Effects of celecoxib on the expression of osteoprotegerin, energy metabolism and cell viability in cultured human osteoblastic cells

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Abstract

Background and Objective

The selective COX-2 inhibitor celecoxib is widely used to treat pain and inflammation in rheumatoid arthritis, osteoarthritis, and ankylosing spondylitis. The drug has well-known important effects on immune cells but its direct and/or indirect influence on osteoblasts has not yet been explored in detail. This study aimed to investigate the dose-dependent effects of celecoxib on cell viability, energy metabolism and bone remodeling processes in cultured human osteoblastic cells.

Methods

Primary human osteoblasts and MG-63 cells were incubated with celecoxib (2, 10, 50μM). Cell viability and apoptosis were determined by trypan blue, 7AAD and Annexin-V staining. Effects on cellular oxygen consumption were measured amperometrically using a Clark electrode. mRNA expression of GLUT-1 and OPG was determined by RT-PCR; OPG protein secretion by ELISA and HIF-1α protein expression by immunoblotting.

Results

While celecoxib at a concentration of 2 and 10μM showed only marginal effects, a suprapharmacological concentration of 50μM influenced viability and energy metabolism, as well as OPG expression and secretion of osteoblastic cells. Cell viability was significantly reduced by celecoxib treatment. Celecoxib at 50μM stimulated oxygen consumption significantly. Corresponding experiments with the protonophore FCCP suggest that this effect is due to mitochondrial uncoupling. After 24h, GLUT-1 mRNA expression was significantly increased. HIF-1α protein was not expressed under any of our experimental conditions. We also showed that celecoxib at 50μM significantly inhibits OPG protein secretion leading to a compensative increase of mRNA expression.

Conclusion

Pronounced effects of celecoxib on cell viability (reduction), oxygen consumption (stimulation), GLUT-1 mRNA expression (stimulation) and OPG protein secretion (inhibition) in osteoblastic cells were observed only at 50μM – a concentration not reached by therapeutic doses giving plasma concentrations less than 10μM. On the contrary, celecoxib at 2 and 10μM showed only marginal effects, suggesting that celecoxib administration is probably safe with respect to bone metabolism in cases requiring potent treatment of pain and inflammation. However, higher intracellular concentrations, which might occur through accumulation, necessitate investigations with high concentrations.

Key words

Osteoblasts, celecoxib, osteoprotegerin, oxygen consumption, glucose transporter-1, hypoxia-inducible-factor-1α, cell viability.

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Introduction

Destructive alteration of the articular cartilage and bone tissue in peripheral joints is characteristic of many inflammatory joint diseases; rheumatoid arthritis (RA) is by far the most relevant condition in this respect. These changes are due to a disturbed multifactorial balance between aggressive and protective factors, mediated by different cytokines and growth factors (1). However, patients with RA show not only juxta-articular osteopenia and bone erosion but also generalized osteoporosis at sites distant from inflamed joints. Further pathogenetic factors include side effects of anti-inflammatory therapies, in particular glucocorticoids and possibly COX-2 inhibitors like celecoxib (2). NSAIDs are used to minimize the clinical symptoms of RA by inhibiting the synthesis of inflammatory mediators such as prostaglandins (3, 4). Continuous use of NSAIDs also reduces radiographic progression in ankylosing spondylitis (AS) (5) and effectively prevent heterotopic ossification after total hip arthroplasty (6-8). Direct and/or indirect effects on osteoblasts are therefore likely.

The glycoprotein osteoprotegerin (OPG) plays a pivotal role in bone remodeling processes (9, 10). OPG is a member of the tumour necrosis factor receptor (TNFR) superfamily, secreted by osteoblastic lineage cells. OPG binds and thus inactivates the soluble receptor activator of nuclear factor-κB ligand (RANKL). RANKL represents the essential osteoblast-derived factor required for osteoclast formation and activation leading to bone loss. These effects are blocked by OPG so that bone resorption is prevented in various physiological and pathophysiological situations (11). OPG expression is modulated by numerous osteotropic agents, *e.g.* it is positively regulated by estrogen, TNF-α and TGF-β, and negatively by PTH and glucocorticoids (11-13).

It has been demonstrated previously that COX-2 inhibitors have significant effects on cellular energy metabolism by uncoupling oxidative phosphorylation and inhibiting ATP turnover (14). Additionally, diclofenac and other COX inhibitors are known to inhibit the electron transport chain in isolated mitochondria (15-19). Several mechanisms, including growth factors and glucose transporter expression in response to the inhibition of oxidative phosphorylation or hypoxia, are stimulated. In consequence, enhanced glycolysis is associated with the increased expression of GLUT-1 (16, 20, 21). It should be noted that inhibition of oxidative phosphorylation *per se* results in stabilization of GLUT-1 mRNA (22). Normoxic conditions lead to ubiquitination and subsequent degradation of the hypoxia inducible transcription factor (HIF-1α) in less than 5 minutes (23) because oxygen is utilized for energy production and increased glycolysis is not needed. In contrast, under hypoxic conditions (O_2 <5%) HIF-1α is stabilized, leading to the formation of a functional transcription factor, and is able to regulate genes involved in homeostasis of glucose metabolism (GLUT-1) or angiogenesis (VEGF) (23).

In addition to their anti-inflammatory potential, COX inhibitors have positive therapeutic effects in the prevention and treatment of colorectal cancer. These effects could be caused by inhibition of cell proliferation as well as induction of apoptosis (24-26). But it remains unclear whether similar effects are also seen in other cell types and could, for example, influence bone metabolism.

