The cyclo-oxygenase-2 inhibitor celecoxib perturbs intracellular calcium by inhibiting endoplasmic reticulum Ca^{2+} -ATPases: a plausible link with its anti-tumour effect and cardiovascular risks

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Substantial evidence indicates that the cyclo-oxygenase-2 (COX-2) inhibitor celecoxib, a widely prescribed anti-inflammatory agent, displays anti-tumour effect by sensitizing cancer cells to apoptosis. As part of our effort to understand the mechanism by which celecoxib mediates apoptosis in androgen-independent prostate cancer cells, we investigated its effect on intracellular calcium concentration ($[\text{Ca}^{2+}]_i$). Digital ratiometric imaging analysis indicates that exposure of PC-3 cells to celecoxib stimulates an immediate $[\text{Ca}^{2+}]_i$ rise in a dose- and time-dependent manner. Kinetic data show that this Ca^{2+} signal arises from internal Ca^{2+} release in conjunction with external Ca^{2+} influx. Examinations of the biochemical mechanism responsible for this Ca^{2+} mobilization indicate that celecoxib blocks endoplasmic reticulum (ER) Ca^{2+} -ATPases. Consequently, inhibition of this Ca^{2+} reuptake mechanism results in Ca^{2+} mobilization from ER stores followed by capacitative calcium entry, leading to $[\text{Ca}^{2+}]_i$ elevation. In view of

the important role of Ca^{2+} in apoptosis regulation, this Ca^{2+} perturbation may represent part of the signalling mechanism that celecoxib uses to trigger rapid apoptotic death in cancer cells. This Ca^{2+} -ATPase inhibitory activity is highly specific for celecoxib, and is not noted with other COX inhibitors tested, including aspirin, ibuprofen, naproxen, rofecoxib (Vioxx®), DuP697 and NS398. Moreover, it is noteworthy that this activity is also observed in many other cell lines examined, including A7r5 smooth muscle cells, NIH 3T3 fibroblast cells and Jurkat T cells. Consequently, this Ca^{2+} -perturbing effect may provide a plausible link with the reported toxicities of celecoxib such as increased cardiovascular risks in long-term anti-inflammatory therapy.

Key words: apoptosis, PC-3 prostate cancer cell.

INTRODUCTION

The discovery of the two isoforms of cyclo-oxygenase (COX) has prompted the development of selective COX-2 inhibitors for the treatment of chronic inflammatory diseases in light of their significantly reduced gastrointestinal toxicity [1–3]. In addition to the anti-inflammatory utility, recent epidemiological and animal model studies demonstrate the potential use of COX-2 inhibitors as chemopreventive agents [4–6]. An array of *in vitro* evidence has correlated this anti-tumour activity with the ability of these molecules to induce apoptosis in cancer cells [7–17]. Together, these findings have led to the recent approval by the U.S. Food and Drug Administration of celecoxib (Celebrex®) for the adjuvant treatment of familial adenomatous polyposis. In addition, celecoxib has been tested in numerous advanced clinical trials against a variety of epithelial malignancies including colon, oesophagus, skin and bladder cancers. However, the mechanism by which COX-2 inhibitors mediate apoptosis in cancer cells remains elusive [18]. Given the importance of pro-inflammatory prostaglandins in carcinogenesis [4,19–21], one school of thought attributes the anti-tumour effect of COX-2 inhibitors to the inhibition of COX-2 enzyme activity. However, an expanding body of evidence from this and other laboratories suggests that these molecules mediate apoptosis via a multitude of signalling targets other than the COX-2 enzyme itself [22,23].

