Arachidonic Acid Enhances Caffeine-Induced Cell Death via Caspase-Independent Cell Death

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Caffeine is a globally consumed psychostimulant but can be fatal to cells at overdose exposures. Although caspase-dependent apoptosis plays a role in caffeine-induced cell death, the responsible intracellular signalling cascade remains incompletely understood. The cellular slime mould, Dictyostelium discoideum, does not possess caspase-dependent apoptotic machinery. Here, we observed that ablation of D. discoideum plaA, which encodes a phospholipase A2 (PLA2) homolog, leads to a decreased rate of cell death under high caffeine concentrations and to enhanced cell death with the addition of arachidonic acid. Moreover, the inhibition of PLA2 activity lead to a recovery of the survival rate in caspase-inhibited Hela cervical carcinoma cells under high caffeine concentrations, indicating that caffeine-induced cell death is enhanced via PLA2-dependent signalling. Our results indicate that arachidonic acid may be a general second messenger that negatively regulates caffeine tolerance via a caspase-independent cell death cascade, which leads to multiple effects in eukaryotic cells.

Results

Caffeine-induced PLA2 activation enhances cell death via a caspase-independent cell death pathway. High caffeine concentrations are cytotoxic to D. discoideum (Fig. 1a). However, we found that a mutant strain lacking plaA, which encodes a calcium-independent patatin-like phospholipase A2, showed less sensitivity to high caffeine concentrations (Fig. 1b). Since AA is a primary product of PLA2, we tested the effect of AA on caffeine tolerance in plaA mutants and observed that the addition of 20 μM AA significantly reduced caffeine tolerance in the null mutant, as well as in wild-type cells (Fig. 1c, d). Furthermore, AA production could be measured in wild-type cells upon stimulation with 20 mM caffeine, whereas no measurable AA was observed in plaA mutant cells (Fig. 1e), indicating that plaA is the gene responsible for AA production by caffeine stimulation. These observations indicate that caffeine activates D. discoideum PLA2 and that the resultant AA leads to the suppression of survival under high caffeine concentrations.

Next, we tested the tolerance to caffeine in Hela cervical carcinoma cells, a mammalian cell line culture. Hela cells were found to be more tolerant of caffeine than D. discoideum cells. Nonetheless, the survival rate of Hela cells gradually decreased with 25–50 mM caffeine treatment over the course of 24 h (Fig. 2a). Bromoenol lactone...
(BEL) is a widely used, general PLA2 inhibitor. We tested the survival of Hela cells in the presence of 50 mM caffeine with or without BEL. The addition of BEL significantly suppressed the caffeine-induced reduction in cell survival, indicating that the activation of PLA2 reduces cell viability under high caffeine concentrations (Fig. 2b). A genomic study has predicted that no caspase-dependent apoptosis cascade is present in D. discoideum. Hence, the caffeine-dependent intracellular signalling pathway in this organism is assumed to be independent of the caspase-dependent apoptotic signalling found in mammalian cells. Furthermore, stimulation with high concentrations of caffeine has been reported to cause a greater than 4-fold increase in AA levels in a mammalian culture cell-line.

Therefore, we measured the survival rate in Hela cells in the presence of the cell-permeable general caspase inhibitor, Q-VD-OPh (Fig. 2b). After 24-h incubation in a medium containing 50 mM caffeine, the survival rate was reduced to 21.8 % ± 6.2 % in the absence of this inhibitor but recovered to 75.9 % ± 5.1 % in its presence, indicating that caffeine-induced cell death involves a caspase-dependent apoptosis cascade, as previously described.

