Cytochrome *c*-binding "*proteo-dendrimers*" as new types of apoptosis inhibitors working in HeLa cell systems†

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The suppressive effects of synthetic dendrimers on mitochondrial apoptosis were first demonstrated in human epithelial carcinoma HeLa cells. The employed proteo-dendrimers included polyanionic hepta(glutamic acids), a fluorescent zinc porphyrinate core, hydrophilic polyether surface and nonpeptide hydrophobic dendrons, and electrostatically interacted with cytochrome *c* in aqueous solutions. The ceramide analogue, (2*S*,4*E*)-2-acetylamino-3-oxo-4-octadecen-1-ol (**C2-ketoCer**) induced mitochondrial apoptosis into HeLa cells and the cell viability was significantly recovered by pretreatment with some dendrimers. Among a series of proteo-dendrimers, the second-generation dendrimer **2a** had the lowest cytotoxicity and the highest solubility. When the cells were treated with this dendrimer, a decrease in the protein levels of active caspase-3 and proteolytically cleaved PARP was remarkably observed. Since cytochrome *c* release from the mitochondria to the cytoplasm was unaffected in the presence of dendrimer **2a**, the observed suppressive effects probably indicate that the proteo-dendrimer trapped cytochrome *c*, not only in the aqueous solutions but also in the living cells.

Introduction

Apoptosis is an evolutionary conserved process of preprogrammed and highly regulated cell death. This critically occurs during development and tissue homeostasis, but excess apoptosis accounts for the pathogenesis of a variety of human disorders such as hepatitis, spinal cord injury and Parkinson's disease.[1] Mostly, apoptotic signaling processes converge on the mitochondria and the pro-apoptotic members of the Bcl-family (Bak, Bad, Bax and Bid) are activated by various apoptotic stimuli through the mitochondrial signaling pathway.[2] During apoptosis by various stimuli such as UV irradiation, anticancer drugs and oxidative stress, cytochrome *c*, AIF, endo G, and Smac/DIABLO are released from the mitochondrial intermembrane space to the cytosol. The released cytochrome *c*, together with the adapter protein Apaf-1 and dATP (ATP), is required for the activation of pro-caspase-9 in the death inducing complex called the "Apoptosome", while caspase-9 triggers the effector caspase cascade that is required for the execution of apoptosis.[3]

In this work, we examine the ability of synthetic dendrimers as new types of anti-apoptotic reagents using human epithelial carcinoma HeLa cells. Cytochrome *c* has a positively charged region composed of four protonated lysines and forms a stable 1 : 1 supramolecular complex with cytochrome b_5 having several carboxylate anions on its surface. Recently, we reported a new series of "proteo-dendrimer" receptors of the polycationic cytochrome *c* (Fig. 1, **1a**–**4a**).[4] These dendrimers comprise a zinc porphyrinate core, a hydrophilic polyether surface, a hydrophobic inner environment, and eight asymmetrically distributed polyanionic -CO_2^- groups to interact with cytochrome *c*. The proteo-dendrimers **2a** and **3a** formed 1 : 1 and 1 : 2 complexes (cytochrome *c* : dendrimer)

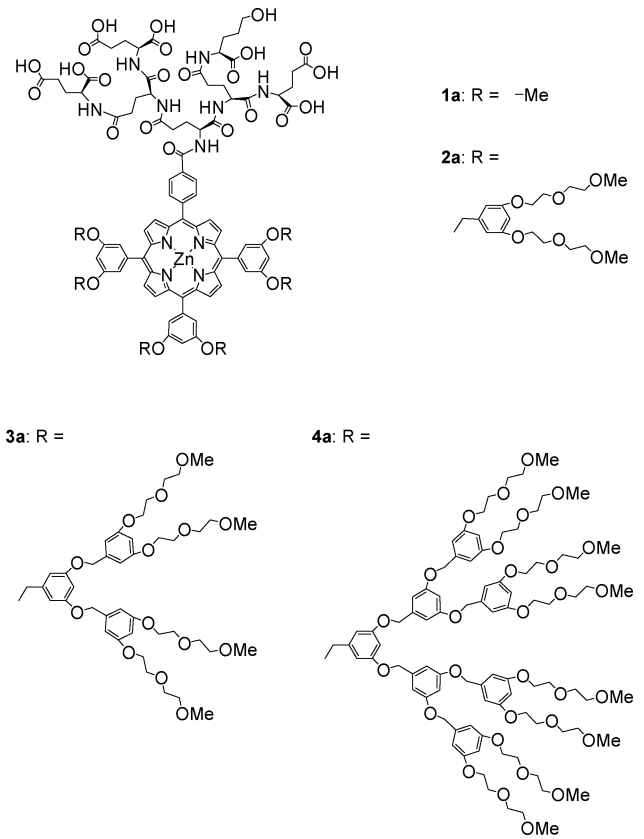

Fig. 1 Structures of proteo-dendrimers.[4]

