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Inhibition of Agonist-Induced p42 and p38 Mitogen-Activated Protein Kinase Phosphorylation and CD40 Ligand/P-Selectin Expression by Cyclic Nucleotide-Regulated Pathways in Human Platelets

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ABSTRACT. Platelet activation and adhesion to endothelial cells and extracellular matrix proteins are crucial events in the development of arterial cardiovascular diseases. Platelet activation is initiated by stimulation of intracellular signaling cascades, including the p42 mitogen-activated protein kinase (MAPK) and p38 MAPK pathways, followed by major changes in the platelet cytoskeleton and expression and activation of platelet surface receptors, such as P-selectin (CD62P) and CD40 ligand (CD40L). Activated platelets directly bind to vascular endothelial cells via CD40L/CD40 interactions and induce inflammatory reactions that initiate or aggravate atherosclerotic lesions. The aim of this study was to investigate effects of two known platelet inhibitors—the cAMP-elevating prostaglandin E_1 (PG-E_1) and the cGMP-elevating sodium nitroprusside (SNP)—on platelet p42 MAPK and p38 MAPK activation as well as on surface expression of CD62P and CD40L. MAPK activation was analyzed by Western blot experiments using phosphorylation-specific antibodies, and surface CD40L and CD62P expression was determined by flow cytometry analysis. PG-E_1 and SNP strongly inhibited p42 and p38 MAPK phosphorylation as well as CD40L and CD62P expression in response to thrombin, a thromboxane A_2 analog, and ADP. These data indicate that adenosine and guanosine 3′,5′-cyclic monophosphate-dependent protein kinases not only inhibit platelet pathways leading to activation and aggregation, but also those resulting in enhanced surface expression of protein ligands involved in inflammation. Expression of CD40L and CD62P was found to be independent of MAPK activation, since it was not inhibited by specific MAPK inhibitors. Inhibition of platelet-induced inflammatory responses including CD62P- and CD40L-mediated interaction of platelets with leukocytes and endothelial cells, respectively, is suggested to be an important component of the long-term vasoprotective effects of cyclic nucleotide-elevating prostaglandins and NO donors. BIOCHEM PHARMACOL **60**;9:1399–1407, 2000. © 2000 Elsevier Science Inc.

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Platelet activation and aggregation are crucial initial pathological events for the development of cardiovascular diseases or severely aggravate existing pathological alterations in the cardiovascular system. A variety of agents (e.g. ADP and TXA_2§) that are released from activated platelets or injured cardiovascular cells have been recognized as major factors in the development of arterial thrombosis [1, 2]. Platelet activation induces major changes in the platelet cytoskeleton, leading to shape change, activation of platelet adhesion receptors, and expression of proinflammatory surface proteins such as P-selectin (CD62P) or CD40 ligand (CD40L) [3, 4]. This expands the role of platelets from involvement in haemostasis to the induction of inflammatory responses at sites of injured vessel wall.

These changes in platelet surface receptors are tightly regulated by a complex network of intracellular activatory and inhibitory signaling cascades. Initially, most platelet agonists bind to G protein-linked receptors, resulting in dissociation of G_α and $G_{\beta\gamma}$ subunits and activation of protein tyrosine kinases (among them pp60src, pp72syk, or pp125FAK) and an isoform of phosphotidylinositol 3-kinase [5–7]. Activation of serine–threonine kinases such as protein kinase C (PKC) and of guanine nucleotide ex-

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§ *Abbreviations:* cAMP, adenosine 3′,5′-cyclic monophosphate; cAMP-PK, cAMP-dependent protein kinase; cGMP, guanosine 3′,5′-cyclic monophosphate; cGMP-PK, cGMP-dependent protein kinase; FITC, fluorescein isothiocyanate; IgG, immunoglobulin G; MAPK, mitogen-activated protein kinase; NO, nitric oxide; PG-E_1, prostaglandin E_1; PRP, platelet-rich plasma; SNP, sodium nitroprusside; TXA_2, thromboxane A_2; and VASP, vasodilator-stimulated phosphoprotein.

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change factors (Rho, Rac, and cdc42Hs) are further indispensible steps for platelet activation (reviewed in [8]). However, very little is known about the precise function and regulation of MAPKs in platelets. p42 MAPK and p38 MAPK are rapidly phosphorylated and activated during the early phase of platelet activation in response to various agonists such as thrombin or TXA_2 [9–13]. It has been shown that low doses of agonist (collagen and arachidonic acid, or collagen and U46619, respectively) induce platelet aggregation and secretion through p42 MAPK- or p38 MAPK-dependent pathways. Under these conditions, specific inhibitors of MAPK kinase (which activates p42 MAPK) or p38 MAPK are able to inhibit aggregation and secretion, an effect that can be overcome by increasing doses of agonist [13, 14]. These studies suggested that p42 MAPK and p38 MAPK activation provide a crucial signal in early stages of platelet activation that might be necessary for surface receptor expression/activation and aggregation in response to minimal concentrations of platelet agonists or extracellular matrix proteins. MAPKs contain a dual phosphorylation site (TXY motif, amino acids 185–187 (p42 MAPK), 202–204 (p44 MAPK), 180–182 (p38 MAPK)) in their activation loop. Phosphorylation of both sites, the threonine and the tyrosine residue, is required for MAPK activation [15]. The availability of a monoclonal di-phospho-specific antibody directed against activated p42/p44 MAPKs and a polyclonal di-phospho-specific antibody directed against activated p38 MAPK allows easy quantitation of MAPK phosphorylation by Western blotting.

Inhibition of platelet activation is essential for prevention and therapy of various cardiovascular diseases. Potent inhibitors of platelet activation are endogenous factors released from vascular endothelial cells such as the endothelium-derived NO and prostacyclin ($PG\text{-}I_2$). In platelets, the effects of cAMP-elevating prostaglandins ($PG\text{-}I_2$, $PG\text{-}E_1$) and cGMP-elevating NO donors (authentic NO, sodium nitroprusside) are mediated by cAMP- and cGMP-dependent protein kinases, respectively [16–20]. The effects of cyclic nucleotide-dependent protein kinases on platelet MAPK activity and on expression of important inflammatory molecules such as CD40L are still unknown.

In this study, the effects of cyclic nucleotide-elevating vasodilators on agonist-induced platelet p42/p38 MAPK activation and platelet CD40L and CD62P surface expression was studied. Specific inhibitors of p42 and p38 MAPK pathways were used to evaluate whether MAPK activation is directly linked to CD40L/CD62P surface expression.

MATERIALS AND METHODS

Materials

Antibody 12D4 directed against threonine^{202}/tyrosine^{204} di-phosphorylated p42/p44 MAPK and the monoclonal anti-p42 MAPK antibody were obtained from nanoTools. Antibody 16C2 directed against serine^{239}-phosphorylated VASP has been described [21]. Polyclonal antisera against p38 MAPK and the phosphorylated form of p38 MAPK were obtained from New England Biolabs. A monoclonal antibody against p44 MAPK, a FITC-conjugated monoclonal antibody against CD62P (P-selectin), and the monoclonal anti-CD40L (anti-CD154) antibody were obtained from Pharmingen. FITC-conjugated goat anti-mouse IgG was obtained from Sigma. Kinase inhibitors PD 98059 and SB 203580 were obtained from Alexis Biochemicals.

