Expression of COX-2 in platelet-monocyte interactions occurs via combinatorial regulation involving adhesion and cytokine signaling

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Tight regulation of COX-2 expression is a key feature controlling eicosanoid production in atherosclerosis and other inflammatory syndromes. Adhesive interactions between platelets and monocytes occur in these conditions and deliver specific signals that trigger inflammatory gene expression. Using a cellular model of monocyte signaling induced by activated human platelets, we identified the central posttranscriptional mechanisms that regulate timing and magnitude of COX-2 expression. Tethering of monocytes to platelets and to purified P-selectin, a key adhesion molecule displayed by activated platelets, induces NF-κB activation and *COX-2* promoter activity. Nevertheless, COX-2 mRNA is rapidly degraded, leading to aborted protein synthesis. Time-dependent signaling of monocytes induces a second phase of transcript accumulation accompanied by COX-2 enzyme synthesis and eicosanoid production. Here, generation of IL-1β, a proinflammatory cytokine, promoted stabilization of COX-2 mRNA by silencing of the AU-rich mRNA decay element (ARE) in the 3′-untranslated region (3'UTR) of the mRNA. Consistent with observed mRNA stabilization, activated platelets or IL-1β treatment induced cytoplasmic accumulation and enhanced ARE binding of the mRNA stability factor HuR in monocytes. These findings demonstrate that activated platelets induce COX-2 synthesis in monocytes by combinatorial signaling to transcriptional and posttranscriptional checkpoints. These checkpoints may be altered in disease and therefore useful as targets for antiinflammatory intervention.

Introduction

Cell-cell interactions are critical in inflammation and thrombosis and can involve paracrine, juxtacrine, and autocrine signaling, frequently in combinatorial fashion (1). Cellular interactions between platelets and leukocytes provide an important mechanism for intercellular communication and information transfer in host defense and in disease (2, 3). These include adhesive interactions of platelets with circulating monocytes and neutrophils (1–3). Human monocytes adhere to platelets deposited at sites of vascular injury, and platelet-monocyte aggregates form in the blood of subjects with a variety of inflammatory syndromes (2–12). Atherosclerosis and its acute complications provide prime examples. Experimental evidence demonstrates that platelet interactions with circulating monocytes mediate atherosclerotic progression (13). Furthermore, clinical studies indicate that heterotypic platelet-monocyte aggregates influence the natural history of atherosclerosis and its complications and are increased in patients with coronary artery disease (4–7, 9, 10). Formation of platelet-monocyte aggregates is an established link between inflammation and thrombosis in acute coronary syndromes and related disorders (10). Interruption of signaling pathways that are triggered when platelets adhere to and activate monocytes may be a new target for molecular intervention (14, 15). Signaling in platelet-monocyte aggregates and in related platelet-monocyte interactions can be characterized in informative in vitro model systems (5, 15–19), which also serve as models of human gene expression (19).

Interactions of myeloid leukocytes and activated platelets involve specific adhesion molecules and coordinate activation of surface receptors by ligands delivered by the platelet (16–21). One key molecular event is binding of P-selectin on activated platelets to P-selectin glycoprotein ligand (PSGL-1) on the leukocytes (2, 22). In many cases, engagement of leukocyte adhesion molecules such as PSGL-1 serves not only to tether the cell to other cells or to matrix structures at sites of inflammation but also to transmit or integrate transmission of outside-in signals that alter gene expression pathways and other functional responses (16, 18, 19, 23). PSGL-1 signals to both transcriptional and posttranscriptional checkpoints in human myeloid leukocytes (2, 16–22), providing diversity in adhesive signaling that is comparable to that of surface integrins (18, 23, 24). Signaling via PSGL-1 acts in concert with surface receptor pathways to modulate integrated synthesis of chemokines, cytokines, and other factors by the adherent monocytes (16–22). These molecular mechanisms operate in platelet-monocyte interactions (5, 10, 16–19, 21).

Nonstandard abbreviations used: ActD, actinomycin D; ARE, AU-rich mRNA decay element; ATF2, activating transcription factor 2; GSK3, glycogen synthase kinase 3; HuR, Hu-antigen R; IL-1 RA, IL-1β receptor antagonist; p-, phosphorylated; PGE_2, prostaglandin E_2; PSGL-1, P-selectin glycoprotein ligand; 3′UTR, 3′-untranslated region.

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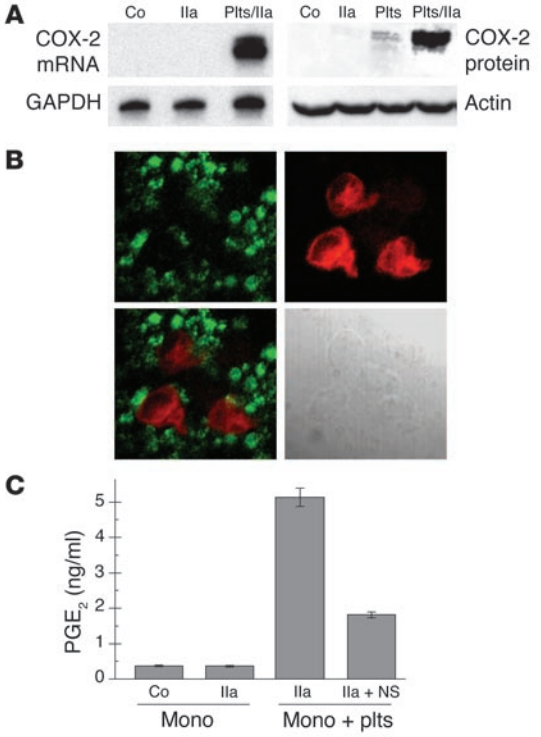

Figure 1
Activated platelets induce COX-2 expression in monocytes. (**A**) Activation of platelets (plts) is required for COX-2 mRNA (left panel) and protein (right panel) expression. Monocytes (10^6 cells) were incubated with control M199 medium (co), thrombin (IIa), inactivated platelets (10^8 cells), or thrombin-activated platelets. After 18 hours, COX-2 mRNA was detected by RNase protection assays. GAPDH mRNA is shown as a control for RNA loading. COX-2 protein was detected by immunoblot of 50 μg of total cell lysate. β-actin was detected on the same blot as a control for protein loading. Data shown represent 3 experiments. (**B**) Immunofluorescent detection of COX-2 and P-selectin in thrombin-activated platelet-monocyte aggregates. Freshly isolated platelets were incubated with monocytes for 18 hours and examined by immunocytochemical analysis. Immunofluorescent staining for COX-2 is shown in red (top right); immunofluorescent staining for P-selectin, a platelet marker, is shown in green (top left). Merged immunofluorescence and phase contrast images are shown (bottom left and right). (**C**) COX activity in monocytes (mono) and monocyte/platelet suspensions (mono+plts) treated with medium (co) or thrombin (IIa) for 18 hours. COX-2 inhibition was accomplished by pretreating cells for 1 hour with 10 μM NS-398 (NS). PGE_2 levels were measured by ELISA in the medium containing arachidonic acid and indicate the average of duplicate experiments.

Expression of highly regulated gene products is thought to be both transcript and cell-type dependent (25). The mechanisms of regulation in primary human cells and critical cell-cell interactions are, however, largely uncharacterized. The inducible form of cyclooxygenase, COX-2, is an immediate-early response gene with complex regulation that has important roles in vascular homeostasis and inflammatory responses (26, 27). COX-2 and the constitutive isoform COX-1 govern the production of eicosanoids under physiologic and pathologic conditions (26, 27). Substantial evidence indicates that unregulated COX-2 expression and prostaglandin synthesis influence chronic inflammatory conditions, including atherosclerosis and its complications (26–30). Furthermore, iatrogenic manipulation of COX-2 activity can have both beneficial and untoward clinical effects (26, 27). Nevertheless, the mechanisms that regulate COX-2 expression by inflammatory cells remain obscure. Based on studies in other cell types, one component may involve rapid transcriptional induction in response to inflammatory stimuli and growth factors (31–40). In addition, there is evidence indicating that COX-2 expression is also regulated at posttranscriptional checkpoints, a feature that allows for precision in the timing of synthesis and abundance of critical proteins (41). Recently, we identified an AU-rich mRNA decay element (ARE) in the 3′-untranslated region (3′UTR) of COX-2 messenger RNA that confers posttranscriptional regulation by controlling both mRNA decay and translational efficiency (42–44). This *cis*-acting element regulates levels of COX-2 protein and associated prostaglandin levels in neoplastic cells and epithelial cell lines, and defects in ARE function result in COX-2 overexpression (31, 42–45). These and other findings demonstrate the importance of posttranscriptional regulation in preventing excess activity of COX-2 (42–45), which is aberrantly expressed and has critical effects in atherosclerotic syndromes, other inflammatory diseases (26–30, 46–48), and cancer (49). How these mechanisms operate in specific inflammatory cells and the signals that regulate them are unknown.

