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Nicotinamide phosphoribosyltransferase inhibitor APO866 induces C6 glioblastoma cell death *via* autophagy

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APO866 is a potent inhibitor of nicotinamide phosphoribosyltransferase (NAMPT), and inhibits nicotinamide adenine dinucleotide (NAD) synthesis. Our previous study showed that APO866 inhibits the proliferation of C6 glioblastoma cells, but failed to induce apoptosis. Since APO866 inhibits cellular metabolism and such metabolic stress is closely related with autophagy, thus we determined whether APO866 can induce autophagy in C6 glioblastoma cells and whether the autophagy induced by APO866 is pro-death or pro-survival. Using LC3 immunofluorescence imaging and transmission electron microscopy detection, we found that APO866 at 1-100 nM induced autophagy in C6 glioblastoma cells. APO866 at 1 nM mainly induced initial autophagic vacuoles. Whereas APO866 at 100 nM induced degrading autophagic vacuoles, as well as induced nuclei malformation and mitochondria swelling. In addition, APO866 concentration-dependently decreased the cell viability of C6 glioblastoma cells, and this effect was attenuated by autophagy inhibitors, including 3-methyladenine and LY294002. APO866 concentration-dependently decreased intracellular NAD level. Interestingly, APO866 at 1 nM slightly decreased intracellular NAD level, but dramatically increased autophagy-positive cells. The dramatical cell viability decreasing required the decreasing of intracellular NAD level to a very low threshold. Thus, our results indicated that APO866 induced pro-death autophagy in C6 glioblastoma cells by decreasing intracellular NAD, and low concentration of APO866 can be used as an autophagy inducer in autophagic-death sensitive glioblastoma.

1. Introduction

Glioblastoma is the most common malignant brain tumor, and it is the most lethal adult brain tumor. The treatment of glioblastoma include maximal surgical resection, followed by radiotherapy and/or chemotherapy. However, due to the natural and inducible resistance, effective chemotherapeutic agents are extremely deficient (Messaoudi et al. 2015; Okonogi et al. 2015).

APO866, also known as FK866 or WK175, is a potent nicotinamide phosphoribosyltransferase (NAMPT) inhibitor (Wosikowski et al. 2002). NAMPT is a rate-limiting enzyme in the mammalian salvaging pathway for the synthesis of nicotinamide adenine dinucleotide (NAD). It has been known that NAMPT is highly expressed in neoplastic cells to produce high level of NAD, which is critical to fulfill their increased levels of glycolysis (Khan et al. 2007; Bi and Che 2010). Accordingly, NAMPT has been recognized as an attractive target for anticancer drug discovery (Khan et al. 2007). Encouragingly, NAMPT inhibitors especially APO866 exhibit broad chemotherapeutic activities both *in vivo* and *in vitro* against several tumors. The underlying mechanisms include anti-angiogenesis (Drevs et al. 2003; Cea et al. 2013), apoptosis (Hasmann and Schemainda 2003; Muruganandham et al. 2005), anti-proliferation (Zhang et al. 2012) and autophagy (Billington et al. 2008; Travelli et al. 2011; Cea et al. 2012, 2013; Ginet et al. 2014).

Although it has been reported that APO866 induces apoptosis of neoplastic cells, it has been shown that APO866 induces autophagic cell death rather than apoptosis in many cell lines derived from various tumors. For example, NAD depletion by APO866 induces autophagic death but not apoptosis in SH-SY5Y neuroblastoma cells (Billington et al. 2008; Travelli et al. 2011), in lymphoma (Ginet et al. 2014), in multiple myeloma cells (Cea et al. 2012, 2013). Thus, autophagy is essential for APO866 cytotoxic activity on cells especially from hematological malignancies, and it can be a new way to enhance the anti-tumor activity of APO866 and related agents (Ginet et al. 2014).