Further, in the presence of Q-VD-OPh, a significant increase in the survival rate was observed by co-incubation with BEL, suggesting that PLA2 activity also negatively contributes to survival under high concentrations of caffeine.
cAMP is not involved in the caffeine-resistant signalling pathway. RegA encodes a cAMP PDE that is responsible for cytosolic CAMP concentrations. The deletion mutant shows elevated intracellular cAMP and, thus, precocious development\textsuperscript{11}. When regA null cells were allowed to develop on caffeine-containing, non-nutrient agar plates, smaller but normal fruiting bodies were formed (Fig. 4a). This indicates that regA is also a suppressor gene that can rescue the caffeine-dependent inhibition of development in \textit{D. discoideum}, although the extent of the suppression was weaker than that of the \textit{plaA} null mutant. Next, to investigate whether regA is involved in survival tolerance under high caffeine concentrations, we measured the survival rate of the regA null mutant in the presence of 30 mM caffeine. Unlike the \textit{plaA} null mutant, the regA null mutant did not show a survival rate that was significantly different from that of wild-type cells (Fig. 4b). Furthermore, co-incubation with the cell-permeable cAMP analogue, 8-Br-cAMP, which can potentially behave like cAMP in intracellular spaces, did not improve the caffeine-induced cell death in wild-type (Fig. 4c), suggesting that the elevated intracellular cAMP in the regA null mutant is not the cause of the increased caffeine tolerance under high caffeine concentrations. Therefore, \textit{PLA2-AAA} was potentially participating in another molecular pathway to reduce the caffeine-induced cell death, in addition to its role in inhibiting intracellular cAMP PDE.

**Discussion**

Caffeine has been shown to induce apoptosis via the PI3K/Akt/mTOR pathway\textsuperscript{13}. Moreover, caffeine triggers autophagy through the inhibition of the PI3K/Akt/mTOR path pathway and activation of the ERK1/2 pathway. Recently, AA was reported to effectively activate both mTOR complex 1 (mTORC1) and mTORC2 in cultured breast cancer cells\textsuperscript{14}. Interestingly, AA-stimulated mTORC1 activation is independent of PI3-K, indicating that the AA produced upon caffeine stimulation causes mTORC1 activation. These observations suggest that complex cross-talk signalling occurs between AA and the PI3K/Akt/mTOR/p70S6K signalling pathways. Further investigations of the possible relationships between the two pathways are required to unravel the signalling cascade evoked by caffeine.

In this study, we show a novel function for \textit{PLA2-AAA} in caffeine-induced cell death via a caspase-independent cell death signalling pathway. Because caffeine is known as a potent enhancer of
Figure 3 | Effect of caffeine on the development of plaA null cells. (a) Development of wild type AX2 and plaA null mutants on 4 mM caffeine-containing, non-nutrient agar plates. Bar, 1 mm. (b, c, d) Spontaneous production of cAMP in wild type AX2 (filled circle) and plaA null mutant (filled triangle) cells without caffeine (b), with 4 mM caffeine (c), and with 4 mM caffeine and 20 μM AA (d). (e, f, g) cAMP production by 5.0 μM 2′-deoxy-cAMP stimulation with no cAMP PDE inhibitors (e), with 5 mM dithiothreitol (DTT), an extracellular cAMP PDE inhibitor (f), and with 3 mM 3-isobutyl-1-methylxanthine (IBMX), an intracellular cAMP PDE inhibitor (g) in wild type AX2 (filled circle) and plaA null mutant (filled triangle) cells. Data in b, c, and d are presented as a representative experiment in 3 independent experiments. Data in e, f, and g are presented as the mean ± s.d. of triplicated determinations of a representative experiment in 3 independent experiments. **P < 0.05, ***P < 0.001 (t-test).
anticancer drugs\textsuperscript{15}, it is important to note that strengthening the PLA\textsubscript{2}-AA cascade might lead to the enhancement of antitumor potency, without inducing the caspase-dependent apoptosis cascade. The molecular mechanism of how caffeine inhibits \textit{D. discoideum} development has been unravelled\textsuperscript{4}. In this study, we also show that the PLA\textsubscript{2}-AA cascade is at least partly involved in the inhibition of development by caffeine through elevating intracellular cAMP PDE activity, which did not appear to play a role in PLA\textsubscript{2}-AA enhanced cell death. A number of caffeine-related genes has been isolated in \textit{Saccharomyces pombe} in a genome-wide survey, but most of the resistant mutants were revealed to be constitutively activated for the oxidative stress-dependent \textit{pap1} pathway. No PLA\textsubscript{2} gene was isolated in the screen\textsuperscript{16}. AA is a well-known precursor of eicosanoids such as prostaglandin \textit{E} and \textit{F}, which are potent mediators of inflammation and immunity and which function as second messengers in the central nervous system\textsuperscript{17,18}. However, in \textit{D. discoideum}, no cyclooxygenase (COX) gene is present in the genome. This suggests that in \textit{D. discoideum}, AA itself may play a role as a potent second messenger in the caffeine-induced signalling pathway, while in a mammalian system, both activation of PLA\textsubscript{2} and inhibition of COX genes may augment the production of AA, which mediates caffeine-induced cell death. It will be important to investigate the role of the AA downstream pathway to resolve the above issue.

