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C.-R. Jan · W.-C. Chen · Y.-H. Lee · J.-K. Huang
H.-C. Ou · C.-J. Tseng

Allopurinol blocks shock-wave-induced rises in cytosolic calcium levels in MDCK cells

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Abstract Allopurinol has been reported to ameliorate the side effects in patients following shock wave lithotripsy (SWL); however, the mechanism has not been studied. We have examined the protective effect of allopurinol on Madin-Darby canine kidney (MDCK) cells after shock wave exposure (SWE) by determining the release of aspartate aminotransferase (ASAT) and lactate dehydrogenase (LD), and the resting cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). In SWE-treated cells, the release of ASAT and LD increased immediately, but largely transiently, by approximately 23% and 5-fold over control, respectively. Within 1–6 h after SWE there was a gradual rise in the resting $[Ca^{2+}]_i$ of 16–137% above control. Allopurinol did not affect the transient enzyme release but blocked the long-term rises in the resting $[Ca^{2+}]_i$. The transient changes in $[Ca^{2+}]_i$ evoked by two hormones, ATP and bradykinin, and a drug that releases Ca^{2+} from internal Ca^{2+} stores, thapsigargin, were only slightly affected in allopurinol-treated cells. We conclude that the protection conferred by allopurinol on patients treated with SWL might involve a direct protection of the kidney cells by maintaining a normal resting $[Ca^{2+}]_i$.

Key words MDCK cells · Allopurinol · Shock waves · Lithotripsy · Calcium signalling

C.-R. Jan (✉) · J.-K. Huang · H.-C. Ou · C.-J. Tseng
Department of Medical Education and Research,
Veterans General Hospital-Kaohsiung, 386 Ta Chung 1st Rd,
Kaohsiung, Taiwan 813, Republic of China;
Fax: 011-886-7-3468056, e-mail: crjan@isca.vghks.gov.tw

W.-C. Chen[1] · Y.-H. Lee · J.-K. Huang
Division of Urology, Department of Surgery, Veterans General Hospital-Kaohsiung, Taiwan 813, Republic of China

[1] *Present address*:
Ping Tung Christian Hospital, Ping Tung,
Taiwan, Republic of China

Introduction

Shock wave lithotripsy (SWL) has been used for disintegrating kidney stones in the treatment of urolithiasis since 1980 [3]; however, there are a number of possible side effects [9, 12]. In addition to local hematomas and edema [2] and a risk of hypertension [14], renal function can be altered, including a transient decrease in filtration rate [5], an increased excretion of α_1- and β_2-microglobulin and *N*-acetyl-β-glucosaminidase, and a decreased excretion of Tamm-Horsfall protein [1, 20, 27].

Allopurinol is a drug used in treating hyperuricemia and gout [7]. It lowers blood uric acid levels by inhibiting xanthine oxidase, which produces uric acid from hypoxanthine and xanthine. It has been reported that patients taking allopurinol showed a lower excretion of β_2-microglobulin and albumin and a greater excretion of Tamm-Horsfall protein [13]. However, the mechanism of this protective effect of allopurinol has not been studied.

To investigate whether allopurinol could directly protect the kidney cells during SWL, we have examined the effect of allopurinol on shock-wave-induced injury of Madin-Darby canine kidney (MDCK) cells, a renal cell model originating from the dog kidney [8], by measuring release of two cellular enzymes, aspartate aminotransferase (ASAT) and lactate dehydrogenase (LD), from cell suspensions; and the resting cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in individual cells after transient shock wave exposure (SWE).

Materials and methods

Chemical reagents

The reagents for cell culture were from Gibco (Grand Island, N.Y.). All other reagents were from Sigma (St. Louis, Mo.).

Cell culture

MDCK cells obtained from American Type Culture Collection (CRL-6253, Rockville, Md.) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO_2-containing humidified air. Only passages 70–80 were used.

SWE of cell suspensions

Confluent cells in flasks were trypsinized, centrifuged and suspended in DMEM (10^7/ml). For SWE, a 15-ml polyethylene tube containing 1 ml of cell suspension was immersed in a water-filled plastic bag by a custom-made holder. A cork was squeezed into the tube to completely cover the suspension in order to minimize the possibility that air-liquid interface effects might be responsible for membrane damage [21]. Shock waves were generated by a Lithostar II (Siemens, Germany) lithotripter with the energy level set at 18 kV. The cell-containing tube was adjusted to the second focus of the ellipsoid by adjusting the holder.