Our research has focused on the effect of COX-2 inhibitors on apoptosis in prostate cancer cells [16,17,23]. Our data indicate that celecoxib mediates apoptosis in prostate cancer cells by

targeting signalling components essential to cell survival such as Akt and extracellular signal-regulated kinase 2 (ERK2). It is noteworthy that this induction of apoptosis is irrespective of androgen responsiveness, p53 functional status and Bcl-2 expression levels. In this study, we report a new pharmacological effect of celecoxib on intracellular calcium concentration ($[\text{Ca}^{2+}]_i$), which may provide a link with its anti-tumour and/or toxicological effects. Evidence indicates that celecoxib perturbs intracellular Ca^{2+} by blocking endoplasmic reticulum (ER) Ca^{2+} -ATPases (also called SERCAs). As Ca^{2+} is taken into the ER lumen by ER Ca^{2+} -ATPases [24], inhibition of this Ca^{2+} reuptake results in Ca^{2+} mobilization from ER stores, thereby leading to $[\text{Ca}^{2+}]_i$ elevation. Ca^{2+} plays a central role in apoptosis via a plethora of plausible mechanisms (see Discussion). It has been hypothesized that androgen-insensitive prostate cancer cells survive androgen ablation-induced apoptosis by preventing intracellular Ca^{2+} elevation [25]. The Ca^{2+} rise by celecoxib may account for its effectiveness in triggering apoptotic death in both androgen-dependent and androgen-independent prostate cancer cells. It is noteworthy that this Ca^{2+} -perturbing effect is not limited to prostate cancer cells. It is also observed in many other cell lines examined, including A7r5 smooth muscle cells, NIH 3T3 fibroblast cells, MCF-7 breast cancer cells, Jurkat T cells and HepG2 hepatoma cells. This general phenomenon thus raises a concern of potential Ca^{2+} -related toxicities in patients with reduced clearance of celecoxib. Data from the recent Celecoxib Long-Term Arthritis Safety Study (CLASS; 8059 patients) show an increased myocardial infarction rate with celecoxib as com-

Abbreviations used: AEBF, 4-(2-aminoethyl)benzenesulphonyl fluoride; AM, acetoxymethyl ester; $[\text{Ca}^{2+}]_i$, intracellular calcium concentration; COX, cyclo-oxygenase; DTT, dithiothreitol; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; IP_3 , *d*-myo-Inos(1,4,5) P_3 .

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pared with the placebo group ($P = 0.02$) [26]. Other reported side effects of celecoxib include elevated blood pressure and leucocyte adherence in a rat model [27].

MATERIALS AND METHODS

Reagents

Celecoxib and rofecoxib were obtained from commercial Celebrex® and Vioxx® capsules, respectively, by solvent extraction followed by recrystallization. The identity and purity of both molecules were verified by NMR and MS. DuP697 was a kind gift from Professor Hsin-Hsiung Tai (University of Kentucky, Lexington, KY, U.S.A.). NS398, fluo-3 acetoxymethyl ester (AM), fluo-3, fura 2/AM, thapsigargin, Ruthenium Red, ryanodine, saponin and bromo-A23187 were purchased from Calbiochem (San Diego, CA, U.S.A.). D-*myo*-Ins(1,4,5) P_3 (IP₃) was synthesized according to a procedure previously reported by this laboratory [28].

Cell culture

Human prostate cancer cell lines LNCaP and PC-3, A7r5 smooth muscle cells, NIH 3T3 fibroblast cells, MCF-7 breast cancer cells, Jurkat T cells and HepG2 hepatoma cells were purchased from American Type Culture Collection (Rockville, MD, U.S.A.). Cells were cultured in RPMI 1640 or Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C in a humidified 5% CO₂ incubator. Cells were replenished daily with a new medium and were passaged 1:4 with fresh medium every 3 days.

Ratiometric digital imaging of [Ca²⁺]_i

Cells were seeded on round (25 mm) glass coverslips with a density of 5000 cells/coverslip, and were cultured in RPMI 1640 supplemented with 10% fetal bovine serum at 37 °C in a humidified 5% CO₂ incubator. Assay buffer contained 118 mM NaCl, 5 mM KCl, 1.6 mM CaCl₂, 1.2 mM MgCl₂, 1.2 mM Na₂HPO₄, 25 mM Hepes and 10 mM glucose, pH 7.54. Before each experiment, the cells were loaded with 1 μM fura-2/AM in assay buffer for 30 min at 37 °C. After loading, the cells were washed twice with assay buffer; each wash lasted 15 min. Coverslips were placed in a chamber (Molecular Probes) and covered with 500 μl of assay buffer. Drugs were added at 2 × concentration to obtain maximal mixing. Experiments were imaged using an inverted microscope (Nikon) equipped with a CCD camera (CoolSnap HQ) and a ×40 lens. Capture and analysis was done with the aid of MetaFluor software (Universal Imaging). Images were obtained every 1 s with excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Regions of interest containing 10 cells were isolated and the average fluorescence intensity at 340 and 380 nm was converted to a ratio. The cells were monitored for 2 min from the time they made contact with celecoxib. Using the Grynkiewicz equation, the ratio data were calibrated to calcium concentration based on minimal (calcium-free buffer) and maximal (1 μM bromo-A23187) ratio values [29].

