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Opposite effects of rofecoxib on NF-κB and AP-1 activation[1]

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Running title: AP-1 activation and NF-κB inhibition through rofecoxib

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Abbreviations: AP, activating protein; NF, nuclear factor; COX, cyclooxygenase, NOS, nitric oxide synthase; TNF, tumor necrosis factor; LPS, lipopolysaccharide

Abstract

Rofecoxib is a selective cyclooxygenase-2 (COX-2) inhibitor approved for the treatment of pain and inflammation in rheumatoid- and osteoarthritis. Daily doses between 12.5 and 50 mg were found to reduce pain and inflammation, however without a clear dose effect relationship. Interestingly, rofecoxib treatment is associated with an unexpected incidence of renal adverse events as compared to other COX-inhibitors. Here, the effects of rofecoxib on the transcription factors NF-κB and AP-1 were analysed to find out whether transcriptional changes might explain the lack of clear dose dependency and the occurrence of renal side effects. In vitro, rofecoxib dose-dependently inhibited DNA binding capacity of NF-κB at doses of 10-100 μM whereas the binding activity of AP-1 was considerably increased at 100 μM. In vivo, the anti-inflammatory effect of rofecoxib was equal at 1 and 10 mg/kg whereas 50 mg/kg caused a significant further reduction of a zymosan induced paw edema. This was associated with a clear decrease of iNOS protein expression in the spinal cord at this dose. At 1 and 10 mg/kg however, iNOS was increased but COX-2 was decreased. Thus, the expression of pro-inflammatory proteins was similarly inconsistent as transcription factor regulation.

In conclusion the opposite effects of rofecoxib on AP-1 and NF-κB may explain the lack of clear dose dependency with rofecoxib in clinical studies or animal experiments. The effects on AP-1 may possibly affect renal sodium transport since certain renal sodium channels are regulated through AP-1. Transcription factor regulation might therefore influence both wanted and unwanted effects of rofecoxib.

Rofecoxib is one of the first selective cyclooxygenase-2 (COX-2) inhibitors approved for the treatment of pain and inflammation in osteoarthritis. In this context it has been described to provide efficacy advantages over acetaminophen and the selective COX-2 inhibitor celecoxib (Geba et al., 2002). It has also been found to be effective in rheumatoid arthritis and as an analgesic in the treatment of acute inflammatory pain following third molar extraction (Morrison et al., 2000) or orthopedic surgery (Reuben and Connelly, 2000). Since rofecoxib does not inhibit COX-1 activity at doses up to 500 mg/d (10 times the highest recommended dose) (Matheson and Figgitt, 2001) and thus does not affect physiological prostaglandin synthesis in the gastrointestinal tract it causes considerably less GI toxicity than non-selective COX-inhibitors such as naproxen or diclofenac (Bombardier et al., 2000; Hawkey et al., 2000; Gretzer et al., 2001). However, it was noted that rofecoxib causes a relatively strong sodium and water retention (Kammerl et al., 2001; Whelton et al., 2001) and reduction of the glomerular filtration rate (GFR) (Swan et al., 2000) as compared to other selective and non-selective non-steroidal anti-inflammatory drugs (celecoxib and diclofenac, ibuprofen, indomethacin, respectively) (Zhao et al., 2001). In a recent study based on spontaneous reports of adverse drug reactions in the World Health Organization/Uppsala Monitoring Centre safety database rofecoxib treated patients experienced a significant higher incidence of peripheral (low-extremity) edema than patients treated with celecoxib or unselective NSAIDs (Swan et al., 2000; Zhao et al., 2001). Another group found no difference in the incidence of peripheral edema when half-life associated dosing patterns of rofecoxib (25 mg once daily), celecoxib (200 mg twice daily) and naproxen (500 mg twice daily) (Schwartz et al., 2001) have been compared. In addition, the VIGOR study has revealed that rofecoxib treatment is associated with an increase of the systolic and diastolic blood pressure which is more pronounced than that observed with the non-selective COX-inhibitor, naproxen (Mukherjee et al., 2001). Furthermore, in

some studies there were some hints that rofecoxib may increase the risk of cardiovascular diseases including myocardial infarction as compared to naproxen treatment (Bombardier et al., 2000; Rainsford, 2001). Thus, it is discussed that rofecoxib causes an imbalance of prothrombotic and antithrombotic arachidonic acid metabolites which does not occur with unselective COX inhibitors because non-selective NSAIDs simultaneously inhibit prothrombotic thromboxane synthesis in platelets (through COX-1) and antithrombotic prostacyclin synthesis in endothelial cells (through COX-2) whereas COX-2 selective drugs inhibit only the latter (for review see (Hinz and Brune, 2002)). Hence the COX-2 selectivity of rofecoxib might at least partly contribute to the increase of the cardiovascular risk. Another feature with rofecoxib is the lack of a clear dose effect relationship concerning its clinical analgesic and also its anti-inflammatory efficacy (Day et al., 2000; Truitt et al., 2001). Thus, there is no reliable information about which dose will probably work for an individual patient. In some studies there were no differences in efficacy between doses of 12.5 mg, 50 mg and 125 mg rofecoxib (Ehrich et al., 1999; Schnitzer et al., 1999).

We have recently observed that the COX-2 mediated anti-inflammatory activity of the other clinically available COX-2 inhibitor, celecoxib is negatively affected at high doses because at high concentrations celecoxib activates the transcription factor NF-κB thereby causing an increase of NF-κB regulated pro-inflammatory genes such as COX-2 and TNFα (Niederberger et al., 2001). The clinical data of rofecoxib together with the previous results obtained with celecoxib prompted us to hypothesize that the lack of a dose dependency with rofecoxib and its renal unwanted effects might be mediated through alterations of certain transcription factors. We therefore evaluated effects of rofecoxib on the transcription factors NF-κB and AP-1 and some of their target genes and assessed the anti-inflammatory effects of rofecoxib at low, medium and very high

doses of the drug to find a possible explanation for the special clinical features of rofecoxib outlined above.

