Dipyridamole Selectively Inhibits Inflammatory Gene Expression in Platelet–Monocyte Aggregates

Andrew S. Weyrich, PhD; Melvin M. Denis, BA†; Jennifer R. Kuhlmann-Eyre, BA; Elliott D. Spencer, PhD; Dan A. Dixon, PhD; Gopal K. Marathe, PhD; Tom M. McIntyre, PhD; Guy A. Zimmerman, MD; Stephen M. Prescott, MD

Background—Drugs that simultaneously decrease platelet function and inflammation may improve the treatment of cardiovascular disorders. Here, we determined whether dipyridamole and aspirin, a combination therapy used to prevent recurrent stroke, regulates gene expression in platelet–monocyte inflammatory model systems.

Methods and Results—Human platelets and monocytes were pretreated with dipyridamole, aspirin, or both inhibitors. The cells were stimulated with thrombin or activated by adhesion to collagen, and gene expression was measured in the target monocytes. Thrombin-stimulated platelets increased monocyte chemotactic protein-1 (MCP-1) expression by monocytes. Dipyridamole but not aspirin attenuated nuclear translocation of NF-κB and blocked the synthesis of MCP-1 at the transcriptional level. Dipyridamole delayed maximal synthesis of interleukin-8 but did not alter cyclooxygenase-2 accumulation. Adherence to collagen and platelets also increased the expression of matrix metalloproteinase-9 (MMP-9) in monocytes, a response that was inhibited by dipyridamole. In this case, however, dipyridamole did not block transcription or distribution of MMP-9 mRNA to actively translating polysomes, indicating that it regulates the expression of MMP-9 protein at a postinitiation stage of translation. Dipyridamole also blocked MCP-1 and MMP-9 generated by lipopolysaccharide-treated monocytes, indicating that at least part of its inhibitory action is unrelated to its antiplatelet properties.

Conclusions—These results indicate that dipyridamole has selective antiinflammatory properties that may contribute to its actions in the secondary prevention of stroke. (Circulation. 2005;111:633-642.)

Key Words: dipyridamole ■ gene expression ■ monocytes ■ platelets

…

To prevent cardiovascular disease and its complications, patients typically receive antiplatelet therapy to suppress thrombotic events; however, the inflammatory arm of treatment has not received as much attention. Although antiplatelet drugs such as aspirin have proven efficacy in reducing the incidence of myocardial infarction, they may not have equal efficacy in preventing disorders such as secondary stroke. In the European Stroke Prevention Study-2 (ESPS-2), aspirin plus extended-release dipyridamole showed a 23.1% reduction in the relative risk of stroke events compared with aspirin alone, indicating that the addition of dipyridamole improves patient outcomes. The mechanism to explain the apparent superiority of combined therapy over aspirin alone is not known. We tested the hypothesis that these agents in combination have antiinflammatory properties. We found that dipyridamole but not aspirin differentially blocked gene expression in stimulated leukocytes. These results indicate…
that in addition to its well-known antiplatelet properties, dipyridamole inhibits the expression of gene products involved in the progression and end-stage complications of atherosclerosis. Dipyridamole is reported to alter cytokine expression in murine cardiac cells and macrophages, but modulation of inflammatory gene expression in human platelet–monocyte interactions, which are critical in several phases of atherosclerosis and its complications, has not previously been observed.

Methods

Cell Isolation

Human platelets and monocytes were isolated according to procedures described previously. In brief, whole human blood was withdrawn into acid-citrate dextrose, and platelet-rich plasma was placed in separate tubes for platelet isolation (see below). Saline, in volumes proportionate to the volume of platelet-rich plasma, was added to the remaining red and white cells, and this mixed cellular pack was sedimented on dextran as previously described. After the sedimentation procedure, the leukocytes were pelleted, and the remaining red blood cells were removed by hypotonic lysis. The red cell–free leukocytes were separated by density centrifugation, and the mono-nuclear fractions were further purified by countercurrent elutriation. Purified monocytes (85% to 90%) were placed in M199 media (37°C) containing 1% human serum albumin. During the leukocyte isolation procedure, washed human platelets were isolated from the platelet-rich plasma as previously described. The platelet-rich pelleted were resuspended in M199 medium (37°C) containing 1% human serum albumin.

