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Metformin attenuates cadmium-induced neuronal apoptosis *in vitro* via blocking ROS-dependent PP5/AMPK-JNK signaling pathway

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Abstract

Cadmium (Cd), a toxic environment contaminant, induces reactive oxygen species (ROS)-mediated neuronal apoptosis and consequential neurodegenerative disorders. Metformin, an anti-diabetic drug, has recently received a great attention owing to its protection against neurodegenerative diseases. However, little is known regarding the effect of metformin on Cd-induced neurotoxicity. Here we show that metformin effectively prevented Cd-evoked apoptotic cell death in neuronal cells, by suppressing Cd activation of c-Jun N-terminal kinases (JNK), which was attributed to blocking Cd inactivation of protein phosphatase 5 (PP5) and AMP-activated protein kinase (AMPK). Inhibition of JNK with SP600125, knockdown of c-Jun, or overexpression of PP5 potentiated metformin's inhibitory effect on Cd-induced phosphorylation of JNK/c-Jun and apoptosis. Activation of AMPK with AICAR or ectopic expression of constitutively active AMPK α strengthened the inhibitory effects of metformin on Cd-induced phosphorylation of JNK/c-Jun and apoptosis, whereas expression of dominant negative AMPK α weakened these effects of metformin. Metformin repressed Cd-induced ROS, thereby diminishing cell death. N-acetyl-L-cysteine enhanced the inhibitory effects of metformin on Cd-induced ROS

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Conflicts of interest

The authors declare that they have no conflicts of interest.

and apoptosis. Moreover, using Mito-TEMPO, we further demonstrated that metformin attenuated Cd-induced cell death by suppressing induction of mitochondrial ROS. Taken together, these results indicate that metformin prevents mitochondrial ROS inactivation of PP5 and AMPK, thus attenuating Cd-induced JNK activation and apoptosis in neuronal cells. Our data highlight that metformin may be a promising drug for prevention of Cd-induced oxidative stress and neurodegenerative diseases.

Keywords

Metformin; Cadmium; Neuronal cells; ROS; PP5; AMPK; JNK

1. Introduction

Heavy metal contamination, one of the greatest global problems, not only negatively affects environment but also endangers humans and animals. Cadmium (Cd), an occupationally and environmentally heavy metal pollutant, can accumulate in multiple organs and tissues due to its long half-life (15~20 years) in the human body, thus resulting in a high incidence of related diseases, including impaired renal function, decreased bone mineral density, increased calcium excretion, reproductive toxicity, and neurotoxicity (Akesson et al., 2006; Johri et al., 2010; Wang and Du, 2013; Xu et al., 2017). Upon absorption into the bloodstream, Cd can easily traverse the blood-brain barrier and accumulate in the brain, manifesting with symptoms including headache and vertigo, olfactory dysfunction, slowed vasomotor function, peripheral neuropathy, decreased equilibrium, decreased ability to concentrate, and learning disabilities (Fowler, 2009; Okuda et al., 1997; Satarug and Nazar, 2010). Merging data have demonstrated that Cd exposure can induce increased levels of reactive oxygen species (ROS) and trigger neuronal apoptosis (Chen et al., 2011; Di Carlo et al., 2012; Yang et al., 2007). Excessive ROS induced by Cd have been recognized as an important pathogenic factor in the development of neuronal cell death and consequential neurodegenerative diseases, including Parkinson's disease (PD), Alzheimer's disease (AD) and Huntington's disease (HD) (Ben Mimouna et al., 2019; Mendez-Armenta and Rios, 2007; Okuda et al., 1997; Peng et al., 2017; Rigon et al., 2008; Williams et al., 2010).

Mitogen-activated protein kinases (MAPKs), a group of evolutionarily highly conserved cascade of dual (tyrosine and serine/threonine) protein kinases, can be activated by different extracellular stimuli such as cytokines, neurotransmitters, hormones and cellular stresses, and play a crucial role in signal transduction and regulate numerous cellular events such as cell growth and survival (Kyriakis and Avruch, 2012). MAPKs in mammalian cells possess at least three distinct groups, including the extracellular signal-regulated kinases (Erk1/2, Erk3/4, Erk5, and Erk7/8), the c-Jun *N*-terminal kinases (JNK1/2/3), and the p38 MAPKs (p38 α / β / γ / δ) (Kyriakis and Avruch, 2012). It is well known that the phosphorylation of MAPKs is balanced by specific MAPK kinases and phosphatases (Kim and Choi, 2010; Kyriakis and Avruch, 2001). The protein phosphatase 5 (PP5) negatively regulates the JNK pathway involved in oxidative stress-induced apoptosis (Chen et al., 2008a; Chen et al., 2009; Han et al., 2012; Morita et al., 2001). AMP-activated protein kinase (AMPK) is a key regulator of energy homeostasis and cell survival in response to inflammation and oxidative

stress (Chiang et al., 2018). Studies have documented that AMPK inactivation or activation due to oxidative stress is involved in neurodegenerative diseases (Chiang et al., 2018; Jiang et al., 2013; Vingtdoux et al., 2011). Our group has observed that Cd activates JNK pathway contributing to cell death by inducing ROS inactivation of PP5 in neuronal cells (Chen et al., 2008a; Xu et al., 2017). We have also demonstrated that Cd triggers ROS-dependent inactivation of AMPK leading to apoptosis of neuronal cells (Chen et al., 2011; Zhang et al., 2017). Hence, we postulated that a compound that can regulate ROS-dependent PP5-JNK and/or AMPK pathways might be useful to prevent Cd neurotoxicity.

Several studies have shown resveratrol, celastrol or nobiletin protection against Cd neurotoxicity by blocking ROS, involving AMPK or JNK pathways (Chen et al., 2014a; Qu et al., 2018; Shati, 2019; Zhang et al., 2017). Anti-diabetic drug metformin (N, N-dimethyl biguanide), a well-known AMPK activator, results in clear benefits in relation to glucose metabolism and diabetes mellitus-related complications (Barzilai et al., 2016; Lu et al., 2016; Singh et al., 2016). It is emerging that metformin may be also an effective drug to protect neurons from injuries and improve behavior in neurodegenerative diseases, such as memory function in a mouse model of Alzheimer's disease, the risk of Parkinson's disease in diabetes and MPTP-induced Parkinson's disease in mice (Patil et al., 2014; DiTacchio et al., 2015; El-Mir et al., 2008; Meng et al., 2016). Metformin has been demonstrated to exert protective effects against several aging-related diseases in humans by decreasing insulin levels, activating AMPK, inhibiting the mammalian target of rapamycin (mTOR), inhibiting mitochondrial complex 1 in the electron transport chain, reducing endogenous ROS production (Barzilai et al., 2016; Duca et al., 2015; Kickstein et al., 2010; Rotermund et al., 2018; Zheng et al., 2012). This prompted us to investigate whether and how metformin protects against Cd-induced neuronal apoptosis. Here, for the first time, we show that metformin ameliorates Cd-evoked neuronal apoptosis via blocking ROS-dependent PP5/AMPK-JNK signaling pathway. The results improve our understanding of the molecular mechanism by which metformin has a potential for the prevention of oxidative stress and neurodegeneration promoted by Cd toxicity.