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with horse heart cytochrome *c* and 4th generation dendrimer **4a** formed only 1 : 1 complex. Furthermore, some dendrimers formed more stable complexes than cytochrome b_5 at neutral pH, by competitive binding experiment. Therefore, it is likely that these dendrimers trap the released cytochrome *c* from mitochondria induced by apoptotic stimuli and inhibit the downstream signaling for the formation of apoptosome complex.

We characterize below the inhibition effects of the proteo-dendrimers on the ceramide analogue-induced mitochondrial apoptosis into HeLa cells. The ceramides (*N*-acyl-D-*erythro*-sphingosines), the metabolites or precursors of sphingolipids, are an important second messenger involved in mediating the apoptosis.[5] Some biosubstances and environmental stresses, such as the anti-Fas antibody,[6] TNF-α[7] and ionizing radiation,[8] promote intracellular ceramide generation by enhancing the mitochondrial sphingomyelinase-mediated hydrolysis of sphingomyelin.[9] Some exogenous cell-permeable ceramides such as *N*-acetyl- (**C2-Cer**) and *N*-octanoyl-D-*erythro*-sphingosine (**C8-Cer**) induce mitochondrial apoptosis, and are widely used as standard tools for the study of apoptosis.[10] Recently, we demonstrated that short-chain 3-oxo ceramide ((2*S*,4*E*)-2-acetylamino-3-oxo-4-octadecen-1-ol, **C2-ketoCer**) has a high apoptotic activity against human leukemia HL-60 cells compared with **C2-Cer**.[11] **C2-ketoCer** induced apoptosis *via* the mitochondrial signaling pathway and the antioxidant glutathione, one of the most popular ROS scavengers, pretreatment was able to largely suppress the leakage of cytochrome *c* from mitochondria and caspase-3 activation. We observe below the significant suppressive effects of the proteo-dendrimers on mitochondrial apoptosis using human epithelial carcinoma HeLa cells. This is the first example of synthetic dendrimers that worked as effective apoptosis inhibitors.

Results and discussion

Apoptogenic activity of C2-ketoCer against HeLa cells

We investigated whether **C2-ketoCer** acted as a potent mitochondrial apoptosis inducer against HeLa cells. Evaluation of the cell-death inducing activity of **C2-ketoCer** was made by an MTT assay. After the HeLa cells were treated with 10 μM of **C2-ketoCer** for various periods, the cell viabilities were represented as a percentage of living cells compared to the untreated control cells. As Fig. 2 shows, the percentage of cell viability decreased in a time-dependent manner and about 90% of the cells were brought to death in 6 hours.

Caspases, a family of aspartate-specific cysteine proteases, play a crucial role in apoptotic cell death by cleaving a specific site of numerous cellular targets in the execution phase; in particular, caspase-3 was activated by the proteolytic processing of pro-caspase-3 in response to exogenous apoptosis inducers.[12] To determine if the **C2-ketoCer**-induced cell death process for the HeLa cell is apoptosis, we analyzed the level of caspase-3 activated in response to exogenously added **C2-ketoCer** by the Western blot analysis employing a polyclonal anti-caspase-3 antibody. Fig. 3A displays time courses of 2 to 6 hours for the generation of active caspase-3 induced by the addition of **C2-ketoCer** (10 μM), indicating that **C2-ketoCer** activated caspase-3 within 2 hours. We also investigated whether poly(ADP-ribose) polymerase (PARP), a proteolytic target of the activated caspase-3, was cleaved in the

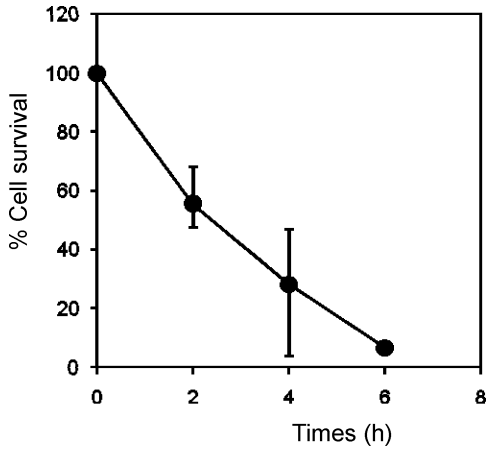

Fig. 2 Effect of short-chain 3-ketoceramide on viability of HeLa cells. Cells were treated with 10 μM **C2-ketoCer** for the indicated time and their viability was measured by MTT assay.