Isolation, Stimulation, and Sample Preparation of Platelet-Rich Plasma or Washed Human Platelets

PRP or washed human platelets were prepared as described previously [16, 17]. For Western blot experiments, washed platelets were resuspended in PBS with 1 mM EDTA, 5.5 mM D-glucose, and 1 mM $MgCl_2$. For flow cytometry experiments, PRP or (in the case of thrombin stimulation) washed platelets resuspended in PBS with 5.5 mM D-glucose, 0.5% BSA, 1 mM $MgCl_2$, and 1 mM $CaCl_2$ were used. PRP or washed platelets were preincubated with a final concentration of 100 μM aspirin for 45 min at 37°, and (except for ADP experiments) 1 U/mL of apyrase was added. Platelets were preincubated with buffer, 10 μM $PG\text{-}E_1$, or 100 μM SNP for 1 min, followed by activation with 0.5 U/mL of thrombin, 2 μM of the TXA_2 analog U46619, or 30 μM ADP. Kinase inhibitors PD 98059 and SB 203580 were used in a final concentration of 10 μM each and preincubated for 30 min at 37°. At various time points, aliquots of the cell suspension were removed and reactions were stopped for Western blot analysis by addition of 30% final concentration of SDS stop solution, as described previously [22]. For flow cytometry, 1% (final concentration) of methanol-free formaldehyde was added, and samples were allowed to fix for 10 min at room temperature.

Western Blotting and Quantification

Western blotting was performed as described previously with minor modifications [22]. Briefly, proteins were separated on 10% SDS–polyacrylamide gels and blotted onto polyvinylidene fluoride (PVDF) membranes, which were blocked with 6% phosphatase-free milk powder in phosphate-buffered saline with 0.05% Tween (PBS/Tween). Antibodies were diluted in 3% milk to a final concentration of 0.4 μg/mL (antibody 12D4, anti-phospho-p42/44), 0.1 μg/mL (anti-p42), 0.5 μg/mL (anti-p44), 0.5 μg/mL (antibody 16C2), and incubated overnight at 4°. Anti-phospho-p38 MAPK antibody and anti-p38 MAPK antibody were used in 1:500 and 1:1000 dilutions, respectively. Immunoreactivity was determined using peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Bio-Rad) and the enhanced chemiluminescence (ECL) reaction (Amersham). Intensity of the Western blot bands was quantified as described previously [22].

Flow Cytometry Analysis

Fixed human platelets were pelleted for 1 min at 2700 *g* and resuspended in PBS with 5.5 mM D-glucose and 0.5%

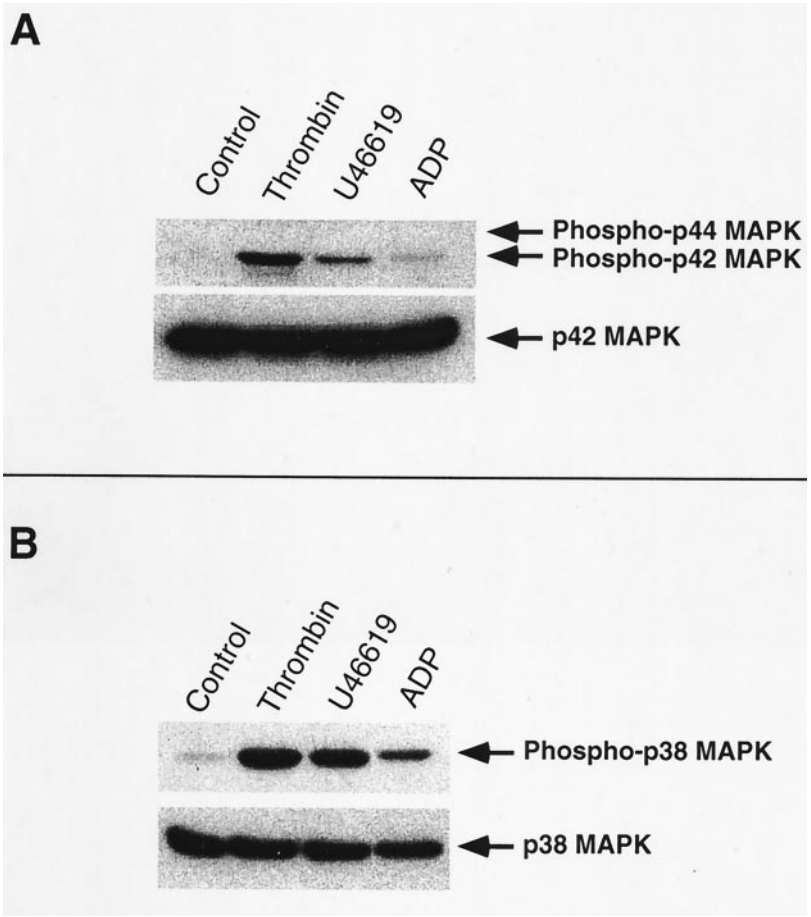

FIG. 1. Phosphorylation of p42 and p38 MAPKs by platelet agonists. Washed human platelets were incubated with buffer (control), 0.5 U/mL of thrombin, 2 μM U46619, or 30 μM ADP for 2 min and analyzed for threonine/tyrosine phosphorylation of p42/p44 MAPK (panel A, upper autoradiograph) and of p38 MAPK (panel B, upper autoradiograph) by Western blotting using phosphorylation-specific antibodies as described in Methods. Approximately equal loading of each lane was confirmed by using phosphorylation-independent antibodies against p42 or p38 MAPKs (lower autoradiographs in panels A and B, respectively). Autoradiographs shown are representative of at least three independent experiments.

bovine serum albumin (PBS/BSA). Staining was performed with a final concentration of 10 μg/mL anti-CD62P–FITC or 10 μg/mL anti-CD40L antibody for 20 min at room temperature in the dark. Platelets incubated with anti-CD40L were stained with 30 μg/mL of FITC-conjugated goat anti-mouse antibody for another 30 min in the dark. Then, platelet suspensions were diluted with PBS/BSA and analyzed as described [22].

