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Ferutinin Induces in Vitro Eryptosis/Erythroptosis in Human Erythrocytes through Membrane Permeabilization and Calcium Influx

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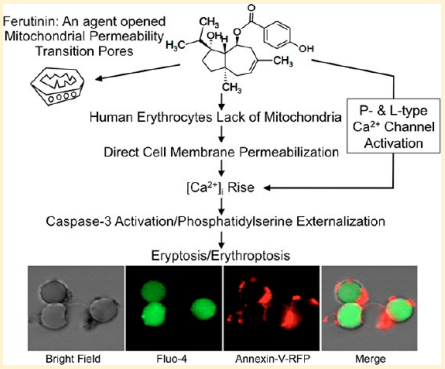
ABSTRACT: Ferutinin, isolated from the root of *Ferula hermonis* and proposed to be used as an antiosteoporosis phytoestrogen, has death promoting activities in a number of cancer cells. However, the effect of ferutinin on the induction of apoptosis in human red blood cells (RBCs), also known as eryptosis or erythroptosis, remains unclear. Given that ferutinin is a small molecule that can induce apoptosis in the cancer cells by opening the mitochondrial permeability transition pores, we therefore hypothesized that the effect of ferutinin to elicit apoptosis in human RBCs devoid of mitochondria would be minimal. This study tried to determine the in vitro effect of ferutinin on the induction of apoptosis in human RBCs. Eryptosis/erythroptosis after ferutinin treatment was examined for phosphatidylserine (PS) externalization, calcein leakage, and other apoptotic feature events by flow cytometry and confocal microscopy. Contrary to our prediction, ferutinin caused eryptosis/erythroptosis in human RBCs and simultaneously increased caspase-3 activity and the cytosolic free Ca^{2+} ion level ($[Ca^{2+}]_i$). Yet, Ca^{2+} seems not to be the sole mediator in ferutinin-mediated eryptosis/erythroptosis because depletion of the external Ca^{2+} could not eliminate the apoptotic effect from ferutinin. Subsequent replenishment of the external Ca^{2+} was able to promote PS externalization, caspase-3 activation, and rise of $[Ca^{2+}]_i$. Also, ferutinin at high dose (40 μM or above) was able to permeabilize the membrane of RBC ghosts in a way similar to that of digitonin. At low dose, ferutinin activated the P- and L-type Ca^{2+} channels as the ferutinin-mediated $[Ca^{2+}]_i$ rise was suppressed by the P-type (ω-agatoxin IVA) and L-type (verapamil and diltiazem) Ca^{2+} channel blockers. Taken together, we report here for the first time that ferutinin induces in vitro apoptosis in human RBCs. Mechanistically, eryptosis/erythroptosis is mediated by membrane permeabilization and upregulation of $[Ca^{2+}]_i$ with the activation of caspase-3.



INTRODUCTION

Ferutinin, also known as jaeschkeanadiol *p*-hydroxybenzoate with a formula of $C_{22}H_{30}O_4$, is isolated from the root of the *Ferula* genus, which is commonly found in the Mediterranean region.[1] Ferutinin has a structure similar to that of estrogen in three-dimensional space, and therefore, this terpenoid can bind to estrogen receptors.[1,2] Because of this feature, ferutinin is proposed to be used as an antiosteoporosis phytoestrogen as well as a supplement that affects the sexual desire and performance in males and females.[3−5] Also, many studies indicate that ferutinin can be used as an anticancer agent to treat cancers. For example, ferutinin has anticancer activities in human colon cancer and pro-apoptotic effects in the human T cell leukemia line through the activation of the intrinsic apoptotic pathway by opening the mitochondrial permeability transition pores.[6,7] Ferutinin is indeed able to increase Ca^{2+} permeability in the mitochondria,[8] possibly acting as an electrogenic Ca^{2+} uniporter on the mitochondrial membrane that provokes Ca^{2+} overloading and mitochondrial depolarization.[9] Yet, the cytotoxic effect of ferutinin in normal cells such as human erythrocytes remains to be elucidated.

In view of the fact that ferutinin is a Ca^{2+} mobilizing agent in many cells and in the mitochondria,[6−10] it will be of interest to examine whether ferutinin exerts any apoptosis-inducing effect in the human red blood cells (RBCs), which are devoid of the mitochondria and nuclei. In fact, human RBCs have their unique cell death program called eryptosis/erythroptosis to remove senescent, defective, potentially harmful, and parasite-infected RBCs to prevent leakage of cellular proteins such as hemoglobin (Hb) which trigger inflammation and other complications.[11,12] Though without the mitochondria and nuclei, human RBCs are able to show apoptotic events like cell shrinkage, membrane blebbing, and membrane phospholipid scrambling.[11,12]

Mechanistically, triggers of eryptosis/erythroptosis include the formation of ceramide through the activation of sphingomyelinase, influx of Ca^{2+} ions, K^+ efflux through the activation of K^+ channels, and the subsequent Cl^- efflux followed by osmotic efflux of water molecules leading to cell shrinkage. Also, the elevated cytosolic Ca^{2+} level induces the externalization of phosphatidylserine (PS) at the cell surface.[11−13] Among these events, the externalization of the PS from the inner to the outer leaflet of the plasma membrane marks the early stage of eryptosis/erythroptosis, and the externalized PS serves as an "eat

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me" signal to attract macrophages to engulf and degrade the defective erythrocytes for cell clearance and to limit the growth of parasites in the malaria-infected RBCs.[14−16] In this study, the in vitro effect of ferutinin on the induction of eryptosis/erythroptosis in human RBCs was investigated, and our results indicated that ferutinin is able to act on cell membranes to trigger the cell death program in human RBCs.

MATERIALS AND METHODS

Materials. Ferutinin was purchased from Enzo Life Sciences (Farmingdale, NY). Calcein/AM and fluo-4/AM were purchased from Invitrogen (Carlsbad, CA). Annexin-V-RFP was synthesized in-house and purified by a nickel affinity column. Calcium ionomycin was obtained from Merck (Darmstadt, Germany). Digitonin was purchased from Sigma-Aldrich (St. Louis, MO). ω-Agatoxin IVA, nifedipine, verapamil, and diltiazem were purchased from Tocris Bioscience (London, UK). Ferutinin was dissolved in dry dimethyl sulfoxide (DMSO) to make up stock solutions at 5 mM. In the experimental assays, ferutinin was further diluted to various concentrations as specified in the assay with HEPES buffers with or without calcium.

Isolation of Human Erythrocytes. This study was approved by the Clinical Research Ethics Committee of the Chinese University of Hong Kong (Protocol number: CRE-2012.025), the Hong Kong Special Administrative Region, China. Two to three droplets of fresh human RBCs were obtained from healthy donors following informed consent by puncturing fingertips with a sterile lancet with depth setting. Heparinized RBCs were washed three times with isotonic PBS ((in mM): 136.9 NaCl, 2.7 KCl, 10 Na_2HPO_4, and 1.5 KH_2PO_4, pH 7.4) for experiments. To avoid bias potentially introduced by the use of different batches of RBCs, positive and negative controls were included with the test groups in all assays, and comparison was made within the same cell batch. Independent experiments were repeated. The results shown in this article represent typical responses from 3 to 5 independent trials.