Fluorescence spectrophotometric measurement of [Ca²⁺]_i

[Ca²⁺]_i was monitored by the change in the fluorescence intensity of fluo-3-loaded cells. PC-3 cells (1 × 10⁷ cells) were suspended in 1 ml of assay buffer consisting of 4.3 mM Na₂HPO₄, 24.3 mM NaH₂PO₄, 4.3 mM K₂HPO₄, 113 mM NaCl and 5 mM glucose, pH 7.4, plus 0.5% BSA and 2 mM probenacid, and incubated with 10 μM fluo-3/AM in the dark for 1 h at 37 °C. The cells

were then pelleted by centrifugation at 1000 *g* for 10 min, washed with the assay buffer twice, and resuspended at approx. 8 × 10⁵ cells/ml in the same buffer containing 1 mM Ca²⁺. The effect of celecoxib or thapsigargin on [Ca²⁺]_i was examined by fluo-3 fluorescence in a spectrofluorimeter at 37 °C with excitation and emission wavelengths at 506 and 526 nm, respectively. The maximum fluo-3 fluorescence intensity ($F_{\max}$) in PC-3 cells was determined by adding 1 μM bromo-A23187 and the minimum fluorescence ($F_{\min}$) was determined following depletion of external Ca²⁺ by the addition of 5 mM EGTA. [Ca²⁺]_i was calculated according to the equation $[Ca^{2+}]_i = K_d(F - F_{\min}) / (F_{\max} - F)$, where K_d denotes the apparent dissociation constant (= 450 nM) of the fluorescence dye-Ca²⁺ complex [29].

Preparation of the microsomal and plasma membrane fractions of PC-3 cells

PC-3 cells were collected, and homogenized with a Dounce homogenizer (pestle B; 15 strokes) in 10 vol. of ice-cold buffer A, consisting of 20 mM Tris/HCl, pH 7.2, 110 mM KCl, 10 mM NaCl, 2 mM MgCl₂, 5 mM KH₂PO₄, 2 mM EGTA, 1 mM dithiothreitol (DTT), 2 μg/ml leupeptin, 1 μg/ml pepstatin A and 100 μM 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF). The homogenate was centrifuged at 1000 *g* at 4 °C for 20 min, and the supernatant was first centrifuged at 15000 *g* at 4 °C for 20 min to remove the mitochondria and then pelleted by centrifugation at 100000 *g* at 4 °C for 40 min. The microsomal fraction was resuspended in buffer A without 2 mM EGTA (buffer B), with a protein concentration of 2–3 mg/ml. For the preparation of plasma membranes, collected PC-3 cells were homogenized with a Dounce homogenizer (pestle B; 5 strokes) in 5 ml of ice-cold PM buffer, consisting of 20 mM Hepes, pH 7.2, 110 mM KCl, 10 mM MgCl₂, 5 mM KH₂PO₄, 1 mM DTT, 1 mM EGTA, 1 mM AEBSF and 20 μg/ml leupeptin. The homogenate was centrifuged at 1500 *g* at 4 °C for 10 min. The pellet was resuspended in 3.125 ml of PM buffer and mixed with 5.5 ml of 69% (w/w) sucrose. Sucrose [42.3% (w/w)] was overlaid and centrifuged at 90000 *g* at 4 °C for 2 h in a swinging-bucket rotor. Plasma membranes at the interface of the two phases were collected and resuspended in 5 ml of 10 mM Hepes, pH 7.5, 1 mM DTT, 1 mM AEBSF and 20 μg/ml leupeptin, then spun at 25000 *g* at 4 °C for 10 min. The pellet containing plasma membranes was resuspended in 1 ml of the same buffer and used immediately for the Ca²⁺-ATPase assay.

