4-[1-ethyl-1-methylhexy]-phenol Induces Apoptosis and Interrupts Ca^{2+} Homeostasis via ROS Pathway in Sertoli TM4 cells

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Abstract

Biological effect of an individual nonylphenol (NP) isomer extremely relies upon the side chain structure. This research was designed to evaluate the impact of NP isomer, 4-[1-ethyl-1-methylhexy]-phenol (NP_{65}), on Sertoli cells *in vitro*. Sertoli TM4 cells were exposed to various concentration (0, 0.1, 1, 10, or 20 μM) of NP_{65} for 24 h, and the outcomes indicated that treatment of NP_{65} induced reactive oxygen species (ROS) generation, oxidative stress as well as apoptosis for Sertoli TM4 cells. In addition, it was found that NP_{65} exposure affected homeostasis of Ca^{2+} in Sertoli TM4 cells by increasing cytoplasm $[Ca^{2+}]i$, inhibiting Ca^{2+}-ATPase activity and decreasing cAMP concentration. Pretreatment with ROS scavenger, N-acetylcysteine (NAC), attenuated NP_{65}-induced oxidative stress as well as apoptosis for TM4 cells. Furthermore, NAC blocked NP_{65}-induced disorders of Ca^{2+} homeostasis by attenuating the growth of intracellular $[Ca^{2+}]i$ and the inhibition of Ca^{2+}-ATPase and cAMP activities. Thus, we have demonstrated that NP_{65} induced apoptosis as well as acted as a potent inhibitor of Ca^{2+}-ATPase activity and resulted in disorder of Ca^{2+} homeostasis in Sertoli TM4 cells, ROS participated in the process. Our results supported the view that oxidative stress acted an essential role within the development of apoptosis and Ca^{2+} overload in TM4 cells as a consequence of NP_{65} stimulation.

1. Introduction

Nonylphenols (NPs), the major degraded products of nonylphenol polyethoxylate (NPEOs), were thought to be one of the endocrine disrupting chemicals (EDCs), which could interrupt the endocrine system function of human and animal. Today, NPs and NPEOs have been employed widely in detergents, cosmetics, insecticides, surface-active reagents, food manufacturing, plastics, polyvinyl chloride tubes, as well as other industrial formula products as plasticizers or antioxidants (Bonefeldjørgensen et al. 2007; Cheng et al. 2017; Noorimotlagh et al. 2016). Studies have demonstrated that NPs was seriously threatening reproductive health (Malmir et al. 2020), immune function (Xia et al. 2013) and system of nerves (Li et al. 2019) in human and animals.

Disturbingly, NPs have been investigated in the majority of retailed food, and they might migrate from films for food packaging to simulants of food within the process of microwaving and cooking (Inoue et al. 2001; Kawamura et al. 2017). In addition, NPs could be uptaken by wildlifes in aquatics, particularly by fish followed by accumulation into bodies of human via the food net (Coldham et al. 1998; Kookana 2002). The existence of NPs within the environment has become a growing worry because they have been shown to be estrogenic compounds in addition to the persistence and toxicity, while most of the studies were focused on industrial nonylphenol, which mostly include a combination of para-substituted mono-alkylphenols containing numerous branched and isomeric nonyl groups. However, it was demonstrated that the biological effect of a distinctive nonylphenol isomer is greatly reliant on the side chain structure (Preuss et al. 2006; Kim et al. 2005). As far as we know, there have been few studies to observe the toxicity of NPs from the perspective of isomers specificity, while the mechanism on toxicity of NP isomers has been barely studied.

Recently, some distinct NP isomers have been purified and used to analyze the toxicity as well as fate to environment of NPs. Oestrogenic effects of some artificial isomers of NP have been described (Boehme et al. 2010; Ying et al. 2012; Gabriel et al. 2008;). To justify the relationship of distinct NP isomers in scientific research, Guenther et al. (2006) have built a system of numbering for entirely potential NP isomers, which respects the IUPAC rule for the description of alkylphenols substituents. For example, 4-[1-ethyl-1-methylhexy]-phenol was abbreviated as NP_{65}. Fortunately, Professor Guenther (Institute for Chemistry and Dynamics of the Geosphere, Research Centre Juelich, Germany) donated 12 NP isomers to our laboratory. This may lead us to investigate the environmental hazard of NPs from the perspective of isomer specificity. The effects of 4-[1, 2, 5-trimethylhexyl]-phenol (NP_{42}) as well as 4-[1, 2, 4-trimethylhexyl]-phenol (NP_{41}) on cell receptors and MAPK pathway in mouse Sertoli TM4 cells have been investigated in our laboratory (Liu et al. 2014).

Our previous results have indicated that NP_{65} suppressed the expression of steroid hormone receptor and activated Akt and JNK-MAPK pathways in Mouse Sertoli TM4 cells (Liu et al. 2017). In recent years, it was testified that several EDCs play the role of chemical matter causing apoptosis of cells (Qian et al. 2006; Hallegue et al. 2002). In addition, studies have shown that apoptosis works as a mechanisms of reproductive damage. 4-n-NP applies a toxic effect on the reproductive system during development by inducing apoptosis of TM4 cells, but whether other NP isomers cause the same problem is still unknown. In our research, we hypothesized that NP_{65} can damage the reproductive by inducing cell apoptosis and disturbing Ca^{2+} homeostasis in Sertoli cells. Therefore, the adverse effects of 0–20 μM NP_{65} on Sertoli cells and the underlying mechanism were evaluated in the existed research.

2. Materials And Methods

2.1 Reagents

The pure NP_{65} was from Prof. Guenther as a gift. The structure of NP_{65} was illustrated in Fig. 1 (Gabriel et al. 2008).

Phenol red-free Dulbecco's modified Eagle's medium nutrient mixture F-12 HAM (DMEM/F-12 medium), donor equine serum and charcoal/dextran-treated fetal bovine serum (FBS) were purchased from Hyclone (Waltham, MA, USA). AnnexinV-FITC apoptosis kit was obtained from KeyGEN BioTECH (Nanjing, Jiangsu, China). ELISA kits were purchased from Westang (Shanghai, China). Streptomycin sulfate, 5(6)-carboxy-2'-7'-dichlorofluorescein diacetate (DCFH-DA) and Penicillin were purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). Ca^{2+}-ATPase detection kit, malnoic dialdehyde (MDA) assay kit and superoxide dismutase (SOD) assay kit were obtained from Jiancheng (Nanjing, China). Analytical grade was applied to all other chemicals.

