COX-2-dependent and independent effects of COX-2 inhibitors and NSAIDs on proatherogenic changes in human monocytes/macrophages

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ABSTRACT

It is the second decade of controversy regarding the cardiovascular effects of cyclo-oxygenase-2 (COX-2) inhibitors. At this time, celecoxib is the only available COX-2-specific inhibitor for treatment of pain and inflammation. Therefore, the present study was designed primarily to determine the impact of celecoxib on cholesterol handling (uptake via scavenger receptors and efflux from the cells) and foam cell formation in human THP-1 macrophages, followed by comparison to rofecoxib and other non-steroidal anti-inflammatory drugs (NSAIDs). THP-1 human macrophages and peripheral blood mononuclear cells were incubated with: celecoxib, rofecoxib, naproxen (at 5, 10, 25 μM) and acetaminophen (0.5 mM, 1 mM)±oxidized low-density lipoprotein (oxLDL, 25 μg/mL). Scavenger receptors: CD36, LOX-1, SR-A1, and CXCL16 and cholesterol efflux proteins: ATP-binding cassette transporter (ABC) A1 and G1, and 27-hydroxylase were detected. The adhesion of monocytes to cultured endothelial cells with/ without COX-2 inhibitors/NSAIDs was also analyzed. The presence of celecoxib and rofecoxib (at high concentrations) significantly decreased expression of 27-hydroxylase and ABCA1, interfering with normal cholesterol outflow from macrophages. Acetaminophen and the non-specific COX inhibitor naproxen had no significant effect on these proteins. Only celecoxib had a profound effect on the class B scavenger receptor CD36 and the class E receptor LOX1. We demonstrate that in contrast to celecoxib, rofecoxib and naproxen increased adhesive properties of monocytes to endothelial cells. This work might contribute to our understanding of multiple mechanisms underlying elevated cardiovascular risk upon the use of COX-2 inhibitors and uncover new possibilities to enhance the safety profile of existing COX-2 inhibitors.

Significance of this study

What is already known about this subject?

- The use of cyclo-oxygenase-2 inhibitors is associated with elevated cardiovascular risk.
- Celecoxib alters expression of the CD36 receptor.
- Celecoxib promotes foam cell formation.

What are the new findings?

- Cyclo-oxygenase-2 inhibition alters cholesterol efflux at high concentrations.
- Celecoxib, but not rofecoxib, elevates expression of CD36 and decreases LOX1.
- In contrast to celecoxib, rofecoxib and naproxen increase adhesive properties of monocytes to endothelial cells.

How might these results change the focus of research or clinical practice?

- This study uncovers multiple mechanisms underlying elevated cardiovascular risk upon the use of cyclo-oxygenase-2 (COX-2) inhibitors. It indicates new possibilities to enhance the safety profile of existing COX-2 inhibitors.

INTRODUCTION

Non-selective non-steroidal anti-inflammatory drugs (NSAIDs) and cyclo-oxygenase (COX)-2-specific inhibitors (COX-2 inhibitors) exhibit analgesic and anti-inflammatory efficacy. COX-2 inhibitors have been associated with increased frequency of cardiovascular toxicity and an elevated risk of acute myocardial infarction,[1–4] which has negatively impacted physician perception and prescribing of this drug class. The COX-2 inhibitors rofecoxib and valdecoxib have been withdrawn from the market, and two other COX-2 inhibitors failed to receive approval due to cardiovascular concerns.[5–7] While most of the interest has focused on the role of COX-2 inhibitors, NSAIDs also have the potential to increase the risk of adverse cardiovascular events.[8]

The mechanisms underlying cardiovascular effects of COX-2 inhibitors are uncertain. A number of explanations have been proposed: induction of thrombosis, due to an imbalance between endothelial prostacyclin and thromboxane,[9–11] cardiorenal effects on blood pressure,[12] and others.

Our laboratory has previously demonstrated that the COX-2-selective inhibitor NS398 increases the propensity of cultured THP-1

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human macrophages to form lipid-laden foam cells. This effect occurs due to inhibition of reverse cholesterol transport from the periphery to the liver and the mechanism that drives this effect is reduced expression of cholesterol 27-hydroxylase (CYP27A1) and ATP-binding cassette transporter 1 (ABCA1).[13 14] Furthermore, our group found that inhibition of the COX-2 pathway augments foam cell formation (FCF) due to upregulation of the class B receptor CD36.[15] Since celecoxib is the only commercially available COX-2-specific inhibitor for treatment of pain and inflammation, we investigated a complex effect of celecoxib on the delicate balance between cholesterol uptake and efflux in human monocytes/macrophages with consequent comparison to the effect of rofecoxib and other NSAIDs in the same cell system.

Here, we reveal distinct and multiple effects of COX-2 inhibitors on proatherogenic changes in monocytes/macrophages. At high concentrations, only celecoxib disrupted cholesterol efflux and upregulated oxLDL uptake in THP-1 human macrophages. However, rofecoxib and naproxen increased adhesive properties of monocytes to endothelial cells.

MATERIALS AND METHODS

Cell culture and reagents

Human THP-1 monocytes (American Type Culture Collection, Manassas, Virginia, USA) and peripheral blood mononuclear cells (PBMC) (Lonza, Allendale, New Jersey, USA) were cultured in RPMI 1640 according to methods, standardized in our laboratory.[15 16] Medium was supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and 50 μg/mL of penicillin–streptomycin at 37°C in a 5% CO_2 atmosphere to a density of 10^6 cells/mL. Cell culture media and supplementary reagents were obtained from Invitrogen (Grand Island, New York, USA). Differentiation of the monocytic THP-1 cells into adherent macrophages was stimulated by 48 hour exposure to 100 nM phorbol 12-myristate 13-acetate (PMA), obtained from Sigma-Aldrich (St. Louis, Missouri, USA). When a differentiated phenotype was achieved, the PMA-containing medium was removed, and replaced with complete RPMI 1640 supplemented with 10% FCS for another 24 hours.

Trizol reagent was purchased from Invitrogen (Carlsbad, California, USA). All reagents for reverse transcription–PCR (RT–PCR) were purchased from Applied Biosystems (Foster City, California, USA). SYBR Green Master mix for the quantitative real-time PCR (QRT-PCR) was obtained from Roche Applied Science (Penzberg, Germany). Primers used in amplification reactions were generated by Sigma-Genosys (St. Louis, Missouri, USA).

Experimental conditions

Celecoxib and rofecoxib were obtained from Toronto Research Chemicals (Canada). (*S*)-(+)-6-Methoxy-α-methyl-2-naphtaleneacetic acid (naproxen) and 4′-hydroxyacetanilide (acetaminophen) were purchased from Sigma (St. Louis, Missouri, USA). Stock solutions were prepared in DMSO (Sigma).

THP-1 monocytes/macrophages and PBMC (density 10^6 cells/mL) were subjected to incubation for 20 hours for gene expression analysis and 24 hours for protein evaluation under the following experimental conditions: cell culture media alone; DMSO solvent control; celecoxib (5 μM); celecoxib (10 μM); celecoxib (25 μM); rofecoxib (5 μM); rofecoxib (10 μM); rofecoxib (25 μM); naproxen (5 μM); naproxen (10 μM), acetaminophen (1 mM).