Ca²⁺-release assay

Measurement of free Ca²⁺ concentrations in incubation media was performed by fluo-3 fluorescence spectrophotometry [28]. The assay medium consisted of 40 units of creatine kinase, 20 mM creatine phosphate and 0.5 μM fluo-3 free acid in 2 ml of buffer B containing 0.2–0.25 mg of microsomal proteins. The mixture was incubated for 5 min at 37 °C, and treated with 2 mM ATP to allow loading of Ca²⁺ stores. Until the external Ca²⁺ concentration returned to a near-base level, the microsomes were stimulated with different Ca²⁺-mobilizing agents (IP₃, ryanodine and celecoxib). Excitation and emission wavelengths were 506 and 526 nm, respectively.

Ca²⁺-ATPase assay

Ca²⁺-ATPase activity was determined by a coupled enzyme assay. The assay medium consisted of 30 mM imidazole/HCl, pH 6.9, 6 mM MgCl₂, 100 mM KCl, 20 mM NaCl, 0.4 mM EGTA, 5 mM NaN₃, 1.5 mM phosphoenolpyruvate, 40 units/ml pyruvate kinase, 40 units/ml lactic dehydrogenase, 0.26 mM

NADH, 2 mM ATP and 10 μM free Ca^{2+} [30]. The assay was started by adding 20 μl of the microsomal preparation to 980 μl of the assay medium at 37 $^{\circ}\text{C}$, and reaction rates were measured by the decrease in the absorbance at 340 nm. Plasma membrane Ca^{2+} -ATPases were analysed in the same manner by using assay medium with the addition of 10 μM ouabain and 0.1 μM calmodulin [31,32]. The assay was started by adding 20 μl of the plasma membrane preparation to 980 μl of the assay medium, and reaction rates were measured by the decrease in the absorbance at 340 nm.

RESULTS

Celecoxib stimulates intracellular Ca^{2+} increase

In view of the crucial role of $[\text{Ca}^{2+}]_i$ in regulating apoptosis in androgen-insensitive prostate cancer cells, we investigated the

effect of celecoxib on $[\text{Ca}^{2+}]_i$ in PC-3 cells. PC-3 cells were grown on coverslips, loaded with fura-2/AM and exposed to celecoxib at different concentrations (10 μM to 100 μM). Changes in $[\text{Ca}^{2+}]_i$ were monitored by a digital ratiometric imaging system. Figure 1(A) displays a set of pseudocolour images representing the immediate Ca^{2+} response to 50 μM celecoxib (panels a–f, 0–10 s post-treatment; 2 s per image).

These cells exhibited an increase in $[\text{Ca}^{2+}]_i$ immediately following celecoxib treatment, while no appreciable effect was noted with control cells receiving DMSO vehicles (results not shown). It appears that the nuclear region shows a higher ratio value, suggestive of a high- $[\text{Ca}^{2+}]_i$ environment. However, these hot spots might, in part, be attributable to compartmental accumulation of the dye, which has been cited as a common problem with ester-loaded fura-2 for cultured cells in monolayers [33,34]. Nevertheless, this Ca^{2+} -perturbing effect is unique to