2. Materials and methods

2.1. Reagents

Cadmium chloride, metformin, 4',6-diamidino-2-phenylindole (DAPI), poly-D-lysine (PDL), N-acetyl-L-cysteine (NAC), SP600125 and protease inhibitor cocktail were purchased from Sigma (St Louis, MO, USA), whereas 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) was purchased from MP Biomedicals (Solon, OH, USA). Mito-TEMPO was acquired from ALEXIS Biochemicals (San Diego, CA, USA). 5-amino-4-imidazolecarboxamide ribose (AICAR) was from Enzo Life Sciences (Farmingdale, NY, USA). CellTiter 96@AQueous One Solution Cell Proliferation Assay kit was from Promega (Madison, WI, USA). Enhanced chemiluminescence solution was from Sciben Biotech Company (Nanjing, China). Dulbecco's modified Eagle medium (DMEM), 0.05% Trypsin-EDTA, NEUROBASAL™ Media and B27 Supplement were purchased from Invitrogen (Grand Island, NY, USA). Horse serum and fetal bovine serum (FBS) were supplied by Hyclone (Logan, UT, USA).

Other chemicals were purchased from local commercial sources and were of analytical grade.

2.2. Cell culture

Rat pheochromocytoma (PC12) and human neuroblastoma SH-SY5Y cell lines were from American Type Culture Collection (ATCC) (Manassas, VA, USA), which were seeded in a 6-well plate or 96-well plate pre-coated with (for PC12) or without (for SH-SY5Y) PDL (0.2 µg/ml). PC12 cells were cultured in antibiotic-free DMEM supplemented with 10% horse serum and 5% FBS, whereas SH-SY5Y cells were grown in antibiotic-free DMEM supplemented with 10% FBS. Cells were maintained in a humid incubator (37°C, 5% CO₂). Primary murine neurons were isolated from fetal mouse cerebral cortexes of 16–18 days of gestation in female ICR mice (being pregnant) as described (Chen et al., 2009; Chen et al., 2010), and seeded in a 6-well plate or 96-well plate coated with 10 µg/mL PDL for experiments after 6 days of culture. All procedures used in this study were approved by the Institutional Animal Care and Use Committee, and were in compliance with the guidelines set forth by the Guide for the Care and Use of Laboratory Animals.

2.3. Recombinant adenoviral constructs and infection of cells

The recombinant adenoviruses expressing HA-tagged wild-type (wt) human PP5 (Ad-PP5) (Morita et al., 2001) (a gift from Dr. Hidenori Ichijo, University of Tokyo, Tokyo, Japan), FLAG-tagged dominant negative c-Jun (FLAG- 169) (Ad-dn-c-Jun) (Whitfield et al., 2001) (a gift from Dr. Jonathan Whitfield, Eisai London Research Laboratories, University College London, London, UK), myc-tagged constitutively active mutant of rat AMPKα1 (T172D) (Ad-AMPKα-ca) (Zang et al., 2004) (a gift from Dr. Kenneth Walsh, Boston University School of Medicine, Boston, MA, USA), HA-tagged dominant-negative mutant of human AMPKα1 (D159A) (Ad-dn-AMPKα) (Lu et al., 2008) (a gift from Dr. Nicholas J.G. Webster, University of California, San Diego, CA, USA), and the control virus expressing the green fluorescent protein (GFP) (Ad-GFP) were described previously (Chen et al., 2008a; Chen et al., 2008b; Chen et al., 2010; Liu et al., 2010). For experiments, PC12 cells were grown in the growth medium and infected with the individual adenovirus for 24 h at 5 of multiplicity of infection (MOI = 5). Afterwards, cells were used for experiments. Cells infected with Ad-GFP served as a control. Expression of HA-tagged PP5 or dn-AMPKα, FLAG-tagged dn-c-Jun, or myc-tagged AMPKα-ca was assessed by Western blot analysis with antibodies to HA, FLAG or myc.

2.4. Drugs administration and Cd treatments

Experimental treatments and time line were shown in Fig. 1. In brief, PC12 cells, SH-SY5Y cells and/or primary neurons, or PC12 cells infected with Ad-PP5, Ad-dn-c-Jun, Ad-dn-AMPKα, Ad-AMPKα-ca or Ad-GFP, respectively, were seeded in a PDL-uncoated or -coated 6-well plate (1×10^6 cells/well for Western blotting) or 96-well plate (1×10^4 cells/well for MTS assay and caspase-3/7 activity assay), or at a density of 5×10^5 cells/well in a 6-well plate containing a PDL-uncoated or -coated glass coverslip per well (for DAPI/TUNEL staining, Immunofluorescence staining and ROS imaging). The next day, cells were treated with/without Cd (10 and/or 20 µM) for 4 h or 24 h following pre-incubation with/without metformin (1 mM) for 24 h and/or with/without a JNK inhibitor SP600125 (20 µM)

(Chen et al., 2008b; Chen et al., 2009), an AMPK activator AICAR (2 mM) (Chen et al., 2011), an antioxidant and ROS scavenger NAC (5 mM) (Chen et al., 2008a; Chen et al., 2011) or a mitochondria-targeted antioxidant Mito-TEMPO (10 μ M) (Yeh et al., 2014) for 1 h with 5 replicates of each treatment. Subsequently, cells were conducted for the below analysis.

2.5. Assays for cell viability and caspase-3/7 activity

The indicated cells, after treatments, were incubated with MTS reagent (one solution reagent) (20 μ l/well) for 4 h, and then the viability of the cells was assayed by measuring the optical density (OD) at 490 nm using a Victor X3 Light Plate Reader (PerkinElmer, Waltham, MA, USA). Caspase-3/7 activity was determined according to the supplier's instructions of Caspase-Glo® 3/7 Assay Kit (Promega, Madison, WI, USA).