Platelet–Monocyte Model Systems

Purified platelets and monocytes were incubated with one another using models described previously. Before incubation, platelets and monocytes were pretreated for 30 minutes with vehicle (see below), dipyridamole (5 μg/mL), aspirin (625 ng/mL), or a dipyridamole/aspirin cocktail (8:1 5 mM MgCl2, pH 9.0, and 10 mM). Dipyridamole (Boehringer-Ingelheim) was dissolved in sodium carbonate (0.8%, pH 7.4). Vehicles containing diluted tartaric acid and further diluted in 0.9% saline. Aspirin (Boehringer-Ingelheim) was diluted in ethyl alcohol and further diluted in sodium carbonate (0.8%, pH 7.4). Vehicles containing diluted tartaric acid, ethyl alcohol, or a combination were used in all studies and did not alter gene expression profiles (see the figures below). The concentrations of aspirin and dipyridamole used in these in vitro studies approach peak plasma levels observed when the drugs are coadministered in patients. For studies involving MCP-1, IL-8, and cyclooxygenase-2 (COX-2) expression, treated cells were coincubated on tissue culture wells that were coated with type I collagen. The cells were allowed to adhere and form platelet–monocyte aggregates. The cell-free supernatants and lysates were collected for quantification of MMP-9 mRNA and protein levels were measured as described below. For measurement of MMP-9, treated cells were coincubated on tissue culture wells that were coated with type I collagen. The cells were allowed to adhere for designated periods of time, and the cell-free supernatants and lysates were collected for quantification of MMP-9 mRNA and protein. In separate experiments, monocytes were pretreated with actinomycin D (5 μg/mL) to prevent transcription of new mRNA. Monocytes were also stimulated with lipopolysaccharide (LPS) to examine the inhibitory effect of dipyridamole in a model of leukocyte gene expression that eliminates contributions from platelets. In select studies, the monocytes were also pretreated with exogenous adenosine deaminase (1.0 U/mL; Calbiochem) to catalyze the hydrolysis of extracellular adenosine to inosine and ammonia. In these studies, cell lysates and supernatants were collected at designated time points to measure MCP-1 and MMP-9 protein levels and NF-κB translocation.

Experimental Data Analyses

Most of the experimental analyses have been described in previous publications. Brief descriptions follow.

Interacellular Contact

Treated cells were stimulated with thrombin or placed on collagen-coated chamber slides as previously described. The cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature. Thrombin-activated cells were centrifuged onto Vectabond-coated cover glass with a Shandon cytopin apparatus. The fixed cells subsequently were permeabilized with PBS containing 0.1% Triton-X100 for 5 minutes at room temperature. After the cells were washed, they were stained with phalloidin and propidium iodide. The stained cells were subsequently examined with an Olympus confocal microscope.

Vasodilator-Stimulated Phosphoprotein Phosphorylation, Phosphodiesterase 4A Phosphorylation, cGMP, and TxB2

Phosphorylation of vasodilator-stimulated phosphoprotein (VASP), a substrate of cGMP- and cAMP-dependent kinases, was measured in platelets alone or platelets and monocytes treated with dipyridamole using antibodies directed against serine 157 and 239 phosphorylation sites (New England BioLabs, Cell Signaling Technology). Phosphorylation of phosphodiesterase (PDE4A) was measured in activated platelets treated with dipyridamole using a phosphospecific antibody that recognizes multiple PDE4A isoforms (FabGennix Inc.). cGMP was measured in platelet cellular lysates treated with vehicle or dipyridamole using a cGMP direct enzyme immunoassay (Amersham). TxB2, was measured in the cell-free supernatants of platelets treated with aspirin using an enzyme immunoassay kit (Assay Designs, Inc.).

IL-8, MCP-1, and MMP-9 Protein

IL-8 and MCP-1 were measured with DuoSet ELISAs purchased from R&D Systems. Total and active MMP-9 was measured by immunoassay (R&D). The ELISAs exhibited no cross-reactivity with one another or other cytokines.

COX-2 Protein

In specific experiments, cell pellets were placed in Laemelli reducing buffer and separated by SDS-PAGE electrophoresis. The gels were transferred to nitrocellulose, and COX-2 expression was examined by Western blot analysis. The COX-2 antibody used in these studies was purchased from Santa Cruz Biotechnology, Inc.

