JPP JOURNAL OF Pharmacy and Pharmacology
ROYAL PHARMACEUTICAL SOCIETY
JPP
Journal of Pharmacy
And Pharmacology
Research Paper

Ouabain exerts cytoprotection by diminishing the intracellular K^+ concentration increase caused by distinct stimuli in human leukemic cells

Masayuki Takechi, Tetsuyuki Wada, Hideki Yagi, Takashi Masuko and Atsufumi Kawabata

Faculty of Pharmacy, Kinki University, Higashiosaka, Osaka, Japan

Keywords
cytoprotection; flow cytometry; human leukemic cells; intracellular K^+ concentration; ouabain

Correspondence
Masayuki Takechi, Faculty of Pharmacy, Kinki University, Higashiosaka, Osaka 577-8502, Japan.
E-mail: mt471019@gmail.com

Received January 27, 2014
Accepted August 3, 2014

doi: 10.1111/jphp.12317

Abstract

Objectives We tested if modulation of cytosolic K^+ levels by ouabain, an inhibitor of Na^+/K^+-ATPase, exerts cytoprotection against distinct stressful stimuli in human leukemic cells.

Methods The cytosolic K^+, Na^+ or Ca^{2+} levels and the cytotoxicity were evaluated by flow cytometry.

Key findings Various cytotoxic chemicals and ultraviolet irradiation induced cell death and increased intracellular concentrations of K^+, Na^+ or Ca^{2+}. Ouabain reduced the cytotoxicity and the elevation of cytosolic levels of K^+ but not those of Na^+ or Ca^{2+}.

Conclusions Our data thus suggest that elevated cytosolic K^+ levels are associated with the cytotoxicity in response to distinct stressful stimuli and that ouabain exerts cytoprotection most probably by regulating intracellular K^+ levels.

Introduction

Ouabain, a Na^+/K^+-ATPase inhibitor, at high concentrations enhances anti-Fas-induced apoptosis of Jurkat cells by inhibiting the uptake of K^+.[1] A Fas ligand stimulates $K_v1.3$, a K^+ channel, in Jurkat cells undergoing apoptosis.[2] Some other studies indicate that the efflux of intracellular K^+ might be critical for apoptosis and that preventing this ion loss could protect cells from apoptosis.[3–5] In contrast, K^+ leak channels, namely two-pore domain K^+ channels, may play a protective role,[6] while overexpression of these channels enhances cell viability by inhibiting the activation of intracellular apoptotic pathways. We have reported that cardiac glycosides and steroids exhibit antiherpetic activity in human amnion cells.[7] It is likely that the inhibition of Na^+/K^+-ATPase by those chemicals and subsequent decrease in intracellular K^+ levels might enhance the cell viability against infection with the virus. Thus, the decreased cytosolic K^+ levels could be cytotoxic or cytoprotective, depending on experimental conditions including the type of stressful stimuli. To clarify the impact of altered cytosolic K^+ levels on cell viability or death, we thus determined intracellular concentrations of K^+ in comparison with Na^+ and Ca^{2+}, and cell viability in human leukemic cells exposed to distinct cytotoxic chemicals or ultraviolet (UV) irradiation, and then asked if ouabain at a non-toxic concentration modulates them.

Materials and Methods

Cultured cells and chemicals

Human leukemic cells such as HL60, Jurkat, Molt4, K562 and Daudi cells (American Type Culture Collection, Manassas, VA, USA) were cultured at 37°C in RPMI-1640 medium (Nissui Pharmaceutical, Tokyo, Japan) containing 8% heat-inactivated fetal bovine serum (ICN Biochemicals, Aurora, OH, USA) in a humidified CO_2 incubator. Vincristine, digitoxin, staurosporine, mitoxantrone, methotrexate, cytarabine, etoposide, wortmannin, retinoic acid and hydroxyurea (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in dimethyl sulfoxide (DMSO) and used at 0.15, 0.16, 0.17, 0.18, 2.2, 3.3, 4.2, 12, 67 and 530 μM, respectively, after dilution with the culture medium (the final concentration of DMSO was 0.1%). Arsenic trioxide and hydrogen peroxide (Wako Pure Chemical Industries, Osaka, Japan) were dissolved in ultrapure water, and used at 25 and 440 μM, respectively, by dilution with the culture medium. Since ouabain (Sigma-Aldrich), dissolved in 0.1%

