Toxicology Letters 208 (2012) 275–285

Contents lists available at SciVerse ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Involvement of oxidative stress-induced ERK/JNK activation in the Cu^{2+}/pyrrolidine dithiocarbamate complex-triggered mitochondria-regulated apoptosis in pancreatic β-cells

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ARTICLE INFO

Article history:
Received 29 June 2011
Received in revised form 29 October 2011
Accepted 31 October 2011
Available online 7 November 2011

Keywords:
Copper (Cu^{2+})
PDTC
Pancreatic β-cell
Oxidative stress
Apoptosis
ERK1/2
JNK

ABSTRACT

Oxidative stress was demonstrated to promote the progression of diabetes mellitus (DM). It has been suggested that copper may play a specific role in the progression and pathogenesis of DM. Pyrrolidine dithiocarbamate (PDTC), a widely apply to the medicine, was known to be capable of enhancing copper accumulation. In this study, we investigated the effect of submicromolar-concentration Cu^{2+}/PDTC complex on pancreatic β-cell damage and evaluated the role of oxidative stress in this effect. $CuCl_2$ (0.01–300 μM) did not affect the cell viability in β-cell line RIN-m5F cells. However, combination of $CuCl_2$ (0.5 μM) and PDTC (0.3 μM) markedly reduced RIN-m5F cell viability. Cu^{2+}/PDTC complex could also increase the LPO and decrease the intracellular reduced GSH levels, and display several features of apoptosis signals including: increase in sub-G1 cell population, annexin-V binding, and caspase-3 activity, mitochondrial dysfunctions, and the activation of PARP and caspase cascades, which accompanied with the marked increase the intracellular Cu^{2+} levels. These apoptotic-related responses of Cu^{2+}/PDTC complex-induced could be effectively prevented by antioxidant *N*-acetylcysteine (NAC). Furthermore, Cu^{2+}/PDTC complex was capable of increasing the phosphorylations of ERK1/2 and JNK, and its upstream kinase MEK1/2 and MKK4, which could be reversed by NAC. Transfection with ERK2- and JNK-specific si-RNA and specific inhibitors SP600125 and PD98059 could inhibit ERK1/2 and JNK activation and attenuate MMP loss and caspase-3 activity induced by the Cu^{2+}/PDTC complex. Taken together, these results are the first report to demonstrate that the Cu^{2+}/PDTC complex triggers a mitochondria-regulated apoptosis via an oxidative stress-induced ERK/JNK activation-related pathway in pancreatic β-cells.

1. Introduction

The prevalence of diabetes mellitus (DM) is an increasingly global problem. From the multivariable DM risk score, the number of adults at high risk of developing DM has risen from 38.4 million in 1991 to 49.9 million in 2000 in the United States, and the total number of worldwide DM cases is projected to reach to 366 million in 2030 (Mainous et al., 2007; Wild et al., 2004). Uncontrolled industrious pollutions have resulted in a very wide segment of the human population or environment being exposed to toxic agents, which have the high risk and/or potential to cause or exacerbate the diseases development such as DM (Grandjean et al., 2011; Joshi and Shrestha, 2010; Meliker et al., 2007). Recently, the growing studies

Abbreviations: DM, diabetes mellitus; PDTC, pyrrolidine dithiocarbamate; LPO, lipid peroxidation; GSH, glutathione; PARP, poly (ADP-ribose) polymerase; NAC, *N*-acetylcysteine; MAPKs, mitogen-activated protein kinases; ERK, extracellular signal-regulated kinases; JNK, c-jun N-terminal kinases; MEK, mitogen-activated kinase/ERK kinase; MKK, mitogen-activated protein kinase kinase; si-RNA, small interference-RNA; MMP, mitochondrial membrane potential; ROS, reactive oxygen species.

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doi:10.1016/j.toxlet.2011.10.022

have indicated that toxic heavy metals such as mercury, arsenic, and cadmium are environmental risk factors for development of DM and/or pancreatic β-cell damage (Chen et al., 2009; Jomova and Valko, 2011; Yen et al., 2007).

Copper (Cu^{2+}) is an essential transition metal ion, which modulates the many biological activities and structural motifs of a multitude of proteins, including enzyme cofactors and other cellular constituents, leading to a potential for toxicants-induced damages (Stern, 2010; Valko et al., 2005). In general, Cu^{2+} can be absorbed from the human diet (~2 mg/day) or drinking water (2 mg/L) across the small intestine and contain 1 mg/L (about 16 μM) in serum levels (Graham et al., 1991; Sadhra et al., 2007). However, Cu^{2+} is also widespread present in the environment as an industrial pollutant, and an excess accumulation of Cu^{2+} may cause deleterious effects and/or diseases production in mammals (Bleackley and Macgillivray, 2011). It has been shown that Cu^{2+} is dangerous due to its ability to production of oxidative stress, and Cu^{2+} overload (1000 ppm through drinking water) causes the severe destruction of the anti-oxidant defense system (depressed superoxide dismutase and glutathione (GSH) levels and increase malondialdehyde concentration) in rat brain tissue leading to development of neurological disorders (Ozcelik and Uzun, 2009; Jomova and Valko, 2011). Recently, clinical studies have indicated that serum concentrations of Cu^{2+} are higher in diabetic patients than in the healthy population. This suggests that Cu^{2+} may play a specific role in the progression and pathogenesis of DM (Hasan, 2009; Serdar et al., 2009).

Dithiocarbamates (DCs), the molecules chemically defined by possession of a (R_1) (R_2) N–C(S)–SR_3 functional group, have widespread applications in agriculture (as pesticides), manufacturing, and in medicine for treatment of AIDS and as a chelating agent used to treat nickel intoxication (Schreck et al., 1992; Sunderman, 1981; WHO, 1988). Food crop residues, groundwater contamination, and industrial contact are potential routes for exposure of humans to DCs (Alexeeff et al., 1994; Vettorazzi et al., 1995). Pyrrolidine dithiocarbamate (PDTC) is a synthetic compound derivative from DC produced that has been largely used in the biochemical investigations (ranged from 2.5 μM to 100 μM) as an antioxidant, an NFγ-B and inflammatory cytokines inhibitor, the providing protection against many diseases (including AIDS, cancer, obstructive uropathy, etc.), and in chelating therapy for metals poisoning (Chen et al., 2008a; Chuang et al., 2009; Malaguarnera et al., 2003; Schreck et al., 1992; Sunderman, 1981). More importantly, the powerful toxicological effects of PDTC in enhancing the accumulation of Cu^{2+} in *in vitro* and *in vivo* systems have been reported which is capable of enhancing the cytotoxic effect of Cu^{2+} (at the physiological concentration) by about 700–1000-fold (Chen et al., 2000, 2008b; Valentine et al., 2006). Because Cu^{2+} is present throughout the environment, and PDTC has also many functions in the biological systems and applications in the clinical medicine, chronic exposure to a combination of Cu^{2+} and PDTC could cause severe damage in mammals. To our knowledge, there is no literature focused on clarifying the important role of the Cu^{2+}/PDTC complex in pancreatic β-cell damage and/or the progression of DM and its complications.

Reactive oxygen species (ROS) eliciting oxidative stress induces a wide variety of undesirable biological reactions that may lead to cell apoptosis and development of human diseases (Jomova and Valko, 2011; Lu et al., 2010). It has been recently shown that oxidative stress plays a crucial role in the progression of diabetes by causing pancreatic β-cell dysfunction and/or cell death through the action of cytokines and autoimmune attack in type 1 DM (Hotta et al., 2000; Newsholme et al., 2007). Interestingly, compared to other cells, pancreatic β-cells are more vulnerable to oxidative stress damage and have increased sensitivity to apoptosis (Chen et al., 2006; Kajimoto and Kaneto, 2004). Contrastingly, oxidative stress triggers many cellular responses by activation of protein phosphorylation pathways such as mitogen-activated protein kinases (MAPKs). The MAPKs are activated by regulation of many important cellular functions in mammals, including: survival, cell growth and proliferation, differentiation, and apoptosis (Chang and Karin, 2001; Ichijo et al., 1997). The growing numbers of studies have proposed that abnormalities or deviations from the controlled MAPKs signaling are implicated in the development of many human diseases, including DM (Gehart et al., 2010). Recently, oxidative stress-induced activation of MAPKs has been found to cause pancreatic β-cell dysfunction and death upon exposure to environmental stimuli or toxic chemicals (Henriksen et al., 2011; Hou et al., 2008). However, the molecular mechanisms underlying the toxicological effects of the Cu^{2+}/PDTC complex in pancreatic β-cells with respect to apoptosis are not well understood. In this study, we try to investigate the toxicological effects and possible mechanisms of Cu^{2+}/PDTC complex-induced pancreatic β-cell damage. Our results showed that low concentrations of Cu^{2+} (a physiological levels) plus PDTC exerted a significantly toxic effects to cause pancreatic β-cell death and that induced oxidative stress damage, mitochondrial dysfunction, exceed the intracellular Cu accumulation, and the activation of caspase cascades. The underlying toxicological mechanism in Cu^{2+}/PDTC complex-induced pancreatic β-cell apoptosis was through oxidative stress-induced ERK/JNK activation, which regulated the mitochondria-dependent apoptosis signaling pathway.

