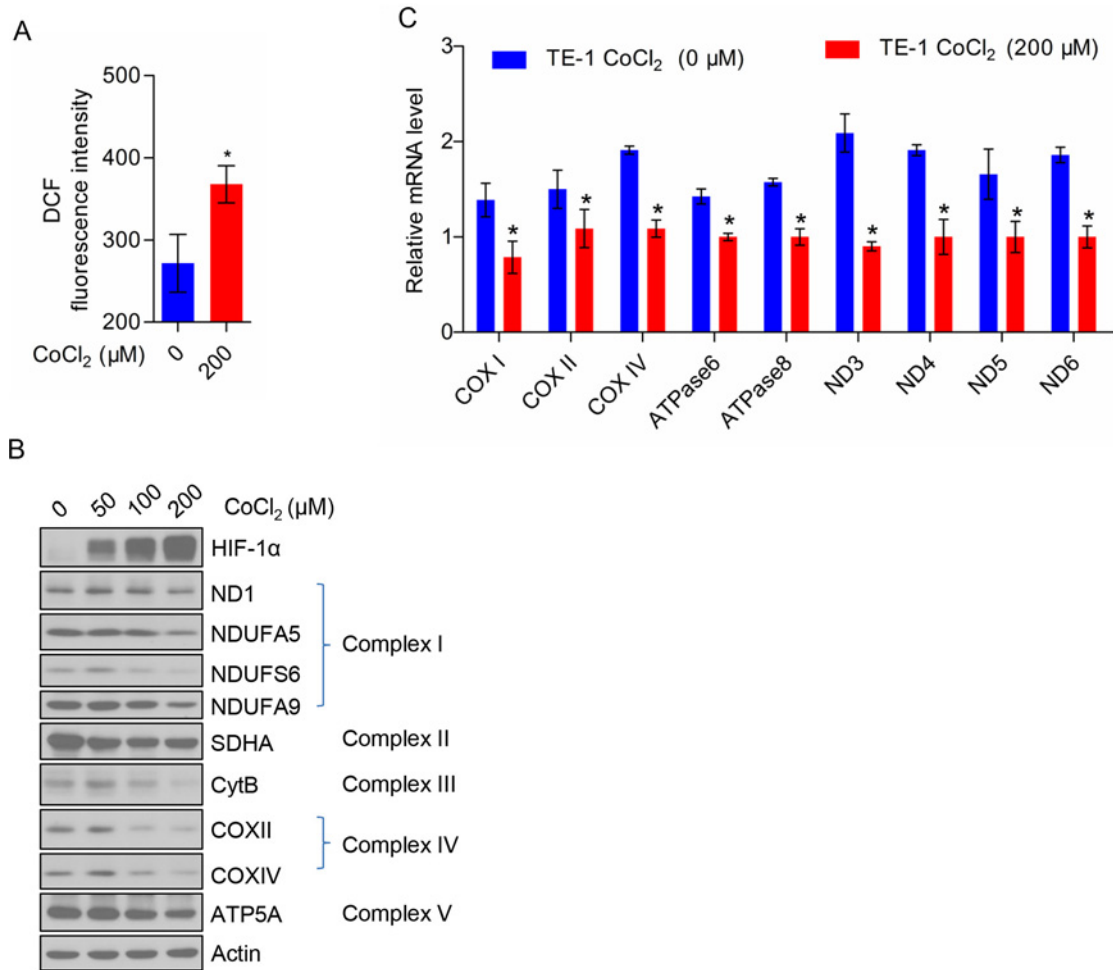
## Statistical analysis

All experiments were performed at least three times and the data obtained were analyzed using SPSS version 16.0 (SPSS, Chicago, U.S.A.). The values are presented as the mean ± S.D. For the evaluation of two groups, Student's *t* test was used. *P* < 0.05 was considered to indicate a statistically significant difference.

## Results

### Effects of CoCl<sub>2</sub> on the expression of mitochondrial respiratory chain complex subunits

To determine the effect of CoCl<sub>2</sub> on TE-1 cells, we first measured the intracellular ROS level in CoCl<sub>2</sub>-treated TE-1 cells by flow cytometry. CoCl<sub>2</sub> treatment significantly increased the ROS production of TE-1 cells as shown in Figure 1A. Next, we treated TE-1 cells with the indicated concentration of CoCl<sub>2</sub> (0, 50, 100, and 200 µM) and analyzed the expression of HIF-1α, complex I subunits (ND1, NDUFA5, NDUFS6, and NDUFA9), complex II subunit (SDHA), complex III subunit (Cyt. B), complex IV subunits (COX II and COX IV) and complex V subunit (ATP5A) in TE-1 cells by Western blot. We found that the expression of HIF1-α protein increased in a dose-dependent manner of COCl<sub>2</sub>. However, the expression of ND1, cytochrome *b* and COX II encoded by mtDNA and NDUFA5, NDUFS6, NDUFA9, SDHA, COX IV, and ATP5A encoded by nuclear gene were decreased in a dose-dependent manner of