Immunocytochemistry

Immunocytochemistry for nuclear translocation of NF-κB and phosphorylation of VASP was done as previously described. The antibody for NF-κB, directed against the p65 subunit, was obtained from Santa Cruz. Phosphorylation of VASP was done using the antibody against serine 157 (see above).

mRNA Expression

TRizol was used to isolate total RNA that was subsequently used to generate cDNA. The cDNA was used as a template for target amplification using Assays-on-Demand Gene Expression Products (Applied Biosystems). AmpliTaq Gold DNA polymerase was used to amplify target cDNA using sequence specific primers and TaqMan MGB probe (6-FAM dye labeled). The primer sets for MCP-1 (Hs00269479) and MMP-9 (Hs00234579) were purchased from Applied Biosystems. mRNA levels for specific gene products were subsequently determined by real-time polymerase chain reaction (PCR) analysis with an ABI Prism 7700 Sequence Detection System (Applied Biosystems).

Ribosomal Profiling

Platelets and monocytes were prepared for ribosomal profiling using a modification of our previously published methods. In brief, 100 μg/mL cycloheximide was added to the cell suspensions just before lysis. The cells were centrifuged, and the pellet was resuspended in 375 μL low-salt buffer (20 mMmM Tris Base, 10 mMmM NaCl, 3 mMmM MgCl2, pH 9.0, and 10 μL of 0.1 mmOL DTT, 1 μL of 100
mg/mL cycloheximide, and 20 mL RNAsin) and placed on ice for 5 minutes. After this incubation period, the cells were placed in 125 μL low-salt lysis buffer (20 mmol/L TrisBase, 10 mmol/L NaCl, 3 mmol/L MgCl₂, pH 9.0, and 1.2% Triton-X100, and 200 mmol/L sucrose), mixed with a pipette tip on ice, and centrifuged. The cytoplasmic extract was removed, mixed with NaCl (150 mmol/L), and carefully overlaid onto 15% to 50% linear sucrose gradients (5 mL). The extracts were resolved by centrifugation with a Beckman SW55Ti rotor at 46 krpm for 65 minutes. The gradients were passed through a continuous-flow chamber and monitored at 254 nm with an UV absorbance detector (ISCO UA-6) to obtain ribosomal profiles.

**Display of Data and Statistics**

Experiments for protein analysis (ELISA and Western blot) were performed at least 3 times using cells from different donors. Each experimental ELISA point was measured in duplicate. The mean±SEM values from ELISA analysis are presented when indicated. Representative experiments that depict typical results are shown for Western analysis and time course experiments. ANOVA was used to determine differences among groups. If significant differences were found, post hoc t tests were used to determine the exact location of the difference; values of P<0.05 were considered statistically significant. Representative experiments for cell staining studies, quantification of mRNA levels, and ribosomal profiling are shown. In most cases, representative experiments were chosen from a total of at least 3 experiments.

**Results**

**Efficacy of In Vitro Treatment With Aspirin and Dipyridamole**

The concentrations of dipyridamole and aspirin used in our in vitro studies approximate the peak plasma concentrations achieved 2 hours after the combination therapy is administered to patients. We found that aspirin (625 ng/mL) blocked thrombin-induced generation of TxB₂ (Figure 1A), although complete blockade required a preincubation period of 30 minutes. Dipyridamole (5 μg/mL) modestly increased the phosphorylation of VASP on serine 157 and 239 in quiescent and thrombin-stimulated platelets (Figure 1B). Dipyridamole (5 μg/mL) also elevated intracellular cGMP levels in quiescent platelets by 10-fold (∼175 to >1800 fmol/1×10⁸ platelets plus dipyridamole). When platelets and monocytes were coincubated with one another, there was a modest increase in VASP phosphorylation that was visibly reduced in the presence of thrombin or collagen (Figure 1C). However, in dipyridamole-treated cells, VASP phosphorylation...
tion on serine 157 and 239 was markedly increased (Figure 1C). Immunocytochemistry experiments demonstrated that VASP was phosphorylated on serine 157 (Figure 1D) and serine 239 (data not shown) in platelets adherent to monocytes. However, we found no evidence of VASP phosphorylation in monocytes (Figure 1D).