RESULTS

Phosphorylation of p42 MAPK and p38 MAPK by Platelet Agonists

Initially, the potency of various platelet agonists to phosphorylate/activate p42 MAPK and p38 MAPK was tested (Fig. 1). Unstimulated platelets showed no significant phosphorylation of either p42 or p38 MAPKs. Thrombin (0.5 U/mL) induced rapid and strong p42 MAPK phosphorylation, whereas p42 MAPK phosphorylation by 2 μM U46619 was only 45% of the thrombin response (upper panel of Fig. 1A). With 30 μM ADP, only weak p42 MAPK phosphorylation (18% of the thrombin response) was detectable. No significant phosphorylation of p44 MAPK could be detected in the stimulated platelets using antibody 12D4, which is directed against a peptide representing the di-phosphorylated forms of both p42 and p44 MAPKs (Fig. 1A, upper panel). p38 MAPK phosphorylation was maximal after thrombin stimulation (upper panel of Fig. 1B). U46619 (2 μM) and ADP (30 μM) reached 81% and 35%, respectively, of the maximum of thrombin-induced p38 MAPK phosphorylation. Staining of these blots with phosphorylation-independent anti-p42 MAPK or anti-p38 MAPK antibodies, respectively, demonstrated the presence of approximately equal amounts of p42 or p38 MAPK (lower blots of Fig. 1, A and B).

To exclude the possibility of additional/synergistic MAPK activation due to thromboxane or ADP released from activated platelets, control experiments were performed with platelets preincubated with 100 μM aspirin and (with the exception of ADP stimulation) stimulated in the presence of 1 U/mL of apyrase. Under these conditions, MAPK phosphorylation by thrombin, U46619, and ADP was unchanged, indicating that MAPK phosphorylation under our experimental conditions was a direct answer to the agonist and not due to secondary effects (data not shown).

Quantitative Analysis of Vasodilator-Induced Inhibition of p42 MAPK and p38 MAPK Phosphorylation

The effects of vasodilators on platelet MAPK phosphorylation were analyzed in washed human platelets preincubated for 1 min with 10 μM PG-E_1 or 100 μM SNP. Under these conditions, PG-E_1 and SNP were previously demon-

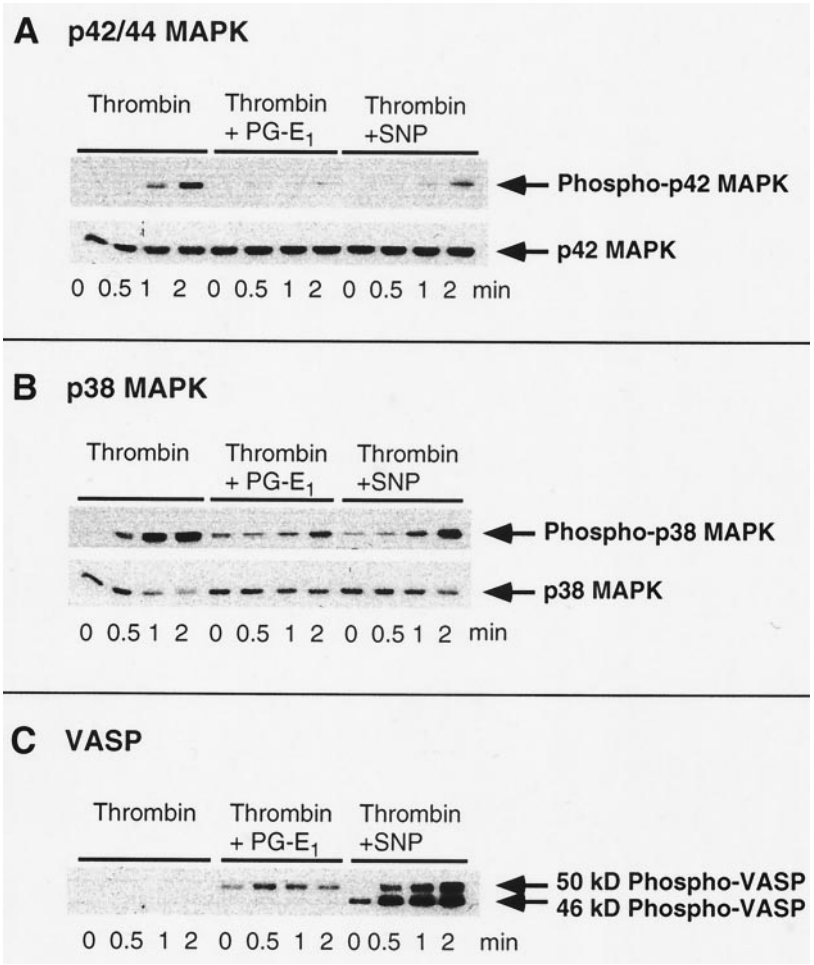

FIG. 2. Inhibition of thrombin-induced p42 and p38 MAPK phosphorylation by cyclic nucleotide-elevating vasodilators in human platelets. Washed human platelets were preincubated with buffer, 10 μM PG-E_1, or 100 μM SNP for 1 min, then stimulated with 0.5 U/mL of thrombin. Reactions were stopped at the time points indicated and analyzed for threonine/tyrosine phosphorylation of p42/p44 MAPK (upper blot of panel A) and of p38 MAPK (upper blot of panel B) by Western blotting using phosphorylation-specific antibodies as described in Methods. Approximately equal loading of each lane was confirmed by using phosphorylation-independent antibodies against p42 and p38 MAPKs (lower blots in panels A and B, respectively). To confirm activity of cAMP-PK and cGMP-PK, phosphorylation of VASP at serine^{239} was detected by the phosphorylation-specific antibody 16C2 (panel C). Autoradiographs shown are representative of at least three independent experiments.

strated to selectively activate cAMP-PK or cGMP-PK, respectively [16–19]. Then, platelets were stimulated with 0.5 U/mL of thrombin, 2 μM U46619, or 30 μM ADP, and at different time points analyzed for MAPK phosphorylation by the Western blot technique as described in Methods. For each agonist, platelet MAPK phosphorylation at 1 min in the absence of vasodilators was defined as MAPK phosphorylation standard (100%), and MAPK phosphorylation at other time points was expressed as a percentage of this standard.

Figure 2 shows original Western blots of a representative experiment with thrombin-stimulated platelets. Thrombin induced a rapid and time-dependent phosphorylation of p42 and p38 MAPKs (Fig. 2, A and B). Preincubation with 10 μM PG-E_1 or 100 μM SNP inhibited phosphorylation of p42 MAPK completely at 0.5 min of stimulation, whereas at later time points inhibition was partially overcome, resulting in a delayed time–course and reduced maximum of MAPK phosphorylation. At 1 min of thrombin stimulation, phosphorylation in the presence of PG-E_1 and SNP was found to be 18% and 30%, and at 2 min 35% and 70%, respectively (Figs. 2A and 3A, left panel). For U46619, PG-E_1-inhibited phosphorylation of p42 MAPK was found to be between 8% and 23%, whereas phosphorylation in the presence of SNP was between 22% and 47% (Fig. 3A, middle panel). ADP-induced p42 MAPK phosphorylation was almost completely inhibited for all time points of incubation (Fig. 3A, right panel).

p38 MAPK phosphorylation in platelets stimulated with 0.5 U/mL of thrombin was completely inhibited at 0.5 min by both PG-E_1 and SNP, whereas inhibition at 1 and 2 min was partially overcome, resulting in 48% and 75% phosphorylation, respectively, for PG-E_1, and 56% and 81% phosphorylation, respectively, for SNP (Figs. 2B and 3B, left panel). For U46619, PG-E_1-inhibited phosphorylation of p38 MAPK was found to be between 20% and 38%, and phosphorylation in the presence of SNP was between 26% and 61% (Fig. 3B, middle panel). For ADP, PG-E_1- or SNP-inhibited phosphorylation of p38 MAPK was found to be between 26% and 32% (Fig. 3B, right panel). Inhibition of thrombin-, U46619-, and ADP-induced p42 and p38 MAPK phosphorylation was significant for all time points for both vasodilators.