(final concentration) DMSO, itself showed cytotoxicity for 24 h at 86–170 nM, but not in a range of 0–43 nM in the preliminary experiments (Figure S1), we evaluated the cytoprotective activity of ouabain at 43 nM in the following experiments. For example, 43 nM ouabain showed remarkable cytoprotective effect against 3.3 μM cytarabine (see Figure S1).

Stressful stimuli

Human leukemic cells were incubated with 0.1% DMSO (Con) or with one of those distinct cytotoxic chemicals in the absence or presence of 43 nM ouabain, 10 mM KCl or 4 μM 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis-acetoxymethyl ester (BAPTA-AM) (Sigma-Aldrich) for 24 h. Each cytotoxic chemical at the concentration employed showed 30–80% cytotoxicity in the preliminary experiments. Human leukemic cells were also preincubated for 2 h in the absence or presence of 43 nM ouabain and then irradiated with a 15 W UV lamp (Toshiba GL15, 254 nm, Tokyo, Japan) at a distance of 40 cm for 10 min, and thereafter incubated for 24 h.

Flow cytometry

To determine intracellular K^+, Na^+ or Ca^{2+} concentrations in human leukemic cells after cytotoxic challenges, the cells were loaded with 0.5 μM potassium ion-binding benzofuran isophthalate-acetoxymethyl ester (PBFI-AM) (Molecular Probe, Eugene, OR, USA), 3 μM sodium ion-binding benzofuran isophthalate-acetoxymethyl ester (SBFI-AM) (Molecular Probe) or 1 μM Fura-2-AM (MoBiTec, Goettingen, Germany), respectively, in the presence of 0.005% Pluronic F-127 (Molecular Probe) for 1 h at 37°C. The cells were then washed and resuspended in the culture medium containing 10 μg/ml propidium iodide (PI, Sigma-Aldrich) for determination of cell viability. More than 10 000 cells were analysed by flow cytometry for each sample using a BD-LSR flow cytometer (Becton-Dickinson, Sunnyvale, CA, USA) equipped with CellQuest software. Excitation and emission wavelengths were set at 350 nm and 380–470 nm for PBFI-AM, SBFI-AM or Fura-2-AM, and 488 nm and 562–588 nm for PI, respectively. A gate was set on a forward scatter (FS) vs PI dot plot to examine living cells (R1 region in Figure S2a) that exhibited both high FS and low PI fluorescence, and the mean FS value of the living cells was measured. The intracellular K^+ concentration in the living cells (red dots in Figure S2b linked to the R1 region in Figure S2a) was determined from P/F, which was the geometrical mean value of PBFI fluorescence divided by the mean FS value of the living cells. The PF ratio was defined as the ratio of the P/F to that of the control, which was assumed to be 1.00. Likewise, to monitor intracellular Na^+ and Ca^{2+} concentrations, the SF and CF ratios were deduced from S/F and C/F, using SBFI-AM and Fura-2-AM, respectively. Cytotoxicity (%) was calculated from an equation, '(whole cells – living cells)/(whole cells)'.

Western blotting

The treated HL60 cells with 0, 21 or 43 nM ouabain for 24 h were collected with the lysis buffer containing 1% Nonidet P-40 (Nakarai Tesque, Kyoto, Japan), protease inhibitor cocktail (Nakarai Tesque) and benzonase (Merck KGaA, Darmstadt, Germany) in PBS for 60 min on ice. The protein samples were separated by electrophoresis on a 4–20% sodium dodecyl sulfate-polyacrylamide gradient gel and transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore Corporation, Billerica, MA, USA). The primary antibodies used were anti-Bcl-2 from Cell Signaling Technology (Beverly, MA, USA) and anti-β-tubulin (Tub 2.1) from Sigma-Aldrich. Horseradish peroxidase (HRP)-linked antirabbit and antimouse IgG (Jackson ImmunoResearch Lab., West Grove, PA, USA) was used as a secondary antibody, respectively. Proteins were detected using Chemi-Lumi One Super, HRP detection substrate (Nakarai), and analysed using ImageQuant RT ECL (GE Healthcare, Buckinghamshire, UK).

