Effects of dexamethasone and celecoxib on calcium homeostasis and expression of cyclooxygenase-2 mRNA in MG-63 human osteosarcoma cells

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Abstract

Objective

Glucocorticoids and selective COX-2 inhibitors are potent anti-inflammatory agents. They are also suggested to influence bone physiology and remodeling. Here we searched for effects of dexamethasone and celecoxib on crucial parameters of bone physiology that could be therapeutically relevant.

Methods

The human osteosarcoma cell line MG-63 was used to measure effects of these drugs on (i) intracellular calcium concentration ($[Ca^{2+}]_i$) using a microfluorometric technique, (ii) alkaline phosphatase and osteocalcin levels (EIA) and (iii) the expression of cox-2 mRNA (quantitative real time PCR). Measurements were performed in Vitamine D-incubated quiescent cells and in cells stimulated with TNF-α and IL-1β.

Results

We found the cytokine-stimulation to increase $[Ca^{2+}]_i$ which was prevented by dexamethasone already after 30 min and still after 48 h. Dexamethasone was without any effect on $[Ca^{2+}]_i$ in quiescent cells. Celecoxib had no measurable short-term or long-term effects neither in quiescent nor in stimulated cells. Vitamin D stimulated the expression of cox-2 mRNA which was further enhanced by TNF-α/IL-1β. Dexamethasone did not have any measurable effects on COX-2 expression after 30 min, but a pronounced inhibition was seen after 48 h. In contrast, celecoxib had no effect on COX-2 expression. Neither of the drugs had any effect on the secretion of alkaline phosphatase and osteocalcin.

Conclusion

We found dexamethasone to inhibit the $[Ca^{2+}]_i$ increase in MG-63 cells following stimulation and to reduce considerably COX-2 expression via the genomic pathway. In contrast, celecoxib did not show any measurable short-term or long-term effects on the parameters of bone physiology measured.

Key words

Osteoblasts, dexamethasone, celecoxib, inflammation, intracellular Ca^{2+} concentration.

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Introduction

Destructive alterations of articular cartilage and bone tissue in rheumatic diseases are based on a disturbed balance between aggressive and protective factors as mediated by different cytokines and growth factors. Glucocorticoids and COX 2 inhibitors are therapeutically used to minimize clinical signs and symptoms by inhibiting the synthesis of mediators of inflammation such as prostaglandins in primary and secondary immune cells. However, it is unknown yet whether there are also direct effects on bone cells. These drugs are usually given for longer periods such as days and weeks (1), therefore rapid as well as long-term effects may occur. In this study we raised the question, whether drugs with effects on the COX-2 enzyme such as glucocorticoids and selective COX-2 inhibitors have rapid (within 30 min) and/or long-term (within 48 h) effects on calcium homeostasis, the secretion of alkaline phosphatase and osteocalcin, and the expression of cyclooxygenase (COX-2) mRNA in MG-63 human osteosarcoma cells.

Glucocorticoids are known to inhibit bone formation on the basis of a decrease in osteoblastic cell replication and differentiation, and an increase in the apoptosis of mature osteoblasts (for an overview see (2)). They have also been shown to stimulate differentiated functions of osteoblasts (3) and to induce the promotion of osteoblastic phenotype and matrix mineralization in osteoblastic cell cultures (4). We investigated dexamethasone which is known to produce strong genomic, but also intense rapid non-genomic glucocorticoid effects. Moreover, we have designed our study to distinguish between these different glucocorticoid effects on cytokine stimulated osteoblasts.

For therapeutic glucocorticoid action the inhibition of COX-2 synthesis is just one aspect. In contrast, selective COX-2 inhibitors like celecoxib seem to mediate their therapeutic effects almost exclusively via inhibiting this enzyme. COX-2 catalyzes the reactions of arachidonic acid to form prostaglandin E_2 (5), but its detailed role in bone physiology and remodeling is still unclear (6). Prostaglandins are known to have both stimulatory as well as inhibitory effects on bone metabolism and, therefore, COX-2 may have a role in bone formation and resorption (7, 8). It is clear, however, that COX-2 mediates the induction of bone formation and regulates mesenchymal cell differentiation into osteoblasts (9). Therefore, several studies suggest that COX-2 may have a critical role in bone repair and fracture healing (9, 10). Given this background, we examined whether celecoxib had any effects on crucial parameters of quiescent and stimulated MG-63 human osteosarcoma cells.