Figure 1 Effect of celecoxib on intracellular calcium in PC-3 cells

(A) Digital ratiometric imaging Ca^{2+} analysis. PC-3 cells grown on coverslips were loaded with fura-2/AM and exposed to 50 μM celecoxib. $[\text{Ca}^{2+}]_i$ of the cells was monitored using a digital ratiometric imaging station as described in the Materials and methods section. Shown are pseudocolour ratiometric images taken every 2 s post-treatment (a–f, 0–10 s). (B) Dose- and time-dependent effect of celecoxib on $[\text{Ca}^{2+}]_i$. Fura-2-loaded PC-3 cells were treated with different levels of celecoxib, ranging from 0 to 100 μM , and were subjected to digital ratiometric Ca^{2+} analysis as described in the Materials and methods section. (C) Celecoxib does not perturb the permeability of lipid vesicles to Ca^{2+} . Fluo-3-loaded multilamellar lipid vesicles were prepared as follows. A mixture of 50 mol% phosphatidylcholine, 30 mol% cholesterol, 10 mol% phosphatidylserine and 10 mol% phosphatidylethanolamine was dissolved in chloroform, and dried under a stream of N_2 followed by vacuum overnight. The lipids were dispersed in 0.1 M HEPES, pH 7.0, containing 10 μM fluo-3, warmed up to 40 $^{\circ}\text{C}$ for 5 min, and vortexed for 30 s. The suspension was centrifuged at 12 000 g for 10 min, and the liposomal pellet was suspended in the assay buffer (described in the Materials and methods section) containing 1 mM CaCl_2 . The fluo-3-loaded liposomes were treated with 50 μM celecoxib or 1 μM bromo-A23187, and the influx of Ca^{2+} was monitored by fluo-3 fluorescence spectrophotometry. The traces are the representative of three independent experiments.

Figure 2 Fluo-3 fluorimetric analysis of the effect of celecoxib on $[Ca^{2+}]_i$ in PC-3 cells

(A) Time course of $[Ca^{2+}]_i$ changes in cells treated with 50 and 100 μ M celecoxib. (B) Kinetics of the Ca^{2+} response to the sequential addition of celecoxib. Traces a and b depict the effect of two tandem additions of 50 μ M celecoxib to untreated cells and to cells treated with 5 mM EGTA, respectively. (C) Inhibition of celecoxib-induced $[Ca^{2+}]_i$ increase by thapsigargin pretreatment. In the presence of 5 mM EGTA, fluo-3-loaded PC-3 cells were treated with thapsigargin (1 μ M; TG), followed by celecoxib (50 μ M) when $[Ca^{2+}]_i$ returned to the baseline. The traces are representative of three independent experiments.

celecoxib since other COX inhibitors examined, including aspirin, ibuprofen, naproxen, rofecoxib, DuP697 and NS398, failed to elicit an appreciable effect on $[Ca^{2+}]_i$ even at 100 μ M after a prolonged exposure (results not shown).

The Ca^{2+} imaging data show that the Ca^{2+} -perturbing effect of celecoxib was dose- and time-dependent (Figure 1B). At 50 μ M or lower, the $[Ca^{2+}]_i$ rapidly reached a plateau after celecoxib exposure. The maximum $[Ca^{2+}]_i$ elicited by 10, 25 and 50 μ M celecoxib was approx. 200, 250 and 300 %, respectively, of the baseline. However, the kinetics of Ca^{2+} response differed when celecoxib concentrations exceeded 50 μ M. At 75 and 100 μ M, a biphasic pattern of $[Ca^{2+}]_i$ response was observed, i.e. a sharp increase to 100 nm followed by a more gradual rise, depending on the drug dose, to approx. 280 nm. It is noteworthy that the threshold for eliciting Ca^{2+} perturbation was approx. 10 μ M, which is in line with that required to trigger apoptotic cell death [16].

We also examined this Ca^{2+} -perturbing effect in other cell lines that included androgen-responsive LNCaP prostate cancer cells, A7r5 smooth muscle cells, NIH 3T3 fibroblast cells, MCF-7 breast cancer cells, Jurkat T cells and HepG2 hepatoma cells. All of these cells were subjected to celecoxib-induced $[Ca^{2+}]_i$ rise with the same level of susceptibility (results not shown), indicating that it was a general phenomenon among all the cell lines examined.

Moreover, our data demonstrate that the increase in $[Ca^{2+}]_i$ was not attributable to the disturbance of membrane permeability

by celecoxib (Figure 1C). To examine the effect of celecoxib on membrane integrity, fluo-3-loaded multilamellar vesicles with a lipid composition similar to that of the plasma membrane were exposed to celecoxib (50 μ M) vis-à-vis the Ca^{2+} ionophore bromo-A23187 (1 μ M) in the presence of 1 mM $CaCl_2$. While bromo-A23187 caused a rapid and robust Ca^{2+} inflow into liposomes, celecoxib did not elicit any appreciable effect on fluo-3 fluorescence. This observation excluded the possibility that celecoxib caused a $[Ca^{2+}]_i$ increase by perturbing membrane permeability.