Statistical analysis

The data are shown as means ± standard deviation ($n = 5$). Tukey's multiple comparison test was used for statistical analysis, and a value of $P < 0.05$ was considered statistically significant.

Results

All of the chemicals exhibited cytotoxicity and enhanced the PF ratio, an indicator of cytosolic K^+ levels, in HL60 cells (Figure 1a and 1b). Ouabain, an inhibitor of Na^+/K^+-ATPase, significantly diminished the cytotoxicity and decreased the elevated PF ratio in response to those stressful chemicals other than digitoxin, another Na^+/K^+-ATPase inhibitor. The cytotoxicity and PF ratio increase caused by digitoxin were even rather enhanced by ouabain (Figure 1a and 1b).

UV irradiation exhibited cytotoxicity and enhanced the PF ratio, an indicator of cytosolic K^+ levels, in HL60 cells. Ouabain significantly suppressed those increases in the cytotoxicity and PF ratio in response to the UV irradiation (Figure 2). Similar results were observed in other human leukemic cells including Jurkat, Molt4, K562 and Daudi cells (Figure 2).

Further, SF and CF ratios in HL60 cells, indicators of cytosolic Na^+ and Ca^{2+} levels, were also elevated by all of the stressful chemicals (Figures 3 and 4). Ouabain significantly reduced the SF ratio elevated by etoposide or arsenic triox-

