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Apoptotic Cell-Derived Sphingosine-1-Phosphate Promotes HuR-Dependent Cyclooxygenase-2 mRNA Stabilization and Protein Expression

Axel M. Johann,* Andreas Weigert,* Wolfgang Eberhardt,† Anne-Marie Kuhn,* Vera Barra,* Andreas von Knethen,* Josef M. Pfeilschifter,‡ and Bernhard Brüne²*

Removal of apoptotic cells by phagocytes is considered a pivotal immune regulatory process. Although considerable knowledge has been obtained on the postphagocytic macrophage phenotype, there is little information on molecular mechanisms, which provoke macrophage polarization. In this study, we show that human apoptotic Jurkat cells (AC) or AC-conditioned medium (CM) rapidly induces cyclooxygenase-2 (COX-2) expression in mouse RAW264.7 macrophages via sphingosine-1-phosphate (SIP). Pharmacological inhibition of SIP release from AC or using CM from cells with a knockdown of sphingosine kinase 2 in human MCF-7 cells abrogates this effect. Expression of COX-2 resulted from an increase in mRNA stability via its 3′-untranslated region (UTR), shown by COX-2-3′-UTR and AU-rich element-driven reporter assays. Western analysis corroborated increased nucleocytoplasmic shuttling of the RNA-binding protein HuR after CM treatment. RNA EMSA analysis revealed an SIP- and CM-mediated increase in HuR-RNA binding to a COX-2-specific UTR, whereas HuR knockdown pointed to its importance for SIP in CM-induced COX-2 expression. Immunofluorescence microscopy of phospholipase A₂ (PLA₂) and ELISA analysis of PGE₂ revealed activation of PLA₂ and production of PGE₂ in response to CM but not SIP. SIP, released from AC, uses HuR to stabilize COX-2 mRNA and thus to increase COX-2 protein expression. However, only CM also activates PLA₂ to provide the substrate for COX-2.


C

tells entering the route of programmed cell death, i.e., apoptosis, are rapidly recognized and removed by professional phagocytes. It is commonly believed that uptake of dying cells before they lose their cell membrane integrity averts an inflammatory response in the surrounding tissue by preventing contact of neighboring cells with potentially proinflammatory cellular constituents of dying cells, such as DNA or proteases (1, 2). Lately, it became apparent that phagocytosis of apoptotic cells (AC) by macrophages is an immune regulatory process that shapes the pro- vs anti-inflammatory cellular macrophage phenotype and thereby provokes cell desensitization. This is characterized by, among other factors, the release of anti-inflammatory mediators such as TGF-β or PGE₂, combined with suppression of proinflammatory cytokine production (2–4). Furthermore, recognition of AC by macrophages attenuates the production of radicals such as NO (5) or superoxide, with the latter mechanism requiring activation of peroxisome proliferator-activated receptor-γ (6). Recently, it was shown that sphingosine-1-phosphate (SIP) is released from AC via sphingosine kinase 2 (SphK2) to promote macrophage survival (4). Interestingly, SIP has also been identified to induce cyclooxygenase 2 (COX-2) (7). Although the clearance of AC can be considered an immune regulatory process, it seems not to be species specific because major determinants for recognition of AC are conserved between human and murine cells (8, 9).

COXs are cardinal enzymes for the production of prostanooids such as PGE₂. Two COX isoenzymes are known (COX-1 and COX-2). In addition, several COX-splicing variants are reported, such as COX-3 in the cerebrum or “partial COX” (PCOX)-1a, which remains inactive because its lacks the catalytic domain (10). COX-1 as well as COX-2 are regulated via transcription/translation (10, 11). An oversimplification implies that COX-1 is constitutively expressed and/or is involved during differentiation. In contrast, COX-2 responds to a number of hormones, growth factors, cytokines, or physical stress with immediate protein expression, often associated with inflammatory responses (11). A further regulatory mechanism, established for COX-2, is the posttranscriptional modification of mRNA stability (11–13). The COX-2 gene contains a large 3′-untranslated region (3′-UTR), which includes several conserved regions and 22 copies of an AUUUA motive (14), which can be targeted by several mRNA-bounding factors. Posttranscriptional regulation of gene expression via mRNA stability regulation is a fast and tightly regulated mechanism, under the control of mRNA-stabilizing and -destabilizing proteins, which target their distinct mRNA motives (15). Several of these factors

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3 Abbreviations used in this paper: AC, apoptotic Jurkat cell; SIP, sphingosine-1-phosphate; SphK2, sphingosine kinase 2; COX, cyclooxygenase; UTR, untranslated region; CM, conditioned medium; Cyt D, cytochrome D; ARE, AU-rich element; wt, wild type; mt, mutant; DAPI, 4′,6-diamidino-2-phenylindole; DMS, dimethylsphingosine; VC, viable cell; NC, necrotic cell; siRNA, small interfering RNA.

