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Non-thermal plasma with metformin synergistically induces cell death via upregulating AMPK to suppress energy metabolism

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Abstract

Non-thermal plasma (NTP) has a selective killing effect on a variety of cancer cells *in vivo* and *in vitro*, and is considered a potential tumor therapy. In this study, we found that metformin, which has shown promising antitumor effects in previous studies, with NTP synergistically increased cell death including apoptosis in colon cancer cell lines via reducing mitochondrial oxidative phosphorylation and intracellular adenosine triphosphate exhausting cellular energy, mediated through activation of AMP-activated protein kinase (AMPK). Reactive oxygen species was revealed to modulate the activation of AMPK after NTP exposure. Considering the safety and wide clinical use of metformin, the combination of metformin and NTP showed great potential in cancer therapy.

Supplementary material for this article is available online

Keywords: non-thermal plasma, metformin, AMP-activated protein kinase, energy metabolism

(Some figures may appear in colour only in the online journal)

1. Introduction

Non-thermal plasma (NTP) is an ionized gas with a near room temperature, which consists of reactive species, ions, electrons, neutral particles, ultraviolet, visible light, etc. In the past decade, owing to its notable advantages to effectively and selectively kill various types of cancer cells and to cause distinctly less damage to normal cells *in vitro* and *in vivo* [1–10], treatment with NTP has been identified as a potential technique of cancer therapy. Furthermore, there was increased evidence that reactive oxygen species (ROS) played an important role in NTP-induced apoptosis *in vitro* [11–16]. In general, NTP-induced ROS induced oxidative stress to DNA, proteins and other cellular components, which could ultimately lead to cell death [17].

The incidence and mortality of colon cancer has been increasing. Treatments on colon cancers based on surgery supplemented with radio- and chemotherapy often show insufficient efficacy since most patients are already at the middle or advanced stage at the time of onset. In relation, new therapeutic strategies such as NTP therapy are being developed to improve the curative effect. Numerous studies have revealed that NTP is a potential method for colon cancer therapy. Kim *et al* found that NTP induced growth arrest and apoptosis of colon cancer cells and at the same time reduced cell migration and invasion [18]. Madduma *et al* elucidated that apoptosis induced by NTP in human colon cancer cells was mediated by endoplasmic reticulum stress, mainly through accumulating mitochondrial calcium and expressing unfolded protein response proteins [19]. In addition, NTP also induced immunogenic cell death in murine CT26 colon tumors [20].

Metformin, a member of the biguanide family, is the most commonly used oral hypoglycemic agent for the treatment of type 2 diabetes. Apart from this, metformin can also reduce the risk of cancers via inhibiting the growth of leukemia, pancreatic, colon and hepatocellular carcinoma cells, etc [21, 22]. Metformin directly inhibits the mitochondrial electron transport chain complex I (ETCI) [23, 24], leading to a decrease in ETCI activity and oxidative phosphorylation (OXPHOS) in cells [25, 26], and the inhibition of ETCI results in reduced proliferation of cancer cells *in vitro* and *in vivo* [27]. Consequently, metformin regulates the changes in adenosine monophosphate (AMP)/adenosine triphoshate (ATP) ratios, mitochondrial transmembrane potential and calcium levels, which are correlated with increased oxidative stress by targeting ETCI [28].

In this study, we found that metformin with NTP synergistically reduced the mitochondrial oxidative phosphorylation and intracellular ATP content in colon cancer cells and then increased cell death via ROS-mediated AMP-activated protein kinase activation. These results hinted the potential application of metformin in the NTP cancer therapy.

2. Methods

2.1. Cell culture and reagents

Three human colon cancer cell lines (RKO, SW480 and HCT116) were purchased from the ATCC (Manassas, VA, USA) and were cultured in DMEM (Dulbecco's modified eagle's medium; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) and 100 U ml^{-1} penicillin-streptomycin (Gibco, Carlsbad, CA, USA). All cell cultures were maintained in 5% CO_2 and humidified air at 37 °C. Metformin was added at 12 h prior to NTP treatment.