2. Materials and methods

2.1. Materials

All chemical reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA). Cell culture used plates and plastics were obtained from BD Falcon™ (BD Biosciences, CA, USA). RPMI-1640 medium, fetal bovine serum (FBS), antibiotics (penicillin and streptomycin), and Lipofectamine RNAi MAX were purchased from Gibco BRL, Life Technologies (Gibco/Invitrogen, Carlsbad, CA, USA). Lipid peroxidation (LPO) assay kit was obtained from Calbiochem (La Jolla, CA, USA). CaspACE™ fluorometric activity assay kit, AMV RTase (reverse transcriptase enzyme), RNasin (RNAase inhibitor) were purchases from Promega Corporation (Madison, WI, USA). Mouse- or rabbit-polyclonal antibodies specific for cytochrome *c*, JNK-1, ERK1/2, p38, MEK-1/2, MKK-4, Bcl-2, Bcl-$_X$L, Bax, Bak, β-actin, and secondary antibodies (goat anti-mouse or anti-rabbit IgG-conjugated horseradish peroxidase (HRP)) were purchased from Santa Cruz Biotechnology Inc., and PARP, caspase-3, caspase-7, caspase-9, phosphor-JNK, phosphor-ERK1/2, phosphor-p38, phosphor-MEK-1/2, phosphor-MEK-4, and ERK2- and JNK-specific small interference-RNA (si-RNA) were purchased from Cell Signaling Technology Inc.

2.2. Cell culture

RIN-m5F rat insulioma pancreatic β-cell line is a clone derived from the RIN-m rat islet cells (Bhathena et al., 1984). Cells were purchased from American Type Culture Collection (ATCC, CRL-11605; with Mycoplasma test: negative) and maintained in RPMI-1640 medium supplemented with 10% FBS and ntibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin) in a humidified chamber with a 5% CO_2–95% air mixture at 37 °C.

2.3. Cell viability assay

Cells were seeded at 2×10^4 cells/well in 96-well culture plates and allowed to adhere and recover overnight. The cells were changed to fresh media and then treated with $CuCl_2$ and PDTC alone or in combination (Cu^{2+}/PDTC complex) for 24 h. After incubation, the medium was aspirated and fresh medium containing 30 μL of 2 mg/mL 3-(4,5-dimethyl thiazol-2-yl-)-2,5-diphenyl tetrazolium bromide (MTT) was added. After 4 h, the medium was removed and replaced with blue formazan crystal dissolved in 100 μL dimethyl sulfoxide (DMSO). Following mixing, and an enzyme-linked immunosorbent assay reader (Bio-Rad, model 550, Hercules, CA, USA) was used for measurement the absorption at 570 nm.

2.4. LPO analysis

RIN-m5F cells were seeded at 1×10^6 cells/well in 6-well culture plates and allowed to adhere and recover overnight. The cells were changed to fresh media and treated with $CuCl_2$ and PDTC alone or in combination (Cu^{2+}/PDTC complex) in the absence or presence of 1 mM NAC (prior to incubate with Cu^{2+}/PDTC complex). After 24 h incubation, the cells were harvested and homogenized in ice-cold 20 mM Tris–HCl buffer, pH 7.4, containing 0.5 mM butylated hydroxytoluene to prevent

sample oxidation. LPO levels of equal volumes sample were measured the generation of malondiadehyde (MDA) by using a commercial LPO assay kit as described by Lu et al. (2010). The protein concentration of each sample was determined using the bicinchoninic acid protein assay kit (Pierce). LPO level was expressed as nanomoles (nmol) MDA per milligram protein and estimated from the standard curve.

2.5. Analysis of intracellular reduced GSH levels

Reduced GSH, a tripeptide (γ-gultamyl-cysteinylglycine), is the major free thiol in most living cells and the key antioxidant in mammalian cells. Reduced GSH levels of RIN-m5F cells were measured using a cell-permeant and GSH-specific and sensitive fluorescent dye: monochlorobimane (mBCl), which is widely used for quantifying the intracellular reduced GSH levels (Rice et al., 1986; Sanchez-Fernandez et al., 1997; Yen et al., 2007). In briefly, cells were seeded at 2×10^5 cells/well in 24-well culture plates and treated with Cu^{2+}/PDTC complex in the same manner as for LPO analysis. After 4 h incubation, the cells were washed twice with PBS, and then a new medium, which contained 60 μM mBCL (Sigma–Aldrich) was added and incubated for further 30 min at 37 °C. After loading the culture cells with mBCL, the supernantants were discarded, cells were washed twice with PBS, and the measurement the intracellular GSH levels were performed as described previously (Yen et al., 2007).

2.6. Flow cytometry analysis of apoptotic cells

2.6.1. Measurement of sub-G1 DNA content

RIN-m5F cells were seeded and treated with Cu^{2+}/PDTC complex in the same manner as for analysis of intracellular GSH contents. At the end of the treatment period (for 24 h), the cells were detached and washed with PBS, then resuspended in 1 mL of cold 70% (v/v) ethanol, and stored at 4 °C for 24 h. After they were washed with PBS, the cells were stained with propidium (PI, 50 μg/mL PI and 10 μg/mL ribonuclease (Rnase) in PBS) at 4 °C for 30 min in dark conditions. The cells were washed and subjected to flow cytometry analysis of DNA content (FACScalibur, Becton Dickinson). Nuclei displaying hypodiploid, sub-G1 DNA contents were identified as apoptotic. The sample of each group was collected more than 10,000 individual cells.

2.6.2. Determination of phosphatidyl serine externalization: annexin-V fluorescein isothiocyanate (FITC) binding assay

The externalization of phosphatidyl serine is an early event in apoptosis. Flow cytometry analysis was performed to determine this event using the annexin-V-FITC binding assay kit (BioVision). Cells were seeded and treated with Cu^{2+}/PDTC complex in the same manner as measurement of sub-G1 DNA content. Then, the cells were washed twice with PBS and stained with annexin-V-FITC for 20 min at room temperature. The cells were washed twice PBS, and the apoptosis level was determined by measuring the fluorescence of the cells by flow cytometry analysis (FACScalibur, Becton Dickinson). More than 10,000 individual cells were collected for each group.

2.7. Measurement of caspase-3 activity

Caspase-3 activity was determined using the CaspACE™ fluorometric activity assay kit as previously described (Lu et al., 2010). In brief, the cells were seeded at 2×10^5 cells/well in 24-well culture plates and treated with Cu^{2+}/PDTC complex in the absence or presence of 1 mM NAC or 20 μM Z-DEVD-FMK for 1 h (prior to incubate with Cu^{2+}/PDTC complex). After 24 h incubation, the cell lysates were incubated at 37 °C with 10 μM Ac-DEVD-AMC, a caspase-3/CPP32 substrate, for 1 h. The fluorescence of the cleaved substrate was measured by a spectrofluorometer (Spectramax, Molecular devices) with an excitation wavelength at 380 nm and an emission wavelength at 460 nm. Protein levels of cell lysate samples were determined using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA) with an absorption band of 570 nm to normalize the cell numbers between control and different drug-treated groups.

2.8. Determination of mitochondrial membrane potential (MMP)

MMP was analyzed using the fluorescent probe $DiOC_6$, with a positive charge of a mitochondria-specific fluorophore. Briefly, RIN-m5F cells were seeded and treated with Cu^{2+}/PDTC complex in the same manner as for analysis of intracellular GSH contents. After incubation, cells were harvested and loaded with 40 nM DiOC6 for 30 min and analyzed with FACScan flow cytometer (Becton Dickinson). The sample of each group was collected more than 10,000 individual cells.