2.6. DAPI and TUNEL staining

For the indicated cells, after treatments, the cells with fragmented and condensed nuclei were determined using DAPI staining as described (Chen et al., 2008b). For the cells using DAPI/TUNEL staining, following DAPI staining, TUNEL staining was conducted according to the manufacturer's protocols of *In Situ* Cell Death Detection Kit® (Roche, Mannheim, Germany). Finally, photographs for all stained samples were recorded under a fluorescence microscope (200 $\times$) (Leica DMI8, Wetzlar, Germany) equipped with a digital camera. For quantitative analysis of the fluorescence intensity using TUNEL staining, the integral optical density (IOD) was determined by Image-Pro Plus 6.0 software (Media Cybernetics Inc., Newburyport, MA, USA).

2.7. Immunofluorescence and imaging

The indicated cells on the coverslips, after treatments, were fixed with 4% paraformaldehyde and incubated with 3% normal goat serum to block non-specific binding. Next, the cells were incubated with rabbit anti-phospho-JNK (Thr183/Tyr185) antibody (Cell Signaling, Danvers, MA, USA, 1:50, diluted in PBS containing 1% BSA) overnight at 4°C, washed 3 times with PBS, and further incubated with FITC-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:500, diluted in PBS containing 1% BSA) for 1 h at room temperature. The cells were then washed 3 times with PBS. Finally, slides were mounted in glycerol/PBS (1:1, v/v) containing 2.5% 1,4-diazabicyclo-(2,2,2) octane. Cell images and IOD for fluorescence intensity were captured under a fluorescence microscope (200 $\times$) and quantitatively analyzed as described above, respectively.

2.8. Cell ROS imaging

For the indicated cells, after treatments, intracellular ROS fluorescence was imaged using an oxidant-sensitive probe, CM-H₂DCFDA, and quantitatively analyzed as described (Xu et al., 2016).

2.9. Western blot analysis

The indicated cells, after treatments, were lysed, followed by western blotting, as described previously (Chen et al., 2010). The blots for detected protein were semi-quantified using

NIH Image J software (National Institutes of Health, Bethesda, MD, USA). The following antibodies were used: phospho-AMPK α (p-AMPK α) (Thr 172), phospho-acetyl-CoA carboxylase (p-ACC) (Ser79), ACC, JNK, cleaved-caspase-3, poly (ADP-ribose) polymerase (PARP) (Cell Signaling Technology, Danvers, MA, USA), p-c-Jun (Ser63), PP5 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), p-JNK (Thr183/Tyr185), c-Jun, AMPK α (Epitomics, Burlingame, CA, USA), FLAG, HA, myc epitope, β -tubulin, goat anti-rabbit IgG-horseradish peroxidase (HRP), goat anti-mouse IgG-HRP, and rabbit anti-goat IgG-HRP (Sciben Biotech Company, Nanjing, China).

2.10. Statistical analysis

Results were expressed as mean $\pm$ SEM. The normality of data was determined using Shapiro-Wilk tests. Student's *t*-test for non-paired replicates was used to identify statistically significant differences between treatment means. Group variability was compared using one-way ANOVA followed by Bonferroni's post-tests to compare replicate means. Significance was accepted at $p < 0.05$.

3. Results

3.1. Metformin attenuates Cd-induced apoptotic cell death in neuronal cells

Recent reports have suggested that metformin possesses protective effects against several aging-related diseases in humans (Barzilai et al., 2016; Duca et al., 2015; Kickstein et al., 2010; Zheng et al., 2012). Metformin, in clinically relevant concentrations, at 0.5–2 mM, substantially protected neuronal PC12 cells from hydrogen peroxide (H₂O₂)-induced cell death (Zhao et al., 2019). Based on the above finding and our pre-experiment results in PC12 cells, SH-SY5Y cells and primary neurons pretreated with metformin (0–1.5 mM) for 24 h and then exposed to Cd (10 and 20 μ M) for 24 h (data not shown), we selected 1 mM metformin as an appropriate concentration for this research. Here we observed that pretreatment of neuronal cells (PC12 cells, SH-SY5Y cells and primary neurons) with metformin (1 mM) for 24 h attenuated apoptotic cell death induced by 24-h exposure with Cd (10 and 20 μ M). This is evidenced by the following findings. 1) Cell viability in the Cd/metformin group was significantly higher than that in the Cd alone group (Fig. 2A), as determined by MTS assay. 2) Pretreatment with metformin for 24 h dramatically diminished the percentage of the cells with nuclear fragmentation and condensation (arrows) and the number of TUNEL-positive cells with fragmented DNA (in green) in PC12 cells, SH-SY5Y cells and primary neurons exposed to Cd (Fig. 2B–D), as imaged and quantified by DAPI and TUNEL staining. 3) Treatment with Cd for 4 h resulted in robust cleavages of caspase-3 and PARP in PC12 cells, SH-SY5Y cells and primary neurons, which were strikingly attenuated by addition of metformin (Fig. 2E and F). 4) Metformin profoundly decreased Cd-elicited activation of caspases-3/7 in the cells (Fig. 2G), as detected by the caspase-3/7 activity assay. Therefore, our results strongly support the notion that metformin can prevent against Cd-induced neuronal cell death.

3.2. Metformin suppresses Cd-induced activation of JNK pathway, preventing apoptosis of neuronal cells

Numerous studies have reported that metformin may alter the activity of JNK under various conditions, thereby affecting cell survival and apoptosis (Chen et al., 2016; Ota et al., 2007; Wu et al., 2011; Zhu et al., 2016). We have recently demonstrated that Cd induces neuronal apoptosis in part through activation of JNK signaling pathway (Xu et al., 2017). Therefore, we reasoned that metformin might ameliorate Cd-evoked neuronal apoptosis by blocking activation of JNK pathway. In this connection, PC12 cells, SH-SY5Y cells and primary neurons were pretreated with metformin (1 mM) for 24 h, and then exposed to Cd (10 and 20 μ M) for 4 h, followed by Western blot analysis. As predicted, pretreatment with metformin effectively inhibited Cd-induced phosphorylation of JNK (Thr183/Tyr185) and its downstream molecule, protein expression and phosphorylation of c-Jun (Ser63) in the cells (Fig. 3A and B). To confirm this, we next conducted phospho-JNK (p-JNK) (Thr183/Tyr185) immunofluorescence staining in PC12 cells, SH-SY5Y cells and primary neurons, respectively. Treatment with Cd (10 and 20 μ M) for 24 h elicited a remarkable increase in p-JNK (Thr183/Tyr185) (in green), which was significantly hindered by metformin pretreatment (Fig. 3C and D).