Gene-silencing via transfection of small-interfering RNA

Transfection of THP-1 macrophages (70% confluence) was carried out after their differentiation followed by 24 hours incubation in complete RPMI. Transfection was performed in serum-free OPTI medium using small-interfering RNA (siRNA) transfection reagent (Santa Cruz, California, USA, sc-29528). Cells were transfected for 6 hours with 100 nM of human COX-2 siRNA (Santa Cruz, California, USA, sc-29528), or irrelevant non-targeting control siRNA-A (Santa Cruz, California, USA, sc-37007) according to the manufacturer's protocol. Cells were then further incubated for 24–72 hour under standard growth conditions. At 24–72 hour post-transfection, depletion of COX-2 was confirmed by QRT-PCR and immunostaining.

RNA isolation and QRT-PCR

Immediately after the incubation period, total RNA was isolated with the Trizol reagent and dissolved in nuclease-free water.[15 16] The quantity of total RNA from each condition was measured by absorption at 260 and 280 nm wavelengths by ultraviolet spectrophotometry (Hitachi U2010 spectrophotometer).

QRT-PCR analysis was performed using the FastStart SYBR Green Reagents Kit according to the manufacturer's instructions on the Roche Light Cycler 480 (Roche Applied Science, Indianapolis, Indiana, USA). cDNA was copied from 1 μg of total RNA using Murine Leukemia Virus reverse transcriptase primed with oligo dT. Equal amounts of cDNA were taken from each reverse transcription reaction mixture for real-time PCR amplification using gene-specific primers for 27-hydroxylase, ABCA1, ABCG1, LXR-α, PPAR-γ, ScR-B1, ScR-A1, CD36, CXCL16 and LOX-1 as well as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (table 1).

Table 1 List of specific primers used for QRT-PCR

Gene	Primer
ABCA1	F 5′-GAAGTACATCAGAACATGGGC-3′ R 5′-GATCAAAGCCATGGCTGTAG-3′
ABCG1	F 5′-CAGGAAGATTAGACACTGTGG-3′ R 5′-GAAAGGGGAATGGAGAGAAG-3′
27-hydroxylase	F 5′-AAGCGATACCTGGATGGTTG-3′ R 5′-TGTTGGATGTCGTGTCCACT-3′
ScR-B1	F 5′-GGTCCCTGTCATCTGCCAA-3′ R 5′-CTCCTTATCCTTTGAGCCCTTT-3′
CD36	F 5′-GAGAACTGTTATGGGGCTAT-3′ R 5′-TTCAACTGGAGAG-GCAAAGG-3′
LOX-1	F 5′-TTACTCTCCATGGTGGTGCC-3′ R 5′-AGCTTCTTCTGCTTGTTGCC-3′
ScR-A1	F 5′-CTCGTGTTTGCAGTTCTCA-3′ R 5′-CCATGTTGCTCATGTGTTCC-3′
CXCL16	F 5′-ACTACACGACGTTCCAGCTCC-3′ R 5′-CTTTGTCCGAGGACAGTGATC-3′
GAPDH	F 5′- ACCATCATCCCTGCCTCTAC-3′ R 5′-CCTGTTGCTGTAGCCAAAT-3′

QRT-PCR was performed using techniques standardized in our laboratory.[15 16] Each reaction was performed in triplicate. To correct for differences in cDNA load among samples, the target PCRs were normalized to a reference PCR involving the endogenous housekeeping gene GAPDH.

Non-template controls were included for each primer pair to check for significant levels of any contaminants. A melting curve analysis was performed to assess the specificity of the amplified PCR products.

cDNA synthesis and atherosclerosis RT2 Profiler PCR Array

Human THP-1 macrophages were incubated with celcoxib and rofecoxib (10 μM) for 18 hours in cell culture. Immediately after the incubation period, total RNA was isolated with the Trizol reagent and dissolved in nuclease-free water. One microgram total RNA was amplified using the RT^2 First Strand Kit (Qiagen, Valencia, California, USA). As per the manufacturer's protocol, genomic DNA was eliminated and the subsequent RNA mixed with the kit's reverse-transcription mix. cDNA synthesis proceeded at 42°C for 15 min and the reaction halted by incubation at 95°C for 5 min. Samples were diluted fivefold with RNase-free water prior to PCR amplification.

Gene expression profiling was performed using the RT^2 Profiler PCR Array kit for human atherosclerosis (Qiagen). For each reaction, 10 ng of cDNA was amplified to analyze target and housekeeping gene expression. Cycling conditions provided by the manufacturer were specific to the Roche LightCyler 480 instrument. Relative fold changes were calculated using Ct values entered into Qiagen's Excel analysis templates.

Protein isolation and Western blot analysis

Cellular extracts were prepared for Western immunoblotting using radioimmunoprecipitation assay (RIPA) lysis buffer (98% PBS, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS), supplemented with 10 μL/mL of protease inhibitor cocktail (Sigma). Protein content was measured in triplicate using the BCA Protein Assay Kit by absorption at 562 nm (Pierce Biotechnology, Rockford, Illinois, USA). Cellular extracts for Wes Protein analysis were prepared with Lysis Kit—RIPA Buffer followed by immunoreactive protein detection with Wes Assay kit (ProteinSimple, Santa Clara, California, USA).

For classical Western blot,[16] protein samples (20 μg/lane) were boiled for 5 min, and fractionated on 8% SDS–PAGE, and transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, California, USA). The membrane was stained with Ponceau red (Sigma) to verify uniformity of protein loading in each lane. The membrane was blocked for 1 hour at room temperature in blocking solution (3% non-fat dry milk (Bio-Rad) in 1× Tris-buffered saline/1% Tween 20 (TTBS)) and then immersed in a 1:500 dilution of primary antibody overnight at 4°C. The following day, the membrane was washed and then incubated in a 1:5000 dilution of ECL horseradish peroxidase-linked species-specific whole antibody in blocking solution. The immunoreactive proteins were detected using Pierce ECL Western Blot substrate system, and film development in SRX-101A (Konica Minolta Holdings, Tokyo, Japan). Stripping and reprobing of the membranes were performed according to the manufacturer's protocol (ECL kit instructions, Thermoscientific, Rockford, Illinois, USA).

For Wes protein assay, cellular extracts were prepared with Lysis Kit—RIPA Buffer (ProteinSimple) and separated with Wes protein analysis system.

Rabbit antihuman ABCA1 (sc-20794) (Santa Cruz, California, USA) and rabbit antihuman ABCG1 (ab-36969) (Abcam, Cambridge, Massachusetts, USA) were used as primary antibodies for detection of ABCA1 and ABCG1, respectively. Rabbit antihuman LOX1 (ab60178), CD36 (ab64014), SR-A1 (ab36625), and CXCL16 (ab101404) were purchased from Abcam. As a loading control, GAPDH was detected using rabbit antihuman GAPDH antibody (ab9485). Anticholesterol 27-hydroxylase antibody is an affinity-purified rabbit polyclonal antibody raised against residues 15–28 of the cholesterol 27-hydoxylase protein.[17]

Band intensities for Western blot protein samples were quantified using Kodak Digital Science 1D, V.2.0.3, after imaging with Kodak Digital Science Electrophoresis Documentation and Analysis System 120. Quantization of proteins analyzed with Wes Assay kit were performed with Compass Software (ProteinSimple).

Oxidized LDL uptake and FCF assay

THP-1 monocytes and PBMC (10^6 cells/mL) were transferred into 8-well glass-chamber slides, and treated with PMA (100 nM, 48 hours, 37°C) to stimulate differentiation into macrophages. THP-1 macrophages and MDM were then further incubated in fresh RPMI 1640 for 24 hours, followed by incubation for 20 hours under conditions described above. Following incubation, medium was aspirated, and slides were washed with PBS and fixed in 4% paraformaldehyde in water for 15 min.