Autophagy is a highly conserved energy-dependent response, through which cellular components can be degraded and thus the cells can maintain homeostasis during metabolic stress. However, autophagy is a double-edged sword for cells, besides autophagy can lead cells to death, it can also contribute to cancer cell survival (Apel et al. 2009). For example, glycyrrhetinic acid can trigger a protective autophagy in hepatocellular carcinoma cells, which might attenuate the anticancer effects of glycyrrhetinic acid (Tang et al. 2014). Induction of autophagy counteracts the anticancer effect of cisplatin and 5-fluorouracil in human esophageal cancer cells (O'Donovan et al. 2011).

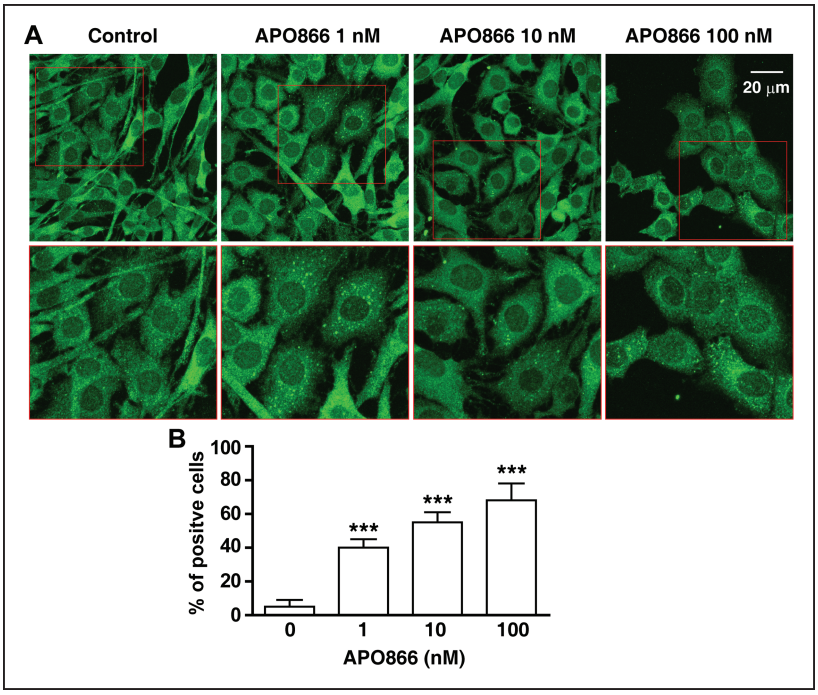

Fig. 1: APO866 induced autophagy in C6 glioblastoma cells. APO866 at 1, 10 or 100 nM APO866 was applied for 72 h. (A) Representative images of LC3-positive autophagic vacuoles determined by using immunofluorescence in C6 glioblastoma cells. (B) Analyzed percentage of autophagy-positive cells (cells containing five or more autophagic vacuoles). N = 4. $^{***}P < 0.001$, compared with no APO866 (control), One-way ANOVA.