2.2 Cell culture

The mouse Sertoli TM4 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in F-12/ DMEM medium free of phenol red, with 5% (v/v) donor equine charcoal stripped serum, 1% penicillin-streptomycin and 2.5% (v/v) dextran-charcoal stripped FBS in an atmosphere of 100% relative humidity as well as 5% CO_2 at 37°C. Cells were pretreated with 5 mM N-acetylcysteine (NAC) in 20 μM NP_{65} group for 30 min, followed by the exposure towards 0, 0.1, 1, 10, or 20 μM of NP_{65} for 24 h.

2.3 Assay of reactive oxygen species (ROS) production

TM4 cells were seeded with a same number (2×10^4/well in 200 μL of growth medium) for each group on a 96-well black microplate, and cultured for 6 h, NP_{65} was employed to the cells in 100 μL basic medium for 24 h, then the medium is aspirated and phosphate buffered saline (PBS) is used to flush the wells. The wells experienced incubation with 25 μM DCFH-DA dissolved in minimal medium for 1 h, then the relative fluorescent intensity was detected by Varioskan Flash (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 525 nm for emission and 485 nm for excitation.

2.4 Determination of SOD activity and MDA content

For detecting the activity of intracellular SOD as well as content of MDA, TM4 cells were seeded in a six well plate with a number of 2×10^5/well, cells were treated with NP_{65} for 24 h, then cells were washed with ice cold PBS twice, followed by harvesting from the plates into PBS and homogenized. Whole cell homogenate was harvested for 10 min by centrifugation at 4°C, 12,000 g. The supernates were harvested and immediately assayed for SOD activity and MDA content with commercial kits with an automated microplate reader (Varioskan Flash, Thermo Scientific, USA), according to the instructions of manufacturer.

2.5 Apoptosis analysis with Annexin V–FITC and PI staining

Sertoli TM4 cells were seeded at a density of 2×10^5/well on 6-wells plate. Cells were harvested followed by washing with cold PBS thrice after exposed to NP_{65} for 24 h, and then the cells experienced staining with 5 μL Annexin V-FITC as well as 5 μL PI in 100 μL binding buffer based on the instructions of manufacturer. The flow cytometer was used for flow cytometry (FACS Calibur, Becton-Dickson, San Jose, CA).

2.6 Determination for release of Ca^{2+} in TM4 cells

The intra-cellular calcium ion ($[Ca^{2+}]i$) was also determined by flow cytometry (Fengling et al. 2012). NP_{65} treated cells were harvested followed by washing thrice with cold PBS, then loading with 5 μM of Fulo-3/AM at 37°C for 40 min at dark. Finally, the extracellular Fluo-3/AM dye was removed and the relative fluorescence intensity within the cells were detected by flow cytometry.

2.7 Determination of Ca^{2+}-ATPase activity, cAMP and cGMP concentration

NP_{65} challenged cells were collected and homogenized. Concentration of cAMP and cGMP in the supernatants were measured by ELISA kits (Westang, Shanghai, China). The Ca^{2+}-ATPase activity was also detected by a commercial kit (Jiancheng Bioengineering Institute, Nanjing, China). The whole measurements were conducted according to the instructions of manufacturer, and the absorbance was determined via an automatic microplate reader.

2.8 Reverse transcription polymerase chain reaction (RT-PCR) analysis

Trizol Reagent (Invitrogen, Carlsbad, CA, USA) was employed for extraction of RNA in total based on the protocol from manufacturer, and 2 μg of the RNA sample was applied for synthesize cDNA by using ReverAid™ First Strand cDNA Synthesis Kit (Thermo Scientific, Maryland, USA). PCR amplification was carried out by employing approximately 1 μg cDNA as a template. The sequences for primer for PCR were shown within Table 1. PCR program included 3 min at 94°C, 30 s at 60°C for annealing, 30 cycles of 30 s at 94°C for denaturation, and 2 min at 72°C for extension, plus 10 min at 72°C as final extension. Products from PCR were examined on 2% (w/v) agarose gel by GoldView-agarose gel electrophoresis, followed by visualization with ChemDoc XRS+. The target genes' signals were examined via scanning densitometry followed by normalization to β-actin with Quantity One software.

Table 1
Sequences of primers used for reverse transcription polymerase chain reaction (RT-PCR).

Genes	Sequence (5'->3')	Product size (bp)
β-actin	Forward: TGGCACCACACCTTCTACAATG Reverse: CCTGCTTGCTGATCCACAATCTG	800
Bcl-2	Forward: AATGCAAGAACGTTGTGCCC Reverse: TCTGCTTCCGGGGGTATGTA	181
Bax	Forward: CTGTCCAGCCACGAATCAGT Reverse: CCTCATCCCTGTCCAGAACG	548
SERCA 2	Forward: TGGATCAGGGGTGCCATCTA Reverse: AAC ATC CTG CAC ACG GAC AT	239

2.9 Statistical analysis

The statistical analysis was conducted on the software of SPSS with the version 19.0 for Windows. One-way analysis of variance with LSD test was employed for determination of the statistical differences among groups with different treatments. Values are presented as mean ± S.D., the differences between two means were considered to be statistically significant if a value of P was less than or equal to 0.05.

3. Results

3.1 Generation of ROS induced by NP_{65} in Sertoli TM4 cells

Pretreatment for cells was carried out with 5 mM NAC in 20 μM NP_{65} group for 30 min, followed by consecutive exposure to NP_{65}, and later the amount of ROS was analyzed via an automatic microplate reader. Results indicated that comparing control group, NP_{65} increased generation of ROS in the cells in a manner of dose dependence, and achieved the maximum at 20 μM NP_{65}. However, the increase was diminished by NAC pretreatment in 20 μM NP_{65} group (Fig. 2).

3.2 Effects of NP_{65} on SOD activity and MDA content

We thus further investigated the influences of NP_{65} upon the activity of SOD as well as MDA content in Sertoli TM4 cells. Compared with control group, 20 μM of NP_{65} treatment dramatically down-regulated the levels of SOD activities in cells ($P < 0.01$, Table 2). On the contrary, content of MDA was rised remarkably in 20 μM NP_{65} treatment groups comparing group of control (Table 2, $P < 0.01$). Interestingly, pretreatment of NAC mitigated the decline of SOD activity and the growth of MDA content induced by NP_{65}. Thus, we proposed that NP_{65} induced lipid peroxidation in TM4 cells, which finally resulted in oxidative stress in NP_{65} treated TM4 cells.