To corroborate the observation that metformin's inhibition of Cd-induced neuronal apoptosis is attributed to its blockage of JNK/c-Jun activation, SP600125, a JNK inhibitor (Chen et al., 2008b; Chen et al., 2009), was employed. When PC12 cells and primary neurons were treated with SP600125 (20 μ M) for 1 h alone, or co-treated with metformin (1 mM) for 24 h in the presence or absence of SP600125 (20 μ M) for 1 h, and then exposed to Cd (10 μ M) for 4 h or 24 h, we found that SP600125 or metformin alone obviously suppressed the phosphorylation of JNK (Thr183/Tyr185) and c-Jun (Ser63), as well as the cleavage of caspase-3 in the cells in response to Cd (Fig. 4A and B). Especially, co-treatment with metformin/SP600125 exhibited a stronger inhibitory effect on Cd-induced p-JNK, p-c-Jun and cleaved-caspase-3 (Fig. 4A and B). Consistently, the combination of metformin with SP600125 also demonstrated more potent suppression of Cd-evoked apoptosis than metformin or SP600125 alone, as evidenced by the decreased percentage of cells with fragmented nuclei (Fig. 4C).

In addition, we verified the above finding using a genetic approach. PC12 cells, infected with Ad-dn-c-Jun and Ad-GFP (as control), respectively, were pretreated with metformin (1 mM) for 24 h, followed by exposure to Cd (10 μ M) for 4 h or 24 h. The results revealed that ectopic expression of dn-c-Jun, but not GFP, almost completely blocked Cd-induced p-c-Jun and cleavage of caspase-3 (Fig. 4D and E). Of importance, overexpression of dn-c-Jun significantly potentiated the inhibitory effect of metformin on Cd-induced apoptosis in PC12 cells (Fig. 4F). Collectively, our data underline the concept that metformin suppresses Cd activation of JNK/c-Jun-dependent neuronal apoptosis.

3.3. Metformin prevents Cd activation of JNK/c-Jun-dependent apoptosis by blocking Cd inactivation of PP5 in neuronal cells

PP5, as a physiological inhibitor of ASK1-JNK signaling, is well-known to negatively regulate JNK pathway, involved in stress response, cell survival and apoptosis (Chen et al.,

2008a; Chen et al., 2009; Han et al., 2012; Morita et al., 2001; Xu et al., 2017). Our previous research has demonstrated that Cd activates JNK pathway leading to apoptosis in part by inhibition of PP5 in neuronal cells (Chen et al., 2008a), and this study has found that metformin inhibited Cd activation of JNK pathway contributing to neuronal cell death (Fig. 3 and Fig. 4). Therefore, we postulated that metformin suppresses Cd activation of JNK pathway by preventing Cd from inactivating PP5. To this end, PC12 cells, SH-SY5Y cells and primary neurons were pretreated with/without metformin (1 mM) for 24 h, and then exposed to Cd (10 and 20 μ M) for 4 h, followed by Western blot analysis. As shown in Fig. 5A and B, Cd-reduced PP5 expression was indeed partly rescued by metformin in PC12 cells, SH-SY5Y cells and primary neurons. To define the role of PP5 in metformin's blockage of Cd-induced JNK activation and neuronal apoptosis, PC12 cells, infected with recombinant adenovirus expressing HA-tagged wild-type human PP5 (Ad-PP5) or Ad-GFP (as control), were pretreated with metformin (1 mM) for 24 h, followed by exposure to Cd (10 μ M) for 4 h or 24 h. By Western blot analysis, we observed over-expression of HA-tagged PP5 in the cells infected with Ad-PP5 (Fig. 5C and D). In line with our previous findings (Chen et al., 2008a), exposure to Cd decreased PP5 expression, and correspondingly increased the phosphorylation of JNK/c-Jun in the control cells infected with Ad-GFP (Lane 3 vs. Lane 1) (Fig. 5C and D). Overexpression of PP5 partially blocked Cd-induced p-JNK/p-c-Jun (Lane 7 vs. Lane 3) (Fig. 5C and D). Moreover, overexpression of PP5 also reinforced the protective effect of metformin against Cd-induced cleavage of caspase-3 (Fig. 5C and D). Using MTS assay and DAPI staining, we further exhibited that overexpression of PP5 in part reversed Cd-induced cell viability reduction and apoptosis, and strengthened the protective activity of metformin (Fig. 5E and F). These results indicate that metformin prevents Cd activation of JNK/c-Jun-dependent neuronal apoptosis in part by blocking Cd inactivation of PP5.

3.4. AMPK α -mediated JNK pathway is involved in metformin's prevention of Cd-induced apoptosis in neuronal cells

We have recently shown that Cd induces neuronal apoptosis through inactivation of AMPK α (Chen et al., 2011; Zhang et al., 2017). Metformin is a well-known AMPK activator (Singh et al., 2016). Therefore, we reasoned that metformin prevents Cd-induced neuronal apoptosis partly by blocking Cd inactivation of AMPK α . To this end, PC12 cells, SH-SY5Y cells and primary neurons were exposed to Cd (10 and 20 μ M) for 4 h following pretreatment with/without metformin (1 mM) for 24 h. Western blotting results showed that metformin markedly attenuated Cd-reduced phosphorylation of AMPK α (Thr172) and its substrate ACC (Ser79) (Fig. 6A and B). Next, we determined whether metformin inhibits Cd-induced neuronal apoptosis by preventing Cd inactivation of AMPK α . For this, PC12 cells and primary neurons were treated with/without Cd (10 μ M) for 4 h or 24 h following pre-incubation with/without metformin (1 mM) for 24 h and/or with/without AICAR (2 mM), an AMPK activator (Chen et al., 2011), for 1 h. In line with our previous finding (Chen et al., 2011), Cd markedly evoked the decline of p-AMPK α and p-ACC, and the elevation of cleaved-caspase-3, which was attenuated by AICAR (Fig. 6C and D). Interestingly, co-treatment with metformin/AICAR rescued the cells from Cd-induced caspase-3 cleavage, viability reduction and apoptosis more potently than metformin or AICAR alone (Fig. 6C–F). Our observation that AICAR powerfully reinforced the inhibitory

effect of metformin on Cd-elicited events supports the idea that metformin ameliorates Cd-induced cell death in part by activating AMPK, which plays a significant role in various aspects in neuronal cells. In addition, we found that Cd-induced phosphorylation of JNK was, to some extent, inhibited by pretreatment with AICAR or metformin alone, and more potently inhibited by co-treatment with metformin/AICAR (Fig. 6C and D). These results imply that AMPK α -mediated JNK pathway may be involved in metformin's prevention of Cd-induced neuronal cell death.