2.9. Real-time quantitative reverse-transcribed polymerase chain reaction (RT-PCR) analysis

The expression of apoptosis-related genes was evaluated by real-time quantitative RT-PCR, as previously described (Lu et al., 2010). Briefly, intracellular total RNA was extracted from the cerebral cortex using RNeasy kits (Qiagen, Hilden, Germany) and reverse transcribed into cDNA using the AMV RTase (reverse transcriptase enzyme; Promega Corporation, Pty. Ltd.) according to the manufacture's instructions. Each sample (2 μL cDNA) was tested with Real-time Sybr Green PCR reagent (Invitrogen, USA) with mouse specific primers (as follows: Bcl-2, forward: 5′-CTTTGTGGAACTGTACGGCCCCAGCATGCG-3′ and reverse: 5′-ACAGCCTGCAGCTTTGTTTCATG-GTACATC-3′, Bax, forward: 5′-GGGAATTCTGGAGCTGCAGAGGATGATT-3′ and reverse: 5′-GCGGATCCAAGTTGC-CATCAGCAAACAT-3′, Bak, forward: 5′-TTTGGCTACCGTCTGGCC-3′ and reverse: 5′-GGCCCAACAGAACCACACC-3′ (Bozec et al., 2004), β-actin, forward: 5′-ATTGTAACCAACTGGGACG-3′ and reverse: 5′-TCTCCAGGGAGGAAGAGG-3′ (Liu et al., 2009) in a 25 μL reaction volume, and amplification was performed using an ABI StepOnePlus sequence detection system (PE, Applied Biosystems, CA, USA). Data analysis was performed using StepOne™ software (Version 2.1, Applied Biosystems). All amplification curves were analyzed with a normalized reporter (R_n: the ratio of the fluorescence emission intensity to the fluorescence signal of the passive reference dye) threshold of 0.2 to obtain the C_T values (threshold cycle). The reference control genes were measured with four replicates in each PCR run, and their average C_T was used for relative quantification analyses (the relative quantification method utilizing real-time PCR efficiencies (Pfaffl et al., 2002)). TF expression data were normalized by subtracting the mean of reference gene C_T value from their C_T value (ΔC_T). The fold change value was calculated using the expression $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T$ represents $\Delta C_{T\text{-condition of interest}} - \Delta C_{T\text{-control}}$. Prior to conducting statistical analyses, the fold change from the mean of the control group was calculated for each individual sample.

2.10. Western blot analysis

The cellular lysates were prepared and Western blotting was performed as previously described (Chen et al., 2006). In brief, equal amounts of proteins (50 μg per lane) was subjected to electrophoresis on 10% (w/v) SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked for 1 h in PBST (PBS, 0.05% Tween-20) containing 5% nonfat dry milk. After blocking, the membranes were incubated with mouse or rabbit anti-rat antibodies in 0.1% PBST (1:1000) for 1 h at room temperature. After they were washed in 0.1% PBST followed by two washes (15 min each), the blots were subsequently incubated with secondary antibody (1:1000) for 1 h. The antibody-reactive bands were revealed by enhanced chemiluminescence reagents (Perkin-Elmer™, Life Sciences) and were exposed on the Fuji radiographic film.

2.11. Determination of intracellular copper contents

RIN-m5F cells were seeded at 1×10^6 cells/well in 6-well culture plates and treated with Cu^{2+}/PDTC complex in the same manner as for analysis of intracellular GSH contents. After incubation, cells were harvested and washed with PBS three times followed by addition of 0.1% nitric acid, and the mixture was vortexed and frozen at −20 °C for 2 h or overnight. Tubes were thawed at 37 °C for 20 min and centrifuged at $1000 \times g$ at 4 °C for 10 min. The supernatant (the intracellular copper levels) was determined by inductively coupled plasma mass spectrometry (ICP-MS). The detection limit for copper was ~0.08 ppb (μg/L).

2.12. Plasmid and si-RNA transfection

Specific si-RNA against rat p42 MAPK (ERK2) and JNK si-RNA were purchased commercially from Cell Signaling Technology. RIN-m5F cells were seeded in 6- or 24-well culture plates and transfected with the si-RNA using Lipofectamine RNAi MAX (Gibco/Invitrogen) according to the manufacturer's instructions. Cellular levels of the proteins specific for the si-RNA transfection were checked by Western blot, and all experiments were performed at 24 h after transfection.

2.13. Statistical analysis

Data are presented as means ± standard deviations (S.D.). The significance of difference was evaluated by the Student's *t*-test. When more than one group was compared with one control, significance was evaluated according to one-way analysis of variance (ANOVA) was used for analysis, and the Duncans's post hoc test was applied to identify group differences. The *p* value less than 0.05 was considered to be significant. The statistical package SPSS, version 11.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis.

3. Results

3.1. Effects of the Cu^{2+}/PDTC complex on cell viability and oxidative stress damage in RIN-m5F cells

To investigate Cu^{2+}/PDTC complex-induced pancreatic β-cell cytotoxicity, we first examined the effects on cell viability of $CuCl_2$ (Cu^{2+}) and PDTC alone or in combination with each other in the form of the Cu^{2+}/PDTC complex using MTT assay in RIN-m5F cells (the rat pancreatic β-cell-derived cell line). Treatment of RIN-m5F

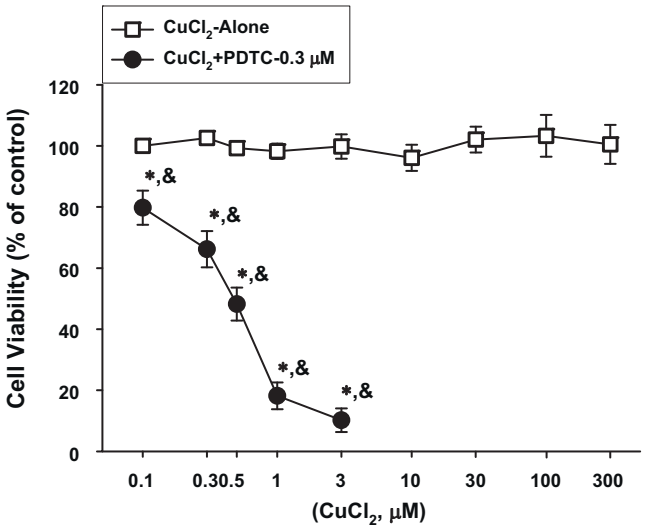

Fig. 1. The cytotoxic effects of $CuCl_2$ and Cu^{2+}/pyrrolidine dithiocarbamate (PDTC) complex in pancreatic β-cells. RIN-m5F cells were treated with $CuCl_2$ (0–300 μM) alone or combination with 0.3 μM PDTC (Cu^{2+}/PDTC complex) for 24 h, and cell viability was determined by MTT assay. All data are presented as mean ± S.D. for four independent experiments with triplicate determinations. $^{*}p < 0.05$ as compared with untreated control; $^{\&}p < 0.05$ as compared with Cu^{2+} group.

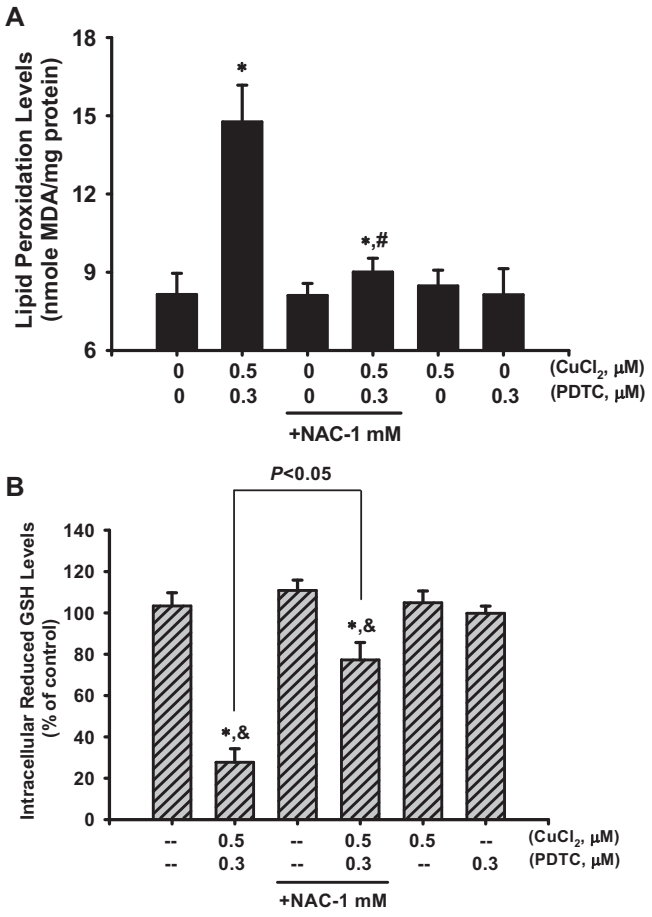

Fig. 2. Effects of the Cu^{2+}/PDTC complex on LPO production and intracellular GSH levels in pancreatic β-cells. RIN-m5F cells were treated with 0.5 μM $CuCl_2$ and 0.3 μM PDTC alone or in combination (Cu^{2+}/PDTC complex) in the absence or presence of 1 mM NAC for 1 h (prior to incubate with Cu^{2+}/PDTC complex). (A) Oxidative damage to membrane lipid (LPO) was detected the levels of MDA (for 24 h), and (B) intracellular GSH levels were determined using a sensitive fluorescent probe: mBCl (for 4 h) as described in Section 2. $^{*}p < 0.05$ as compared with vehicle control; $^{\&}p < 0.05$ as compared with Cu^{2+} group; $^{\#}p < 0.05$ as compared with Cu^{2+}/PDTC complex group.

cells with high concentrations of Cu^{2+} (0.1–300 μM) did not produce a change in cell viability (Fig. 1) and 0.3 μM PDTC was found to be essentially nontoxic (99.53 ± 2.74% of control). However, low concentrations of Cu^{2+} (0.1–3 μM) in combination with 0.3 μM PDTC significantly potentiated the decrease in the number of viable cells over a 24 h period, and the LD_{50} was determined to be approximately 0.5 μM Cu^{2+} plus 0.3 μM PDTC (Cu^{2+}/PDTC complex).