Metformin was obtained from Sigma-Aldrich. (St. Louis, MO, USA). Primary antibodies for poly (ADP-ribose) polymerase (PARP), Caspase 3, Caspase 9, AMPK, phosphorylated-AMPK (Thr-172), and β-actin were purchased from Cell Signaling Technology (Beverly, MA, USA). Secondary IRDye-labeled goat anti-mouse and anti-rabbit IgG antibodies were purchased from LI-COR Biosciences (LI-COR, Lincoln, NE, USA).

2.2. Non-thermal dielectric barrier discharge plasma treatment

Non-thermal dielectric barrier discharge (DBD) plasma was employed in the present work, and the experimental setup was described in detail in our previous studies [29]. Briefly, the device mainly consisted of three parts: plasma reaction chamber, high voltage power supply and gas source. The plasma reaction chamber consisted of four high voltage electrodes, each of which consisted of a copper column with a diameter of 32 mm. A quartz glass (1 mm thickness) was placed under the copper column as an insulating layer, and four copper columns (37 mm in diameter) were used as the negative electrode. There were five air inlets above the reaction chamber and one air outlet on the side, and helium (purity: 99.99%, 120 l h^{-1} flow rate) was used as the working gas and injected 5 min before the treatment to expel air out of the reaction chamber. Cells were exposed to NTP for 0–120 s, and the discharge gap between medium surface and the bottom of the quartz glass was 5 mm. The voltage was 12 kV (peak to peak) at a frequency of 24 kHz, and the discharge power density was 0.9 W cm^{-2}.

2.3. Cell viability detection

The cells were seeded with a density of 3×10^5 cells per 35 mm dish in triplicates, and then returned into the incubator for the adherence on the dish surface one night before the treatment of metformin. At 12 h after metformin treatment, the cells were exposed to NTP without changing the culture medium. At 24 h after NTP exposure, the cell viability was detected with CCK-8 kit (Cell Counting Kit-8; Beyotime, Shanghai, China) following the manufacturer's instructions and the absorbance was measured at 450 nm with Varioskan Flash microplate reader (Thermo Fisher Scientific, Rockford, IL, USA). The results are expressed as percentage (%) cell viability compared to control.

2.4. Apoptosis detection

After treatment, harvested the cells and washed twice with cold PBS, and suspended with 1 × Annexin V binding buffer (500 μl), then stained with Annexin V-FITC and PI from the Apoptosis Detection Kit I (BD Biosciences, Bedford, MA, USA). The apoptosis was detected with a flow cytometer (FACS Calibur, Becton-Dickinson) and analyzed with the FACS suite software.

2.5. ATP measurement

The cellular ATP was detected with the ATP assay kit (Solarbio, Beijing, China) according to the manufacturer's instruction. The luminescence was measured with an Illuminometer (Promega Corporation, Fitchburg, WI, USA). The results were plotted as fold changes from control samples.

2.6. Measurement of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR)

The OCR and the ECAR were measured with Seahorse XF24 Analyzer (Seahorse Bioscience, North Billerica, MA, USA). Briefly, when metformin was used alone, 3×10^4 cells were plated in the 24-well microplate specifically designed for XF24 Analyzer and then metformin was added with a final concentration of 6 mM after cell attachment. The treatment with metformin maintained for 12 h in an incubator until OCR and ECAR were measured.

As regards the combination of metformin and NTP, 3×10^5 cells were plated in 35 mm dishes and treated with metformin in the same manner but the cells were treated with NTP (30 s) at 12 h after metformin treatment and trypsinized immediately, then plated into the XF24 microplate with the original culture medium in. After 12 h incubation, OCR and ECAR were measured. The plate was washed twice with XF medium (OCR: supplemented with glucose, glutamine, and sodium pyruvate; ECAR: glutamine only) and then returned to incubator for 1 h. Sequential injections were performed with different compounds (OCR: oligomycin, FCCP, rotenone + antimycin A; ECAR: D-glucose, oligomycin, and 2-deoxyglucose). Normalization was performed by measuring the protein content in each well and the data were analyzed with the Seahorse Wave software.

2.7. Measurement of mitochondrial membrane potential (MMP)

The cellular MMP was measured with the Mito Probe JC-1 assay kit (Beyotime, Shanghai, China) according to manufacturer's instruction. Briefly, the cells were stained with JC-1 (5 g ml^{-1}) for 20 min at 37 °C in dark, washed twice with PBS and resuspended in fresh medium without serum and then detected with flow cytometer (FACS Calibur, Becton-Dickinson).

