The Effects of an Inhibitor of Diglyceride Lipase on Collagen-Induced Platelet Activation

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ABSTRACT

Human platelet activation by collagen occurs in a dose-dependent manner. High concentrations of collagen bind to a pair of receptors, the α2β1 integrin and glycoprotein (GP)IV/Fc-receptor γ-chain (FcRγ), which stimulate a cascade of events including Syk, LAT, Btk, Gads, and phospholipase Cγ2, leading to calcium release and protein kinase C (PKC) activation. Calcium and PKC are responsible for a range of platelet responses including exocytosis and aggregation, as well as the cytosolic phospholipase A2 (cPLA2)-mediated release of arachidonic acid, which is converted to thromboxane (Tx)A2. In contrast, low concentrations of collagen are acutely aspirin-sensitive, and calcium release and aggregation are TxA2-dependent. Under these conditions, cPLA2 is not involved and it has been suggested that phospholipase C generates 1,2-diacylglycerol (DG) from which arachidonic acid is liberated by diglyceride lipase (DGL). Here a novel DGL blocker (OMDM-188) inhibited collagen-, but not arachidonic acid–induced aggregation and TxA2 synthesis. Furthermore, OMDM-188 inhibited collagen-induced arachidonic acid release. Finally OMDM-188 inhibited collagen-induced p38MAPK phosphorylation, but not extracellular signal-regulated kinase (ERK) phosphorylation, with no effect on the phosphorylation of either enzyme in response to arachidonic acid. Taken together, these data suggest a role for a pathway involving phospholipase C liberating DG from membrane phospholipids in response to minimally activating concentrations of collagen. The DG serves as a substrate for DGL, potentially under the regulations of p38MAPK, to release arachidonic acid, which is subsequently converted to TxA2, which mediates the final platelet response.

Introduction

Collagen is the most thrombogenic component of the subendothelial matrix. Endothelial damage results in the exposure of the collagen to circulating blood, in particular to platelets, resulting in hemostatic or atherothrombotic consequences. Multiple collagen receptors have been identified on the platelet surface, including glycoprotein (GP)IV, the α2β1 integrin, immunoglobulin superfamily member GPVI, which is noncovalently coupled to the Fc-receptor γ-chain (FcRγ), the receptor-like protein tyrosine phosphatase CD148, and C-type lectin-like receptor 2 (CLEC-2) (Tandon et al., 1989; Nieswandt and Watson, 2003; Suzuki-Inoue et al., 2006; Farndale et al., 2007; Surin et al., 2008; Ellison et al., 2010). These individual receptors likely play specific roles to mediate collagen-induced platelet adhesion, activation, and consolidation (Li et al., 2010; Kauskot and Hoylaerts, 2012).