In this report, we demonstrate that expression of COX-2 is induced in human monocytes in response to activated platelets, resulting in increased eicosanoid synthesis, and define the mechanisms involved. These findings offer what we believe are new insights into mechanisms that govern adhesion-dependent signaling and coordinate transcriptional and posttranscriptional pathways critical for expression of COX-2 and other inflammatory genes.

Results

Activated platelets induce COX-2 and prostaglandin synthesis in monocytes. We asked whether activated platelets promote COX-2 expression in monocytes. As shown in Figure 1, human monocytes expressed COX-2 mRNA and protein when incubated with platelets stimulated by thrombin (coagulation factor IIa) for 18 hours. Untreated monocytes or those treated with thrombin did not express COX-2; a small increase in COX-2 protein was observed in monocytes incubated with unstimulated platelets (Figure 1A), presumably due to spontaneous low-level platelet activation with extended ex vivo incubation times. We did not detect COX-2 mRNA or protein in untreated or thrombin-stimulated platelets (data not shown), identifying monocytes as the sole source of COX-2 under the conditions of this experiment. Consistent with previous results (16), we observed that thrombin-activated platelets adhere to monocytes and form rosette-like aggregates (Figure 1B). Blocking PSGL-1 engagement inhibits platelet adhesion to monocytes and aggregate formation both in suspension and at matrix surfaces (16, 17). Immunofluorescence analysis of COX-2 protein demonstrated its expression only in monocytes in platelet-monocyte aggregates, suggesting that platelet adhesion is a critical component in signaling COX-2 gene expression (Figure 1B and data not shown). Cyclooxygenase activity was examined in acti-

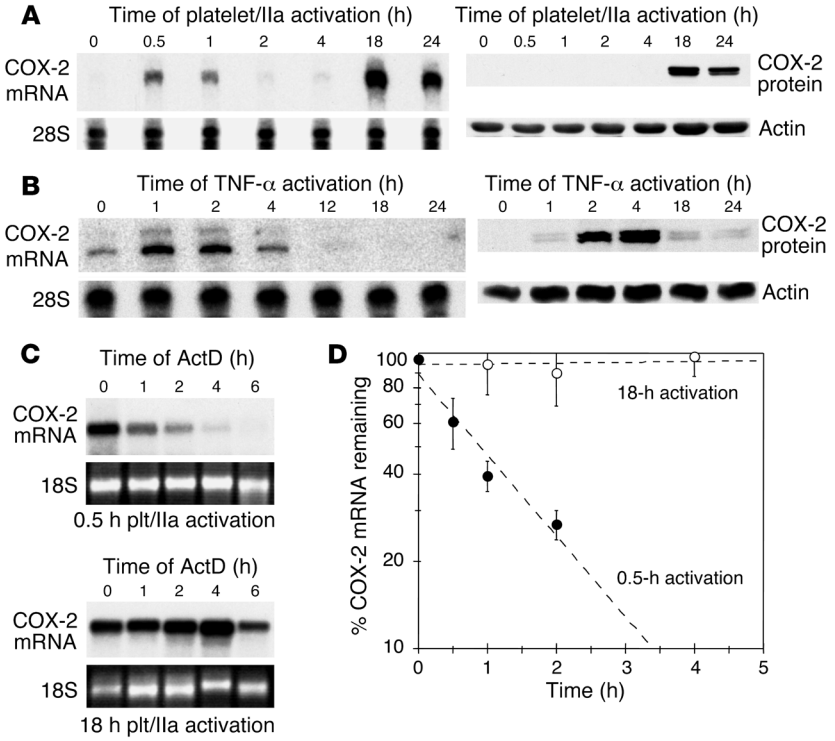

Figure 2
Prolonged adherence to activated platelets modulates COX-2 synthesis and mRNA stability in monocytes. (**A**) Time courses of COX-2 mRNA (left panel) and protein (right panel) expression in monocytes. Platelets (10^8 cells) were incubated with monocytes (10^6 cells) in the presence of thrombin. At the indicated times, COX-2 mRNA was detected by RNase protection assay. 28S RNA is shown as a control for RNA loading. COX-2 protein was detected by immunoblot of total cell lysates. β-actin was detected on the same blot as a control for protein loading. Data shown represent 3 experiments. (**B**) Time courses of COX-2 mRNA (left panel) and protein (right panel) expression in monocytes treated with TNF-α (100 U/ml) were assayed as described in **A**, except platelets were omitted. (**C**) Assays of COX-2 mRNA $t_{1/2}$ were accomplished by adding 5 μg/ml ActD to platelet-monocyte suspensions treated with thrombin for 0.5 hours (top panel) or 18 hours (bottom panel). Total RNA was prepared at the indicated time points and COX-2 mRNA decay was analyzed by Northern blot probing for COX-2. 18S RNA is shown as a control for RNA loading. (**D**) Summary of COX-2 mRNA $t_{1/2}$ data obtained from platelet-monocyte suspensions treated with thrombin for 0.5 hours (filled circles) and 18 hours (open circles) showing the average and range in duplicate experiments.

vated platelet-monocyte suspensions by determining the profile of prostaglandins produced. Thromboxane B_2 and prostaglandin E_2 (PGE_2) were the predominant eicosanoids generated; prostaglandin $F_{2\alpha}$ and prostaglandin D were also present in the medium at lower levels whereas 6-keto–prostaglandin $F_{1\alpha}$ was not detected (not shown). PGE_2 levels were routinely measured to evaluate the activity of monocyte COX-2. As shown in Figure 1C, monocytes incubated with thrombin-stimulated platelets produced over 10-fold more PGE_2 than control- or thrombin-treated monocytes. Treatment of cells with COX-2 inhibitor NS-398 reduced PGE_2 levels substantially, indicating that a majority of its synthesis in monocytes is via COX-2.

Prolonged adherence to platelets modulates COX-2 mRNA stability in monocytes. The kinetics of COX-2 induction in monocytes stimulated by activated platelets was next examined. Incubation of activated platelets with monocytes triggered a rapid biphasic pattern of COX-2 mRNA expression (Figure 2A). Consistent with the profile of an immediate-early response gene, COX-2 mRNA expression was observed as early as after 0.5 hours of incubation, but rapid accumulation of the transcript was transient. A second phase of COX-2 mRNA accumulation was initiated at approximately 8 hours of incubation with activated platelets and continued to 24 hours (Figure 2A and data not shown). Strikingly, COX-2 protein was detected only during the second phase of mRNA expression during incubation with activated platelets (Figure 2A). Monocyte-dependent PGE_2 synthesis correlated with COX-2 protein expression (data not shown). This pattern of COX-2 expression depended on signals delivered by adherent platelets since monocytes stimulated with TNF-α in the absence of platelets yielded a transient pattern of COX-2 mRNA and protein induction that peaked at 2 and 4 hours, respectively (Figure 2B).

A central mechanism that controls synthesis of COX-2 protein involves rapid degradation of the COX-2 transcript (42). To determine whether prolonged adhesion of platelets to monocytes influences rapid mRNA decay, the $t_{1/2}$ of COX-2 mRNA was assessed by Northern blot analysis after actinomycin D (ActD) was added to monocytes to halt transcription (42) at 0.5 hours and 18 hours of incubation with activated platelets. The results shown in Figure 2C demonstrate that rapid COX-2 mRNA decay was seen after 0.5 hours of incubation with activated platelet yielding a $t_{1/2}$ of 54 minutes (Figure 2D). In contrast, COX-2 mRNA was stabilized in monocytes during the second phase of expression induced by adherence to activated platelets such that mRNA decay was not observed until after 4 hours following ActD treatment (Figure 2D). These findings demonstrate the ability of activated platelets to influence a posttranscriptional checkpoint in COX-2 regulation that controls synthesis of COX-2 enzyme at later time points.