Next, to examine the effects of Cu^{2+}, PDTC, and the Cu^{2+}/PDTC complex on oxidative stress damage, we treated RIN-m5F cells with Cu^{2+} (0.5 μM) and PDTC (0.3 μM) alone or in combination (Cu^{2+}/PDTC complex), and measured LPO production (an index of oxidative stress damage) and intracellular reduced GSH levels. As shown in Fig. 2A, the exposure of cells to the Cu^{2+}/PDTC complex, not Cu^{2+} or PDTC alone, for 24 h significantly induced an increase in MDA levels in the cell membrane (0.5 μM Cu^{2+}: 8.47 ± 0.20; 0.3 μM PDTC: 8.13 ± 0.33; Cu^{2+}/PDTC complex: 14.76 ± 0.44; control: 8.14 ± 0.25 nmol MDA/mg protein). Furthermore, the levels of intracellular reduced GSH were markedly decreased after treatment with the Cu^{2+}/PDTC complex for 4 h (Cu^{2+}/PDTC complex: 27.76 ± 3.25% of control) (Fig. 2B). *N*-acetylcysteine (NAC, 1 mM), an antioxidant and GSH precursor, could effectively reduce Cu^{2+}/PDTC complex-induced oxidative stress damage (Fig. 2). The decrease in cell viability induced by the Cu^{2+}/PDTC complex in RIN-m5F cells could also be reversed by NAC (Cu^{2+}/PDTC complex: 39.90 ± 0.72; with 1 mM NAC: 101.68 ± 4.29% of control, $n = 6$, $p < 0.05$ as compared with the Cu^{2+}/PDTC complex alone).

3.2. Cu^{2+}/PDTC complex caused cell death is mediated by a mitochondria-dependent apoptotic signaling pathway in RIN-m5F cells

To investigate whether Cu^{2+}/PDTC complex-induced pancreatic β-cell death occurs through an apoptosis, we first analyzed the sub-G1 hypodiploid cell population (using a fluorescent probe: PI) and the externalization of phosphatidylserine (PS) (using an annexin-V-FITC binding assay) by flow cytometry, which are the hallmarks of apoptosis. RIN-m5F cells treated with the Cu^{2+}/PDTC complex for 24 h exhibited a significant increase in the sub-G1 hypodiploid cell population (Fig. 3A). It was also remarkably triggered the annexin-V-binding fluorescence intensity (Fig. 3B). Moreover, caspase-3 activity (an integral step in the majority of apoptotic events) was markedly induced after treatment of RIN-m5F cells with the Cu^{2+}/PDTC complex, which could be reversed by pre-treatment with a caspase-3 inhibitor (Z-DEVD-FMK, 20 μM) (Fig. 3C). These results indicate that exposure of RIN-m5F cells to the Cu^{2+}/PDTC complex could induce apoptosis.

Next, to determine whether apoptosis induced by the Cu^{2+}/PDTC complex was mediated through a mitochondria-dependent pathway, we analyzed the effect of the Cu^{2+}/PDTC complex on the MMP using flow cytometry with a cationic dye $DiOC_6$. As shown in Fig. 4A, treatment of RIN-m5F cells with the Cu^{2+}/PDTC complex effectively induced MMP loss in a time-dependent manner (for 6 h, 82.92 ± 5.59; for 24 h, 52.05 ± 1.89% of control). We also investigated the release of cytochrome c from the mitochondria into the cytosol in RIN-m5F cells treated with the Cu^{2+}/PDTC complex. Treatment of cells for 24 h significantly increased the cytochrome *c* levels in the cytosolic fraction (Fig. 4B). We also investigated the change in mRNA and protein expressions of Bcl-2 family members. Treatment of cells with the Cu^{2+}/PDTC complex for 24 h markedly induced a decrease in *Bcl-2* (anti-apoptotic) and an increase in *Bax and Bak* (pro-apoptotic) mRNA levels (Fig. 4C – *a*), which accorded with proteins expression (Fig. 4C – *b*) and led to a shift in the anti-apoptotic/pro-apoptotic expression

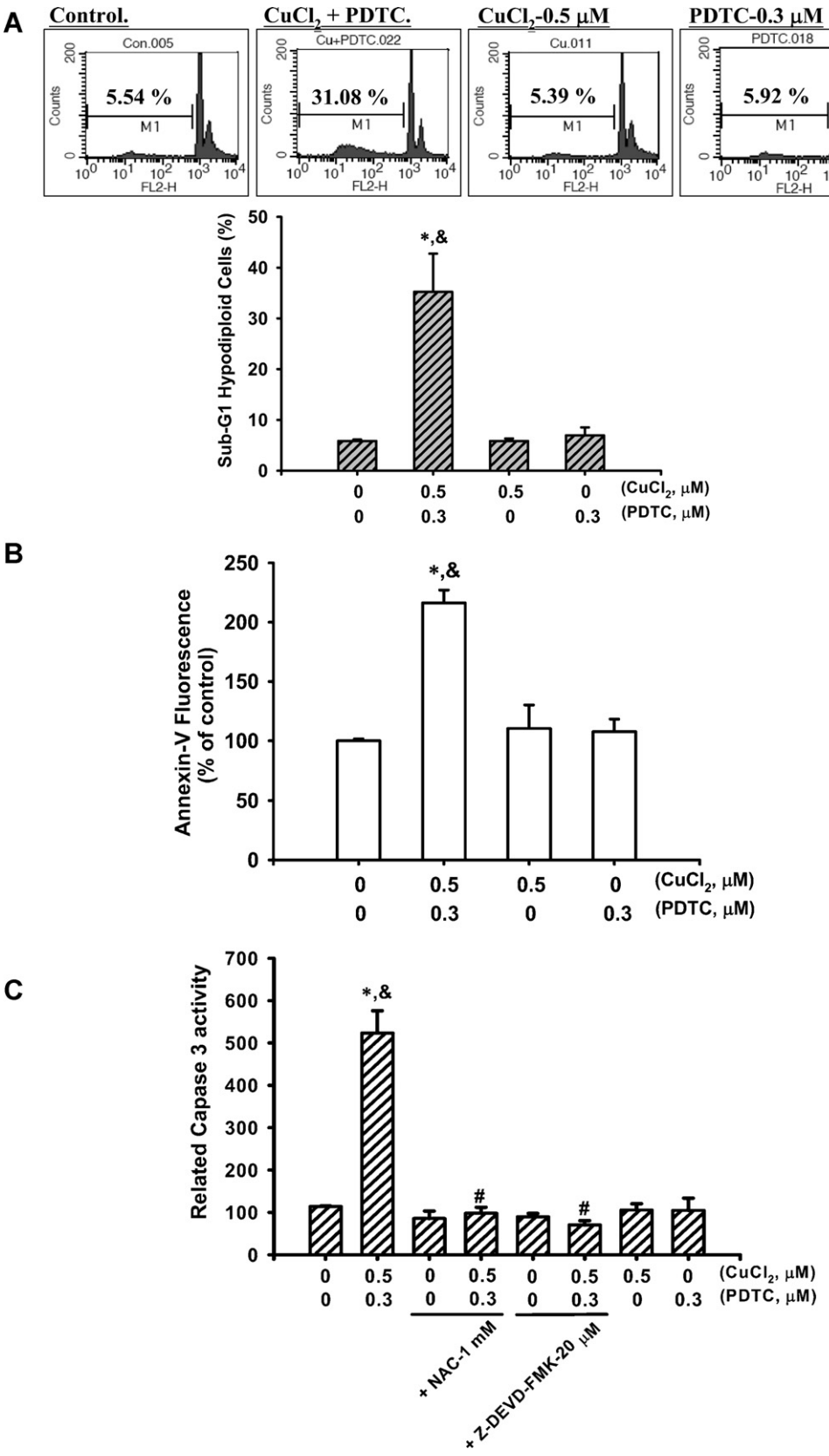

Fig. 3. Cu^{2+}/PDTC complex induced apoptosis in pancreatic β-cells. RIN-m5F cells were treated with 0.5 μM $CuCl_2$ and 0.3 μM PDTC alone or in combination (Cu^{2+}/PDTC complex) for 24 h, and (A) sub-G1 hypodiploid cell population (genomic DNA fragmentation), and (B) phosphatidylserine exposure on the outer cellular membrane leaflets by staining with annexin-V-FITC fluorescent probe were measured using flow cytometry analysis. In addition, the effect of NAC (1 mM) or Z-DEVD-FMK (20 μM) on Cu^{2+}/PDTC complex-induced caspase 3 activity was examined by CaspACE™ fluorometric assay kit as described in Section 2. $^{*}p < 0.05$ as compared with vehicle control; $^{\&}p < 0.05$ as compared with Cu^{2+} group; $^{\#}p < 0.05$ as compared with Cu^{2+}/PDTC complex group.