Methods

Materials

Antibodies for rat COX-2, NF-κB and I-κB were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). The antibody and the cDNA probe for rat iNOS and the cDNA probe for COX-2 were a kind gift from Prof. J. Pfeilschifter (University of Frankfurt, Germany) (Xie and Nathan, 1993) and Prof. D. DeWitt (Michigan, U.S.A.) (DeWitt and Meade, 1993), respectively. Rofecoxib was a gift from Prof. W.J. Wechter (Loma Linda University, U.S.A.). Etoricoxib, used as an analytical internal standard, has been synthesized by WITEGA (Berlin). Identity and purity were checked by MS and ^{1H}NMR and was greater than 98.5%. For the animal experiments the commercially available suspension of rofecoxib (Vioxx®)(5mg/ml) was used.

Zymosan evoked inflammation

Male Sprague Dawley rats (Charles River, Sulzfeld, Germany) weighing 250-300 g were used. They were housed in groups of five in standard cages and maintained in climate- and light-controlled rooms (22 ± 0.5 °C, 12/12 h dark/light cycle). Unilateral hind paw inflammation was induced by subcutaneous injection of 1.25 mg zymosan (Sigma-Aldrich, Steinheim, Germany) suspended in 100 µl phosphate buffer into the midplantar region of the right hind paw (Meller and Gebhart, 1997). The paw volume was measured before zymosan injection (time 0) and at 0.25, 0.5, 1, 2, 4, 6, 8, 24, 30 and 96h using a plethysmometer (Ugo Basile, Varese, Italy) according to the manufacturer's instructions. At each time point four measurements of the paw volume were taken and the median was used to calculate the percentage increase of the paw volume as compared to the value before zymosan injection (ΔPW). At completion of the

experiments rats were deeply anesthetized and killed by cardiac puncture. The spinal cord was rapidly excised and tissue samples from lumbar spinal cord were snap frozen in liquid nitrogen and kept at –80°C until further analysis. In all experiments the ethics guidelines for investigations in conscious animals were obeyed and the procedures were approved by the local Ethics Committee for animal research.

Drug treatment and data analysis

24h before drug administration, animals were deprived of food with free access to tap water. Rofecoxib suspension (Vioxx®)(2,5 mg/ml or 5mg/ml) was administered by gastric gavage at doses of 1, 10 and 50mg/kg. 50 mg/kg was the highest dose that could be administered (2.5 – 3ml of the suspension) to the animals without causing regurgitation or serious diarrhea. Controls received the appropriate volume of tylose suspension. Six to 10 rats were used in each group. The drugs were administered 15 minutes prior to the intraplantar injection of zymosan.

To compare drug effects the area under the "paw volume increase" versus "time" curve ($AUC_{\Delta PW}$) was calculated employing the linear trapezoidal rule. Statistical evaluation was done by SPSS 9.02 for Windows. AUCs from 0-24h were submitted to univariate analysis of variance (ANOVA) with subsequent t-tests employing a Bonferroni α-correction for multiple comparisons. α was set at 0.05.

Rofecoxib plasma concentrations

For determination of the plasma concentrations blood was taken at time points 1, 2, 3, 5,8 and 25 h after drug treatment. Rat plasma samples were extracted using a liquid-liquid extraction method. An aliquot of 100 µl plasma were mixed with the internal standard, etoricoxib and 500 µl 0.1 M sodium carbonate buffer (pH 9.8) and extracted

for 15 min with 2 ml *t*-butylmethylether. The organic layer was separated and evaporated under a gentle stream of nitrogen. The residue was reconstituted in 100 µl mobile phase.

Extracted plasma samples were analyzed by HPLC with post-column photochemical derivatization and fluorescence detection according to a procedure described earlier (Woolf et al., 1999). The HPLC System consisted of a Gastorr 153 degaser, a Jasco LG-980-02S ternary gradient unit, a Jasco PU-1580 pump a Jasco AS 951 autosampler (Jasco, Groß-Umstadt, Germany), a Beam Boost chemical reaction unit (ict, Bad Homburg, Germany) and a Jasco FP-920 fluorescence detector. The HPLC worked in the isocratic mode. The detector was set at an excitation wavelength of 250 nm and an emission wavelength of 375 nm. The column used was a Nucleosil C18 column (125x4 mm, 5µm particle size, 100 Å pore size). The mobile phase consisted of acetonitrile/0.02M phosphate buffer (35:65 v/v) pH 3.0 and was adjusted to a flow rate of 1.2 ml/min. A 50µl sample was injected onto the column. Rofecoxib and etoricoxib eluted at 7.4 min and 3.9 min, respectively. The analytical data output was processed by Jasco Borwin software (version 1.50).

Cell Culture

RAW 264.7 mouse macrophages (courtesy of Prof. J. Pfeilschifter, University of Frankfurt, Germany) were cultured and incubated in RPMI 1640 medium containing 10% FCS and 1% penicillin/streptomycin. At this serum concentration about 10% of the administred rofecoxib concentration was sequestred by protein binding as determined by measuring free and total rofecoxib concentrations in culture medium with HPLC.

Analysis of PGE_2, nitrite/nitrate and TNFα

RAW-cells were stimulated with 10 µg/ml lipopolysaccharide (LPS) for 24h in the absence or presence of various concentrations of rofecoxib. PGE_2 concentrations in culture supernatants were assessed using a commercially available enzyme immunoassay (Biotrend Chemicals, Köln, Germany) according to the manufacturer´s protocol. The reliable limit of quantification was 36 pg/ml and the mean percentage deviation over the calibration range of 36-5000 pg/ml was less than 15%.

The release of NO was assessed by measuring concentrations of nitrite and nitrate using the Griess method (Green et al., 1982). 200 µl of each supernatant was mixed with 50 µl of 0.4% sulphanilamide in 1 N hydrochloric acid and 50 µl of 0.6% naphtylethylendiamine dihydrochloride in water. The absorbance of the mixture was measured photometrically at 540 nm. The reliable limit of quantification was 1 µM and the mean percentage deviation over the calibration range of 1-50 µM was less than 15%.