2.8. Western blot

Briefly, the cells were harvested, washed twice with cold PBS and lysed with RIPA lysis buffer (Beyotime, Shanghai, China). The lysate was then centrifuged at 12 000 × g for 10 min at 4 °C and the supernatants were collected. The protein concentration was determined with BCA Protein Assay Reagent Kit (Beyotime, Shanghai, China). Equivalent amounts of protein were separated with 8%–12% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore Corporation, Bedford, MA, USA). The membranes were then blocked with 5% non-fat milk powder, incubated with special primary antibodies overnight at 4 °C, and labeled with IRDye-conjugated secondary antibodies for 1 h at room temperature. Finally, the membranes were measured with Odyssey-CLx (LI-COR, USA).

2.9. Detection of superoxide anion

To detect the cellular superoxide anion, the cells were stained with its specific fluorescent probe, dihydroethidium (DHE, 5 μM, Molecular Probes) for 1 h in the dark, and then photographed with a fluorescence microscope (Leica DMI 40 008, Germany) or quantified with flow cytometer (FACS Calibur, Becton-Dickinson).

2.10. siRNA transfection

The si-RNA target sequences of AMPK were: RNAi#1, 5′-CGGGAUCAGUUAGCAACUATT-3′; RNAi#2, 5′-GGUUGGCAAACAUGAAUUG-3′. Briefly, cells seeded in 35 mm dishes (50%–60% confluency) were transfected with Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA) according to manufacturer's instruction, and the transfection mix (siRNA and lipofectamine 2000 diluted in Opti-MEM) was then added to the culture media gently. After 48 h incubation, the cells were harvested for further experiments.

2.11. Statistical analysis

All experiments were repeated at least three times and data were expressed as mean ± standard deviation (S.D.). Differences between the results of two groups were calculated with the student's *t*-test and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Metformin with NTP synergistically increases cell death

As shown in figure 1(A), NTP exposure reduced cell viability distinctly in RKO, SW480 and HCT116 cells in a dose-dependent manner. In subsequent experiments, the exposure time of 30 s was chosen as a representative dose for NTP treatment. The treatment with metformin alone only decreased the cell viability of RKO, SW480 and HCT116 slightly. However, after NTP exposure (30 s), the groups with metformin pretreatment significantly reduced the cell viability in all three cell lines when compared to the groups treated with metformin or NTP alone. (figure 1(B)). These results indicate that metformin with NTP synergistically increased cell death.

3.2. Metformin with NTP increases apoptosis synergistically

As an early event of apoptosis [30], the change of MMP was detected with the JC-1 detection kit. Results in figure 2(A) showed that, after NTP exposure, the percentage of MMP changes in RKO and SW480 cells with metformin treatment (6 mM) were significantly higher than those treated with metformin or NTP alone. This result indicates that metformin combined with NTP significantly prompted the depolarization of mitochondrial membrane potential.

Moreover, upon NTP exposure, the apoptosis rate of RKO and SW480 cells with metformin pretreatment (6 mM) was also significantly higher than those treated with metformin or NTP alone (figure 2(B)). A similar trend was found in the expression of PARP, Caspase 9 and Caspase 3 (figure 2(C)). Taken together, metformin with NTP synergistically increased apoptosis.