Protein Expression

Incubation of thrombin-stimulated platelets with monocytes increased the synthesis of MCP-1 by monocytes (Figure 2A) similar to previous observations.11 Dipyridamole (5 μg/mL) completely blocked MCP-1 synthesis (Figure 2B). Aspirin alone (625 ng/mL) did not inhibit MCP-1 production (Figure 2B), and increasing the concentration of aspirin by 10-fold did not alter MCP-1 or MMP-9 accumulation (data not shown). A lower concentration of dipyridamole reduced but did not abolish MCP-1 generated by monocytes adherent to thrombin-activated platelets (Data Supplement Figure I). The inhibitory properties of dipyridamole (5 μg/mL) on MCP-1 accumulation were maintained over 24 hours (Figure 2C).

In parallel experiments, we determined whether dipyridamole exerted its inhibitory effect on other gene products by measuring IL-8 and COX-2 synthesis by monocytes incubated with thrombin-stimulated platelets. Synthesis of IL-8 was observed as early as 4 hours and continued to increase over 24 hours (Figure 2D). Although dipyridamole delayed the onset of IL-8 synthesis, it did not significantly alter IL-8 accumulation at 4 or 18 hours (Figure 2D and Data Supplement Figure II A and II B). Aspirin had no effect on IL-8 production. Activated platelets also increased COX-2 expression in monocytes (D.A.D., G.A.Z., unpublished data). However, COX-2 expression was not inhibited by dipyridamole or aspirin (Data Supplement Figure III).

In the next set of studies, we determined whether aspirin or dipyridamole inhibited expression of MMP-9, a plaque-destabilizing matrix metalloproteinase, in a platelet–monocyte system in which adherence to extracellular matrix enhances MMP-9 expression.3 We found that monocytes adherent to collagen and platelets increased the production of total MMP-9 (Figure 3A). Dipyridamole but not aspirin attenuated the generation of total MMP-9 (Figure 3A). The percentage of total MMP-9 converted to its active form was similarly inhibited by dipyridamole (Figure 3B).

Inhibition of Gene Expression Is Not a Result of Decreased Heterotypic Cellular Interactions

Adhesion between platelets and monocytes is required for the induction of inflammation and gene expression by monocytes.3,11 Therefore, we determined whether dipyridamole regulates intercellular interactions between platelets and monocytes. We observed minimal cell adhesion in unstimulated cell suspensions (Figure 4A and 4B). As previously reported, thrombin-activated platelets adhered to one another and monocytes, yielding platelet–monocyte rosettes (Figure 4C).11 Dipyridamole (Figure 4D) did not decrease intercellular interactions between thrombin-activated platelets and monocytes. Consistent with this finding, thrombin-induced expression of the adhesion molecule P-selectin on platelets was not blocked by dipyridamole (data not shown). When platelets and monocytes were coincubated on immobilized collagen, platelets and monocytes readily adhered to this extracellular matrix, and platelet spreading and platelet–platelet adhesion were easily observed (Figure 4E). Platelet–monocyte clustering also was visible as platelets bridged monocytes to one another (Figure 4E).13 Dipyridamole did not alter cell adherence, platelet spreading, or intercellular interactions in this platelet–monocyte model system (Figure 4F).

To test whether dipyridamole inhibited synthetic patterns in monocytes independently of any platelet stimulatory factors, we measured gene expression in monocytes stimulated...
with LPS. LPS-treated monocytes synthesized MCP-1 and MMP-9, and this response was markedly attenuated by dipyridamole (Figure 5A and 5B). In contrast, COX-2 expression was not blocked by dipyridamole (Figure 5C).

Because MCP-1 is, in part, regulated by NF-κB, we examined the cellular distribution of this transcription factor in LPS-stimulated monocytes. In quiescent cells, NF-κB was located in the cytoplasm (Figure 5C, left). LPS induced a marked increase in nuclear expression of this transcription factor, a response that was attenuated but not completely blocked by dipyridamole (Figure 5C, middle and right). Similar intracellular distribution patterns were seen in monocytes exposed to thrombin-stimulated platelets or collagen-adherent platelets (data not shown).

