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BRIEF COMMUNICATION

Pro-caspase-3 protects cells from polymyxin B-induced cytotoxicity by preventing ROS accumulation

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Abstract

Polymyxin B (PMB), a last-line antibiotic used against antibiotic-resistant superbugs, causes undesirable cytotoxic side effects. However, its mechanisms remain unknown. In this study, we unexpectedly found that caspase-3, a main executor of apoptosis, plays a protective role in PMB-induced cytotoxicity. Caspase-3 knockout (KO) cells exhibited higher susceptibility to PMB-induced cytotoxicity compared with wild-type (WT) cells, accompanied by increased levels of reactive oxygen species (ROS). Interestingly, co-treatment with the antioxidant *N*-acetylcysteine (NAC) rescued cell viability to a similar extent as WT cells. Furthermore, PMB failed to facilitate the processing of inactive caspase-3 (pro-caspase-3) into active forms, suggesting that pro-caspase-3 nonenzymatically suppresses PMB-driven ROS accumulation and its cytotoxicity. Thus, our findings that demonstrate the potential ability of PMB to stimulate ROS generation, but which is normally masked by pro-caspase-3-dependent mechanisms, may provide novel insights into the mechanisms of PMB-induced side effects.

Polymyxin B (PMB), a polypeptide antibiotic that is approved for the treatment of Gram-negative bacterial infections, exerts antibiotic activity by disrupting bacterial membrane integrity [1–3]. It is known that PMB is one of the few drugs that are active against multidrug-resistant Gram-negative bacteria such as *Pseudomonas aeruginosa* [4, 5]. On the other hand, it is widely known that PMB causes undesirable cytotoxic side effects including nephrotoxicity [6, 7]. A possible mechanism to explain the PMB-induced cytotoxicity is that PMB stimulates the activation of caspase-mediated apoptotic pathways [8]. However, the PMB-induced cellular responses associated with its cytotoxicity are largely unknown. In this study, we thus investigated the cellular responses induced by PMB to understand the precise mechanisms of PMB-induced cytotoxicity.

At first, to confirm previous studies that have demonstrated that PMB induces cytotoxicity [8], we performed the cell viability assays in various culture cell lines, as previously described [9]. As shown in Fig. 1a, PMB exhibited decreased viability of all cell lines, including human embryonic kidney (HEK) 293A cells, mouse embryonic fibroblasts (MEF), and human fibrosarcoma HT1080 cells, in a dose-dependent manner. We next assessed whether the reduction of cell viability is responsible for growth inhibition or cytotoxicity. Lactate dehydrogenase (LDH) activity assay is commonly used to evaluate cytotoxicity [10], which revealed that PMB causes cytotoxicity in a dose-dependent manner (Fig. 1b). To verify the results of past paper demonstrating that PMB induces cytotoxicity through apoptosis mediated by the caspase cascade [8], we examined whether PMB activates caspases by evaluating the caspase activation using immunoblot analysis as previously described [11]. In general, caspases are present in enzymatically inactive forms called pro-caspases, and the activation of caspases are induced by cleavage of pro-caspases into active forms [12]. However, we could not detect the cleaved (activated) forms of representative caspases such as