The aim of this study was, therefore, to investigate the dose-dependent effects of celecoxib on cell viability, energy metabolism, and bone remodeling processes in cultured human osteoblastic cells.

Materials and methods

Cell culture

The following human osteoblastic cells were used: 1) MG-63 human osteosarcoma cell line (American Type Culture Collection (27), and 2) primary human osteoblasts (phOB) prepared from trabecular bone explants of four female patients undergoing joint replacement. The local ethics committee approved the study. First passage phOB cells were used as previously described (28, 29). The MG-63 cells as well as the phOB cells (plating density $4x10^3$ cells/cm^2) were maintained in minimum essential

medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 200μM glutamine, 100μM Na-pyruvate and nonessential amino acids, 10 units/ml penicillin and 10μg/ml streptomycin (all Gibco, Eggenstein, Germany). After reaching confluence, phOB cells were further cultured for 10d and subsequently incubated in serum free MEM supplemented with 0.125% BSA for 4d before starting experiments (12). Dexamethasone and carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) (all Sigma, Munich, Germany) were used; celecoxib (SC-58635) was kindly provided by Pfizer, USA. Celecoxib and dexamethasone were dissolved in DMSO (0.1%) (Roth, Karlsruhe, Germany).

Cell viability and apoptosis analysis
Viability was analyzed by trypan blue (0.2%) staining to identify dead cells (quantified using a Neubauer chamber). Continuative analysis of dead and proapoptotic cells was performed by Annexin-V-PE and 7AAD staining (BD Pharmingen) according to the manufacturer's protocol and analyzed by flow cytometry.

Energy metabolism:
1. Oxygen consumption
Oxygen consumption was measured amperometrically with a Clark electrode (0.3-0.4 x10^7 cells/ml). The suspension was magnetically stirred (37°C) in the Perspex incubation chamber of the electrode. Respiration was measured up to 3min (respiration rate of untreated cells). After the addition of DMSO (negative control), the respiration rate was determined again and defined as basal oxygen consumption. The respiration rate was determined for another 3min after addition of celecoxib at different concentrations, as described previously (30) and measured after 1 and 24h. Furthermore, changes in respiration rates were quantified in cells treated with the protonophore FCCP at 0.5μM.

Energy metabolism:
2. GLUT-1 mRNA expression
Total RNA was extracted (RNeasy Mini Kit, Qiagen, Hilden, Germany) and cDNA was obtained by reverse transcription using the TaqMan® Reverse Transcription Reagents (Applied Biosystems, New Jersey, USA) according to the manufacturer's instructions. Quantification of ACTB and GLUT-1 expression was performed by real-time PCR (SYBR Green system, Roche LightCycler, Roche, Mannheim, Germany). GLUT-1 mRNA expression was normalized to the mRNA expression of ACTB. Data are shown as relative ratios to DMSO-treated cells. Results from DMSO-treated cells were given a value of 1. Primers (TIB Molbiol, Berlin, Germany): ACTB forward-primer, 5'-GACAGGATGCAGAAGGAGATCACT-3'; ACTB reverse-primer, 5'-TGATCCACATCTGCTGGAAGGT-3'; GLUT-1 forward-primer, 5'-ACGCTCTGATCCCTCTCAGT-3'; GLUT-1 reverse-primer, 5'-GCAGTACACACCGATGATGAAG-3'. The accuracy determined by amplification efficiency (E) was assessed by measuring a dilution series of cDNA (ACTB: E=1.92±0.103; OPG: E=2.00±0.038; GLUT-1: E=1.93±0.084).

Energy metabolism:
3. HIF-1α Western blotting
Cell lysates (2x10^6 cells) were separated on a 10% polyacrylamide gel and blotted onto a PVDF membrane (Millipore, Germany). After blocking (5% skimmed milk in TBS/Tween20 (0.05%)) the membrane was incubated with α-human-HIF1α (1:300, Becton Dickinson Heidelberg, Germany) or α-β-actin as primary antibody (1:10000, Sigma, Munich, Germany). The secondary antibody was the horseradish peroxidase conjugated α-mouse-IgG (1:10000, PROMEGA). The membrane was developed using ECL™ (Amersham Biotech, UK).

Osteoprotegerin measurements:
1. OPG mRNA expression
Quantification of OPG mRNA was performed as described for GLUT-1 mRNA: OPG forward-primer, 5'-AGCACCCTGTAGAAAACACAC-3'; OPG reverse-primer, 5'-ACACTAAGCCAGTTAGGCGTAA-3'.

Osteoprotegerin measurements:
2. OPG protein ELISA
OPG protein levels were determined by ELISA (Immundiagnostik, Bensheim, Germany) according to the manufacturer's instructions, lower limit of detection was 0.14pmol/l; intra-assay CV (n=16) was 9.0%. OPG protein secretion is expressed as relative ratio to DMSO-treated cells (control).

Statistical analysis
Data are presented as mean ± standard error of the mean (SEM) of at least duplicate determinations. The Wilcoxon test and ANOVA with subsequent Bonferroni α correction were performed using SPSS 12.0 or Prism. *P*-values less than 0.05 were considered significant.