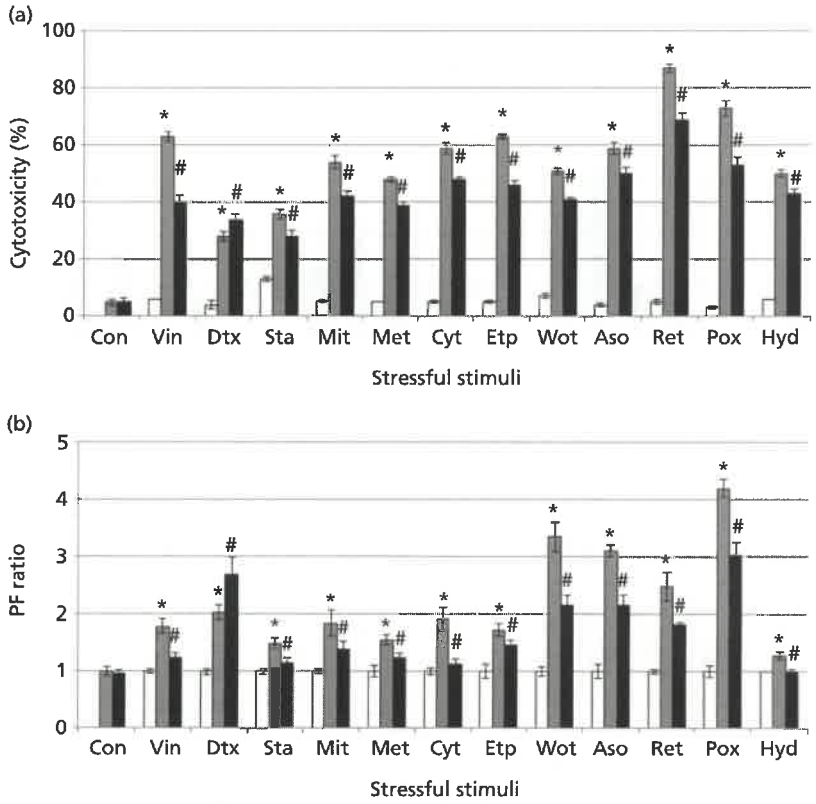

Figure 1 Cytotoxicity and increased cytosolic K^+ levels in HL60 cells exposed to stressful chemicals in the absence or presence of ouabain. The cells were stimulated with vehicle (Con) or chemicals consisting of vincristine (Vin), digitoxin (Dtx), staurosporine (Sta), mitoxantrone (Mit), methotrexate (Met), cytarabine (Cyt), etoposide (Etp), wortmannin (Wot), arsenic trioxide (Aso), retinoic acid (Ret), hydrogen peroxide (Pox) and hydroxyurea (Hyd), at 0.15, 0.16, 0.17, 0.18, 2.2, 3.3, 4.2, 12, 25, 67, 440 and 530 μmol/l, respectively. (a) Cytotoxicity and (b) PF ratio, an indicator of cytosolic K^+ levels, was determined after 24 h chemical stimuli. Ouabain at 43 nM was added before stressful chemicals. White column, no stimuli; grey column, stressful chemicals; black column, stressful chemicals + ouabain. $*P < 0.05$ vs no stimuli; $\#P < 0.05$ vs stressful chemicals only.

ide (Figure 3) and the CF ratio elevated by staurosporine or retinoic acid (Figure 4), whereas it did not reduce the SF and CF ratios in the cells exposed to other stressful chemicals (Figures 3 and 4). It is noteworthy that ouabain rather enhanced the digitoxin-induced increases in the SF and CF ratios (Figures 3 and 4). Thus, the effects of ouabain on the alterations in the SF and CF ratios did not necessarily parallel with its cytoprotective activity.

The addition of 10 mM KCl to the medium significantly facilitated or tended to enhance the increased cytotoxicity and the elevated PF ratio in response to the stressful chemicals other than digitoxin, while the cytotoxicity and PF ratio increases caused by digitoxin were even rather diminished by the addition of 10 mM KCl (Figure 5). The cytosolic calcium chelator, BAPTA-AM, did not significantly decrease the cytotoxicity of HL60 cells by hydrogen peroxide or UV treatment (Figure S3).

Finally, we determined if ouabain affects the expression of Bcl-2, an anti-apoptotic protein, as described elsewhere.[8] The expression level of Bcl-2 protein in HL60 cells tended to increase after 24 h incubation with 21 or 43 nM ouabain, as assessed by the Western blotting (Figure S4).

Discussion

Because ouabain suppressed the increases in cytotoxicity and PF ratio of HL60 cells caused by the stressful chemicals other than digitoxin (see Figure 1a and 1b), the increased cytosolic K^+ levels would be related to the cytotoxicity. This notion is in agreement with our finding that ouabain further enhanced the cytotoxicity and PF ratio increase caused by digitoxin. Both ouabain and digitoxin inhibit Na pump, and this is why ouabain at a non-cytotoxic concentrations would decrease the cytotoxicity and intracellular K^+ concentration caused by drugs other than digitoxin, but increase those caused by digitoxin at a cytotoxic concentration. Furthermore, ouabain inhibited the augmentations in cytotoxicity and PF ratio evoked by the UV irradiation not

