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Exploration of Niflumic Acid's Action on Ca^{2+} Movement and Cell Viability in Human Osteosarcoma Cells

Wei-Chuan Liao[1, &], Chiang-Ting Chou[2], Wei-Zhe Liang[3, 4, &], Lyh-Jyh Hao[5, &], Chun-Chi Kuo[6], Ko-Long Lin[7], Jue-Long Wang[7], and Chung-Ren Jan[3]

[1]Department of Surgery, Kaohsiung Veterans General Hospital, Kaohsiung 81362,
[2]Department of Nursing, Division of Basic Medical Sciences, Chang Gung University of Science and Technology, Chia-Yi 61363,
[3]Department of Medical Education and Research, Kaohsiung Veterans General Hospital, Kaohsiung 81362,
[4]Department of Pharmacy, Tajen University, Pingtung 90741,
[5]Department of Metabolism, Kaohsiung Veterans General Hospital Tainan Branch, Tainan 71051, and
[6]Department of Nursing, Tzu Hui Institute of Technology, Pingtung 92641, and
[7]Department of Rehabilitation, Kaohsiung Veterans General Hospital, Kaohsiung 81362, Taiwan, Republic of China

Abstract

Niflumic acid, a drug used for joint and muscular pain, affected Ca^{2+} signaling in different models. However, the effect of niflumic acid on Ca^{2+} homeostasis and Ca^{2+}-related physiology in human osteosarcoma cells is unknown. This study examined the effect of niflumic acid on cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) in MG63 human osteosarcoma cells. Intracellular Ca^{2+} concentrations in suspended cells were monitored by using the fluorescent Ca^{2+}-sensitive dye fura-2. Cell viability was examined by using 4-[3-[4-Iodophenyl]-2-4(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate] water soluble tetrazolium-1 (WST-1). In MG63 cells, niflumic acid at concentrations of 250-750 μM evoked $[Ca^{2+}]_i$ rises concentration-dependently. Niflumic acid-evoked Ca^{2+} entry was confirmed by Mn^{2+}-induced quenching of fura-2 fluorescence. This entry was inhibited by nifedipine, econazole, SKF96365, the protein kinase C (PKC) activator phorbol 12-myristate 13 acetate (PMA), but was not affected by the PKC inhibitor GF109203X. In Ca^{2+}-free medium, treatment with the endoplasmic reticulum Ca^{2+} pump inhibitor thapsigargin (TG) inhibited niflumic acid-evoked $[Ca^{2+}]_i$ rises. Conversely, treatment with niflumic acid abolished TG-evoked $[Ca^{2+}]_i$ rises. Inhibition of phospholipase C (PLC) with U73122 also partly reduced niflumic acid-evoked $[Ca^{2+}]_i$ rises. Niflumic acid killed cells at 200-500 μM in a concentration-dependent fashion. Chelating cytosolic Ca^{2+} with 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid/AM (BAPTA/AM) did not reverse niflumic acid-induced cytotoxicity. Collectively, our data suggest that in MG63 cells, niflumic acid induced $[Ca^{2+}]_i$ rises by evoking PLC-dependent Ca^{2+} release from the endoplasmic reticulum, and Ca^{2+} entry via PKC-sensitive store-operated Ca^{2+} entry. Niflumic acid also induced Ca^{2+}-independent cell death.

Key Words: Ca^{2+}, endoplasmic reticulum, human osteosarcoma cells, niflumic acid, viability

Corresponding Authors: [1] Dr. Ko-Long Lin, Department of Rehabilitation, Kaohsiung Veterans General Hospital, Kaohsiung 81362, Taiwan, ROC; [2] Dr. Jue-Long Wang, Department of Rehabilitation, Kaohsiung Veterans General Hospital, Kaohsiung 81362, Taiwan, ROC; and [3] Dr. Chung-Ren Jan, Department of Medical Education and Research, Kaohsiung Veterans General Hospital, Kaohsiung 81362, Taiwan, ROC. Tel: +886-7-3422121 ext. 1509. Fax: +886-7-3468056. Email: crjan@isca.vghks.gov.tw. &Contributed equally to this work.
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Introduction

Niflumic acid is clinically used in rehabilitation science for relieving joint and muscular pain. It is categorized as an inhibitor of cyclooxygenase-2 (16). *In vitro*, niflumic acid affected different physiological responses in various models. It has been shown that niflumic acid induced additional currents of the human glial L-glutamate transporter EAAT1 in a substrate-dependent manner (27). Furthermore, niflumic acid has been shown to activate Slo2.1 channels (8) and modulate glutamate and glycine transporters in *Xenopus laevis* oocytes (12). Regarding the effect of niflumic acid on Ca^{2+} homeostasis in various cell models, it has been shown that niflumic acid blocked T-type Ca^{2+} channels in HEK 293 cells (1), and inhibited Cl^- conductance of rat skeletal muscle by directly inhibiting the CLC-1 channel and by increasing intracellular Ca^{2+} (19). In addition, niflumic acid has been shown to regulate store-operated Ca^{2+} channels in K562 cells (17), Ca^{2+}-dependent Cl^- channel in rabbit coronary arterial myocytes (18), and release Ca^{2+} from intracellular stores in rat pulmonary artery smooth muscle cells (6). However, the effect of niflumic acid on Ca^{2+} homeostasis in human osteosarcoma cells is not clear.