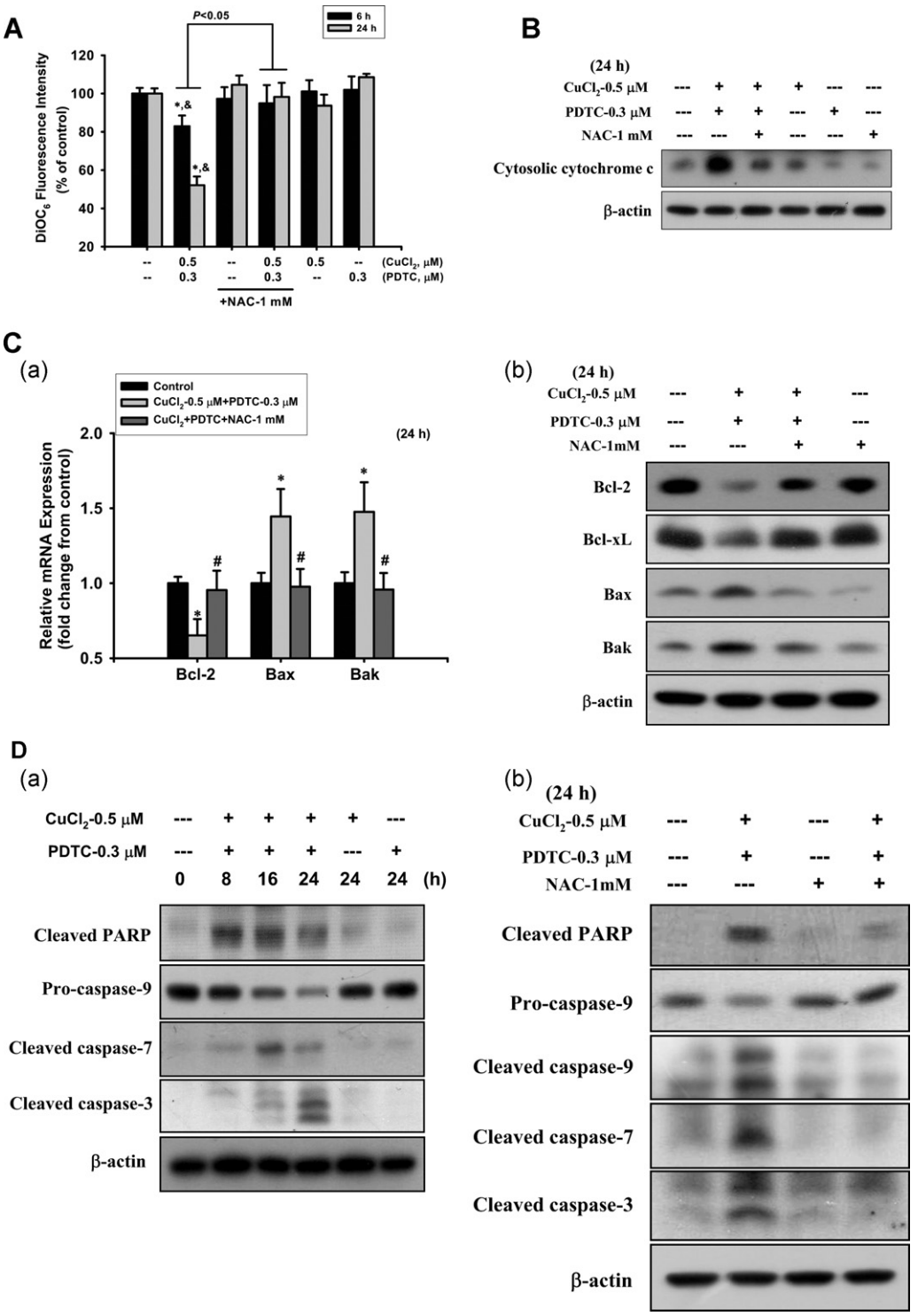

Fig. 4. Changed in mitochondrial membrane potential (MMP), cytochrome c release, Bcl-2 families mRNA expressions, and PARP and caspase cascades activations in the Cu^{2+}/PDTC complex treated pancreatic β-cells. RIN-m5F cells were treated with 0.5 μM $CuCl_2$ and 0.3 μM PDTC alone or in combination for different time intervals in the absence or presence of 1 mM NAC for 1 h (prior to incubate with Cu^{2+}/PDTC complex). MMP depolarization was determined by flow cytometry (A); cytosolic cytochrome c release was examined by Western blot analysis (B); the expressions of anti-apoptotic (*Bcl-2*) and pro-apoptotic (*Bax and Bak*) genes (*a*) and proteins (*b*) were analyzed by real-time quantitative RT-PCR and Western blot, respectively (C); and PARP cleavage and caspase-3, -7, and -9 expressions were examined by Western blot analysis (D) as described in Section 2. Data in A and C are presented as mean ± S.D. for four independent experiments with triplicate determination. $^{*}p < 0.05$ as compared with vehicle control; $^{\&}p < 0.05$ as compared with Cu^{2+} group; $^{\#}p < 0.05$ as compared with Cu^{2+}/PDTC complex group.

ratio toward an apoptosis-associated state. Furthermore, to further evaluate the apoptotic signaling induced by the Cu^{2+}/PDTC complex-induced, the activation of PARP and caspase cascades (the crucial biomarkers for apoptosis that represent both the initiation and execution of cell death) were examined. As shown in Fig. 4D, the treatment of RIN-m5F cells with the Cu^{2+}/PDTC complex for 8–24 h caused a marked increase in the active form of PARP fragment. We also observed the

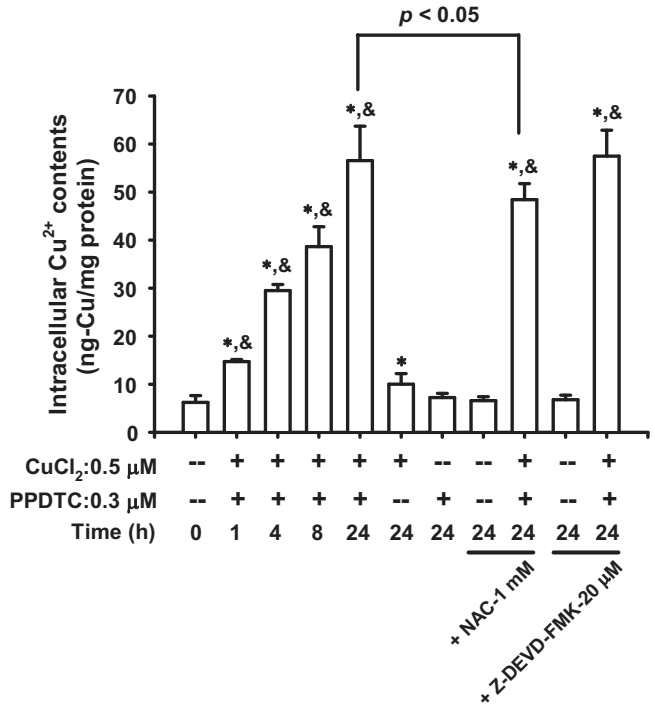

Fig. 5. Intracellular copper content in pancreatic β-cells treated with Cu^{2+}/PDTC complex. RIN-m5F cells were treated with the Cu^{2+}/PDTC complex (0.5 μM $CuCl_2$ combined with 0.3 μM PDTC) for 0–24 h. In addition, the cells by pre-treated with 1 mM NAC or 20 μM Z-DEVD-FMK for 1 h and then incubated with Cu^{2+}/PDTC complex for 24 h were measured. Intracellular copper content was detected by inductively coupled plasma mass spectrometry (ICP-mass) as described in Section 2. Data are presented as mean ± S.D. for four independent experiments with triplicate determination. $^{*}p < 0.05$ as compared with vehicle control; $^{\&}p < 0.05$ as compared with Cu^{2+} group.

significant increase in the activation of caspase-3, caspase-7, and upstream caspase-9 in cells treated with the Cu^{2+}/PDTC complex (Figure 4D – *a*). These Cu^{2+}/PDTC complex-induced apoptosis-related responses (including: loss of MMP, increased the cytochrome *c* release, changed in anti-apoptotic/pro-apoptotic Bcl-2 members expressions, and caspase cascades activations) could be effectively reverse by pre-treated with NAC (1 mM) (Figs. 3C, 4A–C, and D – *b*).