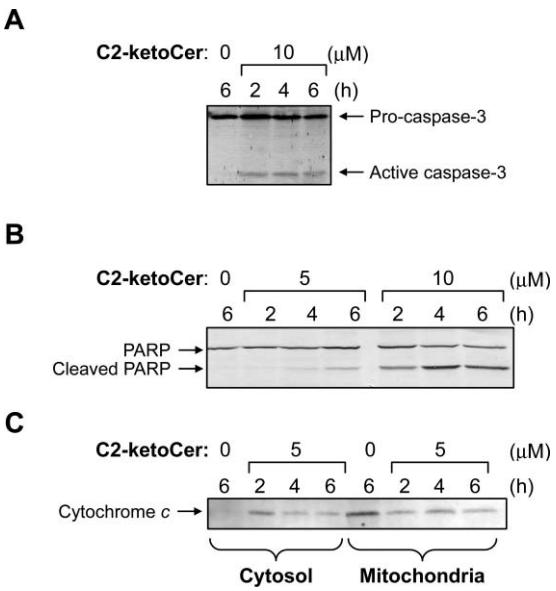

Fig. 3 Cytochrome *c* release, activation of caspase-3 and PARP cleavage induced by 3-ketoceramide. HeLa cells were treated with **C2-ketoCer** (A: 10 μM, B: 5 and 10 μM) for various periods (2–6 h). Each cell extract was subjected to Western blot analysis using *anti*-caspase-3 or *anti*-PARP antibody; (C) HeLa cells were treated with **C2-ketoCer** (5 μM) for the indicated times, and both the cytosolic and mitochondrial fractions were subjected to Western blot analysis using anti-cytochrome *c* antibody.

presence of **C2-ketoCer** (5 and 10 μM). As Fig. 3B indicates, the cleaved PARP was obviously detected even at concentrations of 5 μM in 4 hours.

In order to determine the contribution of the mitochondrial pathway when HeLa cells were treated with **C2-ketoCer**, both the cytosolic and mitochondrial fractions were subjected to Western blot analysis. Fig. 3C shows that the mitochondrial cytochrome *c* release from the mitochondria to the cytoplasm takes place after 2 hours treatment with 5 μM **C2-ketoCer**. Thus, the immunoblot demonstrates that **C2-ketoCer** potently induced mitochondrial apoptosis in HeLa-cells as well as human leukemia HL-60 cells.

Anti-apoptotic effect of the proteo-dendrimers

Fig. 4 compares the cytotoxicity of each proteo-dendrimer against HeLa cells. The dose-dependent decrease of cell viability was only observed in dendrimer **1a**-treated cells, while no remarkable cytotoxicity was detected in **2a**–**4a**-treated cells. Although our previous work has shown that dendrimers **2a** and **3a** have higher binding activity to cytochrome *c* than **1a** and **4a**, the solubility of **3a** in DMSO used as a vehicle was lower than that of **2a**, thus, dendrimer **2a** was selected to assay for the anti-apoptotic effects.

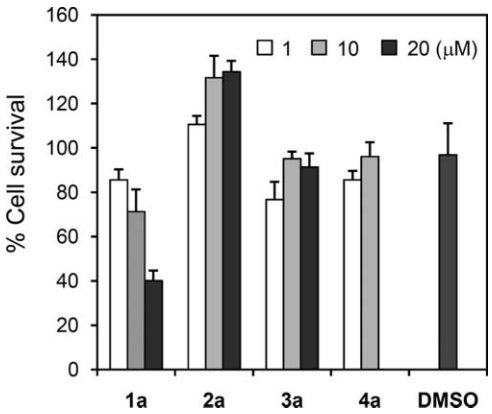

Fig. 4 Evaluation of cytotoxicity of proteo-dendrimers **1a**–**4a** on HeLa cells by WST assay. Cells were treated with indicated concentrations of each dendrimer for 6 h.