mRNA Expression and Translational Distribution
Because dipyridamole blocked synthesis of MCP-1 and markedly reduced MMP-9 protein synthesis in response to signals by platelets and by LPS, we determined whether it was exerting its inhibitory effect by blocking the transcription of corresponding mRNAs in platelet–monocyte systems. Monocytes were incubated with platelets for 2 hours, and cells subsequently were collected to quantitatively determine mRNA levels by real-time PCR. Thrombin-stimulated platelets increased mRNA levels for MCP-1 in monocytes compared with unstimulated (baseline) cells (Figure 6A, top). MCP-1 mRNA levels were markedly suppressed in dipyridamole-treated cells (Figure 6A, right). MMP-9 mRNA levels were increased in monocytes adherent to collagen and costimulated with platelets compared with unstimulated (baseline) cells (Figure 6B). Dipyridamole did not block the increase in MMP-9 mRNA expression, but actinomycin D, a transcriptional inhibitor, did (Figure 6C, left). However, both actinomycin D and dipyridamole blocked the synthesis of MMP-9 protein (Figure 6C, right).

These results indicated that dipyridamole exerts its inhibitory effect at the transcriptional level for MCP-1 and posttranscriptional level for MMP-9. Therefore, we determined the distribution of MCP-1 and MMP-9 mRNA in ribosomal profiles. Monocyte extracts alone exhibited a
characteristic distribution of ribosomal peaks corresponding to the 40S ribosomal subunit, 60S ribosomal subunit, 80S monosome (1 ribosome), and polysomes (multiple ribosomes) (see tracings in Figure 7A). Platelets, at cell numbers used in this study, did not have detectable ribosomal peaks (data not shown). When the monocytes were coincubated with thrombin-activated platelets, there was a clear decrease in the 60S ribosomal subunit with a corresponding increase in 80S monosome peak, indicating an increase in the initiation and subsequent translation of mRNA transcripts (see tracings Figure 5).

Figure 5. Dipyridamole attenuates LPS-induced MCP-1 and MMP-9 synthesis. Monocytes were stimulated with LPS (100 ng/mL) for 18 hours in the presence of vehicle or dipyridamole (Dip; 5 μg/mL). Synthesized MCP-1 (A), MMP-9 (B), or COX-2 (C) protein was measured. A, B, Bars represent mean±SE for 5 experiments. *P<0.05 vs control; **P<0.05 vs LPS-stimulated cells. C, Blot represents 2 independent experiments. D, Monocytes were left quiescent (left) or were stimulated with LPS (100 ng/mL) for 2 hours in the presence of vehicle (middle) or dipyridamole (Dip; 5 μg/mL; right). Intracellular localization of NF-κB was detected as previously described.11,22 D, Representation of 3 independent experiments.

Figure 6. Dipyridamole differentially inhibits mRNA expression. Platelets and monocytes were stimulated with 0.1 U/mL thrombin (A) or placed on immobilized collagen (B) for 2 hours in the presence or absence of dipyridamole (Dip; 5 μg/mL). Total RNA was isolated to generate cDNA for use in quantitative real-time PCR experiments. Left, mRNA expression for MCP-1 (A) and MMP-9 (B). In parallel, GAPDH mRNA levels were measured in the same samples (middle). Right, Fold change in copy number over baseline. GAPDH was used as normalization factor to calculate fold change in copy number. A, B, Representation of 3 independent experiments. C, Platelets and monocytes were placed on immobilized collagen (Coll) in the presence of vehicle, actinomycin D (Act D; 5 μg/mL), or dipyridamole (5 μg/mL), and MMP-9 mRNA expression, as determined by real-time PCR, and total MMP-9 protein were determined. Left, Fold change in copy number of MMP-9 mRNA normalized to GAPDH. These samples were measured at baseline or after 2 hours of adherence and represent 3 independent experiments. Total MMP-9 protein (right) was measured after 18 hours of adherence. Right, Bars are mean±SEM of 4 independent experiments. *P<0.05 vs baseline; **P<0.05 vs vehicle.
The formation of the 80S ribosomal subunit was slightly attenuated but not eliminated in cells treated with dipyridamole (see tracings in Figure 7B). The total amount of MCP-1 mRNA was increased in monocytes coincubated with thrombin-stimulated platelets compared with quiescent monocytes, consistent with enhanced transcription (see bar graphs in Figure 7A and Figure 6A, right). The newly transcribed MCP-1 mRNA was detected primarily in the polysomes, consistent with active translation and increased expression (see bar graphs in Figure 7A). Consistent with the observation that dipyridamole blocked transcription of MCP-1 mRNA (Figure 6), MCP-1 transcripts were not detected in polysome fractions after dipyridamole treatment (see bar graphs in Figure 7B).