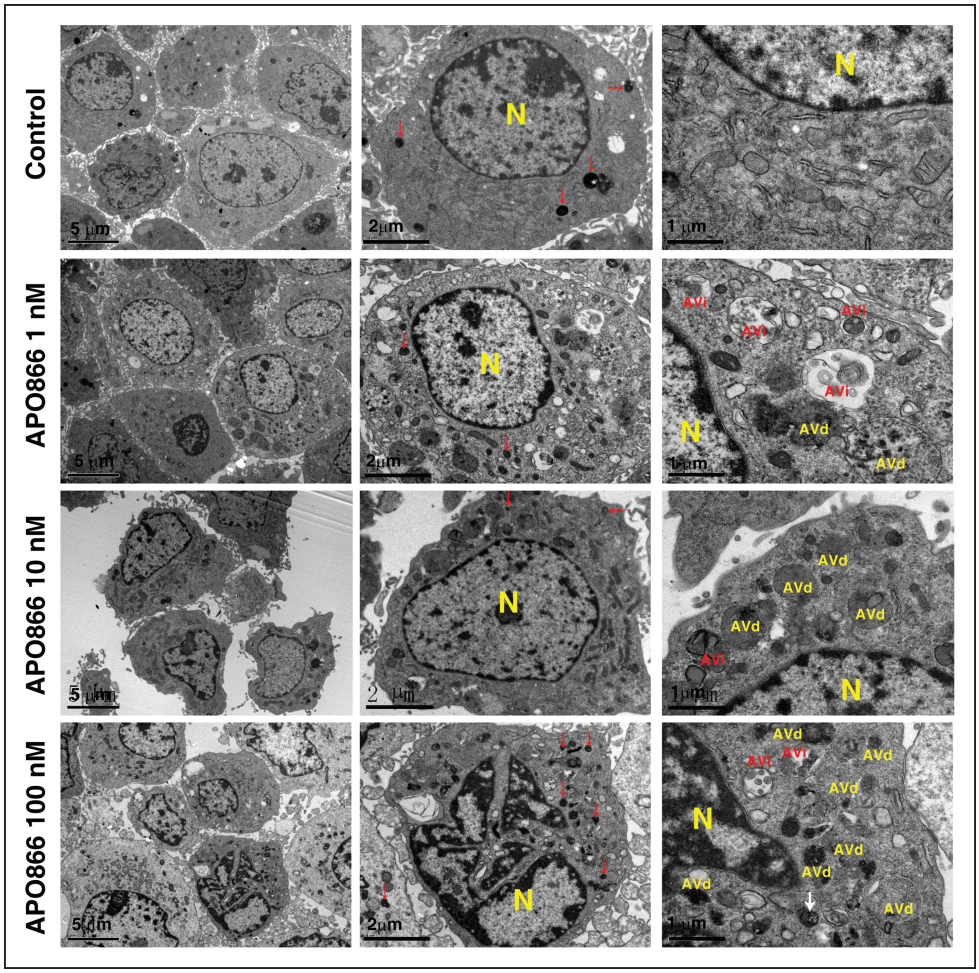

Fig. 2: APO866 induced autophagic vacuoles in C6 cells. Images were taken under electromicroscope after the cells were treated with APO866 for 72 h. N, neuclei. AVi, initial autophagic vacuoles. AVd, degrading autophagic vacuoles. Red arrows indicate lysosomes. White arrow indicates swelling mitochondria.