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have been characterized. Specifically, in RAW 264.7 cells, the involvement of mRNA-binding proteins, such as TIA1, AU1-F, HuR, and TIA-1, has been suggested to affect COX-2 mRNA stability via interaction with its 3' UTR (13, 16-18).

In macrophages, the production of PGE2, following recognition of AC is thought to play a crucial role in desensitization (3). Indeed, COX-2 is thought to play a crucial role in desensitization (3). Indeed, only CM also activates phospholipase A2 (PLA2) and thus modulating macrophage immune responses, it was our intention to characterize molecular mechanisms provoking COX-2 expression in macrophages upon contact with AC. AC or conditioned medium (CM) from AC elicited a fast increase in COX-2 mRNA and protein as well as PGE2 production in RAW264.7 macrophages. We propose that SIP, released from AC, activates nucleocytoplasmic shuttling of the mRNA-stabilizing protein HuR, which in turn stabilizes COX-2 mRNA and elicits COX-2 protein expression. However, only CM also activates phospholipase A2 (PLA2) and thus delivers substrate to COX-2 with concomitant PGE2 synthesis.

Materials and Methods

Materials

Staurosporine and cytochalasin D (Cyt D) were purchased from Sigma-Aldrich. Murine rIFNγ was obtained from Roche Diagnostics. Culture supplements and FCS were ordered from PAA Laboratories. Oligonucleotides were synthesized from Whatman-Biometra. The anti-COX-2 Ab was obtained from Upstate Biotechnology. Anti-HuR, -AUF-1, -HuB, and -cPLA2 Ab were ordered from Santa Cruz Biotechnology. The anti-actin Ab was purchased from Amersham Bioscience. The anti-COX-2 Ab was obtained from Upstate Biotechnology. Anti-HuR, -AUF-1, -HuB, and -cPLA2 Ab were ordered from Santa Cruz Biotechnology. RNA oligonucleotides were synthesized from Whatman-Biometra.

Cell culture

The mouse monocyte/macrophage cell line RAW264.7, MCF-7 breast carcinoma cells, and the human Jurkat T cell line were maintained in RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FCS. Cells where regularly tested to be free of mycoplasma. For induction of apoptosis, medium without FCS was used.

Human monocyte isolation and culture

To isolate human monocytes, we followed established protocols (21). Briefly, cells were isolated from 50 ml ofuffy coats (Deutsches Rotes Kreuz-Blutspendedienst Baden-Württemberg-Hessen, Institut für Transfusionsmedizin und Immunhämatologie, Frankfurt am Main, Germany). Blood was diluted 1/2 with PBS and layered on a Ficoll-Isopaque gradient (p = 1.077 g/ml). The interphase containing PBMC was obtained following centrifugation (445 x g, 35 min). Cells were recovered, washed in PBS, and allowed to adhere to culture dishes (Primaria 3072, BD Biosciences) for 1 h at 37°C. Nonadherent cells were removed. The medium was changed to fresh RPMI 1640 containing 10% heat-inactivated human AB serum (Sigma-Aldrich) and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin). Monocytes (5 x 10⁶) were cultured in a volume of 10 ml/plate at 37°C, 5% CO₂ in a humidified atmosphere. Medium was changed every 2-3 days. After 7 days of culture, monocytes acquired a macrophage-like phenotype (22) and were used for additional experiments.

Generation of apoptotic and necrotic cells

To generate AC Jurkat or MCF-7 cells (2.5 x 10⁶ cells/ml) were seeded in 10-cm dishes in RPMI 1640 without FCS, supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were incubated for 3 h with 0.5 μg/ml staurosporine and afterward were washed twice with medium. Necrotic cells were generated by heating 2.5 x 10⁶ Jurkat cells/ml for 30 min at 56°C. In all experiments, the ratio of apoptotic or necrotic cells to macrophages was kept at a ratio of 5:1. Apoptotic vs necrotic cell death was confirmed by flow cytometry, using Annexin VFITC/propiodipod iodine staining (Immunotech).

AC CM was obtained by incubating 2.5 x 10⁶ AC/ml of medium. After 2 h, cells were centrifuged for 15 min at 1000 x g. The supernatant was removed and passed through a 0.2-μm cellulose syringe filter (Roth). The filtrate was taken as AC CM. In the experiments using CM, culture medium was removed and replaced with CM.

To obtain AC CM without S1P (CM/dimethylsphingosine (DMS)), Jurkat cells were preincubated with 20 μM DMS, an inhibitor of sphingosine kinases. Following the addition of DMS for 4 h, Jurkat cells were stimulated with 0.5 μg/ml staurosporine in FCS-free medium for 3 h to initiate apoptosis, however, not allowing the formation of S1P. AC were washed two times, further incubated in full medium for 2 h, centrifuged, and the cell supernatant was filtered as described for the preparation of CM. The absence of S1P was confirmed by liquid chromatography tandem mass spectrometry determinations (4).