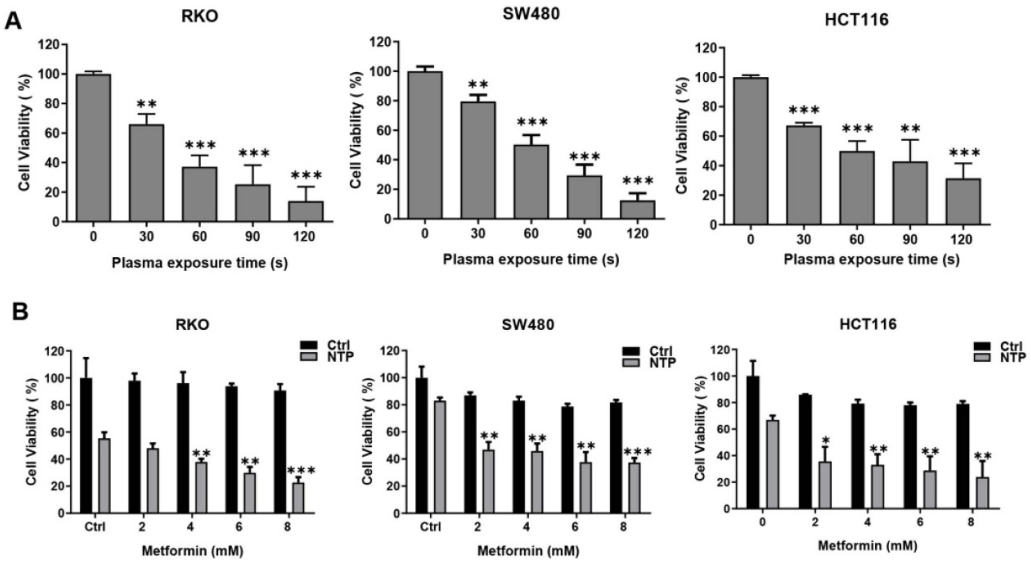

Figure 1. Metformin with NTP synergistically enhances cell death. (A) Cell viability of RKO, SW480 and HCT116 cells. (B) Cell viability of RKO, SW480 and HCT116 cells (metformin: 6 mM; NTP: 30 s). The significance was compared with control NTP group. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

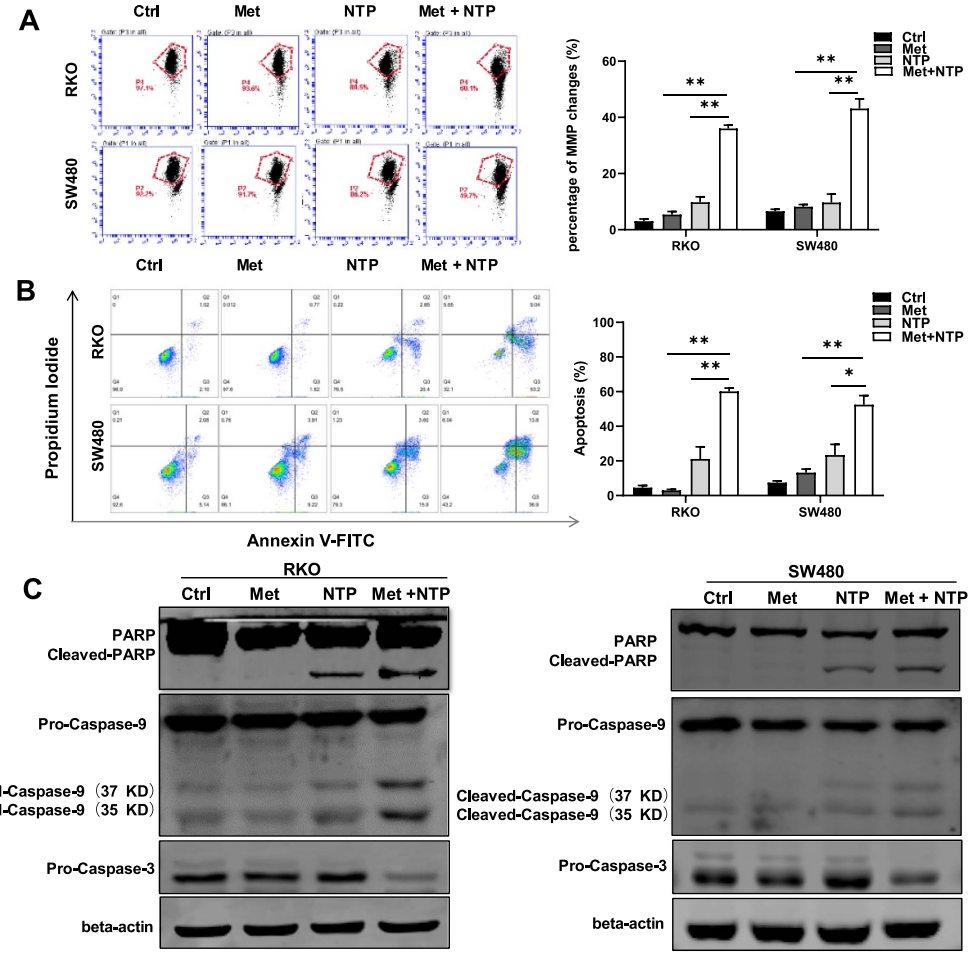

Figure 2. Metformin with NTP synergistically induces apoptosis. (A) MMP of RKO and SW480 cells. (B) Apoptosis of RKO and SW480 cells. (C) Expression level of PARP, caspase-3 and caspase-9. (* $p < 0.01$, ** $p < 0.001$).