**Figure 1. CoCl<sub>2</sub> inhibits the expression of mitochondrial respiratory chain complex subunits**

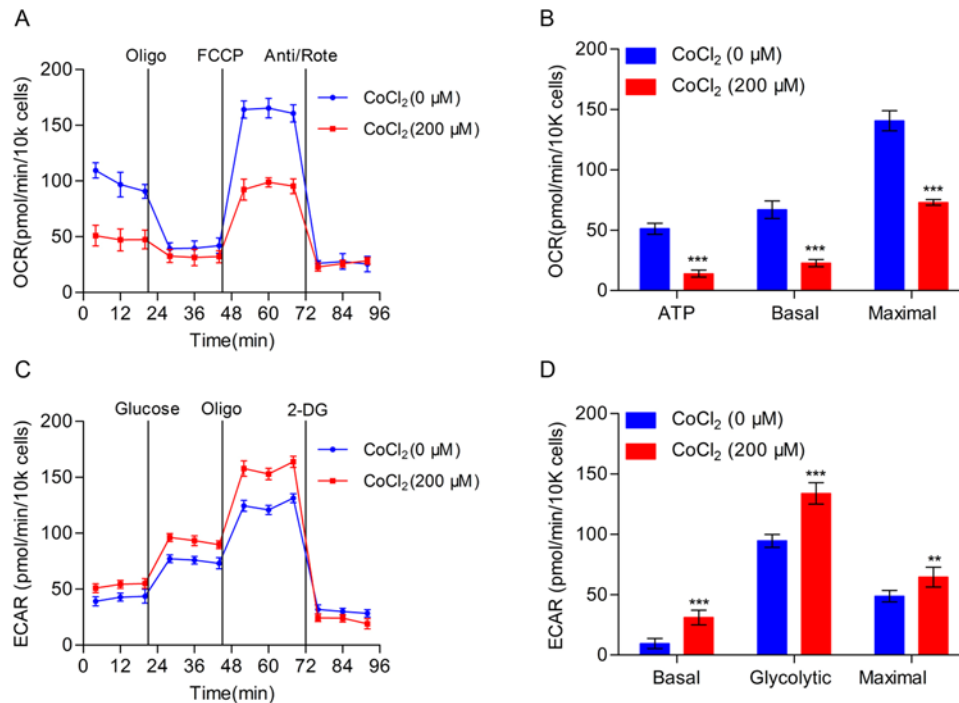
(A) CoCl<sub>2</sub> (200 μM) induces ROS production in TE-1 cells. (B) The expression profile of mitochondrial respiratory chain complex subunits and HIF-1α in TE-1 cells treated with a gradient concentration of CoCl<sub>2</sub>. (C) CoCl<sub>2</sub> (200 μM) reduces the mRNA level of mitochondrial respiratory chain complex subunits of TE-1 cells.

CoCl<sub>2</sub> treatment (Figure 1B). To clarify whether CoCl<sub>2</sub> regulates the protein expression or transcription, we further examined the mRNA levels of these proteins (Figure 1C).

Taken together, our findings indicated that CoCl<sub>2</sub> may inhibit mitochondrial respiration in TE-1 cells.

## Effect of CoCl<sub>2</sub> on TE-1 cell bioenergetics metabolism

In order to further study the effect of CoCl<sub>2</sub> on cellular bioenergetics metabolism, we used Seahorse XF96 Extracellular Flux Analyzers to detect the OCR and found that OCR in TE-1 cells decreased significantly after treating with CoCl<sub>2</sub> for 24 h (Figure 2A). The production of ATP, basal respiration, and maximal respiration was markedly reduced and the difference was statistically significant (Figure 2B). In addition, we detected the ability of glycolysis in TE-1 cells when treated with CoCl<sub>2</sub>, as result showed that when compared with the negative control, the glycolysis ability of TE-1 cells significantly increased under the treatment of CoCl<sub>2</sub> and the difference was statistically significant (Figure 2C,D).