When the platelets and monocytes were coincubated on collagen, there was also an increase in the formation of the 80S ribosomal subunit compared with quiescent monocytes, a response that was not significantly altered by dipyridamole (see tracings in Figure 7C and 7D). MMP-9 mRNA was increased in the polysomes of monocytes coincubated with platelets compared with quiescent monocytes (see bar graphs in Figure 7C). Dipyridamole did not alter the distribution of MMP-9 mRNA across ribosomal fractions (see bar graphs in Figure 7D), indicating that it regulates the expression of MMP-9 protein at a posttranscriptional checkpoint downstream of translation initiation.

Discussion
Myocardial infarction and stroke are 2 of the most frequent causes of death in the United States.25 One of the inciting events for both syndromes is atherosclerotic plaque rupture, a process that activates platelets and forms thrombi in diseased vessels.26 Antiplatelet agents are the mainstays of preventive care because they decrease the incidence of end-stage vessel occlusion that is responsible for most myocardial infarctions and strokes. In addition to thrombosis, however, it is now appreciated that inflammation contributes to the development of atherosclerosis and its complications.1,2,4 In some cases, inflammatory pathways promote thrombosis, and conversely, thrombotic events often exacerbate inflammatory reactions. Thus, drugs that simultaneously block thrombotic occlusion and reduce inflammation may have added benefits in the treatment of cardiovascular disease.
In the present study, we demonstrate that dipyridamole differentially inhibits the synthesis of inflammatory gene products produced by human platelet–monocyte aggregates. In current therapeutic practice, dipyridamole by itself is seldom used. However, an extended-release form of dipyridamole (200 mg) and aspirin (25 mg), designed to take advantage of the additive antiplatelet effects of both drugs, is used for secondary stroke prevention.27 Dipyridamole inhibits the uptake of adenosine by platelets at therapeutic concentrations (0.5 to 5 μg/mL), thereby enhancing platelet adenylate cyclase activity and increasing platelet cAMP levels.27 Through this mechanism, dipyridamole attenuates platelet aggregation in response to moderate agonists such as platelet-activating factor, soluble collagen, and ADP. Dipyridamole also inhibits PDE in some tissues, and we found that it reduces phosphorylation of PDE4A in thrombin-activated platelets (see Data Supplement Figure IV). Therapeutic levels of dipyridamole also inhibit cGMP-PDE, thereby augmenting the increase in cGMP produced by nitric oxide.27 In our studies, dipyridamole modestly enhanced the phosphorylation of VASP in platelets, a response that was further increased when platelets were coincubated with monocytes. Phosphorylation of both serine 157 and 239 residues of VASP was increased, indicating that CAM and cGMP kinases, respectively, were activated.23 VASP phosphorylation was not seen in monocytes adherent to thrombin-stimulated or collagen-adherent platelets (Figure 1D). Aspirin irreversibly inhibits platelet cyclooxygenase and thus inhibits the generation of TXA2, a powerful inducer of platelet aggregation and vasoconstriction.28 In vivo, aspirin concentrations that approximate those used in this study elicit comparable antiplatelet effects compared with full-dose aspirin treatment; the only apparent difference is that low-dose aspirin takes ~45 minutes longer to achieve full platelet inhibition.29