As shown in Fig. 1c and d, treatment with AA was insufficient to restore caffeine sensitivity in the \textit{plaA} null mutant to the level observed in wild type. This indicates that the PLA\textsubscript{2} enzyme may have another function in caffeine tolerance, in addition to its production of AA. Furthermore, as shown in Fig. 2b, PLA\textsubscript{2} inhibitor does not completely block the caffeine-induced cell death, indicating that the PLA\textsubscript{2}-independent pathway is likely involved in this process, probably through autophagy via the PI3K/Akt/mTOR/p70S6K signalling pathway.

Interestingly, extracellular AA acts as a chemoattractant in \textit{D. discoideum}\textsuperscript{19}. Furthermore, \textit{plaA} was originally isolated as a gene involved in chemotaxis, since \textit{plaA} mutants result in the loss of chemotaxis in the PI3K/PTEN null background\textsuperscript{20}. How PLA\textsubscript{2}-AA

Figure 4 | Independence of intracellular cAMP signalling cascade on caffeine tolerance. (a) Development of \textit{regA} null mutant on 4 mM caffeine-containing, non-nutrient agar plates. Bar, 1 mm. (b) The survival of \textit{regA} null cells in 30 mM caffeine. **P < 0.01 versus AX2 (two-way ANOVA). (c) The survival of wild-type cells in 30 mM caffeine with 10 mM 8-Br-cAMP, a cell-permeable cAMP analogue. All data are presented as the mean ± s.d. of triplicated determinations of a representative experiment in 3 independent experiments.
functions in intracellular chemotaxis signalling pathway is not unravelled yet, but studying the mode of the action by caffeine may enlighten its molecular mechanism. Considering that chemotaxis is a composite biological phenomenon comprising cell migration, cell polarity, and gradient sensing, caffeine may be also effective in inflammation and cancer invasion because chemotaxis plays a critical role in those cases.

Thus, in this study, we show that AA increases the sensitivity to caffeine-induced cell death. Since caffeine has been found to enhance the effect of anticancer agents, our discovery underscores that, in the chemotherapeutic treatment on cancer, caffeine increases its potential effect as the enhancer of anticancer agents in combination with the compulsory activation of AA production.

Methods
Wild-type Dictyostelium discoideum AX2 and plaA and regA null mutant cells were cultivated using a standard method. The survival rate of D. discoideum cells was measured by shaking the cells in phosphate buffer (PB; 10 mM Na2HPO4 and 10 mM NaH2PO4 [pH 6.5]) at 21 °C with the indicated concentrations of caffeine and 5 μM propidium iodide (Sigma) with or without 10 μM Q-VD-Oph (R&D Systems, Inc.) and 5 μM bromoeno lactone (CAYMAN). Cell death was calculated by the ratio of the number of cells with fluorescent-stained nuclei to that of the total cells deduced from 15 DIC and fluorescent images, each of which contained 658–1955 cells for one estimation. Images of D. discoideum development were collected using OLYMPUS SZX12 with DP Controller software. Images of Hela cells were collected using OLYMPUS IX71 with IPLab software.

Acknowledgements
We thank Dr. A. Nagasaki for assistance with the cultivation of Hela cells. We also thank Drs. H. Urushihara and K. Mohri for helpful discussions. RegA null mutant was supplied from National Biosources Project (NBRP) Nenkin, Japan. This work was supported by Grants-in-Aid for Scientific Research (C) (no. 22510202) from the Japan Society for the Promotion of Science.

Author contribution
HK designed and performed all the experiments and drafted the manuscript.

Additional information
Competing financial interests: The author declares that he has no competing financial interests.

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How to cite this article: Kuwayama, H. Arachidonic Acid Enhances Caffeine-Induced Cell Death via Caspase-Independent Cell Death. Sci. Rep. 2, 577; DOI:10.1038/srep00577 (2012).