Phosphorylation of VASP, a common substrate of both cAMP-PK and cGMP-PK, was used as a control for PG-E_1- and SNP-induced phosphorylation of these kinases (Fig. 2C) [16]. The pattern of VASP phosphorylation observed in this study confirms the selective activation of cAMP-PK and cGMP-PK by PG-E_1 and SNP, respectively [21].

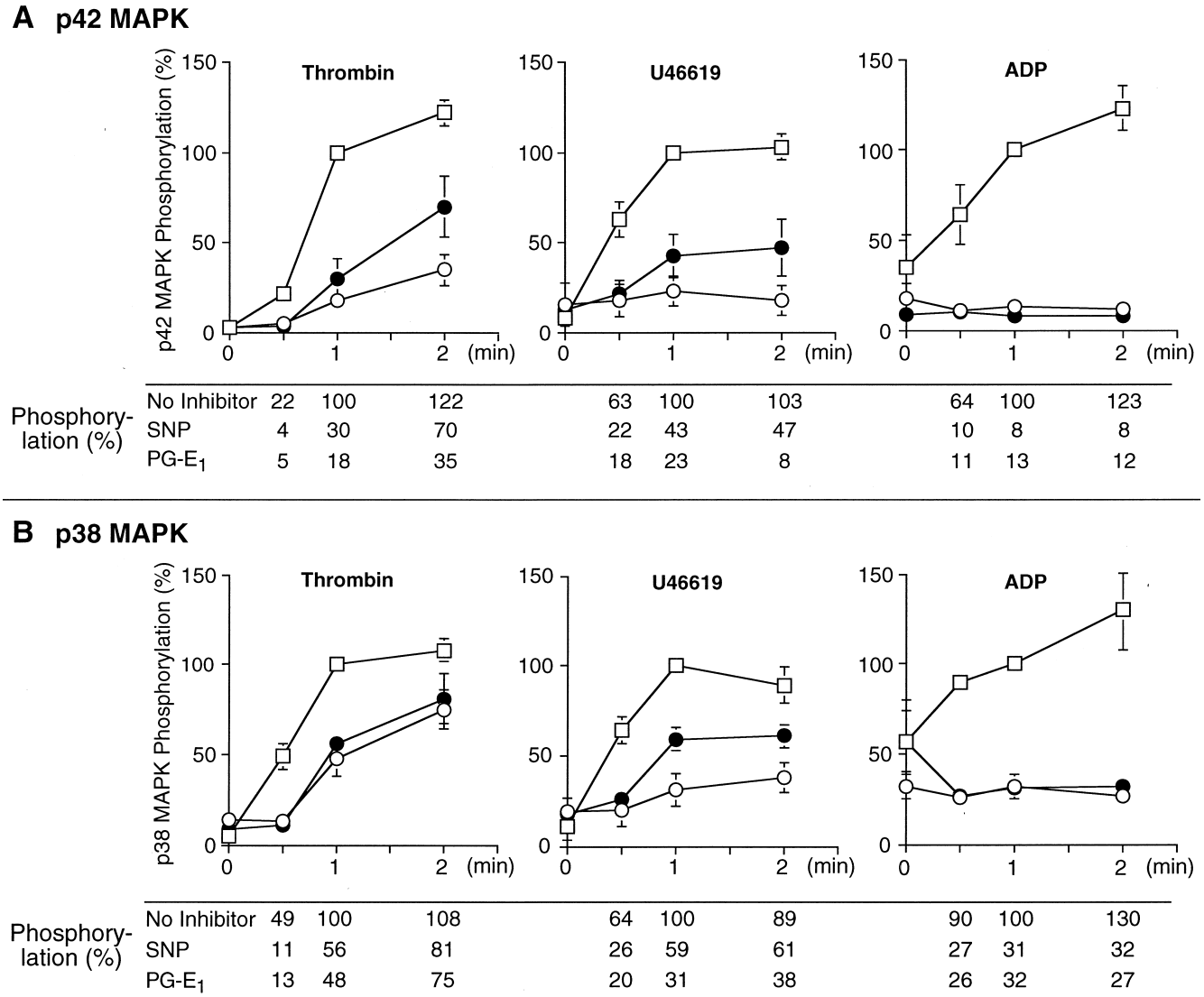

FIG. 3. Quantitative analysis of vasodilator-mediated inhibition of agonist-induced p42 MAPK and p38 MAPK phosphorylation in human platelets. PRP or washed human platelets (in the case of thrombin stimulation) were preincubated with buffer (□), 10 μM PG-E_1 (○), or 100 μM SNP (●), stimulated with 0.5 U/mL of thrombin, 2 μM of the TXA_2 analog U46619, or 30 μM ADP, and analyzed for p42 MAPK and p38 MAPK phosphorylation (panel A and B, respectively) as described in Methods. As demonstrated in Fig. 1, the potency of platelet agonists to activate p42/p38 MAPKs was thrombin > TXA_2 > ADP. For quantitative analysis, image analysis was performed as described in Methods, and agonist-induced MAPK phosphorylation after 1-min stimulation was referred to as 100%. MAPK phosphorylation at other time points was expressed as a percentage of this standard and is indicated below each curve. Data shown are mean values ± SEM of at least three independent experiments.

Regulation of Surface Expression of CD40 Ligand and P-selectin by PG-E_1 and SNP

A rapid increase in platelet CD40L and CD62P expression was observed after stimulation with 0.5 U/mL of thrombin, 2 μM U46619, or 30 μM ADP (Fig. 4, A and B). Maximal expression of CD40L induced by thrombin, U46619, and ADP was comparable for all agonists used, although expression with U46619 and ADP followed a faster time–course (Fig. 4A). Surface expression of CD62P induced by thrombin was 3-fold higher than after stimulation with U46619, whereas ADP induced only weak expression of CD62P (Fig. 4B). Furthermore, CD40L and CD62P surface expression followed different time–courses.