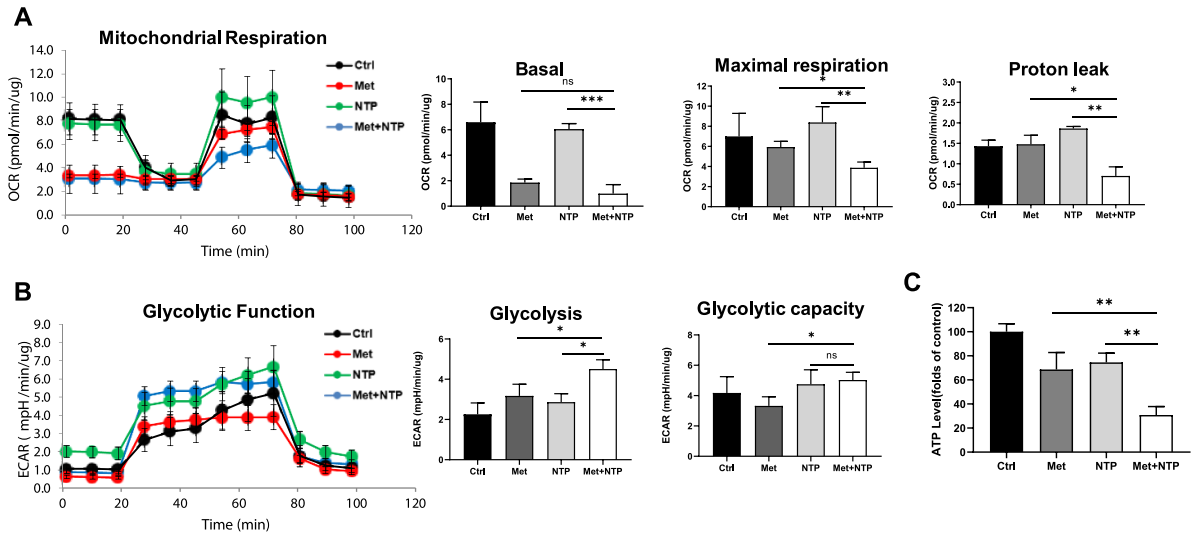

Figure 3. Metformin with NTP synergistically reduces mitochondrial oxidative phosphorylation and intracellular ATP level. (A) OCR and (B) ECAR with or without metformin (6 mM) pretreatment after NTP exposure (30 s). (C) Cellular ATP levels in RKO cell line. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.)

3.3. Metformin with NTP synergistically reduces mitochondrial oxidative phosphorylation and intracellular ATP level in colon cancer cells

Since metformin has been reported to have an inhibition effect on mitochondrial respiratory chain complex, we further measured the bioenergetic profiles with Seahorse analyzer. The results in figures S1(A) and S1(B) (available online at stacks.iop.org/JPhysD/53/385203/mmedia) showed that metformin treatment decreased OCR and increased ECAR in RKO cells, which demonstrated a reduced oxidative phosphorylation and a metabolic shift towards glycolysis. After treatment with metformin and NTP, the RKO cells showed significantly decreased levels of maximum respiration and proton leakage compared to those treated with metformin or NTP alone (figure 3(A)), the basal respiration also reduced without significant difference to the group treated with metformin only. These results indicated that the combination treatment promoted the inhibition of the mitochondria energy metabolism. Similarly, the combination treatment also increased glycolysis in cells (figure 3(B)), which indicated that the basal glycolysis rate and maximum glycolysis rate of the cells were prompted. These results suggested that mitochondrial electron transfer and aerobic oxidation process were blocked, and the required energy could only be obtained through anaerobic respiration, which produced far less ATP than aerobic respiration. Coincidently, the ATP level in RKO and SW480 cells treated with metformin and NTP was distinctly lower than those treated with metformin or NTP only (figure 3(C), figure S1(C)). Taken together, it could be concluded that metformin with NTP synergistically reduced mitochondrial oxidative phosphorylation and intracellular ATP consumption in colon cancer cells.