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ABBREVIATIONS: COX, cyclo-oxygenase; cPLA2, cytosolic phospholipase A2; DG, 1,2-diacylglycerol; DGL, diacylglyceride lipase; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; FcRγ, Fc-receptor γ-chain; FPI, 5-fluoro-2-indolyl des-chlorohalopemide; GP, glycoprotein; IgG-HRP, horseradish peroxidase–conjugated anti-immunoglobulin G–conjugated antibody; IP3, inositol (1,4,5)-trisphosphate; ITAM, immunoreceptor tyrosine-based activation motif; MAPK, mitogen-activated protein kinase; OMDM-188, N-formyl-L-isoleucine-(1S)-1-[[2S,3S]-3-hexyl-4-oxo-2-oxetanyl][methyl]dodecyl ester; p38MAPK, p38 isoform of MAPK; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; RHC-80267, 1,6-bis(cyclohexyloxoiminocarbonylamo)hexane; Tx, thromboxane; w.r.t., with respect to.
However, many of these observations have been made at high concentrations (≥20 μg/ml) of collagen, at low concentrations (1–2 μg/ml), collagen-induced PLC activity, liberation of intracellular Ca^{2+}, and aggregation are all sensitive to cyclo-oxygenase (COX) inhibition and are thus TxA2-mediated (Narita et al., 1985; Kito et al., 1986; Pollock et al., 1986; Elvers et al., 2012), reflecting the highly aspirin-sensitive nature of platelet activation in vivo (Seymour et al., 1984; Gerrard et al., 1989). Agonist-induced synthesis of TxA2 involves the release of arachidonic acid from membrane phospholipids followed by the sequential actions of cyclo-oxygenase and Tx-synthetase. Borsch-Haubold et al. (1995, 1997) demonstrated that cytosolic sequential actions of cyclo-oxygenase and Tx-synthetase. In contrast, although inhibition of cPLA2 by a pharmacological inhibitor, arachidonyl trifluoromethyl ketone (AACOCF3), attenuated TxA2 synthesis in response to low collagen concentrations (McNicol et al., 1998), there was no accompanying effect on arachidonic acid release in response to high doses of collagen and this was modulated by pathways involving several kinases, including the p38 isoform of mitogen-activated protein kinase (p38MAPK). In contrast, although inhibition of cPLA2 by a pharmacological inhibitor, arachidonyl trifluoromethyl ketone (AACOCF3), attenuated TxA2 synthesis in response to low collagen concentrations McNicol et al. 1998, there was no accompanying effect on arachidonic acid release (Lockhart et al., 2001), consistent with an action on COX rather than on cPLA2 (McNicol and Nickolaychuk, 1995; Leis and Windischrofer, 2008). Similarly, inhibition of p38MAPK had no effect on TxA2 synthesis or arachidonic acid release in response to low collagen concentrations (Saklatvala et al., 1996; McNicol et al., 1998). Taken together, this is therefore unlikely that a Ca^{2+}/p38MAPK/cPLA2 pathway accounts for the release of arachidonic acid in response to low collagen concentrations.

Arachidonic acid can also be liberated from platelet membranes by the action of a second enzyme, diglyceride lipase (DGL), on DG (Bell et al., 1979; Rittenhouse-Simmons, 1980). Two potential DG-generating pathways are present in platelets: first the PLC pathway and second the consecutive actions of phospholipase D (PLD) and phosphatidic acid phosphohydrolase. Although both pathways have been reported to be engaged in platelets activated by low concentrations of collagen (Chiang, 1994; Lockhart et al., 2001), the significance of DGL in collagen-induced platelet arachidonic acid release and subsequent aggregation remains unclear. In part this is due to a paucity of pharmacological tools, notably the nonselective nature of DGL inhibitors (Bross et al., 1983; Oglesby and Gorman, 1984). However, a novel tetradehydrolipstatin analog OMDM-188 has recently been synthesized (Ortar et al., 2008) and shown to potently inhibit DGL (Min et al., 2010). Therefore in the current study we have evaluated the effects of OMDM-188 on low dose collagen-induced platelet activation.

Materials and Methods

OMDM-188 (N-formyl-i-isoleucine-(1S)-1-[(2S,3S)-3-hexyl-4-oxo-2-oxetanyl]methyl(dodecyl ester) was synthesized as previously reported (Ortar et al., 2008). Arachidonic acid enzyme–linked immunosorbent assay (ELISA) and Thromboxane enzyme immunoassay (EIA) kits were purchased from Caymen Chemicals (Ann Arbor, MI), collagen and arachidonic acid were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). Antibodies to p38MAPK and phospho-p38MAPK were purchased from New England Biolabs (Beverly, MA), and antibodies to extracellular signal-regulated kinase (ERK) and phospho-ERK, as well as anti-mouse horseradish peroxidase–conjugated anti-immunoglobulin G–conjugated antibody (IgG-HRP) and anti-goat IgG-HRP, were all obtained from Santa Cruz Biotechnology (Dallas, TX). Enhanced chemiluminescence (ECL) reagents and hyperfilm were obtained from Amersham Pharmacia Biotechnology (Baie D’Ur, QC). All electrophoresis and immunoblotting supplies were from Bio-Rad (Mississauga, ON, Canada). All other chemicals and materials were of the highest grade available.

Blood Collection. The study was approved by the Research Ethics Board of the University of Manitoba, and informed consent was obtained from all volunteers. Blood was collected by venipuncture of human volunteers who had denied taking medication known to interfere with platelet function within the previous 2 weeks, into acid/citrate/dextrose anticoagulant (ACD; 3.8 mM citric acid, 7.5 mM trisodium citrate, 125 mM dextrose; 1.8 mM anticoagulant/8.2 ml whole blood). Plasma-free platelet suspensions were obtained, in appropriate buffers, as previously reported (McNicol et al., 1998; McNicol and Jackson, 2003; Jackson and McNicol, 2010).