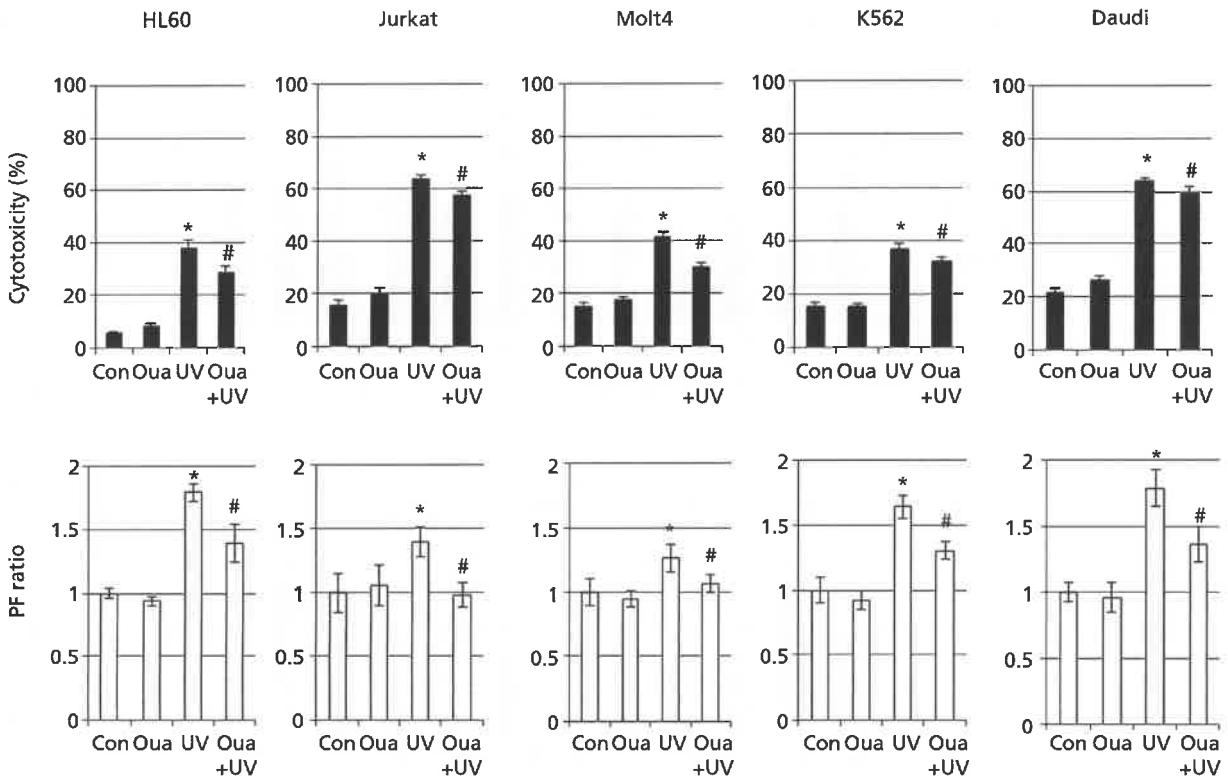

Figure 2 Cytotoxicity and increased cytosolic K^+ levels in HL60, Jurkat, Molt4, K562 or Daudi cells treated by the ultraviolet (UV) irradiation in the absence or presence of ouabain. For the UV irradiation, these cells were preincubated with 43 nM ouabain for 2 h and then irradiated with a 15-W UV lamp at a distance of 40 cm for 10 min. Cytotoxicity and PF ratio, an indicator of cytosolic K^+ levels, was determined 24 h after the UV irradiation. Con, vehicle; Oua, ouabain. $*P < 0.05$ vs Con; $\#P < 0.05$ vs UV.

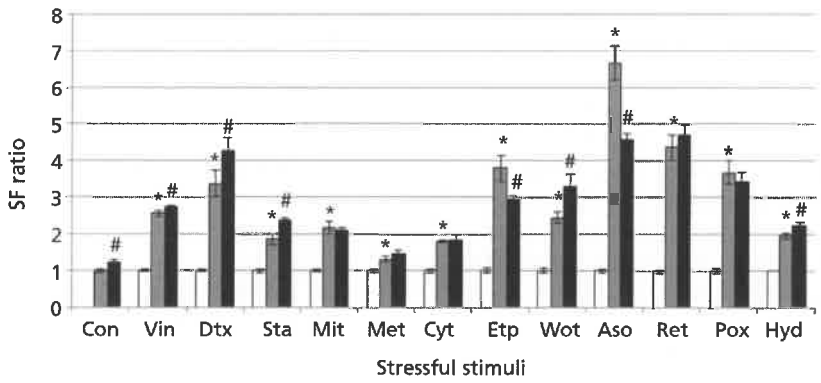

Figure 3 Increased cytosolic levels of Na^+ in HL60 cells exposed to stressful chemicals in the absence or presence of ouabain. SF ratio, an indicator of cytosolic Na^+ levels, was determined after 24 h chemical stimuli. Ouabain at 43 nM was added before chemical stimuli. Other experimental conditions and explanation of abbreviations are shown in the legend for Figure 1. White column, no stimuli; grey column, stressful chemicals; black column, stressful chemicals + ouabain. $*P < 0.05$ vs no stimuli; $\#P < 0.05$ vs stressful chemicals only.