TNFα concentrations in cell culture supernatants and spinal cord homogenates were assessed by a commercially available enzyme immunoassay (Amersham Pharmacia Biotech, Freiburg, Germany) according to the manufacturer´s protocol. The reliable limit of quantification was 10 pg/ml and the mean percentage deviation over the calibration range of 10-2500 pg/ml was less than 15%.

Drug effects were statistically compared by univariate analysis of variance with subsequent t-tests employing a Bonferroni α-correction for multiple comparisons. α was set at 0.05. Separate ANOVAs were performed for PGE_2, nitrite/nitrate and TNFα.

Preparation of crude protein extracts

RAW 264.7 cells were seeded in 10 cm dishes at a density of $5 \cdot 10^5$ cells/dish. At 80% confluency cells were stimulated with LPS for 24h in the presence or absence of

rofecoxib. Unstimulated cells were used as controls. At the end of the incubation period cells were washed with PBS and then scraped with a rubber policeman and collected in 1.5 mm tubes. After short centrifugation, the pellet was resuspended in lysis buffer (10 mM Tris/HCl buffer, pH 7.4 containing 20 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS), 0.5 mM EDTA, 1 mM DTT, 0.5 mM PMSF and 1 µM Pefabloc (Alexis, Grünberg, Germany)) and kept on ice for 30 min. After sonication for 10 sec the suspension was centrifuged at 13,000 rpm in an Eppendorf centrifuge and the supernatant was stored at -80°C until further analysis.

Tissue samples from lumbar spinal cord were homogenized in 10 volumes of lysis buffer. After removal of cellular debris the suspension was centrifuged at 40,000 *g* for 1h and the supernatant was stored at –80°C until further analysis. Protein concentrations were assessed using the Bradford method.

Preparation of nuclear and cytosolic cellular fractions

Cells were incubated for 30 min with rofecoxib and were then stimulated for 30 min with 10 µg/ml LPS. Cell pellets were resuspended in 1 ml lysis buffer I (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM PMSF, 2 mM DTT) and incubated for 10 min on ice. After addition of NP-40 (final concentration 0.5%) the solution was vortexed and centrifuged at 400*g* for 5 min. The supernatant was kept as the cytosolic fraction. The nuclear pellet was washed with lysis buffer I. Pellets were then resuspended in 2 vol. lysis buffer II (20 mM HEPES-KOH, pH 7.4, 600 mM KCl, 0.2 mM EDTA, 1 mM PMSF, 2 mM DTT) and incubated for 30 min on ice. After centrifugation (10,000*g* for 10 min) the supernatant was diluted by the addition of 1 vol. lysis buffer III (20 mM Hepes-KOH, pH 7.4, 0.2 mM EDTA, 0.5 mM PMSF, 2 mM

DTT). Glycerol was added to obtain a final concentration of 20 % and aliquots were stored at –80°C.

Western Blot analysis

Proteins of cell lysates (50 µg), nuclear extracts (20 µg) or spinal cord homogenates (30 µg) were separated electrophoretically by 10% or 12% SDS-PAGE and then transferred onto nitrocellulose membranes by semi-dry blotting. The membrane was incubated overnight at 4°C in blocking buffer (5% skimmed milk in PBS/0.3% Tween 20). It was then incubated with the primary antibodies diluted 1:100 in blocking buffer for 90 min at room temperature. After washing in PBS/0.3% Tween 20 it was incubated for 60 min with a secondary antibody conjugated with peroxidase. Protein-antibody complexes were detected with the Enhanced Chemiluminescence System (Amersham-Pharmacia, Freiburg, Germany). Densitometric analysis of the blots were performed with Quantity One Software (Bio-Rad, Munic, Germany)

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts (5 µg) were incubated in 10 % glycerol, 10 mM Hepes-KOH, pH 7.9, 50 mM KCl, 4 mM $MgCl_2$, 4 mM Tris-HCl, 0.5 mM DTT, 0.5 mM EDTA, 1 µg BSA, 1 µg poly (dI-dC) together with 25 fmol of $\gamma[^{32}P]$-ATP-labeled-oligonucleotide in a final volume of 20 µl for 30 min at room temperature. The oligonucleotide sequence corresponds to the NF-κB binding site (5´-AGTTGAGGGGACTTTCCCAGGC-3´) and the AP-1 binding site (5´-CGCTTGATGACTCAGCCGGAA-3´), respectively. For competition experiments a 100-fold molar excess of unlabeled probe was added to the reaction 15 min before addition of the radiolabeled probe. The nucleotide/protein complex was separated on a 6% native polyacrylamide gel in 0.25 x TBE buffer (0,5×:

Tris-Borate 45mM, EDTA 1mM) at 100 V at room temperature. The gel was dried and radioactive bands were detected by autoradiography.

Northern Blot analysis

Total RNA was isolated from the cells by the method of Chomczynski (Chomczynski, 1993). 20 µg total RNA were dissolved in 10 µl H_2O, mixed with 10 µl of denaturing solution (500 µl formamide, 162 µl 37% formaldehyde, 100 µl 0.2 M MOPS [3-(N-morpholino)-propanesulfonicacid]) and incubated at 60°C for 15 minutes. 4 µl RNA loading dye were added. Total RNA was separated on a 1% agarose gel containing 1% formaldehyde (80 V, 4h). The RNA was then transferred to a nylon membrane over night with 10x SSC (1.5 M sodium chloride, 0.15M sodium citrate) and immobilized with a UV transilluminator (254 nm, 150 mJoule).

The membrane was prehybridized for 3 hours at 42°C in hybridization solution (5 ml formamide, 1 ml 20x SSC, 1 ml 10% SDS, 3 ml H_2O). Probes for iNOS and COX-2 were radiolabeled by random oligonucleotide priming with the "High Prime" system (Roche diagnostics, Mannheim, Germany). Hybridization was performed overnight at 42°C. The membrane was washed 4 times with 2x SSC/0.1% SDS (sodium dodecyl sulfate) and twice with 0.2x SSC/0.1% SDS, each time rotating for 20 minutes at 42°C. Autoradiography was performed using an automated detector system (BAS 1500 of Fuji film; Raytest, Straubenhardt, Germany). 18S rRNA was detected as control for equal RNA loading.