Platelet Aggregation. Platelet aggregation in HEPES-buffered Tyrode solution (134 mM NaCl, 12 mM NaHCO3, 2.9 mM KCl, 0.34 mM Na2HPO4, 1 mM HEPES, pH 7.4) was measured as increased light transmission in a Payton dual channel aggregometer (Payton Assoc., Scarborough, ON, Canada), as previously described (McNicol et al., 1998; McNicol and Jackson, 2003; Jackson and McNicol, 2010) and analyzed by OriginPro 8 software (OriginLab Corporation, Northampton, MA).

Arachidonic Acid Release. Aggregation was terminated by the addition of an equal volume of 2 mM EDTA, 100 μM indomethacin, and the platelets removed by centrifugation (McNicol et al., 1998). The supernatants were analyzed for arachidonic acid by ELISA following the manufacturer’s instructions.

Thromboxane Synthesis. Aggregation was terminated by the addition of an equal volume of acid/citrate, 10% dextran, and the platelets removed by centrifugation (McNicol et al., 1998). The supernatants were analyzed for Thromboxane B2, the stable metabolite of TxA2, by ELISA following the manufacturer’s instructions.

Immunoblotting. Aggregation was terminated by the addition of an equal volume of reducing buffer (62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% (w/v) bromophenol blue) and the samples denatured by boiling for 10 minutes. Proteins were separated on a 10% polyacrylamide/SDS vertical slab gel and transferred to nitrocellulose (Mississauga, ON, Canada). Levels of MAPK phosphorylation were determined as previously described (McNicol and Jackson, 2003; Jackson and McNicol, 2010). Following blocking in a 5% nonfat powdered Carnation milk solution in Tris-buffered saline (TBS; 20 mM Tris base, 130 mM NaCl, pH 7.4) containing 0.1% Tween-20 (TBS-T), the blots were incubated with antibodies to either phospho-p38MAPK or phospho-ERK then to the corresponding horseradish peroxidase (HRP)–conjugated secondary antibody, and the proteins visualized using the enhanced chemiluminescence Western blotting detection system. The immunoblot was subsequently stripped and reprobed with an antibody to the corresponding nonphosphorylated protein (ERK or p38MAPK) to ensure equal sample loading, as previously reported (McNicol and Jackson, 2003; Jackson and McNicol, 2010).

Statistical Analysis. Interexperiment comparisons in platelet aggregation and detection of soluble factors for the different conditions were analyzed by means of the paired t-test or Tukey’s multiple comparison test. All values are reported as means ± S.E.M. and a P value < 0.05 was considered to be significant.

Results

The Effects of OMDM-188 on Agonist-Induced Platelet Aggregation. The addition of submaximal agonist concentrations of 1 μg/ml collagen and 60 μM arachidonic acid caused platelet aggregation (Fig. 1), as previously reported (McNicol and Jackson, 2003). Previous studies have shown...
that 2 μM OMDM-188 has a selective inhibitory action on DGL activity (Min et al., 2010). Therefore the effects of similar concentrations of OMDM-188 on agonist-induced platelet aggregation were examined.

Preincubation (2 minutes) with 3 μM and 10 μM OMDM-188 inhibited collagen-induced aggregation by 12 ± 4% (n = 5; P < 0.05) and 87 ± 10% (n = 5; P < 0.01), respectively. In contrast 3 μM OMDM-188 had no effect on arachidonic acid–induced aggregation, whereas 10 μM OMDM-188 inhibited arachidonic acid–induced aggregation by 50 ± 16% (n = 5; P < 0.05) (Fig. 1).

The Effects of OMDM-188 on Agonist-Induced Arachidonic Acid Release. Collagen (1 μg/ml) stimulated the release of a significant amount of arachidonic acid when compared with the saline control (1.05 ± 0.02 vs. 1.43 ± 0.14 ng/ml; P < 0.05, n = 3), which was equivalent to the release elicited by 0.1 IU/ml thrombin (1.37 ± 0.08 ng/ml). The arachidonic acid release in response to collagen was significantly inhibited by pretreatment for 2 minutes with 10 μM OMDM-188 (1.12 ± 0.05 ng/ml; P < 0.05) but not with either 1 μM OMDM-188 (1.39 ± 0.14 ng/ml) or with 3 μM OMDM-188 (1.23 ± 0.15 ng/ml) (Fig. 2).