In the case of the mitochondrial pathway, caspase-3, an executor of apoptosis, is activated by formation of the apoptosome complex followed by caspase-9 activation. Since our previous data showed that dendrimer **2a** has an ability to bind to horse heart cytochrome *c* in pH 7 phosphate buffer, dendrimer **2a** is expected to act as an inhibitor of caspase cascade to trap the released cytochrome *c* and inhibit the formation of the apoptosome complex. Two commercially available caspase inhibitors, Ac-LEHD-MCA (a caspase-9 inhibitor) and Ac-DEVD-MCA (a caspase-3 inhibitor) were also used for comparison. The HeLa cells were pretreated with Ac-LEHD-MCA (10 μM), Ac-DEVD-MCA (10 μM) or dendrimer **2a** (10 μM) for 30 min and then treated with **C2-ketoCer** (5 μM) for 4 hours. As Fig. 5A indicates, Ac-LEHD-MCA and Ac-DEVD-MCA attenuated the **C2-ketoCer**-induced cell death and the former was more effective than the latter. Dendrimer **2a** was confirmed to dose-dependently inhibit the **C2-ketoCer**-induced cell death (Fig. 5B).

The released cytochrome *c* induces the formation of a caspase-9 activating complex, apoptosome with Apaf-1, dATP and pro-caspase-9. The binding of cytochrome *c* and dATP (ATP) to the twelve WD-40 repeats and nucleotide-binding domain of Apaf-1, respectively, induces conformational changes of Apaf-1 and allows pro-caspase-9 to form the apoptosome.[13] Various regulators of apoptosome formation have been reported.[14] For instance, heat shock proteins Hsp-70[15] and Hsp-90[16] inhibit the formation of the active complex by binding to Apaf-1, and the interaction of acetylcholinesterase with the released cytochrome *c* is required for the interaction between cytochrome *c* and Apaf-1.[17] Lademann *et al.* demonstrated that some diarylurea compounds prevent the formation of the apoptosome complex by preventing Apaf-1 oligomerization.[18]

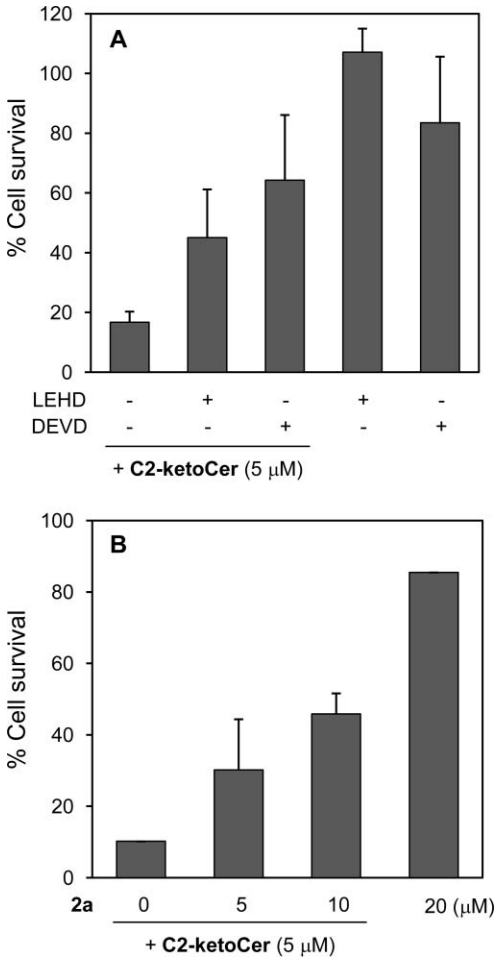

Fig. 5 Cell death suppressive effects of the commercially available caspase inhibitors and dendrimer **2a** on **C2-ketoCer**-induced apoptosis. Cells were pretreated with (A) Ac-LEHD-MCA (LEHD, 10 μM) and Ac-DEVD-MCA (DEVD, 10 μM), or (B) dendrimer **2a** (5 or 10 μM) for 30 min and then treated with **C2-ketoCer** (5 μM) for 4 hours and their viability was measured by WST assay.