Adhesion to P-selectin induces rapid COX-2 transcription in monocytes. Previously, we demonstrated that engagement of PSGL-1 by P-selectin transmits outside-in signals that induce expression of proinflammatory genes by monocytes (16, 18–21). To determine whether this mechanism regulates COX-2, monocytes were allowed to adhere to purified immobilized P-selectin under conditions that specifically engage PSGL-1 (19, 21), and COX-2 mRNA levels were examined over an 18-hour time course (Figure 3A). Rapid transcription of *COX-2* was observed as early as after 0.5 hours of incubation and was transient with no detectable COX-2 mRNA at the later incubation times (18 hours). In contrast, COX-2 protein was not detected by immunoblot analysis at any of these time points (data not shown). This pattern is similar to that seen at early time points when adherent platelets signal monocytes (Figure 2A). In parallel experiments, a reporter construct containing a 1.8-kb fragment of *COX-2* promoter was introduced into U937 myelomonocytic cells, and its activity was examined when the leukocytes were adherent to P-selectin (Figure 3B). The mag-

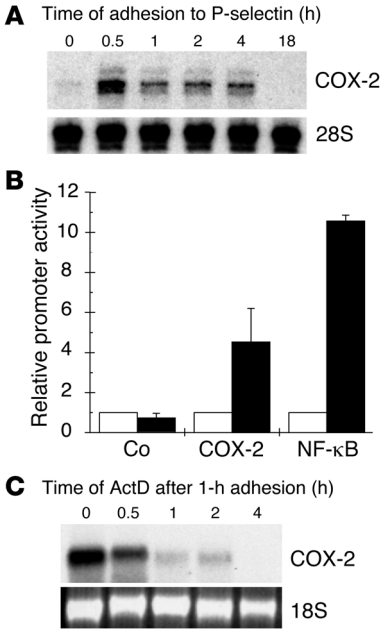

Figure 3
Adhesion of monocytes to P-selectin induces rapid COX-2 transcription. (**A**) Monocytes (10^6 cells) were incubated on plates coated with immobilized P-selectin, and total RNA was prepared at the indicated times (h). COX-2 mRNA was detected by RNase protection assay using 28S RNA as a control for RNA loading. (**B**) Adhesion to P-selectin induces COX-2 promoter activity in U937 cells. A LUC reporter construct containing the 1.8-kb COX-2 promoter was transfected into U937 cells, which were subsequently kept in suspension (white bars) or allowed to adhere to purified, immobilized P-selectin (black bars) for 48 hours. In parallel, NF-κB promoter activity was determined using a LUC reporter construct containing 5 consensus NF-κB–binding elements. The pGL3 control vector containing the SV40 promoter and enhancer elements was used to evaluate specificity of P-selectin–mediated transcriptional induction. Relative activity was assessed as LUC activity normalized to total protein. All values shown are normalized to expression of LUC by transfected cells in suspension and indicate the averages of 3 experiments. (**C**) COX-2 mRNA $t_{1/2}$ in monocytes adherent to immobilized P-selectin. ActD (5 μg/ml) was added after monocytes had been incubated in P-selectin–coated plates for 1 hour, and total RNA was prepared at the indicated time points. COX-2 mRNA decay was analyzed by Northern blot probing for COX-2. 18S RNA is shown as a control for RNA loading. The blot shown is from 1 of 2 experiments with similar results.

nitude of *COX-2* promoter induction was substantial although not as great as that seen with a consensus NF-κB reporter construct, consistent with nuclear translocation of NF-κB triggered by P-selectin/PSGL-1 engagement (16, 19) and consequent rapid *COX-2* transcription. Control incubations using cells transfected with a reporter construct containing the SV40 promoter and enhancer did not show increased activity of the construct with P-selectin/PSGL-1 engagement.

The pattern of COX-2 mRNA accumulation, coincident with absent protein expression at early time points when monocytes adhered to activated platelets (Figure 2) and when monocytes and myelomonocytic cells adhered to purified P-selectin (Figure 3), indicates that P-selectin/PSGL-1 engagement alone is not sufficient to signal mRNA stabilization. To test this, monocytes were allowed to adhere to purified P-selectin for 1 hour, after which ActD was added and COX-2 mRNA $t_{1/2}$ was assessed. Figure 3C demonstrates that rapid decay of COX-2 mRNA ($t_{1/2}$ = 23 min) occurs under these conditions, yielding a $t_{1/2}$ similar to that observed after adhesive signaling by activated platelets for 0.5 hours (Figure 2, C and D). These findings demonstrate the ability of PSGL-1 engagement to signal rapid *COX-2* transcription, but in the absence of an additional stimulus, subsequent rapid decay of the transcript limits COX-2 protein synthesis.

Activated platelets induce IL-1β expression by monocytes. We then examined the nature of the second signal (23) that is required for COX-2 protein expression at later time points. Based on the ability of the IL-1 family of cytokines to promote synthesis of COX-2 by activating posttranscriptional mechanisms (50–52), we explored the role of IL-1β in COX-2 expression when monocytes interact with platelets. IL-1β participates in juxtacrine and paracrine interactions of activated platelets with target cells (reviewed in refs. 1–3) (53) and, consistent with a previous observation based on incubations of activated platelets with unfractionated leukocytes (5), we found that IL-1β was present in substantial amounts in the samples from platelet-monocyte aggregates whereas incubations of monocytes or platelets alone with thrombin yielded much lower levels (Figure 4A). More significantly, IL-1β expression was maximal after prolonged adherence of monocytes to activated platelets (18–24 hours; Figure 4A) at time points at which COX-2 mRNA stabilization occurred (Figure 2, C and D). Consistent with the time course of COX-2 induction in monocytes stimulated by activated platelets (Figure 2A), the ability of exogenous IL-1β to induce COX-2 expression in monocytes was apparent at prolonged incubation times of 18 to 24 hours (Figure 4B). However, IL-1β–induced *COX-2* transcription was delayed compared with rapid transcriptional induction seen with P-selectin/PSGL-1 engagement (Figure 3A). Furthermore, we observed attenuation of COX-2 protein accumulation with the addition of IL-1β receptor antagonist (IL-1 RA) to suspensions of activated platelets and monocytes (Figure 4C). IL-1 RA–mediated inhibition of COX-2 expression was concentration dependent with complete inhibition observed at 500 ng/ml. These results indicate that synthesis of IL-1β and its signaling of monocytes mediates COX-2 protein synthesis under these conditions.

P-selectin and IL-1β act in concert to promote COX-2 mRNA stabilization. Activated platelets express several cytokines, chemokines, and growth factors that could potentially influence gene expression in leukocytes (1–3, 16, 53). To further explore the role of IL-1β in COX-2 expression in platelet-monocyte aggregates, monocytes adherent to purified P-selectin were treated with unfractionated platelet supernatant or factors known to be secreted by thrombin-stimulated platelets. As shown in Figure 5A, adhesion to P-selectin for 0.5 hours induced rapid COX-2 mRNA expression, but addition of supernatant collected from platelets after 1 hour of activation did not increase accumulation of the transcript at this time point or promote mRNA accumulation after 18 hours of adhesion. This indicates that products rapidly released by platelet degranulation do not provide a sufficient second signal for COX-2 expression. In agreement, treatment of adherent monocytes with the chemokine RANTES or TGF-β, factors rapidly secreted from activated platelets at high levels and that alter monocyte responses (16, 54), produced a similar result. In contrast, the addition