For the FCF assay, differentiated macrophages were cholesterol-loaded with 50 μg/mL acetylated LDL or 50 μg/mL oxidized LDL (Inracel, Frederick, Maryland, USA) for 24 hours, and subjected to all conditions described above for another 24 hours in the presence/absence of modified LDL. For Oil Red O staining, cells fixed in 4% paraformaldehyde were then washed and stained with 0.2% Oil Red O (Sigma) for 30 min. After the PBS wash, cell nuclei were stained with hematoxylin (Sigma) for 5 min. After a final wash with PBS, coverslips were mounted on slides using Permount solution (Sigma). For the DiI-oxLDL internalization assay, cells pretreated for 24 hours with oxLDL were subjected to all experimental conditions in the presence of 5 μg/mL 1,1′-dioctadecyl-3,3,3′,3'-tetramethylin docarbocyaninet (DiI)-oxLDL (Inracel) for 3 hours. After incubation, accumulation of DiI-oxLDL in cells was determined by fluorescent intensity of accumulated DiI-oxLDL with a Nikon A1 microscopy unit with 40× magnification and photographed with DS-Ri1 digital camera. Fluorescent intensity was quantified from at least three random fields (1024×1024 pixels) per slide, from three slides per experimental condition and graphed.

Foam cells, recognized as macrophages stained with Oil Red O, were visualized via light microscopy (Axiovert 25; Carl Zeiss, Gottingen, Germany) with 40× magnification and photographed using a DC 290 Zoom digital camera (Eastman Kodak, Rochester, New York, USA). The number of foam cells formed in each condition was calculated in triplicate manually and presented as percentage of total cells.

Cell adhesion assay

Human umbilical vein endothelial cells (HUVEC) (Lonza, Basel, Switzerland) were cultured until confluence in LGM-3 lymphocyte growth medium (Lonza, Basel, Switzerland). Cells were grown in 35 mm glass bottom microwells. THP-1 human monocytes were exposed to DMSO (solvent control), 25 μM celecoxib, 25 μM rofecoxib, or 10 μM naproxen for 18 hours. After membrane labeling using the Green Fluorescent Cytoplasmic Membrane Staining Kit (Promokine, Heidelberg, Germany), monocytes were resuspended in mixed growth media (50% RPMI 1640/50% LGM-3) and incubated in the HUVEC-lined microwell dishes for 18 hours to allow for cell adherence. The dishes were then washed with sterile PBS to eliminate non-adherent cells and monocyte attachment examined under fluorescent microscopy. Cells were analyzed at 40× magnification using a Nikon A1 microscopy unit and photographed with DS-Ri1 digital camera. Quantitative analysis was performed averaging total fluorescence in three random fields of a view for each sample.

Statistical analysis of experimental data

Statistical analysis was performed using Graphpad Prism, V.5.01 (GraphPad Software, San Diego, California, USA). Data were analyzed by one-way analysis of variance with a Bonferroni post hoc test to evaluate the statistical significance of intergroup differences in all the tested variables. FCF and cell-to-cell adhesion assay were analyzed by unpaired Student's t-test to evaluate levels of significance; the p values of <0.05 were considered statistically significant.

RESULTS

Effect of COX-2 inhibitors and COX-2 silencing on cholesterol efflux proteins in THP-1 monocytes and macrophages and in human MDM

The balanced flow of cholesterol into and out of the macrophage is necessary to avoid lipid overload, and ultimately, atheroma development. Our laboratory has previously demonstrated that the COX-2-selective inhibitor NS398 decreased expression of cholesterol 27-hydroxylase (CYP27A1) and ABCA1, proteins involved in reverse cholesterol transport from the periphery to the liver for metabolism.[13 17] Introduction of siRNA produces COX-2 gene inhibition to 44.0±2.0% of the silencing control (n=3, $p<0.01$). Downregulation of COX-2 leads to diminution of ABCA1 message, providing proof of principle that loss of COX-2 activity impairs reverse cholesterol transport mechanisms (figure 1). Thus, transfection of THP-1 macrophages with COX-2 siRNA decreased ABCA1 message to 62.9±4.9% of control (n=3, $p<0.05$). Introduction of siCOX-2 reduced 27-hydroxylase message to a similar level (64.5±2.4%, n=3, $p<0.05$). However, the expression of another ABC transporter—ABCG1, involved in the net efflux of cellular free cholesterol and phospholipid to lipid-free HDL[18]—was not affected by COX-2 knockdown.

First, we compared the effect of COX-2 inhibitors and NSAIDs at the concentrations they reach in the bloodstream after administration (5 μM).[19] When THP-1 monocytes/macrophages and PBMC were cultured with celecoxib, rofecoxib, naproxen at 5 μM, and acetaminophen at 1 mM, no significant changes were detected in expression of the proteins involved in cholesterol efflux.

When tested at higher concentrations (10 and 25 μM), we observed that celecoxib and rofecoxib downregulated the expression of reverse cholesterol transport proteins in a concentration-dependent manner. However, celecoxib and rofecoxib significantly reduced message level of the ABCA1, but not the ABCG1, transporter in naïve THP-1 human macrophages only at 25 μM. ABCA1 mRNA was decreased by celecoxib and rofecoxib reaching 60.9±5.0% and 71.7±3.5%, respectively, for mRNA versus solvent control set at 100.0% for mRNA (n=6 each, $p<0.01$) (figure 2A). We observed compensatory upregulation of expression of ABCG1 by more than twofold in the presence of 25 μM of celecoxib to 233.7±31.3% versus solvent control, while no significant changes were seen in SR-B1 (figure 2A). These results were confirmed by

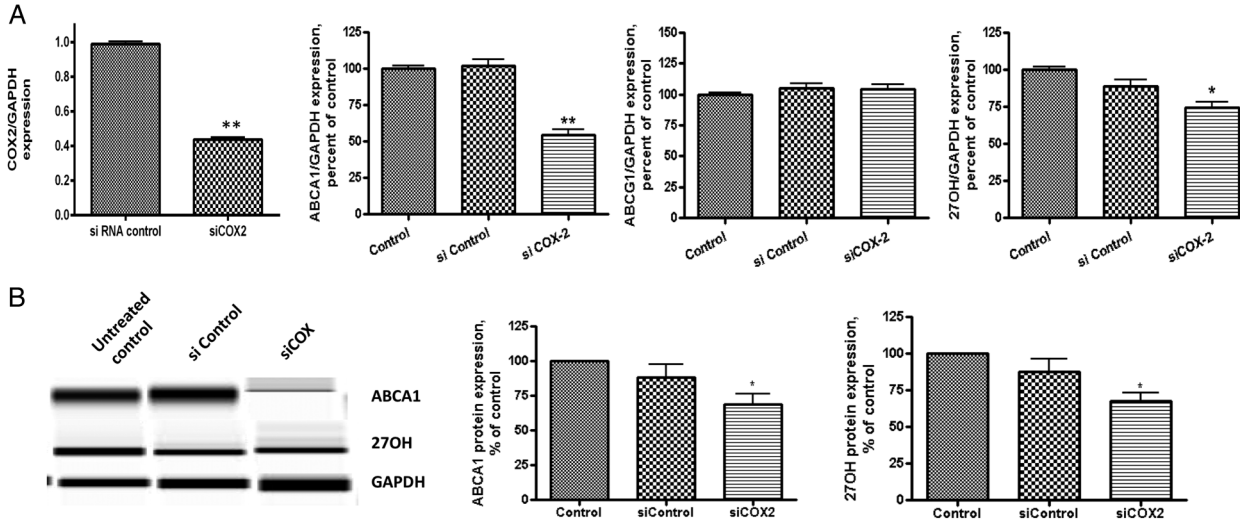

Figure 1 Effect of cyclo-oxygenase-2 (COX-2) silencing on cholesterol efflux protein expression in THP-1 macrophages. Introduction of siCOX2 into THP-1 macrophages inhibits gene (A) and protein (B) expression of the ABCA1 transporter and 27-hydroxylase (27-OH) in THP-1 macrophages. Values are means±SEM of three independent experiments. *p<0.05, **p<0.01 versus siControl.