Generation of sphingosine kinase (SphK2) knockdown cells

For SphK2 knockdown, sequence-specific small interfering RNA (siRNA) oligonucleotides (forward 5'-GATCCAACTAACAAGCATTGTTACCC AAGAGATGTTCAACGTGTTGTTATA 3', reverse: 5'-AGCTTAA ACTAACCAGGTGTTACCTCCTTGAAAGGTCAACAGCTGTTTAG TG-3') were ligated into the pSilencer 4.1-CMV neo vector (Ambion) according to the manufacturer’s instructions. pSilencer-siSphK2 was then transfected into MCF-7 cells using Nucleofector technology (Amaxa) as described previously (23).

PGE2 ELISA

PGE2 ELISA was performed with the PGE2 ELA kit from Cayman Chemical, according to the manufacturer’s manual. The mid-detection range of the assay is 10–1000 pg/ml. If required, samples containing PGE2 were diluted. The lower detection limit was set to 10 pg/ml.

Western blot analysis

Expression of COX-2, HuR, and actin were quantified by Western analysis. Briefly, following individual incubations, cells were washed twice with ice-cold PBS, scraped off, lysed in 200 μl lysis buffer A (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1 mM PMSF, 1 μg protease inhibitor mix (Roche), pH 7.5), incubated on ice for 15 min, sonified, vortexed, and kept on ice for 20 min, followed by centrifugation (15,000 x g, 15 min). Proteins (100 μg/sample) were resolved on 10% SDS-polyacrylamide gels and blotted onto nitrocellulose by a semidry transfer cell. Goat anti-COX-2 (1/1,000), mouse anti-HuR (1/1,000), or rabbit anti-actin Ab (1/2,000) was added and incubated overnight at 4°C. Afterward, nitrocellulose membranes were washed three times for 5 min each with Tween 20 and TBS. For protein detection, blots were incubated with a HRP-labeled sheep anti-mouse secondary Ab (1/2,000), HRP-labeled goat anti-rabbit secondary Ab (1/2,000), or donkey anti-goat Ab (1/2,000) followed by ECL detection. For the generation of COX-2-positive controls (Fig. 1a), IFN-γ was added to the culture medium at a final concentration of 100 U/ml.

RNA extraction and quantitative real-time PCR

RNA from RAW264.7 macrophages was extracted using peqGOLD RNApure (Puelab), according to the protocol supplied by the distributor. For reverse transcription reactions of mouse COX-2 and β₂-microglobulin transcripts, we used the Advantage reverse transcription-for-PCR kit (BD Clontech/BD Biosciences). Quantitative real-time PCR was performed using the MyiQ real-time PCR system (Bio-Rad) and the Absolute QPCR SYBR Green Mix (Abgene) according to the manufacturer’s instructions. Sense and antisense primer (Metabion) sequences and PCR product sizes were as follows: murine COX-2, Taqman probe: 60°C; 5'-ATG TTC TCT CGA GCT GTG CTG C-3'. 5'- ATG CTG GGA GGT GCC CAG TCA-3'; 448 bp; β₂-microglobulin, Taq: 52°C; 5'-ACT CAG GCC CCT GTA TGC-3'; 5'-AGA CGG CCT TGG GCT CG-3'; 298 bp. Annealing temperatures were calculated using the primer design program Oligo (MBI). Controls of isolated RNA omitting reverse transcription during PCR were used to guarantee genomic DNA-free RNA preparations (data not shown). Quantification of real-time PCR results was performed using the Gene Expression Manager (version 1.1) from Bio-Rad, taking β₂-microglobulin expression as the internal control.

COX-2 AU-rich element (ARE) and 3'-UTR luciferase reporter gene assay

For reporter activity plasmids (provided by D. Dixon, University of Utah, Salt Lake City, UT) which contain the full-length COX-2 and β₂-microglobulin transcripts, we used the Advantage reverse transcription-for-PCR kit (BD Clontech/BD Biosciences). Quantitative real-time PCR was performed using the MyiQ real-time PCR system (Bio-Rad) and the Absolute QPCR SYBR Green Mix (Abgene) according to the manufacturer’s instructions. Sense and antisense primer (Metabion) sequences and PCR product sizes were as follows: murine COX-2, Taqman probe: 60°C; 5'-ATG TTC TCT CGA GCT GTG CTG C-3'. 5'- ATG CTG GGA GGT GCC CAG TCA-3'; 448 bp; β₂-microglobulin, Taq: 52°C; 5'-ACT CAG GCC CCT GTA TGC-3'; 5'-AGA CGG CCT TGG GCT CG-3'; 298 bp. Annealing temperatures were calculated using the primer design program Oligo (MBI). Controls of isolated RNA omitting reverse transcription during PCR were used to guarantee genomic DNA-free RNA preparations (data not shown). Quantification of real-time PCR results was performed using the Gene Expression Manager (version 1.1) from Bio-Rad, taking β₂-microglobulin expression as the internal control.
FIGURE 1. COX-2 expression and PGE2 production in response to AC. a and b, RAW264.7 macrophages were incubated with AC (ratio 1:5), 100 U/ml IFN-γ, AC in combination with IFN-γ, or they remained untreated for times indicated. Western analysis was used to follow COX-2 expression as described under Materials and Methods. For statistical evaluation, densitometric analysis was performed. c, RAW264.7 macrophages were incubated with AC (ratio 1:5) for times indicated. Cells were harvested, mRNA was isolated, and real-time PCR analysis was performed as described in Materials and Methods. d, Primary human macrophages were incubated for 4 h with AC (ratio 1:5) or remained as controls. Western blot and real-time PCR analysis were performed. e, RAW264.7 macrophages were incubated with AC (ratio 1:5), a combination of AC and NS 398 (100 µM), or remained as controls for 24 h. PGE2 ELISA was performed as described in Materials and Methods. Data represent the mean ± SEM of at least three individual experiments (*, p ≤ 0.05 vs untreated controls).

