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Effects of the selective COX-2 inhibitors celecoxib and rofecoxib on human vascular cells

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Abstract

Rheumatoid arthritis (RA) is associated with a reduced life expectancy considered to be partly caused by cardiovascular events. A growing concern is that accelerated atherosclerosis is driven by inflammatory mechanisms similar to those responsible for RA. Therefore, selective COX-2 inhibitors, which are widely used for the symptomatic treatment of pain and inflammation in RA, may have an impact on atherosclerotic processes. Their anti-inflammatory properties might provoke anti-atherogenic effects but on the other hand, selective inhibition of anti-thrombotic prostacyclin and COX-2 independent effects might promote the risk of increased prothrombotic activity.

In the current study, the effects of the presently marketed selective COX-2 inhibitors celecoxib and rofecoxib on vascular cells have been investigated. Celecoxib inhibited the proliferation of human umbilical vein endothelial cells (HUVECs) in a concentration-dependent manner. At high concentrations, it induced apoptosis and the modulation of inhibitory cell cycle proteins. In contrast rofecoxib—even at high concentrations—had no effect on cell proliferation, apoptosis or cell cycle distribution indicating that celecoxib and rofecoxib do not affect the same signal transduction pathways in endothelial cells. Both drugs did not affect apoptosis induction or cell cycle proliferation in human vascular smooth muscle cells. The observed effects on endothelial cells appear to be COX-independent since both drugs selectively inhibited COX-2-activity and the applied concentrations lay beyond the IC_{50} for inhibition of prostacyclin production. Regarding endothelial apoptosis as a relevant event in the initiation and progression of atherosclerosis the present data put forward the hypothesis that the presently marketed COX-2 inhibitors have a different impact on atherosclerotic processes.

Keywords: Atherosclerosis; Apoptosis; Endothelial cells; Inflammation; Cyclooxygenase; Vascular smooth muscle cells

1. Introduction

Patients with rheumatoid arthritis (RA) have a reduced life expectancy compared with the general population. Cardiovascular death is considered to be the leading cause of mortality in patients with RA and seems to be responsible for about 50% of deaths in this patient group. Moreover, age-specific cardiovascular mortality in persons with RA occurs about four-fold in excess of what would be expected in persons without RA [1]. There is a growing concern and indeed evidence showing that the premature mortality due to the cardiovascular disease is caused by accelerated atherosclerosis [2,3] promoted by inflammatory mechanisms similar to those responsible for the development of RA. Patients exhibit increased concentrations of inflammatory cytokines and cell adhesion factors as well as high concentrations of lipids and these may influence the initiation of atherosclerosis and the formation of atherosclerotic plaques [4–6].

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used for the treatment of pain and inflammation in rheumatoid arthritis. The analgesic and anti-inflammatory effects and also partly the chemopreventive effects of these agents are attributable to the inhibition of cyclooxygenase (COX) enzymes, which convert arachidonic acid to prostaglandins. Two isoforms of cyclooxygenases are

Abbreviations: NSAID, non-steroidal anti-inflammatory drugs; COX, cyclooxygenase; HUVEC, human umbilical vein endothelial cells; VSMC, vascular smooth muscle cells

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known, one of which, COX-1, is expressed constitutively in most tissues and is mainly responsible for the basal physiological production of prostaglandins and the other, COX-2, is rapidly induced at sites of inflammation [7]. The non-selective NSAID aspirin has been reported to inhibit vascular smooth muscle cell proliferation (a marker of atherosclerosis) by arresting the cell cycle in the G_1-phase at high doses [8] and to attenuate atherosclerosis in Apo $E^{-/-}$ mice [9]. On the other hand, the clinical use of classical NSAIDs is often limited by side-effects such as gastrointestinal ulcers thought to result from COX-1-inhibition. Therefore, COX-2-selective inhibitors which exhibit a reduced rate of gastrointestinal side-effects have been developed and are now preferentially used for the long-term treatment of RA. Celecoxib and rofecoxib are COX-2-selective drugs approved by the FDA for the treatment of arthritis (osteoarthritis and rheumatoid arthritis for celecoxib, osteoarthritis for rofecoxib) and acute pain. Interestingly, the VIGOR [10] study suggested an increased risk of cardiovascular events [11] for rofecoxib but this finding has prompted considerable controversy. The selective inhibition of COX-2 diminishes the production of vasodilatory prostacyclin, whereas the COX-1-generated production of thromboxane remains unaffected. The resulting imbalance between pro- and anti-thrombotic factors might constitute a cardiovascular risk [2,12]. Recent studies have shown that COX-2 is expressed by endothelial cells, smooth muscle cells and macrophages in human atherosclerotic lesions [13,14]. It thus might be expected that COX-2-selective inhibitors would have beneficial effects on these lesions.

There is currently no evidence indicating that long-term treatment with COX-2-selective NSAIDs causes pro- or anti-atherogenic effects in RA patients. Since endothelial cell injury and turnover as well as proliferation of vascular smooth muscle cells are key factors in the pathogenesis of atherosclerosis [15,16], in the present study, we have investigated the effects of the selective COX-2 inhibitors, celecoxib and rofecoxib, on human vascular cells. Their effects on such cells may reflect a possible role in the initiation and progression of atherosclerosis.

2. Materials and methods

2.1. Cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained from Cell Systems/Clonetics and cultured in endothelial growth medium (EGM) supplemented with 1% penicillin/streptomycin, 2% fetal calf serum, hydrocortisone, hEGF, hFGF, VEGF, R3-IGF-1, ascorbic acid, GA-1000 and heparin (Cell Systems/Clonetics). For cell culture experiments passages 2–4 have been used. Human vascular smooth muscle cells (hVSMC) were purchased from ATCC and cultured in Ham's F12 medium (F12K) with the supplements recommended by ATCC.

2.2. Drugs

Celecoxib and rofecoxib were synthesized by WISTA. Their identity and purity was checked by MS- and ^{1H}NMR and was >99.5%. Both drugs were dissolved in DMSO. The DMSO concentration in the experiments was adjusted to 0.1% which has been previously assured not to damage the cells or to disturb the experimental setting.