On the other hand, after treatment of RIN-m5F cells with Cu^{2+}/PDTC complex for 1 h, a remarkable increase in the concentration of intracellular Cu was observed, and the levels of Cu were more significantly increased after a 24 h treatment period (Cu^{2+}/PDTC complex: 56.56 ± 7.16 vs. control: 6.22 ± 1.41 ng Cu/mg protein) (Fig. 5). It was also found that the effect of Cu^{2+}/PDTC complex-induced the dramatic accumulation of intracellular Cu levels could not be changed by pre-treated with Z-DEVD-FMK (caspase-3 inhibitor, for 1 h). Moreover, pre-treatment with NAC for 1 h (prior to Cu^{2+}/PDTC complex) could attenuate Cu^{2+}/PDTC complex-induced the intracellular Cu accumulation, but that was a little efficacy (with NAC: 48.42 ± 3.34 ng Cu/mg protein (the decrease in 14.38% of Cu^{2+}/PDTC complex group), $p < 0.05$). These results indicate that the treatment with low concentration of Cu^{2+} plus PDTC could cause significant amounts of Cu^{2+} to enter the cells, which accompanied by Cu^{2+}/PDTC complex-induced apoptotic signals in pancreatic β-cells.

3.3. ERK1/2 and JNK signaling played important roles in Cu^{2+}/PDTC complex-induced apoptosis in RIN-m5F cells

MAPKs play important roles in many apoptosis signaling, and oxidative stress is known to activate members of the MAPK families (ERK, JNK, and p38) by protein phosphorylation. To elucidate the link between oxidative stress damage induced by the Cu^{2+}/PDTC complex and the subsequent activation of the MAPKs, we investigated the effect of treatment of RIN-m5F cells with the Cu^{2+}/PDTC complex on the phosphorylation of ERK1/2, JNK, and p38-MAPK by Western blot. As shown in Fig. 6, the levels of protein phosphorylation of ERK1/2 and JNK, but not that of p38, were significantly increased (for 15 min and 30 min). Moreover, upstream MEK1/2 and MKK4 were also markedly activated after treatment of cells with Cu^{2+}/PDTC complex (for 5–30 min). These responses induced by the Cu^{2+}/PDTC complex could be abrogated by pre-treatment with NAC (1 mM) (Fig. 6).

To further evaluate the relationship between induction of the apoptotic signaling pathway by the Cu^{2+}/PDTC complex in pancreatic β-cells and MAPKs activation, the cells were pre-treated with the specific ERK inhibitor PD98059, JNK inhibitor SP600125, or p38 inhibitor SB203580 for 1 h and then incubated with the Cu^{2+}/PDTC complex. It was found that the Cu^{2+}/PDTC complex-induced phosphorylation of ERK1/2 and JNK, but not that of p38, in RIN-m5F cells were effectively reversed by their respective inhibitors (Fig. 7A–C). Loss of MMP and the increase in caspase-3 activity induced by treatment of the cells with the Cu^{2+}/PDTC complex could also be prevented (Fig. 7D – *c* and D – *d*). Furthermore, transfection of RIN-m5F cells with ERK2- and JNK-specific si-RNA, respectively, was found to significantly attenuate the phosphorylation of ERK1/2 and JNK (Fig. 7D – *a*, and D – *b*), the depolarization of MMP (Fig. 7D – *c*), and the increase in caspase-3 activity (Fig. 7D – *d*), which occurred in cells treated with the Cu^{2+}/PDTC complex. These results indicate that oxidative stress-mediated ERK1/2 and JNK activation plays a crucial role in Cu^{2+}/PDTC complex-induced pancreatic β-cell apoptosis.

4. Discussion

DM is growing a global health problem with and becoming a serious threat to human health. Many epidemiological studies have reported that exposure to toxic metals (such as As, Hg, Cu, etc.) are associated with increased rates of serious health problems or progressive of diseases development such as DM (Chen et al., 2009; Gallagher et al., 2011; Jomova and Valko, 2011; Joshi and Shrestha, 2010). Cu^{2+} is an essential trace element and its deficiency or excess cause to several diseases. It has been indicated that exposure to overload Cu^{2+} induces cell death (≥10 μM) through the formation of ROS and the impact of ERK and p38 signaling *in vitro*, and causes hepatic dysfunction (100 mg/L, in water) by produced oxidative stress damage and changed in lipid prolife *in vivo* (Nawaz et al., 2006; Ozcelik et al., 2003). Recently, a growing number of studies have examined the relationship between Cu^{2+} levels in the serum and development of DM. The results indicated that the levels of Cu^{2+} in the serum of diabetic patients are higher than those in healthy individuals (about: 31.5 μM vs. 21.3 μM) (Flores et al., 2011; Kazi et al., 2008; Serdar et al., 2009). On the other hand, PDTC, a thiol compound derived from DCs, has widespread applications in many biological reactions and functions and in clinical medicine *in vitro* and *in vivo* (Chuang et al., 2009; Furuta et al., 2002; Malaguarnera et al., 2003). However, it has been shown that PDTC can induce or enhance cell damage upon exposure to physiological concentrations of Cu^{2+} (1–20 μM) in various cells (including rat cortical astrocytes, leukemia cells, and breast cancer cells), which is sequentially activated JNK and/or transcriptional factors (NFκ-B, AP-1, and p53) or proteasome inhibitor leading to cell death and accompanied by marked accumulation of intracellular Cu^{2+} accumulation (Chen et al., 2000, 2008a,b; Daniel et al., 2005; Furuta et al., 2002), and increase the production of LPO causing functional and morphological degeneration of peripheral