Extraction of cytoplasmic proteins

Extraction of cytoplasmic proteins was accomplished as described previously (25). Briefly, cytoplasmic lysates were prepared from RAW264.7 macrophages by lysis in 300 µl of a hypotonic extraction buffer containing 10 mM HEPES (pH 7.6), 10 mM KCl, 0.1 mM EDTA, 1 mM EGTA, 1 mM DTT, and protease inhibitor mix (Roche). After a 15-min incubation on ice, 20 µl of Nonidet-P40 was added and vortexed following centrifugation at 13,000 × g and 4°C. The supernatant contained the cytosolic fraction. To achieve RNase-free conditions, all buffers used were prepared by preincubating the binding reaction for 30 min with different dilutions (1/1000; 1/100, 1/10, and 1/1) of a RNA oligonucleotide stock solution.

Supershift analysis was performed as described (25) by adding 200 ng of Ab, 15 min after the addition of the radioactively labeled RNA oligonucleotide and further incubations for 15 min at room temperature.

Immunofluorescence staining

Immunofluorescence staining was basically performed as described (28). To determine intracellular PLA2 localization, we seeded RAW 264.7 macrophages directly on coverslips. After 24 h, cells were treated as indicated and fixed on the slides by overnight incubations in 4% paraformaldehyde at 4°C. Thereafter, cells were permeabilized in PBS containing 0.5% Triton X-100 for 15 min. After a washing step in PBS, cells were incubated for 2 h with a 1/250 dilution of a mouse PLA2 Ab (Santa Cruz Biotechnology) at 4°C. After three 5-min washing steps with PBS, cells were incubated with a secondary goat anti-mouse Ab (1/250) labeled with Alexa Fluor 488 (Invitrogen Life Technologies) for 2 h at 4°C. Again, cells were washed three times with PBS and counterstained with 4',6'-diamidino-2-phenyldole (DAPI; 1 µg/ml in PBS for 15 min). After a final 5-min washing step in PBS, cells were covered with Vectashield mounting medium (Linaris) and a coverslip. PLA2 localization was determined using an AxioScope fluorescence microscope (Carl Zeiss; lens ×63/0.6 NA; ocular ×10) at room temperature, documented by a charge-coupled device camera (Carl Zeiss) and AxioVision Software (Carl Zeiss).

Statistical analysis

Each experiment was performed at least three times, and statistical analysis was performed using the two-tailed Student t test. Otherwise, representative data are shown.

Results

AC increase mRNA stability and subsequent protein expression of COX-2

In a first set of experiments, we examined the time dependency of COX-2 expression in RAW264.7 macrophages following the treatment with IFN-γ, an agonist known to stimulate COX-2 transcription/translation (29), compared with treatment with AC (Fig. 1a). Western analysis revealed the absence of COX-2 protein in untreated macrophages. When cells were exposed to 100 U/ml IFN-γ, COX-2 protein was absent at 2 h but became visible after 4 h. In contrast, stimulation of macrophages with AC allowed to detect COX-2 at 2 h, with a more pronounced expression at 4 h. Costimulation of RAW264.7 cells with AC and IFN-γ revealed slightly higher rates of COX-2 expression at the 4-h time point,
compared with IFN-γ and AC to express COX-2.

To determine the rate at which AC promote COX-2 expression, we followed protein accumulation within 2 h after stimulating macrophages with AC (Fig. 1b). COX-2 was absent in control cells but was seen 60 min after adding AC. Thereafter, protein expression further increased up to 120 min after AC addition. Fast COX-2 expression in response to AC may indicate posttranscriptional/translational control process.