An immunoblot analysis was carried out to examine whether dendrimer **2a** acts downstream of the released cytochrome *c* signaling in mitochondrial apoptotic processes, and the results are displayed in Fig. 6 and 7. Fig. 6 indicates that pretreatment of cells with 10 μM dendrimer **2a** indeed decreased both the levels of active caspase-3 and proteolytically cleaved PARP. Since Fig. 7

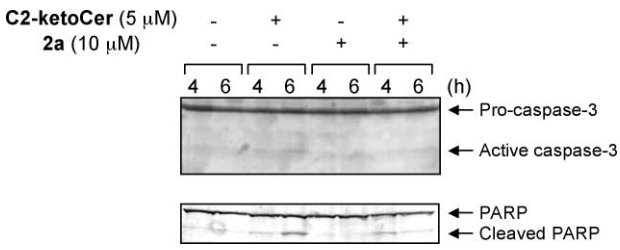

Fig. 6 Inhibition effect of 3-ketoceramide-induced caspase-3 activation and PARP cleavage by treatment with dendrimer **2a**. HeLa cells were pretreated with dendrimer **2a** (10 μM) for 30 min and then incubated with **C2-ketoCer** (5 μM). Each cell extract was subjected to Western blot analysis using *anti*-caspase-3 or *anti*-PARP antibody.

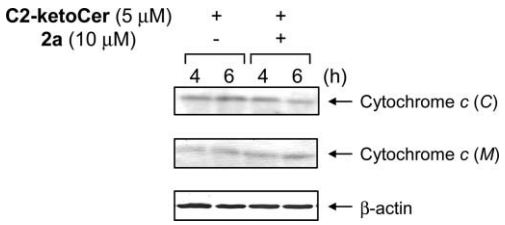

Fig. 7 Inhibition effect of 3-ketoceramide-induced cytochrome *c* release from mitochondria to cytosol by treatment with dendrimer **2a**. HeLa cells were pretreated with dendrimer **2a** (10 μM) for 30 min and then incubated with **C2-ketoCer** (5 μM) for various periods, and both the cytosolic (*C*) and mitochondrial (*M*) fractions were subjected to Western blot analysis using anti-cytochrome *c* antibody.

indicates that the cytochrome *c* release from the mitochondria to the cytoplasm was unaffected by the presence of dendrimer **2a**, dendrimer **2a** was demonstrated to suppress caspase-3 activation after cytochrome *c* release. Although we did not find direct evidence to support that dendrimer **2a** prevents the formation of the apoptosome complex, dendrimer **2a** could suppress caspase-3 activation after cytochrome *c* release. The trapping of the released cytochrome *c* by the dendrimer followed by inhibition of the binding of cytochrome *c* to the WD-40 repeats of Apaf-1 and down-regulation of a downstream caspase cascade were strongly suggested.

Conclusion

In summary, the present results demonstrate for the first time that synthetic dendrimer-type receptors have the ability to protect against mitochondrial apoptosis. Bcl-family proteins are well known to regulate mitochondrial cytochrome *c* release and are considered as attractive targets for cancer therapy. The present proteo-dendrimers are considered as new types of apoptosis inhibitors and also promising candidates as therapeutic agents for certain apoptosis-mediated diseases such as hepatitis.

Experimental section

All materials used were obtained commercially (guaranteed reagent grade). Proteo-dendrimers **1a–4a**[4] and **C2-ketoCer**[11] were prepared according to our previous report. Protease inhibitors, *Z*-VAD-fmk and Ac-IETD-CHO, were purchased from the Peptide Institute (Osaka, Japan) and Sigma Chemical Co. (St. Louis, MO, USA), respectively. Polyclonal antibody for caspase-3 and monoclonal antibody for cytochrome *c* were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal antibodies for PARP and for β-actin were purchased from Trevigen (Gaithersburg, MD, USA) and Abcam (Cambridge, UK), respectively. Anti-rabbit and anti-mouse IgGs coupled to alkaline phosphatase were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture

HeLa cells (human epithelial carcinoma) were grown in RPMI 1640 medium containing 10% heat-incubated fetal bovine serum at 37 °C with 5% CO_2 atmosphere. **C2-ketoCer** was dissolved in EtOH at 5 or 10 mM, and added to the cells as EtOH solutions. Proteo-dendrimers were dissolved in DMSO at 0.5–20 mM. The final concentrations of both EtOH and DMSO were 0.1%, respectively. Control experiments were performed with EtOH (0.1%) and DMSO (0.1%) as the vehicle.