Real-time PCR

RNA was isolated as described above. 2µg of total RNA has been used for the reverse transcription which was performed with a Qiagen Reverse Transcription Kit.

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Expression of iNOS and COX-2 mRNA expression was assessed related to 18S mRNA. Therefore the following specific pimers were used:

COX-2: FW 5´-AGACACTCAGGTAGACATGATCTACCCT-3´,

RV 5´-GGCACCAGACCAAAGACTTCC-3´

iNOS: FW 5´-TCACCCACACTGTGCCCATCTACGA-3´,

RV 5´-CAGCGGAACCGCTCATTGCCAATGG-3´,

100 ng RNA equivalent were subjected to real-time PCR in an Applied Biosystems sequence detection system 7700 with Sybr Green fluorescence staining. The cycle number at which the fluorescence signals cross a defined threshold (Ct-value) is proportional to the number of RNA copies present at the start of the PCR. The threshold cycle number for the specific mRNA was standardized by substracting the Ct-value of 18S from the Ct-value of COX-2 or iNOS of the same sample, respectively. Relative quantitative level of samples was determined by standard $2^{(-\Delta\Delta Ct)}$ calculations and expressed as fold-change of a single reference control sample (LPS treated control).

Results

Effects of rofecoxib on transcription factor regulation of NF-κB and AP-1 in RAW 264.7 cells

The transcription factor NF-κB consists of two dimers (p50/p65) which are bound to the inhibitor I-κB and thus sequestered in the cytoplasm in unstimulated cells. Upon stimulation I-κB is phosphorylated by I-κB kinases, subsequently ubiquitinylated and then degraded by a proteasome complex. Degradation of I-κB allows NF-κB to translocate into the nucleus where it binds to the promoter region of various genes such as COX-2, TNFα or iNOS and activates their transcription (for reviews see (Baeuerle, 1998; Pahl, 1999)). In unstimulated control cells DNA binding of NF-κB was minimal whereas treatment with LPS considerably increased its DNA binding activity. The specificity of the NF–κB DNA binding was confirmed by the reversal of the binding by a 100fold molar excess of unlabeled probe (data not shown). Rofecoxib dose-dependently (1, 10 and 100μM) reduced the LPS stimulated DNA binding activity of NF-κB. At 100 μM, effects of rofecoxib were indistinguishable from those of the positive control Bay 11-7085 which is an inhibitor of I-κB kinase. Figure 1A shows the results of a representative experiment.

We additionally assessed the nuclear translocation of the p65 subunit of NF-κB and the degradation of I-κB in the cytosol by Western Blot analysis. In cells treated with LPS we observed a significant increase of p65 in the nucleus as compared to untreated control cells (Figure 1B). Simultaneously, the cytosolic I-κB protein signal disappeared. Pre-incubation with rofecoxib at 1, 10 and 100 μM did neither alter nuclear concentrations of NF-κB nor cytosolic I-κB as compared to the LPS-treated controls.

Besides NF-κB a variety of other transcription factors are activated during inflammatory processes including activating protein-1 (AP-1). AP-1 is of particular

importance in rheumatoid diseases (one of the main indications of rofecoxib) because it regulates the transcription of several cytokines and matrix metalloproteinases which contribute to the destruction of cartilage and bone. It is a protein complex containing products of the jun and fos oncogene family and is activated in response to a number of inflammatory stimuli including LPS and IL-1β. Because of the key regulatory role of AP-1 on collagenase expression and synovitis we additionally assessed effects of rofecoxib on the DNA binding activity of this transcription factor.

Specific AP-1 binding activity was almost absent in non-LPS treated control cells and considerably increased after stimulation of macrophages with 10 µg/ml LPS for 30 min. This effect was slightly decreased at a concentration of 10 µM rofecoxib, but clearly enhanced after incubation with 100µM rofecoxib, leading to a 1.8 fold increase of the LPS induced AP-1 DNA binding activity as detected by densitometric analysis (Figure 1c). Western Blot experiments of nuclear c-fos and c-jun protein concentrations showed similar results (Figure 1d).

Expression of NF-κB and AP-1 regulated genes in RAW 264.7

To evaluate whether the observed inhibition of NF-κB and the activation of AP-1 affected the expression of pro-inflammatory genes we assessed the expression of COX-2 and iNOS. Northern blot analyses as well as real-time PCR (Figure 2A) revealed that iNOS and COX-2 mRNA which were only slightly detectable in unstimulated RAW 264.7 cells considerably increased after stimulation with LPS. This was associated with a significant increase of NO_2^-/NO_3^- and PGE_2 release (Figure 2B, C). When cells were treated with the positive control dexamethasone, both COX-2 and iNOS mRNA expression was reduced. Incubation with rofecoxib also resulted in a reduction of iNOS and COX-2 mRNA. However, this inhibitory effect showed no clear dose-dependency.

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Prostaglandin-synthesis was, as expected, dose-dependently inhibited by rofecoxib. The IC_{50} value was calculated by linear regression to 7 nM. At doses of ≥ 100 nM the PGE_2 production was below that of unstimulated control cells. NO_2^-/NO_3^- release was also reduced with 0.1-100 µM rofecoxib but without any dose-dependency (Figure 2 B, C).

Effects of rofecoxib on zymosan evoked inflammation in rats

Because, in contrast to celecoxib, rofecoxib has not activated NF-κB but rather inhibited its DNA binding activity we hypothesized that its anti-inflammatory activity should not be abolished at high doses. We tested this hypothesis in the zymosan-induced paw inflammation model in rats. In vehicle treated rats intraplantar injection of 1.25 mg zymosan led to a maximum increase of the paw volume of 110.3 ± 5.3% (mean ± sem). As hypothesized rofecoxib inhibited paw inflammation at doses of 1, 10 and 50 mg/kg (Figure 3A). Statistical comparison of the area under the "paw volume increase" versus "time" curves ($AUC_{\Delta PW}$ from 0-24h) revealed statistically significant differences in treatment means: $F(3, 25)=13.54$, $p<0.001$. Results of the post hoc analysis are shown in figure 3B. Interestingly, 50 mg/kg produced significantly stronger anti-inflammatory effects than 1 and 10 mg/kg whereas the anti-inflammatory effect of 10mg/kg rofecoxib was not stronger than that with 1mg/kg.