Table 2
Effects of NP_{65} on the activity of SOD and MDA content

NP_{65} Concentration (μM)	NAC (μM)	SOD (U/mg protein)	MDA (μM/mg protein)
—	—	15.86 ± 2.48	19.31 ± 2.92
0.1	—	15.58 ± 0.20	19.22 ± 3.34
1	—	10.61 ± 1.76*	19.61 ± 1.03
10	—	8.66 ± 3.92**	20.14 ± 2.41
20	—	8.44 ± 3.48**	21.84 ± 3.07*
20	20	13.86 ± 0.38$^{\#}$	19.95 ± 2.22
Results are presented as mean ± S.D. with triplicate measurements.			
$^{*}P < 0.05$, $^{**}P < 0.01$ vs. Control group; $^{\#}P < 0.05$ vs. 20 μM NP_{65} group.			

3.3 Induced apoptosis after NP_{65} exposure in TM4 cells

Apoptosis induced by NP_{65} was detected by flow cytometry in Sertoli TM4 cells (Fig. 3). Comparing control group, there was no sharp variation in the percentage of apoptotic cells in 0.1 as well as 1 μM of

NP_{65} treatment groups. On contrary, the portion of apoptotic cells was raised remarkably in 10 or 20 μM NP_{65} group ($P < 0.05$). Similarly as the ROS generation, pretreatment with NAC minimized the apoptosis of cells as comparing 20 μM NP_{65} group ($P < 0.01$), indicating that NP_{65}-induced apoptosis in TM4 cell was related to ROS overproduction.

3.4 Alteration in mRNA expression of Bax and Bcl-2 in TM4 cells

As shown in Fig. 4, the mRNA expression levels of Bax increased significantly in 1–20 μM of NP_{65} treatment ($P < 0.05$). For comparison, the levels of Bcl-2 mRNA dramatically declined at 10 and 20 μM NP_{65} exposure groups ($P < 0.05$). In addition, NAC attenuated NP_{65}-induced rise of Bax and decrease of Bcl-2, which implied that ROS production or oxidative stress was related to the apoptosis process in TM4 cells, and that the process of apoptosis should be mediated with mitochondrial pathway.

3.5 Impact of NP_{65} on intracellular Ca^{2+} in TM4 cells

As illustrated in Fig. 5, comparing control group, fluorescence intensity of $[Ca^{2+}]i$ was rised in the groups treated with NP_{65}. Additionally, our data showed that NAC pretreatment attenuated NP_{65} enhanced $[Ca^{2+}]i$ increase in TM4 cells, suggesting that ROS is related to NP_{65} induced $[Ca^{2+}]i$ alternation in TM4 cells.

3.6 NP_{65} inhibited the activity of Ca^{2+}-ATPase and SERCA expression in TM4 cells

The influences of NP isomer upon Ca^{2+} homeostasis disorder were determined concerning Ca^{2+}-ATPase activity and SERCA function in TM4 cells. Ca^{2+}-ATPase activity was down-regulated by NP_{65} in a manner of dose-dependence ($P < 0.01$, Fig. 6A). Furthermore, the data of this research indicated that the decrease of Ca^{2+}-ATPase activity induced by NP_{65} was influenced by pretreatment of NAC, which improved NP_{65}-induced decrease of Ca^{2+}-ATPase activity in the cells ($P < 0.01$). Consistent with the alteration of Ca^{2+}-ATPase activity, the expression level of mRNA of SERCA 2 in TM4 cells was significantly decreased by 20 μM of $NP_{65,}$ which was rescued by NAC (Fig. 6B&C).

3.7 Concentration of cAMP and cGMP in Sertoli TM4 cells

The results illustrated that the concentration of cAMP was significantly reduced by NP_{65} at 1, 10 and 20 μM ($P < 0.05$). However, the decline of cAMP content induced by NP_{65} at 20 μM was up-regulated by NAC (Fig. 7A). On the other hand, it was found that NP_{65} showed no significant effects on cGMP secretion (Fig. 7B), indicating that the injury of NP_{65} to TM4 cells may be through inhibiting cAMP pathway but not cGMP pathway.

4. Discussion

NP was reported to cause intracellular accumulation of ROS in multiple cell types (Qi et al. 2013; Okai et al. 2004; Gong and Han 2006), and induce oxidative stress within testis of rats (Chitra and Mathur 2004). Our interesting findings implied that NP_{65} treatment caused ROS over generation and oxidative stress in TM4 cells, as proven by rapid increase of MDA content and the marked decrease of SOD activity in NP_{65} stimulated cells. Oxidative stress was thought to be the underlying mechanism for the death of apoptotic cells (Kitazawa et al. 2001). The current results demonstrated that NP in TM4 cells could induce oxidative stress, which might lead to cellular apoptotic damages potentially. Apoptosis accompanied by alteration in the mRNA expression of Bcl-2 family, suggesting that in TM4 cells, mitochondrial pathway should be contributed to NP_{65} induced apoptosis.

Ca^{2+}, as the widely employed intracellular messenger, can encode a variety of cellular information based on the regulation of signals of Ca^{2+}. Signals of Ca^{2+} have pivotal roles within various cellular behaviours including differentiation, proliferation as well as transcription of genes (Bootman et al. 2012). In response to different stimuli, increase of the Ca^{2+} concentration within the cytosol induces plenty of types of events, which plays substantial role in cell apoptosis. Wang et al. (2005) have found that Ca^{2+} elevation and Ca^{2+}-independent cell death could be induced by NP in MG63 human osteosarcoma cells. Studies have found that Ca^{2+} release and homeostasis disorder were involved in the apoptosis induced by alkylphenol in TM4 cells (Michelangeli et al. 2008), and NP caused Ca^{2+}-dependent apoptosis in SCM1 human gastric cancer cells (Kuo et al. 2010). Analysis of flow cytometry illustrated that NP_{65} dramatically rised intracellular Ca^{2+} level in TM4 cells through a manner of dose dependence, and ROS scavenging contributed to weakening NP-induced Ca^{2+} overloading. The results suggested that ROS generation was involved in Ca^{2+} release in NP_{65} challenged TM4 cells, and Ca^{2+} homeostasis disorder might act an important role in apoptosis of cells induced by NP_{65}.