Hemolysis Assay. For the hemolysis assay, RBCs (1.5×10^7/mL) were treated with different agents at 37 °C. After treatment, absorbance of the supernatant was determined against the blank at 415 nm with an ELISA plate reader (Bio-Rad) for the leakage of Hb. Total cell lysis was obtained by adding Triton-X-100 (final concentration 0.1% (v/v)) into wells to release Hb into the medium.

Flow Cytometric Assays and Confocal Microscopic Assays. To evaluate eryptosis/erythroptosis in human RBCs, flow cytometric analysis using annexin-V-RFP and calcein/AM was employed. After treatment, RBCs were labeled or loaded with annexin-V-RFP and calcein/AM (1 μM) at room temperature for 30 min. Annexin-V-RFP is able to label the PS on the outer leaflet of the plasma membrane. Calcein/AM is a nonfluorescent dye. After diffusing into the cytoplasm across the plasma membrane, the ester linkage between calcein and the AM group is cleaved by the cytosolic esterases so that the hydrophilic fluorescent calcein cannot diffuse out. However, in apoptotic cells, calcein molecules are leaked out through the damaged membrane, and less calcein is kept in the cytosol. Annexin-V-RFP signals were then determined with a 605 nm (50 nm) band-pass filter after excitation at 543 nm, and calcein signals were measured by a 550 nm (50 nm) band-pass filter with a 488 nm laser.

In some experiments, RBCs after treatment in Ca^{2+}-free HEPES buffer ((in mM): 140 NaCl, 5 KCl, 10 HEPES, 2 EGTA, 10 glucose, and 0.1% (w/v) BSA (bovine serum albumin), pH 7.4) were stained with the Ca^{2+}-dependent protein annexin-V-RFP for 30 min in the Ca^{2+}-containing HEPES buffer ((in mM): 140 NaCl, 5 KCl, 10 HEPES, 2.5 $CaCl_2$, 10 glucose, and 0.1% (w/v) BSA, pH 7.4). After washing, cells were then subject to flow cytometric analysis.

To determine the change of cytosolic free Ca^{2+} ion concentration ($[Ca^{2+}]_i$), RBCs were loaded with fluo-4/AM (1 μM) at room temperature for 30 min. After the cleavage of the AM group similar to the case of calcein/AM, hydrophilic fluo-4 is kept in the cytosol. The fluorescence from the Ca^{2+}-fluo-4 complexes was measured at single cell level by flow cytometry with an excitation at 488 nm. The $[Ca^{2+}]_i$ in the cytosol was reported semiquantitatively.

For the measurement of caspase-3 activity, cells (1×10^6/mL; 0.3 mL) were incubated with a fluorogenic caspase-3 FAM-DEVD-FMK assay (APO LOGIX, Mountain View, CA) for 40 min according to the manufacturer's instructions (Cell Technology Inc., Mountain View, CA, USA), then cells were mixed with annexin-V-RFP and incubated for 20 min. After washing, cells were submitted to flow cytometric analysis.

Flow cytometric analysis was performed on a FACSCanto flow cytometer (BD Biosciences, San Jose, CA), using WinMDI 2.9 software for data acquisition and analysis. Green and red fluorescence after excitation at 488 or 543 nm from a minimum of 10,000 cells were determined. In the two-variant plots, each dot represents one single cell and the figure at each corner represents the percent of total cell population in the quadrant.

For the confocal microscopic analysis, cells after treatment were labeled with fluorochromes as mentioned above and the signals observed under confocal microscope after washing. Bright field and confocal images of cells were acquired with a confocal laser-scanning microscope (Fluoview FV100, Olympus, Tokyo, Japan) fitted with argon (488 nm) and a HeNe-Green (543 nm) laser for excitation. For fluorescence determination, band-pass filters for green (550 ± 25 nm, excited by the argon laser) and red (605 ± 25 nm, excited by the HeNe-Green laser) fluorescence were used. Images and fluorescence intensities were processed by FluoView application software FV10-ASW 3.0 (Olympus, Tokyo, Japan).

Ghost Assay. Ghosts from RBCs were prepared as described previously.[17,18] In this assay, digitonin was used as a positive control. Digitonin is a nonionic detergent that selectively reacts with membrane cholesterol.[19] At low concentrations, it permeabilizes the plasma membrane while leaving the internal membrane (e.g., nuclear envelope and endoplasmic reticulum) with less cholesterol intact.[20] Digitonin in high dose is able to induce hemolysis in erythrocytes[21] and ghosts.[22] After treatments, ghost cells were analyzed by flow cytometry.

Statistical Analysis. Results were expressed as the mean ± SD of three to five determinations from the blood of different donors of several (>3) independent trials. Data were compared using Student's *t*-test; *p*-values less than 0.05 were considered statistically significant.

RESULTS

Acute Cytotoxicity of Ferutinin in Human RBCs. Figure 1 shows the toxicity of ferutinin in human erythrocytes measured by hemolysis assay 24 h after the treatment. As shown in Figure 1,

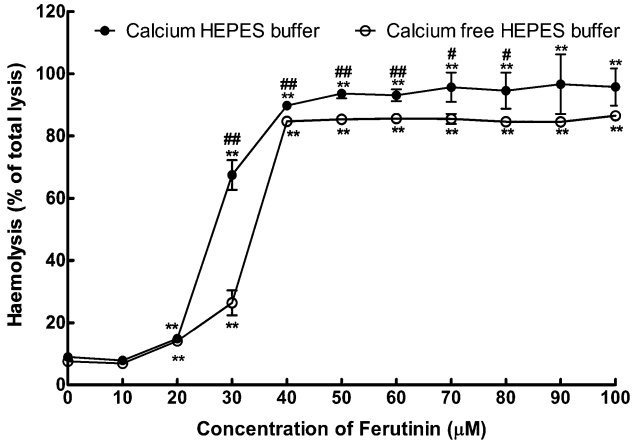

Figure 1. Cellular sensitivity of human RBCs to ferutinin. RBCs (1.5×10^7/mL) were cultured with ferutinin at the concentration indicated in the Ca^{2+}-containing (●) or Ca^{2+}-free (○) buffer at 37 °C for 24 h. Hemolysis was then determined by measuring the optical density of the supernatant containing Hb at OD 415 nm. Triton-X-100 (0.1%) was used for the total lysis. Results are the mean ± SD ($n = 3$). $^{**}p < 0.01$, relative to the corresponding control; $\#p < 0.05$ and $\#\#p < 0.01$, relative to the response at the same ferutinin concentration in Ca^{2+} -free buffer.