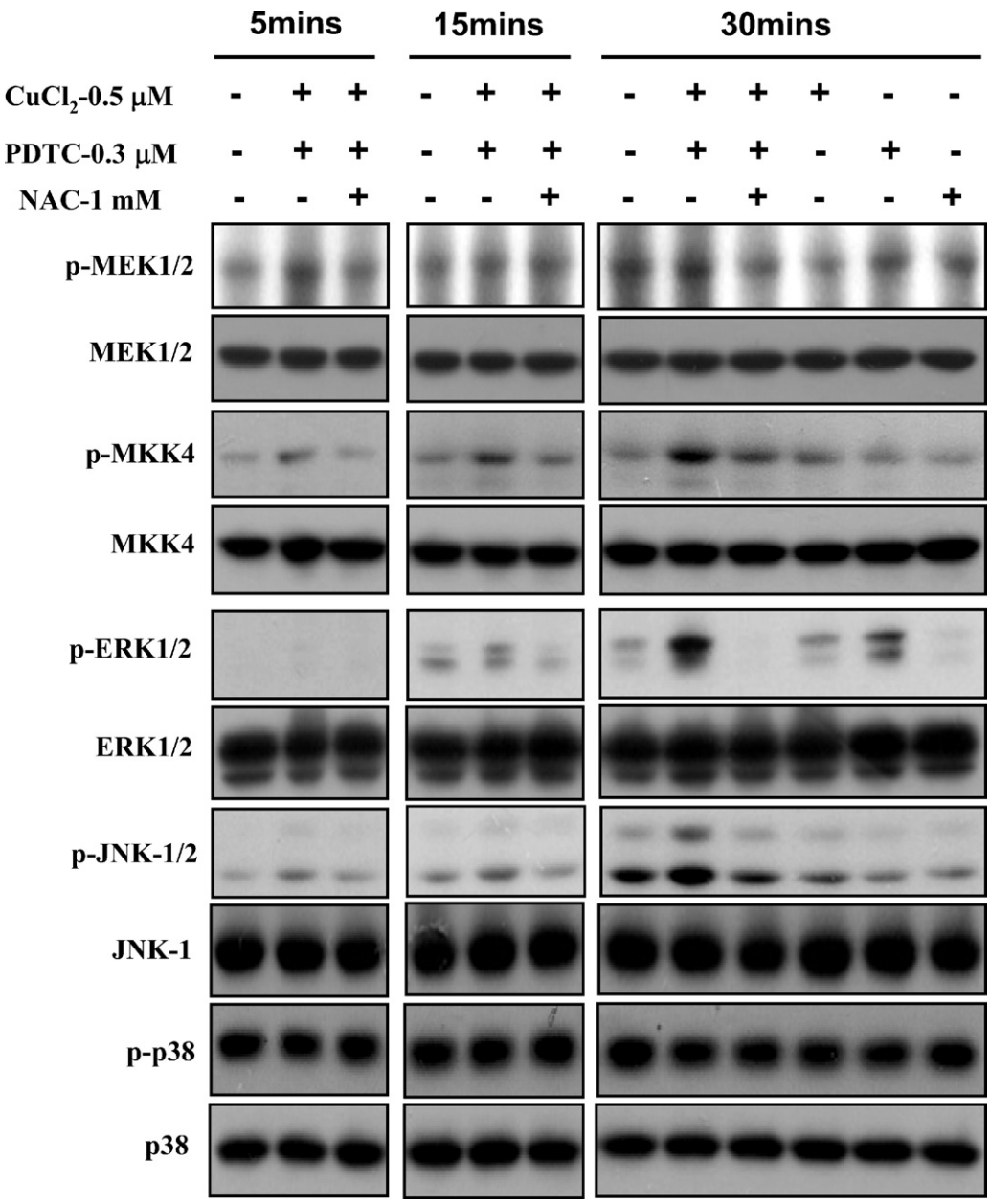

Fig. 6. Effects of mitogen-activated protein kinases (MAPKs) activation in pancreatic β-cells treated with the Cu^{2+}/PDTC complex. RIN-m5F cells were treated with or without Cu^{2+}/PDTC complex (0.5 μM $CuCl_2$ combined with 0.3 μM PDTC) for 5, 15, and 30 min, and the levels of phosphorylation of ERK1/2, JNK, and p38-MAPK, and upstream MEK1/2 and MKK4 proteins were analyzed by Western blot. Additional treatment included pre-incubation with NAC (1 mM) for 1 h prior to Cu^{2+}/PDTC complex for 30 min.

nerves (Calviello et al., 2005; Valentine et al., 2006). Despite the fact that previous studies have indicated that PDTC can increase the deleterious effects of Cu^{2+} by inducing oxidative stress damage and subsequently causing cell death in mammalian cells, the precise mechanisms underlying the induction of pancreatic β-cell death by the Cu^{2+}/PDTC complex, to the best of our knowledge, have not been clarified. The main findings of this study were that exposure of pancreatic β-cells to submicromolar concentrations of Cu^{2+} (0.5 μM) in the presence of PDTC (0.3 μM) caused a significant decrease in cell viability, oxidative stress damage (LPO production of cell membrane and intracellular reduced GSH depletion), and several characteristics of apoptosis (including: increase in gemonic DNA fragmentation, mitochondrial dysfunction, and PARP and caspase cascades activation), which was accompanied by gradual accumulation of intracellular Cu^{2+} content in a time-dependent manner over a period ranging from 1 to 24 h. Pre-treatment with antioxidant NAC could effectively reverse the Cu^{2+}/PDTC complex-induced apoptosis-related responses. These findings indicate that the Cu^{2+}/PDTC complex can enter the intracellular fraction and induce oxidative stress, which involves in pancreatic β-cell apoptosis induced by the complex.

It has been shown that oxidative stress plays an important role in inducing pancreatic β-cell damage and pathogenesis of DM that may be due to the excessive levels of mitochondrial ROS generation and the presence of fewer antioxidant enzymes in pancreatic β-cells (Kajimoto and Kaneto, 2004; Newsholme et al., 2007). Mitochondria are very sensitive to oxidative stress damage by various environmental stimuli, and excess of ROS induce mitochondrial dysfunction has been demonstrated and affected to cause pancreatic β-cell apoptosis leading to type-1 DM (Lee et al., 2009; Luft, 1994). Once apoptosis is initiated, mitochondrial transmembrane potential ($\Delta\Psi_m$) is disrupted (depolarization), and that

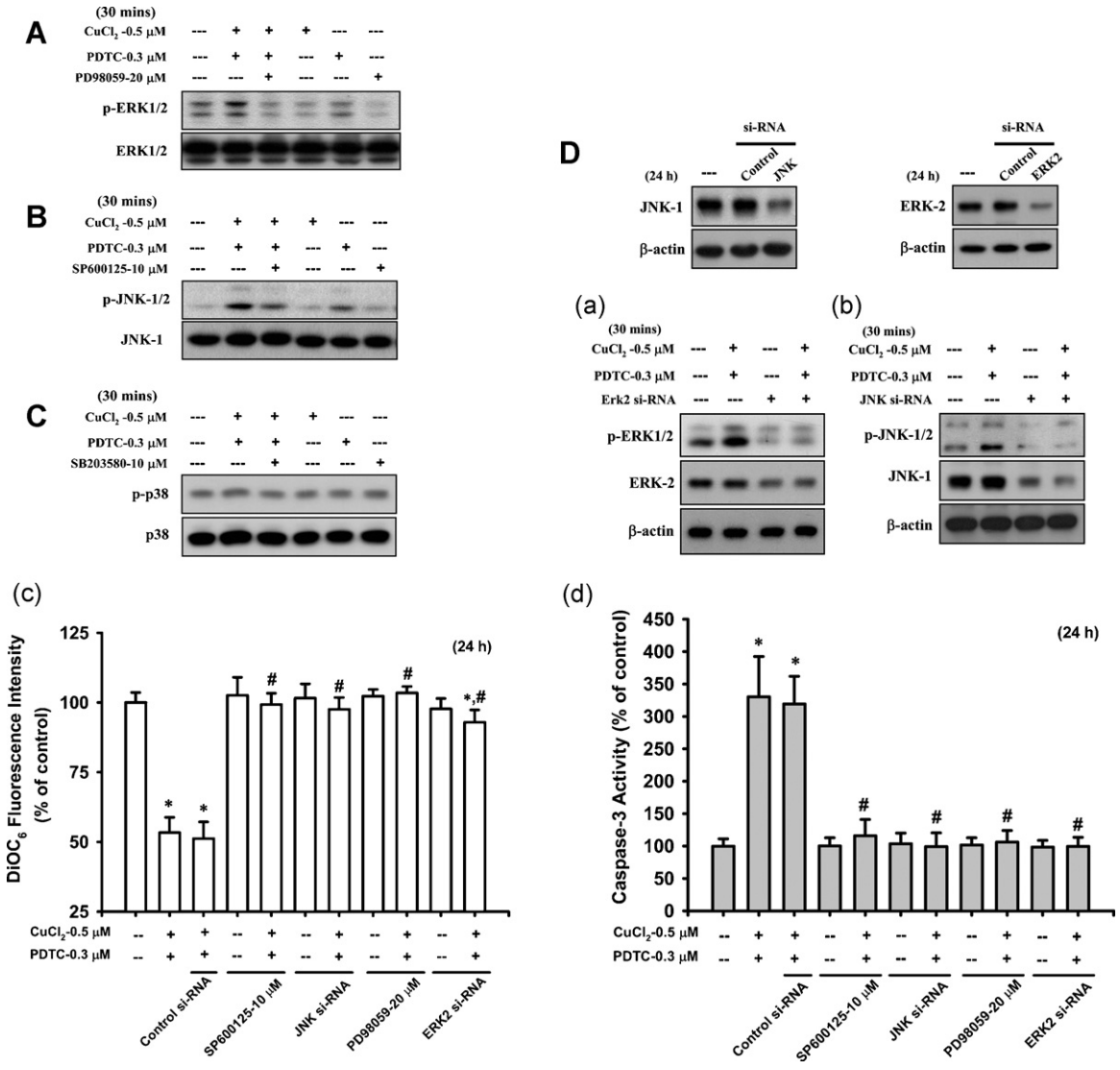

Fig. 7. ERK- and JNK-mediated signaling played critical roles in Cu^{2+}/PDTC complex-induced apoptosis in pancreatic β-cells. RIN-m5F cells were treated including pre-treatment with specific ERK inhibitor (PD98059-20 μM, A), JNK inhibitor (SP600125-10 μM, B), or p38 inhibitor (SB203580-10 μM, C) for 1 h prior to Cu^{2+}/PDTC complex (0.5 μM $CuCl_2$ combined with 0.3 μM PDTC) for 30 min, and the phosphorylation of ERK1/2 and JNK proteins was analyzed by Western blot. In addition, (D) cells were transfected with si-RNA specific to ERK2 or JNK. Following 24 h incubation (ERK-2 or JNK-1 expression was examined by Western blot, upper panel), cells were treated with Cu^{2+}/PDTC complex, and the phosphorylation of ERK1/2 (D – *a*) and JNK (D – *b*, for 30 mins) proteins was detected by Western blot; MMP depolarization was determined by flow cytometry (D – *e*, for 24 h), and caspase-3 activity was measured by CaspACE™ fluorometric assay kit (D – *d*, for 24 h) as described in Section 2. Data in D – *e* and D – *d* are presented as mean ± S.D. for four independent experiments with triplicate determination. $^{*}p < 0.05$ as compared with vehicle control; $^{\#}p < 0.05$ as compared with Cu^{2+}/PDTC complex group.