Maximum plasma concentrations of rofecoxib in rats treated with 1, 10 and 50 mg/kg were 0.66±0.17, 2.87±0.42 µM and 14.47 ± 0.95 µM, respectively. They were reached at 1h following the 1 mg/kg dose and at about 5h after administration of 10 or 50 mg/kg (Figure 4). The terminal elimination half life was calculated to be 6.1 ± 0.5 h. It was independent of the dose administered.

In Western Blot experiments we observed a reduction of the zymosan-induced increase of COX-2 expression in lumbar spinal cord when rats were treated with 1 and 10 mg/kg rofecoxib. At doses of 50 mg/kg however, the COX-2 signal did not significantly differ

from that of zymosan-treated control rats. Thus, the inhibitory effect of rofecoxib on COX-2 expression was lost at this dose. In contrast to COX-2 the zymosan-induced iNOS protein expression in the lumbar spinal cord further increased after treatment with 1 and 10 mg/kg rofecoxib but decreased at 50 mg/kg (Figure 5A). Thus COX-2 and iNOS expression in the spinal cord are oppositely regulated by rofecoxib. Even more complex were the effects on zymosan-stimulated TNFα production in lumbar spinal cord: TNFα was not altered at 1 mg/kg, decreased at 10 mg/kg and again unaltered at 50 mg/kg (Figure 5B).

Discussion

Rofecoxib is a selective COX-2 inhibitor with anti-inflammatory and analgesic efficacy and a low gastrointestinal toxicity as compared to conventional NSAIDs. Although rofecoxib does not inhibit COX-1 at therapeutically relevant doses, its use is associated with a relatively high incidence of renal side effects. This may be partly – although not exactly defined – due to the constitutive expression of COX-2 in the kidney. Depending on the study design rofecoxib treatment is controversially discussed to be more frequently associated with salt and water retention, reduction of the GFR, reports of acute renal failure and increase of blood pressure as compared to other selective (celecoxib) or unselective NSAIDs (Swan et al., 2000; Kammerl et al., 2001; Schwartz et al., 2001; Whelton et al., 2001; Zhao et al., 2001). Another feature with rofecoxib is that its effects are somewhat arbitrarily associated with the concentration or dose, i.e. it is not clear whether a high dose will provide higher efficacy than a lower one. In the present study we assessed the influence of rofecoxib on transcription factor regulation as a potential explanation for the above mentioned controversial discussion.

The major finding of the present study is that rofecoxib inhibited NF-κB but increased AP-1 DNA binding activity. Since NF-κB and AP-1 regulate similar genes but not necessarily in the same direction the simultaneous action on both transcription factors is probably the cause for the here observed at the first view confusing pattern of up- and downregulation of pro-inflammatory genes including COX-2, TNFα and iNOS. For example, the simultaneous inhibition of NF-κB and activation of AP-1 might have caused the significant decrease of iNOS protein expression in the spinal cord at the highest rofecoxib dose, because iNOS transcription is stimulated by NF-κB but inhibited by AP-1 (Kleinert et al., 1998). Thus, the observed inhibition of iNOS expression is probably the sum of both effects and might have contributed to the strong

anti-inflammatory effects of the highest rofecoxib dose (50 mg/kg), particularly in the late phase of the zymosan-induced paw edema. Nevertheless, it cannot be excluded that the stronger effect of rofecoxib at 50 mg/kg as compared to 1 and 10 mg/kg might be caused by higher plasma concentrations and therefore longer inhibition of COX-2 activity rather than by additional effects on transcription factors.

The effects of rofecoxib on NF-κB are directly opposed to those observed with the other less potent "coxib", celecoxib in a previous study. In that study, celecoxib was shown to activate NF-κB at high concentrations. This resulted in a complete loss of its anti-inflammatory efficacy at high doses (100-200 mg/kg) (Niederberger et al., 2001). Since rofecoxib has no NF-κB activating effects it does not surprise that rofecoxib did not lose its anti-inflammatory efficacy even at very high doses. For rofecoxib, the 50 mg/kg dose was the maximum that could be safely administered to rats and considering its about 10 times higher potency in terms of COX-2 inhibition this dose is even more potent to inhibit COX-2 than the celecoxib doses of 100-200 mg/kg at which anti-inflammatory effects of celecoxib were found to be abolished. These data demonstrate that there are considerable differences among these two COX-2 inhibitors. In terms of NF-κB inhibition, the effects of rofecoxib are more similar to those of acetylsalicylic acid, salicyclic acid or flurbiprofen which have been previously found to inhibit NF-κB activation (Muller et al., 2001; Tegeder et al., 2001). In contrast to these drugs however, rofecoxib did not inhibit the nuclear translocation of NF-κB but prevented its DNA binding activity suggesting that although the resultant inhibition of NF-κB dependent gene transcription is similar, the step at which NF-κB activation is inhibited is obviously different. Rofecoxib's stimulating effects on AP-1 also clearly differ from those of other NSAIDs, since both acetylsalicylic acid (Huang et al., 1997) and flurbiprofen (Tegeder et al., 2001) were found to inhibit AP-1 DNA binding. This may

explain why flurbiprofen had no effect on iNOS protein expression whereas rofecoxib reduced its zymosan-induced upregulation at high doses.