Enzyme assay

After SWE, the cell suspensions were centrifuged and the supernatant was assayed for ASAT and LD with a kit from Human (cat. no. 12011, Taunusstein, Germany). A mixture of 10 μl of supernatant and 50 μl of assay solution was added to a cuvette and the absorbance at 340 nm was detected by a Beckman DU640 spectrophotometer. The concentrations (U/l) of ASAT and LD were calculated from the absorbance according to the equation: concentration = ΔAbsorbance/min × 952 (ASAT) or 8095 (LD).

Cell viability assay

Cell viability was determined by trypan blue exclusion immediately after SWE.

Optical measurements of $[Ca^{2+}]_i$ in individual cells

Trypsinized cells were centrifuged and suspended in DMEM. Cells were seeded on polylysine-coated glass coverslips at an appropriate density allowing imaging of 30–40 single cells. The polylysine coating made cells attach to the coverslips within 30 min. The attached cells were loaded with 2 μM fura-2/AM (Molecular Probes, Eugene, Ore.) for 30 min at 25°C. The coverslip was transferred to a chamber (25°C) on the stage of a Nikon Diaphot microscope and viewed under bright light and UV illumination via a ×40 (1.3 NA) oil immersion fluorescence objective (Nikon Fluor). Solutions were applied as a 3.5-ml bolus to the chamber (containing 0.5 ml of solution). This method allowed rapid and complete change of solution (removed by continuous aspiration as the volume of the chamber solution temporarily exceeded 0.5 ml for the new solution).

The MiraCal imaging system (Life Science Resources, Cambridge, UK) in conjunction with a slow scan cooled CCD camera (CMCA Nikon DF/SB ×0.45 Wildfield) was used for digital video imaging of the changes of $[Ca^{2+}]_i$ in individual cells. $[Ca^{2+}]_i$ was calculated to 8-bit accuracy (256 grey levels) every 2 s. Data were analyzed for $[Ca^{2+}]_i$ changes by measurement of the 340 nm (F_{340}) and 380 nm (F_{380}) of excitation signals and emission signal at 510 nm. Ratio values were converted to an estimate of $[Ca^{2+}]_i$ using the formula

$$[Ca^{2+}]_i = K_d \beta (R - R_{min})/(R_{max} - R)$$

where R is the ratio F_{340}/F_{380}, R_{min} and R_{max} are the minimum and maximum values of the ratio, attained at zero and saturated Ca^{2+} concentrations, respectively; F_{340} is the fluorescence emitted by the dye when excited at 340 nm and F_{380} is the fluorescence emitted by the dye when excited at 380 nm; and β is the ratio of fluorescence intensities for Ca^{2+}-free and Ca^{2+}-bound indicator measured with 380 nm excitation. These constants were obtained by addition of 10 μM ionomycin in solutions containing 2 mM Ca^{2+} ($R_{max} = 2.1$), or no added Ca^{2+} plus 10 mM EGTA ($R_{min} = 0.2$). The value of β is 3.13 and a K_d of 155 nM was assumed [10].

All experiments in this study were done at 23–25°C.

Statistical analysis

Values are reported as the mean ± SE. Statistical comparisons were done using Student's two-tailed t-test. A significant difference was accepted when $P < 0.05$.

Results

Increased release of ASAT and LD from MDCK cells after SWE

We first examined the effect of SWE on ASAT and LD release from MDCK cells. Consistent with previous studies [21, 22], an immediate release of ASAT and LD was found from cells exposed to 16–126 impulses of shock waves (not shown). The net release did not increase within 12 h after SWE. Trypan blue exclusion assay showed that the percentage and absolute number of live cells in the cells exposed to either 64 or 126 impulses were similiar to that of control (> 95% viability). Thus, an impulse rate of 64 was chosen for the following experiments.

Figure 1 shows that cells that had been exposed to 64 impulses of shock waves significantly increased their release of ASAT by 9 ± 1 U/l, equivalent to 22% over control which was 40 ± 3 U/l ($n = 9$; $P < 0.01$), and their release of LD by 1210 ± 32 U/l, equivalent to 5-fold over control which was 250 ± 23 U/l ($n = 9$; $P < 0.01$) immediately (0 h) after SWE, implying that cell membrane integrity was perturbed. The induced release was transient because it did not significantly increase within 6 h after SWE, except that LD release at 1, 3 and 6 h appeared to be larger than at 0 h; however, the differences were not statistically significant ($P > 0.05$).