Ca^{2+} ions play a crucial role in different biological responses. A rise in $[Ca^{2+}]_i$ can initiate many pathophysiological cellular processes (2). However, an uncontrolled $[Ca^{2+}]_i$ rise induces dysfunction of enzymes, apoptosis, and proliferation, etc. (5). A $[Ca^{2+}]_i$ rise can be due to Ca^{2+} entry from external medium and/or Ca^{2+} release from intracellular organelles (2, 5). It is important to explore the mechanisms of an agent-induced Ca^{2+} entry and Ca^{2+} release in order to understand the impact of this agent on physiology of the cells.

A previous study (23) has shown that niflumic acid-induced skeletal fluorosis is associated with iatrogenic disease and may provide therapeutic perspective for osteoporosis. Therefore, the effect of long-term use of niflumic acid on bone physiology should be cautioned. This study was aimed to explore the effect of niflumic acid on cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) and cell viability in human osteosarcoma cells, and to explore their relationship. In order to explore the effect of niflumic acid on physiology in human osteosarcoma cells, the MG63 cell was used because it produces considerable $[Ca^{2+}]_i$ rises upon stimulation. Literature shows that in this cell, the stimulation of compounds such as NPC-14686 (4), sertraline (20), and 3,3'-diindolylmethane (21) evoked $[Ca^{2+}]_i$ rises and cytotoxicity.

In this study, fura-2 was used as a Ca^{2+}-sensitive dye to measure $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ rises were characterized, the concentration-response plots were established, the mechanisms underlying niflumic acid-evoked Ca^{2+} entry and Ca^{2+} release was examined. The effect of niflumic acid on cytotoxicity and its relationship to Ca^{2+} was also explored.

Materials and Methods

Chemicals

The reagents for cell culture were from Gibco® (Gaithersburg, MD, USA). Aminopolycarboxylic acid/acetoxy methyl (fura-2/AM) and 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid/acetoxy methyl (BAPTA/AM) were from Molecular Probes® (Eugene, OR, USA). Niflumic acid and other reagents were from Sigma-Aldrich® (St. Louis, MO, USA).

Cell Culture

MG63 human osteosarcoma cells obtained from Bioresource Collection and Research Center (Taiwan) were cultured in minimum essential medium (MEM) medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

Solutions Used in $[Ca^{2+}]_i$ Measurements

Ca^{2+}-containing medium (pH 7.4) had 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 5 mM glucose. Ca^{2+}-free medium contained similar chemicals as Ca^{2+}-containing medium except that $CaCl_2$ was replaced with 0.3 mM ethylene glycol tetraacetic acid (EGTA) and 2 mM $MgCl_2$. Niflumic acid was dissolved in ethanol as a 2 M stock solution. The other chemicals were dissolved in water, ethanol or dimethyl sulfoxide (DMSO). The concentration of organic solvents in the experimental solutions did not exceed 0.1%, and did not affect viability or basal $[Ca^{2+}]_i$.

$[Ca^{2+}]_i$ Measurements

The $[Ca^{2+}]_i$ was measured as previously described (4, 20, 21). Confluent cells grown on 6 cm dishes were trypsinized and made into a suspension in culture medium at a concentration of 10^6 cell/ml. Cell viability was determined by trypan blue exclusion (adding 0.2% trypan blue to 0.1 ml cell suspension). The viability was greater than 95% after the treatment. Cells were subsequently loaded with 2 μM fura-2/AM for 30 min at 25°C in the

same medium. After loading, cells were washed with Ca^{2+}-containing medium twice and was made into a suspension in Ca^{2+}-containing medium at a concentration of 10^7 cell/ml. Fura-2 fluorescence measurements were performed in a water-jacketed cuvette (25°C) with continuous stirring; the cuvette contained 0.5 million cells suspended in 1 ml of medium. Fluorescence was monitored with a Shimadzu RF-5301PC spectrofluorophotometer immediately after 0.1 ml cell suspension was added to 0.9 ml Ca^{2+}-containing or Ca^{2+}-free medium, by recording excitation signals at 340 nm and 380 nm and emission signal at 510 nm at 1-s intervals. For calibration of $[Ca^{2+}]_i$, after completion of the experiments, the detergent Triton X-100 (0.1%) and $CaCl_2$ (5 mM) were added to the cuvette to obtain the maximal fura-2 fluorescence. Then the Ca^{2+} chelator EGTA (10 mM) was added to chelate Ca^{2+} in the cuvette to obtain the minimal fura-2 fluorescence. Control experiments showed that cells bathed in a cuvette had a viability of 95% after 20 min of fluorescence measurements. $[Ca^{2+}]_i$ was calculated as previously described (11).