To test the influence of activation of cAMP- and cGMP-dependent protein kinases on CD40L and CD62P surface expression, platelets were preincubated with 10 μM PG-E_1 or 100 μM SNP, followed by stimulation with thrombin, U46619, or ADP. CD40L surface expression was strongly reduced by PG-E_1 and SNP in thrombin-stimulated platelets (between 74% and 98% inhibition) and in U46619-stimulated platelets (between 56% and 84% inhibition). However, inhibition of ADP-induced CD40L expression in the presence of PG-E_1 and SNP was only found to be between 21% and 69% (Fig. 4A). CD62P surface expression was strongly reduced by PG-E_1 and SNP in thrombin-stimulated platelets (between 88% and 100%

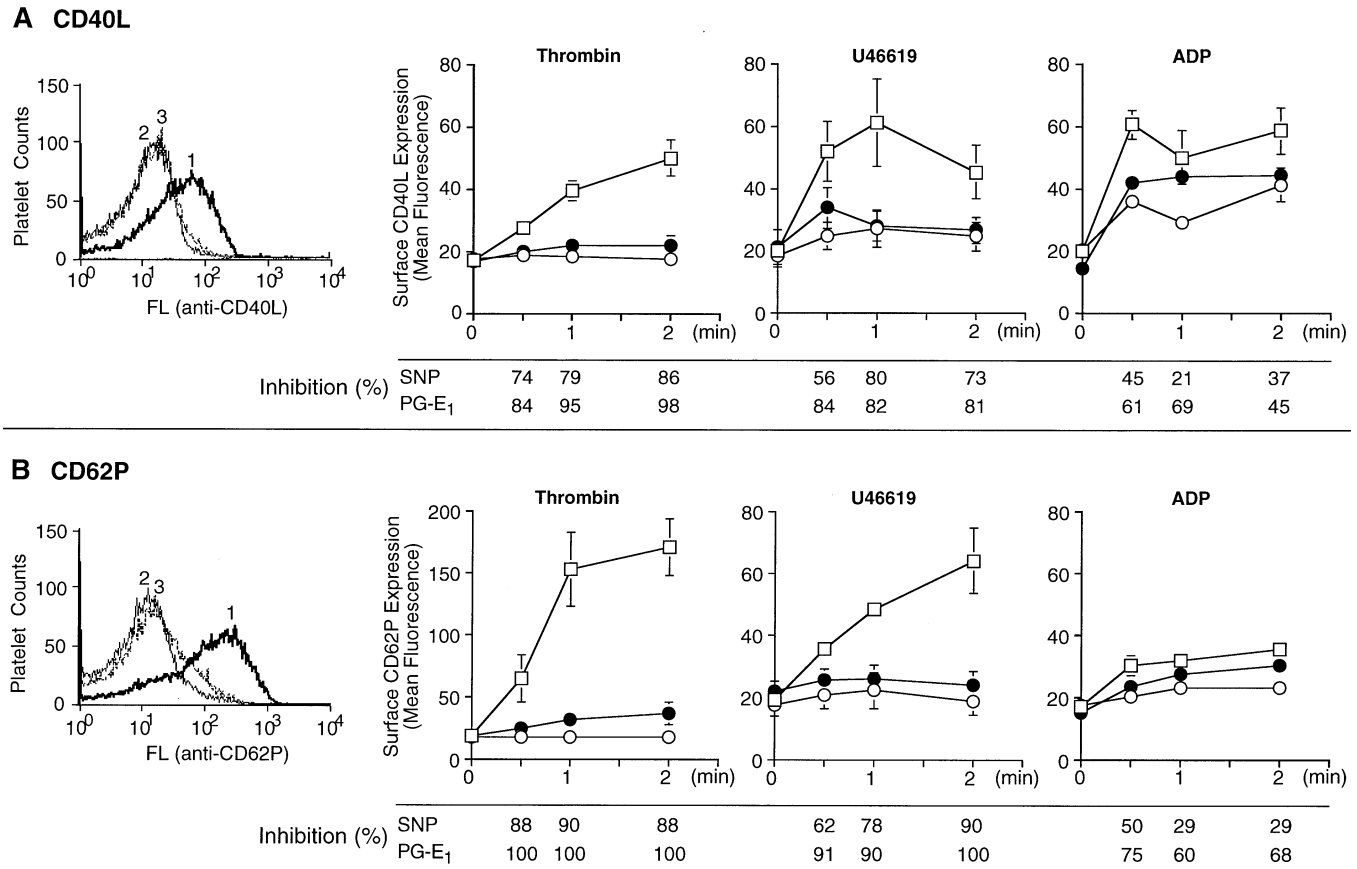

FIG. 4. Flow cytometry analysis of vasodilator-mediated inhibition of agonist-induced platelet surface expression of CD40L and CD62P (P-selectin). PRP or washed human platelets (in the case of thrombin stimulation) with 100 μM aspirin and 1 U/mL of apyrase (except for ADP stimulation) were preincubated with buffer, 10 μM PG-E_1, or 100 μM SNP, stimulated with 0.5 U/mL of thrombin, 2 μM of the TXA_2 analog U46619, or 30 μM ADP for the indicated time points, and then analyzed for surface expression of CD40L (panel A) or CD62P (panel B) by flow cytometry as described in Methods. The left side of both panels shows representative original flow cytometry histograms of antibody binding to platelets preincubated with buffer (curve 1), PG-E_1 (curve 2), or SNP (curve 3) after 2 min of thrombin stimulation. The right side of the panels indicates the time–courses of mean fluorescence of specific antibody binding to thrombin-, U46619-, or ADP-stimulated platelets preincubated for 1 min with buffer (□), PG-E_1 (○), or SNP (●). Note the different y-axis scale for thrombin in panel B. Below the curves, the percent inhibition of agonist-induced CD40L/CD62P surface expression by SNP or PG-E_1 is indicated. Data shown are mean values ± SEM of at least three independent experiments.

inhibition) and in U46619-stimulated platelets (between 62% and 100% inhibition). The weak ADP-induced CD62P expression was inhibited between 29% and 75% in the presence of PG-E_1 or SNP (Fig. 4B).

Influence of p42/p38 MAPK Inhibition on CD40L and CD62P Expression

MAPK activation was reported to be important for platelet activation when platelets were activated with low concentrations of collagen or U46619 [13, 14]. To test whether p42 or p38 MAPK activation are upstream events of CD40L or CD62P surface expression, PRP with 100 μM aspirin and 1 U/mL of apyrase was preincubated with 10 μM each of the MAPK kinase (MEK) inhibitor PD 98059 and the p38 MAPK inhibitor SB 203580 for 30 min, and stimulated for 1 min with 2 μM U46619. Under these conditions, U46619 induced a less than 2-fold increase in CD40L/CD62P expression. PRP incubated with inhibitors showed no significant reduction in CD40L or CD62P expression as compared to control platelets with the exception of a slight, but significant reduction in basal CD62P expression (Fig. 5). To ensure activity of the MAPK inhibitors, inhibition of p42 MAPK activation by PD 98059 was confirmed by Western blotting using the phosphorylation-specific antibody 12D4 (data not shown).

DISCUSSION

The major new findings of this study are: (i) Cyclic nucleotide-induced activation of either cAMP-PK or cGMP-PK inhibits agonist-induced phosphorylation of both p42 and p38 MAPKs in human platelets, as well as agonist-induced platelet surface expression of proinflammatory molecules including CD40L; and (ii) Our experimental data do not support the conclusion that p42 and p38 MAPK activation are involved in CD40L/CD62P expression.