Effect of allopurinol on SWE-induced enzyme release

Figure 1 shows that allopurinol (0.2 mM) did not significantly inhibit SWE-induced enzyme release. At 6 h, ASAT release was even enhanced by allopurinol (100%; $P < 0.05$). At 3 and 6 h, LD release appeared to be reduced by allopurinol; however, the differences were not statistically significant ($P > 0.05$).

SWE-induced rises in the resting $[Ca^{2+}]_i$

Since the integrity of the cell membrane was perturbed by SWE, it is logical to suspect that there was a rise in the resting $[Ca^{2+}]_i$ due to extracellular Ca^{2+} influx across the plasma membrane and/or internal

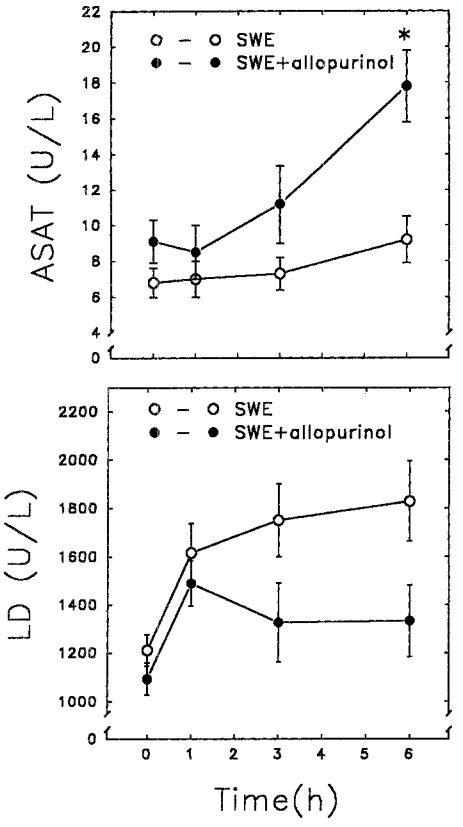

Fig. 1 Effect of allopurinol on shock wave exposure (SWE)-induced release of aspartate aminotransferase (ASAT) and lactate dehydrogenase (LD) from Madin-Darby canine kidney (MDCK) cell suspensions. ASAT (*top*) and LD (*bottom*) release was determined in suspended cells within 6 h after SWE (64 impulses). *Open circles* SWE-treated group after correction for control values from non-SWE-treated group; *filled circles* SWE- and allopurinol-treated group. Allopurinol (0.2 mM) was added to the cell suspension before and during SWE and throughout the enzyme assay. The data were corrected for control values from allopurinol-treated, non-SWE-treated cells. Allopurinol did not affect the control enzyme release. Data are mean ± SE of nine (SWE) or four (SWE + allopurinol) independent experiments. $^*P < 0.01$

Ca^{2+} release from damaged organelles. Thus, we examined the resting $[Ca^{2+}]_i$ in individual MDCK cells. For these experiments, suspended cells which had been exposed to 64 impulses of shock waves were plated on coverslips 1 h before the optical measurements. The cells which were not exposed to shock waves were plated as control. Most of the cells attached firmly within 30 min. The attached cells were loaded with the membrane-permeant, fluorescent indicator dye fura-2/AM [10] for 30 min to monitor $[Ca^{2+}]_i$.

The cells after SWE showed no differences in their ability to attach to coverslips and to sequester and hydrolyze fura-2/AM compared with control cells. Note that unlike the enzyme assay, which was done immediately after SWE, these $[Ca^{2+}]_i$ measurements were performed 1 h after SWE was removed (due to the time needed for cell attachment and fura-2 loading), thus only revealing the long-term effect of SWE within 1–6 h after SWE was stopped. The resting $[Ca^{2+}]_i$ in non-