3.4. Activation of AMPK mediates the synergistic effect of metformin and NTP

Since the ratio of intracellular AMP concentration to ADP concentration ([AMP]: [ADP]) and the ratio ADP concentration to ATP concentration ([ADP]: [ATP]) regulate AMP kinase (AMPK) -thr172 phosphorylation [31], we further investigated the activation of AMPK. The results in figure 4(A) showed that the combination treatment increased the level of p-AMPK in both RKO and SW480 cells, which suggested that the combined treatment prompted the activation of AMPK. To further confirm the role of AMPK, the special siRNA was used to knock down the expression of AMPK. The negative control group were treated with NC-siRNA. The transfection efficiency was confirmed with western blotting. The transient transfection with the siRNAs led to a significant decrease in the AMPK expression (figure 4(B)) and phosphorylation (figure 4(C)). Knocking down AMPK attenuated cell death (figure 4(D)) and apoptosis (figure 4(E)) in RKO cells after the combined treatment. These results indicated that the activation of AMPK mediated the synergistic effect of metformin and NTP in the inhibitory effect of metformin and NTP.

3.5. ROS initiates the activation of AMPK

It was known that ROS could directly regulate AMPK activation and then induced apoptosis [32, 33], so production of intracellular superoxide anion, an important kind of ROS, was measured with its specific fluorescent probe DHE. The results in figures 5(A) and (B) showed that the production of superoxide anion in cells treated with metformin and NTP was significantly higher than those treated with metformin or NTP alone. To further investigate the role of the superoxide anion, N-acetyl-L-cysteine (NAC), a scavenger of ROS, was added 4 h before NTP treatment. Through treatment with NAC, the viability of RKO cells was distinctly higher than those without NAC treatment (figure 5(C)). Moreover, activation of AMPK induced by the combination of metformin and NTP was inhibited by NAC treatment (figure 5(D)). Furthermore, this phenomenon was also reflected in the detection of cell apoptosis (figure 5(E)). These results indicated that activation of AMPK