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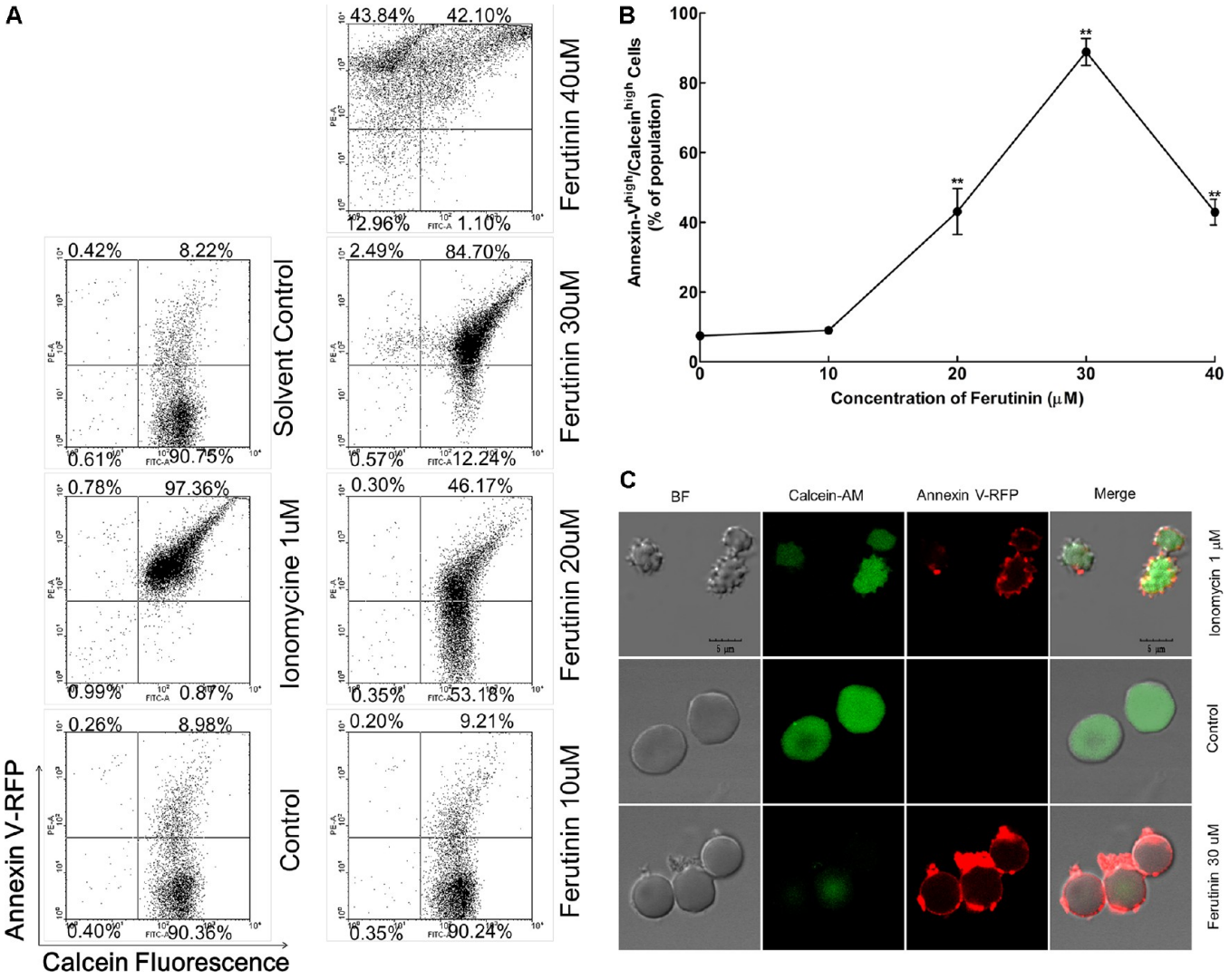

Figure 2. Effect of ferutinin on the induction of eryptosis/erythroptosis in human RBCs. RBCs (1.5×10^7/mL) were treated with ionomycin (1 μM), medium alone, or ferutinin at the concentration indicated at 37 °C for 24 h in the Ca^{2+} HEPES buffer. After treatments, cells (1×10^6/mL) were incubated with annexin-V-RFP and calcein/AM (1 μM) in the dark for 30 min. After washing, cells were subject to flow cytometric analysis. Percentage of cells in the quadrants was then determined (A). Results in B are the mean ± SD ($n = 3$) of the early apoptotic cells (annexin-V^{high}/$calcein^{high}$) in A. $^{**}p < 0.01$, relative to the control. In C, cells after treatments were seeded in a confocal dish, and an optical section was acquired by laser scanning confocal microscopy with corresponding excitation and emission wavelengths. The scale bar represents the cell dimension, 5 μm.

about 10% of cells were hemolyzed in the control group. When cells were incubated with ferutinin, cell lysis was obtained in a dose-dependent manner with an EC_{50} of 26 and 34 μM in the Ca^{2+}-containing or Ca^{2+}-free buffer, respectively. When the ferutinin concentration was increased to 40 μM, more than 80% of cells were lysed in both buffers.

Effect of Ferutinin on the Induction of Eryptosis/Erythroptosis in Human RBCs. To determine whether ferutinin induced eryptosis/erythroptosis, flow cytometric analysis using annexin-V-RFP and calcein/AM was performed to evaluate, respectively, the degree of PS externalization and membrane integrity of the cells in the human RBCs treated with ferutinin.[23] As shown in Figure 2A, cells were treated with ferutinin in Ca^{2+}-containing HEPES buffer for 24 h. As can be seen, most of the cells in the control group were found in the lower right quadrant (90.4%). They were healthy cells with normal PS asymmetry and membrane integrity (annexin-V^{low}/$calcein^{high}$). Similar results were obtained in the solvent control group. To validate the assay, Ca^{2+} ionomycin was used as a positive control that elicits eryptosis/erythroptosis in erythrocytes.[24] In Figure 2A, it is clear that Ca^{2+} ionomycin (1 μM) significantly increased the number of early apoptotic cells (annexin-V^{high}/$calcein^{high}$) in the upper right quadrant from 9.0% (control) to 97.4%, with a concomitant decrease in the number of healthy cells in the lower right quadrant (0.9% vs 90.4% in the control).

When cells were treated with ferutinin for 24 h, the percentage of cell population in the lower right (healthy) and upper right (early apoptotic) quadrants decreased and increased, respectively, in a dose-dependent manner (Figure 2A). When cells were treated with ferutinin at 40 μM, it seems likely that the cells lost the membrane integrity and that the calcein was leaked out into the external buffer giving a lower calcein fluorescence signal in the upper right quadrant (42.1%), and more cells were found in the upper left quadrant (43.8% vs 0.3% in control). Independent assays were repeated thrice, and the response profile in the upper right quadrant is shown in Figure 2B. These results suggest that