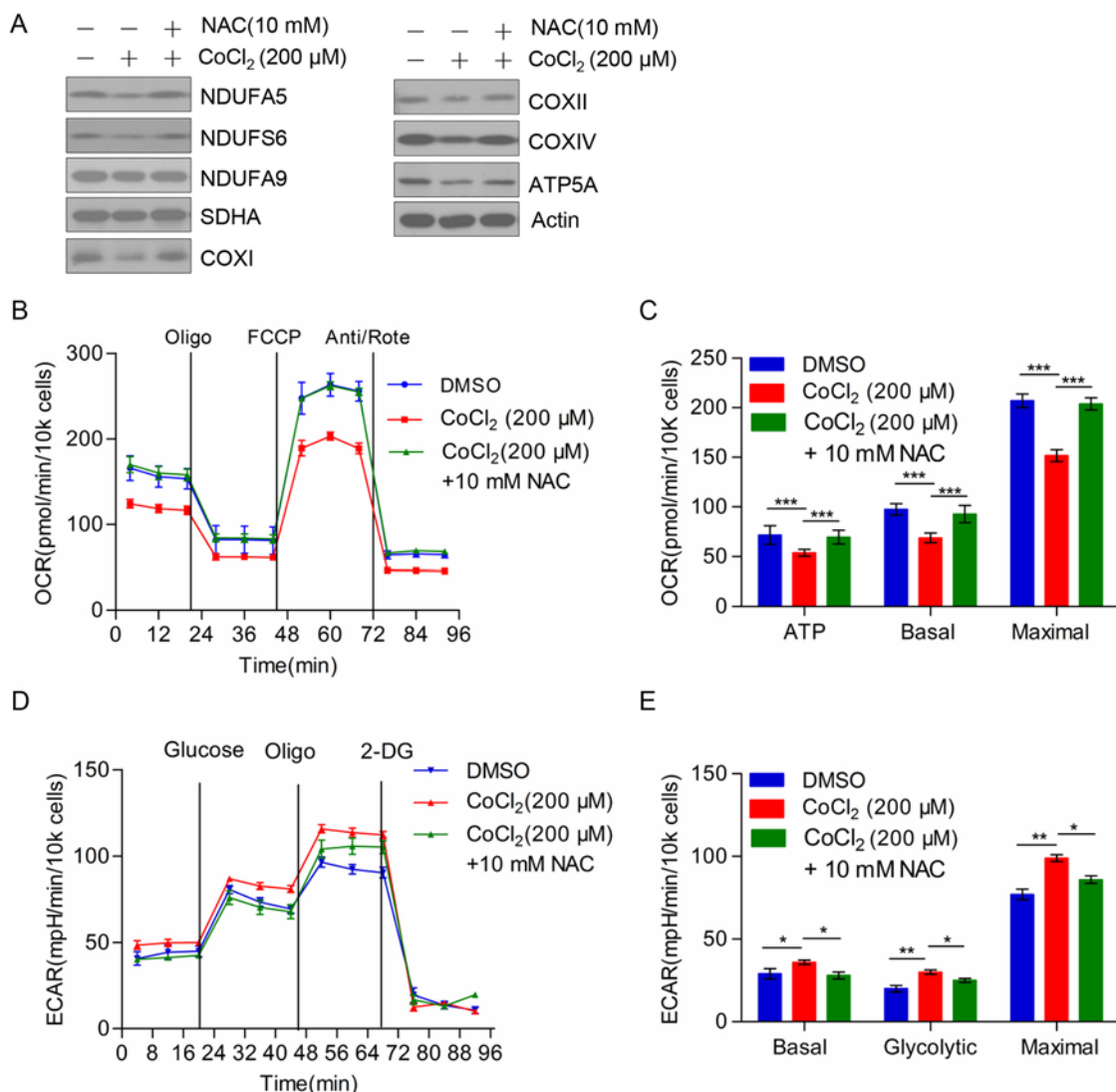


**Figure 2. The effect of CoCl<sub>2</sub> on bioenergetics metabolism in TE-1 cells**

(A) TE-1 cells with or without CoCl<sub>2</sub> (200 μM) treatment for 24 h, and the OCR was measured real-time using Seahorse XF96 Extracellular Flux analyzer. The basal OCR was measured at three time points, and then four chemicals were injected into the medium sequentially: the ATP synthase inhibitor oligomycin (1 μM), the uncoupler FCCP (1 μM), the complex I inhibitor rotenone (1 μM), and complex III inhibitor antimycin (1 μM). (B) Statistical analysis of OCR in TE-1 cells with or without CoCl<sub>2</sub> (200 μM) treatment. ATP production, basal, and maximal respiration were presented as mean ± S.D. of six replicates. (C) TE-1 cells treated with or without CoCl<sub>2</sub> (200 μM) treatment for 24 h. ECAR was detected by the Seahorse XF96 Extracellular Flux Analyzer. Three drugs were added sequentially: glucose (10 mM), oligomycin (1 μM), and 2-DG (100 mM). (D) Statistical analysis of ECAR in TE-1 cell with or without CoCl<sub>2</sub> (200 μM) treatment. Basal ECAR, glycolytic ECAR, and maximal ECAR are presented as mean ± S.D. of six replicates; \*\**P* < 0.01, \*\*\**P* < 0.001.

## NAC could rescue the effect of CoCl<sub>2</sub> on the expression of mitochondrial respiratory chain complex subunits and bioenergetics metabolism of TE-1 cells

HIF-1α was one of the important transcription factors in tumor development and progression, contributed to cell survival, and activation of gene expression under hypoxic condition. The target genes mainly related to metabolism of carbohydrates that include glycolytic enzymes, aldolase A, and glucose transporter protein-1 (GLUT-1). We hypothesized that ESCC cell TE-1 may switch cellular energy metabolism from mitochondrial OXPHOS to glycolysis under hypoxic conditions stimulated by CoCl<sub>2</sub>. On the one hand, TE-1 cells inhibited the expression of mitochondrial complex subunits by increasing ROS level; on the other hand, TE-1 cell enhanced glycolysis ability by increasing the expression of glucose metabolism related enzymes. To demonstrate our hypothesis, we set three groups: the negative control group, CoCl<sub>2</sub> treated group, and both CoCl<sub>2</sub> and N-acetyl cysteine (NAC, ROS scavenger) treated group. Western blot was used to detect mitochondrial complex subunits protein expression in the three groups. We found that the subunits of mitochondrial complex recovered obviously in the group of TE-1 cells treated with CoCl<sub>2</sub> and NAC simultaneously (Figure 3A). Meanwhile, by using Seahorse Bioenergetics Analyzer to measure OCR, we found that NAC could significantly rescue mitochondrial respiration in TE-1 cells treated with CoCl<sub>2</sub> (Figure 3B). The difference was statistically significant (Figure 3C). Additionally, we found that NAC could rescue CoCl<sub>2</sub> induced up-regulation of aerobic glycolysis. As shown in Figure 3D, NAC treatment decreased aerobic glycolysis enhanced by CoCl<sub>2</sub>. Moreover, we assessed the basal glycolytic rate, spare glycolytic, and maximal glycolytic rate, which indicated that NAC treatment could reverse CoCl<sub>2</sub> induced aerobic glycolysis (Figure 3E). Hence these results suggested that