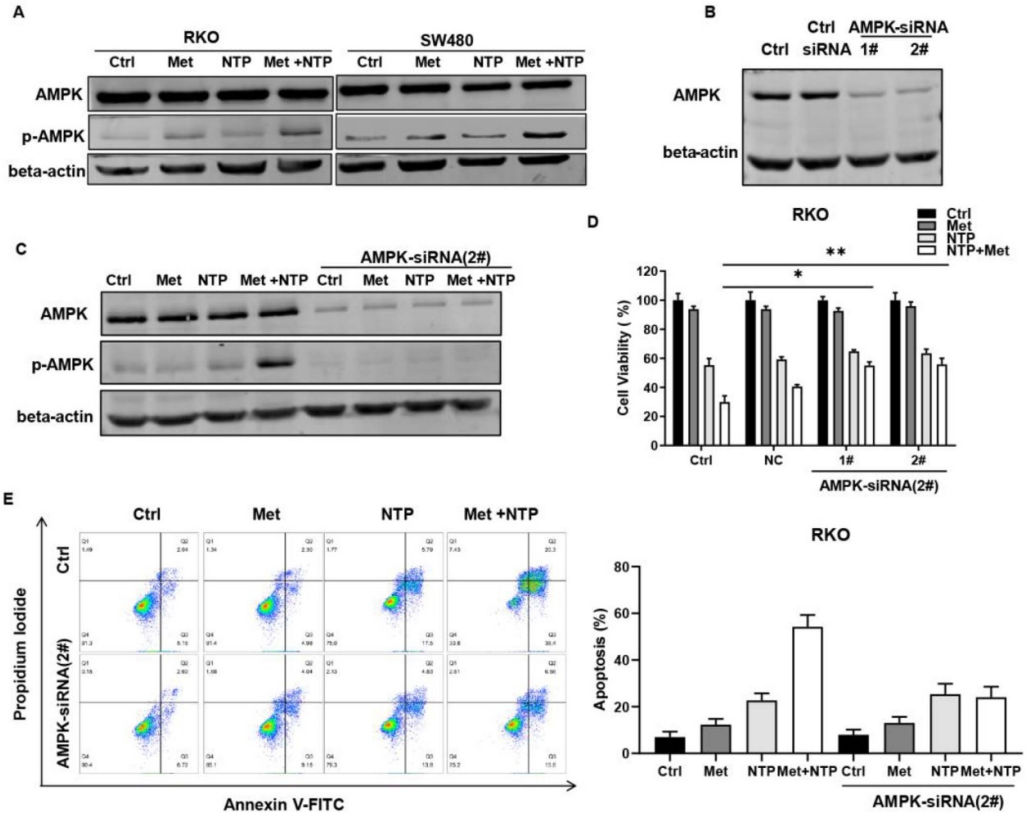

Figure 4. Activation of AMPK mediates the synergistic effect of metformin and NTP. (A) Expression of p-AMPK and AMPK. (B) Effect of knocking down AMPK with siRNA. (C) Phosphorylation of AMPK, (D) cell viability and (E) apoptosis after metformin/NTP treatment. (* $p < 0.01$, ** $p < 0.001$.)

was initiated by the production of ROS induced by metformin and NTP.

4. Discussion

As a promising anti-cancer therapeutic method, NTP induces growth arrest and cell death in various types of cancer cells. Different sensitivities to NTP exposure have been observed in different cancer cells, and researchers have also tried to sensitize the resistant cells to NTP with chemicals or nanoparticles [32–34]. In the present study, we showed that metformin with NTP synergistically effectively killed three human colon cancer cell lines, namely, RKO, SW480 and HCT116.

Apoptosis is a kind of programmed cell death, and its primary role is to maintain homeostasis in multicellular organisms [35–37]. Apoptosis has been reported in the literature to play an important role in NTP-induced cell death. For example, NTP can induce damages to DNA and other biological molecules, which then lead to apoptosis [38, 39]. Our results showed that metformin combined with NTP significantly prompted the depolarization of mitochondrial membrane potential and the ratio of apoptotic cells.

A plenty of studies have shown that metformin exhibits anti-tumor effects. Clinical studies revealed that patients with type 2 diabetes treated with metformin had significantly reduced cancer incidence rate compared to those patients treated with other drugs [40, 41]. A research on patients with endometrial cancer also showed a remarkable correlation between metformin intake and increased recurrence-free survival and overall survival rates [42]. Studies on possible mechanisms revealed that metformin reduced the systemic insulin level and insulin-like growth factor-1 (IGF-1) to inhibit the growth of insulin-dependent tumor cells by inhibiting hepatic gluconeogenesis or by reducing oxidative phosphorylation and other metabolic activities of tumor cells [21, 43]. Our results showed that metformin could also reduce oxidative phosphorylation and enhance glycolysis in colon cancer cells, while aerobic respiration in the cells was further reduced and glycolysis was further increased after the combined treatment of metformin and NTP, indicating a further reduction in the energy supply. Coincidently, Zhuang *et al* found metformin inhibited oxidative phosphorylation and increased glycolysis in an AMPK dependent manner in breast cancer cell lines [44]. Furthermore, Julie *et al* also found that metformin decreased oxygen consumption and mitochondrial-dependent ATP production, and increased glycolytic ATP and lactate production [45].

AMPK, an important downstream target gene of metformin [46], is a low energy checkpoint and a classic metabolic regulator [47]. When hypoxia or nutrient deprivation occurs, intracellular ATP levels decreases and AMP levels increases, leading to conformational changes in AMPK heterotrimer and phosphorylation of the amino subunit threonine at 172, leading to activation of AMPK [48]. A series