only in HL60, but also in Jurkat, Molt4, K562 or Daudi cells (see Figure 2), suggesting that the correlation between cytotoxicity and increased cytosolic K^+ levels is a common phenomenon at least in human leukemic cells. There is a possibility that stressful stimuli might cause functional upregulation of Na^+/K^+-ATPase, resulting in increased intracellular K^+ concentrations. However, this hypothesis is not consistent with the increased cytosolic Na^+ levels caused

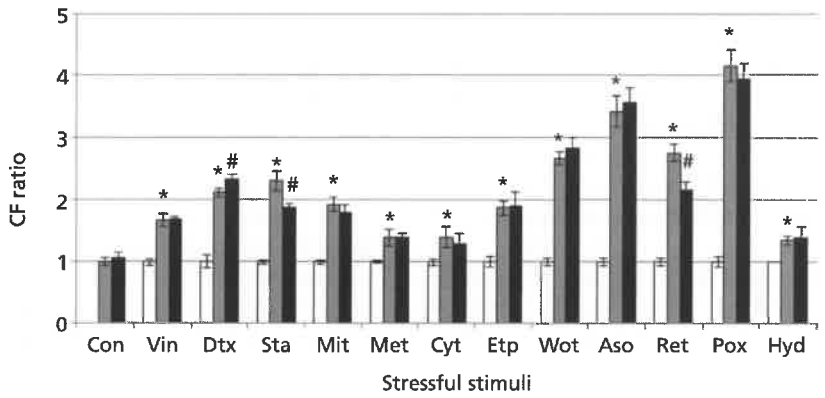

Figure 4 Increased cytosolic levels of Ca^{2+} in HL60 cells exposed to stressful chemicals in the absence or presence of ouabain. CF ratio, an indicator of cytosolic Ca^{2+} levels, was determined after 24 h chemical stimuli. Ouabain at 43 nM was added before stressful stimuli. Other experimental conditions and explanation of abbreviations are shown in the legend for Figure 1. White column, no stimuli; grey column, stressful chemicals; black column, stressful chemicals + ouabain. *$P < 0.05$ vs no stimuli; #$P < 0.05$ vs stressful chemicals only.