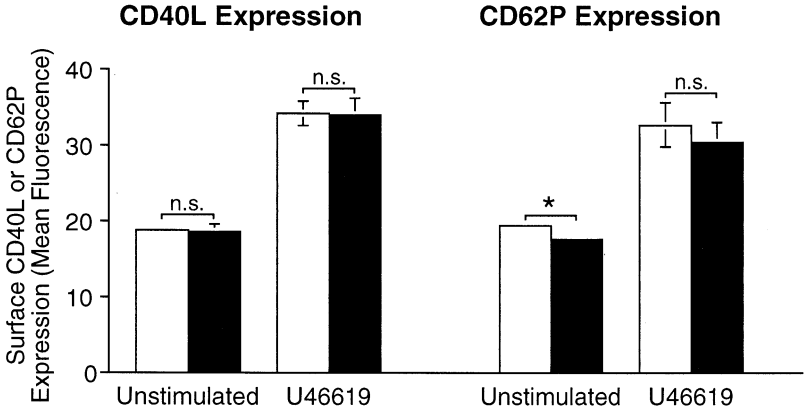

FIG. 5. Effects of the MAPK inhibitors PD 98059 and SB 203580 on U46619-induced CD40L and CD62P expression. PRP with 100 μM aspirin and 1 U/mL of apyrase was preincubated for 30 min with buffer (open bars) or 10 μM PD 98059 and 10 μM SB 203580 (black bars). For analysis of CD40L and CD62P expression, unstimulated PRP and PRP stimulated for 1 min with 2 μM U46619 were analyzed as described in Methods. Data shown are mean values ± SEM of at least 7 independent experiments. (n.s., not significant; *, $P < 0.001$; t-test).

It is well known that development and progression of cardiovascular diseases is critically influenced by intravascular platelet activation followed by expression of platelet surface proteins such as CD62P or CD40L. CD62P is a well-characterized endothelial and platelet adhesion receptor mediating interactions of activated platelets and endothelial cells with leukocytes [2, 23]. This enhances inflammatory responses by initiating leukocyte cytokine production and secretion [24]. CD40L, a type I member of the tumor necrosis factor (TNF) receptor superfamily of proteins, was originally identified on stimulated CD4+ T cells, but has also been found to be rapidly expressed on the surface of activated platelets [4]. CD40L binds to CD40 on endothelial cells inducing endothelial chemokine secretion and up-regulation of tissue factor and endothelial adhesion molecules. These mechanisms initiate a procoagulant phenotype, mediate leukocyte binding, and induce inflammatory responses at sites of injured vessel wall [4, 25].

Potent physiologic inhibitors of platelet activation are endogenous factors released from vascular endothelial cells such as the endothelium-derived relaxing factor (NO/EDRF) and prostacyclin (PG-I_2). These factors, as well as the widely used pharmacological vasodilators (e.g. PG-E_1 or NO donors such as SNP), mediate their effects in human platelets through elevation of intracellular cyclic nucleotides and activation of cyclic nucleotide-dependent protein kinases which phosphorylate a variety of intracellular signaling molecules (reviewed in [18, 19]). Inhibition of CD62P by cAMP and cGMP/NO has been described *in vitro* and *in vivo* [26–29]. Our study demonstrates that in human platelets cAMP- and cGMP-elevating vasodilators strongly inhibited agonist-induced surface expression of both CD62P and CD40L. The inhibition of inflammatory molecules might contribute to the long-term beneficial effects of vasodilators in vascular diseases [30, 31]. However, ADP-induced expression of CD40L was inhibited only partially, an effect that needs further investigation.

Activation of p42 and p38 MAPKs has been found to be a potentially important factor for platelet activation and surface receptor expression. MAPKs are expressed in all eukaryotic cells as an essential part of complex signaling networks. They are activated by diverse stimuli such as chemokines, growth factors, and hormones, leading to cell proliferation or cell differentiation. In platelets, only a few substrates of MAPKs are known. Platelet substrates of p42 MAPK include ribosomal S6 kinase ($p90^{rsk}$) [9], and several studies have suggested a role for p42 MAPK in phospholipase A_2 ($cPLA_2$) activation [32, 33]. p38 MAPK in platelets directly phosphorylates $cPLA_2$ [12, 33] and MAPK-activated protein kinase-2 (MAPKAPK-2), leading to arachidonic acid release and phosphorylation of the small heat shock protein hsp27 [13]. Little is known about the function and regulation of MAPKs in platelets. Both p42 and p38 MAPKs are rapidly activated at an early stage in human platelets in response to a variety of platelet agonists [9–13]. In this study, platelet agonists were compared with regard to their potency to phosphorylate p42 and p38 MAPKs in platelets (Figs. 1 and 3). Thrombin was found to be a strong activator of both p42 and p38 MAPK. Agonists such as U46619 or ADP were less potent to phosphorylate p42 MAPK, whereas phosphorylation of p38 MAPK induced by U46619 was only slightly reduced compared to thrombin. ADP was the weakest activator of both p42 MAPK and p38 MAPK. Several studies have suggested that p42 MAPK and p38 MAPK activation provide a crucial signal in the early stages of platelet activation that might be necessary for surface receptor expression/activation and aggregation in response to minimal concentrations of platelet agonists or extracellular matrix proteins [13, 14]. In our study, using a TXA_2 analog at a concentration that induced only weak platelet activation, we found no effect of p42/p38 MAPK inhibition on platelet CD40L/CD62P expression, indicating that under these conditions MAPKs do not regulate platelet surface receptor expression. However, preincubation with the MAPK inhibitors slightly but significantly reduced basal expression of CD62P, an effect that requires further studies to recognize its effect on platelet function. These experiments were performed in the presence of aspirin to avoid overlying effects due to inhibition of cyclooxygenases by the MAPK inhibitors [34].

cAMP was reported to have both activatory and inhibitory effects on p42/p44 MAPK activity, depending on the cell type investigated. In Swiss 3T3 fibroblasts, COS-7

monkey kidney cells, and a myeloid progenitor cell line, cAMP increased MAPK activity [35, 36], whereas in Rat-1 fibroblasts and human aortic smooth muscle cells MAPK activation was inhibited by cAMP [35, 37]. cGMP can also have opposite effects on p42/p44 MAPK activation in different cell types. Rat mesangial cells and cGMP-PK-expressing rat aortic smooth muscle cells showed stimulation of p42/p44 MAPK activation in response to cGMP analogs [38, 39]. In contrast, cGMP analogs inhibited epidermal growth factor-induced MAPK activation in cGMP-PK-expressing baby hamster kidney cells [40]. p38 MAPK has been reported to be activated by cAMP or cGMP in rat epididymal fat cells or human embryonic kidney fibroblasts, respectively [41, 42]. Here, we have demonstrated inhibition of agonist-induced p42 and p38 MAPK phosphorylation by both cAMP- and cGMP-elevating vasodilators in human platelets. In platelets, various substrates of cAMP-PK and cGMP-PK are known [18, 43]. However, the target substrates of cAMP-PK and cGMP-PK which initiate inhibition of both platelet granule secretion and MAPK activation remain to be identified. Earlier, it was shown that cyclic nucleotides inhibit platelet aggregation at an early step of the activation cascade, presumably at the level of phospholipase C [44]. This supports the hypothesis that platelet degranulation is affected at a level proximal to PKC activation [26, 27]. Our data confirm this model, since PKC activation is also an upstream event in the p42 MAPK cascade [10]. However, p38 MAPK activation was reported to be independent of PKC [45].