Results
Cell viability
Dead cells were determined by trypan blue staining in MG-63 cells treated with DMSO, dexamethasone (10nM) or celecoxib (10μM or 50μM) for different incubation periods (Fig. 1A). The percentage of dead cells was related to DMSO-treated cells (negative control group). The number of dead cells significantly increased after 24h treatment with all drugs (Fig. 1A). In order to investigate cell death more specifically by Annexin-V (marks proapoptotic cells) and 7AAD (marks dead cells) staining on single cell level, we performed a kinetic. We completed our experimental set-up by an additional concentration of celecoxib of 2μM to imitate concentrations found in plasma of humans. Cells were analyzed at 0h and after 12, 24, 36, 48 and 72h (Fig. 1B). While cells treated with celecoxib at 2 and 10μM did not differ in the frequency of $7AAD^+$ (dead) cells from DMSO treated cells, significantly higher frequencies of dead cells were found after 36, 48 and 72h ($p<0.001$, respectively). The highest effect occurred after 72h with 7.7%±1.1% $7AAD^+$ cells, thus more than 90% of the cells were still alive at all conditions within our experimental set-up. The Annexin-V staining revealed a significant increase in the frequency of proapoptotic cells after 36 ($p<0.001$), 48 ($p<0.01$) and 72h ($p<0.001$) (Fig. 1C).

Energy metabolism:
1. Oxygen consumption rate
Selective COX-2 inhibitors have already been shown to influence energy

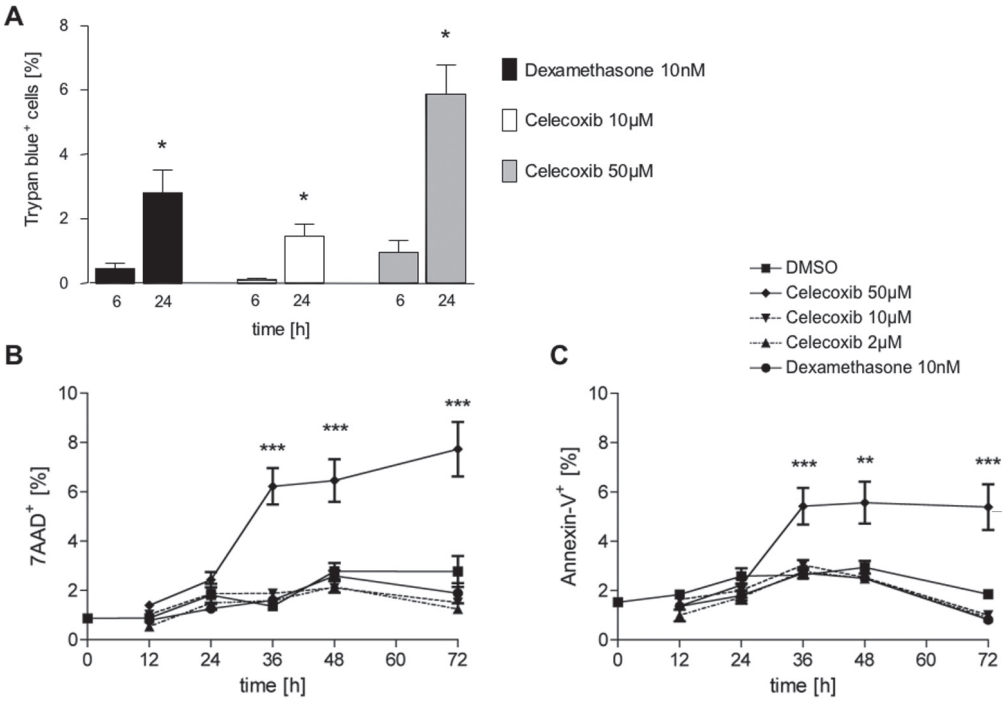

Fig. 1. Effects of dexamethasone and celecoxib treatment on the viability of MG-63 cells. (**A**) Cells were exposed to DMSO (control), dexamethasone (10nM) or different concentrations of celecoxib (10µM, 50µM) for 6 and 24h. Dead cells were determined by trypan blue staining and related to DMSO treated cells. Columns: mean of six different cell passages by triplicate determination; error bars: SEM; *: $p<0.05$, compared with DMSO group using Wilcoxon test.
(**B, C**) MG-63 cells were treated with DMSO and celecoxib at 2, 10 and 50µM. At the indicated time points, cells were analyzed by 7AAD staining to determine dead cells (**B**) and Annexin-V staining to determine proapoptotic cells (**C**) on single cell level by flow cytometry. Illustrated are means ± SEM, n=4, ** $p<0.01$, *** $p<0.001$, compared with DMSO group using 2-way ANOVA with Bonferroni posttests.