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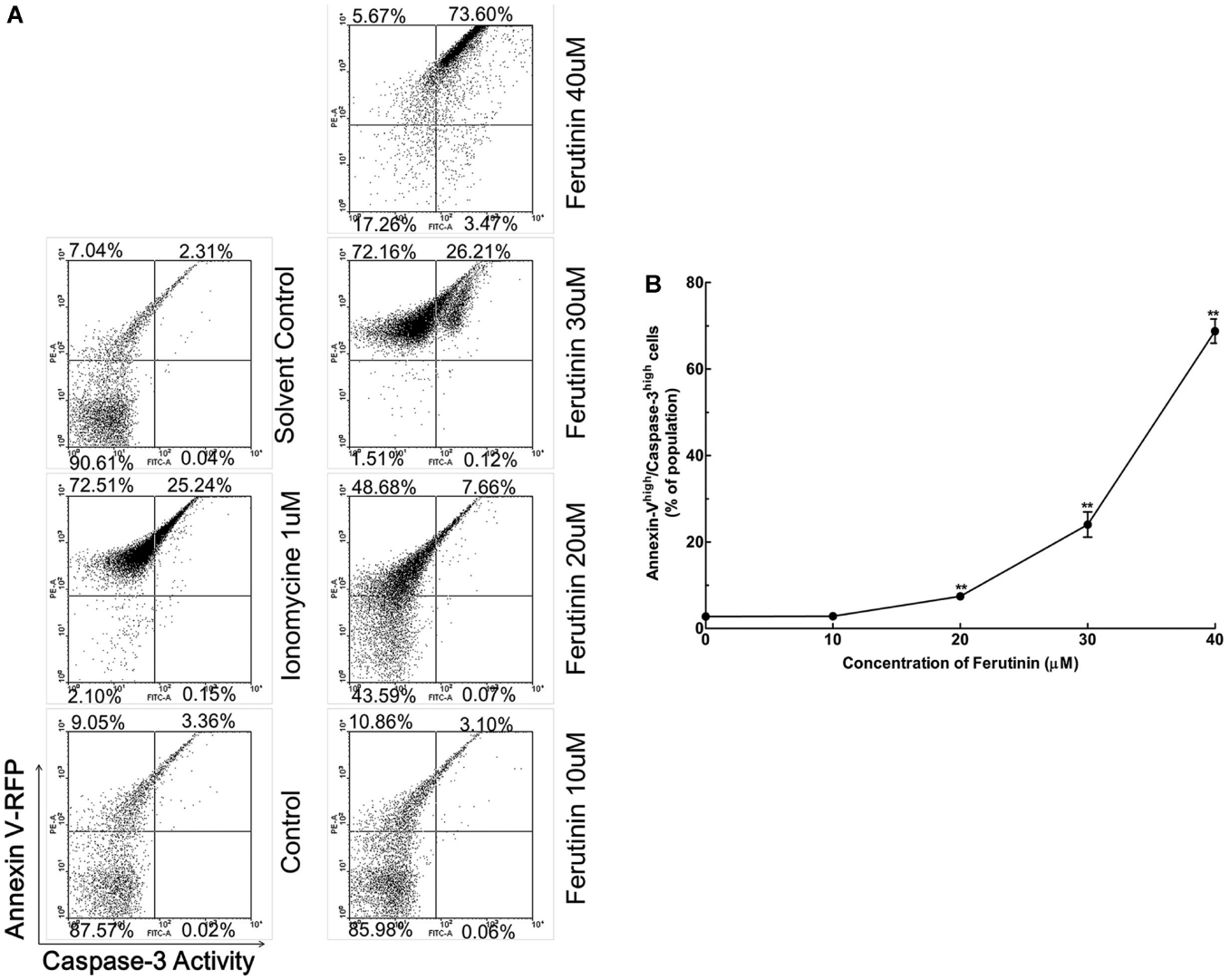

Figure 3. Effect of ferutinin on the activation of caspase-3 in human RBCs. RBCs (1.5×10^7/mL) were treated as described in the Figure 2 legend. After treatments, cells (1×10^6/mL) were incubated with FAM-DEVD-FMK in the dark for 40 min according to the manufacturer's instructions, then mixed with annexin-V-RFP and incubated for 20 min. After washing, cells were subject to flow cytometric analysis and the percentage of cells in the quadrants determined (A). Results in B are the mean ± SD ($n = 3$) of the cells in the upper-right quadrant (annexin-V^{high}/caspase-3^{high}) in A. $^{**}p < 0.01$, relative to the control.

ferutinin is able to induce eryptosis/erythroptosis in human erythrocytes in a dose-dependent manner.

The eryptosis effect of ferutinin was further determined by confocal microscopy with annexin-V-RFP and calcein/AM. As shown in Figure 2C, the human erythrocytes in the control group exhibited the typical shape of biconcave disks with a diameter of ~8 μm containing strong calcein fluorescence without any annexin-V-RFP labeling. After ionomycin treatment (1 μM) for 24 h, the cell size became smaller, and annexin-V signals were obtained at the cell surface with less calcein inside. After ferutinin treatment (30 μM), cells became smaller too. Also, less calcein signals were found in the cytosol, and more annexin-V-RFP signals were observed on the membrane blebs when compared to that of the control. These results were consistent with the flow cytometric assays (Figure 2A).

Role of Ferutinin in Caspase-3 Activity and Cytosolic Ca^{2+} Ion Level in Human RBCs. Because caspase-3 is a crucial executor in apoptosis,[25] we therefore examined the activity of caspase-3 of the human RBCs treated with ferutinin with a caspase-3 fluorogenic substrate FAM-DEVD-FMK. As shown in Figure 3A, ionomycin (1 μM) once more increased the cell population in the upper right quadrant (annexin-V^{high}/caspase-3^{high}) from 3.4% (control) or 2.3% (solvent control) to 25.2%. When cells were challenged with different doses of ferutinin, a dose-dependent increase in the cell number in the upper right quadrant (annexin-V^{high}/caspase-3^{high}) was obtained (Figure 3A and B). In all treatment groups, cells carrying a higher caspase-3 activity also displayed a stronger annexin-V signal. Notably, the effect of ferutinin (40 μM) on the activation of caspase-3 was stronger than that of ionomycin (1 μM) (Figure 3A). These results indicate that ferutinin was a strong casapse-3 activator in human RBCs.

Next, we determined whether ferutinin induced an increase in the $[Ca^{2+}]_i$ in human RBCs since $[Ca^{2+}]_i$ is an important trigger of eryptosis/erythroptosis.[15,24,26,27] Cells with or without ferutinin treatment were dual stained with annexin-V-RFP and fluo-4/AM. Results in Figure 4A show the effects of different doses of ferutinin on the $[Ca^{2+}]_i$ and PS externalization in Ca^{2+}-cotaining HEPES buffer.

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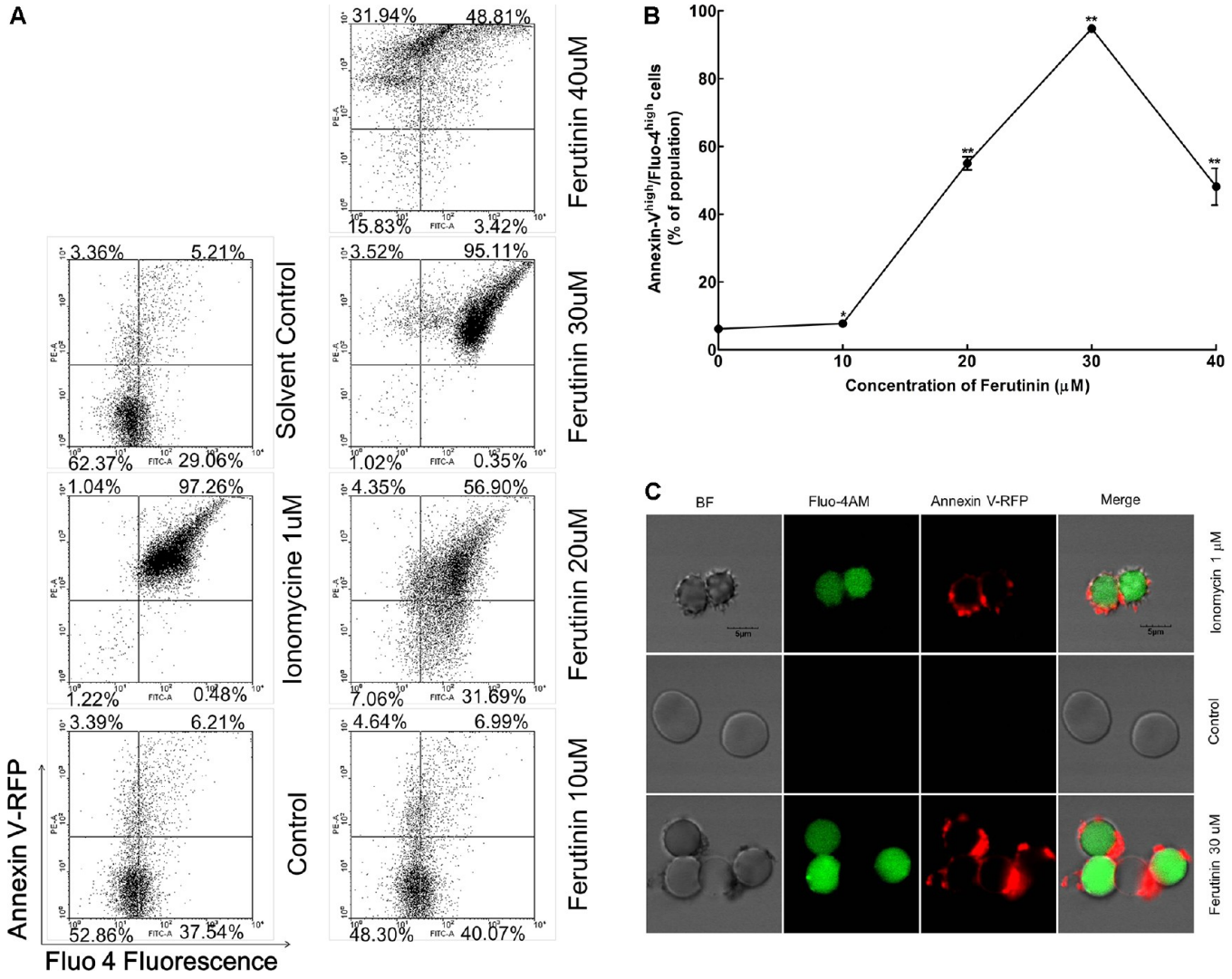