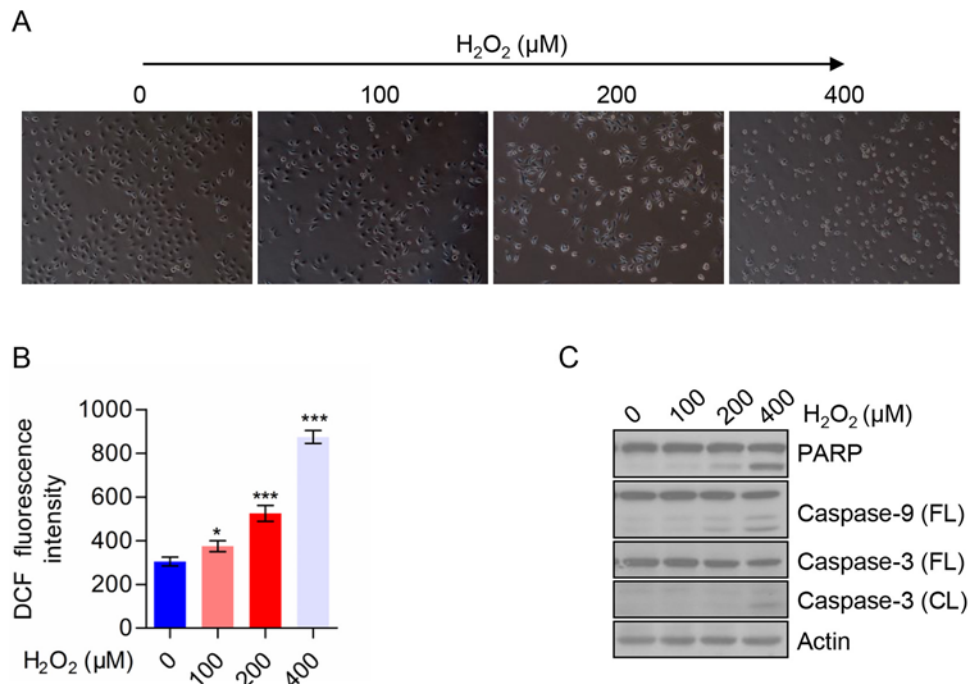
**Figure 3. NAC rescued the effect of CoCl<sub>2</sub> on the expression of mitochondrial respiratory chain complex subunits and bioenergetics metabolism in TE-1 cells**

(A) The expression of HIF-1 $\alpha$  and mitochondrial complex subunits in control group (DMSO), CoCl<sub>2</sub> (200  $\mu$ M) treated group, CoCl<sub>2</sub> (200  $\mu$ M), and NAC (10 mM) treated group. (B) OCR of control group (DMSO), CoCl<sub>2</sub> (200  $\mu$ M) treated group, CoCl<sub>2</sub> (200  $\mu$ M), and NAC (10 mM) treated group in TE-1 cells. (C) ATP production, basal respiration, and maximal respiration were presented as mean  $\pm$  S.D. of six replicates. Quantitation and statistical analysis of data from (B). (D) Overall ECAR of TE-1 cells which was treated by CoCl<sub>2</sub> in the presence or absence of NAC. (E) Basal glycolytic rate, spare glycolytic, and maximal glycolytic rate were assessed according to the manufacturer's protocol; \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001.

ESCC cell TE-1 maintained cell survival by the transformation of energy metabolism under conditions of low oxygen caused by CoCl<sub>2</sub>.

## H<sub>2</sub>O<sub>2</sub> induced TE-1 cells death through generation of ROS

As shown in Figure 4A, we treated TE-1 cells with the indicated concentration of H<sub>2</sub>O<sub>2</sub>. We observed cell morphological changes under phase contrast microscopy. With the increase in the concentration of H<sub>2</sub>O<sub>2</sub>, the cell viability was decreased compared with the control group as well as the cell size became smaller, indicating that the cells tended to apoptotic state. To explore the effect of H<sub>2</sub>O<sub>2</sub> on TE-1 cells, we analyzed the intracellular ROS level by flow cytometry. The result showed that compared with the negative control group, intracellular ROS level increased in a dose-dependent manner in H<sub>2</sub>O<sub>2</sub>-treated TE-1 cells (Figure 4B). In order to further examine the apoptotic rate of cells,



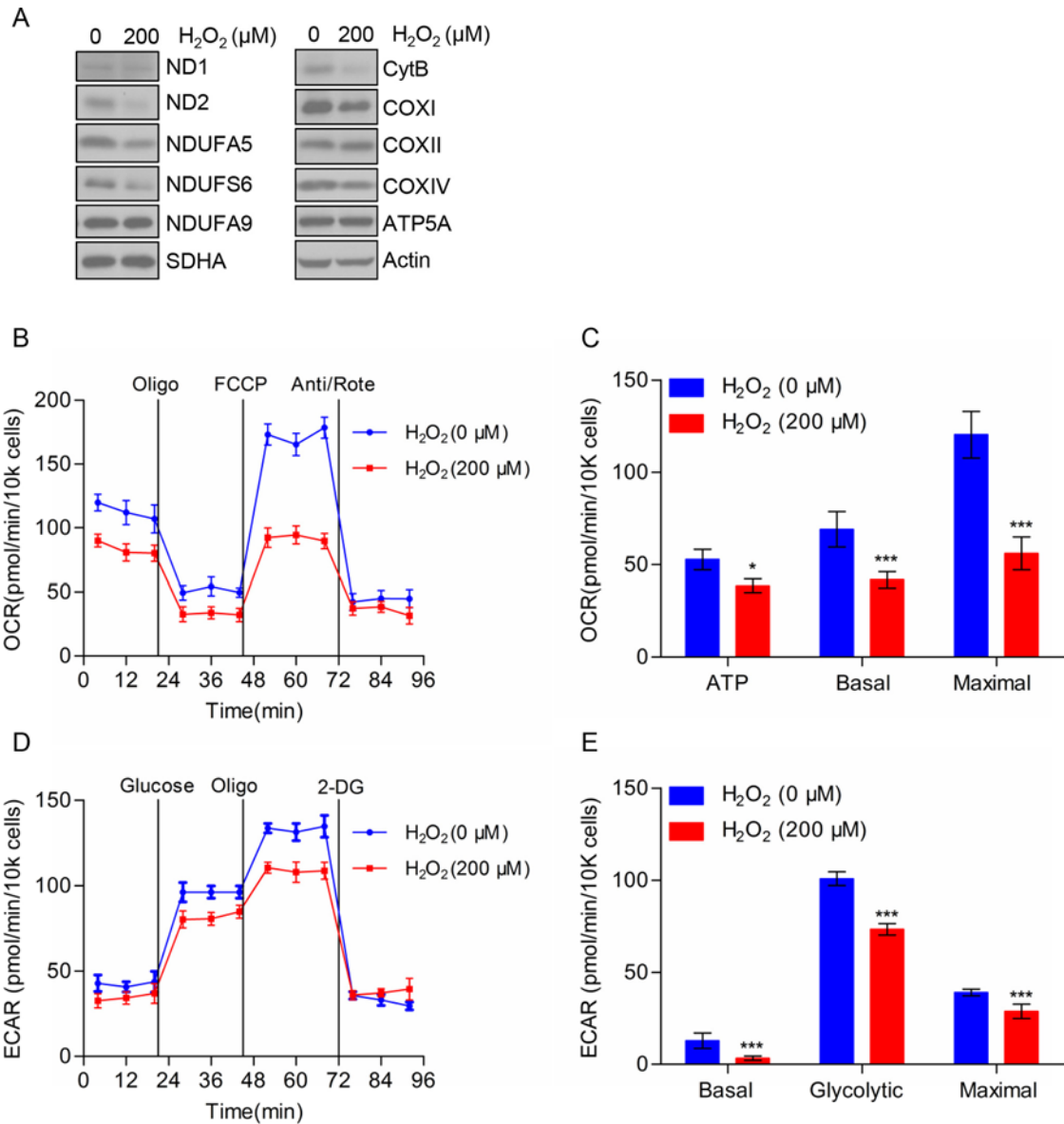
**Figure 4. H<sub>2</sub>O<sub>2</sub> induced TE-1 cells death through generation of ROS**