In conclusion, the physiological role of endothelium-derived vasodilators and platelet inhibitors such as PG-I_2 and NO and the impairment of these systems in diseases associated with endothelial dysfunction are well recognized [46–48]. The findings of this study reveal that pathophysiologically important platelet pathways and molecules involved in inflammation (CD40L, P-selectin), as well as platelet MAPK pathways, are down-regulated by activators of platelet cAMP-PK and/or cGMP-PK. Down-regulation of CD40L expression, a major proinflammatory stimulus, could be of considerable importance for the prevention of cardiovascular diseases. This underlines the physiological role of endothelium-derived vasodilators and the therapeutic potential of cyclic nucleotide-elevating platelet inhibitors for the prevention of the initial development and subsequent progression of inflammatory vascular lesions.

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References

1. Gaardner A, Jonsen J, Laland S, Hellem A and Owen PA, Adenosine diphosphate in red cells as a factor in the adhesiveness of human blood platelets. *Nature* **192:** 531–532, 1961.
2. Born GV, Adenosine diphosphate as a mediator of platelet aggregation *in vivo*: An editorial point of view. *Circulation* **72:** 741–742, 1985.
3. Hsu-Lin S, Berman CL, Furie BC, August D and Furie B, A platelet membrane protein expressed during platelet activation and secretion. Studies using a monoclonal antibody specific for thrombin-activated platelets. *J Biol Chem* **259:** 9121–9126, 1984.
4. Henn V, Slupsky JR, Grafe M, Anagnostopoulos I, Forster R, Muller-Berghaus G and Kroczek RA, CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* **391:** 591–594, 1998.
5. Fox JE, Platelet activation: New aspects. *Haemostasis* **26** (Suppl 4): 102–131, 1996.
6. Zhang J, Falck JR, Reddy KK, Abrams CS, Zhao W and Rittenhouse SE, Phosphatidylinositol (3,4,5)-triphosphate stimulates phosphorylation of pleckstrin in human platelets. *J Biol Chem* **270:** 22807–22810, 1995.
7. Shattil SJ and Brugge JS, Protein tyrosine phosphorylation and the adhesive functions of platelets. *Curr Opin Cell Biol* **3:** 869–879, 1991.
8. Eigenthaler M and Shattil SJ, Integrin signaling and the platelet cytoskeleton. In: *Current Topics in Membranes* (Ed. Nelson WJ), Vol. 43, pp. 265–291. Academic Press, San Diego, 1996.
9. Papkoff J, Chen RH, Blenis J and Forsman J, p42 mitogen-activated protein kinase and p90 ribosomal S6 kinase are selectively phosphorylated and activated during thrombin-induced platelet activation and aggregation. *Mol Cell Biol* **14:** 463–472, 1994.
10. Borsch-Haubold AG, Kramer RM and Watson SP, Cytosolic phospholipase A2 is phosphorylated in collagen- and thrombin-stimulated human platelets independent of protein kinase C and mitogen-activated protein kinase. *J Biol Chem* **270:** 25885–25892, 1995.
11. Kramer RM, Roberts EF, Strifler BA and Johnstone EM, Thrombin induces activation of p38 MAP kinase in human platelets. *J Biol Chem* **270:** 27395–27398, 1995.
12. Kramer RM, Roberts EF, Um SL, Borsch-Haubold AG, Watson SP, Fisher MJ and Jakubowski JA, p38 mitogen-activated protein kinase phosphorylates cytosolic phospholipase A2 (cPLA2) in thrombin-stimulated platelets. Evidence that proline-directed phosphorylation is not required for mobilization of arachidonic acid by cPLA2. *J Biol Chem* **271:** 27723–27729, 1996.
13. Saklatvala J, Rawlinson L, Waller RJ, Sarsfield S, Lee JC, Morton LF, Barnes MJ and Farndale RW, Role for p38 mitogen-activated protein kinase in platelet aggregation caused by collagen or a thromboxane analogue. *J Biol Chem* **271:** 6586–6589, 1996.
14. McNicol A, Philpott CL, Shibou TS and Israels SJ, Effects of the mitogen-activated protein (MAP) kinase kinase inhibitor 2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one (PD98059) on human platelet activation. *Biochem Pharmacol* **55:** 1759–1767, 1998.
15. Rossomando AJ, Payne DM, Weber MJ and Sturgill TW, Evidence that pp42, a major tyrosine kinase target protein, is a mitogen-activated serine/threonine protein kinase. *Proc Natl Acad Sci USA* **86:** 6940–6943, 1989.
16. Eigenthaler M, Nolte C, Halbrugge M and Walter U, Concentration and regulation of cyclic nucleotides, cyclic-nucleotide-dependent protein kinases and one of their major substrates in human platelets. Estimating the rate of cAMP-regulated and cGMP-regulated protein phosphorylation in intact cells. *Eur J Biochem* **205:** 471–481, 1992.
17. Eigenthaler M, Ullrich H, Geiger J, Horstrup K, Hönig-Liedl