Since the highest concentration of celecoxib, 100 μM, killed more than 90% of the cells in the proliferation assay, all further experiments with HUVEC were performed with concentrations up to 50 μM only, while rofecoxib has been used up to 100 μM.

Fura-2AM was purchased from Molecular Probes.

Caspase-inhibitors were purchased from Calbiochem. To cover a broad spectrum of caspases, we used a caspase Inhibitor III (Boc-D-FMK) which inhibits all caspases, a caspase 3-inhibitor (Z-DEVD-FMK) which inhibits caspases 3, 6, 7, 8 and 10 and a caspase 9-inhibitor (Z-LEHD-FMK) specific for caspases 4, 5 and 9.

2.3. Determination of celecoxib and rofecoxib concentrations

Samples for the determination of free (protein unbound) celecoxib or rofecoxib concentrations in cell culture medium were prepared according to Paulson et al. [17]. Briefly, HUVEC-medium containing 2% FCS was mixed with either 10 or 50 μM celecoxib or 50 μM or 100 μM rofecoxib, respectively and then centrifuged at 150,000 × g at 37 °C for 15 h in a Sorvall Discovery 90 SE ultracentrifuge (Kendro Laboratory Products) to separate protein-bound from -unbound drug. The supernatant was diluted 1:100 in acetonitrile/H_2O/ammonia solution 25% (50:50:0.1).

Drug concentrations were measured with liquid chromatography tandem mass spectrometry (LC/MS–MS) as described previously [18].

2.4. Cell proliferation assay

For the sulforhodamin B assay, cells were seeded in 24-well plates (3000 per well) and cultured overnight prior to incubation. The cells were then incubated 24 h with various concentrations of celecoxib and rofecoxib (each concentration in quadruplicate). At the end of the incubation period, the cells were fixed with 5% trichloroacetic acid (TCA) for 1 h at 4 °C. The supernatant was discarded; the plates were washed three times with H_2O and then dried overnight. Staining of cell proteins was performed for 30 min at RT with sulforhodamine B (SRB) at a concentration of 0.1% in 1% acetic acid. The plates were washed three times with 1% acetic acid and then dried again overnight. SRB was dissolved in 10 mM Tris, pH 10.5,

and the stained supernatant measured photometrically at 570 nm.

2.5. Detection of thromboxane and prostacyclin concentrations

Thromboxane B_2 (the stable breakdown product of thromboxane A_2) and 6-keto-$PGF_{1\alpha}$—(the hydrolysis product of prostacyclin) concentrations in the supernatants of the cell culture were determined using commercially available ELISA kits (Biotrend Chemicals) according to the manufacturer's protocol. Cells incubated with IL-1β [1 nM] for 24 h served as positive control.

2.6. Detection of cell cycle distribution and apoptosis using flow cytometry

Synchronization of the cell cycle was achieved by starving HUVEC (5×10^5) for 24 h and VSMC for 48 h in FCS-free medium at 37 °C with 5% CO_2. Cells were then treated for 24 h in medium containing 2% FCS with various concentrations of celecoxib (1, 10, 50 μM) or rofecoxib (10, 50, 100 μM). VSMCs were simultaneously stimulated with PDGF (4 ng/ml). Cells were harvested by trypsinization, washed twice with PBS, fixed with ethanol, incubated for 5 min with 0.125% Triton X-100, and stained with propidium iodide (20 μg/ml) in PBS containing 0.2 mg/ml RNAse A. Stained cells were analyzed by flow cytometry (Beckton Dickinson). For each sample, cells were analyzed until 5000 cells had been counted in a predefined G1-gate. The cell cycle distribution (percentage of cells in the G0/G1, S, and G2/M phase) was assessed using WinMDI 2.8 software (http://facs.scripps.edu/software.html). The percentage of apoptotic cells was obtained by calculating the percentage of cells in the sub-G1 phase.

2.7. Ratiometric digital imaging of $[Ca^{2+}]_i$

HUVEC were seeded on round (25 mm) glass coverslips at a density of 1000 cells/coverslip and cultured overnight in complete EGM-medium. Before each Ca^{2+}-measurement, the cells were loaded with 1 μM Fura-2AM in cell culture medium for 1 h at 37 °C. Coverslips were then placed in a chamber and washed with physiological buffer (140 mM NaCl, 5 mM KCl, 0.4 mM $MgSO_4$, 0.5 mM $MgCl_2$, 4.3 mM HPO_4, 0.4 mM KH_2PO_4, 1 mM $CaCl_2$, 5.5 mM Glucose (for Ca^{2+}-free buffer add 2 mM EGTA instead of $CaCl_2$)) for 15 min. Experiments were monitored with an upright microscop (Zeiss) equipped with a 40× water-immersion objective CCD camera (Imago, Till Photonics). Capture and analysis was performed with TillVision software (Till Photonics). The fluorescence of the Fura-2AM was excited alternatively at wavelengths of 340 nm (F_{340}) and 380 nm (F_{380}). Optical images of the emitted light were aquired every 2 s at 510 nm. Fluorescence was monitored 2 min prior to celecoxib treatment to get a basal emission level of intracellular calcium. Celecoxib perfusion was then started and the cells were observed for 2 min. For analysis, the ratio between the average fluorescence at 340 and 380 nm after background fluorescence substraction has been calculated.

2.8. Western blot analysis

Cells were seeded in 10 cm diameter dishes at a density 1×10^6. After 24 h cells were starved for further 24 h in medium without FCS and then incubated for 24 h with various concentrations of celecoxib and rofecoxib as mentioned above. Preparation of protein extracts and Western blot analysis were performed as described previously [19].

2.9. Statistics

Data are pesented as mean ± S.D. Statistical evaluation was done with SPSS 9.02 for Windows. Univariate analysis of variance, with subsequent *t*-tests employing a Bonferroni α-correction for multiple comparisons and α set at 0.05, was used for statistical comparisons. IC_{50} values were calculated by linear regression.