(A) Morphology of TE-1 cells treated with the indicated concentrations of H<sub>2</sub>O<sub>2</sub>. (B) ROS level of TE-1 cells treated with the indicated concentration of H<sub>2</sub>O<sub>2</sub>. (C) The expression of apoptosis-related proteins in TE-1 cells treated with the indicated concentrations of H<sub>2</sub>O<sub>2</sub>. All data are presented as mean ± S.D.; \**P*<0.05, \*\*\**P*<0.001.

we detected the apoptosis-related proteins by Western blot and the results showed that cleaved-PARP, cleaved-Caspase 3, and cleaved-Caspase 9 were increased in a dose-dependent manner with H<sub>2</sub>O<sub>2</sub> treatment from 100 to 400 μM (Figure 4C). These data indicated that H<sub>2</sub>O<sub>2</sub> could induce TE-1 cells death that is mediated through caspase activation. We also analyzed the effects of H<sub>2</sub>O<sub>2</sub> and CoCl<sub>2</sub> on cell growth, and found that both H<sub>2</sub>O<sub>2</sub> and CoCl<sub>2</sub> could suppress cell growth in TE-1 cells. However, H<sub>2</sub>O<sub>2</sub> had a stronger suppressive activity than CoCl<sub>2</sub> on TE-1 cells (Supplementary Figure S1). We further detected the cell death of CoCl<sub>2</sub>-treated cells, however, the treatment of CoCl<sub>2</sub> had no significant effect on cell death of TE-1 cells (Supplementary Figure S2).

## H<sub>2</sub>O<sub>2</sub> reduced the expression of mitochondrial respiratory chain complex subunits and bioenergetics metabolism in TE-1 cells

Next, we assessed the protein level in mitochondrial respiratory chain complex subunits in TE-1 cells treated with H<sub>2</sub>O<sub>2</sub> by using Western blot analysis. As shown in Figure 5A, the expression of complex subunits (ND2, NDUFA5, NDUFS6, and NDUFA9), complex III subunit Cyt. B and complex IV subunits (COXI and COXIV) were decreased in TE-1 cells treated with H<sub>2</sub>O<sub>2</sub>. However, there were no significant difference in the protein expression level of complex II subunit SDHA and complex V subunit ATP5A. To further investigate the effects of H<sub>2</sub>O<sub>2</sub> on TE-1 cellular bioenergetics, we analyzed mitochondrial respiration by determining the OCR (Figure 5B), and found that cells under the treatment of H<sub>2</sub>O<sub>2</sub> displayed a lower basal respiration and a significantly lower maximum respiratory capacity and accompanied by less ATP production compared with the negative control group (Figure 5C). In addition, we found that the glycolysis rates of TE-1 cells were significantly decreased after H<sub>2</sub>O<sub>2</sub> treatment (Figure 5D,E). To further confirm the reduction in respiration and aerobic glycolysis were caused by H<sub>2</sub>O<sub>2</sub> treatment, we used ROS scavenger NAC to investigate whether NAC could rescue these effects. As shown in Figure 6A, we found that NAC treatment dramatically reverses H<sub>2</sub>O<sub>2</sub> induced mitochondrial respiration reduction, as well as ATP production, basal respiration, and maximal respiration (Figure 6B). Moreover, we detected the alterations in ECAR, and found that NAC could also rescue aerobic glycolysis suppressed by H<sub>2</sub>O<sub>2</sub> (Figure 6C). In addition, basal glycolytic rate, spare glycolytic rate, and maximal glycolytic rate were assessed (Figure 6D). Consistently, NAC could reverse these three indexes of aerobic glycolysis. These results indicated that H<sub>2</sub>O<sub>2</sub> could cause the reduction in cellular bioenergetics of TE-1 cells via decreasing the expression of mitochondrial respiratory chain complex subunits.