To confirm the above findings, PC12 cells, infected with recombinant adenoviruses expressing HA-tagged dominant negative AMPK α 1 (Ad-dn-AMPK α), myc-tagged continuous-active AMPK α (Ad-AMPK α -ca) and control virus encoding GFP alone (Ad-GFP), respectively, were exposed to Cd (10 μ M) for 4 h or 24 h following pretreatment with/without metformin (1 mM) for 24 h. We observed a high level of HA-tagged dn-AMPK α in Ad-dn-AMPK α -infected cells and that of myc-tagged AMPK α -ca in Ad-AMPK α -ca-infected cells, but not that in Ad-GFP-infected cells (control) (Fig. 7A and D). As expected, ectopic expression of dn-AMPK α decreased the AMPK activity, while expression of AMPK α -ca increased the AMPK activity, as evidenced by the decreased/increased basal or Cd-induced p-AMPK α and p-ACC levels detected by Western blotting (Fig. 7A, B, D, and E). Of note, expression of dn-AMPK α profoundly enhanced the basal or Cd-increased p-JNK, p-c-Jun, c-Jun, cleaved-caspase-3 and cell apoptosis, and obviously attenuated the inhibitory effect of metformin on Cd-induced events (Figure 7A–C). In contrast, expression of AMPK α -ca remarkably potentiated the inhibitory activity of metformin (Fig. 7D–F). These results demonstrate the importance of metformin's prevention of Cd-inactivated AMPK α in blocking Cd activation of JNK/c-Jun-dependent apoptosis in neuronal cells.

3.5. Metformin ameliorates Cd-induced neuronal apoptosis by suppressing ROS-dependent PP5/AMPK-JNK signaling pathway.

It has been documented that Cd induces excessive ROS contributing to neuronal apoptosis (Chen et al., 2011; Di Carlo et al., 2012; Yang et al., 2007). AMPK inactivation/activation due to oxidative stress is associated with neurodegenerative diseases (Chiang et al., 2018; Jiang et al., 2013; Vingtdeux et al., 2011). Our group has demonstrated that Cd induction of ROS results in inactivation of PP5 and AMPK leading to apoptosis of neuronal cells (Chen et al., 2008a; Chen et al., 2011; Xu et al., 2017; Zhang et al., 2017). Next, we wondered whether metformin suppresses Cd-induced inactivation of PP5/AMPK, activation of JNK/c-Jun, and neuronal apoptosis by reducing Cd-induced ROS level. For this, ROS assay was performed. We observed that pretreatment with metformin (1 mM) for 24 h significantly attenuated Cd-elevated intracellular ROS levels in PC12 cells, SH-SY5Y cells and primary neurons (Fig. 8A and B), as measured by imaging and quantifying using an oxidant-sensitive probe, CM-H₂DCFDA. This was consistent with the observations that Cd induced cell viability reduction and apoptosis (Fig. 2). Further, pretreatment with NAC, an antioxidant and ROS scavenger (Chen et al., 2008a; Chen et al., 2011), clearly repressed Cd-evoked ROS levels (Fig. 8C) and markedly strengthened metformin's intervention in Cd-triggered abnormal ROS accumulation in PC12 cells, SH-SY5Y cells and primary neurons (Fig. 8C). In line with this, co-treatment with metformin/NAC diminished the percentage of cells with fragmented nuclei more potently than treatment with NAC or metformin alone (Fig. 8D).

Further, we exhibited that NAC alone slightly promoted the phosphorylation of AMPK α and ACC (Fig. 8E and F). NAC alone also, to some degree, prevented Cd-induced inactivation of PP5/AMPK, activation of JNK, and cleavage of caspase-3 (Fig. 8E and F). These findings suggest that metformin suppresses Cd elevation of ROS, which links to its prevention of Cd-induced inactivation of PP5/AMPK, activation of JNK, and apoptosis in neuronal cells.

Since metformin induces mitochondrial biogenesis (Singh et al., 2016), whereas Cd induces mitochondrial ROS, we next sought to validate whether metformin protection from Cd-induced neuronal apoptosis is dependent on mitochondrial ROS change. For this, PC12 cells and primary neurons were pretreated with/without metformin (1 mM) for 24 h, followed by exposure to Cd (10 μ M) in the presence or absence of Mito-TEMPO (10 μ M), a mitochondria-targeted antioxidant (Yeh et al., 2014). As expected, Mito-TEMPO significantly repressed Cd-induced ROS levels (Fig. 9A) and cell apoptosis (Fig. 9B), and dramatically strengthened the inhibitory effects of metformin on Cd-induced events in PC12 cells and primary neurons (Fig. 9A and B). In addition, we noticed that Mito-TEMPO reversed the expression of PP5, p-AMPK α , p-ACC, p-JNK, p-c-Jun, c-Jun, as well as cleavage of caspase-3 in the cells in response to Cd (Fig. 9C and D). Especially, co-treatment with metformin/Mito-TEMPO exhibited a stronger intervention for Cd-evoked events in the cells (Fig. 9C and D). Taken together, we conclude that metformin attenuates Cd-induced mitochondrial ROS, thereby preventing Cd inactivation of PP5/AMPK α and activation of JNK pathway, and ultimately protecting against apoptosis in neuronal cells.

4. Discussion

Abundant evidence has shown that smelting, burning of fossil fuels and municipal wastes, refining of metals, and cigarette smoking are the main sources of Cd pollution in water, air and soil (Chen et al., 2008a; Chen et al., 2011; Wang and Du, 2013). So people can easily expose to Cd through daily life. Especially, after entering human body, Cd can readily pass through the blood-brain barrier and accumulate in the brain, hinder the function of the nervous system by its induction of neuronal apoptosis, and thereby contributing to neurodegenerative diseases, such as PD, AD and HD (Ben Mimouna et al., 2019; Mendez-Armenta and Rios, 2007; Okuda et al., 1997; Peng et al., 2017; Rigon et al., 2008; Williams et al., 2010). Our group has shown that Cd evokes ROS overproduction and consequent cell death in neuronal cells (Chen et al., 2008a; Chen et al., 2011; Chen et al., 2014b; Xu et al., 2017; Zhang et al., 2017). Therefore, it is of great importance to find a novel therapeutic strategy to prevent Cd-induced ROS-dependent neuronal apoptosis. Metformin, a well-known antidiabetic drug, has been investigated and proposed to possess neuroprotective activity in the models of neurodegenerative disorders (Foretz et al., 2014; Kickstein et al., 2010; Ma et al., 2007; Singh et al., 2016). Some studies have revealed that metformin acts in protection against several aging-related diseases in humans by activating AMPK and reducing endogenous ROS production (Barzilai et al., 2016; Duca et al., 2015; Kickstein et al., 2010; Zheng et al., 2012). However, little is known about whether and how metformin prevents Cd neurotoxicity. Here, for the first time, we provide evidence that metformin prevents Cd-elicited apoptotic cell death in neuronal cells, by repressing mitochondrial ROS inactivation of PP5 and AMPK, leading to suppression of JNK cascade.