References
1. Ribeiro, J. A. & Sebastião, A. M. Caffeine and adenosine. J. Alzheimers Dis. Suppl 1, S3–15 (2010).
2. Daly, J. W. Caffeine analogs: biomedical impact. Cell Mol. Life Sci. 64, 2153–2169 (2007).
3. Bode, A. M. & Dong, Z. The enigmatic effects of caffeine in cell cycle and cancer. Cancer Lett. 247, 26–39 (2007).
4. Jang, M. H. et al. Calcium-independent phospholipase A2 (PLA2) with the compulsory activation of AA production. Biochim. Biophys. Acta. 1761, 1344–1350 (2006).
5. Golstein, P., Aubry, L. & Levraud, J. P. Cell-death alternative model organisms: why and which? Nat. Rev. Mol. Cell Biol. 4, 798–807 (2003).
6. Kim, D. K. & Jung, K. Y. Caffeine causes glycerophosphorylcholine accumulation through cytidine-inhibitable increase of cellular calcium and activation of phospholipase A2 in cultured MDCK cells. Exp. Mol. Med. 30, 151–158 (1998).
7. Caserta, T. M., Smith, A. N., Gultice, A. D., Reedy, M. A. & Brown, T. L. Q-VD-Oph, a broad spectrum caspase inhibitor with potent antiapoptotic properties. Apoptosis 8, 345–352 (2003).
8. Matsusaka, S., Morigaya, T., Ohara, N. & Maruo, T. Caffeine induces apoptosis of human umbilical vein endothelial cells through the caspase-9 pathway. Gynecol Endocrinol 22, 48–53 (2006).
9. Alvarez-Curto, E., Weening, K. E. & Schaap, P. Pharmacological profiling of the Dictyostelium adenylate cyclases ACA, ACB and ACG. Biochem J. 401, 309–316 (2007).
10. Balsinde, J., Pérez, R. & Balboa, M. A. Calcium-independent phospholipase A2 (PLA2) with the compulsory activation of AA production. Biochim. Biophys. Acta. 1761, 1344–1350 (2006).
11. Roussos, E. T., Condeelis, J. S. & Patsialou, A. Caffeine in cancer. Nat. Rev. Cancer 11, 573–587 (2011).
12. Kuwayama, H. & Kubohara, Y. Differentiation-inducing factor-1 and -2 function also as modulators for Dictyostelium chemotaxis. Dev. Cell 12, 603–614 (2007).
13. Calvo, I. A. et al. Genome-wide screen of genes required for caffeine tolerance in fusion yeast. PLoS One 4, e6619 (2009).
14. Funk, C. D. Prostaglandins and leukotrienes: advances in eicosanoid biology. Science 294, 1871–1873 (2001).
15. Bazinet, R. P. Is the brain arachidonic acid cascade a common target of drugs used to manage bipolar disorder? Biochem. Soc. Trans. 37, 1104–1109 (2009).
16. Schaloske, R. H., Blaesius, D., Schlatterer, C. & Lusche, D. F. Arachidonic acid is a chemoattractant for Dictyostelium discoideum cells. J. Biol. Sci. 32, 1281–1289 (2007).
17. Chen, L. et al. PLA2 and PI3K/PTEN pathways act in parallel to mediate chemotaxis. Dev. Cell 12, 603–614 (2007).
18. Roussos, E. T., Condeelis, J. S. & Patsialou, A. Caffeine in cancer. Nat. Rev. Cancer 11, 573–587 (2011).
19. Kuwayama, H. & Kubohara, Y. Differentiation-inducing factor-1 and -2 function also as modulators for Dictyostelium chemotaxis. PLoS One 4, e6658 (2009).
20. Kuwayama, H., Ecke, M., Gerisch, G. & Van Haastert, P. J. Protection against osmotic stress by cGMP-mediated myosin phosphorylation. Science 271, 207–209 (1996).
21. Kuwayama, H., Ishida, S. & Van Haastert, P. J. Non-chemotactic Dictyostelium discoideum mutants with altered cGMP signal transduction. J. Cell Biol. 123, 1453–1462 (1993).