This study extends our recently published work where we described the effects of dexamethasone and celecoxib on mechanically induced changes in $[Ca^{2+}]_i$ and mitochondrial membrane potential. In this study we found these drugs to induce short-term changes in membrane characteristics and long-term changes in protein synthesis which indicated an influence on cell-to-cell communication and metabolism that may be therapeutically relevant (11). These intriguing results drove us to investigate the effects in a model of simulated inflammation we report here.

Materials and methods

MG-63 cell culture and stimulation

For the experiments presented here, we used the human osteosarcoma cell line MG-63 (American Type Culture Collection). This cell line shows an osteoblastic phenotype, including the responsiveness to calciotropic hormones and cytokines (12).

The cells were maintained in MEM (minimum essential medium; Gibco, Eggenstein, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 200 μM glutamine, 100 μM Na-pyruvate and nonessential amino acids (all Gibco) at 37°C in a 5% CO_2 atmosphere. Standard antibiotics (10 units/ml penicillin, 10 μg/ml streptomycin, Gibco) were used during cultivation. Cells were subcultured into 24-well plates (Nunc, Roskilde, Denmark) for measurement of $[Ca^{2+}]$ and into 25 cm^2 culture flask for RT-PCR studies after reaching 80 – 90 % confluence.

The stimulation of the MG-63 cells was performed by a 4-hour-preincubation (starting at time point T_{-1}; Fig. 1) with the inflammatory cytokines TNF-α and IL-1 β added to the culture medium in order to simulate inflammation.

Drug application

Aliquots from quiescent and cytokine-stimulated MG-63 cells were taken at the beginning of each experiment (time point T0) for baseline measurements of $[Ca^{2+}]_i$, alkaline phosphatase, osteocalcin, and COX-2 mRNA expression. Subsequently the cells were incubated with either dexamethasone or celecoxib, whereas Ca^{2+}/Mg^{2+}-free buffer served as control. After 30 min incubation (time-point T1; Fig. 1) aliquots were taken to measure the parameters mentioned above. Vitamin D3 (Vit D) was added, the cells were further incubated for 48 hours (T_2), and samples were taken again for the measurements described above.

Fluorescence measurements

Microfluorometric experiments were carried out using an imaging system based on an inverse microscope (IX50, Olympus, Hamburg, Germany) with 10x, 40x and 60x objectives (numerical aperture 0.3, 1.15 and 1.20, respectively; Olympus), a xenon light source with a monochromator (Polychrome II; TILL-Photonics, Martinsried, Germany) and a charge-coupled device camera (Imago, TILL-Photonics) (11). The imaging hardware was controlled via commercial software developed by TILL-Photonics on a PC.

For measurements of $[Ca^{2+}]_i$, MG-63 cells were incubated in culture media containing the acetoxymethyl (AM) ester of the dual-wavelength fluorometric Fura-2 (dissolved in DMSO, final concentration 1 μM, Molecular Probes Europe, Leiden, Netherlands) for 10 - 15 min at 37°C. After washing the cells for 15 min at 37°C using fresh Ca^{2+}- and Mg^{2+} free PBS, Fura-2 was excited at 340 nm and 380 nm and fluorescence measured at 510 nm. The calibration of the Fura-2 fluorescence signal was performed by an *in vitro* calibration procedure. Fura-2 as the free acid was added to saline containing

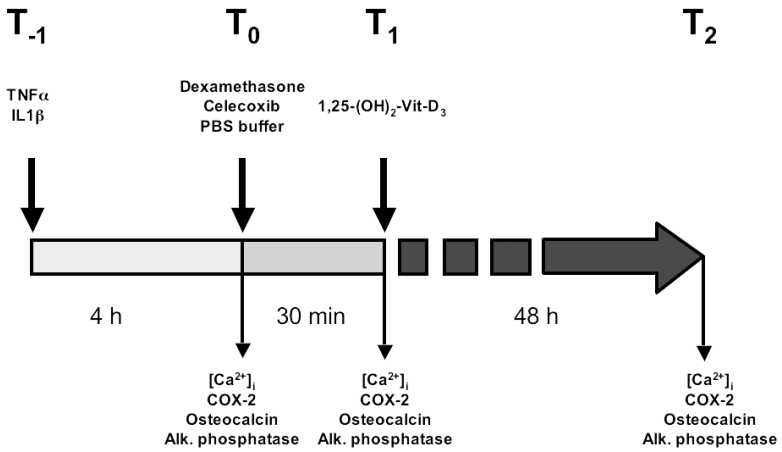

Fig. 1. Incubation procedure.