Celecoxib-stimulated Ca^{2+} response is attributable to ER Ca^{2+} release and capacitative Ca^{2+} entry

The kinetics of celecoxib-induced Ca^{2+} response in PC-3 cells were further examined (Figure 2). Suspended PC-3 cells were loaded with fluo-3/AM, and Ca^{2+} response to celecoxib was monitored by fluorescence spectrophotometry.

Figure 2(A) indicates $[Ca^{2+}]_i$ changes following the treatment with 50 and 100 μ M celecoxib. Exposure to 50 μ M celecoxib stimulated a transient $[Ca^{2+}]_i$ increase, whereas 100 μ M celecoxib gave a robust, sustained $[Ca^{2+}]_i$ elevation with a significantly higher amplitude. This Ca^{2+} response was quantitatively different from that observed with digital ratiometric measurement (Figure 1). This discrepancy might, in part, be accounted for by differences in the cell preparation, i.e. cell suspension versus monolayers, and assay conditions. Figure 2(B) suggests that the Ca^{2+} response elicited by 100 μ M celecoxib was largely due to Ca^{2+} influx from the medium. Trace a (Figure 2B) shows changes in $[Ca^{2+}]_i$ following two tandem treatments of 50 μ M celecoxib. As shown, after the Ca^{2+} transient levelled off to the baseline following the initial treatment, a subsequent challenge stimulated a gradual rise in $[Ca^{2+}]_i$ to a level similar to that elicited by 100 μ M celecoxib alone. The second $[Ca^{2+}]_i$ increase, however, could be abrogated by removing external Ca^{2+} with EGTA (Figure 2B, trace b), whereas the first Ca^{2+} transient was not much affected. Moreover, pretreatment of cells with thapsigargin (1 μ M) in the presence of EGTA to deplete the internal Ca^{2+} stores also blocked $[Ca^{2+}]_i$ increase caused by 50 μ M celecoxib (Figure 2C). These data suggest that the transient elevation in $[Ca^{2+}]_i$ was attributable to Ca^{2+} release from internal Ca^{2+} stores, whereas the second Ca^{2+} signal was due to Ca^{2+} influx from the medium. This premise is in line with the capacitative Ca^{2+} entry theory that the depletion of intracellular Ca^{2+} stores signals an influx of Ca^{2+} across plasma membranes through Ca^{2+} -release-activated Ca^{2+} (CRAC) channels (or store-operated Ca^{2+} channels) [35,36].

Consequently, an important question arose as to how celecoxib induced Ca^{2+} release from internal stores. In principle, internal Ca^{2+} release might be mediated via one of the following Ca^{2+} -transporting mechanisms on ER membranes: (a) IP_3 receptors, (b) ryanodine receptors and (c) ER Ca^{2+} -ATPase (or SERCA) pumps [37,38]. Activation of IP_3 receptors or ryanodine receptors gives rise to Ca^{2+} release from the respective Ca^{2+} stores. With regard to the third mechanism, inhibition of the ATP-dependent Ca^{2+} pump by specific inhibitors such as thapsigargin results in rapid release of ER Ca^{2+} into the cytosol via ER Ca^{2+} leak channels [39]. To test the first and second possibilities, we examined the effect of pharmacological inhibitors specific for IP_3 receptors or ryanodine receptors on celecoxib-induced Ca^{2+} release from microsomes or permeabilized cells.