SERCA has always been accepted as playing a central role in the mechanism of Ca^{2+} transport across the membrane, from the cell cytosol into the endoplasmic reticulum, and this is the reason why low levels of free cytosolic Ca^{2+} are maintained in the cells. Under stimulation, $[Ca^{2+}]i$ becomes higher and remains high if SERCA is blocked. Nonylphenol was reported to influence Ca^{2+} signaling mechanisms within cells by influencing transporters of Ca^{2+} such as Ca^{2+} pumps of SERCA (Hughes et al. 2000). Many estrogenic alkylphenols such as NP have been tested to inhibit the sarcoplasmic reticulum Ca^{2+}-ATPase of skeletal muscle, which functions in a similar manner to the endoplasmic reticulum Ca^{2+}-ATPase (Mason et al. 1993). Therefore, we hypothesized that the evaluation of Ca^{2+} induced by NP_{65} may be related to alteration of SERCA Ca^{2+} pumps function. The inhibition of Ca^{2+}-ATPase activity and SERCA-type Ca^{2+} pumps expression in this study suggested that NP_{65} might act as an inhibitor of SERCA. We proposed that NP_{65} could release Ca^{2+} from intracellular stores by inhibiting the activity of SERCA Ca^{2+} pumps. Ca^{2+} channels were regulated by different kinases, calmodulin-dependent kinase, protein tyrosine kinase,

as well as G protein subunits, etc. (Keef et al. 2001). Since cAMP and cGMP are important for regulation of protein kinase C, cGMP-dependent protein kinase and cAMP-dependent protein kinase to exert vital functions of cell (e.g. differentiation, proliferation, transcription of genes as well as cellular apoptosis,) within all organisms, we concentrated our researches upon the effects of cAMP as well as cGMP in TM4 cells. The decrease of cAMP concentration could be one of the factors that were involved in the elevation of Ca^{2+} level in TM4 cells, although the mechanism was still unclear. Interestingly, the Ca^{2+} disorder was moderately inhibited by NAC, no matter the inhibition of the activity of Ca^{2+}-ATPase or the decrease of cAMP content was weakened by NAC pretreatment. We concluded that ROS pathway was involved in NP_{65} disturbed Ca^{2+} signaling in TM4 cells.

Apoptosis and Ca^{2+} overload were related to the formation of ROS, several antioxidants can block cell apoptosis. NAC has been reported to alleviate damage of cells induced by toxic chemicals (Spagnuolo et al. 2006). In the present study, NAC was employed for investigation of the putative role of ROS production in apoptosis and loss of Ca^{2+} homeostasis induced by NP_{65}. NP_{65}-induced apoptosis and loss of Ca^{2+} homeostasis were blocked by NAC, suggesting that ROS generation was involved in NP_{65} induced apoptosis as well as loss of Ca^{2+} homeostasis. Thus, the current study built a direct linkage between NP_{65} induced generation of ROS and apoptosis as well as Ca^{2+} disorder in TM4 cells.

NPs exerted effects not only through binding to estrogen receptors but also through cell signal pathways. It is known that the the structure of the side chain may influence the estrogenic effect of a single NP isomer to a large extent. The estrogenic potency of NP_{65} was 6.1×10^{-6}, and 4-n-NP showed no estrogen effect (Preuss et al. 2006). Michelangeli et al. (2008) reported that the SERCA inhibition potency of alkylphenols was relevant to length of chain for linear chain alkylphenols, and branched chain alkylpenols in general had higher potencies compared with their counterparts of linear chain. There were different effects between NP_{65} and 4-n-NP on the apoptosis and Ca^{2+} disorder in TM4 cells, which further proved that the effects of NPs to organism were related to their structures or their potential xenoestrogenic activity.

In summary, this study has shown that the NP isomer, NP_{65}, could trigger oxidative stress as well as mitochondrial pathway apoptosis in mouse Sertoli TM4 cells. In addition, NP_{65} was capable of elevating intracellular $[Ca^{2+}]i$ levels in Sertoli TM4 cells. Our study found that ROS was related to both the apoptosis as well as Ca^{2+} disorder induced by NP_{65} in TM4 cells. However, whether NP_{65} evoked Ca^{2+} disorder is related to NP_{65}-induced apoptosis is an important issue, which will be investigated in our further study. Anyway, results in this study offered an alternative vision that NP isomer might disrupt the development of male reproduction by inducing apoptosis and disturbing relative signaling pathways in the cells, but not require to be mediated solely by direct interaction with the estrogen receptor.

5. Abbreviations

cAMP: cyclic guanosine monophosphate; cGMP: cyclic guanosine monophosphate; DCF: 2’-7’-dichlorofluorescein; DCFH-DA: 5(6)-carboxy-2’-7’-dichlorofluorescein diacetate; DMEM/F-12 medium: Dulbecco’s modified Eagle’s medium nutrient mixture F-12 HAM; FBS: fetal bovine serum; MDA: maleic dialdehyde; NAC: N-acetylcysteine; $NP_{65:}$ 4-[1, 2, 4-trimethylhexyl]-phenol; NPs: Nonylphenols; PBS: phosphate buffered saline; RT-PCR: reverse transcription polymerase chain reaction; SERCA: Sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; SOD: superoxide dismutase

6. Declarations

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare no competing interests.

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Author contribution

Xiaozhen Liu contributed to methodology and original draft. Fuxiang Li performed the research. Zhaoliang Zhu and Gaoyi Peng analyzed data. Danfei Huang contributed to discussion and resource provision. Mingyong Xie contributed to review, editing and supervision. All authors approved the final manuscript.

Data availability

All data supporting the conclusions of this study will be made available by the authors, without undue reservation.

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Figures

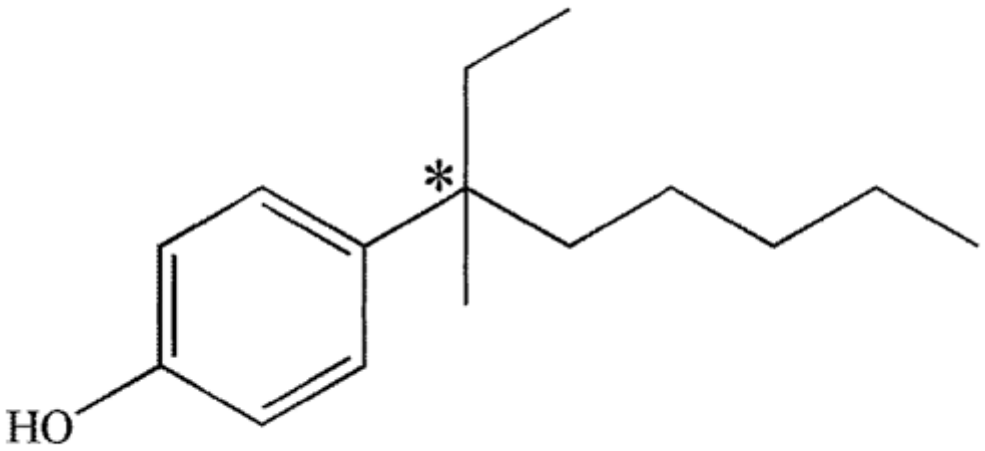

Figure 1

Structure of 4-[1-ethyl-1-methylhexy]-phenol (NP65). * represents chiral carbon atom.