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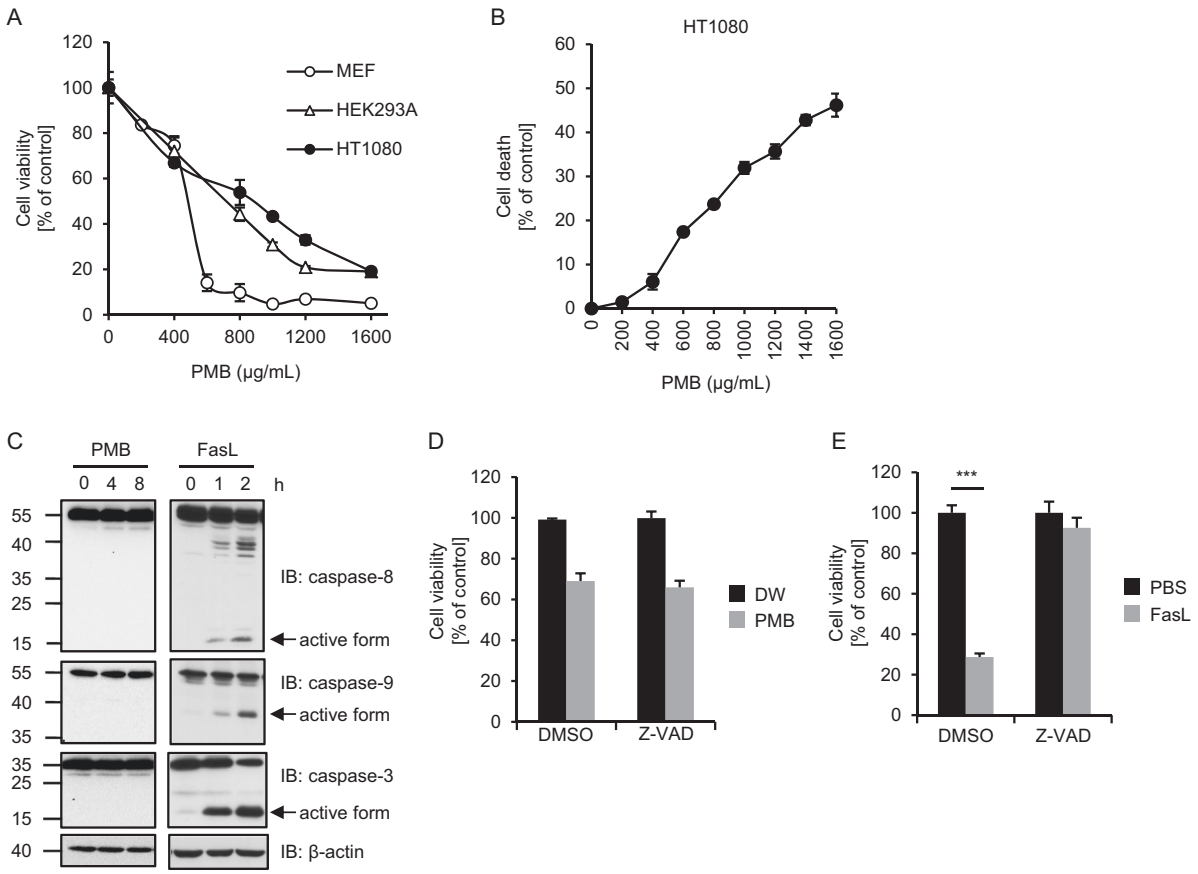
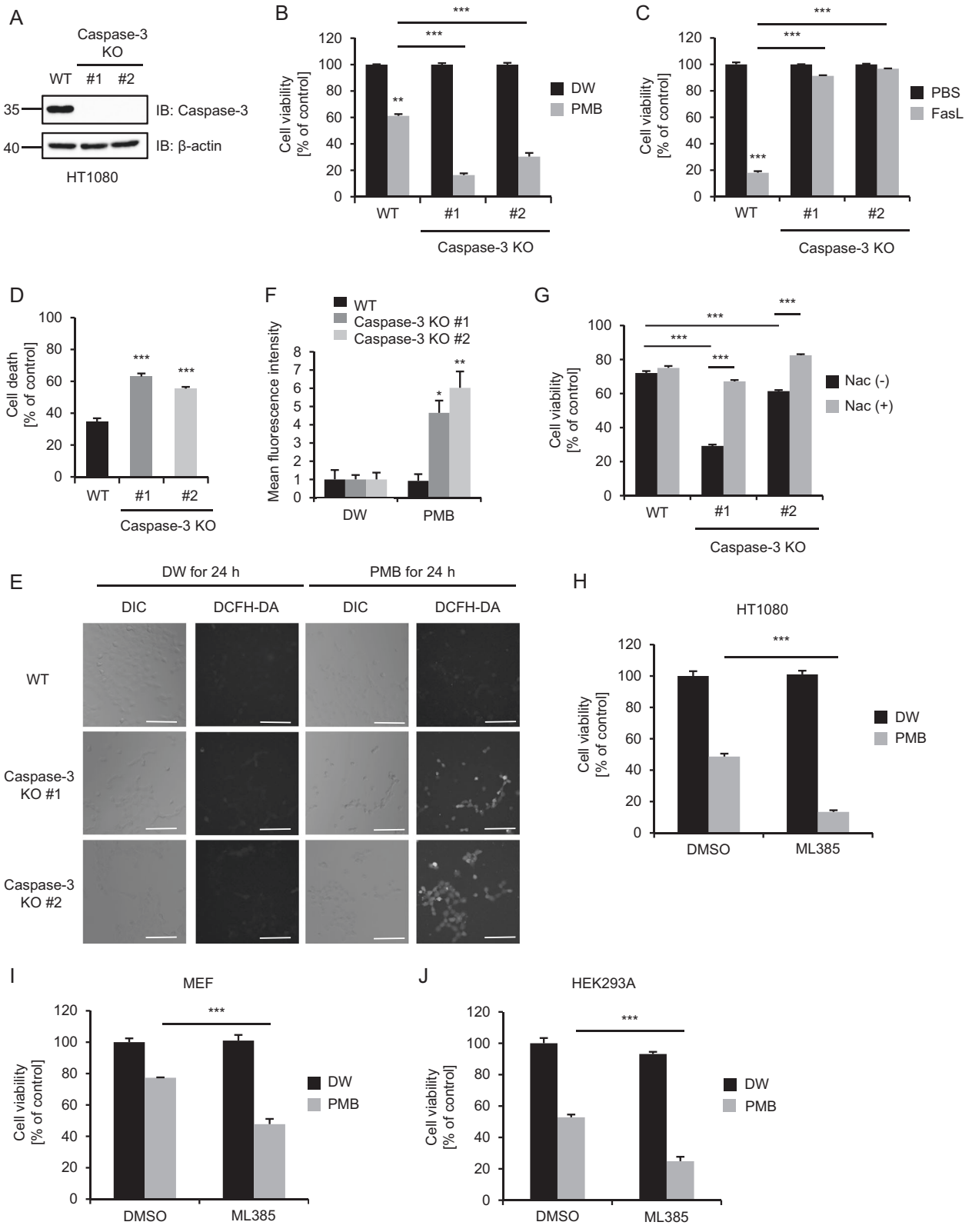