3. Results

3.1. Determination of drug protein binding

A LC/MS–MS-method was used to determine celecoxib and rofecoxib protein binding in serum-containing medium. The results showed that an average of 40% of the applied

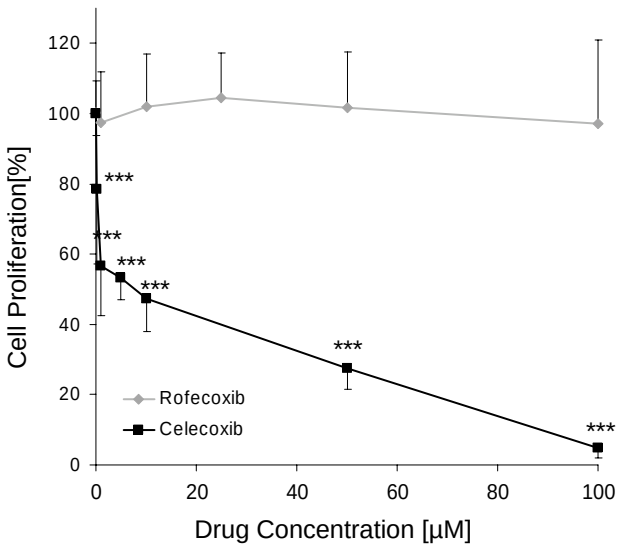

Fig. 1. Sulforhodamin B cell proliferation assay. HUVEC were incubated for 24 h with or without celecoxib or rofecoxib. Subsequently, cells were fixed with TCA and the cellular proteins stained with sulforhodamin B and the absorption was measured at 570 nm. The figure shows the relative proliferation rate as compared to the untreated control which has been set as 100%. Data present mean ± S.D. from at least three independent experiments (*** statistically significant mean difference, $P < 0.001$).

celecoxib remained unbound while 60% is bound to the proteins in the medium. In contrast, rofecoxib showed only about 10% protein binding indicating that 90% of the drug remains unbound in the medium.

3.2. Effects of celecoxib and rofecoxib on cell proliferation

Sulforhodamin B proliferation experiments were performed to determine the proliferation rate of HUVEC with and without the addition of celecoxib and rofecoxib. As shown in Fig. 1, 24 h incubation with celecoxib caused a concentration-dependent reduction of cell proliferation as compared to untreated control cells with a calculated IC_{50} value of 10.6 ± 6.3 μM. There were significant differences between celecoxib-treated and untreated control cells at all celecoxib concentrations used. In contrast, rofecoxib applied for 24 h had no marked effects even up to a concentration of 100 μM.

Since the highest concentration of celecoxib, 100 μM, killed more than 90% of the cells in culture, all further experiments with HUVEC were performed with concentrations up to 50 μM only.

In VSMC, rofecoxib again showed no effect on cell proliferation. Celecoxib only slightly inhibited VSMC proliferation even at a concentration of 100 μM after incubation for 48 h (data not shown).

3.3. Effects of celecoxib and rofecoxib on thromboxane and prostacyclin production in HUVEC

To investigate the COX-dependent and -independent actions as well as the COX-1/COX-2 specificity of micromolar concentrations of celecoxib and rofecoxib, respectively we measured the release of 6-keto-$PGF_{1\alpha}$ (COX-2 product) and thromboxane B_2 (COX-1 product) in HUVEC. In non-stimulated cells, only COX-1 is expressed constitutively whereas COX-2 is not detectable. COX-2 expression can be induced by treating the cells with IL-1β (Fig. 2A). Regarding the thromboxane B_2 measurement, there were no significant differences between the individual samples. As shown in Fig. 2C, thromboxane B_2 release