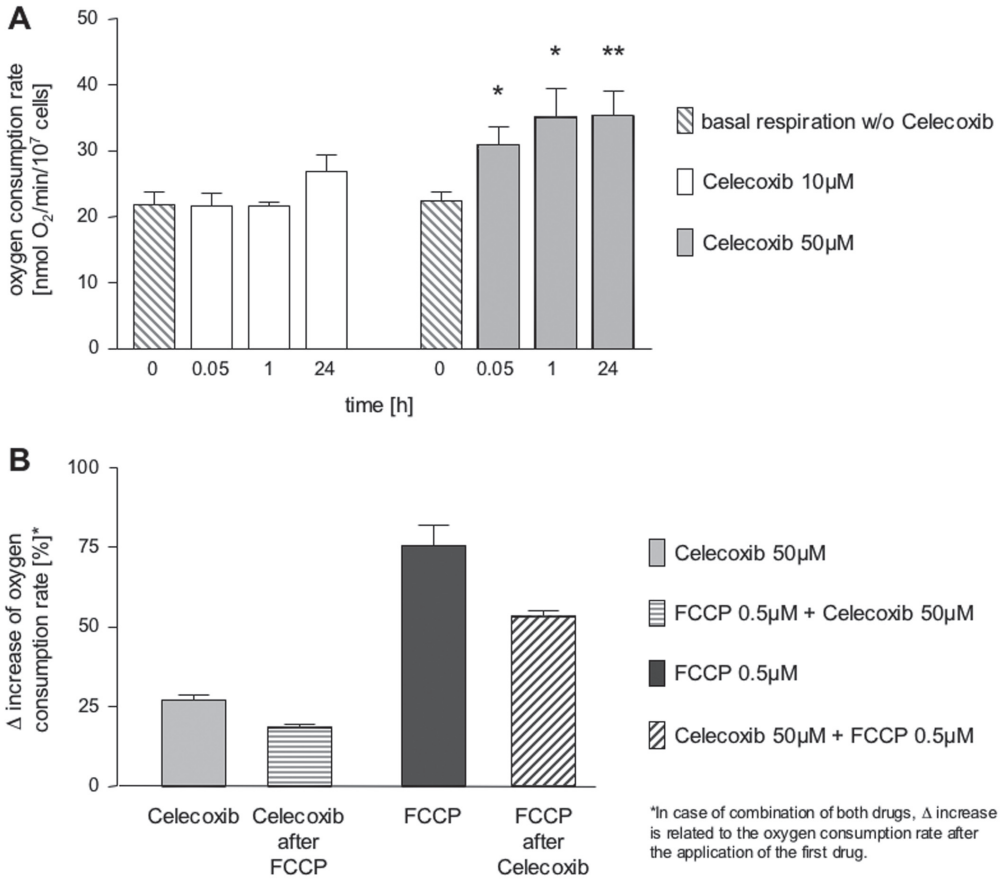

Fig. 2. (**A**) Effects of different celecoxib concentrations (10µM, 50µM) on oxygen consumption rate (nmol O_2/min/10^7 cells) of MG-63 cells after 0.05, 1 and 24h. Results were compared with DMSO-treated cells (basal consumption rate). Columns: mean of six different cell passages by triplicate determination; error bars: SEM; * $p<0.05$; ** $p<0.01$, compared with control group using Wilcoxon test.
(**B**) Effects of celecoxib (50µM) and FCCP (0.5µM) on the oxygen consumption rate of MG-63 cells with single treatment and in combination. First column: application of celecoxib without FCCP; second column: application of FCCP followed 5min later by celecoxib; third column: application of FCCP without celecoxib; fourth column: application of celecoxib followed 5 min later by FCCP. When drugs were applied alone the increase in oxygen consumption was related to the basal oxygen consumption. When the two drugs were applied consecutively, the effect mediated by the second applied drug is shown. Columns: mean of six different cell passages by triplicate determination; error bars: SEM.

metabolism by uncoupling oxidative phosphorylation (14). To address this point as a possible reason for the observed effects on osteoblastic cell survival, we investigated the influence of celecoxib on the energy metabolism of osteoblasts by evaluating the oxygen consumption (Fig. 2A). Administration of celecoxib at 10µM did not have any effect on the oxygen consumption rate, neither immediately nor after 1h incubation. After 24h, celecoxib (10µM)

led to a numerical, but statistically not significant, enhancement of the oxygen consumption rate (Fig. 2A). In contrast, treatment with celecoxib at 50μM stimulated the oxygen consumption rate immediately and significantly by 38% (30.94±2.67 nmol O_2/min/10^7 cells, p<0.05). After 1h the oxygen consumption rate further increased significantly (35.1±4.31 nmol O_2/min/10^7 cells, p<0.05) and remained at 35.41±3.31 nmol O_2/min/10^7 cells after 24h representing a significant increase of nearly 60% (p<0.01).

To investigate if the celecoxib-induced stimulation of oxygen consumption was mediated by a mechanism of uncoupling oxidative phosphorylation, the results described above were compared with effects produced by the protonophore FCCP. Celecoxib (50μM) and FCCP (0.5μM) were applied to MG-63 cells either individually or in combination (Fig. 2B). The percentage increase in oxygen consumption 3min after administration of the first drug was determined. Then the other drug was added: celecoxib was followed by FCCP and vice versa. The increase in oxygen consumption mediated by the second drug was quantified and related to the oxygen consumption level achieved after the first drug. The average increase in oxygen consumption caused by celecoxib alone was 27.2±1.63%. Application of FCCP enhanced the oxygen consumption rate by 75±6.64% (Fig. 2B). Both celecoxib and FCCP stimulated the respiration of osteoblasts. However, when cells were treated with FCCP prior to the celecoxib application, the celecoxib-mediated increase in oxygen consumption amounted to only 18.47±0.91%. This value was considerably lower than the 27% increase caused by celecoxib alone. And, vice versa, celecoxib pretreatment followed by FCCP administration resulted in an FCCP-mediated increase in respiration of only 53.26±1.59%. The FCCP effect in the presence of celecoxib was thus considerably lower than the 75% increase seen with FCCP alone (Fig. 2B). In summary, application of the first drug led to a certain increase in oxygen consumption rate, and the second drug was still able to evoke an additional effect.