Mn^{2+} quenching of fura-2 fluorescence was performed in Ca^{2+}-containing medium containing 50 μM $MnCl_2$. $MnCl_2$ was added to cell suspension in the cuvette 30 sec before the fluorescence recoding was started. Data were recorded at excitation signal at 360 nm (Ca^{2+}-insensitive) and emission signal at 510 nm at 1-s intervals as described previously (22).

Cell Viability Assays

Viability was assessed as previously described (4, 20, 21). The measurement of cell viability was based on the ability of cells to cleave tetrazolium salts by dehydrogenases. Increases in the amount of developed color directly correlated with the number of live cells. Assays were performed according to manufacturer's instructions (Roche Molecular Biochemical, Indianapolis, IN, USA). Cells were seeded in 96-well plates at a concentration of 10^4 cell/well in culture medium for 24 h in the presence of niflumic acid. The cell viability detecting tetrazolium reagent 4-[3-[4-Iodophenyl]-2-4(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate] (WST-1; 10 μl pure solution) was added to samples after niflumic acid treatment, and cells were incubated for 30 min in a humidified atmosphere. In experiments using BAPTA/AM to chelate cytosolic Ca^{2+}, cells were treated with 5 μM BAPTA/AM for 1 h prior to incubation with niflumic acid. The cells were washed once with Ca^{2+}-containing medium and incubated with/without niflumic acid for 24 h. The absorbance of samples (A_{450}) was determined using an enzyme-linked immunosorbent assay (ELISA) reader. Absolute optical density was normalized to the absorbance of unstimulated cells in each plate and expressed as percentage of control value.

Statistics

Data are reported as mean ± standard error of the mean (SEM) of three experiments. Data were analyzed by one-way analysis of variances (ANOVA) using the Statistical Analysis System (SAS®, SAS Institute Inc., Cary, NC, USA). Multiple comparisons between group means were performed by *post-hoc* analysis using the Tukey's honestly significantly difference (HSD) procedure. A *P*-value less than 0.05 was considered significant.

Results

Effect of Niflumic Acid on $[Ca^{2+}]_i$ in MG63 Cells

The effect of niflumic acid on basal $[Ca^{2+}]_i$ was examined. Fig. 1A shows that the basal $[Ca^{2+}]_i$ level was 50 ± 1 nM. Niflumic acid induced $[Ca^{2+}]_i$ rises in a concentration-dependent manner at concentrations between 250 and 750 μM, in Ca^{2+}-containing medium. At a concentration of 750 μM, niflumic acid evoked $[Ca^{2+}]_i$ rises that attained to a net increase of 99 ± 3 nM. The Ca^{2+} response saturated at 750 μM niflumic acid because 1000 μM niflumic acid did not induce a greater response (data not shown). Fig. 1B shows that in the absence of extracellular Ca^{2+}, 250-750 μM niflumic acid induced concentration-dependent $[Ca^{2+}]_i$ rises. Fig. 1C shows the concentration-response relationship of niflumic acid-induced $[Ca^{2+}]_i$ rises. The EC_{50} value was 430 ± 3 μM or 550 ± 2 μM in Ca^{2+}-containing medium or Ca^{2+}-free medium, respectively, by fitting to a Hill equation. Ca^{2+} removal reduced the Ca^{2+} signal by approximately 40%.

Niflumic Acid-Induced Mn^{2+} Influx in MG63 Cells

We further explored whether niflumic acid indeed evoked Ca^{2+} influx. Literature shows that Mn^{2+} and Ca^{2+} move across the cell through identical pathways but the former quenches fura-2 fluorescence at all excitation wavelengths (22). Therefore, quenching of fura-2 fluorescence excited at the Ca^{2+}-insensitive excitation wavelength of 360 nm by Mn^{2+} suggests that niflumic acid induced Ca^{2+} influx. Because niflumic acid-induced Ca^{2+} response saturated at 750 μM, in the following experiments the response induced by 750 μM niflumic acid was used as control. Fig. 2 shows that 750 μM niflumic acid evoked an instant decrease in the