Western blot (figure 2C). Incubation of monocytes/macrophages with naproxen or acetaminophen did not significantly change expression of cholesterol efflux proteins other than ABCA1 after exposure to 10 μM naproxen (123.5±9.4% vs solvent control). Similar to the selective COX-2 inhibitor NS398, celcoxib and rofecoxib decreased expression of 27-hydroxylase to 69.6±1.6% and 82.9 ±7.9%, respectively, versus solvent control in THP-1 macrophages (figure 2B).

When tested at 10 μM in human PBMC, COX-2 inhibitors did not significantly affect expression of proteins involved in cholesterol efflux. However, rofecoxib, at 10 μM concentration, downregulated expression of LXRα, indicating a possible LXRα-dependent change of cholesterol efflux in these cells in long-term and high-concentration exposure.

These results were confirmed by significant inhibition of cholesterol efflux to ApoA1 in the presence of celecoxib, but not naproxen (figure 2D). In THP-1 macrophages labeled with ^{3}H cholesterol, celecoxib significantly downregulated ABCA1-mediated efflux to ApoA1. Thus, the presence of celecoxib (25 μM) significantly inhibited efflux to 4.36% of cholesterol release from cells versus 6.24% ($p \leq 0.0005$, n=3) of solvent control. The presence of naproxen did not affect cholesterol efflux in the same cell system (4.47% vs 5.46%; p=0.29, n=3) (figure 2D).

The effect of pain medication on the expression of scavenger receptors

We examined the effect of COX-2 inhibition on expression of major scavenger receptors (ScR) responsible for cholesterol uptake. Celecoxib was the only COX-2 inhibitor that had a significant impact on the expression of scavenger receptors. Thus, we observed changes in the expression of CD36 and LOX1 to 319.0±120.0% and 55.8%±12.9%, respectively, versus control, but not on SR-A1 (figure 3).

First, we tested the effect of COX-2 inhibitors at 5 μM concentration (similar to the plasma level achieved after administration) for celecoxib, rofecoxib, naproxen and acetaminophen at 1 mM in THP-1 monocytes/macrophages and PBMC. Only celecoxib induced an increase in CD36 gene (167.4±8.78%) (n=5, p<0.05) and protein (to 183 ±78%) (n=5, p<0.05) expression versus solvent control set at 100%. No significant changes in the expression of other scavenger receptors were found.

When tested in THP-1 macrophages at higher concentrations (10 and 25 μM), a significant augmentation of CD36

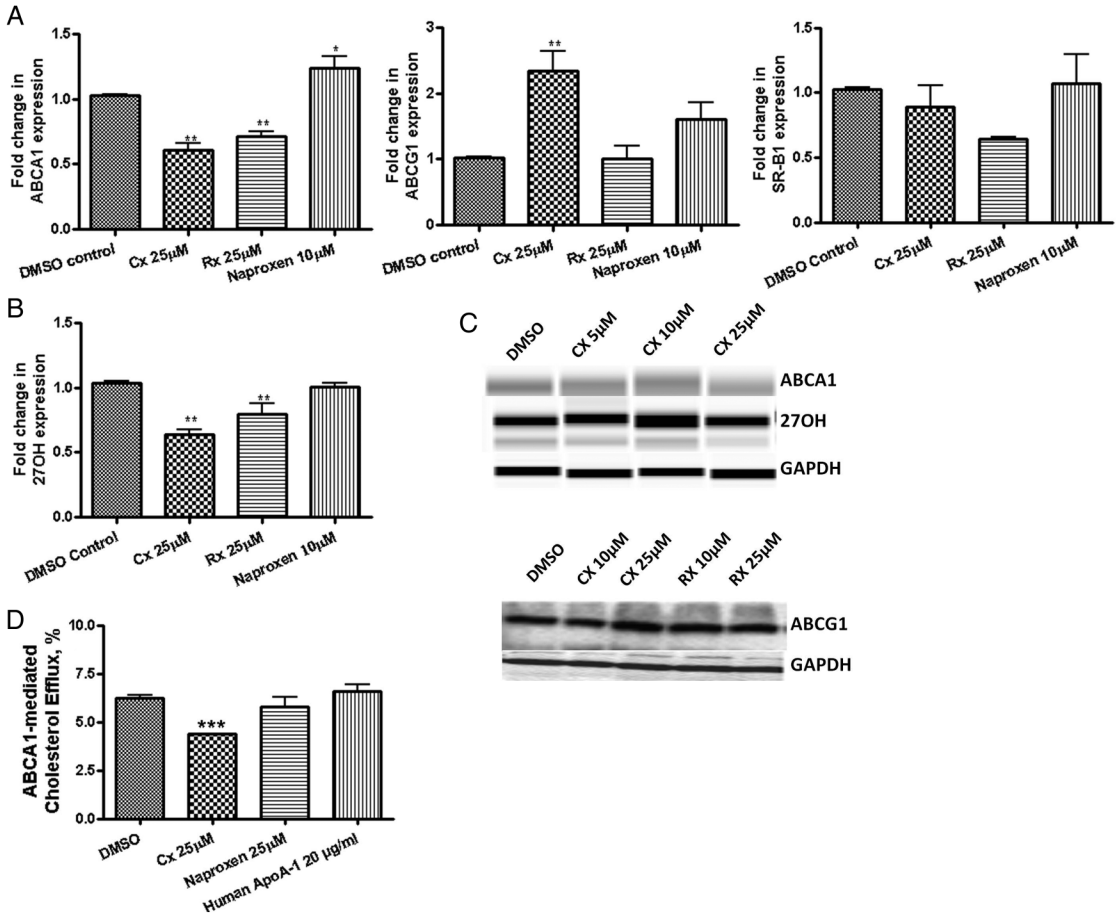

Figure 2 Effect of pain medications on cholesterol efflux protein expression and net cholesterol efflux in THP-1 macrophages. Cyclo-oxygenase-2 inhibitors inhibit gene (A) and protein (B) expression of the ABCA1 transporter and 27-hydroxylase (27-OH) in THP-1 macrophages. Celecoxib augments mRNA level, but not protein expression of the ABCG1 transporter. Celecoxib, but not naproxen, decreases net flux to apoA-1 (C). Values are means±SEM of three independent experiments. *p<0.05, **p<0.01, ***p<0.001 versus solvent control.