To show that AC-induced COX-2 expression correlates with a fast increase in COX-2 mRNA, we performed real-time PCR (Fig. 1c). Exposing RAW264.7 macrophages time dependently to AC showed a roughly 3-fold increase in COX-2 mRNA at 60 min. At 90 min, the mRNA increase was ~20-fold and further increased to roughly 100-fold with incubations lasting 2 h. Data on mRNA expression supported the idea of an immediate response to AC, which resulted in a COX-2 mRNA and protein increase, thus making transcriptional regulation of COX-2 unlikely. COX-2 mRNA and protein expression was also evident in primary human macrophages incubated with AC for 4 h, which implied that the mechanism is conserved among species (Fig. 1d). To underscore the functional consequence of AC-mediated COX-2 expression, we measured macrophage PGE₂ production by ELISA. Exposing macrophages for 24 h to AC increased PGE₂ production to values ~2000 pg/ml, compared with control values of 30 pg/ml. The COX inhibitor NS 398 blocked PGE₂ production completely (Fig. 1e).

Taking into account that mRNA stabilization is an important feature of COX-2 regulation, we performed luciferase reporter assays either by using the COX-2-3'-UTR (pLuc plus 3'-UTR) or COX-2-specific ARE (pLuc plus ARE) luciferase reporter plasmids (Fig. 2a). In these plasmids, either the full-length COX-2-3'-UTR or the ARE-rich region of the COX-2-3'-UTR is fused to the luciferase gene (24). Luciferase expression therefore is indicative of increased 3'-UTR- or ARE-mediated COX-2 mRNA stabilization. RAW264.7 cells were transfected with either pLuc plus 3'-UTR or pLuc plus ARE and treated with AC for 2–24 h (Fig. 2, b and c). With both plasmids, we observed a time-dependent increase in luciferase activity. Activity was significantly increased compared with controls at 4 h and further increased up to a 24-h lasting incubation. Experiments were then reproduced in primary macrophages during 24-h lasting incubations with AC (Fig. 2, d and e). We conclude that a COX-2-3'-UTR-dependent mRNA stabilization mechanism accounts for increased COX-2 expression in AC-treated macrophages.

**AC-derived S1P provokes COX-2 expression without cell-cell contact of AC and macrophages**

We examined whether AC-induced COX-2 expression in macrophages requires cell-cell contacts with AC, or rather is facilitated by AC-derived soluble factors. Therefore, RAW264.7 cells where transiently transfected with the pLuc plus 3'-UTR expression plasmid using jetPEI and treated with Cyt D to block phagocytosis, by inhibition of actin polymerization. In turn, cells were exposed to AC or apoptotic cell-derived CM for 24 h, using luciferase induction as a readout (Fig. 3a). Corroborating our results, AC significantly increased luciferase activity compared with untreated control cells. As a further control for AC specificity, neither viable (VC) nor necrotic (NC) cells provoked significant luciferase induction. Moreover, Cyt D alone did not affect luciferase activity. Stimulation of luciferase activity by AC remained high, when supplied in combination with Cyt D, suggesting that phagocytosis was not required for COX-2 expression. We then tested CM and indeed noticed luciferase induction by CM comparable to AC. These data support the notion that a factor released from AC was able to induce COX-2 in macrophages, not requiring cell-cell contacts of AC with the phagocyte. To prove that CM indeed induces a rapid COX-2 protein expression, we performed Western analysis. CM, added to RAW264.7 macrophages, time dependently provoked COX-2 up-regulation (Fig. 3b). Protein expression was first noticed after 90 min and progressively increased during a 3-h lasting incubation period. To further prove specificity, we used CM from viable cells (CM-VC) and necrotic cells (CM-NC) and incubated them with macrophages for 4 h. As expected, only CM form apoptotic cells significantly increased COX-2 protein, but neither CM-VC nor CM-NC (Fig. 3c). As an additional functional readout, we again measured macrophage PGE₂ production by ELISA (Fig. 3d). Incubating macrophages with CM, CM-VC, or CM-NC for 4 h showed significant production of PGE₂ only when using CM.

Taking into account that S1P is released from AC (4) and that S1P has been described to promote COX-2 expression in macrophages (30), we considered this phospholipid as a candidate for transmission of the COX-2-promoting capability of CM. Therefore, we used CM which does not contain S1P (CM/DMS) (4),
obtained by incubating Jurkat cells with the sphingosine kinase inhibitor DMS during induction of apoptosis (described in Materials and Methods). We compared the potency of CM/DMS to standard CM, in the ability to evoke COX-2 expression in RAW264.7 macrophages (Fig. 4a). Although CM induced pronounced COX-2 expression after 4 h compared with controls, the stimulatory potency of CM/DMS was significantly reduced. These results suggest that S1P, produced in AC, expresses COX-2 in macrophages. To strengthen this assumption, we extracted acidic phospholipids from CM as described in Materials and Methods and exposed macrophages for 4 h to these extracts, or corresponding solvent controls. Only the lipid extract induced COX-2 (Fig. 4b). We went on and incubated macrophages for 4 h with 1/1000 M authentic S1P, which also induced COX-2 protein (Fig. 4c).