Celecoxib does not affect IP_3 - or ryanodine-sensitive Ca^{2+} stores

To test the effect of celecoxib on IP_3 -sensitive Ca^{2+} stores, the microsomal fraction of PC-3 cells was prepared, suspended in an

Figure 3 Evidence that celecoxib does not affect IP_3 -sensitive and ryanodine-sensitive Ca^{2+} stores

(A) Effect of heparin (200 $\mu\text{g}/\text{ml}$) on Ca^{2+} release elicited by IP_3 (10 μM) and celecoxib (50 μM) from Ca^{2+} -loaded microsomes prepared from PC-3 cells. Panel a, untreated microsomes; panel b, heparin-treated microsomes. The traces are representative of three independent experiments. (B) Effect of Ruthenium Red (10 μM) on celecoxib-induced Ca^{2+} signal in permeabilized PC-3 cells. Panels a and b depict the Ca^{2+} responses elicited by celecoxib (50 μM) and ryanodine (50 μM) in untreated cells and cells pretreated with Ruthenium Red. Cell permeabilization was carried out by treating with saponin (30 $\mu\text{g}/\text{ml}$) for 30 min. The traces are representative of three independent experiments.

Figure 4 Dose-response curves for the inhibition of ER Ca^{2+} -ATPases by thapsigargin and celecoxib

Inset, dose-dependent effect of celecoxib (0–100 μM) on ATP-dependent Ca^{2+} uptake in PC-3 microsomes. Each data point represents the mean $\pm$ S.D. ($n = 3$).

assay buffer containing fluo-3, and treated with ATP (2 mM) for Ca^{2+} loading. Addition of IP_3 (10 μM) and subsequent exposure to celecoxib (50 μM) each resulted in immediate Ca^{2+} release into medium (Figure 3A, upper panel). The amplitude of the Ca^{2+} response elicited by celecoxib was greater than that of IP_3 , indicating that IP_3 did not desensitize the Ca^{2+} -release mechanism mediated by celecoxib. Treatment of the microsomes with heparin

(200 $\mu\text{g}/\text{ml}$), an antagonist of IP_3 receptors [40,41], abolished IP_3 -induced Ca^{2+} release, but imposed no effect on celecoxib-mediated Ca^{2+} increase (Figure 3A, lower panel). Taken together, these data suggest that celecoxib did not affect IP_3 -sensitive Ca^{2+} stores.

To test the possibility of whether ryanodine receptors contributed to celecoxib-mediated Ca^{2+} response, we examined the effect of Ruthenium Red, an antagonist of ryanodine receptors [42], in permeabilized PC-3 cells (Figure 3B). Fluo-3-loaded PC-3 cells were permeabilized by treatment with saponin (30 $\mu\text{g}/\text{ml}$), followed by celecoxib (50 μM) and ryanodine (50 μM), in tandem. Both celecoxib and ryanodine gave rise to a robust Ca^{2+} increase (Figure 3B, upper panel). The amplitude of the Ca^{2+} signal elicited by ryanodine remained unchanged with or without celecoxib (results not shown). Pretreatment of the permeabilized cells with Ruthenium Red (10 μM) had no appreciable effect on celecoxib-induced Ca^{2+} increase, while dramatically attenuating the Ca^{2+} response elicited by ryanodine (Figure 3B, lower panel). These data indicated that celecoxib did not activate ryanodine-sensitive Ca^{2+} stores.

Celecoxib selectively inhibits ER Ca^{2+} -ATPases

In view of these data, we turned our attention to the effect of celecoxib on ER Ca^{2+} -ATPases and ATP-dependent Ca^{2+} uptake. The inhibitory profiles of celecoxib on the Ca^{2+} -ATPase activity and Ca^{2+} uptake (inset) were consistent (Figure 4).

These dose-response curves indicate that celecoxib was a modest inhibitor of ER Ca^{2+} -ATPases compared with thapsigargin, with IC_{50} values of 35 μM and 29 nM, respectively, while the IC_{50} for the inhibition of Ca^{2+} uptake was approx. 32 μM . The determined IC_{50} value for thapsigargin was consistent with that reported in the literature [39]. Meanwhile, the effect of celecoxib on ER Ca^{2+} -ATPases was highly specific. Our data

indicate that the drug exerted no inhibition on the plasma membrane Ca^{2+} -ATPase while vanadate completely blocked the enzyme activity (results not shown).