COX-1 inhibition and thereby reduction of thromboxane synthesis and platelet aggregation is the major mechanism underlying the beneficial cardiovascular effects of aspirin. Recently it has been suggested, that the treatment of patients with COX-2 selective drugs may be associated with a higher risk of cardiovascular events (Boers, 2001; Hennan et al., 2001; Mukherjee et al., 2001) probably because these drugs may increase prothrombotic activity by decreasing the vasodilatory and anti-aggregatory prostacyclin production without a simultaneous inhibition of platelet aggregation. This feature however is shared by all COX-2 inhibitors (Zhao et al., 2001). In addition, some studies showed that the incidence of peripheral edemas under treatment with rofecoxib was about twice that observed with celecoxib or other non-selective NSAIDs (Zhao et al., 2001).

Considering that AP-1 regulates the transcription of renal sodium channels (Otulakowski et al., 1999) it may be hypothesized that the rofecoxib-induced activation of AP-1 may be involved in the increase of salt and water retention that occurs during treatment with this drug. The action of rofecoxib on the expression of certain sodium transporters in the kidney remains to be evaluated.

In summary, we show in the present study that the pattern of transcription factor regulation caused by rofecoxib is specific for this “coxib” and may explain the inconsistent pattern of up- and downregulation of pro-inflammatory genes such as COX-2, iNOS and TNFα. This may be the reason for the occurrence of side effects such as salt and water retention and the lack of a linear dose dependency of the analgesic and anti-inflammatory effects of rofecoxib (Day et al., 2000; Truitt et al., 2001).

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Figure legends

Figure 1

A) Electrophoretic mobility shift assay (EMSA). RAW 264.7 cells were preincubated for 30 min with rofecoxib at the indicated concentrations and then stimulated with 10 µg/ml LPS for further 30 min. The nuclear fractions were extracted and 5 µg were subjected to EMSA using a ^{32}P-labeled NF-κB consensus oligonucleotide.

B) Western blot analysis of the p65 subunit of NF-κB in nuclear extracts and I-κB in cytosolic extracts. Cells were treated for 30 min with 1, 10 and 100 µM rofecoxib followed by the stimulation with 10µg/ml LPS for further 30 min.

C) Electrophoretic mobility shift assay (EMSA) with RAW cells preincubated for 30 min with rofecoxib at the indicated concentrations and then stimulated with 10 µg/ml LPS for further 30 min. Nuclear fractions were subjected to EMSA using a ^{32}P-labeled AP-1 consensus oligonucleotide.

D) Western Blot analysis of c-Fos and c-Jun in nuclear extracts of RAW 264.7 macrophages. Cells were treated as described above.

Representative results of 4 experiments are shown.

Figure 2

A) Expression of iNOS and COX-2 mRNA in RAW 264.7 cells stimulated with LPS for 24h in the absence or presence of rofecoxib or dexamethasone at the indicated concentrations.

Upper panel: Northern Blot analysis of iNOS and COX-2; 20µg RNA were separated in a 1% agarose gel, blotted on a nylon membrane and then hybridized with the radioactive labelled probe. The blots show representative results of 4 experiments. 18SRNA was assessed as a loading control.

Lower panel: Real-time RT-PCR, 100ng RNA-equivalent were subjected to real-time PCR in a SDS 7700 with SybrGreen staining. dCt-values were calculated with 18S-RNA as internal standard. The diagramm shows the relative amount of mRNA compared to the LPS stimulated control which was set as 1.

B) Release of nitrite/nitrate in RAW 264.7 stimulated for 24h with 10 µg/ml LPS in the absence or presence of rofecoxib or 0.5 µM dexamethasone (Dex). Nitrite/nitrate concentrations (mean ± s.e.m.) were measured by the Griess method. (* statistical significance mean difference, p=0.05)

C) Effects of rofecoxib on the release of prostaglandin E_2 (PGE_2; mean ± s.e.m.) in RAW264.7. Cells were treated as described above. PGE_2 concentrations were measured by an enzyme immuno assay. (* statistical significance mean difference, p=0.05)

Figure 3

A) Time course of the anti-inflammatory effects of rofecoxib in zymosan induced hindpaw inflammation in rats (mean ± s.e.m.). Six to ten rats were used in each group. Rofecoxib was administered orally 15 min prior to an intraplantar injection of 1.25 mg zymosan. Control animals received the appropriate volume of tylose slime.

B) For statistical comparison of drug effects the areas under the "paw volume increase" versus "time" curves ($AUC_{\Delta PW}$ from 0-24h, mean ± s.e.m.) were calculated using the linear trapezoidal rule and subjected to univariate analysis of variance with subsequent Bonferroni post hoc tests. The box represents the interquartile range, the line within the box the median, the ends of the "whiskers" show the 5^{th} and 95^{th} percentile. Open dots show individual values. (*, *** statistically significant mean difference with $p<0.05$ and <0.001, respectively).

Figure 4

Mean ± s.e.m plasma concentration time curves of rofecoxib in rats following the oral administration of 1 mg/kg (●), 10 mg/kg (■) and 50 mg/kg (Δ).

Figure 5

Expression of COX-2, iNOS and TNFα in rat lumbar spinal cord as assessed by western blot analysis (COX-2, iNOS) or immunoassay (TNF-α); The spinal cord was excised 96h after induction of a peripheral inflammation by injection of 1.25 mg zymosan into one hindpaw. Animals (n=3-4 for each dose) were treated with a single oral dose of 1, 10 and 50 mg/kg rofecoxib 15 min before the zymosan injection.

A upper panel: Western Blot analysis of iNOS and COX-2. The blots show one representative experiment of three.

lower panel: Densitometric analysis of all investigated spinal cord samples (3 per value; mean ± s.e.m.). Zymosan treated control rats were set as 1.

B TNFα expression

(*, ** statistically significant mean difference with $p<0.05$ and <0.01, respectively.)