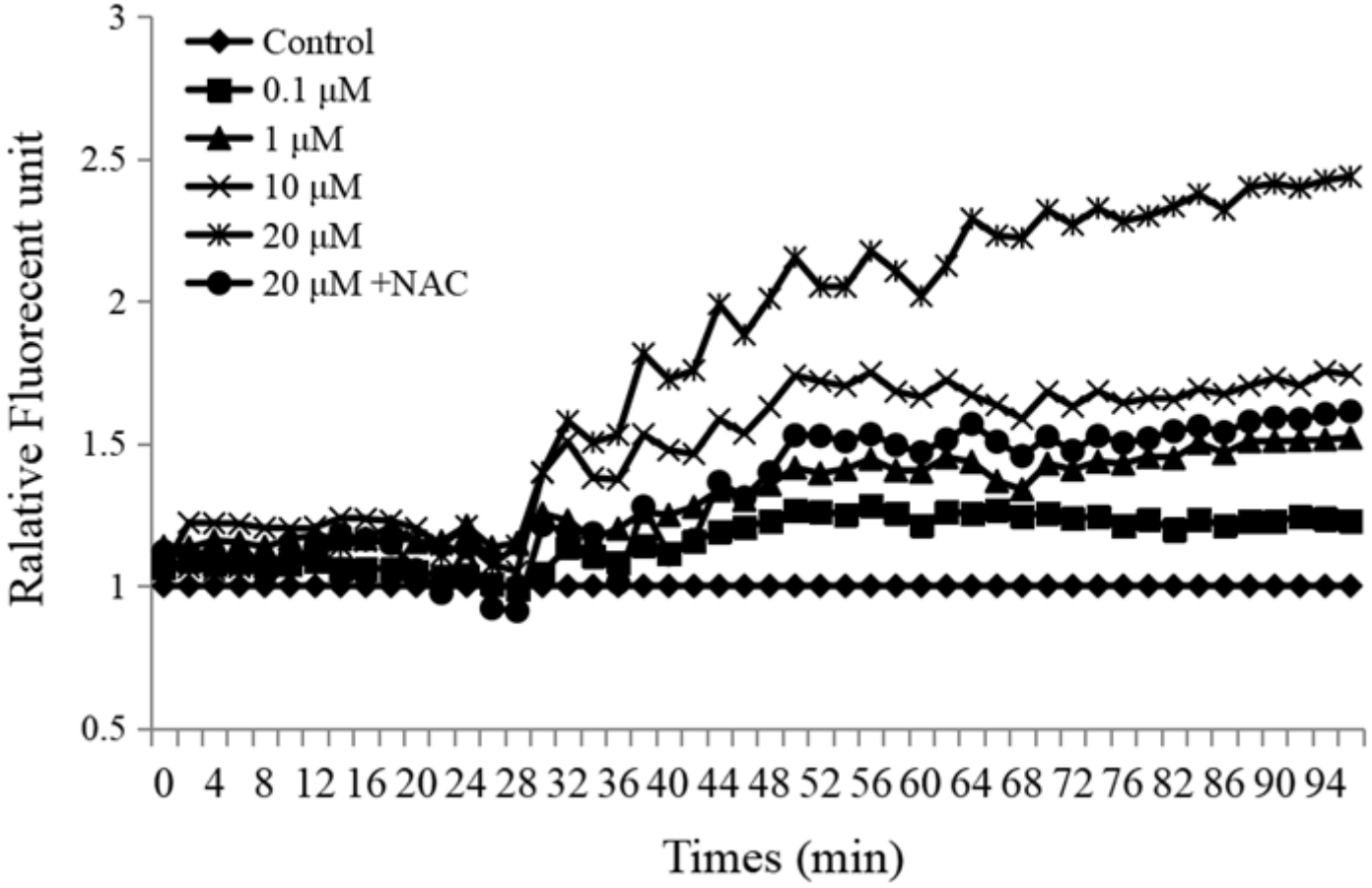

Figure 2

ROS generation in NP65 treated TM4 cells. TM4 cells were seeded in black 96 well plate and exposed to NP65, ROS generation was determined by an automated microplate reader using DCFH-DA.