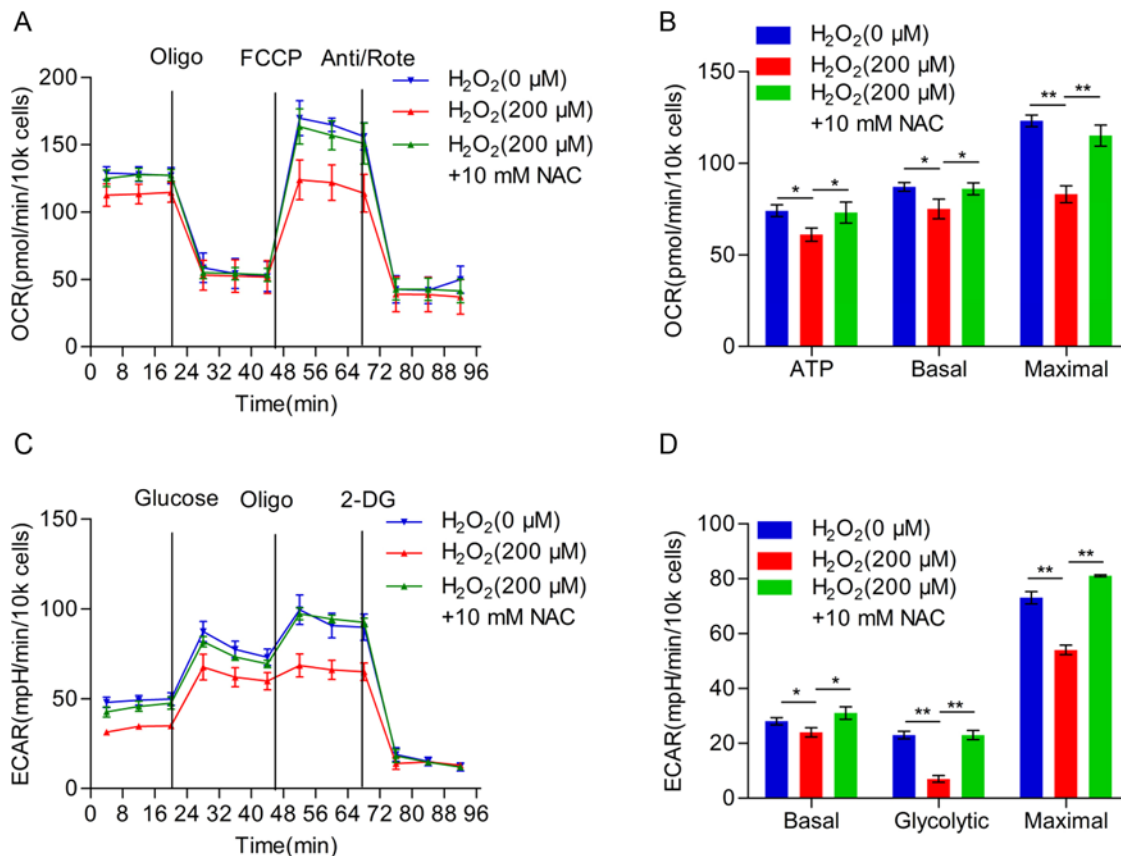
**Figure 5. Effects of H<sub>2</sub>O<sub>2</sub> on the expression of mitochondrial respiratory chain complex subunits and bioenergetics metabolism of TE-1 cells**

(A) TE-1 cells were pretreated with or without 200 μM H<sub>2</sub>O<sub>2</sub> for 24 h and the cells were subjected to Western blot analysis. (B) OCR of TE-1 cells treated with H<sub>2</sub>O<sub>2</sub> (200 μM). (C) ATP production, basal, and maximal respiration are presented as mean ± S.D. of six replicates. (D) ECAR of TE-1 cells treated with H<sub>2</sub>O<sub>2</sub> (200 μM). (E) Quantitation of the ECAR performed on TE-1 cells as shown in (B). The data are presented as mean ± S.D.; \**P*<0.05, \*\*\**P*<0.001.

## H<sub>2</sub>O<sub>2</sub> promotes apoptosis which could be blocked by NAC

Based on the above data, we speculated that H<sub>2</sub>O<sub>2</sub> induced apoptosis in TE-1 cells may be due to cellular energy metabolism disorders that were caused by the increase in ROS. Therefore, we tested the hypothesis by adding ROS scavenger (NAC) simultaneously, and as shown in Figure 7A, TE-1 cells were treated with H<sub>2</sub>O<sub>2</sub> in the absence or presence of NAC, and we observed that apoptosis reduced obviously in the presence of NAC. Moreover, we examined the apoptosis by flow cytometry, with the increased concentration of H<sub>2</sub>O<sub>2</sub>, the apoptosis rate gradually increased, but when TE-1 cells were treated with NAC simultaneously, we found that apoptosis induced by H<sub>2</sub>O<sub>2</sub> was significantly reduced (Figure 7B,C). Thus, we believed that H<sub>2</sub>O<sub>2</sub> may induce the production of ROS and cause progressive oxidative damaged, mitochondrial dysfunction, cell bioenergetics metabolism disorders, and ultimately cell death.





