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

Chin-Ching Wu[a,1], Cheng-Chieh Yen[b,c,1], kuan-I Lee[d,1], Chin-Chuan Su[e,1], Feng-Cheng Tang[f,1], Kuo-Liang Chen[g], Yi-Chang Su[h], Ya-Wen Chen[i,*]

[a] Department of Public Health, China Medical University, Taichung 404, Taiwan
[b] Department of Occupational Safety and Health, College of Health Care and Management, Chung Shan Medical University, Taichung 402, Taiwan
[c] Department of Occupational Medicine, Chung Shan Medical University Hospital, Taichung 402, Taiwan
[d] Department of Emergency, Buddhist Tzu Chi General Hospital, Taichung Branch, Taichung 427, Taiwan
[e] Department of Otorhinolaryngology, Head and Neck Surgery, Changhua Christian Hospital, Changhua 500, Taiwan
[f] Department of Occupational Medicine, Changhua Christian Hospital, Changhua 500, Taiwan
[g] Department of Urology, China Medical University Hospital, Taichung 404, Taiwan
[h] School of Chinese Medicine, College of Chinese Medicine, China Medical University, Taichung 404, Taiwan
[i] Department of Physiology and Graduate Institute of Basic Medical Science, College of Medicine, China Medical University, Taichung 404, Taiwan

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.

* Corresponding author. Tel.: +886 4 22052121x7728; fax: +886 4 22333641.
E-mail address: ywc@mail.cmu.edu.tw (Y.-W. Chen).
[1] These authors contribute equally to this work.

0378-4274/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
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