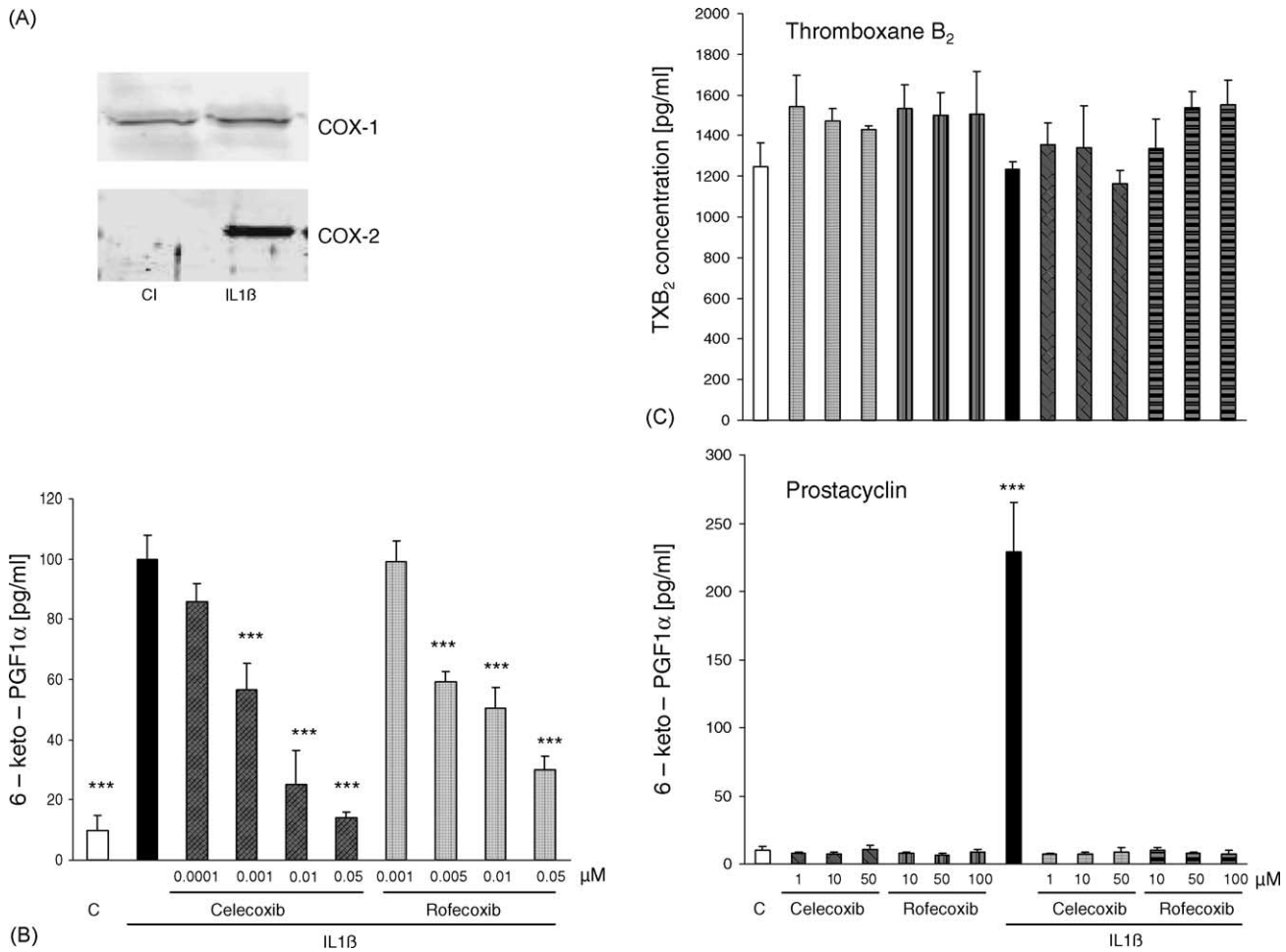

Fig. 2. (A) Western blot analysis of COX-1 and COX-2 protein expression in HUVEC. (B) Release of 6-keto-$PGF_{1\alpha}$ in the supernatant of HUVEC for determination of the IC_{50} values for celecoxib and rofecoxib, respectively (low concentrations have been used) after stimulation of the prostacyclin release with IL 1β. (C) Release of thromboxane B_2 and 6-keto-$PGF_{1\alpha}$ in the supernatant of HUVEC. Cells were incubated for 24 h either with IL1β, celecoxib or rofecoxib or with a combination of IL1β together with celecoxib or rofecoxib, respectively (*** statistically significant mean difference, $P < 0.001$).

was unaffected by IL-1β and treatment with celecoxib or rofecoxib, indicating that the COX-1 activity is not influenced by the COX-2 selective inhibitors even when applied at rather high concentrations. With respect to prostacyclin production only the IL-1β treated control showed a significant increase in 6-keto-$PGF_{1\alpha}$ production. When compared to untreated control cells, cells treated with a combination of IL-1β and celecoxib or rofecoxib showed no increase in 6-keto-$PGF_{1\alpha}$ release, indicating complete inhibition of COX-2 even at the lowest concentrations tested (Fig. 2C). IC_{50} values for the inhibition of IL-1β stimulated 6-keto-$PGF_{1\alpha}$ release were calculated as 3.3 ± 1.5 and 5.5 ± 2.9 nM for celecoxib and rofecoxib, respectively (Fig. 2B).

3.4. Effects on cell cycle progression

Cell cycle distribution was investigated by determining the phase-specific DNA content using flow cytometry (Fig. 3). Treatment of HUVEC with celecoxib caused a concentration-dependent accumulation of cells in the sub-G_1 phase indicating induction of apoptosis. Incubation with 50 μM celecoxib for 24 h caused a significant increase of cells in the subG_1-phase whereas the number of cells in the G_1-phase was significantly decreased (Fig. 4A). This effect was even more pronounced at celecoxib concentrations of 100 μM although at this concentration the cell number was considerably reduced (data not shown).

In contrast, rofecoxib showed no effect on cell cycle progression or induction of apoptosis even at concentrations up to 100 μM (Fig. 4B).

Incubation of VSMC with celecoxib or rofecoxib, respectively, also did not result in a cell cycle shift or apoptosis induction as measured by FACS analysis (Fig. 4C and D) and Western blot with poly-ADP ribose polymerase (PARP) protein (data not shown).

3.5. Induction of apoptosis by celecoxib in HUVEC

Referring to FACS analysis the pathway of celecoxib-induced apoptosis in HUVEC was assessed. At first we investigated the cleavage of poly-ADP ribose polymerase