DISCUSSION

The present study demonstrates that the COX-2 inhibitor celecoxib affects intracellular calcium homeostasis by selectively inhibiting ER Ca^{2+} -ATPases. Together with our previous reports [16,17], these data suggest that celecoxib mediates rapid apoptotic death in prostate cancer cells via an intricate mechanism involving multiple signalling targets, including Akt, ERK2 and Ca^{2+} . The role of Akt and ERK2 in the regulation of cell survival is well understood. In addition, disruption of ER Ca^{2+} regulation may induce apoptosis through several plausible mechanisms. A sustained, modest increase of $[\text{Ca}^{2+}]_i$ for a sufficient time (minutes to hours) leads to the activation of a series of apoptosis-regulating enzymes including Ca^{2+} -sensitive proteases [43,44], $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent endonuclease [45–47], caspases [48] and calcineurin [49,50]. Moreover, sustained $[\text{Ca}^{2+}]_i$ elevations impose an adverse effect on mitochondria since some of the Ca^{2+} released from the ER is rapidly taken up by the organelle [38]. Recent evidence indicates that mitochondrial Ca^{2+} uptake involves the opening of the mitochondrial permeability transition pore, and yields cytochrome *c* release, thereby triggering apoptosis via caspase activation [51]. Moreover, this Ca^{2+} -perturbing effect may account for the effectiveness of celecoxib in sensitizing androgen-independent prostate cancer cells to apoptosis. It has been hypothesized that androgen-insensitive prostate cancer cells are not susceptible to the induction of apoptosis by androgen ablation, in part because such withdrawal did not induce an elevation of Ca^{2+} in these cells [25,52]. In the literature, a number of distinct Ca^{2+} -perturbing agents have been demonstrated to have anti-neoplastic activity. In addition to thapsigargin, a unique example that has received much attention is curcumin, a natural product with both anti-inflammatory and anti-carcinogenic activities [53]. Curcumin has been shown to interfere with multiple cellular targets, including COX-2 expression [54], SERCA pumps ($\text{IC}_{50} = 7\text{--}15\text{ }\mu\text{M}$) [55,56] and IP_3 receptors ($\text{IC}_{50} = 10\text{ }\mu\text{M}$) [57]. Due to its intricate interactions with two distinct Ca^{2+} channels on ER, curcumin displays a dual mode of action on ATP-dependent Ca^{2+} uptake, i.e. stimulation at low concentrations ($5\text{--}10\text{ }\mu\text{M}$) and inhibition at high concentrations ($> 10\text{ }\mu\text{M}$) [55]. In contrast, celecoxib inhibits both Ca^{2+} -ATPases and Ca^{2+} uptake in a consistent manner, indicating the high degree of specificity in the drug action.

It is noteworthy that this calcium-perturbing effect was also noted in smooth muscle cells and many other cell lines examined. This observation might be relevant to the reported side effects of celecoxib, which include elevated blood pressure and leucocyte adherence in a rat model [27], and risk of cardiovascular events like myocardial infarction and stroke [26]. It has been hypothesized that the cardiovascular toxicities of COX-2 inhibitors, in general, may be related to the selective inhibition of prostacyclin (PGI_2) but not thromboxane A_2 [58]. However, it warrants investigation whether the Ca^{2+} -perturbing activity of celecoxib also plays a role in these adverse effects. Our data show that celecoxib as low as $10\text{ }\mu\text{M}$ may cause a rise in $[\text{Ca}^{2+}]_i$. The peak plasma concentration (C_{max}) in normal individuals after taking therapeutic doses of celecoxib ranges between 2 and $8\text{ }\mu\text{M}$ [59]. Conceivably, the C_{max} value may be substantially higher in patients with compromised metabolic function or deficiency in CYP2C9, the major enzyme for celecoxib metabolism.

In summary, the present study proposes a unique pharmacological activity of celecoxib in interfering with intracellular

calcium homeostasis. This Ca^{2+} -perturbing effect may play a role in the apoptotic effect of celecoxib in androgen-independent prostate cancer cells, and provides a plausible link with the reported cardiovascular risk associated with the long-term use of celecoxib.

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