Figure 4. Effect of ferutinin on the $[Ca^{2+}]_i$ in human RBCs. RBCs (1.5×10^7/mL) were treated as described in the Figure 2 legend. After treatments, cells (1×10^6/mL) were incubated with annexin-V-RFP and fluo-4/AM (1 μM) in the dark for 30 min. After washing, cells were subject to flow cytometric analysis and the percentage of cells in the quadrants determined (A). Results in B are the mean ± SD ($n = 3$) of the cells in the upper-right quadrant (annexin-V^{high}/fluo-4^{high}) in A. * $p < 0.05$ and ** $p < 0.01$, relative to the control. In C, cells after treatments were seeded in a confocal dish, and an optical section was acquired by laser scanning confocal microscopy with corresponding excitation and emission wavelengths. The scale bar represents the cell dimension, 5 μm.

In the control group, 52.9% of cells were found in the lower left quadrant (annexin-V^{Low}/fluo-4^{Low}) and 6.2% in the upper right quadrant (annexin-V^{high}/fluo-4^{high}) (Figure 4A). After ionomycin stimulation, most of the cells shifted to the upper right quadrant (97.3%) suggesting that increase in the $[Ca^{2+}]_i$ induced PS externalization. When RBCs were incubated with ferutinin, a dose-dependent increase in the percentage of cell population was found in the upper right quadrant (annexin-V^{high}/fluo-4^{high}) (Figure 4A and B). Again, many cells were found in the upper left quadrant when cells were challenged with ferutinin at 40 μM (31.9%) largely due to the leakage of fluo-4 from the damaged membrane (Figure 4A). Together, these results further link ferutinin with the induction of apoptosis and also shed light on the mechanism that cytosolic Ca^{2+} ion is an important trigger of eryptosis/erythroptosis.

Next, we repeated the experiments by using confocal microscopy. As can be seen in Figure 4C, the human RBCs in the control group again appeared round in shape containing very weak fluo-4 fluorescence without any annexin-V-RFP labeling. After the challenge with ionomycin (1 μM) for 24 h, the cells became smaller with strong fluo-4 and annexin-V signals. After ferutinin treatment, the fluo-4 and annexin-V-RFP signals increased (Figure 4C).

Role of External Ca^{2+} in Ferutinin-Mediated Eryptosis/Erythroptosis in Human RBCs. To verify the role of Ca^{2+} in ferutinin-mediated eryptosis/erythroptosis, cells treated with a single dose of ferutinin (30 μM) in Ca^{2+}-free buffer for 24 h were resuspended in Ca^{2+}-free or Ca^{2+}-containing HEPES buffer (2.5 mM) to examine changes in fluo-4 fluorescence, annexin-V binding, and caspase-3 activity at different time points. We reasoned that if Ca^{2+} is the only trigger for the ferutinin-mediated eryptosis/erythroptosis, depletion of Ca^{2+} ions should block the apoptotic events.

As shown in Figure 5A, the relative fluo-4 intensity of the cells treated with ferutinin in Ca^{2+}-free buffer for 24 h and then resuspended in Ca^{2+}-free buffer for different time intervals was low and was similar to that of the untreated control group in the Ca^{2+}-free buffer. However, resuspension of the ferutinin-treated

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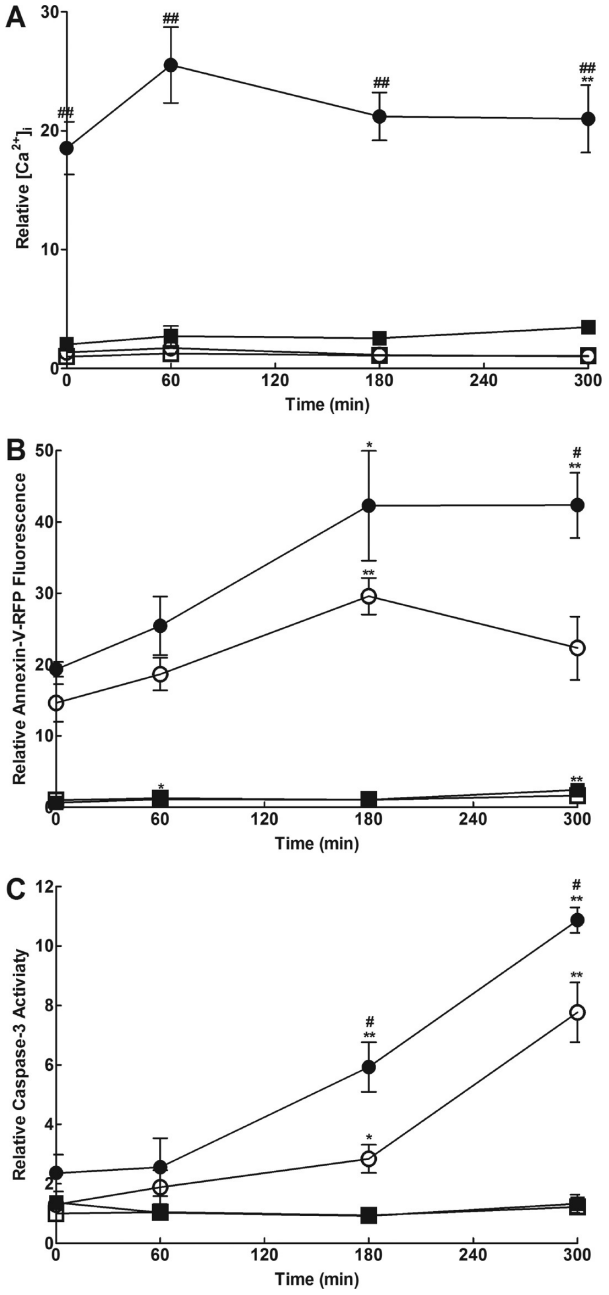