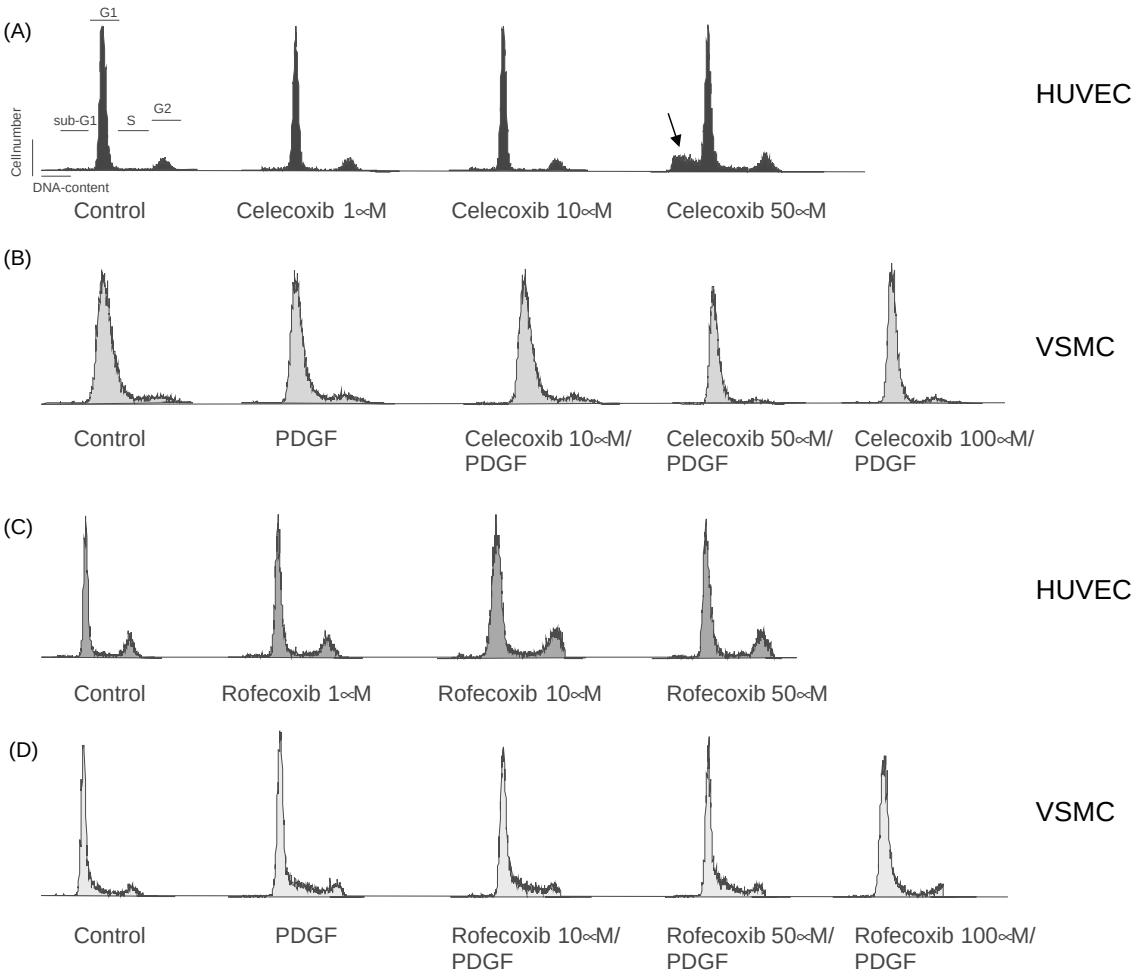

Fig. 3. FACS analysis. HUVEC and VSMCs were starved for 24 and 48 h, respectively for synchronisation and then incubated with celecoxib or rofecoxib for further 24 h. VSMC were simultaneously stimulated with PDGF (4 ng/ml). Cells were then harvested and stained with propidiumiodide for FACS analysis. Five thousand cells were counted in the G_1-phase. (A) HUVEC incubated with celecoxib; (B) VSMC incubated with PDGF and celecoxib; (C) HUVEC incubated with rofecoxib; (D) VSMC incubated with PDGF and rofecoxib.

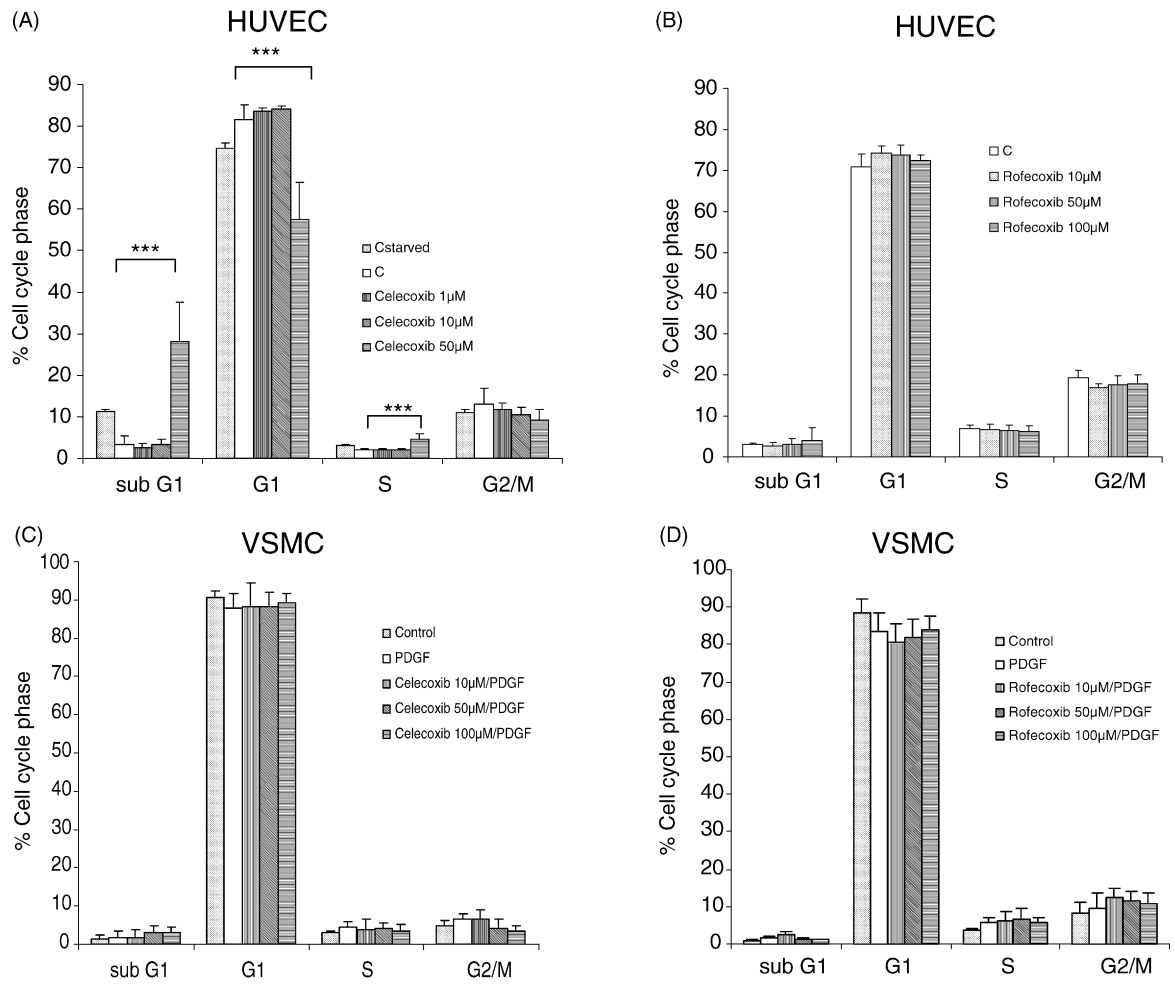

Fig. 4. Cell cycle analysis. FACS data have been analysed and the cell cycle distribution of celecoxib and rofecoxib treated HUVEC (A and B) or VSMC (C and D) calculated and compared to untreated control cells. Data indicate mean ± S.D. of three independent experiments (*** statistically significant mean difference, $P < 0.001$).

by Western blot analysis. PARP is a 112 kDa protein which is cleaved specifically into 85 and 29 kDa fragment by the activation of caspases, an early event in the induction of apoptosis. The PARP antibody used recognizes only the 112 kDa protein and the large fragment of 85 kDa. The cleavage of PARP induced after 24 h incubation with 50 μM celecoxib is clearly shown in Fig. 5A.