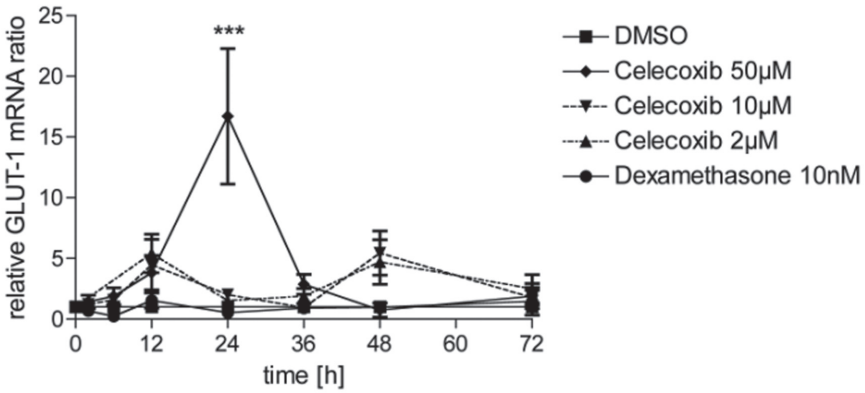

Fig. 3. Effects of celecoxib on the GLUT-1 mRNA expression of MG-63. Cells were exposed to dexamethasone (10 nM) or different concentrations of celecoxib (2, 10, 50μM) for the indicated time points. Changes in GLUT-1 mRNA expression in MG-63 cells are shown for each incubation period after substance administration. Illustrated are means ± SEM, n=4, ***p<0.001, compared with DMSO group using 2-way ANOVA with Bonferroni posttests.

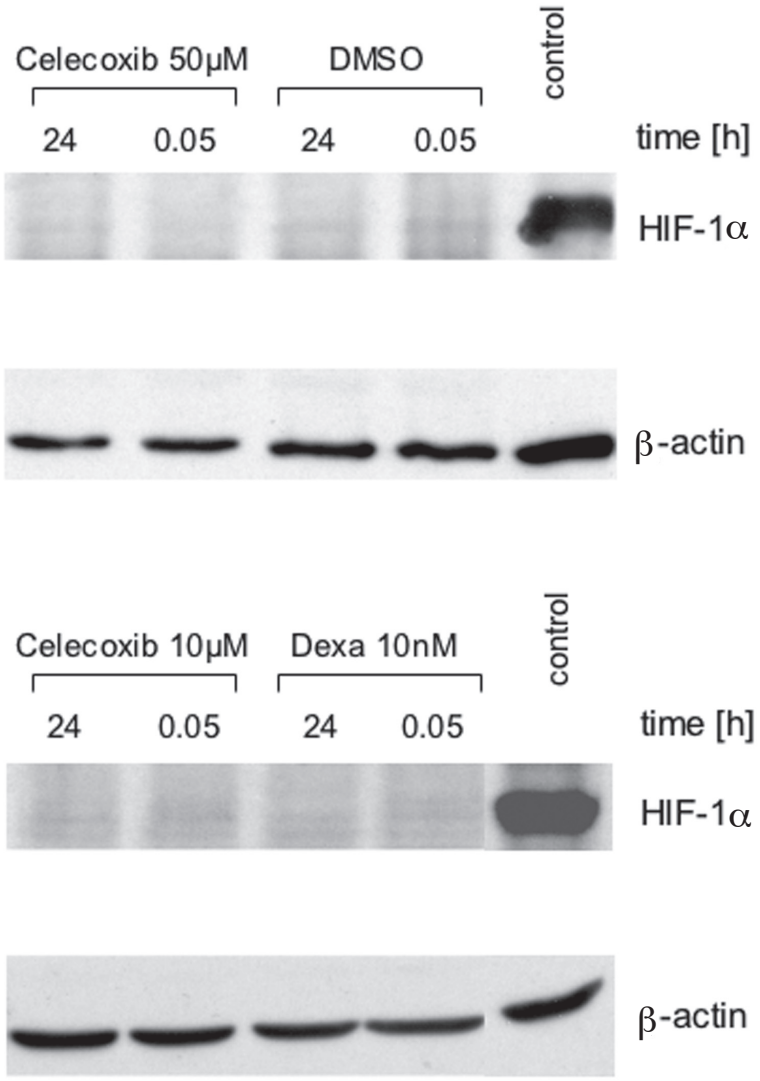

Fig. 4. Effects of DMSO, dexamethasone (Dexa) and celecoxib treatment on HIF-1α expression of MG-63 cells. Cells treated with DMSO, dexamethasone (10nM) or celecoxib (10μM, 50μM) were incubated for the periods indicated. HIF-1α expression was determined using western blots (control: β-actin). All lanes contained 20μg of protein. The positive control reflects a blot of PMA/Ionomycin stimulated $CD4^+$ T-lymphocytes under hypoxic conditions (6h).

However, this effect was clearly smaller than that achieved by the drug applied alone. The observed effect could be a partial uncoupling of oxidative phosphorylation.

Energy metabolism:
2. GLUT-1 mRNA expression

Uncoupling of oxidative phosphorylation necessarily leads to reduced ATP production. It is usually counteracted by increased ATP production via glycolysis. This should be reflected in the stimulated transport of glucose into the cell. To test this, we examined glucose-transporter-1 (GLUT-1) mRNA expression. MG-63 cells treated with DMSO (control), dexamethasone (10nM) or celecoxib (2, 10 or 50μM) were examined for their GLUT-1 mRNA expression (Fig. 3).

Celecoxib (2, 10 and 50μM) led to a numerical but statistically insignificant increase of GLUT-1 mRNA after 12h compared to DMSO. After 24h celecoxib at 50μM caused a high and significant increase in the expression of GLUT-1 mRNA (16.7±5.6, $p<0.001$). At the later time points in this kinetic there were no statistically significant differences. The escalation of GLUT-1 mRNA expression after 24h further supports the assumption that celecoxib increases oxygen consumption via uncoupling. Despite increased oxygen consumption ATP production via oxidative phosphorylation is reduced, which causes the increased demand of the cells for glucose.

Energy metabolism:
3. HIF-1α expression

Among several different effects, HIF-1α is involved in the regulation of glucose metabolism by up-regulating GLUT-1 expression. To rule out the possibility that the observed increase in GLUT-1 mRNA expression (Fig. 3) is due to endogenous hypoxia, we examined HIF-1α expression under the same conditions.