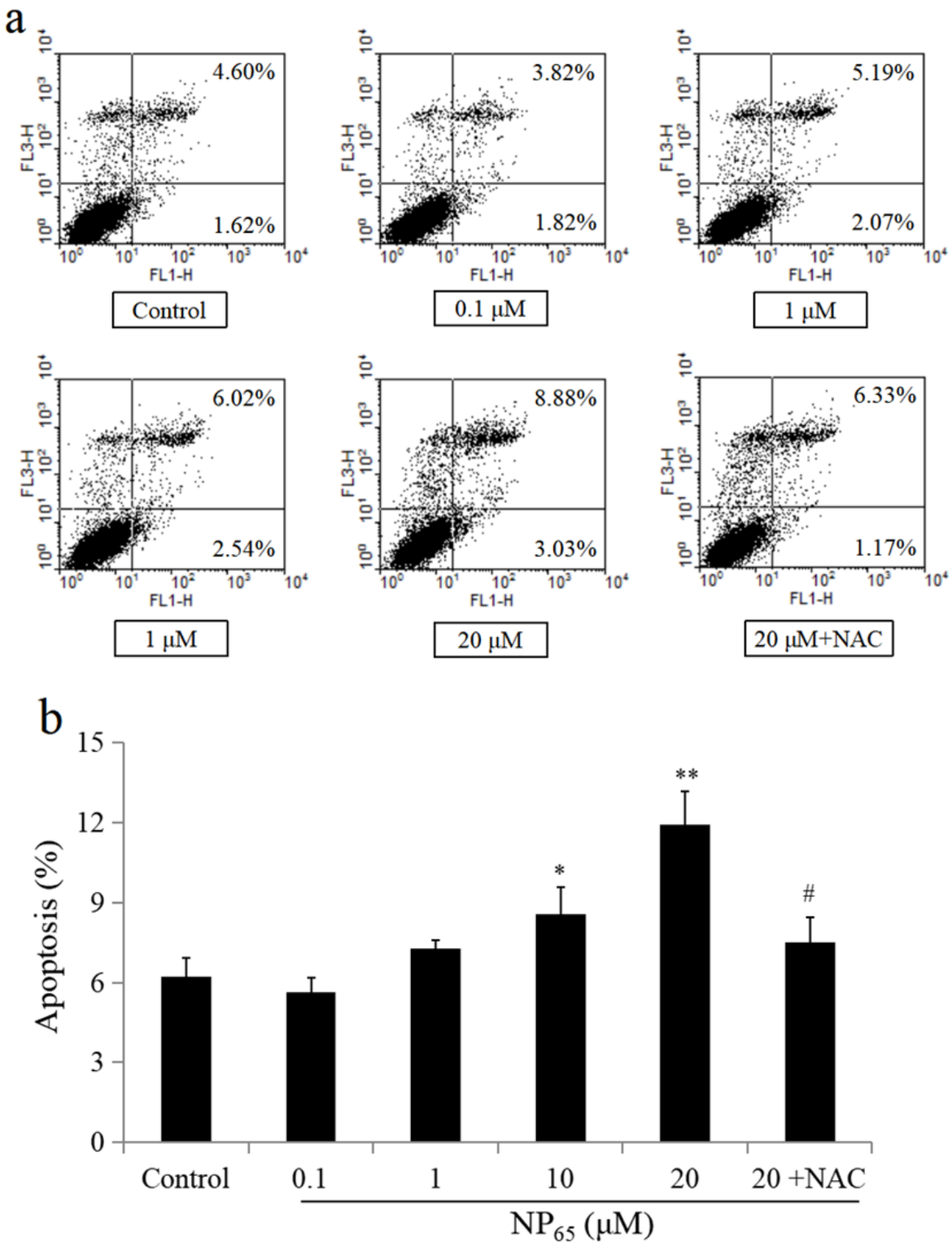

Figure 3

Apoptosis detection in NP65 treated TM4 cells. (a) Flow cytometry for analysis of apoptotic cells with staining of Annexin V-FITC/PI. 20,000 cells were collected for every sample. (b) Histogram represents the proportion of apoptotic cells. Results with triplicate measurements are displayed as mean ± S.D.. *P < 0.05 vs Control group, #P < 0.05 vs. 20 μM NP65 group.

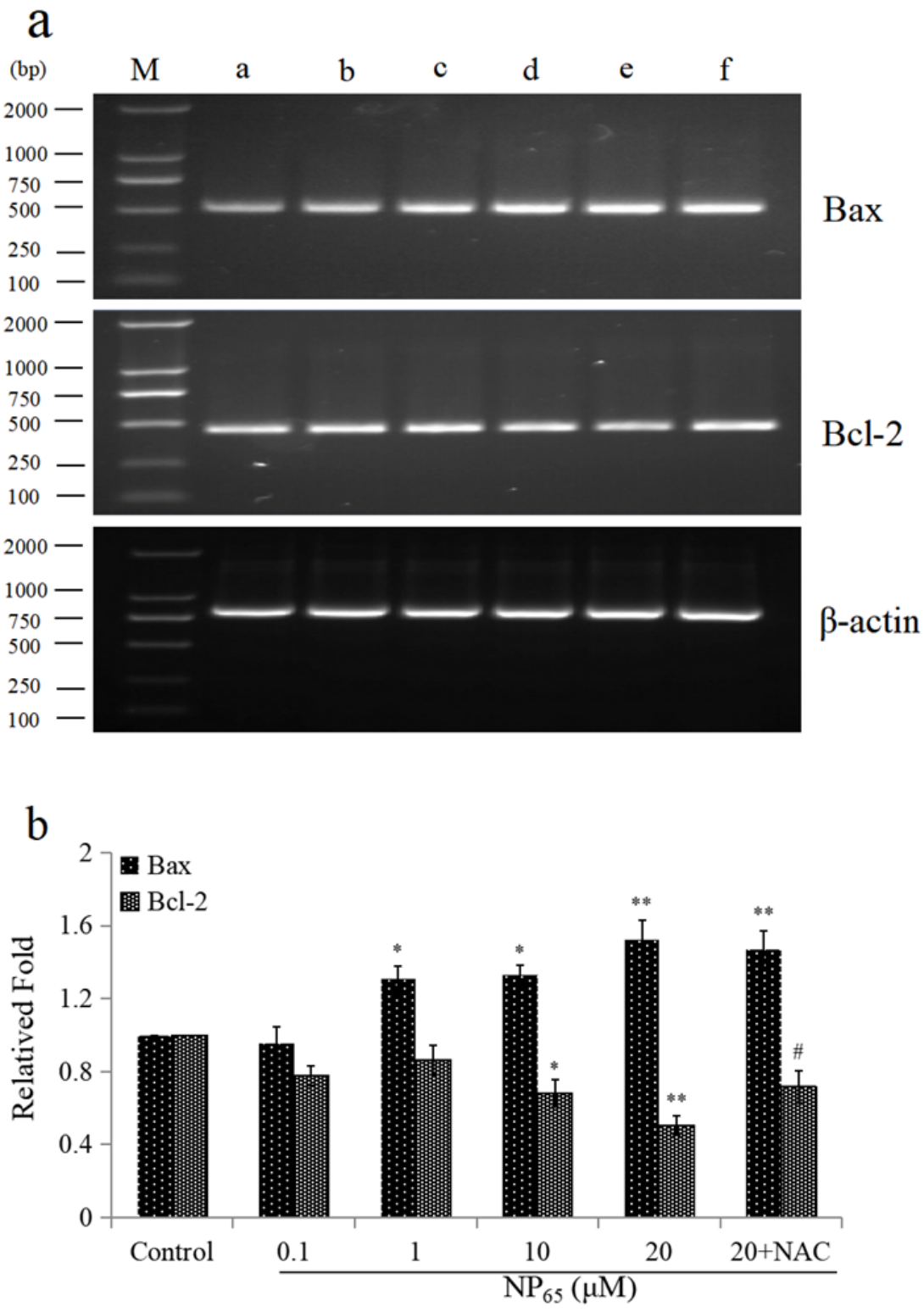

Figure 4

Expression levels of Bcl-2 as well as Bax mRNA in NP65 treated TM4 cells. (a) target genes and β-actin were detected by RT-PCR. (b) Histogram shows quantification of Bax as well as Bcl-2 mRNA levels (define Control cells/β-actin level as 1). Values with triplicate measurements are shown as mean ± S.D. *P < 0.05 and **P < 0.01 vs. Control group, #P < 0.05 vs. 20 μM NP65 group. M: Marker, a: Control, b: 0.1

μM NP65 group, c: 1 μM NP65 group, d: 10 μM NP65 group, e: 20 μM NP65 group, f: 20 μM NP65+NAC group.