Since Ca^{2+} plays a central role in apoptosis induction and in the activation of several caspases, we further investigated the effects of celecoxib on Ca^{2+} homeostasis in HUVEC. Celecoxib induced an increase of the intracellular Ca^{2+}-concentration which could be observed with Ca^{2+}-containing as well as Ca^{2+}-free buffer indicating the release of calcium from intracellular stores (Fig. 5B).

FACS analysis of HUVEC showed an inhibition of apoptosis induction by 50 μM celecoxib if a broad spectra caspase inhibitor (Boc-D-FMK, 25 μM) or a caspase 3-inhibitor (Z-DEVD-FMK, 25 μM) had been added to the cell culture plate for 24 h. Addition of a caspase 9 inhibitor (Z-LEHD-FMK, 25 μM) had no effect on celecoxib-induced apoptosis (Fig. 5C).

3.6. Expression of cell cycle-related proteins

The effects of celecoxib and rofecoxib on cell cycle-related proteins in HUVEC have been investigated using Western blot analysis (Fig. 6).

The inhibitory cell cycle proteins p21 and p53 were upregulated in the presence of 10 μM celecoxib but downregulated at 50 μM. p27 was upregulated at both, 10 and 50 μM celecoxib. Rofecoxib-treated cells showed no detectable change in the expression of p53 and a slight upregulation of the inhibitory proteins p21 and p27 after 50 and 100 μM rofecoxib as compared to untreated control cells.

4. Discussion

The effect of long-term treatment of RA patients with NSAIDs on the development and progression of atherosclerosis is not well established. Since it is becoming increasingly accepted that atherosclerosis is an inflammatory disease, there may be benefits in the administration of