MG-63 cells were treated with DMSO, dexamethasone (10nM) or celecoxib (10μM, 50μM) and HIF-1α protein expression was analyzed immediately and after 24h. We found that none of the drugs caused HIF-1α protein expression at any time (Fig. 4).

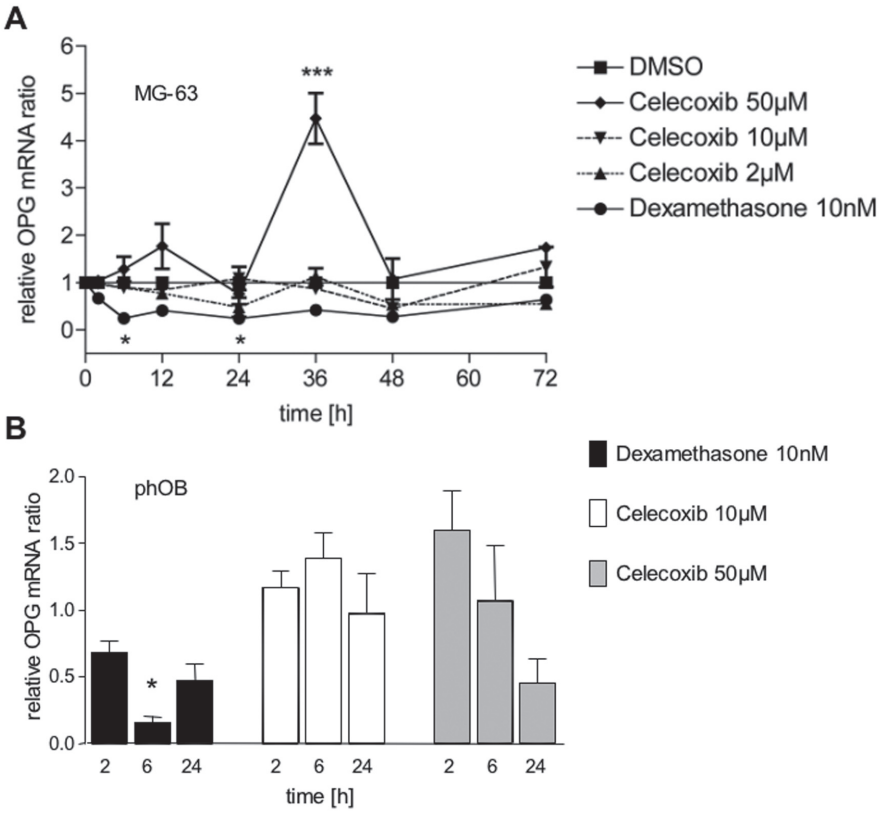

Fig. 5. Effects of celecoxib and dexamethasone on the OPG mRNA expression. (**A**) Changes of OPG mRNA expression in MG-63 cells. Illustrated are means ± SEM, n=4, ***$p<0.001$; *$p<0.05$; compared with DMSO group using 2-way ANOVA with Bonferroni posttests. (**B**) Changes of OPG mRNA expression in phOB. Columns: mean of OPG expression of four different patients (phOB) by triplicate determination; error bars: SEM; *$p<0.05$; compared to control using ANOVA with Bonferroni posttests.

Osteoprotegerin measurements:
1. OPG mRNA expression

The next question concerned the possible impact of the demonstrated effects on bone remodeling. First, we examined OPG mRNA expression in MG-63 cells treated with DMSO, dexamethasone (10nM) or celecoxib (2, 10 or 50μM). OPG mRNA was analyzed after 2, 6, 12, 24, 36, 48 and 72h (Fig. 5A). Dexamethasone inhibited OPG mRNA expression numerically at all time points measured. This effect was significant after 6 and 24h ($p<0.05$, respectively). OPG mRNA expression was not significantly influenced by celecoxib at 2 and 10μM at any time. Celecoxib at 50μM significantly increased the OPG mRNA expression after 36h compared to DMSO control (4.5±0.5, $p<0.001$) (Fig. 5A).

This experimental set-up was repeated with phOB cells (Fig. 5B). OPG mRNA expression of phOB cells was diminished 2, 6 and 24h after dexamethasone administration. This decrease was significant after 6h (0.16±0.04, $p<0.05$). Treatment of phOB cells with celecoxib at 10 and 50μM did not have any significant effect on OPG mRNA expression after 2, 6 and 24h. But in this experimental set-up only the early time points could be measured and we would expect the same effects like for MG-63 cells at later time points.

Osteoprotegerin measurements:
2. OPG protein secretion

To check the observed effects of celecoxib and dexamethasone on OPG mRNA expression at protein level, OPG protein secretion was measured by ELISA (Fig. 6). Dexamethasone (10nM) treatment of phOB cells inhibited the OPG protein secretion gradually; the minimum value was seen after 24h, without reaching significant levels. Treatment with celecoxib at 10μM did not have any obvious effect on OPG protein secretion. In contrast, celecoxib