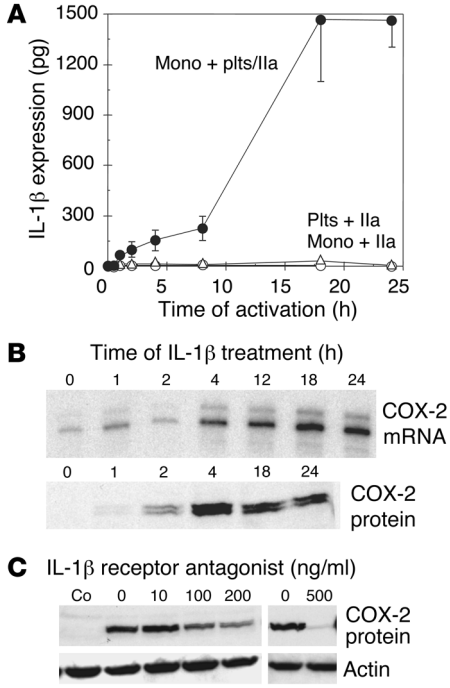

Figure 4
IL-1β is expressed in interactions of monocytes with activated platelets and induces COX-2 synthesis. (**A**) Time course of IL-1β expression in platelet-monocyte (10^8 and 10^6 cells, respectively) incubations (filled circles), monocytes alone (open triangles), and platelets alone (open circles) treated with thrombin. At the indicated times, IL-1β was measured by ELISA in total cell lysates and reported as pg IL-1β present in cell lysate. These data indicate the mean ± SEM for either duplicate or triplicate points; the results shown are representative of 4 independent experiments. (**B**) Time courses of COX-2 mRNA and protein expression in monocytes in suspension treated with IL-1β (10 ng/ml) were assayed by RNase protection assay and immunoblot, respectively, as described above. (**C**) Platelet-monocyte suspensions were treated with thrombin in the presence of increasing amounts of IL-1 RA (0 to 500 ng/ml) for 18 hours. COX-2 protein expression was assayed by immunoblot as described above. COX-2 protein expression in untreated monocytes in suspension for 18 hours is shown (co).

of exogenous IL-1β yielded high levels of COX-2 mRNA after 18 hours of adhesion and appeared to increase the amount of COX-2 mRNA at the earlier 0.5-hour time point (Figure 5A). Consistent with this, COX-2 protein expression was only observed with addition of IL-1β (Figure 5B); the addition of TGF-β, RANTES, or platelet-activating factor did not induce COX-2 protein expression (Figure 5B and data not shown). Our results shown in Figure 4 indicate the ability of IL-1β to promote sustained COX-2 expression in monocytes. To determine whether engagement of PSGL-1 contributes to enhanced COX-2 expression, monocytes were incubated on immobilized P-selectin with medium containing IL-1β in the presence of a P-selectin–blocking peptide (55) for 18 hours. As shown in Figure 5C, the P-selectin antagonist caused dose-dependent inhibition of IL-1β–induced COX-2 expression. Control incubations using monocytes in suspension stimulated with LPS showed no significant change in COX-2 protein expression in the presence of the P-selectin antagonist (Figure 5C). Consistent with this, addition of a PSGL-1–blocking Ab (anti–PSGL-1 mAb PL-1; ref. 20) to monocytes incubated on immobilized P-selectin in the presence of IL-1β led to attenuation of COX-2 protein expression at 18 hours (data not shown).

The ability of IL-1β to induce sustained levels of COX-2 mRNA in monocytes after prolonged adhesion to P-selectin indicated that this cytokine alters rapid decay of the mRNA. Therefore, the $t_{1/2}$ of COX-2 mRNA was examined after 18 hours of adhesion to P-selectin and treatment with IL-1β. As shown in Figure 5D, mRNA stabilization occurs under these conditions ($t_{1/2}$ = 150 min). Treatment of monocytes in suspension with IL-1β yielded a moderately increased $t_{1/2}$ that was not as great as in adherent cells ($t_{1/2}$ = 80 min). Taken together, these results indicate a combinato-

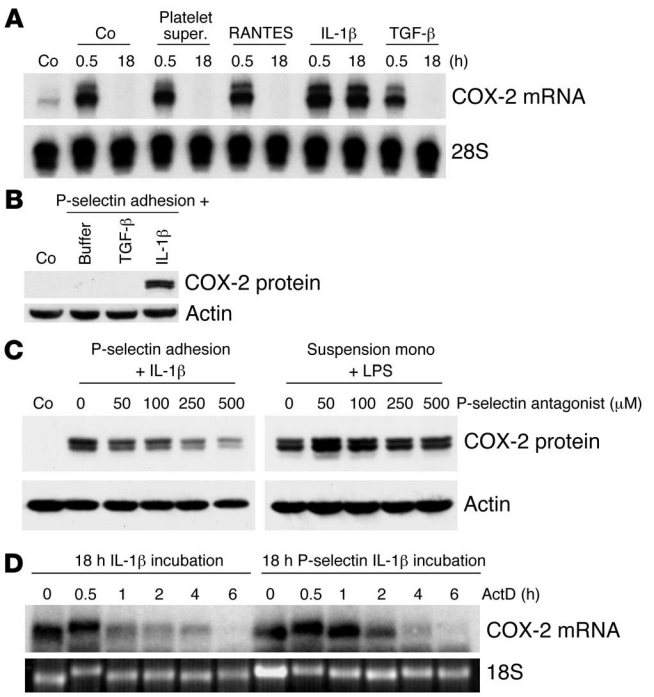

Figure 5
IL-1β induces stabilization of COX-2 mRNA in monocytes. (**A**) Monocytes (10^6 cells) were allowed to adhere to purified, immobilized P-selectin with medium alone (co), supernatant (super.) medium from platelets (10^8 cells) activated with thrombin for 1 hour, medium containing 10 ng/ml RANTES, medium containing 10 ng/ml IL-1β, or medium containing 10 ng/ml TGF-β. After 0.5 and 18 hours of incubation, total RNA was prepared and COX-2 mRNA was detected by RNase protection assay; 28S RNA is shown as a control for RNA loading. COX-2 expression in freshly isolated monocytes is shown (co). (**B**) COX-2 protein expression in monocytes incubated on immobilized P-selectin with medium containing vehicle buffer control, 10 ng/ml TGF-β, or 10 ng/ml IL-1β for 18 hours was assayed by immunoblot. (**C**) Monocytes were incubated on immobilized P-selectin with medium containing IL-1β (10 ng/ml; left panel) or kept in suspension with medium containing LPS (1 μg/ml; right panel) in the presence of increasing amounts of P-selectin antagonist for 18 hours. COX-2 protein expression was assayed by immunoblot. (**D**) COX-2 mRNA $t_{1/2}$ in monocytes in suspension (left panel) or adherent to immobilized P-selectin (right panel) in the presence of IL-1β (10 ng/ml). After 18 hours of incubation, ActD (5 μg/ml) was added and COX-2 mRNA decay was assayed by Northern blot.

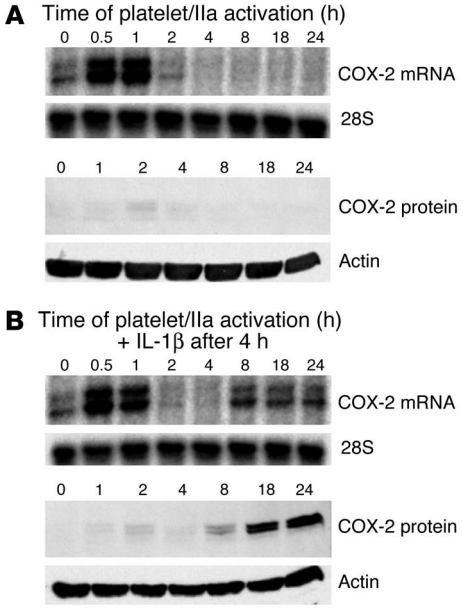

Figure 6
IL-1β is required for synthesis of COX-2 by U937 myelomonocytic cells in interactions with activated platelets. (**A**) Time courses of COX-2 mRNA and protein expression in U937 cells. Platelets (10^8 cells) were incubated with U937 cells (10^6 cells) in the presence of thrombin. At the indicated times, COX-2 mRNA was detected by RNase protection assay. 28S RNA is shown as a control for RNA loading. COX-2 protein was detected by immunoblot of total cell lysates. β-actin was detected on the same blot as a control for protein loading. (**B**) Time courses of COX-2 mRNA and protein expression in U937 cells incubated with platelets in the presence of thrombin were assayed as described above except that exogenous IL-1β (10 ng/ml) was added to time points after 4 hours of incubation.

rial effect of P-selectin and IL-1β in promoting COX-2 expression through mechanisms involving mRNA stabilization.