CM induces nucleocytoplasmic HuR shuttling to stabilize COX-2 mRNA

For the following experiments, we hypothesized that the mRNA-binding protein HuR might play a role in CM-mediated COX-2 mRNA stabilization. As it is known that HuR activation is correlated with its translocation from the nucleus to the cytosol, we performed Western analysis to monitor compartmentalization of HuR in response to CM (Fig. 5a). As a positive control to follow HuR activation, we used 1 µg/ml LPS, which has been described to activate HuR (31), while control cells remained untreated. Both, CM as well as LPS, significantly increased cytosolic HuR, thus pointing to CM-mediated activation of this mRNA-binding protein. Moreover, total HuR expression was not affected by CM (data

FIGURE 3. Soluble factors provoke COX-2 expression and PGE2 release. a, RAW264.7 cells were transiently transfected with pLuc + 3′-UTR. After 24 h, medium was changed and macrophages remained untreated or were incubated for another 24 h with AC, NC, VC (each at a ratio of 1:5), 2 µM Cyt D or CM as indicated. Luciferase activity was analyzed as described under Materials and Methods. b, Time-dependent expression of COX-2 in response to CM. RAW264.7 cells were incubated with CM or remained untreated for times indicated. Western analysis was used to follow COX-2 expression. c, Expression of COX-2 in RAW264.7 cells in response to CM, CM from necrotic (CM-NC) or vital cells (CM-VC) after 4 h. Western analysis was used to follow COX-2 expression. d, RAW264.7 macrophages were incubated with CM, CM-NC, or CM-VC or remained as controls for 4 h. PGE2 ELISA was performed as described in Materials and Methods. For statistical evaluation, densitometric analysis was performed. Data represent the mean ± SEM of at least three different experiments (*, p ≤ 0.05 vs unstimulated controls).

FIGURE 4. DMS attenuated COX-2 expression in response to CM. a, RAW264.7 cells were incubated for 4 h with CM, CM from DMS-treated cells (CM/DMS), or remained as controls. Statistics compare CM- vs CM/DMS-treated cells. b, RAW264.7 cells were incubated for 4 h with lipid extracts from CM (extraction of acidic phospholipids as described in Materials and Methods), solvent, or remained untreated. c, RAW264.7 cells were incubated for 4 h with authentic S1P (1 µM) or remained untreated. COX-2 expression was followed by Western analysis. For statistical evaluation, densitometric analysis was performed. Data represent the mean ± SEM of at least three different experiments (*, p ≤ 0.05 vs unstimulated controls).
not shown). To strengthen the idea that CM-activated HuR shutoff is functionally important, we tested whether stimulation of cells with CM or S1P induces HuR binding to a COX-2-specific ARE-containing mRNA. We used an RNA oligonucleotide encompassing an ARE from the 3′-UTR of COX-2 (COX-2-ARE-2),
which represents an established functional binding element for HuR (27). To this end, cytoplasmic extracts, similar to the ones used for identification of HuR translocation to the cytoplasm (see Fig. 5a), were tested for in vitro RNA binding. We noticed the formation of one prominent complex, which showed enhanced RNA binding upon stimulation of cells with CM (Fig. 5b). Considering that S1P causes COX-2 expression, we dose dependently exposed macrophages to S1P and followed HuR binding to the COX-2-specific ARE by EMSA. All tested concentrations of S1P provoked an increase in the ARE-bound complex formation (Fig. 5c).

To specify the identity of the complex bound to the COX-2-specific ARE site, we performed supershift analysis as described in Materials and Methods, using Abs against AUF-1, HuR, and HuB (Fig. 5d). As expected neither Abs raised against HuB nor AUF-1 shifted the HUR complex, although the latter caused a minor diminution in RNA binding, thus indicating that the mRNA-destabilizing factor AUF-1 participates in COX-2-specific ARE binding. However, only the HuR-specific Ab elicited a real supershift, thereby proving that HuR is a main constituent of the SP1-induced complex. To furthermore demonstrate specificity of ARE binding, we performed competition analysis. CM-induced binding to the COX-2-ARE site 2 was dose dependently competed by adding nonlabeled, wt oligonucleotide (wt-COX-ARE) (Fig. 5e). By contrast, competition with the unlabeled mt oligonucleotide (mt-COX-ARE) was much less effective and caused only a moderate inhibition of complex formation when used at the highest concentration. From these results, we suggest that the ARE motif is a specific target of the CM-induced HuR binding to the 3’-UTR of COX-2 mRNA.