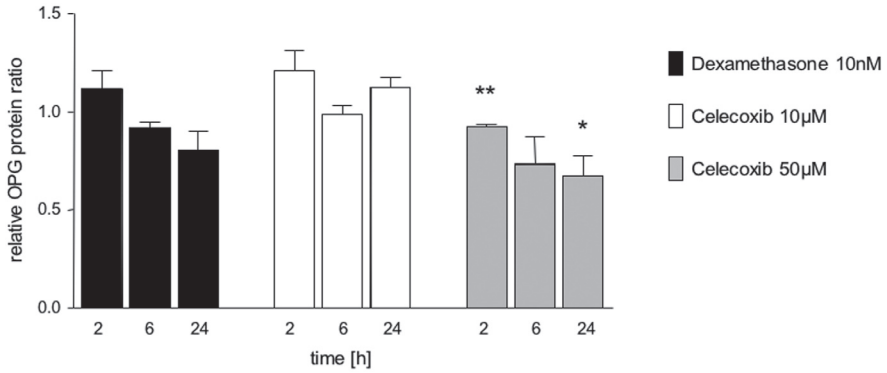

Fig. 6. Effects of celecoxib and dexamethasone treatment on the OPG protein secretion of phOB cells. OPG protein from cell culture supernatants was measured by ELISA. Cells were exposed to different concentrations of celecoxib (10µM, 50µM) or dexamethasone (10nM) for 2, 6 and 24h. Columns: mean of OPG protein production of four different patients by duplicate determination; error bars: SEM; *p<0.05; ** p<0.01, compared to control using ANOVA with Bonferroni posttests.

(50µM) reduced the protein secretion after 2, 6 and 24h. After 2 and 24h the level of significance was reached (0.92±0.025, p<0.01 and 0.68±0.089, p<0.05, respectively). *In vivo* such effects could lead to bone loss with time.

Discussion

The selective COX-2 inhibitor celecoxib is used therapeutically to minimize clinical symptoms of conditions including RA by inhibiting the synthesis of inflammation mediators such as prostaglandins (3, 4). The present studies were performed to investigate different responses of bone cells after celecoxib administration.

We showed that treatment with celecoxib leads to reduced survival of osteoblasts (Fig. 1A, B). Increased frequencies of dead cells (detected by trypan blue) were found in celecoxib-treated cell cultures. The overall number of cells in the cultures did not differ. From this we conclude that, as there is a negligible effect on the proliferation of osteoblasts, the increased number of dead cells cannot be explained by differences in proliferative behaviour. The increased cell death was confirmed on single cell level by 7AAD staining, which detects dead cells. Additionally increased rate of Annexin-V$^+$ proapoptotic cells could be detected after treatment with celecoxib at 50µM. Celecoxib has already been shown to induce apoptosis in different cell lines (25, 26). The proapoptotic effects of celecoxib are among the reasons for its effectiveness in the prevention and treatment of colorectal cancer (25, 26). Other groups have shown that bone cell growth is inhibited with celecoxib administration (31, 32). In fact, it was found that the COX-2 inhibitor rofecoxib decreased the number of osteoblasts and potentially delayed fracture healing in a rabbit model (31). It can be assumed that celecoxib has proapoptotic effects in osteoblasts, which are negligible at a concentration of 2 and 10µM but distinct at a concentration of 50µM. High concentrations of celecoxib could obviously lead to impairment of bone healing processes but only at concentrations that are unlikely to occur *in vivo*. Nevertheless, this possible *in vivo* effect underlines the need for this study in osteoblasts.

We were able to show that celecoxib causes an increase of oxygen consumption (Fig. 2A). COX inhibitors are known to influence cellular energy metabolism by uncoupling oxidative phosphorylation (15-18). Furthermore, we had previously demonstrated that the selective COX-2 inhibitor SC-236 (a compound with similar characteristics to celecoxib but with a longer half-life) has significant effects on cellular energy metabolism by uncoupling oxidative phosphorylation and inhibiting ATP turnover (14). Thus, we assume that the increase of oxygen consumption by celecoxib is likely to be caused by the uncoupling of oxidative phosphorylation. In contrast, Mahmud *et al*. and Tibble *et al*. found no effects of non-acidic selective COX-2 inhibitors on the respiration rate of isolated rat liver mitochondria (18, 19), so we assume that the uncoupling effect probably depends on the cell type. Based on this assumption, we demonstrated for the first time that osteoblasts are among the cell types in which celecoxib influences cellular energy metabolism probably by uncoupling oxidative phosphorylation.

To examine the increased respiration in osteoblasts we compared the effects of celecoxib with those of FCCP, a known protonophore. Protonophores significantly impair the ATP production leading to increase of cellular oxygen consumption (33). As expected, FCCP led to highly increased respiration (by more than 75%) and celecoxib (50µM) treated cells also showed increased oxygen consumption (by 27%) (Fig. 2B). When one of those two drugs was applied after pretreatment with the other one, in each case the increase of oxygen consumption mediated by the second drug was lower than the effect of this drug alone: celecoxib alone (+27%) *vs*. celecoxib after FCCP (only +18%); FCCP alone (+75%) *vs*. FCCP after celecoxib (only +53%). From these results, we conclude that the mechanism of increasing oxygen consumption is probably similar to that of FCCP: the uncoupling of oxidative phosphorylation. This would explain the observed differences between the effects when FCCP or celecoxib is given alone or as the second substance. In fact, COX-inhibitors and protonophores share similar molecular properties, *e.g*. hydrophobic weak acids with aromatic ring structures, therefore accumulating to a higher concentration in membranes and causing uncoupling by proton translocation, as well as by disturbance of the mitochondrial membrane structure (16).