Fig. 1 **a**, **b** Cells were treated with the indicated concentrations of PMB for 24 h, and then subjected to cell viability assay (**a**) or LDH assay (**b**). **c** HT1080 cells were treated with 800 μg ml^{-1} PMB or 50 ng ml^{-1} FasL for indicated periods, and then immunoblot analysis was performed with the indicated antibodies. **d**, **e** HT1080 cells were treated with 800 μg ml^{-1} PMB for 24 h or 50 ng ml^{-1} FasL for 16 h with or without 20 μM Z-VAD-fmk, and then subjected to cell viability assay. DW; distilled water. DMSO; dimethyl sulfoxide. PBS; phosphate-buffered saline. **a**, **b**, **d**, **e** Data shown are the mean ± SD. Significant differences were determined by two-way ANOVA, followed by Tukey–Kramer test; ***$p < 0.001$. All data are representative of at least three independent experiments. Experimental procedures are described in supporting information

caspase-3, -8, and -9, whereas Fas/CD95 ligand (FasL), a cytotoxic cytokine that causes apoptosis, clearly induced the cleavage (activation) of these caspases (Fig. 1c). Moreover, the pan-caspase inhibitor Z-VAD-fmk actually failed to suppress PMB-induced cytotoxicity (Fig. 1d), unlike FasL-treated cells (Fig. 1e). These observations show that apoptosis is not induced at least under our experimental condition, and thus we concluded that not only apoptosis but also other types of cell death including non-programmed cell death (necrosis, etc.) are involved in PMB-induced cytotoxicity.

To further evaluate the involvement of apoptosis in PMB-induced cytotoxicity, we established caspase-3 knockout (KO) HT1080 cells by using CRISPR/Cas9 system [13], and confirmed the loss of caspase-3 protein (Fig. 2a). Given that apoptosis is not responsible for PMB-induced cytotoxicity, we predicted that the extent of cytotoxicity might not be altered in caspase-3 knockout (KO) cells. However, we unpredictably found that caspase-3 KO HT1080 cells displayed significantly reduced viability in response to PMB (Fig. 2b), whereas they were highly resistant to FasL-mediated apoptosis, which is a reasonable result (Fig. 2c). Moreover, LDH assay revealed that PMB-induced cytotoxicity is exacerbated by caspase-3 knockout (Fig. 2d). These observations suggest that caspase-3 plays a protective role in PMB-induced cytotoxicity. Importantly, since PMB failed to cleave caspase-3 into the active form shown in Fig. 1b, pro-caspase-3 may nonenzymatically exert its protective functions against PMB. Recent evidence has strikingly demonstrated the non-apoptotic functions of pro-caspase-3 that contribute to cell survival [14, 15]. Notably, it turned out that pro-caspase-3 plays a role in mitochondrial homeostasis, and the loss of pro-caspase-3 leads to increased levels of reactive oxygen species (ROS) due to mitochondrial dysfunction [14]. On the other hand, recent evidence has implied the involvement of ROS in the toxicity of PMB [16]. We, therefore, speculated that the accelerated ROS generation exacerbates