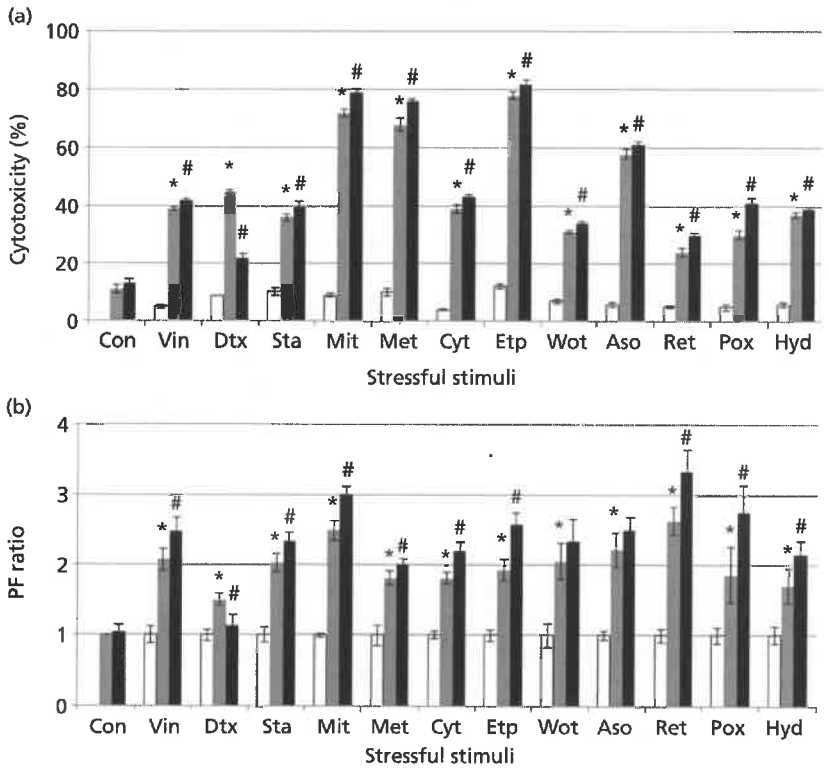

Figure 5 Cytotoxicity and increased cytosolic K^+ levels in HL60 cells exposed to stressful chemicals in the absence or presence of 10 mM KCl. (a) Cytotoxicity and (b) PF ratio, an indicator of cytosolic K^+ levels, were determined after 24 h chemical stimuli. Added before stressful chemicals was 10 mM KCl. Other experimental conditions and explanation of abbreviations are shown in the legend for Figure 1. White column, no stimuli; grey column, stressful chemicals; black column, stressful chemicals + 10 mM KCl. *$P < 0.05$ vs no stimuli; #$P < 0.05$ vs stressful chemicals only.

by the stressful chemicals (see Figure 3). It is likely that the stressful stimuli might cause functional downregulation of K^+ leak channels, namely two-pore domain K^+ channels, leading to elevated intracellular K^+ concentrations, because there is evidence that overexpression of TASK-3 (KCNK9) and related two-pore domain K^+ channels enhances cell viability by inhibiting the activation of intracellular apoptotic pathways.[6] We speculate that the increased

intracellular K^+ concentrations in response to the stressful stimuli would elevate the membrane potential towards depolarization, which might increase the cytosolic Na^+ and Ca^{2+} levels (see Figures 3 and 4). The involvement of membrane depolarization in elevated cytotoxicity by the stressful stimuli is further supported by our results that the addition of KCl tented to further enhance the elevated cytotoxicity and PF ratio by stressful stimuli other than digitoxin (see Figure 5).

Here we propose that ouabain normalizes the increased cytosolic K^+ levels by blocking the Na^+/K^+-ATPase and then exerts cytoprotection in the cells exposed to stressful stimuli. This notion might even interpret the antiherpetic activity of cardiac glycosides and steroids in the mammalian cells in our previous study.[7] There is evidence that low doses of ouabain cause low-frequency Ca^{2+} oscillations that activate the nuclear factor-kappa B pathway, prevent apoptosis[9,10] and block excitotoxic apoptosis through up-regulation of nuclear Bcl-2 *in vivo*,[8] which is supported by this study (see Figure S4). It has also been reported that overexpression of small-conductance calcium-activated K^+ (SK) channels reduces apoptotic cell death[11] and that mcl-1, a member of the Bcl-2 family, causes membrane hyperpolarization via K^+ channel activation.[12] Together, the cytoprotective activity of ouabain might involve the low-frequency Ca^{2+} oscillations followed by SK channel activation and the upregulation of Bcl-2. Interestingly, ouabain appears to stimulate Src kinase[13] by dissociating the Na^+/K^+-ATPase-Src complex,[14] and Src may directly activate K^+ channels.[15] These reports are in agreement with the present results that ouabain suppressed the cytosolic K^+ level increase and cytotoxicity in response to distinct stressful stimuli.

We consider that the cytotoxicity induced by stress caused by a variety of stimuli including chemicals or UV irradiation would be related to ageing or the development of ageing-related diseases including cancer. In our study, we suggest that the excessive depolarization caused by the increase of intracellular K^+ concentration is critical and that ouabain, an Na pump inhibitor, would have cytoprotective effects on various cells by decreasing the intracellular K^+ concentration, in addition to its therapeutic usefulness for treatment of heart failure. On the basis of the present results, we suggest that Na pump inhibitors such as ouabain might be available as antistressful or anti-ageing, for example, they might prevent the development of cancer or diseases caused by virus infection.