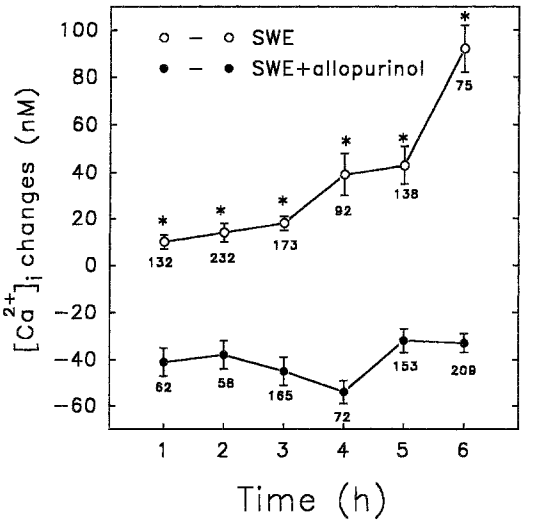

Fig. 2 Inhibition of SWE-induced rises in the resting cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) of individual MDCK cells by allopurinol 1–6 h after SWE. *Open circles* SWE (64 impulses)-treated group after correction for control values from the non-SWE-treated group. Control values were 60–70 nM. All data in the SWE-treated group were significantly increased compared with the control ($P < 0.05$). *Filled circles* SWE- and allopurinol-treated group. Allopurinol (0.2 mM) was added to cell suspensions before and during SWE and throughout the $[Ca^{2+}]_i$ measurements. The data were corrected for control values from non-SWE-treated cells. Data are mean ± SE with the cell number indicated below each data point. $^*P < 0.01$ between the two groups

SWE-treated control cells was 60–70 nM ($n = 76$–193 cells at various time points). In SWE-treated cells the resting $[Ca^{2+}]_i$ was significantly elevated above control at all time points. Figure 2 shows that at 1 h, the net $[Ca^{2+}]_i$ increase was 10 ± 2 nM (16% above control which was 61 ± 2 nM; $n = 132$ cells). This low resting $[Ca^{2+}]_i$ (< 100 nM) suggests that within 1 h after SWE the cells had recovered to the extent that the resting $[Ca^{2+}]_i$ could be maintained at a normal level. However, in the following 5 h there was a significant progressive rise in the resting $[Ca^{2+}]_i$ in SWE-treated cells. At 6 h, the net $[Ca^{2+}]_i$ increase was 92 ± 12 nM, which was 137% above control (67 ± 1 nM; $n = 75$ cells). Because these experiments were performed 1 h after SWE, the results did not reflect the $[Ca^{2+}]_i$ changes occurring during or immediately after SWE.

Allopurinol blocked SWE-induced rises in the resting $[Ca^{2+}]_i$

To test whether allopurinol could inhibit the SWE-evoked $[Ca^{2+}]_i$ rises, we performed the following experiments. Allopurinol was added to cells before and during SWE and throughout the optical measurements. Figure 2 shows that in allopurinol (0.2 mM)-treated cells the resting $[Ca^{2+}]_i$ was maintained at approximately 35–55 nM below control, which was 60–70 nM (as shown by the negative values) 1–6 h after SWE. Given that the SWE-evoked rise in the resting $[Ca^{2+}]_i$

was completely abolished by allopurinol, it was critical to determine whether the $[Ca^{2+}]_i$ transients evoked by physiological agonists were altered by allopurinol. We measured the transient changes in $[Ca^{2+}]_i$ evoked by two physiological agonists, ATP and bradykinin (BK), that interact with plasma membrane receptors; and the drug thapsigargin (TG) [25], which inhibits the endoplasmic reticulum (ER) Ca^{2+}-ATP pump and depletes ER Ca^{2+} stores. In previous studies [19, 28, 29], ATP, BK and TG have been shown to evoke robust $[Ca^{2+}]_i$ rises in MDCK cells. Figure 3 illustrates that in cells 2–3 h after SWE, ATP (10 μM), BK (1 μM) or TG (1 μM) evoked robust transient $[Ca^{2+}]_i$ changes (Fig. 3A–C, left-hand panels). The peak amplitude and kinetics of these $[Ca^{2+}]_i$ changes were largely similar to those from non-SWE-treated cells (not shown). When allopurinol (0.2 mM) was present, the peak amplitude of the $[Ca^{2+}]_i$ changes evoked by ATP was reduced by 17% (571 ± 35 nM, $n = 18$ vs. 684 ± 25 nM, $n = 22$; $P < 0.05$); that evoked by TG was reduced by 31% (301 ± 21 nM, $n = 21$ vs. 437 ± 23 nM; $n = 26$; $P < 0.05$); while the BK-evoked response remained unaffected (555 ± 59 nM; $n = 33$ vs. 537 ± 12 nM, $n = 40$; $P > 0.05$). Similar results were obtained from cells at 6 h after SWE (not shown).