P, Wiebecke D and Walter U, Defective nitrovasodilator-stimulated protein phosphorylation and calcium regulation in cGMP-dependent protein kinase-deficient human platelets of chronic myelocytic leukemia. *J Biol Chem* **268:** 13526–13531, 1993.
18. Eigenthaler M and Walter U, Signal transduction and cyclic nucleotides in human platelets. *Thromb Haemorrh Disorders* **8:** 41–46, 1994.
19. Eigenthaler M, Lohmann SM, Walter U and Pilz RB, Signal transduction by cGMP-dependent protein kinases and their emerging roles in the regulation of cell adhesion and gene expression. *Rev Physiol Biochem Pharmacol* **135:** 173–209, 1999.
20. Massberg S, Sausbier M, Klatt P, Bauer M, Pfeifer A, Siess W, Fassler R, Ruth P, Krombach F and Hofmann F, Increased adhesion and aggregation of platelets lacking cyclic guanosine 3′,5′-monophosphate kinase I. *J Exp Med* **189:** 1255–1264, 1999.
21. Smolenski A, Bachmann C, Reinhard K, Hönig-Liedl P, Jarchau T, Hoschuetzky H and Walter U, Analysis and regulation of vasodilator-stimulated phosphoprotein serine 239 phosphorylation *in vitro* and in intact cells using a phosphospecific monoclonal antibody. *J Biol Chem* **273:** 20029–20035, 1998.
22. Schwarz UR, Geiger J, Walter U and Eigenthaler M, Flow cytometry analysis of intracellular VASP phosphorylation for the assessment of activating and inhibitory signal transduction pathways in human platelets. *Thromb Haemost* **82:** 1145–1152, 1999.
23. Palabrica T, Lobb R, Furie BC, Aronovitz M, Benjamin C, Hsu YM, Sajer SA and Furie B, Leukocyte accumulation promoting fibrin deposition is mediated *in vivo* by P-selectin on adherent platelets. *Nature* **359:** 848–851, 1992.
24. Neumann FJ, Marx N, Gawaz M, Brand K, Ott I, Rokitta C, Sticherling C, Meinl C, May A and Schomig A, Induction of cytokine expression in leukocytes by binding of thrombin-stimulated platelets. *Circulation* **95:** 2387–2394, 1997.
25. Slupsky JR, Kalbas M, Willuweit A, Henn V, Kroczek RA and Muller-Berghaus G, Activated platelets induce tissue factor expression on human umbilical vein endothelial cells by ligation of CD40. *Thromb Haemost* **80:** 1008–1014, 1998.
26. Michelson AD, Benoit SE, Furman MI, Breckwoldt WL, Rohrer MJ, Barnard MR and Loscalzo J, Effects of nitric oxide/EDRF on platelet surface glycoproteins. *Am J Physiol* **270:** H1640–H1648, 1996.
27. Murohara T, Parkinson SJ, Waldman SA and Lefer AM, Inhibition of nitric oxide biosynthesis promotes P-selectin expression in platelets. Role of protein kinase C. *Arterioscler Thromb Vasc Biol* **15:** 2068–2075, 1995.
28. Yoshimoto H, Suehiro A and Kakishita E, Exogenous nitric oxide inhibits platelet activation in whole blood. *J Cardiovasc Pharmacol* **33:** 109–115, 1999.
29. Gries A, Bode C, Peter K, Herr A, Bohrer H, Motsch J and Martin E, Inhaled nitric oxide inhibits human platelet aggregation, P-selectin expression, and fibrinogen binding *in vitro* and *in vivo*. *Circulation* **97:** 1481–1487, 1998.
30. Szczeklik A, Nizankowski R, Skawinski S, Szczeklik J, Gluszko P and Gryglewski RJ, Successful therapy of advanced arteriosclerosis obliterans with prostacyclin. *Lancet* **1:** 1111–1114, 1979.
31. Boger RH, Bode-Boger SM, Thiele W, Creutzig A, Alexander K and Frolich JC, Restoring vascular nitric oxide formation by L-arginine improves the symptoms of intermittent claudication in patients with peripheral arterial occlusive disease. *J Am Coll Cardiol* **32:** 1336–1344, 1998.
32. Borsch-Haubold AG, Kramer RM and Watson SP, Inhibition of mitogen-activated protein kinase kinase does not impair primary activation of human platelets. *Biochem J* **318:** 207–212, 1996.
33. Borsch-Haubold AG, Kramer RM and Watson SP, Phosphorylation and activation of cytosolic phospholipase A2 by 38-kDa mitogen-activated protein kinase in collagen-stimulated human platelets. *Eur J Biochem* **245:** 751–759, 1997.
34. Borsch-Haubold AG, Pasquet S and Watson SP, Direct inhibition of cyclooxygenase-1 and -2 by the kinase inhibitors SB 203580 and PD 98059. SB 203580 also inhibits thromboxane synthase. *J Biol Chem* **273:** 28766–28772, 1998.
35. Faure M and Bourne HR, Differential effects on cAMP on the MAP kinase cascade: Evidence for a cAMP-insensitive step that can bypass Raf-1. *Mol Biol Cell* **6:** 1025–1035, 1995.
36. Lee AW, Synergistic activation of mitogen-activated protein kinase by cyclic AMP and myeloid growth factors opposes cyclic AMP's growth-inhibitory effects. *Blood* **93:** 537–553, 1999.
37. Graves LM, Bornfeldt KE, Raines EW, Potts BC, Macdonald SG, Ross R and Krebs EG, Protein kinase A antagonizes platelet-derived growth factor-induced signaling by mitogen-activated protein kinase in human arterial smooth muscle cells. *Proc Natl Acad Sci USA* **90:** 10300–10304, 1993.
38. Callsen D, Pfeilschifter J and Brune B, Rapid and delayed p42/p44 mitogen-activated protein kinase activation by nitric oxide: The role of cyclic GMP and tyrosine phosphatase inhibition. *J Immunol* **161:** 4852–4858, 1998.
39. Komalavilas P, Shah PK, Jo H and Lincoln TM, Activation of mitogen-activated protein kinase pathways by cyclic GMP and cyclic GMP-dependent protein kinase in contractile vascular smooth muscle cells. *J Biol Chem* **274:** 34301–34309, 1999.
40. Suhasini M, Li H, Lohmann SM, Boss GR and Pilz RB, Cyclic-GMP-dependent protein kinase inhibits the Ras/Mitogen-activated protein kinase pathway. *Mol Cell Biol* **18:** 6983–6994, 1998.
41. Moule SK and Denton RM, The activation of p38 MAPK by the beta-adrenergic agonist isoproterenol in rat epididymal fat cells. *FEBS Lett* **439:** 287–290, 1998.
42. Browning DD, McShane MP, Marty C and Ye RD, Nitric oxide activation of p38 mitogen-activated protein kinase in 293T fibroblasts requires cGMP-dependent protein kinase. *J Biol Chem* **275:** 2811–2816, 2000.
43. Butt E and Walter U, Signal transduction by cyclic nucleotide-dependent protein kinases in platelets. In: *Advances in Molecular and Cell Biology. Vol. 18, The Platelet* (Eds. Bittar EE and Lapetina EG), pp. 311–333. JAI Press Inc., Greenwich CT, 1997.
44. Waldmann R and Walter U, Cyclic nucleotide-elevating vasodilators inhibit platelet aggregation at an early step of the activation cascade. *Eur J Pharmacol* **159:** 317–320, 1989.
45. Ezumi Y, Nishida E, Uchiyama T and Takayama H, Thrombopoietin potentiates agonist-stimulated activation of p38 mitogen-activated protein kinase in human platelets. *Biochem Biophys Res Commun* **261:** 58–63, 1999.
46. Haller H, Modulation of endothelial function: Strategy for long-term cardiovascular protection. *J Hypertens Suppl* **14:** S27–S32, 1996.
47. Holtz J and Goetz RM, Vascular renin-angiotensin-system, endothelial function and atherosclerosis? *Basic Res Cardiol* **89** (Suppl 1): 71–86, 1994.
48. Ross R, Atherosclerosis—an inflammatory disease. *N Engl J Med* **340:** 115–126, 1999.