Although the antiplatelet properties of aspirin and dipyridamole are well documented, antiinflammatory attributes of these drugs are less clear. Here, we found that dipyridamole differentially inhibited the expression of several gene products in human models of cellular inflammation. We focused on gene expression in platelet–monocyte systems because heterotypic aggregates between these cells are prevalent in syndromes of dysregulated inflammation, including stroke and myocardial infarction.3,4,6 Dipyridamole blocked the synthesis of MCP-1 by monocytes that were adherent to activated platelets. MCP-1 is a potent chemokine that regulates the onset, development, and progression of atherosclerosis.13 Inhibition of MCP-1 synthesis by dipyridamole was not a result of decreased formation of platelet–monocyte aggregates, a step that is critical for signaling altered gene expression in target monocytes.3,9,11 In addition, inhibition of MCP-1 synthesis also occurs in LPS-stimulated monocytes, indicating that at least part of the effect involves synthetic pathways in the leukocytes. Dipyridamole blocked MCP-1 protein expression by decreasing transcription of MCP-1 mRNA, identifying this as a point at which it exerts antiinflammatory control. NF-κB acts in concert with other DNA-binding proteins to control the transcription of MCP-1 mRNA,22,30 and we found that nuclear translocation of NF-κB is reduced after dipyridamole treatment, although not completely. Inhibition of MCP-1 gene expression in LPS-stimulated monocytes may be due to increases in extracellular adenosine because exogenous adenosine deaminase reversed the inhibitory effect of dipyridamole (see Data Supplement Figure V). It is known that adenosine can alter LPS-induced gene expression in monocytes.31 In addition to modulating adenosine-dependent mechanisms, dipyridamole regulates other signaling pathways in parallel. In this regard, VASP phosphorylation, an established marker of nitric oxide/cGMP effects,32–34 is markedly increased in platelets that have been treated with dipyridamole. Nitric oxide, which increases cGMP levels in cells, blocks the synthesis of MCP-1 expression at the mRNA and protein levels in keratinocytes and endothelial cells.35,36 PDE4 inhibitors like dipyridamole also block gene expression at the transcriptional level in LPS-stimulated human monocytes.37

Dipyridamole had differential inhibitory effects on inflammatory gene expression in monocytes. There was no effect on COX-2 expression, an inflammatory gene that is expressed in LPS-stimulated monocytes or in monocytes stimulated with activated platelets (D.A.D., D.A.Z., unpublished data). This indicates that its effects on MCP-1, IL-8, and MMP-9 expression were not due to cellular toxicity. Dipyridamole delayed maximal synthesis of IL-8, a cytokine that attracts neutrophils to diseased vessels and downregulates tissue inhibitor of metalloproteinase-1, an antithrombotic metalloproteinase.13 Because NF-κB is critical for the transcription of IL-8 message,38 it is possible that dipyridamole delayed the synthesis of IL-8 protein by reducing nuclear translocation of this transcription factor. Even if this is the case, however, attenuation of nuclear NF-κB was not great enough to thwart IL-8 protein accumulation over time. Finally, we found that dipyridamole markedly suppressed the expression and activity of MMP-9, a matrix metalloproteinase that is produced by monocytes adherent to extracellular matrices and platelets.3 Although it has been reported that platelets also release MMP-9,39 we could not detect this metalloproteinase in human platelets and documented that monocytes are the principal source under these conditions.3 In this system, transcription of MMP-9 mRNA occurs when monocytes adhere to extracellular matrix and the engagement of P-selectin glycoprotein-1 on the monocyte by P-selectin displayed by activated platelets delivers signals that further increase transcript formation and protein production.3 MMP-9 is expressed in mononuclear cells embedded in platelet-rich areas of ruptured atherosclerotic plaques,3 and increased MMP-9 production regulates vascular remodeling and contributes to aneurysm formation in addition to promoting plaque rupture.40–42 MMP-9 is also a strong predictor of ischemic stroke.43–45 Although dipyridamole has well-documented antiplatelet properties, including increased phosphorylation of VASP,44 it did not prevent cellular adherence or spreading on immobilized collagen (Figure 4). Likewise, dipyridamole did not significantly decrease intercellular contact between platelets and monocytes adherent to collagen. Similar to its effects on MCP-1 expression, this indicates that the inhibitory properties of dipyridamole on monocyte MMP-9 production occurred downstream of adherence to extracellular matrix or platelets, a conclusion that is further
supported by attenuation of MMP-9 synthesis in LPS-stimulated monocytes. Reports in other cellular systems demonstrate that increased nitric oxide reduces MMP-9 expression in rat smooth muscle and renal mesangial cells, implying that dipyridamole may block MMP-9 synthesis by increasing cGMP levels in platelets and/or monocytes.