We then asked what effects the uncoupling of oxidative phosphorylation by celecoxib would have on the energy metabolism of osteoblasts. Enhanced glycolysis after treatment with COX-inhibitors (diclofenac and aspirin) has been shown earlier and attributed to the inhibition of oxidative phosphorylation (16). We could confirm this effect in osteoblasts by determining GLUT-1 mRNA. We were indeed able to demonstrate a stimulation of GLUT-1

mRNA expression after treatment with celecoxib, but at 2 and 10μM the effect was not significant. However, the effect of celecoxib at 50μM was detectable after 12 and 24h reaching a significant level after 24h (Fig. 3). Since ATP synthesis via oxidative phosphorylation is significantly impaired due to uncoupling, the cells respond by generating energy from increased glucose utilisation. For that reason, GLUT-1 mRNA expression is up-regulated to enable enhanced glycolytic ATP synthesis (20-22). An approximately 6 kbp DNA fragment located 5' to the GLUT-1 gene transcription start site mediates increased transcription in response to hypoxia in a dual fashion: in response to a decrease in the concentration of oxygen *per se*, as well as to the accompanying inhibition of oxidative phosphorylation (22, 34, 35).

To rule out endogenous hypoxia as a possible reason for increased GLUT-1 mRNA expression (22, 34, 35), we examined HIF-1α expression in osteoblasts after dexamethasone or celecoxib application. We could not detect any expression of HIF-1α neither in control nor in drug-treated cells (Fig. 4). These results demonstrate normoxic conditions during incubation.

Based on our findings that celecoxib caused increased respiration of osteoblasts, but due to the related uncoupling of oxidative phosphorylation, the oxygen could not be utilized to generate energy and the cells increased GLUT-1 expression in order to obtain more glucose for energy generation by anaerobic glycolysis. The increased cell death could be explained by either direct effects and/or loss of energy. As most cells survive, however, we then had to examine the impact of the demonstrated effects on the bone remodeling potential of osteoblasts. We examined effects of celecoxib on OPG mRNA and protein expression. OPG secreted by osteoblastic cells blocks the effects of RANKL by neutralizing it and thus preventing bone loss (11). A shift in the RANKL/OPG ratio in favour of RANKL leads to bone erosion (36). We used osteoblasts treated with dexamethasone as the positive control (12) and could confirm its known effect on bone metabolism (OPG mRNA suppression Fig. 5A). We demonstrated for the first time that celecoxib at 50μM inhibits OPG protein secretion in osteoblastic cells. Interestingly, while celecoxib at 2 and 10μM did not have any obvious effect on OPG mRNA expression, celecoxib at 50μM increased the OPG mRNA expression after 36h. This could be interpreted as being a reaction on the showed effects of celecoxib on cell death and energy metabolism: the impaired energy supply leads to a reduced protein secretion, and the cells in turn try to protect themselves via inducing OPG mRNA production.

Although the RANKL expression was not examined, an imbalance of the RANKL/OPG ratio can be assumed; thus we suppose that celecoxib at suprapharmacological concentrations would lead to resorptive bone processes by OPG inhibition as has already been demonstrated for glucocorticoids. However, Hu *et al*. showed that celecoxib at 50μM enhanced the translocation of the glucocorticoid receptor into the nucleus of neuronal PC-12 cells. The subsequent receptor mediated gene expression via the kinases p38/MAPK pathway was additionally associated with COX-2 inhibition (37). This fact could explain the similar effects of dexamethasone (10nM) and celecoxib (50μM) on bone metabolism.

Based on our *in vitro* data, it appears that a high concentration (50μM) of celecoxib inhibits the survival of osteoblasts and stimulates oxygen consumption by uncoupling oxidative phosphorylation. The osteoblasts induce GLUT-1 to use anaerobic glycolysis but the energy produced is probably required in the first line for basic needs. The increasing lack of energy and the proapoptotic and toxic effect could be the reason for decreased OPG protein secretion followed by a reactive induction of OPG mRNA expression. Thus, celecoxib, at least at suprapharmacological concentrations, reduced osteogenic potential. These effects could also explain the inhibition of heterotopic ossification after total hip arthroplasty mediated by COX-inhibitors (6-8). The lack of energy and cell death caused by celecoxib in osteoblastic cells could, at least partially, contribute to the reduced radiographic progression (evaluated by syndesmophyte formation) observed in patients with AS on continuous COX-inhibitor therapy (5). Nevertheless, great caution should be taken in interpreting the clinical significance of cell culture data, particularly with respect to the drug concentrations used.

Regarding the concentrations of celecoxib (10 and 50μM), it may be argued that the observed *in vitro* effects may not be of clinical relevance. Plasma concentrations in normal individuals who are taking therapeutic doses of celecoxib range between 2μM and 8μM. These concentrations may be notably higher in patients with impaired metabolic function or deficiency of CYP2C9, the major enzyme for celecoxib metabolism (38). In our study, cells were exposed to celecoxib for only short periods; patients with RA or AS are treated long-term, which may lead to higher plasma concentrations. Moreover, proteins that bind to celecoxib and neutralize its activity have been identified in the serum (39). The relevant concentration of celecoxib in the tissue is therefore unclear and it is difficult to correlate the celecoxib concentration used *in vitro* with that which is clinically relevant.

On the one hand, our study showed that celecoxib could lead to impaired fracture healing in multimorbid patients with a limited ability to metabolize celecoxib or on long-term treatment. On the other hand, lower concentrations of celecoxib (2 and 10μM) did not show any negative effects on bone remodeling. It could thus be assumed that the doses of celecoxib used in patients should be harmless with respect to the osteogenic potential. In contrast, in the presence of inflammation with increased osteoblastic bone formation, as in AS (syndesmophytes), the effects of celecoxib on the energy metabolism could contribute to the known effect of preventing syndesmophyte formation. The proapoptotic effect of celecoxib at low concentrations could be much higher in cell types other than osteoblasts, *e.g*. in colorectal adenoma cells, without having any negative effects on bone remodeling at these concentrations, as we have demonstrated.

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