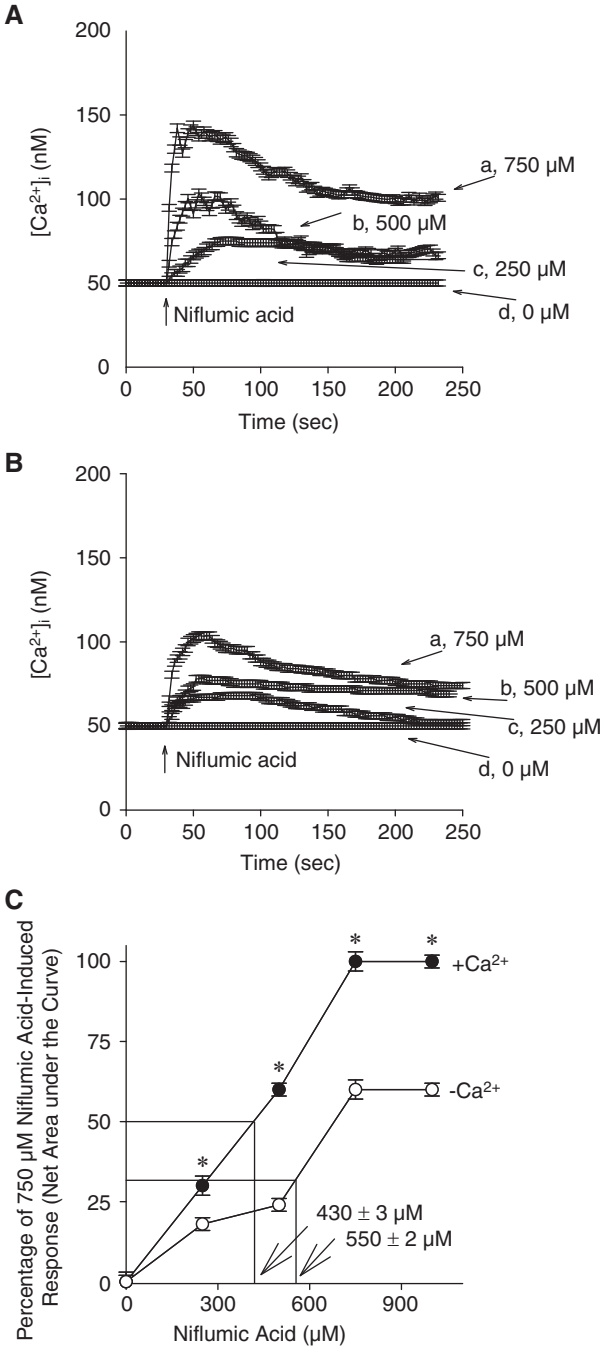

Fig. 1. Effect of niflumic acid on $[Ca^{2+}]_i$ in fura-2-loaded MG63 cells. (A) Niflumic acid was added at 25 s. The concentration of niflumic acid was indicated. The experiments were performed in Ca^{2+}-containing medium. (B) Effect of niflumic acid on $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. Niflumic acid was added at 25 s in Ca^{2+}-free medium. (C) Concentration-response plots of niflumic acid-induced $[Ca^{2+}]_i$ rises in the presence or absence of extracellular Ca^{2+}. Y axis is the percentage of the net (baseline subtracted) area under the curve (25-250 s) of the $[Ca^{2+}]_i$ rises induced by 750 μM niflumic acid in Ca^{2+}-containing medium. Data are mean ± SEM of three experiments. $*P < 0.05$ compared to open circles.

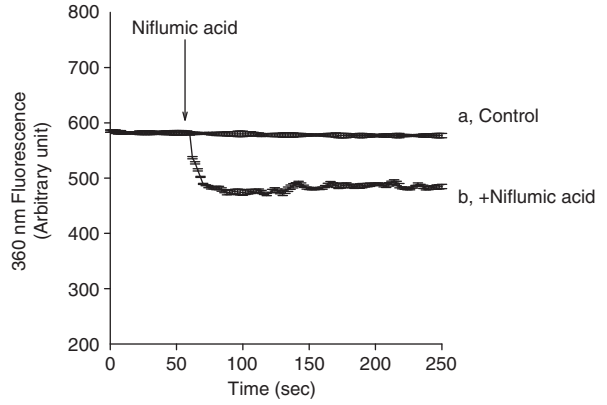

Fig. 2. Effect of niflumic acid on Ca^{2+} influx by measuring Mn^{2+} quenching of fura-2 fluorescence in MG63 cells. Experiments were performed in Ca^{2+}-containing medium. $MnCl_2$ (50 μM) was added to cells 1 min before fluorescence measurements. The y axis is fluorescence intensity (in arbitrary units) measured at the Ca^{2+}-insensitive excitation wavelength of 360 nm and the emission wavelength of 510 nm. Trace a: control, without niflumic acid. Trace b: niflumic acid (750 μM) was added as indicated. Data are mean ± SEM of three separate experiments.