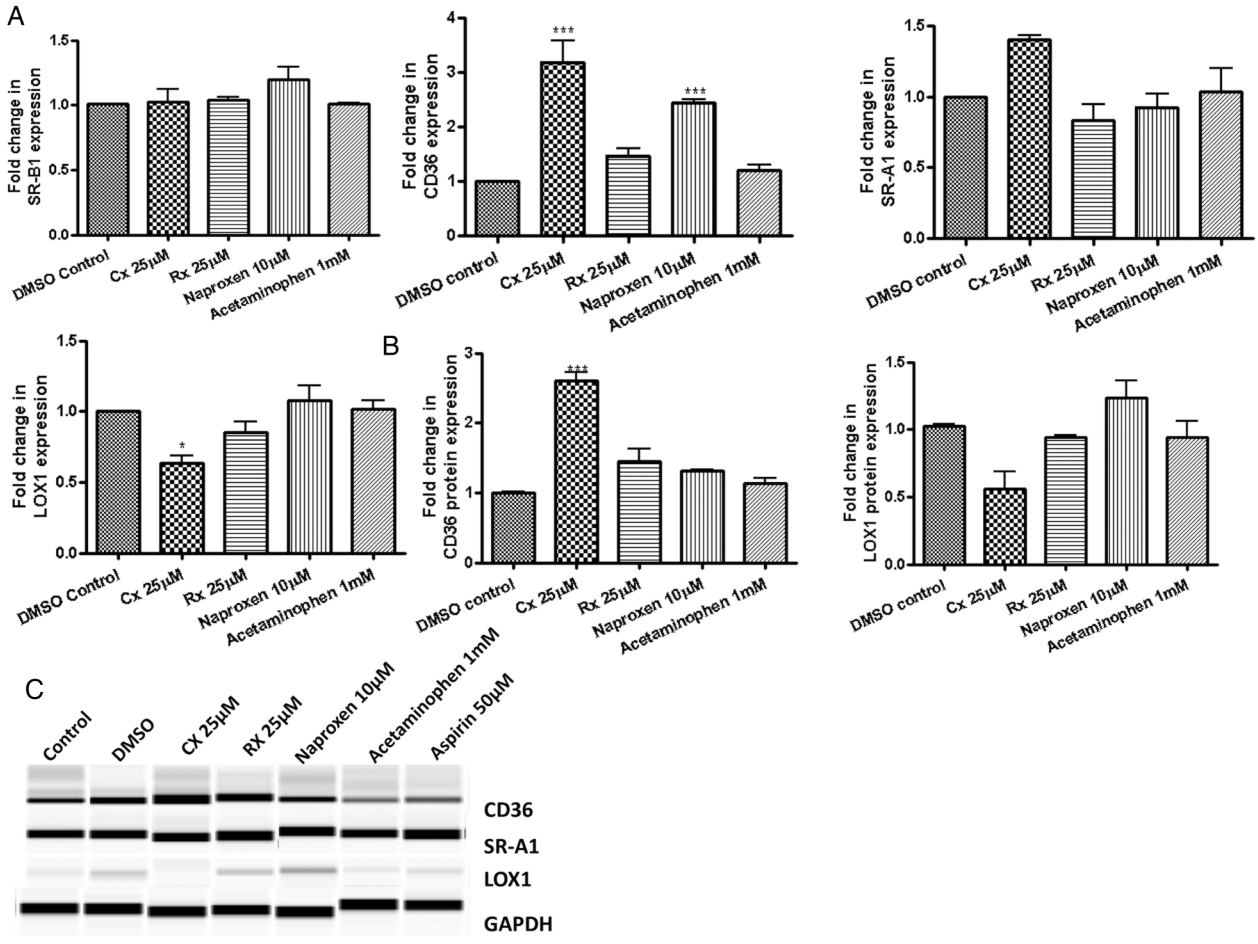

Figure 3 Effect of pain medications on the expression of major scavenger receptors: SR-A1, SR-B1, CD36 and LOX-1. Cyclo-oxygenase-2 (COX-2) inhibitors did not affect expression of SR-A1 and SR-B1. Celecoxib, among all COX-2 inhibitors, amplified CD36 and decreased LOX-1 message (A) and protein (B,C). Naproxen augmented CD36 mRNA level, but not protein expression *p<0.05; ***p<0.001 versus solvent control.

message was detected in the presence of celecoxib and naproxen (3.2±1.2 and 2.2±0.2-fold above solvent control, respectively) (n=5, p<0.001) (figure 3A). Celecoxib consequently increased CD36 protein expression (2.2±0.24) in macrophages (n=5, p<0.05). Naproxen failed to increase CD36 protein level significantly above solvent control (figure 3B, C). Celecoxib, but not rofecoxib, decreased LOX1 mRNA levels to 0.63±0.07 (n=3, p<0.05) and protein expression to 0.56±0.2 (NS) versus solvent control. SR-A1 level did not differ from solvent control in the presence of COX-2 inhibitors. Similar results were obtained when COX-2 inhibitors were tested in PBMC.

COX-2 inhibition with celecoxib, but not rofecoxib, enhanced oxLDL uptake and macrophage foam cell transformation

Celecoxib, but not other COX-2 inhibitors, concentration dependently increased FCF of THP-1 differentiated macrophages. Thus, macrophages exposed to celecoxib displayed augmented FCF: from 1.25-fold increase versus solvent control at 10 μM to 2.1-fold at 25 μM (n=3, p<0.05) (figure 4A). Similarly, inhibition of COX-2 in human MDM with celecoxib and AACOCF3 (an inhibitor for phospholipase A2) elevated FCF compared with untreated cells (figure 4B).

COX-2 inhibition with rofecoxib, but not celecoxib, promoted THP-1 monocyte adhesion to non-inflammatory endothelial cells

We investigated the role of COX-2 inhibition on adhesive properties of THP-1 monocytes to non-inflammatory endothelium. An adhesion assay of fluorescently stained monocytes exposed to specific and non-specific COX-2 inhibitors was employed. This assay revealed significant differences in the effect of COX-2 inhibition with celecoxib and rofecoxib (figure 5). Thus, celecoxib did not alter the adhesive properties of the monocytes. In contrast, rofecoxib twofold increased adhesion of monocytes (n=5, p<0.01) versus solvent control. Similarly, naproxen-treated cells displayed 1.6 times increased adhesion compared with solvent control (n=5, p<0.05).

The effect of COX-2 inhibition by celecoxib and rofecoxib on multiple genes contributing to atherosclerosis progression

We evaluated the impact of COX-2 inhibition by celcoxib and rofecoxib (10 μM) on the expression of multiple genes