Although dipyridamole differentially inhibited MCP-1 and MMP-9 synthesis, it regulated expression of these gene products at different molecular steps. Dipyridamole blocked MCP-1 protein production primarily through decreased transcription of DNA to RNA, presumably through NF-κB–dependent mechanisms (see above). In contrast, dipyridamole did not inhibit transcription of MMP-9 mRNA even though it suppressed protein levels. This is somewhat surprising because MMP-9 gene expression is influenced by NF-κB in an agonist-specific fashion in transfected cell systems, and our data clearly indicate that transcription is requisite for MMP-9 protein synthesis (see Figure 6C). Therefore, we examined the association of MMP-9 mRNA with ribosomes to determine whether dipyridamole blocked initiation, the rate-limiting step of translation in which ribosomes attach to mRNA. As expected, we found that MMP-9 mRNA is associated with polyribosomes in monocytes adherent to platelets and collagen (Figure 7), indicating that the message is being actively translated. Dipyridamole did not block the distribution of MMP-9 mRNA to polyribosomal fractions, indicating that it inhibited expression of MMP-9 protein at a postinitiation checkpoint. This suggests that dipyridamole blocks protein production by “stalling” ribosomes on MMP-9 mRNA or by inducing rapid turnover of MMP-9 protein after it is made. Another possibility is that dipyridamole destabilizes MMP-9 mRNA after it is transcribed, reducing the amount of template available for translation over time. Altered stability of the MMP-9 transcript is reported in rat mesangial cells through cGMP mechanisms. Although the specific mechanism is yet to be determined, our observations indicate an inhibitory role for dipyridamole at a posttranscriptional or posttranslational checkpoint, a unique feature that may be relevant to its antistroke effects.

Inhibition of the synthesis of inflammatory gene products by monocytes indicates that dipyridamole may block important signaling responses in cells besides platelets in vivo. Additional in vitro studies also demonstrate that dipyridamole inhibits superoxide ion generation and decreases tissue factor expressed by leukocytes via increased extracellular adenosine levels. Dipyridamole also blocks the synthesis of leukotrienes B$_3$ and C$_4$ by stimulated leukocytes through mechanisms that are not related to extracellular adenosine. Whether the effective concentration of dipyridamole at cellular membranes in vivo approaches that obtained in vitro is not clear because 91% to 99% of dipyridamole administered in vivo is protein bound. Nevertheless, our studies point to antiinflammatory properties of dipyridamole that were not previously considered. This, combined with the well-known antiplatelet effects of aspirin, may partially explain the beneficial effects of the dipyridamole/aspirin combination therapy in ESPS-1 and ESPS-2. It is not known whether other antiplatelet reagents such as ticlopidine or clopidogrel have similar antiinflammatory effects.

In summary, we demonstrated a clear inhibitory effect of dipyridamole on inflammatory gene markers produced by activated monocytes. Inhibition occurred at transcriptional and posttranscriptional gene regulatory steps, depending on the gene product. It is now recognized that the vascular pathobiology leading to myocardial infarction and stroke is multifactorial and that treatments beyond platelet inhibition are required. In some clinical settings, dipyridamole/aspirin combination therapy may have an advantage over other antiplatelet drugs because of the antiinflammatory properties of dipyridamole.

Acknowledgments

This work was supported by an unrestricted research grant from Boehringer Ingelheim (A.S.W. and S.M.P.), an Established Investigator Award from the American Heart Association (A.S.W.), and National Institutes of Health grants (HL-66277 to A.S.W., HL-44525 to G.A.Z., and HL-44513 to T.M.M.). We thank the technical staff of the Vascular Biology group for help with cell isolation, our colleagues in the Program of Human Molecular Biology and Genetics and elsewhere at the University of Utah for their helpful discussions and critical reading of the manuscript, Michele Czerwinski for revision of the manuscript text, and Diana Lim for preparation of the figures. We also thank W.G. Eisert, MD, PhD, of Boehringer Ingelheim, for a critical review and discussion of our data and manuscript.

References

14. Loftus IM, Naylor AR, Goodall S, Crowther M, Jones L, Bell PR, Thompson MM. Increased matrix metalloproteinase-9 activity in unstable
Dipyridamole Selectively Inhibits Inflammatory Gene Expression in Platelet–Monocyte Aggregates

Andrew S. Weyrich, Melvin M. Denis, Jennifer R. Kuhlmann-Eyre, Elliott D. Spencer, Dan A. Dixon, Gopal K. Marathe, Tom M. McIntyre, Guy A. Zimmerman and Stephen M. Prescott

_Circulation_. 2005;111:633-642; originally published online January 24, 2005; doi: 10.1161/01.CIR.0000154607.90506.45

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/111/5/633

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2005/02/07/01.CIR.0000154607.90506.45.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/