360 nm excitation signal that reached a maximum value of 111 ± 1 arbitrary units at 100 s. This suggests that Ca^{2+} influx participated in niflumic acid-evoked $[Ca^{2+}]_i$ rises.

Regulation of Niflumic Acid-Induced $[Ca^{2+}]_i$ Rises in MG63 Cells

The Ca^{2+} entry modulators [nifedipine (1 μM), econazole (0.5 μM), SKF96365 (5 μM), phorbol 12-myristate 13 acetate (PMA; 1 nM), and GF109203X (2 μM)] were applied 1 min before niflumic acid (750 μM) in Ca^{2+}-containing medium. Except GF109203X, the other chemicals inhibited niflumic acid-induced $[Ca^{2+}]_i$ rises by 22-35% (Fig. 3). This suggests that store-operated and PKC-sensitive Ca^{2+} entry were involved in niflumic acid-induced $[Ca^{2+}]_i$ rises.

Source of Niflumic Acid-Induced Ca^{2+} Release in MG63 Cells

In most cell types, the endoplasmic reticulum has been shown to be the dominant Ca^{2+} store (2, 5). Thus the role of the endoplasmic reticulum in niflumic acid-evoked Ca^{2+} release in MG63 cells was examined. To exclude the contribution of Ca^{2+} influx, the experiments were conducted in Ca^{2+}-free medium. Fig. 4A shows that addition of 1 μM

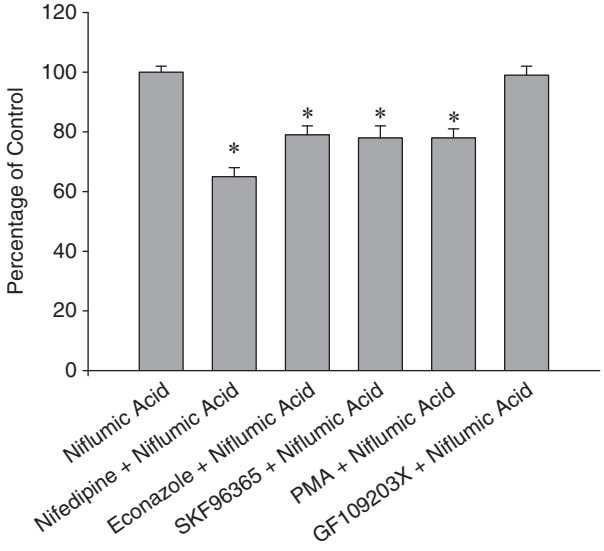

Fig. 3. Effect of Ca^{2+} channel modulators on niflumic acid-induced $[Ca^{2+}]_i$ rises in MG63 cells. In blocker- or modulator-treated groups, the reagent was added 1 min before niflumic acid (750 μM). The concentration was 1 μM for nifedipine, 0.5 μM for econazole, 5 μM for SKF96365, 10 nM for phorbol 12-myristate 13-acetate (PMA), and 2 μM for GF109203X. Data are expressed as the percentage of control (1st column) that is the area under the curve (25-200 s) of 750 μM niflumic acid-induced $[Ca^{2+}]_i$ rises in Ca^{2+}-containing medium, and are mean ± SEM of three separate experiments. $*P < 0.05$ compared to the 1st column.