Ca^{2+}-EGTA buffers giving minimum and saturating levels of Ca^{2+} and hence the minimum (Rmin) and maximum (Rmax) fluorescence ratios and also the ratio of the Ca^{2+}-free and Ca^{2+}-saturated fluorescence excited at 380 nm (β), required for the equation (13):

$$[Ca^{2+}]_i = K_D \beta \frac{R - R_{min}}{R_{max} - R}$$

The fluorescence signals from single osteoblasts were analyzed offline by adjusting individual regions of interest (ROI) using NIH Image (version 1.61, http://rsb.info.nih.gov/nih-image).

Measurement of COX-2 mRNA expression

After incubation, cells were trypsinized at indicated time points. Total RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. RNA quality was assessed by gel electrophoresis, identifying the 18S and 28S rRNA bands. Subsequently, cDNA was obtained by reverse transcription of total RNA with oligo dT primer using the TaqMan® Reverse Transcription Reagents (Applied Biosystems, New Jersey, USA) according to manufacturer's instructions. The quantification of actin and cox-2 transcripts was assessed by real-time PCR based on fluorescence resonance energy transfer (FRET) probe chemistry using the Roche LightCycler instrument and software (Roche, Mannheim, Germany). Cox-2 transcript expression was normalized versus the housekeeping gene β-actin. Primers and probes used in the real-time PCR assay were purchased from TIB Molbiol (Berlin, Germany): β-actin forward-primer, 5'-CCgTgAAAAgATgACCCAgAT-3'; β-actin reverse-primer, 5'-CTCAgCTgTggTggTgAAgC-3'; cox-2 forward-primer, 5'-CCTCCTgTgCCTgATgATTgC-3'; cox-2 reverse-primer, 5'-TggCCCTCgCTTATgATCTg-3'; LightCycler Red 640 fluorophore-5'-end-labeled probes: β-actin, 5'- LC-ACCTggCTggCCgggACCTgA-3' and cox-2, 5' LC-CATTCTTTgCCCAg-CACTTCACgC -3'; fluorescein-3'-end-labeled probes: β-actin, 5' TCTC-CCTCACgCCATCCTgCgTCT-FL-3' and cox-2, 5'-ATCCCCAgggCT-CAAACATgATgT-FL-3'. The accuracy of the PCR assay as determined by the amplification efficiency (E) was assessed by measurement of a dilution series of MG-63 cDNA (β-actin: E = 2.02 ± 0.031; cox-2: E = 1.99 ± 0.045).

Alkaline phosphatase and osteocalcin measurement

To determine alkaline phosphatase and osteocalcin levels, MG-63 cells were cultured and drug-treated as described above. At time points T_0,T_1 and T_2, medium samples were collected, centrifuged to remove cell debris and stored at -20 °C till batch analysis. For measurement of alkaline phosphatase we used the commercially available Hybritech Tandem®-MP Ostase® assay (Beckman Coulter, Krefeld, Germany),

a microplate enzyme immunoassay for bone specific alkaline phosphatase (bone ALP). For the measurements of osteocalcin levels we used the Metra Osteocalcin microplate enzyme immunoassay (Quidel, Heidelberg, Germany).

Drugs and solutions

To analyse changes of $[Ca^{2+}]_i$, dye-loaded cells were incubated in Ca^{2+}- and Mg^{2+}-free PBS. Vitamine D (Vit D; 1,25-cholecalciferol) (Sigma, Deisenhofen, Germany) was used at 10 nM. In order to stimulate MG-63 cells the inflammatory cytokines TNF-α (100 ng/ml) and IL-1β (10 ng/ml) (both: Sigma, Deisenhofen, Germany) were added to the culture media according to similar experimental procedures as described by different authors (14-17). Dexamethasone (Sigma, Deisenhofen, Germany) was used at 100 nM. This concentration is clinically relevant (18) and has been used to mediate significant glucocorticoid effects (19). Celecoxib (Pharmacia Corporation, Peapack; USA) was used at 10 μM as previously described by other authors (20, 21). All experiments were performed at 24-28 °C.

Statistical analysis

Values are given as means ± standard deviation (SD). Statistical differences were assessed by Mann-Whitney-U and Wilcoxon tests. P values < 0.05 were accepted as significant.