The combinatorial roles of P-selectin/PSGL-1 engagement and IL-1β signaling in COX-2 expression were further examined using U937 monocytic leukocytes. Incubation of activated platelets with U937 cells triggered a rapid induction of COX-2 mRNA expression (Figure 6A). In contrast to what we observed with primary monocytes (Figure 2A), a second phase of COX-2 mRNA accumulation was not apparent in U937 cells with extended activated platelet incubations, and COX-2 protein expression was low to undetectable at all time points examined (Figure 6A). This suggests that U937 cells are deficient in IL-1β synthesis in response to activated platelets. Samples from platelet–U937 cell aggregates were then examined for IL-1β expression. Over incubation times shown in Figure 6A, we were unable to detect significant amounts of IL-1β derived from U937 cells incubated with activated platelets whereas addition of phorbol ester or LPS promoted IL-1β expression in U937 cells (data not shown). Furthermore, addition of exogenous IL-1β to activated platelet–U937 cell incubations led to a subsequent increase in COX-2 mRNA expression accompanied by a concomitant increase in COX-2 protein expression that continued to 24 hours (Figure 6B). These results, along with those shown in Figure 4, indicate that IL-1β signaling is a necessary component for expression of COX-2 in platelet-myeloid leukocyte aggregates.

The MAPK ERK, the stress-activated protein kinase p38 MAPK, and kinase Akt/protein kinase B (Akt/PKB) signaling pathways are important in the posttranscriptional regulation of COX-2 expression (31). To determine whether the observed stabilization detected in monocytes correlated with activation of these kinases, we examined their activities in monocytes under conditions that influence COX-2 expression. Adhesion of monocytes to P-selectin alone did not significantly induce kinase activity (Figure 7). IL-1β treatment of monocytes in suspension resulted in modest activation of ERK1/2 and p38 MAPK pathways whereas their activities were further increased 2- to 3-fold when adhesion to P-selectin and IL-1β signaling were combined (Figure 7). Low-level activation of the Akt/PKB pathway was observed in isolated monocytes and was not changed by any of the treatments. These results are consistent with the possibility that stabilization of COX-2 mRNA occurs through the concerted actions of P-selectin/PSGL-1 engagement and IL-1β signaling on checkpoints involving the ERK and p38 MAPK pathways.

Posttranscriptional regulation of COX-2 expression has been shown to be mediated through the class II–type ARE–containing 3′UTR of COX-2 mRNA in cell lines and epithelial cells (42, 44). To determine whether the 3′UTR of COX-2 regulates its expression in myelomonocytic cells, we transfected U937 monocytic leukocytes with LUC/3′UTR reporter constructs containing the full-length COX-2 3′UTR (LUC+3′UTR) or with COX-2 3′UTR deleted (LUCΔ3′UTR), and LUC expression was measured. As in other cell types (42, 43, 45), COX-2 3′UTR reduced LUC expression approximately 10-fold in U937 cells (Figure 8A), consistent with rapid decay of the mRNA. We next investigated the ability of adhesion to P-selectin and IL-1β signaling to influence posttranscriptional regulation mediated through COX-2 3′UTR (Figure 8B). U937 cells transfected with the LUC+3′UTR construct yielded similar levels of expression when the cells were allowed to adhere to P-selectin or were incubated in suspension, consistent with evidence that PSGL-1 engagement alone has little effect on decay of COX-2 mRNA in primary monocytes (Figures 2, C and D, and Figure 3C). In contrast, addition of IL-1β to U937 cells in suspen-

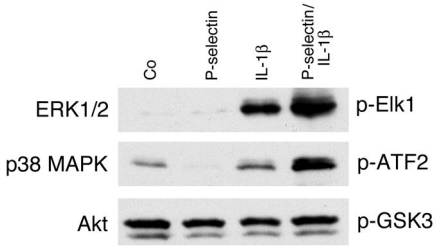

Figure 7
Combinatorial signaling by P-selectin and IL-1β selectively activates ERK and p38 MAPK pathways in human monocytes. Monocytes (10^6 cells) were immediately harvested (co), allowed to adhere to immobilized P-selectin for 0.5 hours (P-selectin), kept in suspension in medium containing 10 ng/ml IL-1β for 18 hours (IL-1β), or incubated on immobilized P-selectin in the presence of IL-1β for 18 hours (P-selectin/IL-1β). Equal amounts of lysates (200 μg) were immunoprecipitated using monospecific ERK1/2, p38 MAPK, or Akt p-specific Abs; the immunoprecipitate was then incubated with ELK1, ATF2, or GSK3 fusion proteins, respectively, in the presence of ATP. Kinase activity was detected by immunoblotting, using Abs against p-ELK1, p-ATF2, or p-GSK3.

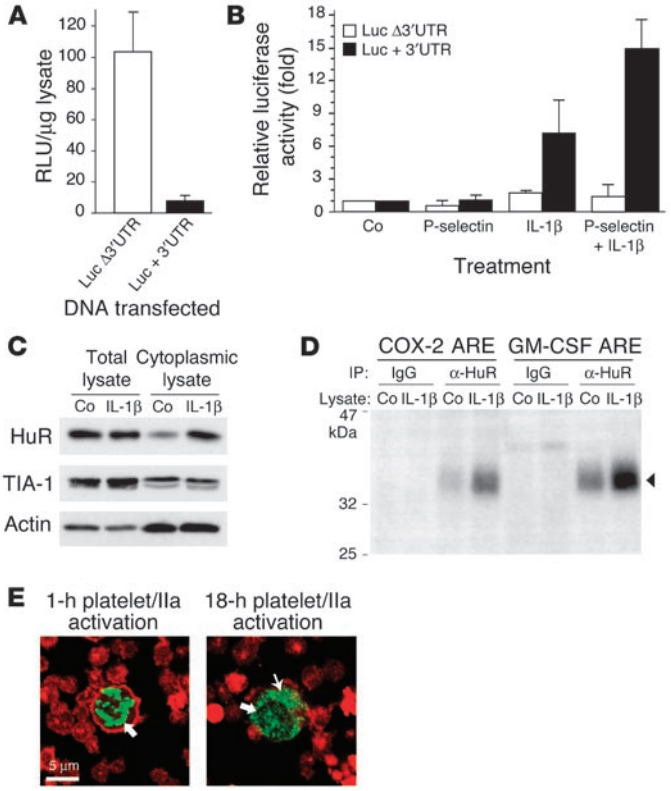

Figure 8
IL-1β regulates COX-2 mRNA decay through the ARE-binding protein HuR. (**A**) LUC/3′UTR-reporter constructs containing no 3′UTR (LUCΔ3′UTR, white bars) or COX-2 3′UTR (LUC+3′UTR, black bars) were transfected into U937 cells. LUC activity (RLU) was normalized to total protein and is the average of 3 experiments. (**B**) U937 cells were transfected with LUC/3′UTR reporter constructs and kept in suspension medium (co), incubated on immobilized P-selectin, kept in suspension in medium containing 10 ng/ml IL-1β, or incubated on immobilized P-selectin in medium containing IL-1β. Relative LUC activity was normalized to total protein, and values shown are based on LUC expression from control transfections. (**C**) U937 cells (10^6 cells) were left untreated (co) or incubated for 18 hours with 10 ng/ml IL-1β. ARE-binding proteins HuR and TIA-1 were examined in 15 μg of total cell lysates and 25 μg of cytoplasmic lysates by immunoblot. β-actin was detected as a loading control. (**D**) Cytoplasmic lysates from untreated (co) or IL-1β–treated U937 cells were incubated with ^{32}P-labeled AREs based on COX-2 or GM-CSF mRNA sequences. Bound proteins were cross-linked to the ARE and immunoprecipitated, using anti-HuR Ab or control IgG. The arrowhead indicates the major immunoprecipitated species. (**E**) Platelets were incubated with monocytes for 1 hour and 18 hours in the presence of thrombin, then examined for HuR localization by immunocytochemical analysis. Immunofluorescence of HuR in platelet-monocyte aggregates is shown in green; platelet and monocyte cytoskeleton are shown in red. Thick arrows indicate monocyte nuclei; thin arrow indicates monocyte cytoplasm. Scale bar: 5 μm.

sion or adherent to P-selectin increased LUC expression 7-fold and 15-fold, respectively (Figure 8B). U937 cells transfected with the LUCΔ3′UTR construct yielded similar levels of expression under all conditions examined. These findings indicate that P-selectin and IL-1β act in concert to promote COX-2 expression through inhibition of rapid mRNA decay mediated by the *cis*-acting COX-2 ARE in the 3′UTR of the transcript.