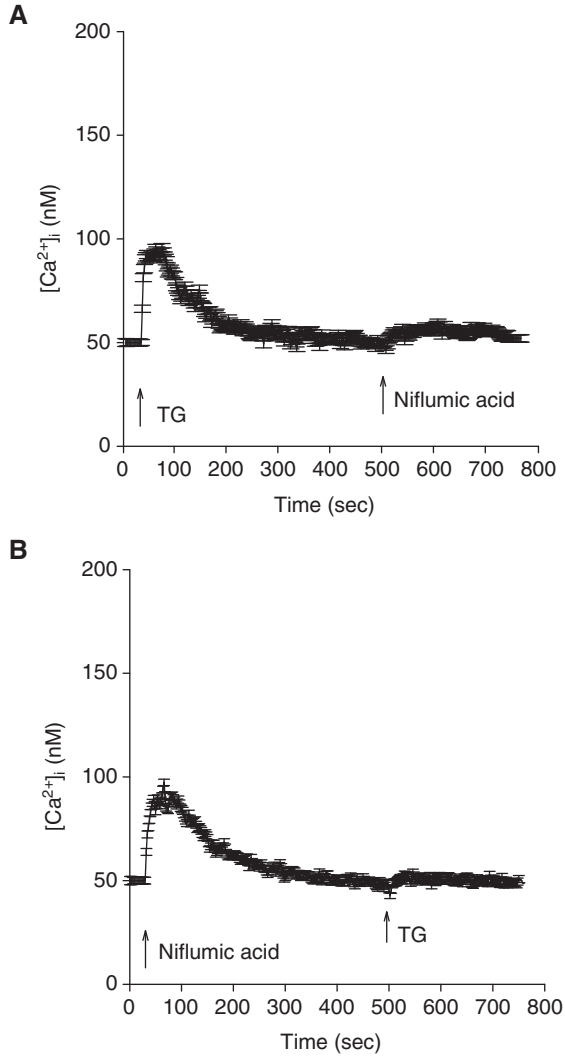

Fig. 4. Effect of thapsigargin (TG) on niflumic acid-induced Ca^{2+} release in MG63 cells. (A), (B) TG (1 μM) and niflumic acid (750 μM) were added at time points indicated. Experiments were performed in Ca^{2+}-free medium. Data are mean ± SEM of three separate experiments.

thapsigargin (TG), an endoplasmic reticulum Ca^{2+} pump inhibitor (28), induced $[Ca^{2+}]_i$ rises of 50 ± 2 nM. Addition of 750 μM niflumic acid afterwards induced tiny $[Ca^{2+}]_i$ rises of 6 ± 2 nM. Fig. 4B shows that after niflumic acid-induced $[Ca^{2+}]_i$ rises, addition of TG failed to induce $[Ca^{2+}]_i$ rises. This suggests that niflumic acid induced $[Ca^{2+}]_i$ rises by releasing Ca^{2+} from the endoplasmic reticulum.

A Role of Phospholipase C (PLC) in Niflumic Acid-Induced $[Ca^{2+}]_i$ Rises in MG63 Cells

PLC is one of the pivotal proteins that regulate the release of Ca^{2+} from the endoplasmic reticulum (2). Because niflumic acid released Ca^{2+} from the endoplasmic reticulum, the role of PLC in this process was explored. U73122 (29), a PLC inhibitor, was applied to explore if the activation of this enzyme was required for niflumic acid-induced Ca^{2+} release. Fig. 5A shows that ATP (10 μM) induced maximum $[Ca^{2+}]_i$ rises of 88 ± 2 nM. ATP is a PLC-dependent agonist of $[Ca^{2+}]_i$ rises in most cell types (10). Fig. 5B shows that incubation with 2 μM U73122 did not change basal $[Ca^{2+}]_i$ but abolished ATP-induced $[Ca^{2+}]_i$ rises. This suggests that U73122 effectively suppressed PLC activity. The data also show that incubation with 2 μM U73122 did not alter basal $[Ca^{2+}]_i$ but inhibited 70% of 750 μM niflumic acid-induced $[Ca^{2+}]_i$ rises. U73343 is a PLC-insensitive structural analog of U73122 and is often used as a control for U73122 activity. Our data show that U73343 (2 μM) did not alter ATP-evoked $[Ca^{2+}]_i$ rises (not shown). This suggests that U73122 inhibited niflumic acid-induced $[Ca^{2+}]_i$ rises partially through acting on PLC.