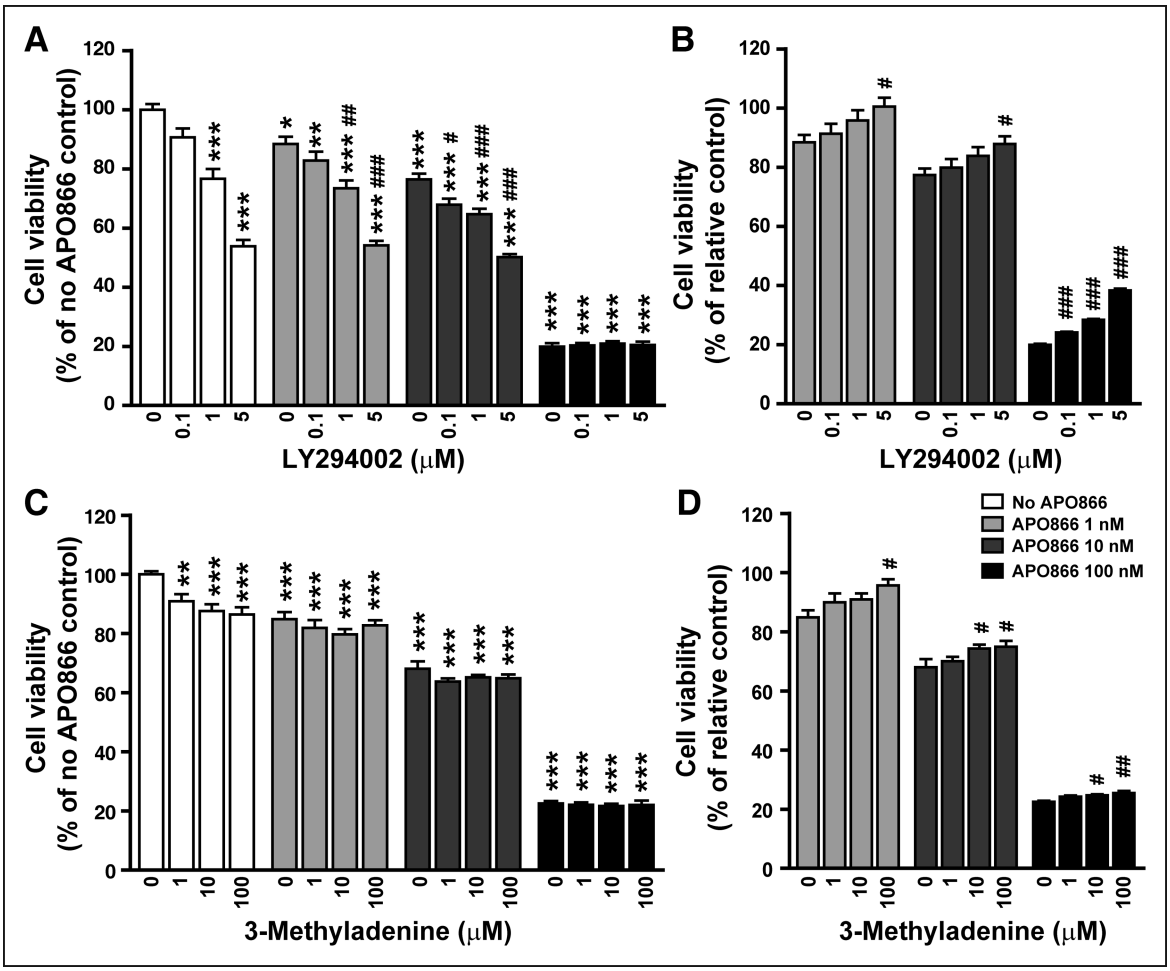

Fig. 3: Autophagy inhibitors decreased APO866-induced cell viability reduction. The LY294002 and 3-methyladenine was applied to medium 30 min before APO866, and cells viability was determined 72 h after the application of APO866. (A and B) Effect of LY294002 on APO866-induced cell viability decreasing. Cell viability was normalized to no APO866 and no LY294002 group (A) or was normalized to no APO866 but with same concentration of LY294002 (B). (C and D) Effect of 3-methyladenine on APO866-induced cell viability decreasing. Cell viability was normalized to no APO866 and no 3-methyladenine group (C) or was normalized to no APO866 but with same concentration of 3-methyladenine 2 (D). N = 12, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$,compared with no APO866 and no LY294002 (or no 3-methyladenine) group, one-way ANOVA. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with no LY294002 (or no 3-methyladenine) but with same concentration of APO866, one-way ANOVA.

Autophagy induced by EGFR inhibitors acts as a cytoprotective response and autophagic cell deathin cancer cells depending on the level of autophagy (Cui et al. 2014). In fact, it is believed that the role of autophagy in cancer treatment depends on the context, type and stage of tumors. Thus, it is important to know whether APO866 can induce autophagy in glioblastoma and whether it leads cell death or survival.

We have found that APO866 effectively inhibited its proliferation, but APO866 did not induce apoptosis of C6 glioblastoma cells (Zhang et al. 2012). At that study, a lag existed between dramatic reduction of NAD level and low cell death. In this study, we found that APO866 induces autophagic death of C6 glioblastoma cells.

2. Investigations and results

2.1. APO866 induced autophagic vacuoles in C6 glioblastoma cells

To determine whether APO866 induces autophagy, we observed the formation of LC3-positive autophagic vacuoles by using LC3 immunofluorescence. In control cells, very few LC3-positive autophagic vacuoles were observed (Fig. 1A and 1B). After treatment with 1, 10 and 100 nM APO866 for 72 h, autophagic vacuoles could be observed in many cells. APO866 at 1-100 nM significantly increased the autophagy-positive cells (containing five or more autophagic vacuoles) in a concentration dependent manner (Fig. 1A and 1B).