COX-2 mRNA is a target of cellular RNA-binding proteins that regulate mRNA stability and translational efficiency through their interaction with the ARE (49). In cancer cells, we have previously shown that altered posttranscriptional regulation of COX-2 is mediated by increased cytoplasmic mRNA binding of the mRNA stability factor Hu-antigen R (HuR) (45) and decreased binding of the translational silencer TIA-1 (43). Under normal conditions, both HuR and TIA-1 are primarily localized in the nucleus. However, in response to cellular signaling, both factors are rapidly translocated to the cytoplasm where they influence mRNA stabilization and translational efficiency (56, 57). Based on this, we sought to determine whether changes in cytoplasmic trafficking of these factors could account for the observed COX-2 mRNA stabilization and protein expression promoted by IL-1β. As shown in Figure 8C, U937 cells treated with IL-1β displayed an increase in cytoplasmic HuR levels whereas similar low levels of cytoplasmic TIA-1 were seen in both control and IL-1β–treated cells. No change in overall HuR or TIA-1 expression levels was observed as a result of IL-1β treatment. To determine whether increased ARE-binding activity of HuR occurs under these conditions, cytoplasmic extracts from control or IL-1β–treated U937 cells were incubated with ^{32}P-labeled COX-2 ARE, and bound proteins were cross-linked by UV light. The lysates were then subjected to immunoprecipitation using Ab against HuR (Figure 8D). This yielded specific immunoprecipitation of a ^{32}P-labeled polypeptide migrating at 36 kDa, the expected molecular weight of HuR. No cross-linked proteins were immunoprecipitated in identical reactions when isotype-matched IgG was used. Approximately 3- to 4-fold more HuR was bound to the radiolabeled COX-2 ARE in IL-1β–treated cells compared with control leukocytes. Similar results were obtained with immunoprecipitation using a ^{32}P-labeled ARE derived from the transcript for GM-CSF, which is known to interact with HuR (58).

Our observations suggest that increased HuR in the cytoplasm promotes enhanced ARE binding and COX-2 mRNA stabilization under conditions in which signaling of myeloid leukocytes by IL-1β occurs. To test this, primary monocytes were incubated with thrombin-activated platelets, and localization of HuR was examined by immunofluorescence (Figure 8E). After 1 hour of incubation, HuR was detected in the nuclei of monocytes in platelet-monocyte aggregates. Similarly, HuR was almost exclusively localized to the nuclei of control, untreated monocytes (data not shown). In contrast, cytoplasmic HuR was robustly detected by immunostaining after 18 hours of incubation with activated, adherent platelets (Figure 8E). These results indicate that HuR is translocated from the nucleus to the cytoplasm under conditions in which monocytes are signaled by activated platelets and that this contributes to COX-2 mRNA stabilization and protein synthesis (Figures 2, 4, and 5).

Discussion

The observations presented here demonstrate that activated platelet interactions with PSGL-1 on the monocytes promote COX-2 expression and increased PGE_2 synthesis by monocytes via mechanisms involving cellular adhesion and induced cytokine signaling.

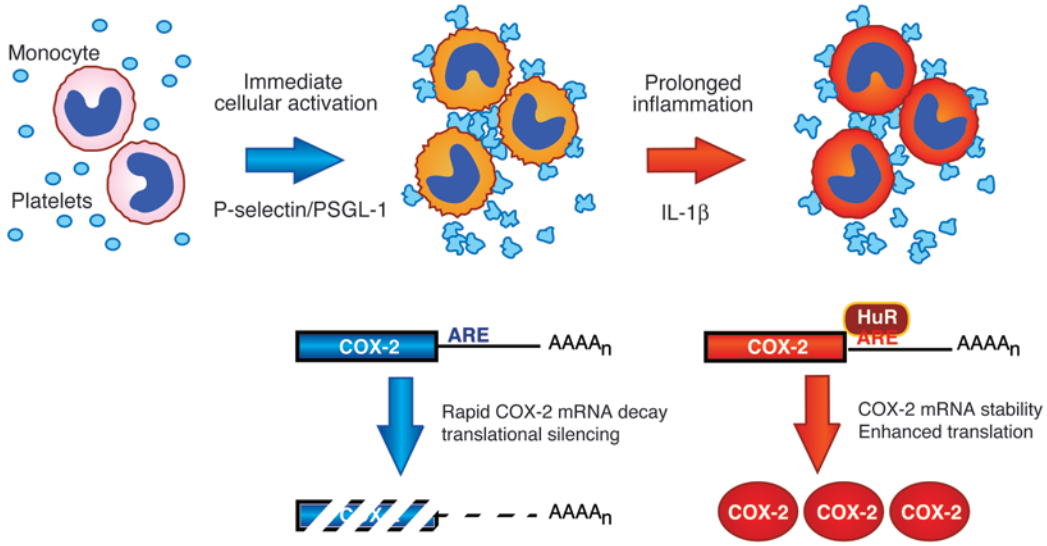

Figure 9

Adhesion-dependent regulation of COX-2 expression in human monocytes by activated platelets. Cellular activation promotes rapid translocation of P-selectin to the platelet surface. Engagement of PSGL-1 on the monocyte surface by P-selectin mediates formation of heterotypic, mixed-cell aggregates and outside-in signaling that induces the transcription of *COX-2*. Subsequent rapid decay and translational silencing of COX-2 mRNA controls protein expression and prostaglandin synthesis. Prolonged adhesion-dependent signaling promotes the expression of IL-1β, which modifies the influence of COX-2 ARE by promoting increased cytoplasmic levels of the mRNA stability factor HuR, resulting in increased mRNA $t_{1/2}$ and enhanced COX-2 protein synthesis. Details are discussed in the text. $AAAA_n$, polyadenylated tail.

A model incorporating the results is shown in Figure 9. Rapid binding of P-selectin on the plasma membranes of activated platelets to PSGL-1 on the monocytes mediates stable interaction between the 2 cell types (16, 59) and, in addition, triggers outside-in signaling that leads to NF-κB activation and *COX-2* transcription. This molecular interaction alone, however, is not sufficient to promote COX-2 protein expression. Due to its inherent ARE-mediated instability, COX-2 mRNA is rapidly degraded. Monocytes adherent to purified, immobilized P-selectin alone failed to synthesize COX-2 protein although accumulation of COX-2 transcript was apparent, indicating that a second signal is required for expression of the protein gene product under these conditions. A similar pattern has been reported for some transcripts in models of monocyte gene expression induced by adhesion to matrix structures via surface integrins (23). A second signal is then provided by IL-1β synthesized in response to cellular interaction and activation. IL-1β contributes to stabilization of COX-2 mRNA by signaling mobilization of the ARE-binding protein HuR from the nucleus to the cytosol and promotes efficient translation and synthesis of COX-2 protein. Thus, fine control of COX-2 expression occurs at both transcriptional and posttranscriptional checkpoints in activated monocytes (Figure 9), consistent with roles of COX-2 enzyme as a potent and highly regulated gene product (26, 27). This precision of control and the requisite mechanisms presumably have evolved for a regulated and physiologic inflammatory response. In contrast, several lines of evidence indicate that unregulated COX-2 expression can have complex and potentially deleterious effects in inflammatory syndromes (28–30, 46–48, 60–62). In atherosclerosis, enhanced expression of COX-2 by monocytes may be a marker of subclinical disease (48) and appears to be an important feature of platelet-monocyte thrombi in culprit lesions of patients with aspirin-insensitive myocardial infarction (F. Cipollone, personal communication). These observations underscore the relevance of the mechanism summarized in this study (Figure 9).

Our results are consistent with the notion that monocytes are a major source of COX-2 and COX-2–dependent eicosanoids in inflammation and thrombosis, although other cell types are also sources (26–28, 63), and that platelet-dependent signaling is a critical mechanism of COX-2 expression. Evidence that unregulated COX-2 expression and consequent eicosanoid production can have deleterious effects in inflammatory syndromes (see above) together with observations that platelet activation and platelet-leukocyte aggregate formation are frequently associated with these disorders (4–12) strongly suggests that alterations in check and balance mechanisms that govern COX-2 synthesis may be part of their pathogenesis (Figure 9). The posttranscriptional components of COX-2 regulation in monocytes may be particularly important, as suggested by emerging evidence that pathologic alteration of posttranscriptional control is a key mechanism of inflammatory disease (18, 25, 41). If so, posttranscriptional control points may be targets for therapeutic intervention. Our prior observations in an in vitro model of atherosclerotic plaque rupture indicate that expression of inflammatory genes that are controlled by transcriptional and posttranscriptional pathways can be differentially modulated by interruption of adhesive interactions and by pharmacologic agents (15, 17).