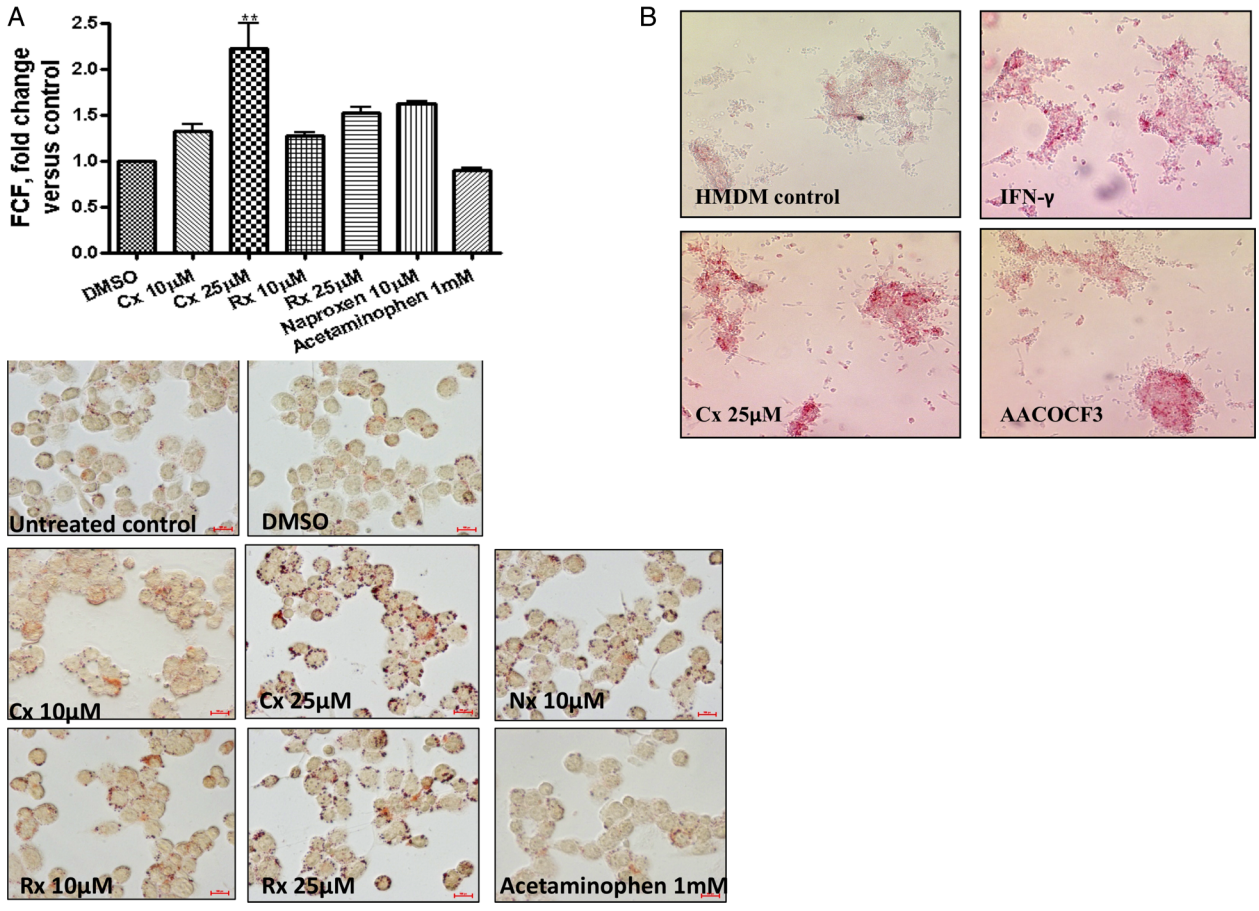

Figure 4 Effect of pain medications on foam cell formation (FCF) in THP-1 macrophages and peripheral blood mononuclear cells (PBMC). Representative photomicrographs at magnification ×40 of oil-red-O staining to detect foam cells in THP-1 human macrophages (A) and PBMC (B). FCF was quantified in oil-red-O stained cells with comparison as fold-difference from cells treated with solvent control (DMSO), which were set at 1.0. All results are expressed as means±SEM of three independent experiments. **p<0.01 versus solvent control. In human PBMC, effects of cyclo-oxygenase-2 inhibition (celecoxib) and cPLA2 inhibition (AACOCF3) were investigated (B).

related to atherosclerosis development in THP-1 human macrophages using the Atherosclerosis RT2 Profiler PCR Array. This array includes genes involved in lipid transport and metabolism, blood coagulation, cell adhesion, transcriptional regulation and inflammatory response (figure 6).

None of the COX-2 inhibitors displayed a significant effect on the expression of ABCA1, ApoA1, ApoB or ApoE (figure 6A). Surprisingly, celecoxib and rofecoxib differed in their effect on expression of the LDL receptor. Thus, celecoxib significantly downregulated expression of the LDL receptor to 0.48-fold (n=3, p<0.001). In contrast, rofecoxib upregulated it to 1.84 (n=3, p<0.05) versus solvent control. Both COX-2 inhibitors increased expression of lipoprotein lipase (LPL) twofold over solvent control (n=3, p<0.05) (figure 6A). The expression of lipoprotein (a) (LPA) was 0.5-fold decreased on exposure to rofecoxib and was not significantly changed in the presence of celecoxib.

None of the COX-2 inhibitors significantly altered expression of transcriptional factors (figure 6B).

We documented celecoxib and rofecoxib effects on coagulation parameters (figure 6C). Celecoxib caused a twofold increase in expression of endoglin, a transmembrane auxillary receptor for transforming growth factor-β, serpin peptidase inhibitor, clade E (SERPINE1) (plasminogen activator inhibitor type 1), and angiotensin I-converting enzyme (ACE) (n=3, p<0.05). Rofecoxib increased expression of SERPINE1 and ACE as well, reaching 1.45 and 1.55, respectively (n=3, p<0.05). In addition, rofecoxib upregulated expression of fibronectin to 1.73 times control (n=3, p<0.05).

COX-2 inhibition notably influenced the NFK-β-mediated cell response, decreasing expression of NFK-β (figure 6D). However, celecoxib and rofecoxib had a different effect of the expression of tumor necrosis factor (TNF-α). Thus, celcoxib reduced its expression, and reofecoxib increased its expression twofold (n=3, p<0.05). In addition, celecoxib significantly increased expression of IL-1α and eNOS (n=3, p<0.05). Rofecoxib, but not celecoxib, affected release of chemokine (C–C motif) ligand 5 (CCL5 or RANTES) and CCL2 or MCP-1, increasing their expression 1.45 and 1.73-fold over solvent control, respectively (n=3, p<0.05).

Celecoxib and rofecoxib had distinctly different effects on the expression of adhesion molecules (figure 6E): celecoxib reduced to 0.25-fold expression of vascular cell adhesion protein (VCAM) and to 0.2-fold leucocyte-endothelial adhesion molecule-1 (LECAM-1) (n=3, p<0.05). In contrast, rofecoxib 1.63-fold increased expression of vascular

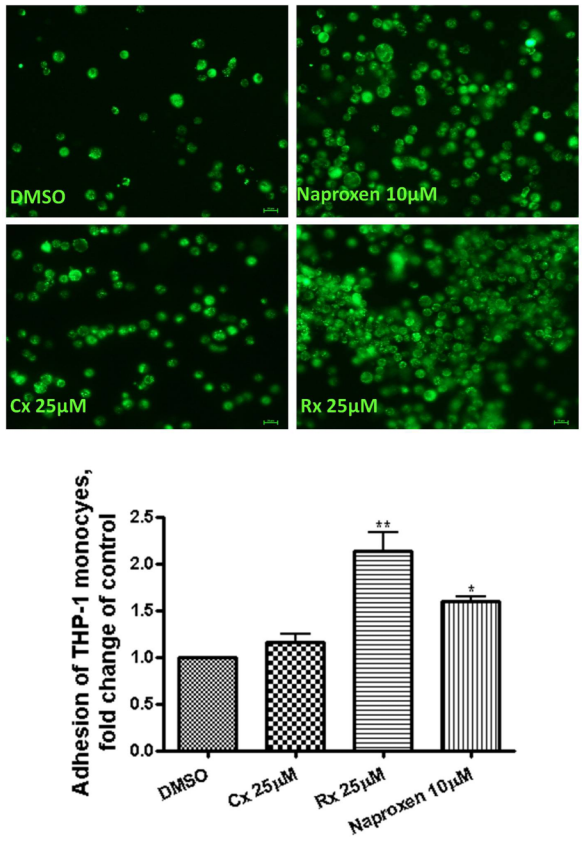

Figure 5 Effect of pain medications on adhesion properties of THP-1 monocytes. Fluorescently marked THP-1 monocytes were exposed to specific and non-specific cyclo-oxygenase-2 inhibitors, followed by incubation in HUVEC layered culture dishes (18 hours). Cells were analyzed and photographed using a Nikon A1 microscopy unit with ×40 magnification (A). Quantitative analysis was achieved by averaging total fluorescence in three random fields of a view for each sample. Average intensity for solvent control was set as 1.0. Adhesion for other conditions was calculated as fold increase/decrease versus solvent control and graphed (B). *p<0.05; **p<0.01 versus solvent control.

endothelial growth factor A (VEGFA) and 1.43 of its receptor (VEGFR) in THP-1 macrophages (n=3, p<0.05).