It has been shown that metformin may alter the activity of JNK under various conditions, and thus affecting cell survival and apoptosis (Chen et al., 2016; Ota et al., 2007; Wu et al., 2011; Zhu et al., 2016). Our previous studies have identified that Cd activates JNK cascade leading to neuronal apoptosis (Chen et al., 2008a; Chen et al., 2008b). In the current study, we found that metformin inhibited Cd-induced phosphorylation of JNK, including protein expression and phosphorylation of c-Jun, the substrate of JNK, in PC12 cells, SH-SY5Y cells and primary neurons (Fig. 3). This was further verified by using pharmacological JNK inhibitor SP600125 or genetic manipulation of c-Jun (Fig. 4).

PP5 negatively regulates JNK pathway, involved in stress response, cell survival and apoptosis (Chen et al., 2008a; Chen et al., 2009; Han et al., 2012; Morita et al., 2001; Xu et al., 2017). Here we revealed that metformin alleviated Cd-induced activation of JNK/c-Jun-dependent neuronal apoptosis through preventing Cd from inhibition of PP5. This is supported by the findings that overexpression of PP5 partially prevented Cd-induced activation of JNK/c-Jun, cleaved-caspase-3 and cell death, and potentiated the inhibitory activity of metformin for Cd-elicited the events (Fig. 5). Based on the entire data from our observations and other literatures, to our knowledge, this is the first report presenting that metformin protects against Cd neurotoxicity partly by rescuing PP5-dependent suppression of JNK pathway.

AMPK activation/phosphorylation is a key biochemical event responsible for cell survival and apoptosis, which is involved in aging and neurodegenerative diseases (Chiang et al., 2018; Jiang et al., 2013; Vingtdeux et al., 2011; Zhao et al., 2019). It is known that metformin is a widely accepted AMPK activator, which can accelerate AMPK phosphorylation at Thr172 site (Barzilai et al., 2016; Lu et al., 2016; Singh et al., 2016). In this study, we observed that metformin obviously blocked Cd-induced decline of p-AMPK α (Thr172) and its substrate p-ACC (Ser79) in PC12 cells, SH-SY5Y cells and primary neurons (Fig. 6), implying that metformin prevents Cd inactivation of AMPK α . Of note, the neuroprotective effect of metformin in primary neurons was found to be more profound at low doses of Cd (10 μ M), probably because of the higher toxicity of 20 μ M Cd. Using AMPK α activator (AICAR) and overexpression of constitutively activated AMPK α (AMPK α -ca) or dominant negative AMPK α (dn-AMPK α), we further demonstrated that metformin inhibited Cd activation of JNK/c-Jun-dependent neuronal apoptosis by preventing Cd inactivation of AMPK α (Fig 6 and 7). Overall, these observations support that metformin protects against Cd neurotoxicity partly by restraining Cd inactivation of AMPK, thereby preventing activation of JNK/c-Jun pathway.

Under pathological conditions, excessive ROS in the mitochondria reduces mitochondrial biogenesis, which, in turn, further promotes ROS induction and mitochondrial dysfunction, and thus commits the cell to undergo apoptosis (Lu et al., 2012; Seo et al., 2010; Zhang et al., 2017). Some reports have shown that mitochondrion is the primary target of metformin within the cell in which metformin transiently inhibits mitochondrial complex 1 in the electron transport chain, promotes mitochondrial biogenesis (Singh et al., 2016; Vancura et al., 2018). Recent evidence exhibits that metformin protects PC12 cells and hippocampal neurons from H₂O₂-induced oxidative insult via activating AMPK pathway, implying metformin resistance to oxidative stress as an anti-oxidative compound (Zhao et al., 2019).

In this study, when metformin-pretreated PC12 cells, SH-SY5Y cells and primary neurons were exposed to Cd for 24 h, intracellular abnormal ROS accumulation was significantly suppressed compared to the vehicle-pretreated cells (Fig. 8A and B). This is in line with the finding that metformin possesses antioxidant activity (Zhao et al., 2019). Using NAC, an antioxidant and ROS scavenger (Chen et al., 2008a; Chen et al., 2011), and Mito-TEMPO, a mitochondria-targeted antioxidant (Yeh et al., 2014), we further observed that addition of NAC or Mito-TEMPO obviously reinforced metformin inhibition of Cd-evoked ROS elevation and apoptosis, in PC12 cells and primary neurons (Fig. 8 and 9). Subsequently, we unveiled that Cd-induced inactivation of PP5/AMPK and consequential activation of JNK/c-Jun and caspase-3 were more powerfully ameliorated in the cells co-treated with metformin/NAC or metformin/Mito-TEMPO than in those treated with metformin, NAC or Mito-TEMPO alone (Fig. 8 and 9). Together, the findings support that metformin prevents Cd-induced apoptosis in neuronal cells by suppressing mitochondrial ROS-dependent PP5/AMPK-JNK signaling pathway.

Given the potential therapeutic effects of metformin, trials have been invested to study counteraction of metformin on neurodegenerative diseases in animals and humans, but its therapeutic use is not yet accepted since the results are often conflicting, such as divergent effects of oral metformin on an inflammatory model of Parkinson's disease in rats, oral metformin's protection on various brain tissues as cortical neurons through activation of AMPK, and metformin's reduction of neurological symptoms in some patients and disease phenotypes in animal and cell models (Rotermund et al., 2018; Tayara et al., 2018). Acute metformin (10 mg/kg, i.p. for 24 h) preconditioning confers neuroprotection against focal cerebral ischemia by activation of AMPK-dependent autophagy in rats (Jiang et al., 2014), and chronic metformin (50 mg/kg daily, i.p. for 3 weeks) preconditioning provides neuroprotection in rats with permanent cerebral ischemia (Zhu et al., 2015). At the molecular level the findings vary depending on the doses of metformin used and duration of treatment, with clear differences between acute and chronic administration (Rotermund et al., 2018; Vancura et al., 2018; Zhang et al., 2016). Metformin's bioavailability in brain has not been reported, but the neuroprotective effects of metformin on neurodegenerative diseases imply that metformin could cross the blood brain barrier. In the last 10 years, the researchers have moved from a simple picture, that metformin improves glycemia by acting on the liver via AMPK activation, to a much more complex picture reflecting its multiple modes of action. More studies on the *in vivo* metformin's prevention of Cd toxicity in animal models are required to truly understand how metformin works on Cd neurotoxicity associated with aging and neurodegenerative diseases.