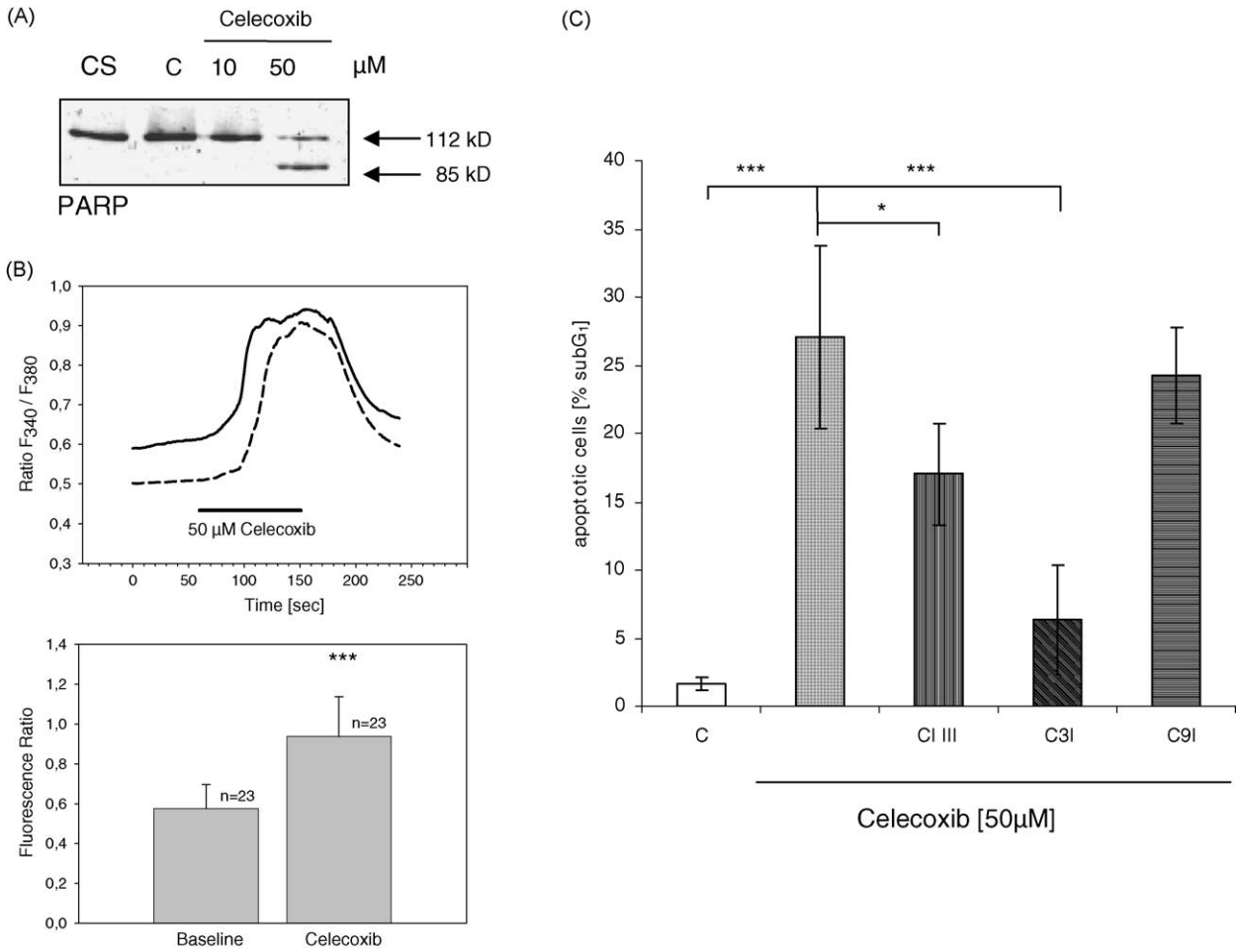

Fig. 5. (A) PARP cleavage as indication for apoptosis induction in HUVEC after incubation with celecoxib which was indicated by the sub-G_1 peak of the cell cycle analysis. (B) $[Ca^{2+}]_i$ increase in HUVEC after addition of celecoxib [50 µM]; upper panel: two representative cells which showed an increase in $[Ca^{2+}]_i$ when celecoxib was added. $[Ca^{2+}]_i$ returned to baseline when celecoxib was washed out; lower panel: statistical analysis showing the fluorescence ratio of cells (n = number of cells measured) incubated with celecoxib as compared to the baseline fluorescence ratio ($P < 0.001$). Results from three independent incubations. (C) Apoptotic cells (sub-G1) observed by FACS analysis of HUVEC after 24 h incubation with or without celecoxib [50 µM] and specific caspase inhibitors (CI III = Boc-D-FMK; C3I = Z-DEVD-FMK; C9I = Z-LEHD-FMK) (25 µM each). Data indicate mean ± S.D. of three independent experiments (*** statistically significant mean difference, $P < 0.001$; * statistically significant mean difference $P < 0.05$).

anti-inflammatory drugs. Several reports indicate an improvement in atherosclerotic disease after treatment of atherosclerotic mice with indomethacin, aspirin or sulindac which inhibited neointimal formation after arterial injury [9,20,21]. There is also evidence that COX-2-selective inhibitors, which are increasingly used for the long-term treatment of RA patients because of their lower gastrointestinal toxicity, reduce the formation of atherosclerotic lesions. Rofecoxib has already been shown to significantly reduce atherosclerosis in LDL-$R^{-/-}$ mice and those mice which also carried the COX-$2^{-/-}$ phenotype developed significantly less atherosclerosis than COX-$2^{+/+}$ mice [22]. Recently, celecoxib has been shown to improve endothelium-dependent vasodilation and to lower C-reactive protein as well as oxidized LDL concentrations in a clinical setting [23]. Hence, these advantageous effects of COX-2-selective drugs may be attributable to inhibition of the COX-2 enzyme which is known to be upregulated in atherosclerosis and thereby by reducing inflammatory responses at the atherosclerotic plaque. On the other hand there are data—although highly controversial—indicating that COX-2 selective inhibitors are associated with an increased risk of cardiovascular events which may be partially linked to an imbalance in pro- and anti-thrombotic factors such as prostacyclin and thromboxane (for review see [11,24]).

Development of atherosclerosis is a complex process involving various cell types such as vascular smooth muscle cells, macrophages and endothelial cells. Injury of the vascular endothelium is a critical event in the initiation of atherosclerosis [25]. Moreover, in addition to the association of endothelial cell apoptosis with the formation of atherosclerotic lesions, it may also be involved in disease progression involving plaque erosion and thrombus formation leading to vessel occlusion and myocardial infarction.