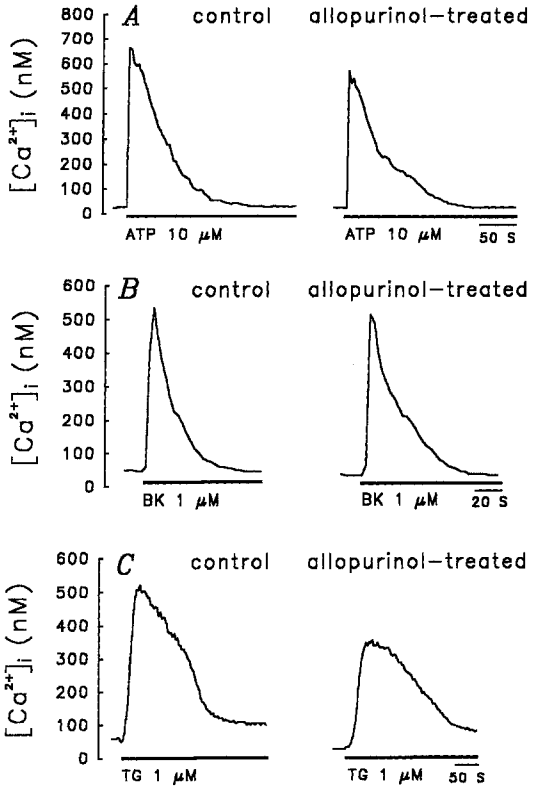

Fig. 3A–C Representative recordings of $[Ca^{2+}]_i$ in individual MDCK cells 2–3 h after SWE (64 impulses). Addition of **A** 10 μM ATP **B** 1 μM bradykinin (BK) or **C** 1 μM thapsigargin (TG) to cells evoked $[Ca^{2+}]_i$ transients in control (SWE-treated) and allopurinol-treated (SWE- and allopurinol-treated) cells. The *long bars* indicate both the reagent that was used and the time at which the reagent was applied. Allopurinol (0.2 mM) was added to cells before and during SWE and throughout the $[Ca^{2+}]_i$ measurements. The *short bars* indicate time scale in seconds (s). Each recording is representative of similar responses from 20–40 cells

Discussion

We have examined the effect of allopurinol on SWE-induced injury in MDCK cells. We have found that after SWE there was a transient release of two cellular enzymes: ASAT and LD. The resting $[Ca^{2+}]_i$ was elevated within 1–6 h after SWE. Allopurinol blocked the SWE-induced rise in the resting $[Ca^{2+}]_i$ with minor inhibition on the $[Ca^{2+}]_i$ changes evoked by hormones. We conclude that the protection allopurinol confers on patients after SWL might involve the ability of allopurinol to maintain a normal resting $[Ca^{2+}]_i$ in the kidney cells.

The cause of the protection of allopurinol appears to be a low resting $[Ca^{2+}]_i$ (~30 nM). Fura-2 experiments revealed a significant long-term rise in the resting $[Ca^{2+}]_i$ 1–6 h after SWE. At 1 h, the resting $[Ca^{2+}]_i$ was 77 ± 2 nM in SWE-treated cells, which was 16% above control. The control resting $[Ca^{2+}]_i$ was 60–70 nM 1–6 h after cells were plated, which was within the normal physiological range for MDCK cells [29]. At 6 h, the net increase in $[Ca^{2+}]_i$ was 137% above control. However, because the measurements were done 1 h after SWE, the results did not reflect the transient $[Ca^{2+}]_i$ changes occurring during SWE. Because cellular enzymes had leaked during SWE, extracellular Ca^{2+} should have flowed into cells due to the Ca^{2+} gradient across the plasma membrane; however, this rise in $[Ca^{2+}]_i$ returned to a normal level (<100 nM) within 1 h after SWE (Fig. 2; 1 h). It was technically impossible to measure the instantaneous $[Ca^{2+}]_i$ changes during SWE with our experimental protocol.