Integration of pathways leading to COX-2 protein synthesis in monocytes responding to signals from adherent, activated platelets (Figure 9) indicate precise regulation of COX-2 gene in a specialized cellular context. One pathway involves binding of P-selectin to PSGL-1 on myeloid leukocytes and leads to differential expression of individual inflammatory genes (16–21). Earlier observations demonstrated that adhesion of monocytes to P-selectin induces NF-κB translocation and activation (16, 19). We found that rapid

induction of *COX-2* transcription occurs through a similar mechanism (Figure 3), presumably via NF-κB *cis*-acting elements present in COX-*2* promoter (64). Although translocation of NF-κB was previously suggested to be a sufficient stimulus for adhesion-regulated immediate-early gene expression (23), we did not observe synthesis of COX-2 protein under these conditions. A major feature explaining this dissociation of mRNA accumulation and protein synthesis, which is a hallmark of posttranscriptional regulation, is rapid degradation of COX-2 transcript (Figures 2, C and D, and Figure 3C). This pattern is similar to that in colonic epithelial cells that have constitutional transcriptional activity of *COX-2* but little or no protein expression due to highly efficient degradation of COX-2 mRNA (45). Here we demonstrate that the ARE-containing 3′UTR of COX-2 mRNA is a potent regulator of gene expression in myelomonocytic cells and that it provides critical sequence information that targets the transcript for rapid degradation in this context (Figure 8A). Adhesion of monocytic cells to P-selectin did not influence the ability of COX-2 3′UTR to suppress expression (Figure 8B) even though engagement of PSGL-1 signals to some posttranscriptional pathways (18, 21). This suggested that an additional checkpoint operates on the 3′UTR to stabilize COX-2 mRNA and facilitate its translation at later time points in interactions of monocytes with activated platelets (Figures 1, 2, and 8). The significance of the initial burst of *COX-2* transcription when monocytes adhere to activated platelets or purified P-selectin (Figures 2A and 3A) is unclear, but such a mechanism may serve to prime the leukocytes for COX-2 protein synthesis after transient cellular interactions, such as adhesion and transmigration across inflamed endothelial cells or monolayers of platelets deposited at sites of vascular injury (1). Whether triggering of *COX-2* transcription similar to that induced by P-selectin presented by adherent platelets can be induced by P-selectin on microparticles (65, 66) or soluble P-selectin (67–71) remains to be determined.

Identification of IL-1β as a second signal that induces COX-2 protein expression at later time points further demonstrates remarkable specialization and integration of pathways (Figure 8) and is consistent with broad and ancient interspecies roles of the IL-1 system in inflammation and thrombosis (3, 72). IL-1β has previously been reported as an immediate-early gene in monocytes that is regulated in part by adhesive interactions and platelet P-selectin (5, 23). Its induction by platelet-monocyte aggregation (Figure 4A) and evidence for signaling of COX-2 expression (Figure 4C) suggest intricate levels of control for this component of the system (Figure 9). Of note, microarray analysis indicates that both IL-1β and IL-1β receptor subtype transcripts are induced in monocytes adherent to P-selectin (21). The platelet-signaling factors and molecular pathways that induce IL-1β synthesis in monocytes are not yet completely defined and may involve elements such as CD40L-CD40 ligation (10, 73, 74) in addition to adhesion via P-selectin (5). IL-1β may also be contributed by activated platelets in platelet-monocyte aggregates, since platelets synthesize this cytokine in a time-dependent fashion (53). Blockade of IL-1β signaling in platelet-monocyte incubations with a competitive antagonist, an approach that is used in clinical antiinflammatory strategies (72), dramatically inhibited COX-2 protein expression (Figure 4C). This demonstrates the importance of IL-1β as a second signal in the cascade leading to synthesis of COX-2 enzyme (Figures 5 and 8). Furthermore, blockage of PSGL-1 engagement with a P-selectin antagonist attenuated IL-1β–mediated COX-2 expression, indicating that the 2 pathways effectively converge to induce COX-2 protein synthesis (Figure 5C). Stabilization of COX-2 mRNA induced by IL-1β in monocytes adherent to P-selectin identified a key mechanism for transcript accumulation and its translation (Figure 5, B and D). These findings are consistent with previous reports of posttranscriptional regulation of COX-2 by this cytokine (50–52), which can act in series with transcriptional regulation (32–40), depending on the cellular context. In monocytes adherent to P-selectin, IL-1β signaling promoted activation of the ERK1/2 and p38 MAPK pathways (Figure 7), consistent with observations that activation of these kinases is a feature that contributes to enhanced COX-2 expression in several cell types (75–79). The extent of mRNA stabilization under these in vitro conditions was, however, less than that observed with activated platelets (Figure 2C), suggesting that additional signals provided by adhesion of activated platelets may also be contributing to posttranscriptional regulation of COX-2.

AREs mediate tight control of synthesis of proteins critical for cell growth and inflammation by targeting their mRNAs for rapid decay and, in some cases, influencing their translational efficiency (41, 49). In specific cells, normal growth is associated with rapid decay of COX-2 mRNA, illustrating the importance of this mechanism (42, 44). The results presented here demonstrate that COX-2 ARE function is modulated by adhesion-dependent signaling at prolonged time points in platelet-monocyte interactions (Figures 5, 8, and 9). This culminates in expression of the enzyme and synthesis of COX-2–dependent eicosanoids (Figure 1) that can then modify the inflammatory milieu. Similar posttranscriptional regulation may also have an impact on other proinflammatory proteins encoded by transcripts that contain class II AREs (80) and that are produced in platelet-monocyte aggregates. We have observed expression patterns similar to that of COX-2 for GM-CSF and TNF-α in monocytes adherent to activated platelets (data not shown).

A key mechanism by which ARE function is controlled is through interaction with RNA-binding proteins (81–84). In other cell types, we have identified HuR and TIA-1 as RNA-binding proteins that recognize COX-2 ARE and exert effects on COX-2 expression by controlling rapid mRNA decay and translational efficiency (43, 45). Our findings here strongly argue for a role of HuR in IL-1β–mediated stabilization of COX-2 mRNA in primary monocytes and myelomonocytic cells. HuR is a nuclear-cytoplasmic shuttling protein (85), and it is generally believed that the ability of HuR to promote mRNA stabilization requires its translocation to the cytoplasm (86, 87). This is supported by observations that IL-1β treatment of myelomonocytic cells mobilizes HuR from the nucleus to the cytoplasm. Furthermore, thrombin-activated platelets induce cytoplasmic trafficking of HuR in primary monocytes at times when there is elevated IL-1β expression and COX-2 mRNA stabilization and protein expression (Figures 1, 4, 5, and 8). Although the specific contributions of individual RNA-binding proteins to COX-2 regulation in adherent monocytes and the influence of platelet signals on these trans-acting factors are not yet fully characterized, the findings are consistent with the possibility that HuR acts as an mRNA stabilizing factor by competing or interfering with other ARE-binding proteins that promote mRNA decay similarly to what is seen in other cell types (56, 81). How these mechanisms and others that influence 3′UTR-mediated posttranscriptional control in monocytes and macrophages are altered in atherosclerosis and other inflammatory diseases (18, 25, 41, 88–90) also remains to be defined.

Methods

Cell isolation and platelet-monocyte incubations. Human monocytes were purified to greater than 90% by countercurrent elutriation to avoid activation events triggered by adhesion to plastic or other substrates as described (16). Washed platelets were isolated from acid-citrate-dextrose-anticoagulated human blood as described (16). Blood samples for monocytes and platelets were collected from normal volunteers after informed consent. These procedures were approved by the University of Utah Institutional Review Board.

Purified platelets were resuspended in serum-free M199 medium (Cambrex) containing 10 μg/ml polymyxin B sulfate (Sigma-Aldrich), and elutriated monocytes were resuspended in the same medium. Each time point typically contained 10^6 monocytes and 10^8 platelets in a volume of 1.5 ml unless designated otherwise. Experiments were initiated with the addition of 0.1 U/ml of thrombin (Sigma-Aldrich), and cells were collected by centrifugation (14,000 *g*, 5 min) at the indicated times. Experiments examining LPS (Sigma-Aldrich), TNF-α, and IL-1β (R&D Systems) stimulation of monocytes were performed similarly, except platelets and thrombin were omitted and monocytes were kept in suspension as indicated.