Results

Intracellular calcium

The intracellular calcium concentration $[Ca^{2+}]_i$ in quiescent MG-63 cells was 106 ± 8 nmol/l at time-point T_0 (treatment with dexamethasone or celecoxib) and stayed constant during the observed time period: T_1: 105 ± 9 nmol/l (Fig. 2 A); T_2: 107 ± 9 nmol/l (Fig. 2 B). In contrast, 4 hour pre-incubation with TNF-α and IL-1 β lead to a significant increase of $[Ca^{2+}]_i$ at time-point T_0 (117 ± 9 nmol/l; $p < 0.001$), which was 10% higher than in quiescent cells. This stimulatory effect stayed constant during the observed time-period, e.g. T_1 (115 ± 7 nmol/l; Fig. 2 C) and T_2 (117 ± 10 nmol/l; Fig. 2 D).

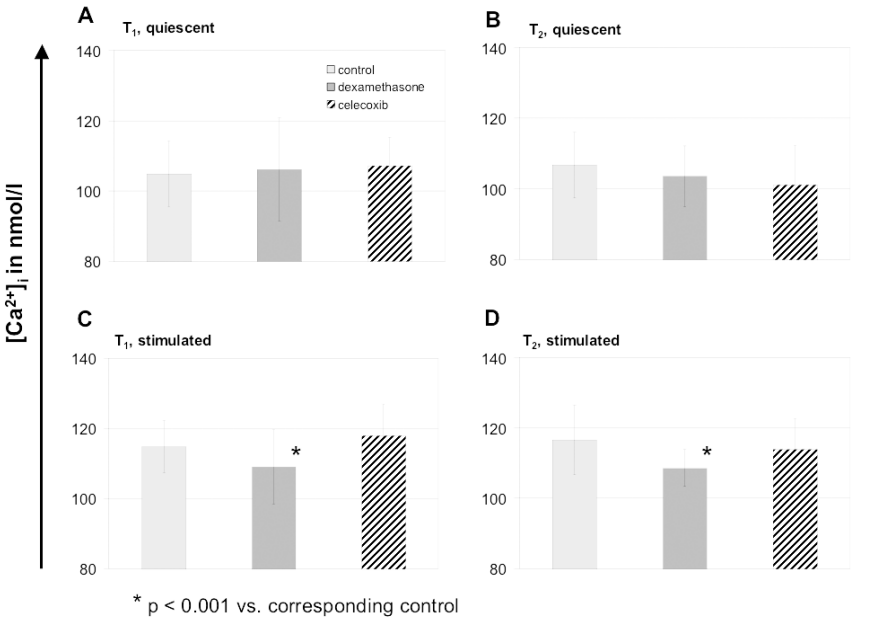

Fig. 2. A-D. Changes in $[Ca^{2+}]_i$ in the presence of dexamethasone and celecoxib compared to control group. $[Ca^{2+}]_i$ given in nmol/l measured in unstimulated cells 30 minutes (T_1; A) and 48 hours (T_2; B) after substance administration, and in stimulated cells 30 minutes (T_1; C) and 48 hours (T_2; D) after drug administration.

Dexamethasone did not affect $[Ca^{2+}]_i$ in quiescent cells neither at time-point T_1 nor at time-point T_2. Compared to quiescent control, there was no significant difference detectable. In contrast, treatment of cytokine stimulated MG-63 cells with dexamethasone decreased $[Ca^{2+}]_i$ after 30min (T_1) to the level of 109 ± 11 nmol/l ($p < 0.001$) and similar to that observed in stimulated control cells (Fig. 2 C) and in quiescent cells at time-point T_1 (Fig. 2 A). This result was also observed at time-point T_2. With the presence of dexamethasone the $[Ca^{2+}]_i$ was with a value of 109 ± 5 nmol/l, significantly lower ($p < 0.001$) than that observed in stimulated control cells (Fig. 2 D) and in quiescent cells at time-point T_2 (Fig. 2 B).

Celecoxib did not influence $[Ca^{2+}]_i$ significantly compared to dexamethasone neither at T_1 or T_2 nor in quiescent or stimulated MG-63 cells (Figs. 2 C and D).

Cox-2 mRNA expression

In quiescent cells, addition of Vit D (T_1) led to a 2.8 fold increase of cox-2 expression after 48 hours (T_2) (indicated as control in Figure 3 A/B). Additionally, 4 hours pre-stimulation of MG-63 cells with TNF-α and IL-1β led to a 2.5 fold increase of cox-2 expression at time-point T_0 and to a 3.4 fold increase respectively at time-point T_1 in comparison to the starting point (Fig. 3 C) ($p < 0.05$). Furthermore, treatment of stimulated cells with Vit D enhanced the cox-2 expression leading to a 7.9 fold increase at time-point T_2. Obviously, both Vit D and pro-inflammatory cytokines act synergistically stimulating on cox 2-expression (Fig. 3 D).