Ultrastructural transmission electron microscopy showed that there are abundant mitochondria and some lysosomes in the cytoplasm of C6 glioblastoma cells (Fig. 2). After treatment with 1 and 10 nM APO866 for 72 h, there was no obvious morphological change in mitochondria and lysosomes. However, many autophagic vacuoles appeared in the perinuclear position (Fig. 2). After treatment with 100 nM APO866 for 72 h, besides the formation of autophagic vacuoles, nucleus malformation and swelling mitochondria were observed (Fig. 2). In addition, the autophagic vacuoles induced by 1 nM APO866 were mainly initial autophagic vacuoles, and they were mainly degrading autophagic vacuoles when induced by 10 and 100 nM APO866 (Fig. 2).

2.2. Autophagy inhibitor attenuated APO866 induced cell viability decreasing of C6 glioblastoma cells

Using MTT assay, we found that APO866 at 1, 10, 100 nM concentration-dependently decreased the cell viability (Fig. 3). LY294002, a phosphoinositide 3-kinase (PI3K) inhibitor used as an autophagy inhibitor, concentration dependently decreased the cell viability (Fig. 3A). However, when combined with APO866,

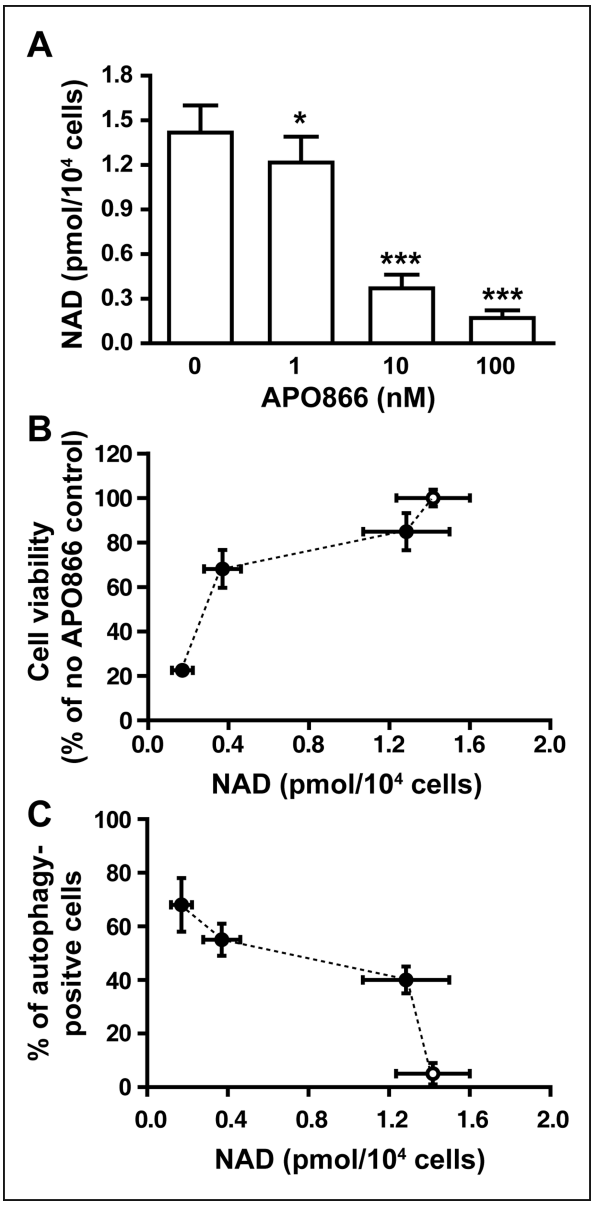

Fig. 4: The correlation of the NAD level, cell viability and percentage of autophagy-positive cells after the treatment with APO866. (A) NAD level after the treatment of APO866. N = 6, $^{*}P < 0.05$, $^{***}P < 0.001$, compared with "0" APO866, one-way ANOVA. (B) Correlation between the NAD level and the cell viability. (C) Correlation between the NAD leveland the percentage of autophagy-positive cells.