In conclusion, we have identified that metformin attenuated Cd-elicited neuronal apoptosis by preventing mitochondrial ROS inactivation of PP5 and AMPK, thus suppressing activation of JNK pathway (Fig. 10). Our results suggest that metformin is a promising drug for prevention of Cd-induced oxidative stress and neurodegenerative diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations:

ACC	acetyl-CoA carboxylase
AD	Alzheimer disease
AICAR	5-amino-4-imidazolecarboxamide ribose
AMPK	AMP-activated protein kinase
Cd	cadmium
CM-H2DCFDA	5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate; DAPI, 4', 6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
FBS	fetal bovine serum
HD	Huntington's disease
H2O2	hydrogen peroxide
JNK	c-Jun N-terminal kinase
MAPK	mitogen-activated protein kinase
NAC	N-acetyl-L-cysteine
PBS	phosphate buffered saline
PD	Parkinson disease
PDL	poly-D-lysine
PP5	protein phosphatase 5
ROS	reactive oxygen species
TUNEL	the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labeling.

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Highlights

- Metformin attenuates Cd-evoked apoptotic cell death in neuronal cells.
- Metformin suppresses Cd-activated JNK pathway, preventing neuronal apoptosis.
- Metformin prevents Cd-elicited mitochondrial ROS inactivation of PP5 and AMPK, thereby suppressing activation of JNK pathway.
- Metformin may be a promising drug for prevention of Cd-induced oxidative stress and neurodegenerative diseases

Fig. 1.

Experimental treatments and time line *in vitro*. PC12 cells, SH-SY5Y cells and/or primary neurons, or PC12 cells infected with Ad-PP5, Ad-dn-c-Jun, Ad-dn-AMPK α , Ad-AMPK α -ca or Ad-GFP, respectively, were treated with/without Cd for 4 h or 24 h following pre-incubation with/without metformin for 24 h and/or with/without SP600125, AICAR, NAC or Mito-TEMPO for 1 h. Subsequently, cells were conducted for the analysis.

Fig. 2.

Metformin attenuates Cd-induced apoptotic cell death in neuronal cells. PC12 cells, SH-SY5Y cells and primary neurons were pretreated with/without metformin (1 mM) for 24 h, followed by exposure to Cd (10 and 20 μ M) for 4 h (for Western blotting) or 24 h (for MTS assay, DAPI/TUNEL staining and caspase-3/7 activity assay). A) Relative cell viability was evaluated by MTS assay. B) Apoptotic cells were evaluated by nuclear fragmentation and condensation (arrows) using DAPI staining (upper panel) and concurrently by *in situ* detection of fragmented DNA (in green) using TUNEL staining (lower panel). Scale bar: 20 μ m. C and D) The percentages of cells with fragmented nuclei and the number of TUNEL-positive cells were quantified. E) Total cell lysates were subjected to Western blotting using indicated antibodies. The blots were probed for β -tubulin as a loading control. Similar

results were observed in at least three independent experiments. F) The relative densities for cleaved-caspase-3, cleaved-PARP to β -tubulin were semi-quantified using NIH image J. G) Caspase-3/7 activities were detected using Caspase-3/7 Assay Kit. Results are presented as mean $\pm$ SEM (n = 3–5). Using one-way ANOVA, * p < 0.05 for difference with no metformin group, ^a p < 0.05 for difference with no Cd group, ^b p < 0.05 for difference with 10 μ M Cd group.

Fig. 3.

Metformin prevents Cd-induced activation of JNK pathway. PC12 cells, SH-SY5Y cells and primary neurons were pretreated with/without metformin (1 mM) for 24 h, followed by exposure to Cd (10 and 20 μM) for 4 h (for Western blotting) or 24 h (for immunofluorescence staining). A) Total cell lysates were subjected to Western blotting using indicated antibodies. The blots were probed for β-tubulin as a loading control. Similar results were observed in at least three independent experiments. B) The relative densities for p-JNK to JNK, and p-c-Jun to β-tubulin were semi-quantified using NIH image J. C and D) Expression of p-JNK (Thr183/Tyr185) was stained and imaged using immunofluorescence, showing that treatment of the cells with Cd for 24 h resulted in lower p-JNK expression (in green), which was obviously reversed by metformin. Scale bar: 20 μm. Results are presented as mean ± SEM (n = 3–5). Using one-way ANOVA, * $p < 0.05$ for difference with no metformin group, ^a $p < 0.05$ for difference with no Cd group, ^b $p < 0.05$ for difference with 10 μM Cd group.

Fig. 4.

Pharmacological inhibition of JNK or expression of dominant negative c-Jun strengthens metformin's inhibition of Cd-induced apoptosis in neuronal cells. PC12 cells and primary neurons, or PC12 cells infected with Ad-dn-c-Jun or Ad-GFP (as control), respectively, were pretreated with/without SP600125 (20 μ M) for 1 h and then metformin (1 mM) for 24 h, or pretreated with/without metformin for 24 h, followed by exposure to Cd (10 μ M) for 4 h (for Western blotting) or 24 h (for DAPI staining). A and D) Total cell lysates were subjected to Western blotting using indicated antibodies. The blots were probed for β -tubulin as a loading control. Similar results were observed in at least three independent experiments. B and E) The relative densities for p-JNK to JNK, and p-c-Jun, cleaved-caspase-3 to β -tubulin were semi-quantified using NIH image J. C and F) Apoptotic cells were evaluated by nuclear fragmentation and condensation using DAPI staining. Results are presented as mean $\pm$ SEM (n = 3–5). Using one-way ANOVA, * p < 0.05 for difference with no metformin group, ^a p <

0.05 for difference with no Cd group, ^b $p < 0.05$ for difference with no SP600125 group;
Using Student's *t*-test, ^c $p < 0.05$ for Ad-dn-c-Jun group versus Ad-GFP group.

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Fig. 5.