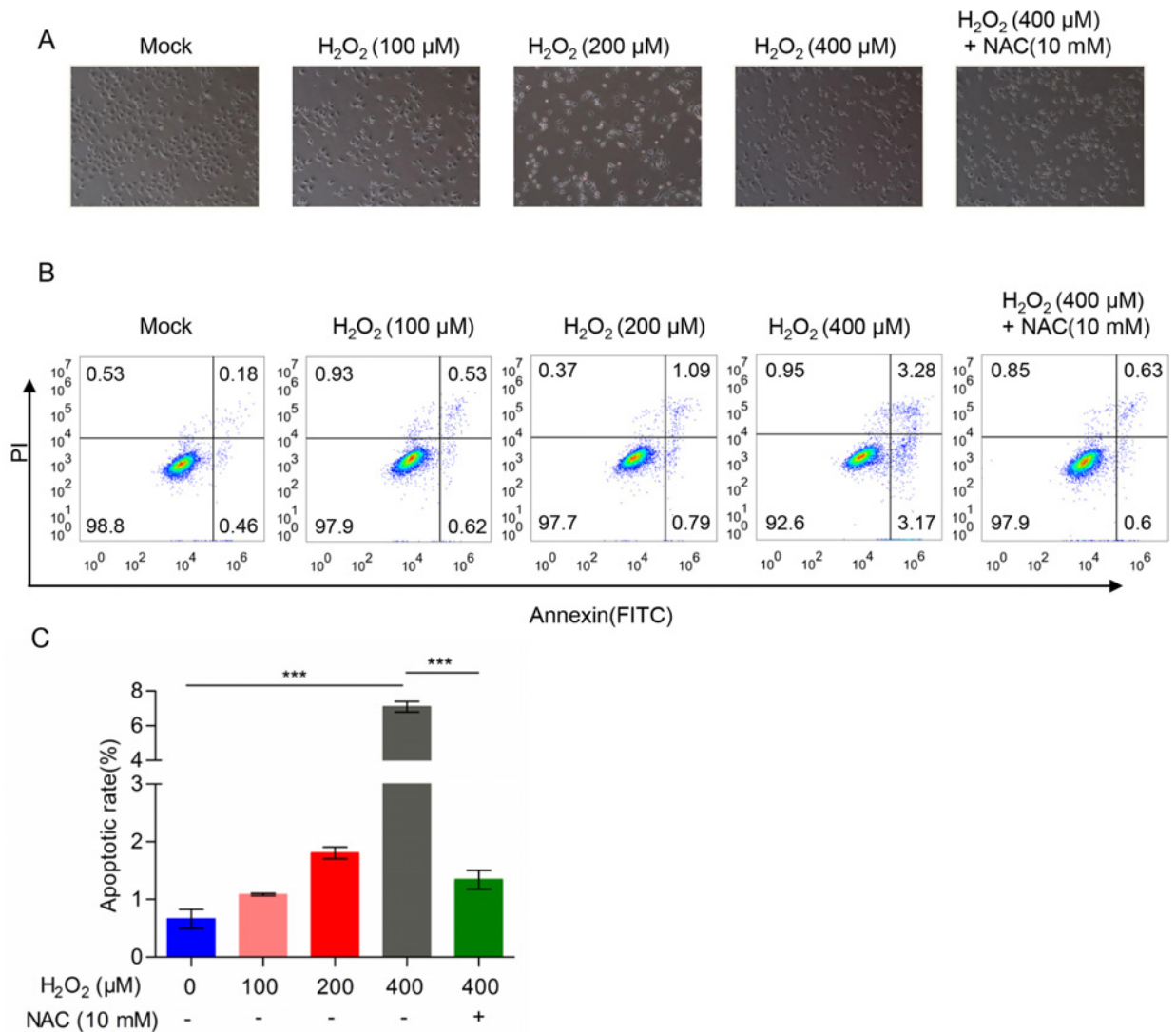
**Figure 6. NAC rescues H<sub>2</sub>O<sub>2</sub> caused reduction in cellular bioenergetics in TE-1 cells**

(A) Overall OCR curve of TE-1 cells treated with H<sub>2</sub>O<sub>2</sub> (200 μM) or H<sub>2</sub>O<sub>2</sub> (200 μM) combined with NAC (10 mM). (B) ATP production, basal respiration, and maximal respiration alterations of TE-1 cells in (A). (C) Overall ECAR curve of TE-1 cells treated with H<sub>2</sub>O<sub>2</sub> (200 μM) or H<sub>2</sub>O<sub>2</sub> (200 μM) combined with NAC (10 mM). (D) Basal glycolytic rate, spare glycolytic rate, and maximal glycolytic rate were assessed according to the treatment as shown in (C); \**P*<0.05, \*\**P*<0.01.

## Discussion

Accumulating studies have shown that the development of malignant tumors is actually a process of tumor cell metabolism reprogramming [20,21], which is one of the hallmarks of most human tumors [22]. Mitochondria as key actors in cancer metabolic reprogramming, not just because these organelles play a significant role in energy production [23] and biosynthetic intermediates formation, as well as occurrence of mutations in both nuclear and mtDNA encoding metabolic enzymes is associated with different types of cancer [24,25]. Glycolysis is an oxygen-independent metabolic pathway that converts glucose into pyruvate via a series of intermediate metabolites and leads to a net production of ATP [26]. OXPHOS is the metabolic pathway in which cells use enzymes to oxidize nutrients, thereby releasing energy which is accounts for high ATP yield [27]. OXPHOS is a critical part of metabolism, however, it also produces ROS which leads to damaging cells and contributes to diseases and ageing. The Warburg effect suggests that the levels of different cellular energy metabolism are not the same [28]. Cancer cells mainly produce ATP by a high rate of glycolysis followed by lactic acid fermentation in the cytosol, rather than by a relatively low rate of glycolysis followed by oxidation of pyruvate in mitochondria as in most normal cells [29]. Previous studies have shown that mutations in mtDNA, mitochondrial fusion and fission dysfunction and mitochondria-related active enzyme mutation can cause normal cells transformed into cancer cells [30,31]. The enzymes involved in glycolysis are also the target of many anticancer drugs that inhibit their activities or regulate gene expression [32].