Conclusions

The increased intracellular K^+ level is associated with the cytotoxicity caused by stressful chemical and UV stimuli, and its inhibition by ouabain exerts cytoprotection in human leukemic cells.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government.

References

1. Bortner CD *et al.* Plasma membrane depolarization without repolarization is an early molecular event in anti-Fas-induced apoptosis. *J Biol Chem* 2001; 276: 4304–4314.
2. Storey NM *et al.* Stimulation of Kv1.3 potassium channels by death receptors during apoptosis in Jurkat T lymphocytes. *J Biol Chem* 2003; 278: 33319–33326.
3. Bortner CD, Cidlowski JA. Cell shrinkage and monovalent cation fluxes: role in apoptosis. *Arch Biochem Biophys* 2007; 462: 176–188.
4. Marklund L *et al.* Cellular potassium ion deprivation enhances apoptosis induced by cisplatin. *Basic Clin Pharmacol Toxicol* 2004; 94: 245–251.
5. Kahlenberg JM, Dubyak GR. Mechanisms of caspase-1 activation by $P2X_7$ receptor-mediated K^+ release. *Am J Physiol Cell Physiol* 2004; 286: C1100–C1108.
6. Liu C *et al.* Protective effects of TASK-3 (KCNK9) and related 2P K channels during cellular stress. *Brain Res* 2005; 1031: 164–173.
7. Takechi M *et al.* Biological activities of synthetic saponins and cardiac glycosides. *Phytother Res* 2003; 17: 83–85.
8. Golden WC, Martin LJ. Low-dose ouabain protects against excitotoxic apoptosis and up-regulates nuclear Bcl-2 in vivo. *Neuroscience* 2006; 137: 133–144.
9. Li J *et al.* Low doses of ouabain protect from serum deprivation-triggered apoptosis and stimulate kidney cell proliferation *via* activation of NF-κB. *J Am Soc Nephrol* 2006; 17: 1848–1857.
10. Zhang S *et al.* Distinct role of the N-terminal tail of the Na,K-ATPase catalytic subunit as a signal transducer. *J Biol Chem* 2006; 281: 21954–21962.
11. Lee AL *et al.* Potassium channel gene therapy can prevent neuron death resulting from necrotic and apoptotic insults. *J Neurochem* 2003; 86: 1079–1088.

12. Wang L *et al.* Protection from cell death by *mcl-1* is mediated by membrane hyperpolarization induced by K^+ channel activation. *J Membr Biol* 1999; 172: 113–120.
13. Haas M *et al.* Src-mediated inter-receptor cross-talk between the Na^+/K^+-ATPase and the epidermal growth factor receptor relays the signal from ouabain to mitogen-activated protein kinases. *J Biol Chem* 2002; 277: 18694–18702.
14. Liang M *et al.* Functional characterization of Src-interacting Na/K-ATPase using RNA interference assay. *J Biol Chem* 2006; 281: 19709–19719.
15. Gomes P *et al.* Identification of a functional interaction between Kv4.3 channels and c-Src tyrosine kinase. *Biochim Biophys Acta* 2008; 1783: 1884–1892.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 Concentration-dependent cytotoxic and cytoprotective activities of ouabain. Data show the cytotoxicity in the absence (white column) and presence (black column) of 3.3 μM cytarabine. $*P < 0.05$ vs 0 nM ouabain without cytarabine; $\#P < 0.05$ vs 0 nM ouabain with cytarabine.

Figure S2 Determination of cytocolic K^+ levels in living cells by the flow cytometry. See the text in detail.

Figure S3 Cytotoxities of hydrogen perxide (Pox) and ultraviolet (UV) treatment in the absence (white column) and presence (black box) of 4 μM BAPTA-AM. The detailed experimental conditions are explained in the legend for Figures 1 and 2. Con, control. $*P < 0.05$ vs control without BAPTA-AM.

Figure S4 The expression of Bcl-2 in HL60 cells after 0, 21 or 43 nM ouabain treatment.