Figure 5. Role of external Ca^{2+} in ferutinin-mediated eryptosis/erythroptosis in the human RBCs. RBCs (1.5×10^7/mL) were treated with medium alone (open or closed square) or ferutinin (30 μM) (open or closed circle) at 37 °C for 24 h in Ca^{2+}-free HEPES buffer. After treatments, cells were resuspended in Ca^{2+}-containing (■ or ●) or -free HEPES buffer (□ or ○) for 0, 60, 180, or 300 min. Cells (1×10^6/mL) were then incubated in dark with fluo-4/AM (1 μM) (A), annexin-V-RFP (B) for 30 min, or FAM-DEVD-FMK for 60 min (C). After washing, cells were subject to flow cytometric analysis. Results (in relative MFI) are the mean ± SD ($n = 3$). * $p < 0.05$ and ** $p < 0.01$, relative to time 0 min; # $p < 0.05$ and ## $p < 0.01$, relative to the cells resuspended in Ca^{2+}-free HEPES buffer.

cells in the Ca^{2+}-containing buffer increased the fluo-4 fluorescence significantly (8 times), when compared to that of controls (Figure 5A). Under this condition, fluo-4 fluorescence was kept high until the end of the experiment. In the Ca^{2+}-free buffer, the control groups, with or without ferutinin (30 μM), showed low fluo-4 fluorescence, and no change was observed throughout the experiment (Figure 5A). Taken together, our results indicate that the 24-h ferutinin treatment in the Ca^{2+}-free buffer had acted on the membrane that allowed Ca^{2+} influx when external Ca^{2+} ions were replenished.

Next, we tried to investigate if the ferutinin-induced $[Ca^{2+}]_i$ increase played a key role in the induction of eryptosis/erythroptosis. For this, we repeated the treatments in Figure 5A, but cells were labeled with annexin-V-RFP in Ca^{2+}-containing buffer since the binding of annexin-V to PS on the cell surface is a Ca^{2+}-dependent process.[28] As shown in Figure 5B, cells in the control group with the Ca^{2+}-free buffer alone for 24 h showed a little annexin-V binding no matter whether Ca^{2+} ions were provided during the annexin-V labeling period. This observation supports the finding that bathing cells in a Ca^{2+}-containing buffer for 30 min from a Ca^{2+}-free environment is too short to trigger apoptosis in human RBCs.[25,29,30]

In the ferutinin-treated group (24 h, Ca^{2+}-free environment), PS were found on the cell surface and the degree of annexin-V binding was higher if external Ca^{2+} ions were supplied (Figure 5B). These high annexin-V readouts should be a result of the ferutinin effect and also the contribution of Ca^{2+} to annexin-V-PS binding. Although we cannot rule out the latter, the strong annexin-V signals in the ferutinin-treated group in the Ca^{2+}-free environment, when compared to those of the corresponding untreated group, indicate that ferutinin is able to induce PS externalization in human RBCs in the absence of external Ca^{2+} ions. This observation also suggests that the Ca^{2+} ion is not the only messenger for the induction of the apoptosis mediated by ferutinin.

These experiments were repeated for caspase-3 activity (Figure 5C). As can be seen, a time-dependent activation of caspase-3 was observed in the ferutinin-treated groups, and the activity was significantly higher when the cells were resuspended in Ca^{2+}-containing buffer (Figure 5C). As human RBCs do not contain any internal Ca^{2+} stores, bathing RBCs in a Ca^{2+}-free buffer should deplete all of the Ca^{2+} ions in the system. Under this condition, removal of Ca^{2+} could not totally eliminate the ferutinin effect, suggesting that Ca^{2+} is important but that it is not the sole messenger for ferutinin-mediated caspase-3 activation. Collectively, ferutinin was able to elicit eryptosis/erythroptosis in a Ca^{2+}-free environment, but the effect was less pronounced in the absence of Ca^{2+} ions. These observations suggest that Ca^{2+} plays a role, at least in part, in ferutinin-mediated eryptosis/erythroptosis.

Role of Ferutinin in Membrane Permeabilization in RBC Ghosts. To complement our findings on the mechanism of ferutinin-mediated eryptosis/erythroptosis, we were next curious to examine the role of ferutinin on the RBC membrane. To determine the degree of membrane permeabilization before and after ferutinin treatment, RBC ghosts were prepared with calcein (non-AM form) as previously described.[31] During membrane permeabilization, the amount of entrapped calcein released from the ghosts to the external buffer should be proportional to membrane permeability. As shown in Figure 6, most of the erythrocyte ghosts in the control group carried a strong calcein signal (70.5% in the selected region). When the ghosts were treated with digitonin (4 μM), a detergent that increases cell permeability by interacting with cholesterols in the cell membrane[32,33] as a positive control, all the ghosts showed a low fluorescence and the histogram moved to the left-hand side (0% in the selected region) (Figure 6). When the ghosts were challenged with ferutinin, a dose-dependent release of calcein was obtained. In particular, an abrupt drop was observed when

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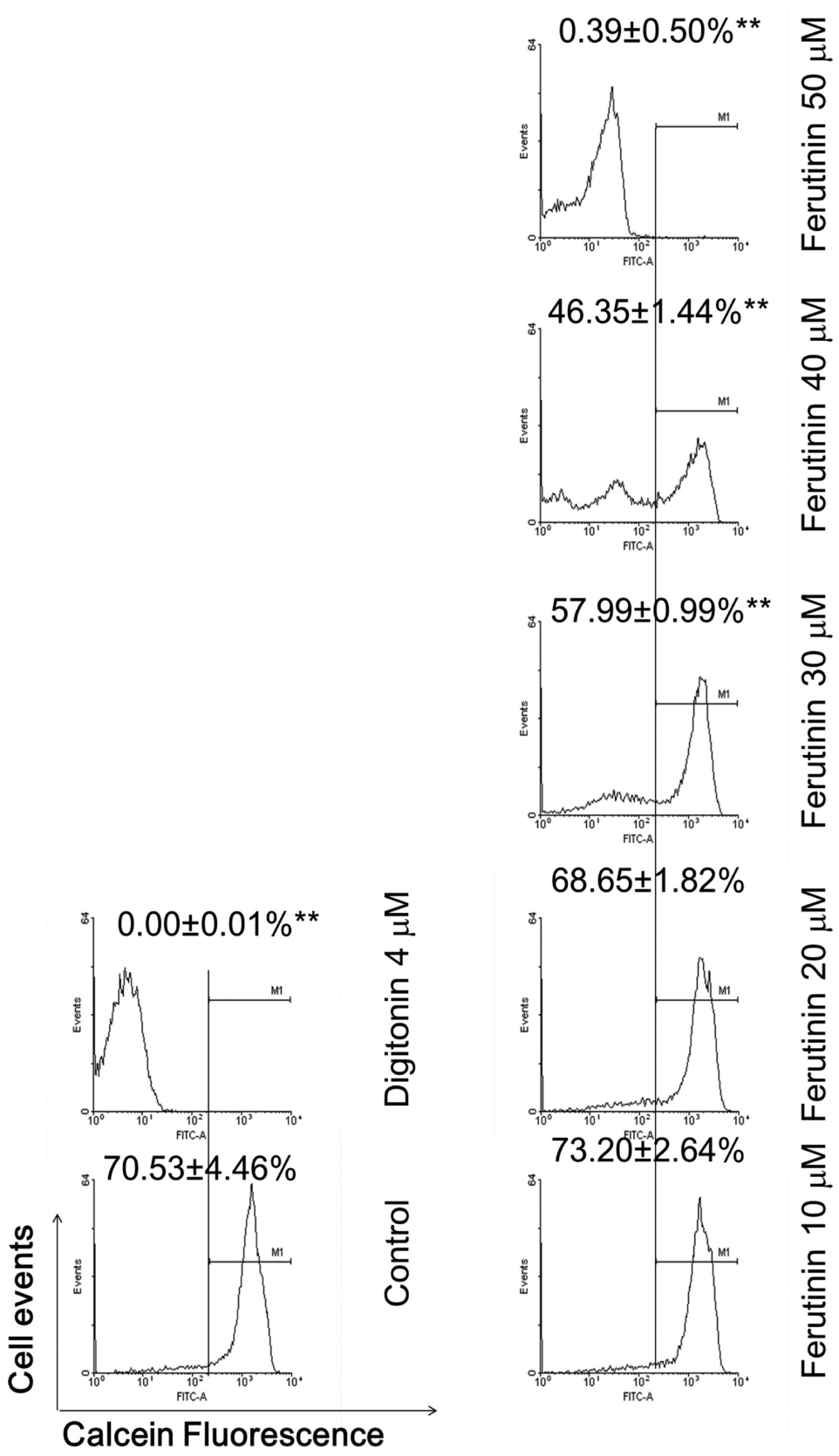