cytochrome *c* and apoptosis-inducing factor (AIF), caspase activating proteins localized to the intermembrane space of unstimulated mitochondria are released from the mitochondria into the cytosol and inducing activation of caspase-9 followed by activation of caspase-3 (Susin et al., 1999). Here, the present work showed that exposure of RIN-m5F cells to the Cu^{2+}/PDTC complex is significantly induced oxidative stress damages (LPO production of cell membrane and intracellular reduced GSH depletion) and subsequently triggered the loss of MMP, cytochrome *c* release from the mitochondria into the cytosol, and activations of PARP and caspase-3/-7/-9. Furthermore, Bcl-2 family proteins have been demonstrated to be the central regulators of the mitochondria-dependent apoptosis signaling pathway with a balance of anti- and pro-apoptotic members involved in the determination of whether a cell lives or dies (Leibowitz and Yu, 2010). It has been indicated that toxic insults can initiate of apoptotic signals through transcriptional regulation of BH3-only (proapoptotic BH3-only members of the Bcl-2 family) genes, which cooperate and up-regulate with BH3 containing proteins Bax and Bak and inhibit Bcl-2/Bcl-xL proteins leading to promptly up-regulate in response to apoptotic signals (including: an increase in the depolarization of MMP, the efflux of cytochrome *c* into sytosol, and downstream caspase cascades activations) (Harris and Johnson, 2001; Scorrano and Korsmeyer, 2003). Our results revealed that a significant expressions of the decrease in Bcl-2 and an increase in both Bax and Bak mRNA and protein levels were also observed by exposed of cells to Cu^{2+}/PDTC complex, which resulted in increase the pro-apoptotic/anti-apoptotic ratio leading to contribute to the promotion of Cu^{2+}/PDTC complex-induced pancreatic β-cell apoptosis. These responses could be effectively prevented by pre-treatment with NAC. Therefore, these results implicate that oxidative stress induced by the Cu^{2+}/PDTC complex causing pancreatic β-cell death through a mitochondria-dependent apoptotic signaling pathway.

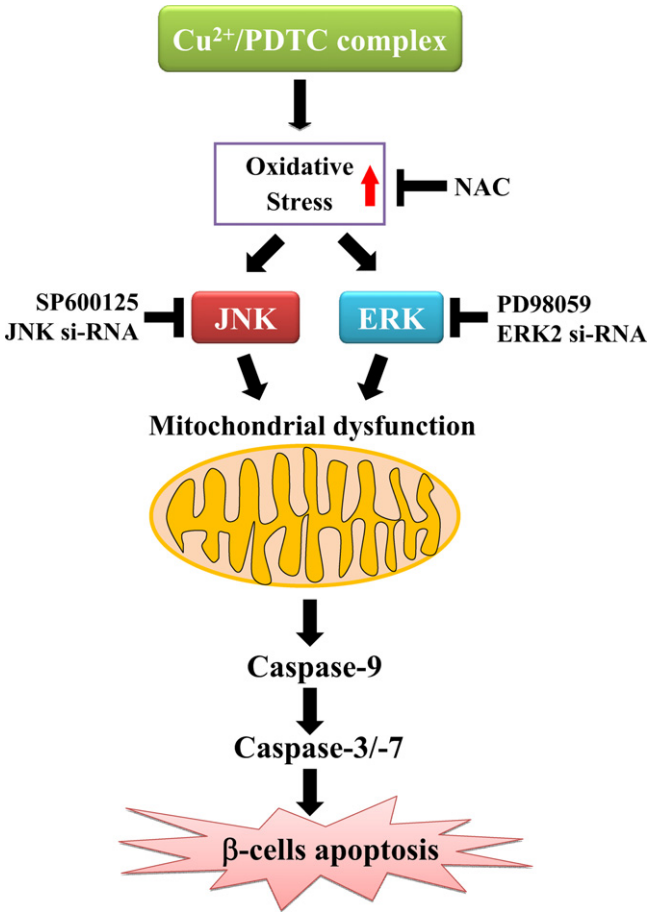

Fig. 8. Schematic diagram of the signal pathways involved in pancreatic β-cell death inducing by the Cu^{2+}/PDTC complex. Proposed models represent that Cu^{2+}/PDTC complex causes β-cell apoptosis through oxidative stress-induced ERK1/2 and JNK activation regulated mitochondria-dependent signaling cascades.

The deleterious effects of oxidative stress have been implicated in a wide variety of activation of intracellular cell death signals and pathophysiological processes, including DM (Finkel and Holbrook, 2000; Kim and Choi, 2010), and found to activate members of the MAPK family (Hou et al., 2008; Matsuzawa and Ichijo, 2005). The activation of JNK pathway induced by oxidative stress is involved in the progression of pancreatic β-cell dysfunction in DM (Kaneto et al., 2002, 2005, 2006). Recent studies have also indicated that ROS-induced pancreatic β-cell death may be regulated by activation of MAPKs (Hou et al., 2008; Lu et al., 2011; Mokhtari et al., 2008). Although, a limited number of literatures have shown that the JNK might be activated by exposure to Cu^{2+}/PDTC complex and caused cell death, including rat cortical astrocyte and human leukemia cell (Chen et al., 2000, 2008b), there are no reports to demonstrate the crucial role of MAPKs activation in Cu^{2+}/PDTC complex-induced oxidative stress which causes mitochondrial dysfunction and leads to pancreatic β-cell apoptosis. Here, our results revealed that the Cu^{2+}/PDTC complex significantly induced phosphorylation of ERK1/2 and JNK, but not p38-MAPK, in pancreatic β-cells. Correlative activation of ERK1/2 and JNK was observed for the upstream kinase MEK1/2 and MKK4, respectively. Pre-treatment with NAC could prevent Cu^{2+}/PDTC complex-induced ERK1/2 and JNK activation. In addition, pre-treatment of pancreatic β-cells with the specific ERK inhibitor PD98059 and JNK inhibitor SP600125 or transfection with ERK2- and JNK-specific si-RNA, respectively, attenuated the phosphorylation levels of ERK1/2 and JNK, which also prevented the depolarization of MMP and the increase in caspase-3 activity in pancreatic β-cells by treated with the Cu^{2+}/PDTC complex. On the basis of these findings, it indicates, for the first time that the oxidative stress-induced ERK/JNK pathway activation downstream-regulated mitochondria-dependent apoptosis is involved in pancreatic β-cell death induced by the Cu^{2+}/PDTC complex.

5. Conclusion

Collectively, this study presents evidence that submicromolar-concentration Cu^{2+}/PDTC complex induces apoptosis through oxidative stress-mediated mitochondria-dependent caspase-activated signaling pathway in pancreatic β-cells by transferring external copper into cells, where the increase in LPO production and the depletion of intracellular reduced GSH (oxidative stress damage), and the decrease in MMP, the downregulation of Bcl-2 and upregulation of Bax and Bak, and cytochrome *c* release into cytosol (mitochondrial dysfunction) are the critical events for apoptosis induction (Fig. 8). More importantly, to our knowledge, this is the first report to demonstrate that ERK/JNK activation-regulated mitochondria-dependent apoptotic signals acts as the crucial apoptotic cell death, and this signaling is tightly affected by oxidative stress generated in the Cu^{2+}/PDTC complex-treated pancreatic β-cells. Therefore, these observations of our research indicates that the Cu^{2+}/PDTC complex, occurring in the clinical application or environmental contamination, may be an important risk factor for the progression of pancreatic β-cell damage and in the development of DM.

Conflict of interest statement

All authors declare that there are no conflicts of interest in this study.

Acknowledgments

This work was supported by research grants form the National Science Council of Taiwan (NSC 98-2320-B-039-014-MY3), the China Medical University Hospital, Taichung, Taiwan (DMR-98-046), the Buddhist Tzu Chi General Hospital, Taichung Branch (TTCRD-10020), the Changhua Christian Hospital, Changhua, Taiwan (100-CCH-IRP-53), and also supported in part by Taiwan Department of Health Clinical Trial and Research Center of Excellence (DOH100-TD-B-111-004).

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