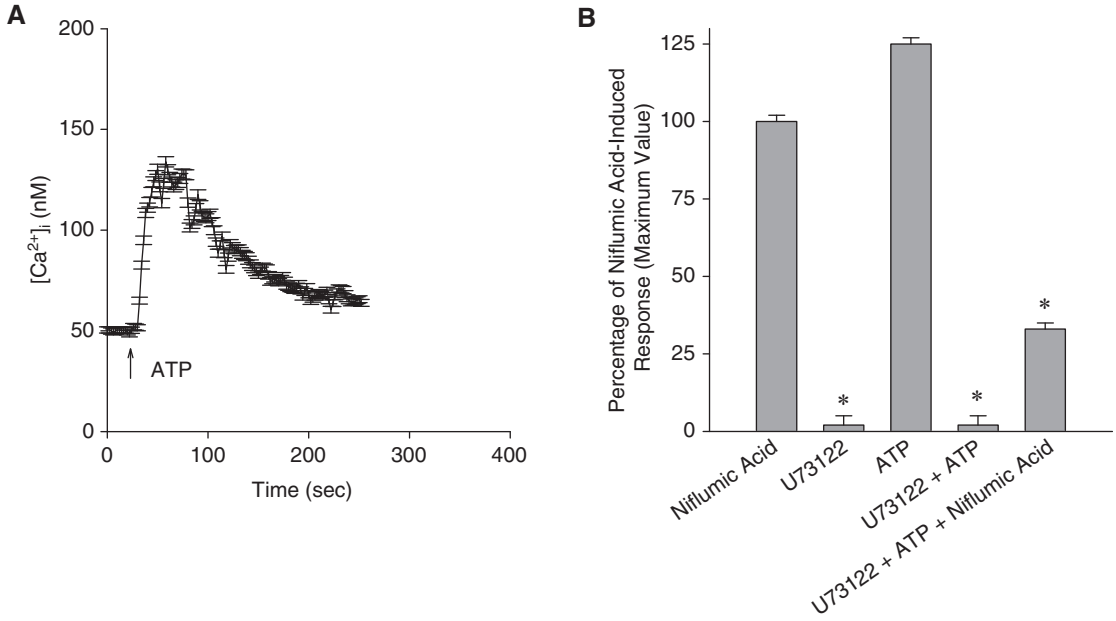

Fig. 5. Effect of U73122 on niflumic acid-induced Ca^{2+} release in MG63 cells. Experiments were performed in Ca^{2+}-free medium. (A) ATP (10 μM) was added as indicated. (B) U73122 (2 μM), ATP, and niflumic acid (750 μM) were added as indicated. Data are mean ± SEM of three separate experiments. *$P < 0.05$ compared to first bar (control). Control is the area under the curve of 750 μM niflumic acid-induced $[Ca^{2+}]_i$ rises (25-190 s).

Effect of Niflumic Acid on Cell Viability in MG63 Cells

Cells were treated with 0-500 μM niflumic acid for 24 h, and tetrazolium assay was performed. In the presence of niflumic acid, cell viability decreased in a concentration-dependent manner between 200-500 μM (Fig. 6). The next question was whether niflumic acid-induced cytotoxicity was related to preceding $[Ca^{2+}]_i$ rises. The intracellular Ca^{2+} chelator BAPTA/AM (5 μM) (30) was used to prevent $[Ca^{2+}]_i$ rises during niflumic acid pretreatment. At 750 μM, niflumic acid did not evoke $[Ca^{2+}]_i$ rises in BAPTA/AM-treated cells (not shown). Fig. 6 shows that 5 μM BAPTA/AM loading did not alter control cell viability. In the presence of 200-500 μM niflumic acid, BAPTA/AM loading did not reverse niflumic acid-induced cell death. Therefore, the data suggest that niflumic acid-induced cell death was not caused by preceding rises in $[Ca^{2+}]_i$.

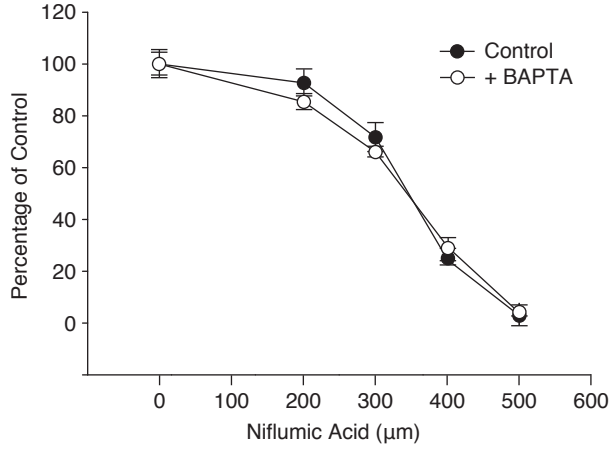

Fig. 6. Niflumic acid-induced Ca^{2+}-independent cell death in MG63 cells. Cells were treated with 0-500 μM niflumic acid for 24 h, and the cell viability assay was performed. Data are mean ± SEM of three separate experiments. Each treatment had six replicates (wells). Data are expressed as percentage of control that is the increase in cell numbers in niflumic acid-free groups. Control had 10,513 ± 711 cells/well before experiments, and had 12,366 ± 755 cells/well after incubation for 24 h. *$P < 0.05$ compared to control. In each group, the Ca^{2+} chelator BAPTA/AM (5 μM) was added to cells followed by treatment with niflumic acid in Ca^{2+}-containing medium. Cell viability assay was subsequently performed.

Discussion

Several reports have shown that niflumic acid affected Ca^{2+} signaling and regulated physiology in various models such as HEK 293 cells (1), K562 cells (17) or rat skeletal muscle (19). However, whether niflumic acid affects Ca^{2+} homeostasis and

causes Ca^{2+}-associated cytotoxicity in human osteosarcoma cells is unknown. This study shows that niflumic acid increased $[Ca^{2+}]_i$ in MG63 cells. Niflumic acid acted by depleting Ca^{2+} stores and causing Ca^{2+} influx. Evidence suggests that niflumic acid induced Ca^{2+} entry because the niflumic acid-induced Ca^{2+} signal was inhibited by removal of extracellular Ca^{2+}. Removal of extracellular Ca^{2+} decreased niflumic acid-induced response throughout the measurement period suggests that Ca^{2+} influx occurred during the whole stimulation period. The Mn^{2+} quenching data also suggest that Ca^{2+} influx occurred during niflumic acid incubation.