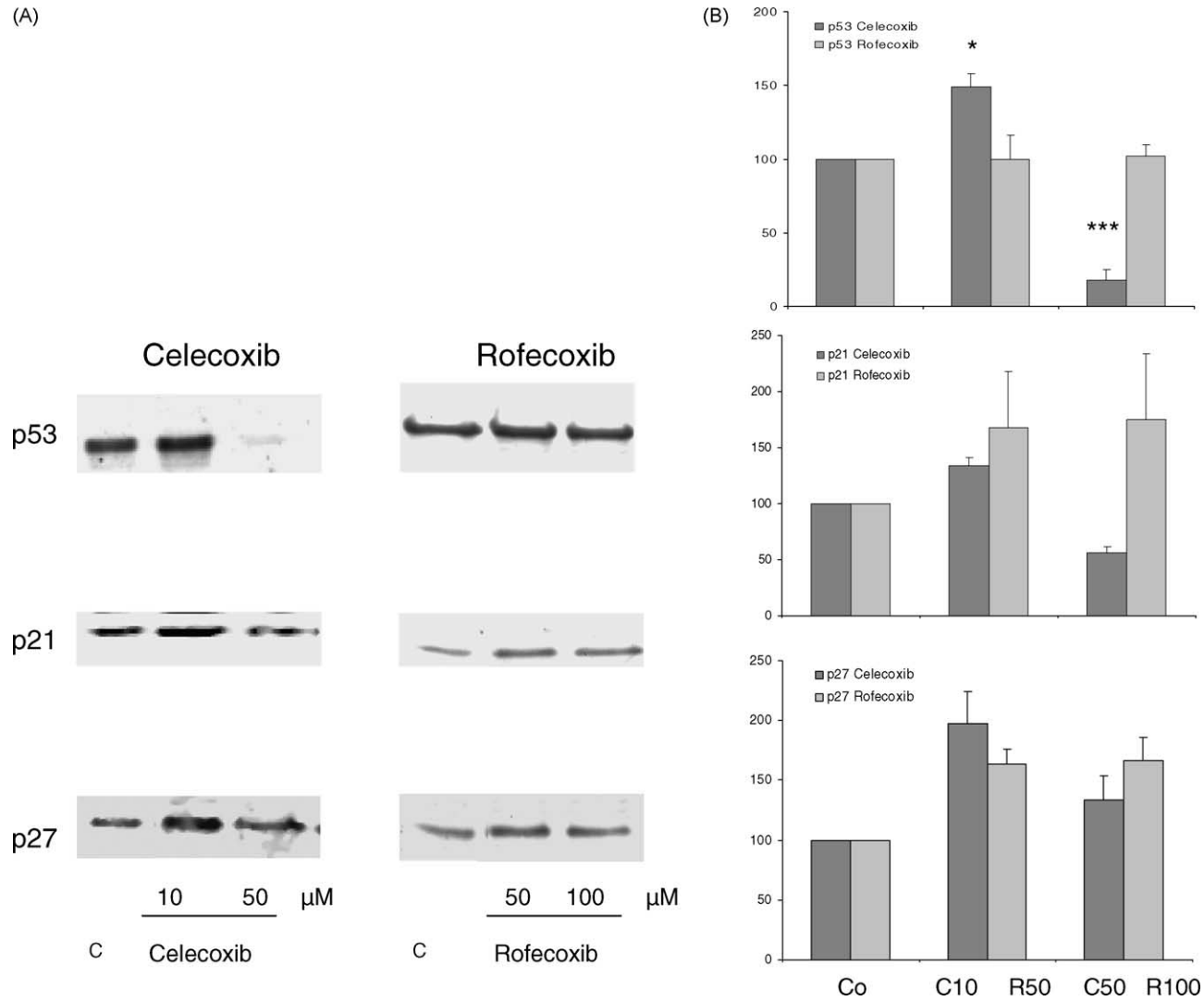

Fig. 6. (A) Western blot analysis of cell cycle-related proteins in HUVEC after 24 h treatment with celecoxib or rofecoxib, respectively. Representative result of three independent experiments. (B) Densitometrical analysis of the Western blots. Data indicate mean ± S.D. of three independent experiments (*** statistically significant mean difference, $P < 0.001$; * statistically significant mean difference $P < 0.05$).

In the current study, we investigated the effects of the presently marketed COX-2 selective inhibitors celecoxib and rofecoxib on human vascular endothelial cells and found that celecoxib: (i) inhibited cell proliferation; (ii) induced apoptosis in the G_1-phase of the cell cycle; and (iii) at sub-apoptotic concentrations, increased the expression of inhibitory proteins during the G_1-phase of the cell-cycle. In contrast, rofecoxib showed no effect on cell proliferation and cell-cycle progression even at higher concentrations. The inhibition of cell proliferation at sub-apoptotic concentrations of celecoxib might be due to upregulation of cell cycle inhibiting proteins. However, the FACS analysis showed no G_1-block of the cell cycle at these concentrations indicating only a weak effect. At higher concentrations, the inhibition of proliferation and the downregulation of cell cycle-related proteins p21 and p53 can be ascribed to the massive induction of apoptosis. The mechanism of apoptosis induction involves a rapid increase in intracellular Ca^{2+}-concentrations and the activation of caspase 3. Similar results with celecoxib have already been shown in rheumatoid synovial fibroblasts [26] and colon adenocarcinoma cells [27].

COX-2 is not or only marginally expressed in non-stimulated HUVEC. After incubation with IL-1β however, COX-2 was upregulated, and the increased prostacyclin production associated with this was completely inhibited by both celecoxib and rofecoxib at all concentrations tested. Thus, it can be assumed that the observed effects of celecoxib and rofecoxib on vascular cells are independent from COX-2-inhibition. Since thromboxane B_2 levels are unchanged, it can also be assumed that celecoxib and rofecoxib at the concentrations used do not affect COX-1 activity. COX-independent mechanisms of COX-inhibitors have already been postulated in several studies involving inhibition of proliferation, induction of apoptosis and effects on transcription factors and cellular kinases

(for review see [28]). Regarding the relatively high concentrations of celecoxib used, it may be argued that the observed in vitro effects may not be of clinical relevance because these concentrations are not reached in vivo. This may hold true when arthritis patients are treated with the recommended dose of 1×200 to 2×200 mg celecoxib per day. Recently, however it has been shown that celecoxib reduced polyp growth in patients with familial adenomatous polyposis (FAP) at doses of 800 mg per day while lower doses had no significant effect in this particular study, suggesting that higher celecoxib concentrations are required for apoptotic and thereby protective effects [29]. Maximal celecoxib concentrations reached in these patients are ~8 μM [30] which is in the range of the IC_{50} value in our proliferation and inhibitory cell cycle assays. Thus, it seems possible that there are no remarkable COX-2-independent effects on vascular cells in RA patients after intake of the recommended anti-inflammatory doses of 100–200 mg b.i.d. but in cases where higher doses of celecoxib are used e.g. in patients who suffer from FAP there might be an impact of celecoxib on vascular endothelium function. Moreover, higher celecoxib concentrations are seen in patients with impaired function or deficiency in *CYP*2C9, the major enzyme for celecoxib metabolism. Interestingly, there are observations suggesting that especially patients with cardiovascular diseases show an enhanced incidence of alterations in CYP expression and activity [31]. Lastly, RA patients are chronically treated with the approved doses of celecoxib resulting in moderate plasma concentrations and long-term exposure of vascular cells, and the biological effects of this are not defined at this time. By contrast, in our experiments HUVECs are exposed with higher concentrations but only for hours. Thus, the ''concentration over time'' product may be comparable in both situations. Nevertheless, cell culture experiments can only reflect an acute model indicating that the in vivo effects remain to be evaluated in both animal and clinical studies.

Previous experiments in our laboratory on the COX-independent effects of celecoxib and rofecoxib using macrophages also showed that the two drugs used have opposite effects. Whereas celecoxib activated the transcription factor NF-κB at high concentrations, and thus lost its anti-inflammatory activity in vivo at high doses, rofecoxib inhibited NF-κB and activated AP-1 resulting in an enhanced anti-inflammatory activity [19,32]. The activation of pro-inflammatory transcription factors in macrophages might also be a risk factor in the development of atherosclerosis since release of inflammatory markers excacerbates the progression of the disease.

Another key event in atherosclerosis progression is the proliferation of vascular smooth muscle cells [16]. Investigations with vascular smooth muscle cells showed no significant effects of both drugs, celecoxib and rofecoxib, on cell cycle progression indicating that the proliferation of these cells is not affected by these COX-2 inhibitors and that HUVECs and VSMCs are differentially sensitive to these agents.

Taken together, our results show that the COX-2-selective drugs celecoxib and rofecoxib have different effects on human umbilical vein endothelial cells while vascular smooth muscle cells are not affected by both drugs. In our study, high celecoxib concentrations impaired endothelial cell homeostasis whereas rofecoxib did not influence proliferation and the induction of apoptosis in HUVEC. Nevertheless, the question if apoptosis of endothelial cells is indeed a proatherogenic event or if it possibly even protects the endothelium from serious damage is still open.

Therefore, animal experiments, clinical trials as well as long-term clinical outcome studies with both selective COX-2 inhibitors are mandatory to ultimately determine whether this differential effect occurs in clinical use.

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