DISCUSSION

Atherosclerosis, an underlying cause of myocardial infarction and stroke, remains the leading cause of death in developed countries.[20 21] It is a chronic inflammatory condition and a disorder of lipid metabolism. The study presented here shows an additional rationale for cardiovascular harm from COX-2 inhibitors: disruption of cholesterol transport through effects on expression of critical genes involved in this process in macrophages.

We postulate that administration of COX-2 inhibitors could stimulate a proatherogenic cholesterol transport profile in human monocytes/macrophages through COX-2-dependent and independent mechanisms (figure 7). Thus, at low concentration of COX-2 inhibitors, representing the level they reach in the bloodstream, inhibition of COX-2 leads to alteration in the expression of scavenger receptor CD36. This receptor is a key receptor involved in oxLDL binding, internalization, and cholesterol ester accumulation. All these events are critical points for triggering of macrophage transformation into foam cells. However, we have not observed any other significant changes in macrophages, when a low concentration was used.

Our studies involve exposure of cells to COX-2 inhibitors over only a matter of hours, while duration of use in persons who become susceptible to cardiovascular events extends over months or years.[7] Cell culture systems come with clear limitations and by using higher doses, we are compressing the timeline for effect. Only future in vivo studies can clarify this issue.

As a confirmation of the idea that high concentrations are a reasonable model of prolonged use, only COX-2 inhibition by siRNA or celecoxib and rofecoxib at high concentrations leads to inhibition of cholesterol efflux via decrease in the expression of ABCA1 and 27-hydroxylase. When COX-2 gene expression was knocked-down, expression of another transporter—ABCG1—was neither directly suppressed, nor increased as a compensatory adaptation. This lack of change in ABCG1 could be explained by the partial rather than complete knockdown of COX-2 expression in our experiment, leaving some residual activity (figure 1). When high concentrations of celecoxib, but not rofecoxib, were used, presumably with complete inhibition of COX-2 activity, we observed a compensatory amplification of ABCG1 transporter simultaneously with downregulation of ABCA1 (figure 2). Such differences might reflect multiple mechanisms of regulation of ABCA1 and ABCG1 transporters in human macrophages.[22] Neither naproxen nor acetaminophen affected any of these genes.

We believe that the use of high concentrations of COX-2 inhibitors in our experiments is relevant since it reflects long-term administration of these medications as an analgesic. Moreover, there are in vivo data to support the theory of intracellular accumulation of celecoxib,[23] which is even more likely with long-term administration in persons who experience elevated risk of myocardial infarction. However, these changes in cholesterol efflux at high concentrations might reflect involvement of non-COX-2-inhibiting offtarget effects as well.[24]

Differences between celecoxib and rofecoxib become apparent on deeper comparison, using high concentrations. Only celecoxib impacted the expression of scavenger receptors: CD36 and LOX1. Consequently, FCF was elevated in THP-1 human macrophages exposed to celecoxib.[25] Interestingly, the expression of the LDL receptor was decreased by celecoxib and enhanced by rofecoxib, as demonstrated by PCR array. NSAIDs are known to be PPAR-α and PPAR-γ agonists. However, at the time and concentration points acquired by the PCR array, we found no significant changes in expression of any transcription factors (figure 6B). This might reflect a complex and multipoint response in the expression of different subtypes of PPAR. Moreover, we have to account for PPAR-independent mechanisms of regulation of the CD36 receptor.[26]

Another distinct point of difference between rofecoxib and celcoxib was their effect on adhesion of monocytes to endothelial cells, an early and critical event in atherosclerotic plaque formation. Adhesion may be accelerated by rofecoxib or naproxen, but not celecoxib. This effect corresponds to a 1.63-fold increased expression of VEGFA and