To verify the functional importance of HuR in the process of CM-induced COX-2 expression, we performed HuR knockdown experiments. RAW264.7 cells were transfected with a HuR-specific siRNA and attenuation of HuR protein expression was confirmed by Western analysis (Fig. 6a). Having established the successful use of siRNA, compared with a non targeting control siRNA (c siRNA), we used the knockdown approach to follow COX-2 expression in macrophages. Nontargeting control siRNA
left the response to CM or 1 μM S1P unaltered and allowed COX-2 expression in RAW264.7 macrophages. However, following HuR silencing, neither CM nor S1P induced COX-2 expression (Fig. 6b). These experiments strongly suggest that CM- or S1P-dependent COX-2 expression involves HuR. The HuR knockdown approach was used further to link HuR to CM- or S1P-induced macrophage PGE$_2$ formation (Fig. 6c). As expected, CM induced significantly higher levels of PGE$_2$ in control siRNA transfected cells compared with the situation with HuR being silenced. To our surprise, 1 μM authentic S1P produced no significant amounts of PGE$_2$ at all. Treatment of nontransfected RAW 264.7 with S1P at concentrations from 0, 1, to 10 μM also revealed no PGE$_2$ production (data not shown). This implies that S1P may contribute to COX-2 expression in response to CM but an additional factor in the conditioned medium apparently is required to allow prostanoid formation.

To further study the role of S1P, we created a constitutive sphingosine kinase (SphK2) knockdown in MCF-7 cells. These cells do not produce S1P during apoptosis (for reference, see Ref. 23). We checked whether CM from these knockdown cells (CM-MCF-siSK2), compared with CM from control MCF-7 cells (CM-MCF) or CM from apoptotic Jurkat cells, induces COX-2 (Fig. 7a). Western analysis showed a COX-2 increase after 4 h when using CM and CM-MCF, but a significantly lower expression with CM-MCF-siSK2. We went on to show that CM-MCF-siSK2 compared with CM-MCF elicited reduced levels of PGE$_2$ upon incubation with macrophages (Fig. 7b). ELISA determination showed that CM-MCF induces the formation of roughly 800 pg/ml PGE$_2$, whereas CM-MCF-siSK2 only produced 300 pg/ml PGE$_2$ during 4-h incubations. Control examinations determined that indeed expression of SphK2 was much lower in MCF-siSK2 cells compared with parent controls (Fig. 7c). Data so far suggest that S1P is a critical factor in CM to induce macrophage COX-2 expression. However, S1P alone seems not to be sufficient to provoke PGE$_2$ production (see Fig. 6c). Knowing that substrate availability is a critical step in PGE$_2$ production, we checked for cPLA$_2$ activation, which provides the substrate, i.e., arachidonate for COX-2. With the notion that a perinuclear, endoplasmic reticulum/Golgi distribution of PLA$_2$ is indicative for its activation (7), we performed immunostaining for PLA$_2$ to examine its localization in response to CM or authentic S1P. RAW264.7 macrophages were treated for 4 h with CM, S1P (1 μM), or LPS (10 μg/ml) as a positive control, followed by immunostaining of nuclei and cPLA$_2$, as described in Materials and Methods. As expected, controls did not show any perinuclear localization of PLA$_2$, because staining for nuclei and cPLA$_2$ was clearly separated. In contrast, LPS treatment provoked PLA$_2$ translocation, because staining for nuclei and cPLA$_2$ overlapped. Interestingly, CM also provoked perinuclear localization of PLA$_2$, while S1P-treated cells again did not show cPLA$_2$ translocation, i.e., activation. These findings suggest that CM induces not only COX-2 expression but also activates PLA$_2$, whereas S1P causes expression of COX-2 as well, but fails to activate PLA$_2$.

**Discussion**

During the last years, it became apparent that phagocytosis of AC conveys immune regulatory functions rather than being considered a simple waste disposal. Following recognition and/or engulfment, i.e., phagocytosis of AC, macrophages are “alternatively” activated, which allows them to acquire an anti-inflammatory phenotype (32–34). One factor implicated in this anti-inflammatory effect is PGE$_2$ (3), which is produced via COX-mediated conversion of arachidonic acid (10). Indeed, earlier work showed that COX-2 inhibitors attenuated anti-inflammatory effects conveyed by AC (20) and we confirmed that the COX-2 inhibitor NS 398 attenuated AC-mediated PGE$_2$ production in RAW264.7 macrophages. However, a molecular mechanism to explain AC-mediated COX-2 expression remained unclear. We present evidence that S1P, released from AC, provoked a rapid COX-2 expression via mRNA stabilization involving HuR activation. However, only CM also activates PLA$_2$ and thus provides substrate to COX-2 for efficient PGE$_2$ formation.

During our studies, we recognized a rapid expression of COX-2 protein following the addition of AC to RAW264.7 macrophages. The response using AC became apparent from 60 min onward and was considerably faster than using the established transcriptional/translational agonist IFN-γ, which produced a COX-2 signal after 4 h, only. Earlier work studied the impact of cytokines and glucocorticoids on COX-2 expression and pointed to mRNA stabilization as an important regulatory process, besides transcriptional regulation (10). Multiple copies of the AUUUA motive in the 3’-UTR of the COX-2 gene are responsible for the process of mRNA stabilization (12). Our data confirm previous findings that mRNA stabilizing, and thus posttranscriptional COX-2 regulatory mechanisms do operate in RAW264.7 macrophages (16). At present, a multiplicity of mRNA-stabilizing and -destabilizing factors such as TIAR, AUF1, HuR, and TIA-1 are known (15) and implicated in COX-2 regulation (16, 17, 35). Previous work also suggested a role of HuR in COX-2 mRNA stabilization after treatment of human mammary epithelial cells with taxanes (13).