Figure 1

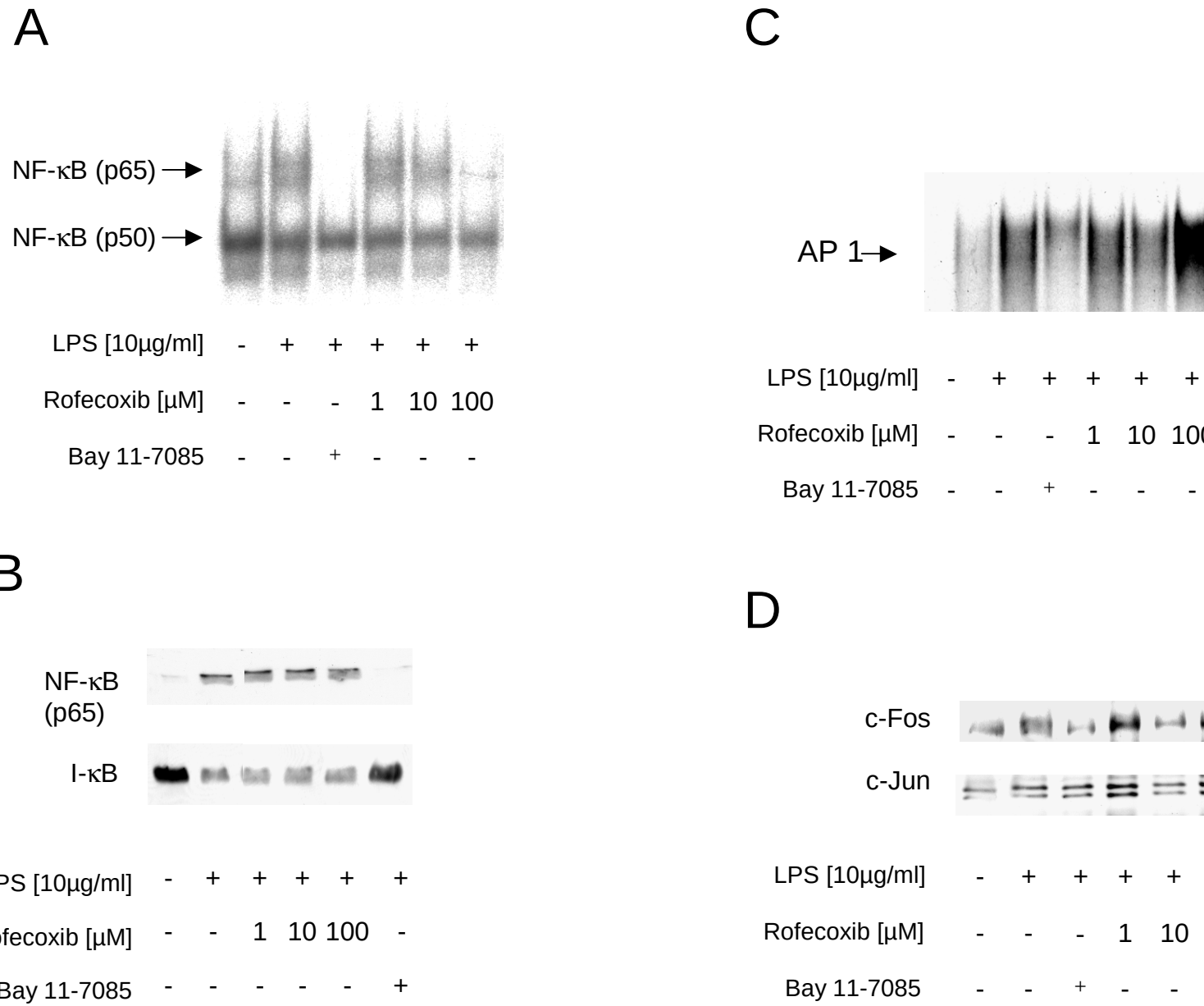

Figure 2

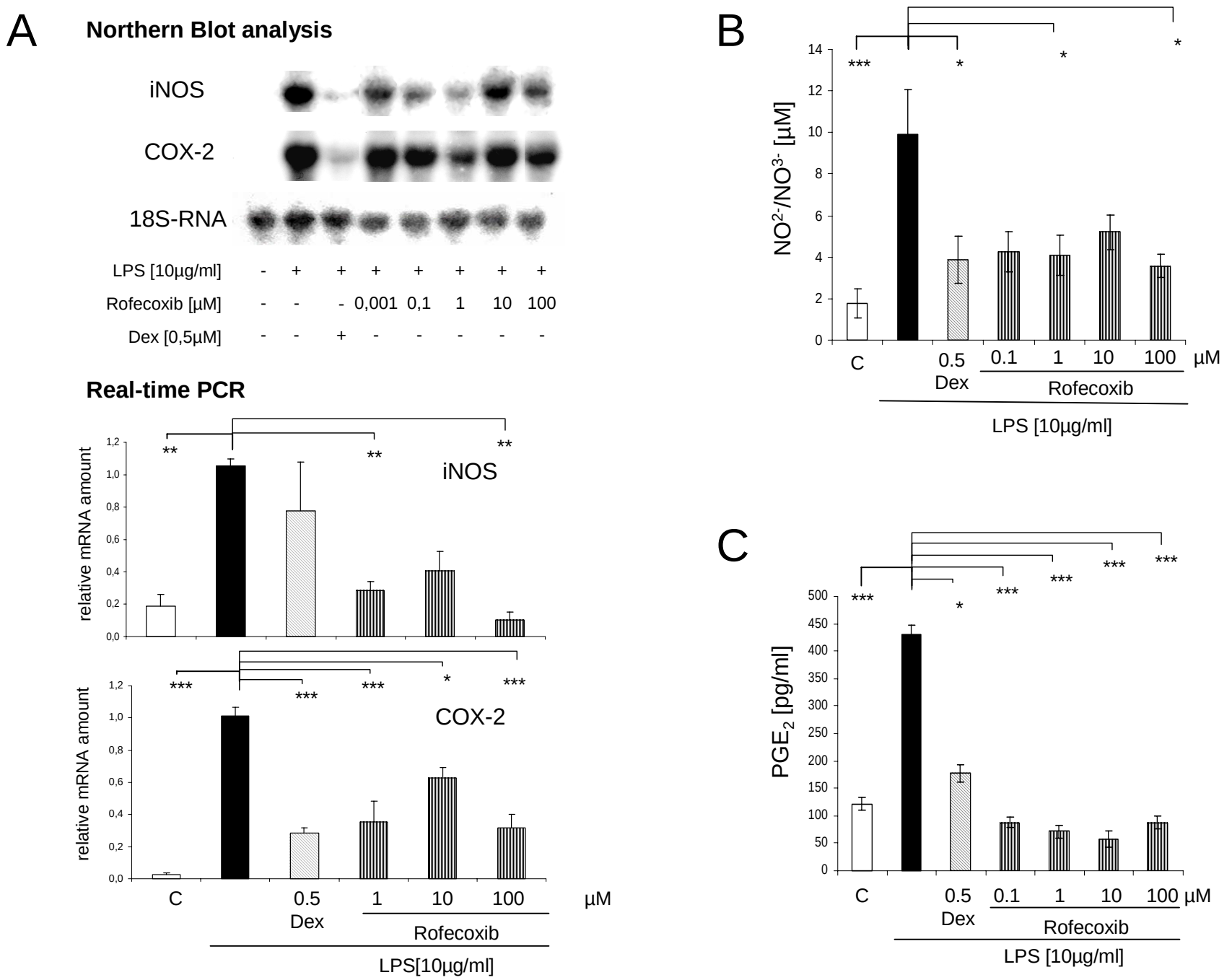