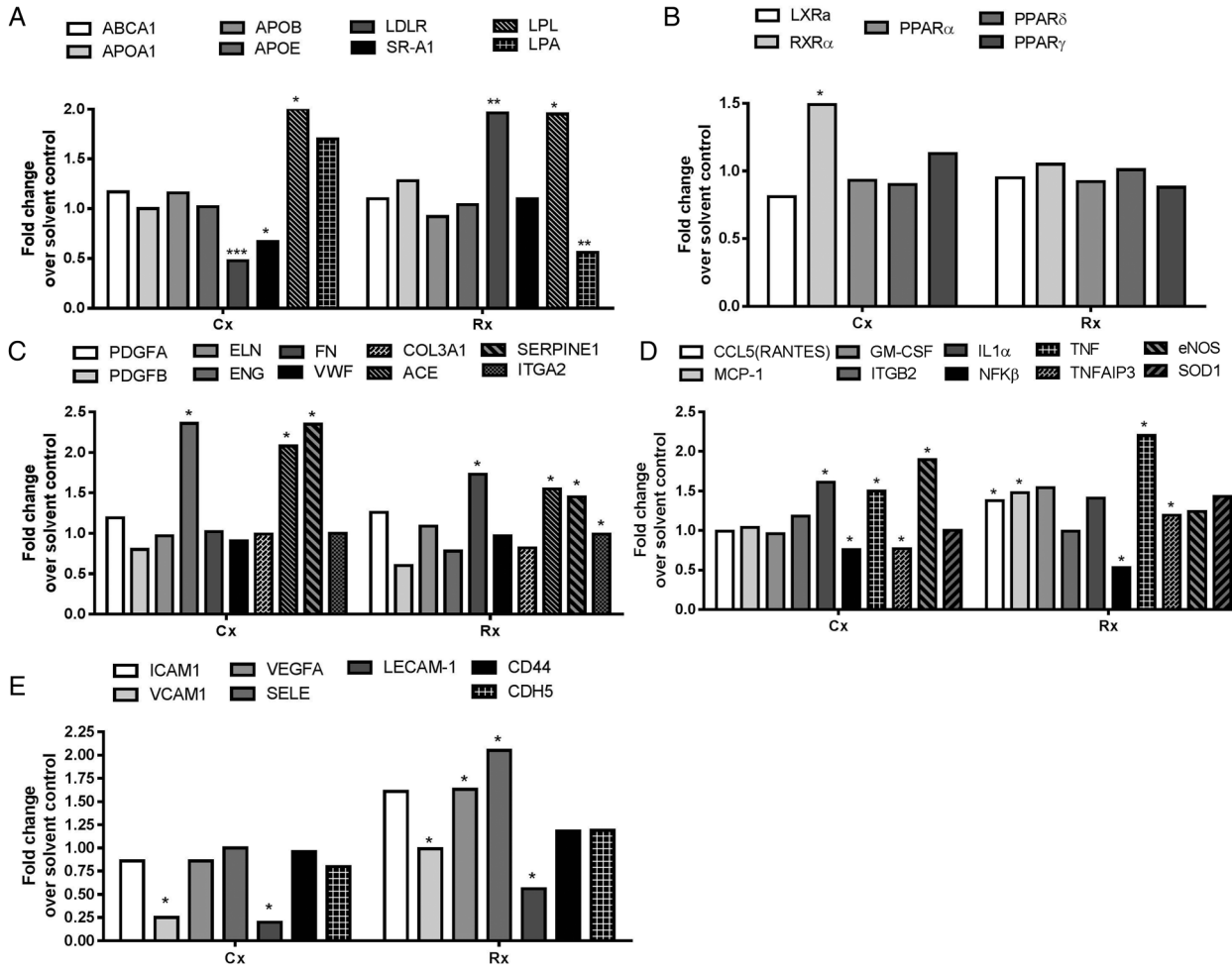

Figure 6 Cyclo-oxygenase-2 inhibition with celcecoxib and rofecoxib and expression of genes related to atherosclerosis. Gene expression analysis performed with RT2 Profiler PCR Array in THP-1 macrophages exposed to celcoxib and rofecoxib (10 μM). This array reflects changes in gene expression of proteins involved in lipid transport and metabolism (A) with corresponding regulation of transcription (B), blood coagulation (C), inflammatory response (D), and cell-to-cell adhesion (E). All results are means for three independent experiments. *p<0.05; **p<0.01 versus solvent control. (A) ABCA1, ATP-binding cassette transporter A1; APOA1, apolipoprotein A–I; APOB, apolipoprotein B; APOE, apolipoprotein E; LDLR, low-density lipoprotein receptor; SRA1, macrophage scavenger receptor A 1; LPL, lipoprotein lipase; LPA, lipoprotein (a); (B) LXR α, liver X receptor α; RXRα, retinoid X receptor α; PPARα, peroxisome proliferator-activated receptor α; PPARδ, peroxisome proliferator-activated receptor δ; PPARγ, peroxisome proliferator-activated receptor γ; (C) PDGFA, platelet-derived growth factor α; PDGFB, platelet-derived growth factor β; ELN, elastin; ENG, endoglin; FN, fibronectin; VWF, Von Willebrand factor; COL3A1, collagen α-1(III) chain; ACE, angiotensin I-converting enzyme; SERPINE1, serpin peptidase inhibitor, clade E; ITGA2, integrin α 2; (D) CCL5 (RANTES), chemokine (C–C motif) ligand 5; MCP1, monocyte chemotactic protein-1; GM-CSF, colony stimulating factor 2; ITGB2, integrin β 2; IL1α, interleukin 1 α; NFKβ, nuclear factor κ-light-chain-enhancer of activated B cells; TNF α, tumor necrosis factor α; TNFAIP3, tumor necrosis factor, α-induced protein 3; eNOS, endothelial nitric oxide synthase; SOD1, superoxide dismutase 1; (E) ICAM1, intercellular adhesion molecule 1; VCAM1, vascular cell adhesion molecule 1; VEGFA, vascular endothelial growth factor A; SELE, selectin E; LECAM1, leucocyte-endothelial adhesion molecule-1.

a 1.43-fold increased expression of its receptor (VEGFR) in THP-1 macrophages, induced by rofecoxib (figure 6E). The difference in this regulation may lay in triggering of COX-2 independent pathways and indicate that these compounds have unique properties, despite being in the same drug class. Several previous papers have noted significant differences between the two drugs as well.[27 28]

Our results are consistent with previous work showing no effect of celecoxib on monocyte adhesion[29 30] and with a study showing that rofecoxib treatment of patients with squamous cell carcinoma increased monocyte adhesion.[31] Such differences might provide an explanation for cardiovascular risk on administration of rofecoxib and celecoxib.[32]

It is important to point out that celecoxib and rofecoxib are distinct molecules that may possess different COX-2 inhibitory properties. Celecoxib's COX-2-mediated augmentation of scavenger receptors, even at low concentrations, leads to a more profound effect on cholesterol efflux when concentration is high. An exhaustive analysis of the differences between the two drugs is beyond the scope of this paper. However, we believe that our report lays the groundwork for future in vivo and in vitro studies as it highlights important points for investigation.

Cardiovascular hazard from COX-2 inhibitors limits pain treatment choices. It has been hypothesized that selective COX-2 inhibition upsets the thrombotic equilibrium,

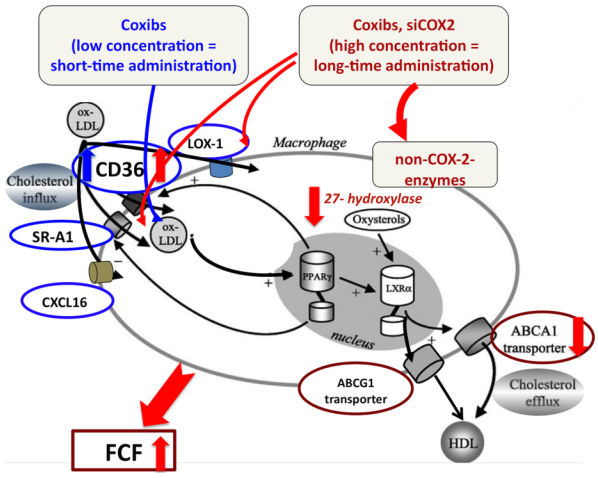

Figure 7 Scheme of cyclo-oxygenase-2 (COX-2) dependent or independent effect of COX-2 inhibitors on lipid handling in monocytes/macrophages. Celecoxib at low concentrations (5 μM), representing the level they reach in the bloodstream, increases expression of CD36 scavenger receptor, elevating uptake of oxLDL. Knockout of COX2 and exposure of macrophages to high concentrations (10 and 25 μM) of COX-2 inhibitors, in addition to increased CD36 expression, compromises outflow of cholesterol, decreasing expression of ABCA1 transporter and 27-hydroxylase. Such imbalance in lipid processing enhances foam cell formation, a crucial early event in atheroma development.

creating an imbalance between antithrombotic and prothrombotic factors. COX-2 protein levels are elevated in endothelial cells, smooth muscle cells, and macrophages in human atherosclerotic lesions, perhaps as a defensive mechanism.[33 34] Heightened cardiovascular risk can also be seen with traditional NSAIDs.[35]

The FDA recently strengthened its warning with regard to NSAIDs, requiring a change in labeling that will make clear that risk for myocardial infarction and stroke can increase even with short-term use and that risk level is dose-dependent (http://www.fda.gov/safety/medwatch/safetyinformation/safetyalertsforhumanmedicalproducts/ucm454141.htm). In light of these warnings, the decision to use these drugs and the choice among multiple medications may be increasingly difficult. NSAIDs and COX inhibitors relieve pain and inflammation. They may be used over long periods of time; for months or years to treat chronic pain.

The problem is particularly complex in patients with autoimmune rheumatic diseases such as rheumatoid arthritis, psoriatic arthritis and systemic lupus erythematosus. These patients often suffer with debilitating pain and achieve some relief with NSAIDs and/or COX inhibitors. Despite advances in treatment, cardiovascular morbidity in this rheumatic disease cohort is enhanced compared with the general population.[36] Therefore, the proinflammatory and proatherogenic background of these patients merits consideration when choosing pain medications.

It is hoped that this study will provide insight into the unique atherogenic properties of individual COX-2 inhibitors and NSAIDs, so that further work can be performed to distinguish the pros and cons of the various medications available and, perhaps, to develop new compounds with a more favorable profile.

Contributors IV, ABR, and SEC contributed to conception and design of research; IV and MJL performed experiments; IV, LJK, and ABR analyzed data; IV, SEC, JDL, LJK, and ABR interpreted results of experiments; IV prepared figures; IV, LJK, and ABR drafted manuscript; SEC, JDL, and IHG edited and revised manuscript; SEC, JDL, and ABR approved final version of manuscript.

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