LY294002 at 0.1, 1 and 5 μM concentration dependently attenuated APO866-induced cell viability decrease (Fig. 3B).
3-Methyladenine is another PI3K inhibitor, and similar as LY294002, 3-methyladenine alone concentration dependently decreased the cell viability, but attenuated APO866-decreased cell viability (Fig. 3C and 3D).

2.3. Correlation between the NAD level, cell viability and percentage of autophagic cells after the treatment of APO866

Treatment of APO866 at 1, 10 and 100 nM for 72 h concentration dependently decreased the intracellular level of NAD (Fig. 4A). Interestingly, the cell viability decreased upon decreasing of the NAD level, but the dramatically decreasing of the cell viability required an NAD level reaching a threshold (Fig. 4B). The percentage of autophagy-positive cells increased upon decreasing of the NAD level, and only slight decreasing of the NAD level induced a dramatical increase in autophagy-positive cells (Fig. 4C).

3. Discussion

In this study, we found that APO866 induced autophagic cell death in C6 glioblastoma cells. The effect of APO866 on inducing autophagy is related to decreased of intracellular levels of NAD.
In our previous study, we have shown that only 100 nM APO866 but not 10 nM APO866 can induce apoptosis, and the percentage of apoptotic cells was only around 15% cells (Zhang et al. 2012). Here we showed that even 1 nM APO866 can induce autophagy of around 40% cells. NAMPT is closely related with cell metabolism (Rongvaux et al. 2002). When NAMPT is inhibited by APO866, metabolic stress takes place. And the metabolic stress is one of the main causes that induce autophagy, thus cellular materials can be incorporated into vesicles and be sent to lysosomes for degradation providing metabolic precursors (Kenific and Debnath 2015). Autophagy has been shown to play opposite functions in distinct cancers, either kill cancer cells or induce resistance to chemotherapeutic agents (Apel et al. 2009; Zhi and Zhong 2015). Thus, it is important to understand the consequence of autophagy in a particular cancer cell. Here our results show that autophagy inhibitors reversed APO866-induced cell viability decrease, indicating that APO866 induced pro-death autophagy but not pro-survival autophagy in C6 glioblastoma cells.
The relationship between metabolism and autophagy has been widely revealed (Jiang et al. 2015; Kenific and Debnath 2015; Kim 2015; Su et al. 2015). APO866 decreases intracellular NAD level, which will be resulted in metabolic stress. It has been known that autophagic cell death can potentiate the chemotherapeutic effects of many classical anti-cancer agents (Travelli et al. 2011; Fulda and Kogel 2015; Shi et al. 2015). For example, in glioma cells, the synergistic effect of temozolomide and chloroquine combination treatment is dependent on autophagy (Lee et al. 2015). Temozolomide is one of the main chemotherapeutic agents used against glioblastoma, and its function is damaging DNA (Kamiya-Matsuoka and Gilbert 2015). The enzymes involved in DNA repair, such as poly(ADP-ribose) polymerase (PARP) and siruins, are NAD-dependent. Thus, the lowering of NAD by APO866 may also provide synergistic effects *via* inducing autophagy when combined with alkylating agents.
Interestingly, our results showed that a slight decrease in NAD levels (decreased by 1 nM APO866) is sufficient to induce autophagy. However, slightly decreased NAD level induced autophagic vacuoles are mostly initial autophagic vacuoles, while dramatically decreased NAD level (decreased by 100 nM APO866) induced autophagic vacuoles are mostly degrading autophagic vacuoles. Besides, 100 nM APO866 induced nuclei malformation and mitochondria swelling, indicating cell apoptosis. This agrees with our previous report that only 100 nM APO866 induced cell apoptosis (Zhang et al. 2012). It has been known that autophagy can be a cell death process linking apoptosis (Su et al. 2015). Thus, our results indicate that the dramatic decrease in intracellular NAD levels will link autophagy to apoptosis, and leads to a robust drop of cell viability.
Taking together, we showed that APO866 induces pro-death autophagy in C6 glioblastoma cells, which is related to intracellular NAD decrease. The concentration for APO866 to induce autophagic cell death was in the nM range. Thus, it might be beneficial to use a low concentration of APO866 as an autophagy inducer in the treatment of autophagic-death sensitive glioblastoma.