Addition of dexamethasone influenced cox-2 expression neither in resting nor in stimulated osteoblasts after 30 min ($p > 0.275$; Figs. 3 A and C). Instead, long-term incubation with dexamethasone for 48 hours led to a significant inhibition of cox-2 expression in comparison to the corresponding controls: Vit D treated, resting and stimulated cells ($p < 0.05$) (Figs. 3 B and D). The level of cox-2 expression in both cases was similar to that observed before drug and Vit D application: 0.75 ± 0.5 in quiescent cells, and 1.33 ± 0.7 in stimulated cells.

In contrast, celecoxib did not have any measurable effects on cox-2 expression after 30 min and after 48 hours in resting and in stimulated cells ($p > 0.275$).

Osteocalcin

Stimulation of MG-63 cells with TNF-α and IL-1β led to a significant in-

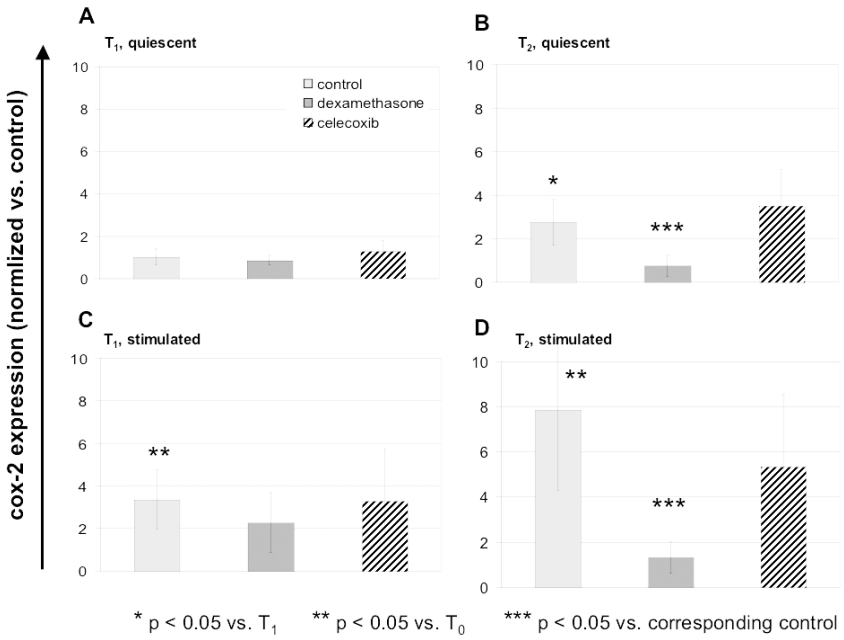

Fig. 3. A-D. Changes in cox-2 expression in the presence of dexamethasone and celecoxib compared to control group. All values normalized against cox-2 expression of unstimulated control cells. Expression measured in unstimulated cells 30 minutes (T_1; A) and 48 hours (T_2; B) after substance administration, and in stimulated cells 30 minutes (T_1; C) and 48 hours (T_2; D) after substance administration.

crease of osteocalcin secretion of 1.69 ± 0.32 µg/l up to 2.66 ± 0.94 µg/l after 48 hours incubation ($p < 0.05$). This effect was neither affected by the addition of dexamethasone nor by Celecoxib (2.42 ± 0.58 µg/l respectively 2.93 ± 1.08 µg/l).

Alkaline phosphatase

Resting osteoblasts showed an alkaline phosphatase level of 4.99 ± 1 µg/l, this level remained unchanged (4.77 ± 0.99 µg/l) even after stimulation with TNF-α und IL-1β. Neither dexamethasone nor celecoxib influenced the alkaline phosphatase level (4.94 ± 1.26 µg/l, and 4.63 ± 1.25 µg/l respectively).

Discussion

The successful clinical application of celecoxib in the treatment of osteoarthritis and rheumatoid arthritis primarily depends on the inhibition of COX-2 and the subsequent diminished synthesis of inflammatory mediators by primary and secondary immune cells (22). Furthermore, additional primary and secondary effects of celecoxib, both COX-2 dependent and -independent on different cell-types and organs have been described. For example, various obviously tissue specific genomic effects such as the impact on the expression of c-Jun and c-Fos as well as on the expression of COX-2 enzyme itself have to be mentioned (23; 24). Additional genomic actions have been described for celecoxib in skin tissue (Rantes, MCP-1), in breast epithelial cells (insulin-like growth factor binding protein-3) and in pancreatic cancer (Sp1 transcription factor activity) (25-27).