Metformin blocks Cd activation of JNK/c-Jun-dependent neuronal apoptosis by suppressing Cd inactivation of PP5. PC12 cells, SH-SY5Y cells and primary neurons, or PC12 cells infected with Ad-PP5 or Ad-GFP (as control), respectively, were pretreated with/without metformin (1 mM) for 24 h, followed by exposure to Cd (10 and/or 20 μ M) for 4 h (for Western blotting) or 24 h (for MTS assay and DAPI staining). A and C) Total cell lysates were subjected to Western blotting using indicated antibodies. The blots were probed for β -tubulin as a loading control. Similar results were observed in at least three independent experiments. B and D) The relative densities for p-JNK to JNK, and PP5, p-c-Jun, cleaved-caspase-3 to β -tubulin were semi-quantified using NIH image J. E) Relative cell viability was evaluated by MTS assay. F) Apoptotic cells were evaluated by nuclear fragmentation and condensation using DAPI staining. Results are presented as mean $\pm$ SEM (n = 3–5). Using one-way ANOVA, $^*p < 0.05$ for difference with no metformin group, $^ap < 0.05$ for difference with no Cd group, $^bp < 0.05$ for difference with 10 μ M Cd group; Using Student's *t*-test, $^cp < 0.05$ for Ad-PP5 group versus Ad-GFP group.

Fig. 6. Metformin prevents Cd activation of JNK/c-Jun-dependent neuronal apoptosis by repressing Cd inactivation of AMPK. PC12 cells, SH-SY5Y cells and/or primary neurons, respectively, were pretreated with/without metformin (1 mM) for 24 h, or pretreated with/without AICAR (2 mM) for 1 h and then metformin for 24 h, followed by exposure to Cd (10 and/or 20 μM) for 4 h (for Western blotting) or 24 h (for MTS assay and DAPI staining). A and C) Total cell lysates were subjected to Western blotting using indicated antibodies. The blots were probed for β-tubulin as a loading control. Similar results were observed in at least three independent experiments. B and D) The relative densities for p-AMPKα to AMPK, p-ACC to ACC, p-JNK to JNK, and p-c-Jun, cleaved-caspase-3 to β-tubulin were semi-quantified using NIH image J. E) Relative cell viability was evaluated by MTS assay. F) Apoptotic cells were evaluated by nuclear fragmentation and condensation using DAPI staining. Results are presented as mean ± SEM (n = 3–5). Using one-way ANOVA, * $p < 0.05$ for difference with no metformin group, ^a $p < 0.05$ for difference with no Cd group, ^b $p < 0.05$ for difference with 10 μM Cd group, ^c $p < 0.05$ for difference with no AICAR group.

Fig. 7.

Ectopic expression of dominant negative AMPKα or continuous-active AMPKα affects metformin preventing Cd activation of JNK pathway and apoptosis in neuronal cells. PC12 cells, infected with Ad-dn-AMPKα, Ad-AMPKα-ca or Ad-GFP (as control), respectively, were pretreated with/without metformin (1 mM) for 24 h, followed by exposure to Cd (10 μM) for 4 h (for Western blotting) or 24 h (for DAPI staining). A and D) Total cell lysates were subjected to Western blotting using indicated antibodies. The blots were probed for β-tubulin as a loading control. Similar results were observed in at least three independent experiments. B and E) The relative densities for p-AMPKα to AMPK, p-ACC to ACC, p-JNK to JNK, and p-c-Jun, cleaved-caspase-3 to β-tubulin were semi-quantified using NIH image J. C and F) Apoptotic cells were evaluated by nuclear fragmentation and condensation using DAPI staining. Results are presented as mean ± SEM (n = 3–5). Using one-way ANOVA, **p* < 0.05 for difference with no metformin group, ^a*p* < 0.05 for difference with no Cd group; Using Student's *t*-test, ^b*p* < 0.05 for Ad-dn-AMPKα group versus Ad-GFP group, ^c*p* < 0.05 for Ad-AMPKα-ca group versus Ad-GFP group.

Fig. 8.

NAC potentiates metformin's protection against Cd-induced ROS-dependent PP5/AMPK inactivation, JNK activation and apoptosis in neuronal cells. PC12 cells, SH-SY5Y cells and/or primary neurons, respectively, were pretreated with/without metformin (1 mM) for 24 h, or pretreated with/without NAC (5 mM) for 1 h and then metformin for 24 h, followed by exposure to Cd (10 and/or 20 μ M) for 4 h (for Western blotting) or 24 h (for cell ROS imaging and DAPI staining). A-C) Cell ROS was imaged and quantified using an oxidant-sensitive probe CM-H2DCFDA, showing that Cd-evoked abnormal ROS fluorescence (in green) was potently suppressed by metformin in the cells. Scale bar: 20 μ m. D) Apoptotic cells were evaluated by nuclear fragmentation and condensation using DAPI staining. E) Total cell lysates were subjected to Western blotting using indicated antibodies. The blots

were probed for β -tubulin as a loading control. Similar results were observed in at least three independent experiments. F) The relative densities for p-AMPK α to AMPK, p-ACC to ACC, p-JNK to JNK, and PP5, p-c-Jun, cleaved-caspase-3 to β -tubulin were semi-quantified using NIH image J. Results are presented as mean $\pm$ SEM (n = 3–5). Using one-way ANOVA, * p < 0.05 for difference with no metformin group, ^a p < 0.05 for difference with no Cd group, ^b p < 0.05 for difference with 10 μ M Cd group, ^c p < 0.05 for difference with no NAC group.

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Fig. 9.

Metformin prevents Cd-induced mitochondrial ROS-dependent PP5/AMPK inactivation, JNK activation and apoptosis in neuronal cells. PC12 cells and primary neurons were pretreated with/without Mito-TEMPO (10 μ M) for 1 h and then metformin (1 mM) for 24 h, followed by exposure to Cd (10 μ M) for 4 h (for Western blotting) or 24 h (for cell ROS imaging and DAPI staining). A) Cell ROS was imaged and quantified using an oxidant-sensitive probe CMH2DCFDA. B) Apoptotic cells were evaluated by nuclear fragmentation and condensation using DAPI staining. C) Total cell lysates were subjected to Western blotting using indicated antibodies. The blots were probed for β -tubulin as a loading control. Similar results were observed in at least three independent experiments. D) The relative densities for p-AMPK α to AMPK, p-ACC to ACC, p-JNK to JNK, and PP5, p-c-Jun, cleaved-caspase-3 to β -tubulin were semi-quantified using NIH image J. Results are presented as mean $\pm$ SEM (n = 3–5). Using one-way ANOVA, * p < 0.05 for difference with no metformin group, ^a p < 0.05 for difference with no Cd group, ^b p < 0.05 for difference with no Mito-TEMPO group.

Fig. 10.

A graphical model of how metformin protects against Cd-induced neuronal apoptosis. Metformin attenuates Cd-elicited neuronal apoptosis by preventing mitochondrial ROS inactivation of PP5 and AMPK, thus suppressing activation of JNK pathway.