Figure 6. Effect of ferutinin on the release of calcein in ghosts. RBC ghosts entrapped with calcein were obtained as described in the Materials and Methods section. After treatments, ghost cells were submitted to flow cytometric analysis for the calcein fluorescence. Results are the mean ± SD ($n = 3$). $^{**}p < 0.01$, relative to the control.

the ghost was challenged with ferutinin of 50 μM. These results strongly indicate that ferutinin has a direct permeabilization effect on the RBC membrane without the involvement of cytosolic messengers.

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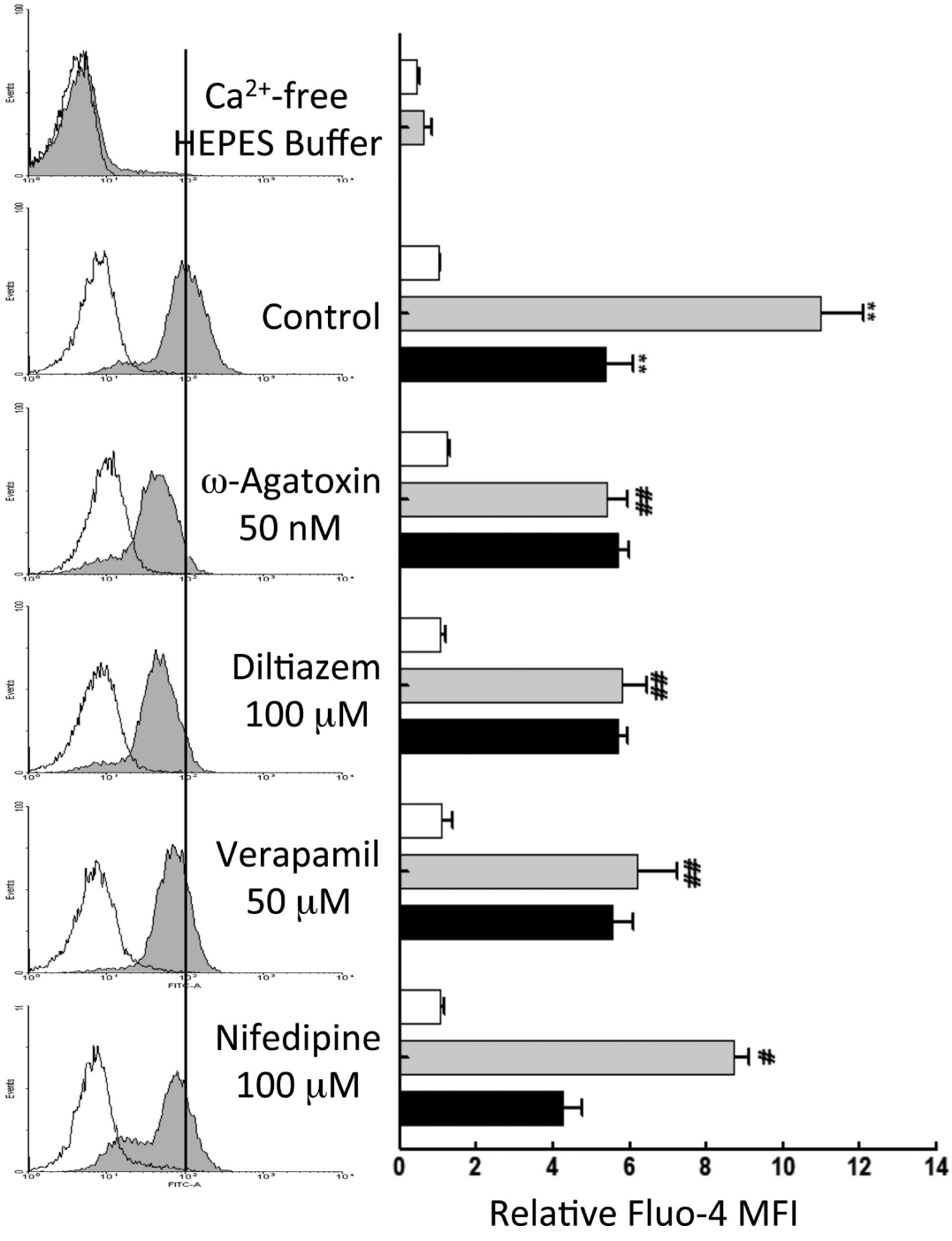

Figure 7. Effect of L-type and P-type Ca^{2+} channel blockers on ferutinin induced Ca^{2+} influx in human RBCs. RBCs (1.5×10^7/mL) were treated as described in the legend to Figure 2 except that the RBCs were pretreated with ω-agatoxin IVA (50 nM), diltiazem (100 μM), verapamil (50 μM), or nifedipine (100 μM) for 30 min before the challenge with ferutinin (30 μM or 40 μM) at 37 °C for 24 h. After treatment, cells (1×10^6/mL) were incubated with fluo-4/AM (1 μM) in the dark for 30 min. After washing, cells were subject to flow cytometric analysis. Histograms are presented on the left and relative mean fluorescence intensity (MFI) on the right (control MFI = 1). Results in the bar chart are the mean ± SD ($n = 3$). Open histogram or open bar, buffer or blockers alone; gray histogram or gray bar, ferutinin (30 μM) with or without blocker; dark bar, ferutinin (40 μM) with or without blocker as indicated. $^{**}p < 0.01$, relative to the control. $\#p < 0.05$ and $\#\#p < 0.01$, relative to 30 μM ferutinin in the control group.