The SWE-induced cell damage resulted in a progressive elevation in the resting $[Ca^{2+}]_i$ in the following 5 h. This rise may represent increased extracellular Ca^{2+} influx and/or internal Ca^{2+} release. Although cell permeability recovered rapidly, the impairment caused by SWE might have weakened the cells and hindered them from maintaining the resting $[Ca^{2+}]_i$ at normal levels. This increased Ca^{2+} influx during the long-term phase after SWE is likely because shock waves are known to enhance free radical production and increase cell permeability [18, 23, 24]. Because the organelle membrane could be damaged, as indicated by reduced ER Ca^{2+} stores (Fig. 3C) and enhanced release of cellular enzymes, the resting $[Ca^{2+}]_i$ might rise due to Ca^{2+} release from damaged organelles and/or impaired ability of the Ca^{2+} pump to pump Ca^{2+} into internal Ca^{2+} stores. $[Ca^{2+}]_i$ levels in many tissues including the kidney often rise when lethal cell injury develops in the presence of extracellular Ca^{2+} [11]. In LLC-PK1 cells, another kidney cell line similar to MDCK cells, ischemia was found to elevate $[Ca^{2+}]_i$ [4]. The fact that in SWE-treated cells allopurinol kept the resting $[Ca^{2+}]_i$ approximately 50% lower than that in non-SWE-treated

cells suggests that allopurinol might protect the kidney cells against SWL-induced damage and, in turn, results in an attenuation of the side effects that patients suffer after SWL. Allopurinol has also been shown to block endotoxin-induced $[Ca^{2+}]_i$ rises in erythrocytes [26].

In cells 2–3 h after SWE, the peak amplitude of the $[Ca^{2+}]_i$ changes evoked by ATP and bradykinin were reduced by 17% or unaltered, respectively, by allopurinol; however, the kinetics of the $[Ca^{2+}]_i$ changes were not affected. The peak amplitude of the $[Ca^{2+}]_i$ changes evoked by TG was more significantly reduced (31%) by allopurinol. This decrease in TG-sensitive ER Ca^{2+} stores reflects damage of organelle membranes.

At least two mechanisms could explain allopurinol's blocking effect on SWE-induced $[Ca^{2+}]_i$ rises: (1) a direct block of Ca^{2+} influx or internal Ca^{2+} release; (2) and indirect block of $[Ca^{2+}]_i$ rises by decreasing cell permeability. The first possibility is unlikely because allopurinol has not been shown previously to interact with Ca^{2+} channels or Ca^{2+} stores. Because shock waves have been shown to produce oxygen radicals and enhance cell permeability in other cell types [18, 23, 24], it is likely that allopurinol could protect MDCK cells by inhibiting xanthine oxidase and reducing oxygen radical production, and results in a decrease in cell permeability after SWE. While direct evidence in SWE is lacking, oxygen radicals are thought to play a key role in perpetuating ischemia-induced cell damage by increasing cell permeability [16, 17].

Additionally, $[Ca^{2+}]_i$ has been shown to modulate enzymatic conversion of xanthine dehydrogenase to xanthine oxidase in the kidney cells, which was potentiated during ischemia [16]. If this is also true following SWL, the protective effect of allopurinol could be amplified if the production of xanthine oxidase in the kidney cells is reduced by the low resting $[Ca^{2+}]_i$.

The $[Ca^{2+}]_i$ changes we measured occurred 1–6 h after SWE when the enyme release had stopped, and were not the instantaneous changes during SWE when the enymes were transiently leaking out. This may explain why in SWE-treated cells, despite the fact that allopurinol blocked the long-term rises in the resting $[Ca^{2+}]_i$, it did not inhibit the transient release of enzymes occurring during SWE. Lack of allopurinol inhibition of LD release has been shown previously in another renal tubule study. Doctor and Mandel [6] demonstrated that in rat renal tubules, LD level was increased by three-fold during anoxia with an additional 8–10% release during reoxygenation; this increase was not inhibited by 0.2 mM allopurinol. Even at 5 mM, allopurinol only delayed hypoxia-induced LD release by 30 min in perfused rat liver [15]. Thus, it appears that the ability of allopurinol to maintain a low resting $[Ca^{2+}]_i$ after SWE does not contradict the fact that it could not block the transient enzyme leak that occurred during SWE.

The side effects induced by SWL in the majority of patients are transient and do not cause permanent damage [13]. However, patients with risk factors, such as pre-existing renal disorders, urinary tract infection, previous lithotripsies and solitary kidneys, are likely to suffer more damage from SWL. Thus, in these patients, allopurinol might prove a useful drug.

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