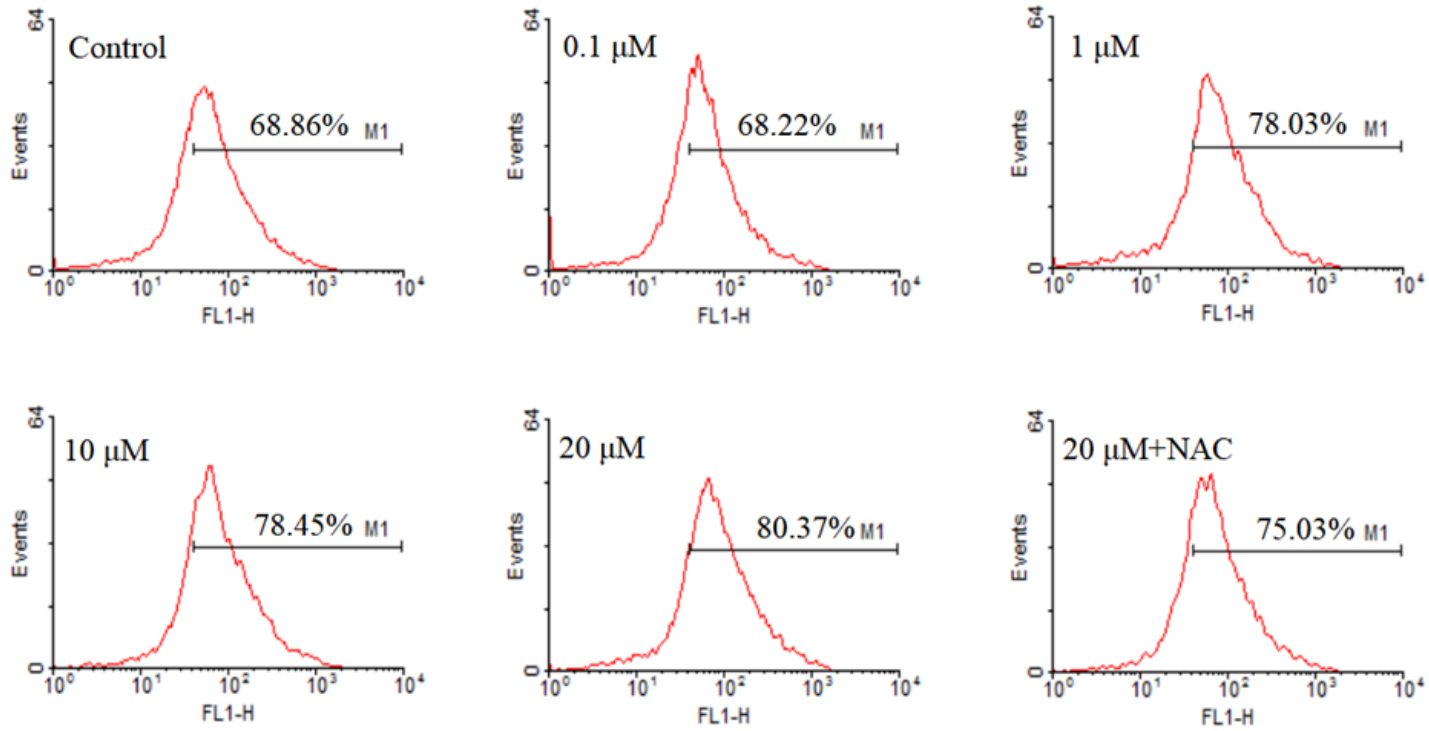

Figure 5

Release of Ca2+ in NP65 treated TM4 cells. The release of Ca2+ was analyzed by flow cytometry with fluorescent probe of Fluo-3/AM. Each sample contains 20,000 cells.