the PMB cytotoxicity in caspase-3 KO HT1080 cells, and performed microscopic analysis using the ROS indicator 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA) as previously described [13]. As expected, PMB enhances ROS generation in caspase-3 KO HT1080 cells when compared with WT HT1080 cells (Fig. 2e, f). Moreover, we

◀ **Fig. 2** **a** HT1080 cells were subjected to immunoblotting with the indicated antibodies. **b–d** HT1080 cells were treated with 800 μg ml^{-1} PMB for 24 h (**b**, **d**) or 50 ng ml^{-1} FasL for 14 h (**c**), and then subjected to cell viability assay (**b**, **c**) or LDH assay (**d**). DW; distilled water. PBS; phosphate-buffered saline. **e**, **f** HT1080 cells were treated with 1 mg ml^{-1} PMB for 24 h, and then treated with 10 μM DCFH-DA. Fluorescence images (**e**) and intensity (**f**) of HT1080 cells were acquired as described in supporting information. Cell morphology was determined by Nomarski differential interference contrast (DIC) microscopy. Scale bar, 50 μm. DW; distilled water. **g** HT1080 cells were treated with 1 mg ml^{-1} PMB for 24 h with or without 1 mM NAC, and then subjected to cell viability assay. DW was used as a negative control for PMB and NAC. **h–j** HT1080 cells (**h**), MEFs (**i**), or HEK293A cells (**j**) were treated with 800 μg ml^{-1} (**h**, **j**) or 500 μg ml^{-1} (**i**) PMB for 24 h in the presence or absence of 20 μM ML385, and then subjected to cell viability assay. DW; distilled water. DMSO; dimethyl sulfoxide. **b–d**, **f–j** Data shown are the mean ± SD. Significant differences were determined by one-way (**b–d**, **f**) or two-way (**g–j**) ANOVA, followed by Tukey–Kramer test; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$. All data are representative of at least three independent experiments. Experimental procedures are described in supporting information

found that the antioxidant *N*-acetylcysteine (NAC) can rescue the viability of caspase-3 KO HT1080 cells to a similar extent to WT HT1080 cells (Fig. 2g). Collectively, these observations show that PMB has the ability to cause ROS-mediated cytotoxicity, but which is normally masked by pro-caspase-3-dependent mechanisms that suppress ROS accumulation. On the other hand, NF-E2-related factor-2 (Nrf2) is a transcription factor that protects cells from ROS-mediated cytotoxicity [17], and its reactivity to ROS in HT1080 cells has been confirmed in our recent study (Suzuki M el al., unpublished data). Interestingly, ML385, the specific inhibitor of Nrf2, exacerbated PMB-induced cytotoxicity in not only HT1080 cells but also MEFs and HEK293A cells (Fig. 2h–j). These findings suggest that blocking of the Nrf2 activation by ML385 allows PMB-driven ROS accumulation regardless of cell types, which affects on the susceptibility to PMB.

In the present study, we demonstrate that pro-caspase-3 contributes to cell survival upon exposure to PMB. As shown in Fig. 2, loss of caspase-3 increased susceptibility to PMB due to ROS accumulation, indicating that the down-regulation or dysfunction of pro-caspase-3 exacerbates the side effects of PMB including nephrotoxicity. In this regard, a previous report has demonstrated that minocycline, a second-generation tetracycline, suppresses caspase-3 expression at mRNA levels [18]. Moreover, immunohistochemical analysis of caspase-3 expression in vivo has shown that expression levels of caspase-3 in the kidney glomeruli are relatively low [19]. These observations raise the possibility that chemical substances enriched by the urine concentration and lesser expression of caspase-3 in the kidney glomeruli affect the onset of the nephrotoxicity induced by PMB. Thus, although further studies are required for the elucidation of the mechanisms by which pro-caspase-3 suppresses PMB-induced ROS generation, our results uncovered novel functions of caspase-3 associated with PMB-induced cytotoxicity, which may help to elucidate the mechanism underlying the cytotoxicity of PMB.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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