It is well known that ROS produced by aerobic cells during metabolic processes and have important roles in cell signaling and homeostasis [33,34]. However, while undergoing a wide variety of environmental stress, ROS levels can increase dramatically thus leading to oxidative damage to cells [35]. Some studies indicate that ROS levels significantly increased in tumor cells and plays a vital role in the occurrence and development of tumors and also participates in



**Figure 7. H<sub>2</sub>O<sub>2</sub> induced the apoptosis and NAC could dramatically rescue H<sub>2</sub>O<sub>2</sub>-induced apoptosis in TE-1 cells**

(A) Morphology of TE-1 cells treated with H<sub>2</sub>O<sub>2</sub> in the absence or presence of NAC. (B) TE-1 cells were pretreated with a gradient concentration of H<sub>2</sub>O<sub>2</sub> (0, 100, 200, 400 μM) or 400 μM H<sub>2</sub>O<sub>2</sub> and 10 mM NAC. (C) Cell apoptotic rate determined by flow cytometry; \*\*\**P*<0.001.

many signaling pathways, such as mitochondrial mediated caspase-dependent apoptotic pathway [36] and regulation of Akt/MAPK-related signaling pathways [37], and even affect the proliferation and differentiation of tumor cells [38-40].

Due to mitochondria being the major sources of ATP or ROS, more and more attention has been focussed on the study of the relationship between energy metabolism and tumor [41-43]. Our present study investigated the mechanism of oxidative stress on the biological energy metabolism of ESCC cells. We used the oxidative stress inducer CoCl<sub>2</sub> and H<sub>2</sub>O<sub>2</sub> to treat TE-1 cells to study the effects of different stress on cellular bioenergy metabolism.

First of all, we utilized CoCl<sub>2</sub> to simulate hypoxic microenvironments. Under hypoxic conditions, HIF-1α is no longer degraded and translocated to the nucleus regulating hundreds of genes, and many of them play important roles in cancer cell metabolism [44]. Our findings demonstrate that CoCl<sub>2</sub> induced the production of ROS and reduced the expression of mitochondria respiratory chain enzyme complex subunits due to reduced gene transcription and protein synthesis. To further confirm the effects of CoCl<sub>2</sub> on the energy metabolism of TE-1 cells, we detected the OCR and ECAR to evaluate the ability of TE-1 cells OXPHOS and glycolysis, our results indicate that the mitochondrial respiration was significantly decreased, accompanied by the increase in glycolysis rates. This is consistent with the

findings in breast cancer [27]. Additionally, we used the ROS scavenger (NAC) to verify our previous hypothesis, and NAC could dramatically rescue from the effects induced by  $\text{CoCl}_2$ , which suggest that tumor cell may undergo the metabolic reprogramming to survive under adverse conditions.

Second,  $\text{H}_2\text{O}_2$  as a widely used inducer of cell damage and apoptosis, is capable of producing large amounts of oxygen free radicals that cause cellular oxidative stress [45,46]. Our data proved that  $\text{H}_2\text{O}_2$  indeed could induce apoptosis and inhibit the expression of mitochondria respiratory chain complex subunits. Moreover, we found that  $\text{H}_2\text{O}_2$  could dramatically suppress the OCR and ATP production and simultaneously inhibited the cellular glycolytic rate. Hence, we proposed that the apoptosis induced by  $\text{H}_2\text{O}_2$  may be due to the energy metabolism dysfunction that is caused by the increase in ROS levels. This was further confirmed that NAC pretreatment significantly abolished  $\text{H}_2\text{O}_2$ -induced apoptosis in TE-1 cells. These results demonstrate that oxidative stress caused by  $\text{H}_2\text{O}_2$  leads to severe impairment in TE-1 cell energy metabolism. Moreover, our findings show that  $\text{H}_2\text{O}_2$  mediates apoptosis of TE-1 cells through activating caspase 3 and caspase 9. Therefore,  $\text{H}_2\text{O}_2$  induced the increase in ROS levels, causing cell oxidative damage, mitochondrial dysfunction, and resulting in cell biological energy metabolism disorder, and ultimately induced apoptosis.

In summary, we found that ESCC TE-1 cells exhibit distinct changes in bioenergy metabolism in response to different oxidative stress. Under hypoxic condition, TE-1 cells are able to switch their metabolic phenotype from OXPHOS to glycolysis as an alternative source of biogenetic substrates to maintain cell survival. However, TE-1 cells are unable to reprogram metabolism when they are exposed to  $\text{H}_2\text{O}_2$ , and eventually leads to cell death due to energy deficiency. This may provide as a novel strategy for treatment of ESCC, and which is also helpful to solve the problems of drug resistance of ESCC by combination with current treatment and realize the specific targeted therapy of tumor cells.

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## Competing interests

The authors declare that there are no competing interests associated with the manuscript.

## Author contribution

B.L. and L.L. conceived the idea, supervised the research, and wrote the manuscript. X.Z., L.L., L.N., J.L., C.L., M.G., S.M., and J.L. performed the experiments. Y.L., L.L., and B.L. performed data analyses. All the authors read and approved the final manuscript.

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## Abbreviations

Cyt. b, Cytochrome b; DCF, Dichlorofluorescein; DCFH-DA, Dichlorodihydrofluorescein diacetate; 2-DG, 2-Deoxy-D-glucose; ECAR, extracellular acidification rate; EGFR, epidermal growth factor receptor; ERK, extracellular regulated protein kinases; ESCC, esophageal squamous cell carcinoma; FCCP, carbonylcyanide-p-trifluoromethoxyphenylhydrazone; HIF-1 $\alpha$ , hypoxia-inducible factor-1 $\alpha$ ; HRP, horseradish peroxidase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NAC, N-acetyl cysteine; OCR, oxygen consumption rate; OXPHOS, oxidative phosphorylation; PARP, poly ADP-ribose polymerase; PI, oropidium iodine; PTK, protein tyrosine kinase; qRT-PCR, quantitative real-time PCR; ROS, reactive oxygen species; RPMI-1640, Roswell Park Memorial Institute-1640; SOD, superoxide dismutase; TBS-T, TBS-Tween.

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