Effect of L-Type and P-Type Ca^{2+} Channel Blockers on the Ferutinin-Induced Influx of Ca^{2+} into Human RBCs. To further determine which type of Ca^{2+} channel ferutinin induced Ca^{2+} influx into human RBCs, RBCs were pretreated with different Ca^{2+} channel blockers (ω-agatoxin IVA, (P-type), nifedipine (dihydropyridine L-type), verapamil, (phenylalkylamine L-type), and diltiazem (benzothiazepine L-type))[34,35] for 30 min before the challenge with ferutinin (30 and 40 μM) for 24 h for the changes of $[Ca^{2+}]_i$. As shown in Figure 7 (open bar), treatment of RBCs alone with these blockers individually did not alter much the fluo-4 fluorescence when compared to that of the control (buffer alone). When cells were pretreated with the blockers and then challenged with ferutinin (30 μM), ω-agatoxin IVA, diltiazem, and verapamil reduced the $[Ca^{2+}]_i$ in the RBCs

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more significantly than nifedipine (Figure 7 gray bar), suggesting that ferutinin at 30 μM activated the classical P-type Ca^{2+} channel and phenylalkylamine and benzothiazepine the L-type Ca^{2+} channels. However, when the human RBCs were treated with ferutinin (40 μM) alone, a lower fluo-4 fluorescence (compared to that with 30 μM) was obtained (Figure 7 solid bar). This observation is consistent with the results reported in Figure 4. Also, pretreatment of cells with these Ca^{2+} channel blockers had no effect on the $[Ca^{2+}]_i$ mediated by ferutinin (40 μM) (compared to that with the 40 μM ferutinin alone control) (Figure 7, solid bar). Taken together with the findings from Figure 6, it is highly likely that ferutinin at 40 μM or above has a membrane permeabilization effect which allows fluo-4 to leak out from cells. At 30 μM, ferutinin induces Ca^{2+} influx by activating the P- and L-type (phenylalkylamine and benzothiazepine) Ca^{2+} channels.

DISCUSSION

Ferutinin, used as folk medicine to treat digestive disorders, headache, and arthritis,[36] was found to induce apoptosis in cancer cells through the activation of the mitochondrial pathway with an effective dose at 50 μM.[6] If the action on the mitochondria is the sole mechanism responsible for the induction of apoptosis, ferutinin should not be able to induce eryptosis/erythroptosis in the human RBCs without mitochondria. In fact, very limited information is available in the literature regarding the effect of ferutinin on the human erythrocytes. To evaluate the potential and proper use of ferutinin, we set out to investigate whether ferutinin, using the doses and experimental settings used in other studies,[8,9] produces any eryptotic effects in human RBCs. To our surprise, ferutinin is able to trigger hemolysis and eryptosis/erythroptosis in the human erythrocytes in a dose-dependent manner starting from 20 μM as confirmed by the annexin-V/calcein release assay that reports PS externalization and loss of membrane integrity after the ferutinin treatment (Figure 2). This finding suggests that the use of ferutinin for cancer treatment may induce anemia in the host through the induction of eryptosis/erythroptosis.

It has been known for a while that eryptosis/erythroptosis can be activated by at least two signaling pathways in a coordinated manner.[11] The first pathway involves the activation of sphingomyelinase that converts sphingomyelin to ceramide. Ceramide then increases the $[Ca^{2+}]_i$. The second pathway proceeds through the activation of Ca^{2+} channels on the cell membrane and inhibition of the (Ca^{2+}-Mg^{2+})-ATPase activity to increase the $[Ca^{2+}]_i$.[37] The ceramide and high $[Ca^{2+}]_i$ promote the translocation of the negatively charged PS from the inner to the outer plasma membrane leaflet. Moreover, the elevated $[Ca^{2+}]_i$ also activates the Ca^{2+}-dependent calpain to proteolyze many cytoskeletal proteins leading to changes in morphology and bleb formation.[14,24,26]

Having established that ferutinin could induce eryptosis/erythroptosis, we next investigated the role of Ca^{2+} in ferutinin-mediated apoptosis. Our results confirm that ferutinin induced the cell death program in human RBCs in part through the Ca^{2+} pathway by activating the P-type and phenylalkylamine and benzothiazepine L-type Ca^{2+} channels which were found in human RBCs before.[34,35] Our conclusion is based on the following observations. First, when the treatment was conducted in Ca^{2+}-free buffer, all of the eryptosis-/erythroptosis-associated events such as PS externalization, increase in the $[Ca^{2+}]_i$ and activation of caspase-3 were reduced when compared to those in a Ca^{2+}-containing environment. These results obviously suggest that the apoptotic pathway in the human RBCs can be activated, at least in part, in the absence of Ca^{2+}. Second, readdition of Ca^{2+} after the treatment in a Ca^{2+}-free buffer for 24 h caused a rapid and immediate influx of Ca^{2+}, activation of caspase-3, and PS externalization, disclosing the role of Ca^{2+} in ferutinin-induced eryptosis/erythroptosis. Third, ω-agatoxin IVA, verapamil, and diltiazem significantly reduced ferutinin-induced Ca^{2+} entry into the RBCs. Intrigued by these results, we tried to explore whether another mechanism exists for ferutinin's cytotoxicity.

In view of the fact that ferutinin is able to increase membrane permeability on the artificial membrane and the mitochondrial membrane,[8] we prepared ghosts devoid of cellular components from RBCs and studied the effect of ferutinin on the ghost membrane. RBC ghosts have been a good tool for studying membrane properties for many years.[38,39] Although the effect was weaker than that of digitonin, results from our study indicate that ferutinin at high dose (e.g., 40 μM) was able to act on ghosts to release the entrapped calcein into external buffer, suggesting that ferutinin is able to act on the cell membrane directly without the involvement of cellular components. Since the $[Ca^{2+}]_i$ is lower than that of the extracellular buffer, this direct effect explains why ferutinin increased the $[Ca^{2+}]_i$ in the ferutinin-treated RBCs via Ca^{2+} influx, apart from the activation of Ca^{2+} channels. This observation is similar to our previous finding from polyphyllin D (PD) that it acts as a detergent on the ghost membrane to release entrapped calcein,[18] although PD and ferutinin do not share structural similarity. Remarkably, results in our study indicate that ferutinin is able to alter the membrane permeability in the human RBCs for the small molecules like calcein and Ca^{2+} ions but not the large complex FAM-DEVD-FMK-caspase-3 (data not show). All together, our results show that ferutinin permeabilizes the RBC membrane and the influx of Ca^{2+} through P-type and phenylalkylamine and that benzothiazepine L-type Ca^{2+} channels activate the apoptotic pathway, at least in part, in the human RBCs. For the role of ceramide, more work is needed to identify whether ferutinin activates the sphingomyelinase for the induction of eryptosis/erythroptosis. Also, the roles of kinases such as p38 kinase,[40] Janus kinase3,[41] AMPK,[42] and G-kinase[43] during ferutinin-induced eryptosis/erythroptosis should be investigated in the future. In conclusion, our results demonstrate that ferutinin is a trigger of eryptosis/erythroptosis in human RBCs without the involvement of mitochondria. We also show the mechanism of how ferutinin acts an apoptogenic agent in the RBCs to increase the $[Ca^{2+}]_i$. In view of the toxic in vitro effect of ferutinin in RBCs and its enhanced stablilty with plasma albumin,[44] more work is needed to examine the eryptotic effect of ferutinin in animals before using it as a phytoestrogen or anticancer drug.

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Notes

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■ ABBREVIATIONS

BSA, bovine serum albumin; $[Ca^{2+}]_i$, intracellular free calcium ion concentration; DMSO, dimethyl sulfoxide; EGTA, ethylene glycol tetraacetic acid; Hb, hemoglobin; PD, polyphyllin D; PS, phosphatidyl-serine; RBCs, red blood cells

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