Figure 3

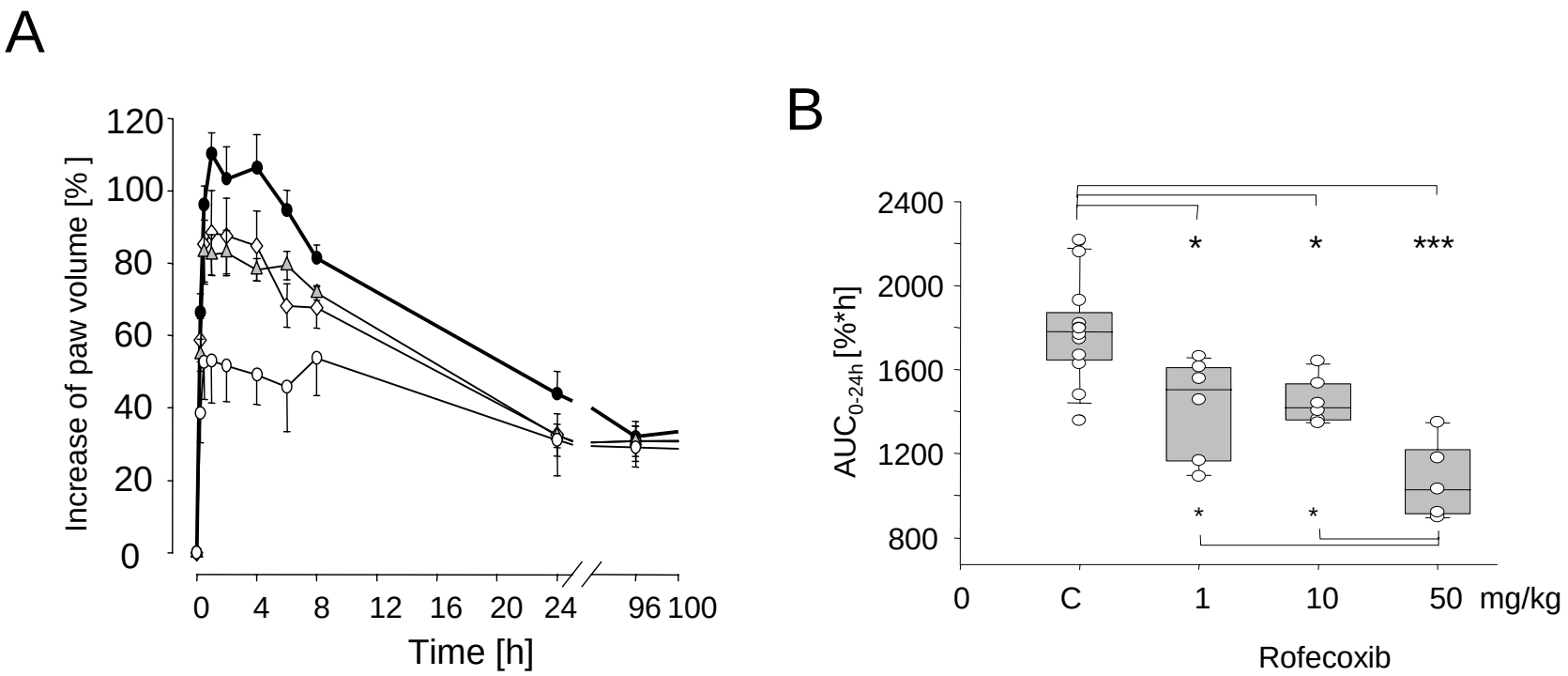

Figure 4

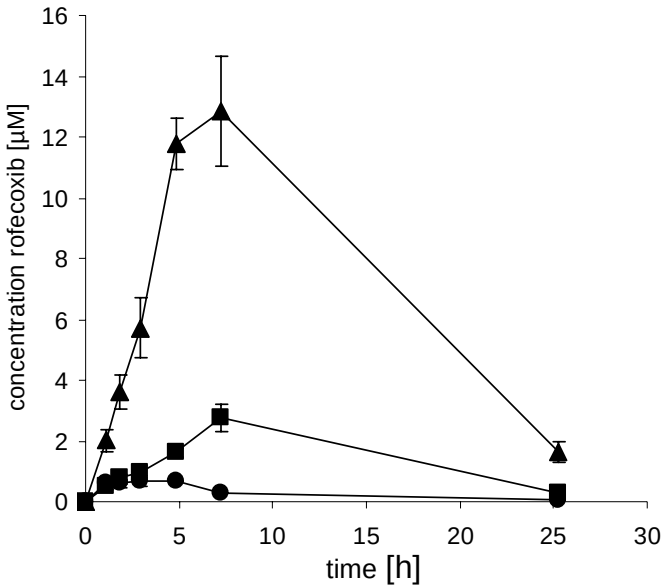

Figure 5