The mechanism of niflumic acid-induced Ca^{2+} influx was explored. Store-operated Ca^{2+} channels have been shown to play a role in $[Ca^{2+}]_i$ rises stimulated with several compounds in MG63 cells such as NPC-14686 (4) and sertraline (20). Nifedipine, econazole and SKF96365 were used to explore whether niflumic acid induced Ca^{2+} influx *via* store-operated Ca^{2+} channels (26). These three compounds have been used to inhibit store-operated Ca^{2+} entry in various cell types, although there are so far no selective inhibitors for this entry (7, 9, 31). Our data show that all of these blockers inhibited niflumic acid-induced $[Ca^{2+}]_i$ rises. Thus it suggests that niflumic acid-induced Ca^{2+} entry was *via* store-operated Ca^{2+} pathway.

The activity of many protein kinases has been known to associate with Ca^{2+} homeostasis. For example, it has been shown that calmodulin and protein kinases are involved in the upstream of cytosolic and nucleic Ca^{2+} signaling induced by hypoosmotic shock in tobacco cells (24). Our data show that niflumic acid-evoked $[Ca^{2+}]_i$ rises were decreased when PKC activity was activated by PMA. Inhibition of PKC with GF109203X had no effect. This suggests that a normally maintained PKC level is needed for niflumic acid to induce a full Ca^{2+} response. The relationship between PKC and store-operated Ca^{2+} entry has been well established in NPC-14686- (4)- or 3,3'-diindolylmethane- (21) treated MG63 cells. Therefore, niflumic acid might cause PKC-sensitive Ca^{2+} influx in MG63 cells.

Regarding the Ca^{2+} stores involved in niflumic acid-evoked Ca^{2+} release, the endoplasmic reticulum stores seemed to be the dominant one. One possible mechanism for niflumic acid to release the endoplasmic reticulum Ca^{2+} is that niflumic acid acts similarly to TG by inhibiting the endoplasmic reticulum Ca^{2+}-ATP pump (28). The data further show that the Ca^{2+} release was *via* a PLC-dependent mechanism, given the release was inhibited by 70% when PLC activity was inhibited. Besides PLC-dependent Ca^{2+} release, the Ca^{2+} release pathways may include nicotinic acid adenine dinucleotide phosphate (NAADP) pathway (25) and cyclic adenosine 5'-diphosphoribose (cADPR) (13). Therefore, niflumic acid-induced Ca^{2+} release in MG63 cells deserves further research.

Our study shows that niflumic acid (200-500 μM) was cytotoxic to MG63 cells in a concentration-dependent manner. Ca^{2+} overloading is known to initiate processes leading to alteration in cell viability (3). Because niflumic acid induced both $[Ca^{2+}]_i$ rises and cell death in MG63 cells, it is crucial to know whether the death occurred in a Ca^{2+}-dependent fashion. Our findings show that 200-500 μM niflumic acid-induced cell death was not reversed when cytosolic Ca^{2+} was chelated by BAPTA/AM. This implies that in this case, niflumic acid-induced cell death was not triggered by $[Ca^{2+}]_i$ rises.

$[Ca^{2+}]_i$ measurements and viability were two totally different assays in this study. $[Ca^{2+}]_i$ measurements were conducted online and terminated within 15 min. After 20 min incubation with niflumic acid, cell viability was still >95%. Conversely, in viability assays, cells were treated with niflumic acid overnight in order to obtain measurable changes in viability. This is why 500 μM niflumic acid decreased cell viability by approximately 95% after overnight treatment whereas 750 μM niflumic acid did not alter viability in $[Ca^{2+}]_i$ measurements.

The pharmacokinetics and availability of niflumic acid in humans were explored previously (14, 15). The plasma level of niflumic acid may reach ~40 μM (14, 15). This level may be expected to go much higher in patients with liver or kidney disorders or taking higher doses. Our data show that niflumic acid at a concentration of 200 μM induced slight cell death. Therefore, our data may be clinically relevant in some groups of patients.

Together, the results showed that niflumic acid evoked $[Ca^{2+}]_i$ rises by Ca^{2+} release from the endoplasmic reticulum in a PLC-dependent manner, and Ca^{2+} entry *via* PKC-sensitive store-operated Ca^{2+} entry in MG63 human osteosarcoma cells. Niflumic acid also evoked cell death in a Ca^{2+}-independent manner. The $[Ca^{2+}]_i$-elevating and cytotoxic effects of niflumic acid in osteosarcoma cells should be taken into account in performing other *in vitro* studies.

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Conflict of Interests

The authors declared no conflicts of interest.

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