HuR binds to AREs in the COX-2–3’-UTR with high affinity and functions as a mRNA-stabilizing factor (36). Overexpression of HuR has been shown to stabilize reporter mRNAs which contain the ARE of COX-2 (37). However, COX-2 mRNA stabilization following HuR overexpression has controversially been discussed, due to an artificial increase in cytoplasmic HuR (36). In our system, we noticed a rapid increase in endogenous cytosolic HuR levels following the addition of CM, which underscores the (patho)physiological relevance of our test system. In this context, it should be noticed that a basal activation level of HuR is not surprising in RAW264.7 cells and has been described before (16). Furthermore, it should be noticed that increased cytosolic HuR levels did not result from an increased HuR expression.

Recently, we showed that S1P derived from AC promotes survival of macrophages that had been exposed to chemotherapeutic agents to provoke apoptosis (4). We now extend the role of S1P as a messenger, released from AC with the ability to express COX-2 in macrophages. Previously, we noticed that SphK2 is responsible for releasing of S1P during apoptosis (4). When formation of S1P in AC was blocked by the sphingosine kinase inhibitor DMS or a SphK2 knockdown approach, the resulting CM showed a greatly diminished potency to induce COX-2. Although the ability of S1P to increase COX-2 expression has previously been described, and a mRNA-stabilizing mechanism has been discussed (30), mechanistic details remained incomplete and the involvement of HuR in S1P-mediated COX-2 expression has not been appreciated before. Lately, it has been suggested that in mouse embryonic fibroblasts S1P increased COX-2 expressions via the S1P receptors S1P$_1$, S1P$_3$ and S1P$_5$, with subsequent activation of NF-κB (38). This mechanisms seems unlikely to be the case in macrophages because they predominantly express S1P$_2$ and S1P$_4$, but no S1P$_3$ (39, 40). Moreover, Cvetanovic et al. (41), as well as our own unpublished data, show that activation of NF-κB is inhibited in macrophages in response to AC. In addition, Kimura et al. (42) observed defective TNF-α-dependent NF-κB activation in THP-1 cells in response to S1P, which makes a NF-κB response in macrophages unlikely. Furthermore, transcriptional activation of NF-κB by S1P in mouse embryonic fibroblasts observed by Ki et al. (38) does not rule out...
an additional and potential synergistic involvement of mRNA stabilization via HuR. Our results imply that S1P, produced in and released from AC, acts as a transmitter to induce COX-2 in macrophages, involving HuR-dependent mRNA stabilization. These new insights provide a so far unrecognized mechanism how dying cells rapidly modulate early immune responses in macrophages, such as PGE2 release. In our previous studies, we measured S1P in CM from Jurkat cells (4) or MCF-7 cells (23). The concentrations of exogenously added S1P are above those found in CM, but it is widely accepted that exogenous concentrations are normally higher because of solubility problems due to, e.g., micelle formation (43).

Seemingly in contrast to our findings, expression of a dominant-negative TGF-β receptor in RAW264.7 macrophages suggested that TGF-β mediates COX-2 expression in response to AC (19). Interestingly, this suggestion might be fully compatible with our observations considering that S1P receptors are G-protein coupled, which not only undergo homodimerization but also heterodimerization and/or oligomerization with other receptor classes (44). Corroborating this phenomenon, Xin et al. (45) provided evidence for a cross-communication between S1P receptors and TGF-β signaling, which was confirmed recently by Keller et al. (46). It can be speculated that elimination of TGF-β signaling might attenuate COX-2 expression by not allowing transmission of functional S1P signals. Further studies need to identify the nature of S1P receptors, transmitting the response to CM. In contrast to CM, the addition of authentic S1P does not seem to be sufficient for PGE2 production in macrophages because PLA2 is not activated. This suggests the existence of a yet unidentified activity in CM that accounts for substrate supply to COX-2. These considerations are in agreement with the work of Pettus et al. (7), which suggested that S1P may induce COX-2, but does not activate PLA2 to provoke arachidonic acid release.

In summary, our findings add new information on fast gene regulatory mechanisms evoked by AC or CM. We provide evidence that AC-derived S1P activates nucleocyttoplasmic HuR shuttling, stabilizes COX-2 mRNA, and provokes COX-2 expression. In combination with substrate release via PLA2, the formation of PGE2 adds to anti-inflammatory postphagocytic phenotype alterations of macrophages. These regulatory circuits help to understand the fast immune-regulatory role of AC and provide insights in the basic mechanisms of the early phase of alternative macrophage activation.

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Disclosures

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References