Adhesion of monocytes to immobilized P-selectin was accomplished as described (16). P-selectin (0.5 μg/ml; R&D Systems) in HBSS was added to 35 × 10 mm plates and incubated overnight at 4°C. The plates were blocked for 4 hours at 25°C with HBSS containing 2% human serum albumin (Baxter) and then washed 3 times with HBSS. Monocytes (10^6 cells), resuspended in serum-free M199 medium, were added to P-selectin–coated plates and maintained at 37°C for the indicated times. Where indicated, P-selectin antagonist (Galloyl-N-gaba-WVDV-OH; EMD Biosciences) was added to incubations. After incubation, cells were scraped from plates on ice and centrifuged at 14,000 *g* for 5 minutes at 4°C.

Cell culture and DNA transfections. U937 cells were purchased from ATCC and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS (HyClone). Transient transfections of U937 cells with LUC+COX-2 3′UTR reporter cDNA constructs (42) or a 1.8-kb human COX-2 promoter/LUC reporter construct (43) were accomplished using SuperFect (QIAGEN) or FuGENE 6 (Roche Diagnostics) according to the vendor's protocol for suspension cells. The plasmids pNF-κB/LUC and pGL3-control LUC reporter plasmids were obtained from Stratagene and Promega, respectively. U937 cells (1×10^6) were transfected in RPMI medium containing 0.5% FBS for 18 hours, after which cells were kept in suspension or added to P-selectin–coated plates for 48 hours; IL-1β (10 ng/ml) was added to specific incubations as indicated in the text. Cells were collected by centrifugation, lysed in reporter lysis buffer (Promega), and assayed for LUC activity using the Luciferase Assay System (Promega). Reporter gene activities were normalized to total protein, and all results represent the average of triplicate experiments.

Immunoblot, ELISA, and prostaglandin analysis. Cell pellets were lysed in RIPA buffer (1 × PBS, 1% Nonidet P40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease inhibitors (Sigma-Aldrich). U937 cell cytoplasmic lysates were obtained by resuspending cell pellets in ice-cold hypotonic lysis buffer (10 mM HEPES pH 7.5, 1.5 mM $MgCl_2$, and 10 mM KCl) containing protease inhibitors and incubating on ice for 10 minutes. Cells were lysed by 10 passages through a 27-gauge needle, and cytoplasmic extracts were separated from nuclei by centrifugation at 3,300 *g* for 10 minutes. Protein content was determined using a BCA protein assay with BSA as standard (Pierce Biotechnology). Lysates were denatured and separated by 10% SDS-PAGE. Following electrophoresis, proteins were transferred to PVDF membranes and probed with Abs against COX-2 (C-20), HuR (3A2), and TIA-1 (C-20) (Santa Cruz Biotechnology Inc.). Blots were stripped and then probed with anti–human β-actin Ab (MP Biomedicals Inc.). Detection and quantitation of blots were performed as described (42).

The expression of IL-1β was assayed in total cell lysates extracted in RIPA buffer. The concentration of IL-1β (pg/ml) was measured using an anti–human IL-1β Ab by ELISA according to vendor's protocol (R&D Systems) and normalized to 10^6 monocytes, 10^8 platelets, or both.

Eicosanoid production was determined in serum-free medium from incubations of monocytes and platelets (10^6 and 10^8 cells, respectively) stimulated with thrombin for 18 hours. Stable isotope dilution techniques employing gas chromatography negative ion chemical ionization mass spectrometry (91) were used to identify eicosanoids using Primary Eicosanoid HPLC Mixture (Cayman Chemical) as standards. PGE_2 synthesis in thrombin-stimulated monocyte-platelet cocultures was examined by ELISA (Cayman Chemical). After 18 hours, cells were pelleted by centrifugation, and the medium was removed. Cells were resuspended in serum-free medium containing 0.1% albumin and 20 μM arachidonic acid (Cayman Chemical) and incubated for 20 minutes at 37°C. COX-2 inhibition was accomplished by pretreating cultures with 10 μM NS-398 (Cayman Chemical) for 1 hour prior to addition of arachidonic acid–containing medium.

mRNA analysis. RNase protection assays were used to detect COX-2, GAPDH, and 28S RNAs in 10 μg of total RNA as previously described (42). COX-2 mRNA decay experiments were initiated by adding ActD (5 μg/ml) to the growth medium and assessed by Northern blot analysis using 10 μg of total RNA. Samples were separated on formaldehyde-agarose gels and blotted onto nylon membranes that were probed with antisense riboprobes for COX-2 mRNA. 18S RNA signals were used as controls to determine RNA integrity and equality of loading in each lane.

Protein kinase assay. Determination of p44/42 (ERK1/2) MAPK, p38 MAPK, and Akt kinase activities was accomplished using respective nonradioactive kits produced by Cell Signaling Technology. Freshly isolated monocytes were treated as indicated and lysed, and equal amounts of lysate were immunoprecipitated using monoclonal phosphorylated-specific (p-specific) ERK1/2, p38 MAPK, or Akt Abs; the immunoprecipitate was then incubated with ELK1, activating transcription factor 2 (ATF2), or glycogen synthase kinase 3 (GSK3) fusion proteins, respectively, in the presence of ATP. Phosphorylation of fusion proteins was detected by immunoblotting using Abs against p-ELK1 (Ser383), p-ATF2 (Thr71), or p-GSK3α/β (Ser21/9).

Analysis of protein-RNA interactions. UV light cross-linking/label transfer experiments were conducted as described (45) to examine HuR protein binding to in vitro–transcribed, ^{32}P-labeled COX-2 or GM-CSF ARE RNAs. Immunoprecipitation of cross-linked HuR was accomplished as follows. After UV cross-linking and RNase digestion, 800 μl of IP buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1% NP-40, 1 mM DTT, and 10% glycerol) was added, and reactions were incubated with equal amounts (1 μg) of HuR mAb (3A2) or mouse IgG for overnight at 4°C. Protein AG+ agarose (Santa Cruz Biotechnology Inc.) was added and incubated for 1 hour at room temperature. Immunoprecipitates were collected by brief centrifugation, washed 4 times with PBS containing 0.1% NP-40, and electrophoresed in 10% SDS-PAGE. The ^{32}P-labeled immunoprecipitated proteins were visualized by autoradiography.

Immunofluorescence. Platelets (10^8 cells) and monocytes (10^6 cells) were incubated in suspension in the presence of 0.1 U/ml of thrombin for the designated times. The cells were fixed in 2% paraformaldehyde for 20 minutes at room temperature and centrifuged onto Vectabond-coated coverglass using a Shandon Cytospin apparatus (Thermo Electron Corp.). Fixed cells were permeabilized with 0.05% Triton X-100 in PBS for 5 minutes at room temperature. For studies involving COX-2, the cells were blocked with 10% filtered horse serum diluted in HBSS for 1 hour at room temperature. Endogenous COX-2 was detected with an anti–COX-2 polyclonal Ab (C-20; 2 μg/ml) diluted in the blocking serum, and goat IgG was used in parallel as a control for nonspecific staining. The cells were incubated

with the anti–COX-2 Ab or goat IgG overnight at 4°C, washed, and then incubated with Alexa Fluor 568 conjugated to anti-goat IgG (2 μg/ml; Invitrogen) for 1 hour at room temperature. P-selectin was detected using a mAb against P-selectin (mAb S12; 1 μg/ml) that was directly conjugated to Alexa Fluor 488 (Invitrogen). Ab S12 was kindly provided by R. McEver (University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, USA). For studies involving HuR, the cells were blocked with 10% filtered goat serum diluted in HBSS for 1 hour. Cells were incubated overnight at 4°C with an anti-HuR mAb (3A2, 0.8 μg/ml) or mouse IgG that was diluted in the blocking serum. The cells were washed and incubated with an anti-mouse secondary Ab conjugated to Alexa Fluor 546 (2 μg/ml; Invitrogen) for 1 hour at room temperature. The cells were counterstained with Alexa Fluor 488–labeled phalloidin. Confocal images were obtained using an Olympus confocal microscope.

Statistics. The data are expressed as the mean ± SD. *P* values less than 0.05 were considered significant.

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