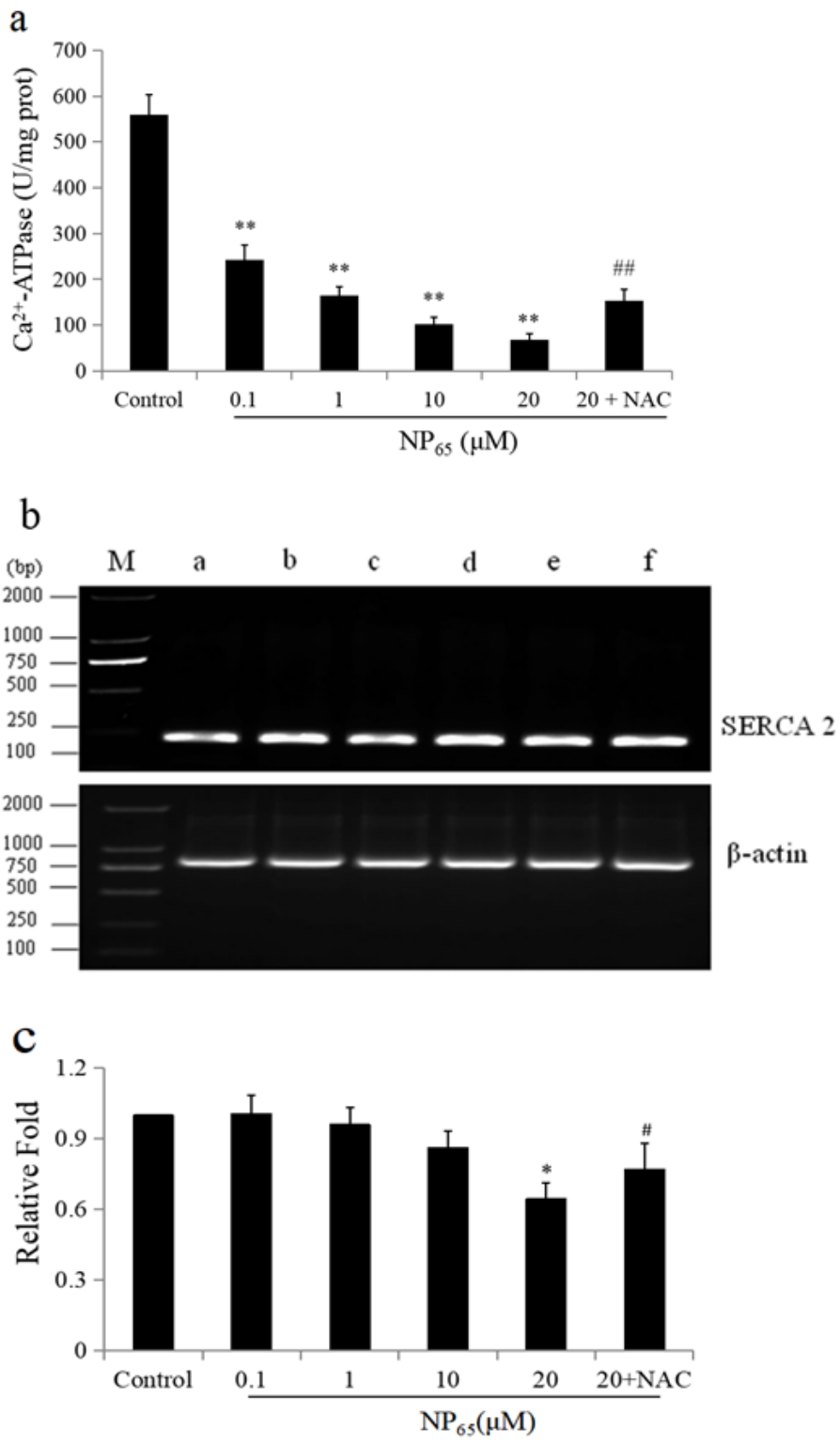

Figure 6

Effects of NP65 on the concentration of Ca2+-ATPase and expression of SERCA 2 mRNA in TM4 cells. (a) Influence of NP65 on Ca2+-ATPase concentration in TM4 cells. (b) SERCA 2 gene and β-actin were detected by RT-PCR. (c) Histogram shows quantification of expression level of SERCA 2 mRNA (define Control cells/β-actin level as 1). Values are shown as mean ± S.D. with triplicate measurements. *P < 0.05 and **P < 0.01 vs. Control group, #P < 0.05, ##P < 0.01 vs. 20 μM NP65 group. M: Marker, a: Control, b:

0.1 μM NP65 group, c: 1 μM NP65 group, d: 10 μM NP65 group, e: 20 μM NP65 group, f: 20 μM NP65+NAC group.

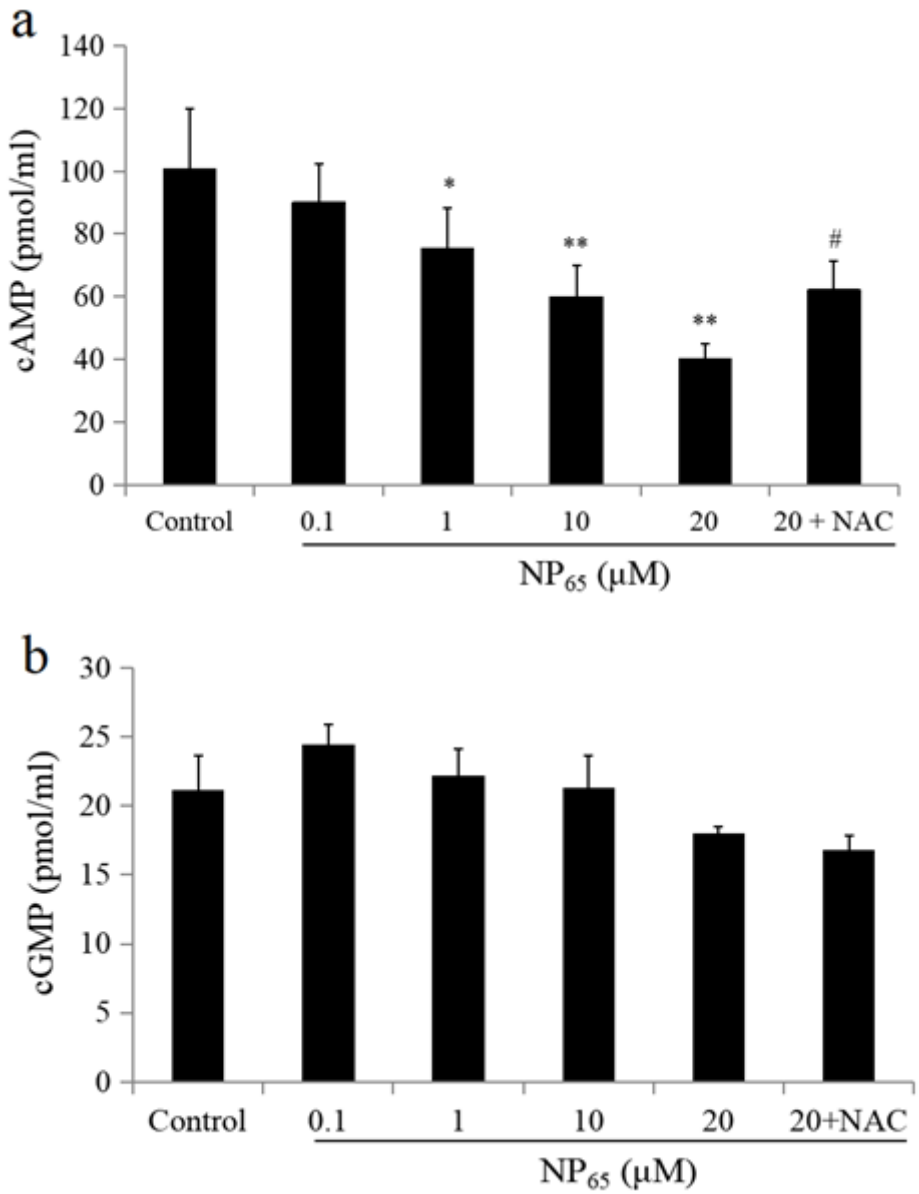

Figure 7

Effects of NP65 on cAMP and cGMP activities in TM4 cells. (a) Influence of NP65 on cAMP activity in TM4 cells. (b) Impact of NP65 on cGMP activity in TM4 cells. Values with triplicate measurements are presented as mean ± S.D. *P < 0.05 and **P < 0.01 vs. Control group, #P < 0.05 vs. 20 μM NP65 group .