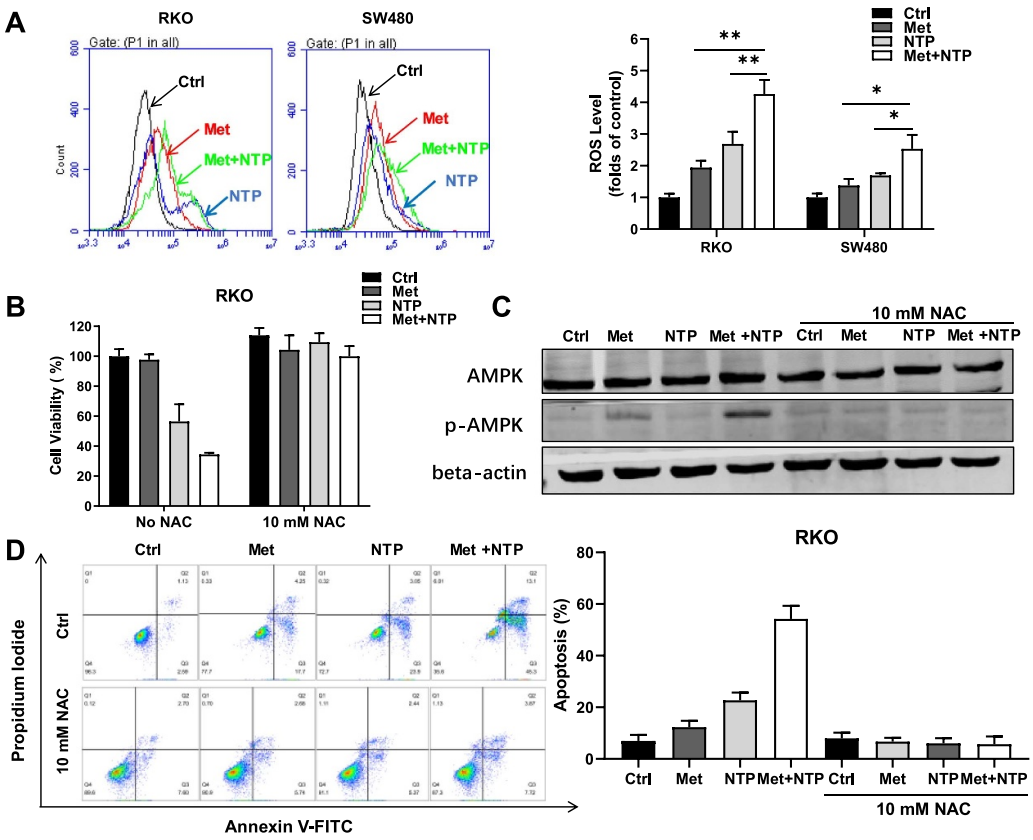

Figure 5. ROS initiates the activation of AMPK. (A) Intracellular ROS level. (B) Cell viability. (C) Protein level of AMPK/p-AMPK. (D) Apoptosis. (* $p < 0.05$, ** $p < 0.01$)

of studies revealed the pro-apoptotic effect of AMPK via inhibiting the degradation of p53, up-regulating the expression of Bim, inhibiting the activity of mTOR and increasing the level of ROS [49, 50]. Furthermore, activation of AMPK with its agonists has also been used to initiate apoptosis in tumor cells [51–53]. In the present study, we were interested in the involvement of activation of AMPK in the synergistic effect of metformin and NTP. An increased ratio of p-AMPK/AMPK was observed in the groups treated with metformin and NTP, indicating enhanced activation of AMPK when compared to those treated with metformin or NTP only. Upon knocking down with specific siRNA of AMPK, the activation of AMPK and the killing effect including apoptosis prompted by the combination of metformin and NTP were significantly attenuated, indicating the important role of AMPK activation.

ROS, a well-known initiator of apoptosis, has also been reported to be induced by the continuous activation of AMPK and lead to apoptosis [54–56]. In this study, we found production of ROS in cells treated with metformin plus NTP was significantly higher than that in cells treated with metformin or NTP only. With NAC treatment, the prompted killing effect including apoptosis occurrence and AMPK activation was attenuated significantly, indicating the involvement of ROS-AMPK.

In conclusion, our study showed that metformin with NTP synergistically killed the colon cancer cells via the axis of ROS-AMPK. Considering the extensive clinical use and safety of metformin, we hope our research contributes to an improved therapeutic effect.

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Conflicts of interest

The authors declare no conflict of interest.

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