The action of celecoxib in human osteoblasts still remains unclear; besides the well-known inhibition of COX-2 activity (28), it is under discussion if the expression of the enzyme itself is influenced. This question is of major interest in particular for stimulated osteoblasts because of the presence of inflamed conditions with a local increase in pro-inflammatory cytokine concentrations found during clinical application of celecoxib (29, 30). Due to these inflamed conditions, the cellular actions of drugs can be extremely changed compared to a non-inflamed microenvironment (31, 32).

Several actions of dexamethasone on human osteoblasts have already been described in a variety of publications (11, 33, 34).

In this study we used dexamethasone as a typical anti-inflammatory drug for direct comparison with celecoxib. The dexamethasone dosage used (100 nM) is therapeutically relevant (18) and has already been shown to affect osteoblastic differentiation (35).

In accordance with Yamamoto *et al.*, we could show that dexamethasone considerably reduced the COX-2 expression in Vit D treated quiescent and cytokine-stimulated osteoblasts after 48 h (but not after 30 min) (36). This effect has already been described for several other cell-types such as vascular endothelial cells, synovial fibroblasts, and HeLa cells (37-39). Obviously, this effect is a result of genomic glucocorticoid action, mediated by a transrepressive mechanism of glucocorticoids (i.e. inhibition of transcriptional activators such as AP-1 and NFκB) (40-42). In addition to the observed genomic glucocorticoid actions, we could show a rapid – already detectable after 30 min – and a long lasting – still detectable after 48 hours – stabilising influence on $[Ca^{2+}]_i$ in MG-63 cells after dexamethasone treatment. Although inflammatory cytokines TNF-α and IL-1β are known to influence second messenger systems such as intracellular calcium and accordingly cellular activity (43), $[Ca^{2+}]_i$ did not change in the presence of dexamethasone. This effect can be explained by cytosolic or membrane bound glucocorticoid receptor mediated specific or non-specific (physicochemical influence on biological membranes) non-genomic glucocorticoid action (for details see ref. 44).

For the first time, we could show in this study that celecoxib had no influence on cox-2 expression or on calcium homeostasis of MG-63 cells. Therefore, these cells lack a negative feedback loop between COX-2 activity and cox-2 expression, which means there is no increase in cox-2 synthesis after inhibition of COX-2 activity in MG-63 cells. In contrast, Hsueh *et al.* demonstrated that inhibition of COX-2 with celecoxib resulted in an increase of cox-2 mRNA expression in neuronal spinal cord cells (23). Obviously, the mechanisms of action of celecoxib on proteomic and genomic level are highly variable in different cell types (23,

25-27). However, in this study we can exclude any effect of celecoxib on the expression of cox-2 in both cytokine-stimulated and quiescent MG-63 cells. This result is of important clinical relevance because COX-2 plays a pivotal role in the signalling cascade leading to osteoblastic proliferation and differentiation (9, 45, 46). In addition to the mediation of inflammatory activity, COX-2 is involved in carcinogenesis, being a central, pro-carcinogenious factor (47-49).

An increase in $[Ca^{2+}]_i$ can be judged as a marker of enhanced cell activity, a result or part of activated signalling cascades. Rapid effects on calcium homeostasis induced by celecoxib application have already been described. For instance, Wang *et al*. showed in human osteoblasts and Johnson *et al.* in prostate cancer cells an immediate increase of $[Ca^{2+}]_i$ after celecoxib treatment (50, 51). In this study, celecoxib did not show any measurable effect on $[Ca^{2+}]_i$ in quiescent cells. Stimulating the cells with TNF-α and Il-1β instead led to a significant increase in $[Ca^{2+}]_i$, which is similar to an enhanced cell activity in an inflammatory situation or an inflamed area. But also under these conditions celecoxib showed neither a diminishing nor an enhancing influence. Additionally, celecoxib had no effect on the cytokine-stimulated secretion of osteocalcin and on the activity of alkaline phosphatase in MG-63 cells.

In summary, our results demonstrate that celecoxib does not influence the cox-2 expression, $[Ca^{2+}]_i$, osteocalcin secretion, and alkaline phosphates activity in MG-63 cells in contrast to dexamethasone. Thus, we can conclude that there is no evidence for any positive or negative effects on human osteosarcoma MG-63 cells induced by inhibition of COX-2 using celecoxib under inflamed conditions.

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