4. Experimental

4.1. Cells and cell culture

Rat-derived C6 glioblastoma cells were purchased from the Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)supplemented with 10% heat inactivated fetal calf serum and 100 U/ml penicillin/streptomycin, at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2.

4.2. Cell viability assay

The MTT assay was used to assess cell viability. After 72 h treatment with APO866 (Provided by NIMH Chemical Synthesis and Drug Supply Program, NIH, USA) alone or with LY294002 (Sigma-Aldrich, St Louis, MO, USA) or with 3-methyladenine (Sigma-Aldrich, St Louis, MO, USA), the cells cultured in 96-well plates were incubated with 0.5 mg/ml MTT for 2 h at 37 °C. The MTT solution was then carefully removed and 100 μl DMSO were added to each well and incubated for 10 min. The absorbance was read at 490 nm in a plate reader (Elx800, Bio-TEK Instrument USA).

4.3. Immunofluorescence of LC3 autophagic vacuoles

After 72 h treatment with APO866, the cells grown on round glass coverslips were fixed by using pre-chilled methanol for 20 min. After permeabilized with 0.% Triton X-100 (in PBS) for 10 min, the cells were blocked by using 5% normal donkey serum for 30 min. Then the cells were incubated with rabbit polyclonal anti-LC3 antibody (1:200, Cell Signaling Technology, Danvers, MA, USA) at 4 °C overnight. After three washing steps with PBS, the cells were incubated with FITC-labeled goat anti-mouse IgG antibody (1:200; Chemicon) for 2 h. Finally, the cells were mounted by using an anti-fade medium (Invitrogen), and were observed under a confocal laser scanning microscope (Nikon, A1, Japan). The cells containing five or more autophagic vacuoles were identified as positive cells.

4.4. Transmission electron microscopy

The cells were gently scraped using a cell scraper (Corning). Then the cells were fixed in 2.5% glutaraldehyde in PBS for 4 °C overnight. The glutaraldehyde was then removed and the cells were washed with PBS. The pellets were post-fixed in 1% osmic acid at 4 °C for 1 h. Then the cells were prestained in 4% uranyl acetate at 4 °C for 30 min, and sequentially rinsed and dehydrated in 50%, 70%, 90%, 100% alcohol and in 100% acetone at 4 °C for 20 min. Cells were infiltrated in 1:1 acetone-EPON812 (Araldite) for 2 h at room temperature, and then infiltrated in 100% EPON812 for 2 h at room temperature. Samples were polymerized at 37 °C for 24 h, 45 °C for 24 h and 60 °C for 48 h. After polymerization, 120 nm thin sections were cut on an Ultracut microtome (Leica). The sections were stained in 4% uranyl acetate for 20 min and 5 min in lead citrate, rinsed and dried. EM images weretaken on 80 keV Philips TECNAI 10 transmission electron microscope equipped with Ganta 794 CCD.

4.5. NAD detection

The intracellular NAD was detected by using NAD + /NADH quantification kit (BioVision, Mountain View, CA, USA). According to the manufacturer's instruction, the cells were subjected to two freeze/thaw cycles (20 min at –70 °C, then 10 min at room temperature) in NADH/NAD extraction buffer. Then the supernatant was obtained by centrifugation at 12,000× g for 5 min, and 50 (l supernatant sample was mixed with100 (l NAD Cycling Mix and 2 (l NADH Cycling Enzyme Mix, and incubated at room temperature for 5 min to convert NAD to NADH. After adding 10 (l NADH developer, the optical density was read at 450 nm every 30 min for 4 h. The amount of sample NAD was calculated according to the NADH standard curve. The total NAD level was expressed as pmol/10^4 cells.

4.6. Statistical analysis

Data were expressed as mean ± S.E.M. Values between groups were compared using one-way ANOVA. GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA) was used for statistical analysis. $P < 0.05$ was considered statistically significant.

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