MTT assay

Cell death inducing activity of **C2-ketoCer** was assessed by the reduction of MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide], as described in our previous paper (17321747). Briefly, HeLa cells were seeded at a density of 10^4 cells per well in 100 μL of the growth medium in 96-well plates. Following 24 h of incubation the medium was removed and washed in PBS, then replaced with fresh serum-free mediums containing **C2-ketoCer** (10 μM) or the proteo-dendrimers (0.5–20 μM). 10 μL of 5 mg mL^{-1} MTT was added to each well 2 h before the end of the culture, and the reaction was stopped by adding 100 μL of 0.04 M HCl in isopropanol. Two optical densities of the mixture were measured at 570 nm and 650 nm. Final values were obtained by subtracting 650 nm from 570 nm. All data were the average of at least three separate determinations.

WST assay

HeLa cells were seeded at a density of 10^4 cells per well in a 96-well microtiter plate. Following 24 h of incubation the medium was removed and washed in PBS, then replaced with fresh serum-free mediums containing the proteo-dendrimers **1a–4a** (1–20 μM). The cells were further incubated for 4 h and then treated with a Cell Counting Kit-8® (Dojindo, Kumamoto, Japan) for 2 h. Cell viability was estimated from the absorbance of medium at 620 and 450 nm. The percentage of cell viability in the absence of a polyelectrolyte was normalized to 100%. All data were the average of at least three separate determinations.

For the apoptosis inhibition assay, HeLa cells were preincubated in serum-free mediums including Ac-LEHD-MCA (10 μM, a caspase-9 inhibitor), Ac-DEVD-MCA (10 μM, a caspase-3 inhibitor) and proteo-dendrimer **2a** (10 μM) for 30 min and then treated with **C2-ketoCer** (5 μM) for various periods.

Immunoblot analysis for whole cell extraction

HeLa cells were seeded at a density of 0.5×10^6 cells per well in 1 mL of the growth medium in 6-well plates and incubated for 24 h. Two wells were used per sample. Following 24 h of incubation the medium was removed and washed in PBS, then replaced with fresh serum-free mediums containing **C2-ketoCer** (1–10 μM) for various periods. After incubation for various periods, cells were collected and centrifuged at 400 × *g* for 5 min at 4 °C, washed twice with phosphate buffer (pH 7.4) and lysed in 100 mL of lysis buffer [62.5 mM Tris-HCl (pH 6.8), 6 M urea, 10% glycerol, 2% SDS, 0.00125% bromophenol blue, 5% β-mercaptoethanol]. Cell lysates were boiled for 3 min and separated on 8% (for PARP) or 14% (for caspase-3) SDS-polyacrylamide gels, transferred to a polyvinylidene difluoride membrane, and probed with rabbit polyclonal anti-caspase-3 antibody or monoclonal anti-PARP antibody, followed by probing with goat anti-rabbit antibody (for caspase-3) or anti-mouse antibody (for PARP) coupled to alkaline phosphatase.

For the apoptosis inhibition assay, HeLa cells were preincubated in serum-free medium including proteo-dendrimer **2a** (10 μM) for 30 min and then treated with **C2-ketoCer** (5 μM) for various periods.

Preparation of cytosolic and mitochondrial fractions and immunoblot analysis

We prepared cytosolic and mitochondrial fractions according to Frey's method.[19] Briefly, HeLa cells (0.5×10^6 cells per mL) were treated as described above although three wells were used per sample. After incubation for various periods, cells were collected and centrifuged at $400 \times g$ for 5 min at 4 °C, and washed twice with PBS. Cells were then treated with a cell extraction buffer (80 mM KCl, 250 mM sucrose, 0.5 mg ml^{-1} digitonin, 1 mM DTT, including protease inhibitor cocktail, 300 μL) for 5 min and centrifuged for 5 min at $10\,000 \times g$ at 4 °C. The supernatant was centrifuged at $10\,000 \times g$ to obtain the cytosolic fraction. The cell pellet was washed once with a cell extraction buffer and then solubilized in a lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EGTA, 300 μL]. Cytosolic and mitochondrial fractions remained frozen in tubes at −80 °C until use. The total amounts of protein in each sample were measured and adjusted with a BCA® Protein Assay Kit (Thermo Fisher Scientific, USA). Western blot analysis was carried out by using mouse monoclonal anti-cytochrome *c* antibody, as described above.

Acknowledgements

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