The Effects of OMDM-188 on Agonist-Induced Thromboxane Release. There were negligible levels of TxB₂, the stable metabolite of TxA₂, in the releasates of platelets stirred with saline alone. Collagen (1 μg/ml) stimulated the release of TxB₂, which was significantly inhibited by pretreatment for 2 minutes with 5 μM OMDM-188 but not with 1 μM OMDM-188. The addition of arachidonic acid (60 μM) to platelets also led to the formation of significant levels of TxB₂; however, in this case the TxB₂ levels were unaffected by pretreatment of up to 100 μM OMDM-188, consistent with OMDM-188 having no effect on cyclo-oxygenase/thromboxane synthetase (Fig. 3). There was an apparent increase in TxB₂, but not of arachidonic (Fig. 2), release in the presence of the low (1 μM) concentration of OMDM-188. The reason for this apparent paradox is unclear and requires further examination.

The Effects of OMDM-188 on Agonist-Induced Protein Phosphorylation. Collagen (1 μg/ml) caused the phosphorylation of p38MAPK (Fig. 4), as previously reported (McNicol and Jackson, 2003; Jackson and McNicol, 2010). This phosphorylation was unaffected by pretreatment for 2 minutes with 1 μM OMDM-188 but significantly reduced by pretreatment with 10 μM OMDM-188. In contrast p38MAPK phosphorylation by arachidonic acid was unaffected by pretreatment with OMDM-188 at concentrations of up to 10 μM (Fig. 4).

Collagen (1 μg/ml) and arachidonic acid (60 μM) each caused the phosphorylation of ERK, which was unaffected by pretreatment (2 minutes) with OMDM-188 at concentrations of up to 1 mM (Fig. 5).

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**Fig. 1.** Effects of OMDM-188 on collagen- and arachidonic acid–induced aggregation. Washed human platelets were incubated for 2 minutes with OMDM-188 at the concentrations indicated, or saline control, prior to the addition of 1 μg/ml collagen (n = 5) or 60 μM arachidonic acid (n = 5). Aggregation was monitored continuously as an increase in light transmission for 3 minutes. The extent of aggregation was quantified using OriginPro 8 software and data expressed as a percentage of the saline control (n = 5; *P < 0.05; **P < 0.01). Insert is a representative example of the effects of OMDM-188 on collagen-induced aggregation.
Discussion

Collagen is accepted to be the most thrombogenic component of the subendothelial matrix. Engagement of collagen receptors triggers intracellular signaling pathways leading to the final platelet response. The current evidence suggests that the primary signaling occurs via GPVI. Activation of GPVI leads to the phosphorylation of FcRγ, which sequentially leads to the activation of Syk and PLCγ2 (Stalker et al., 2012). The effects of collagen are promoted by the release of arachidonic acid by cPLA2 and its subsequent conversion to TxA2 by COX/TxA2 synthetase (Stalker et al., 2012). The upstream mediators of cPLA2 are likely to include intracellular calcium and p38MAPK (Borsch-Haubold et al., 1995, 1997; Stalker et al., 2012) (Fig. 6).

However, studies both in vivo and in vitro have shown that platelet responses to low doses of collagen are highly aspirin-sensitive, and therefore TxA2-mediated (Seymour et al., 1984; Narita et al., 1985; Kito et al., 1986; Pollock et al., 1986; Gerrard et al., 1989). The signaling pathways associated with platelets stimulated by low, submaximal doses of collagen are not well elucidated. Both PLC activity and the elevation of intracellular Ca2+ are inhibited by aspirin and therefore do not precede, but rather are a consequence of TxA2 synthesis (Narita et al., 1985; Kito et al., 1986; Pollock et al., 1986). Similarly, pharmacological inhibition of neither p38MAPK nor cPLA2 had any effect on low dose collagen-induced arachidonic acid release (McNicol et al., 1998; Lockhart et al., 2001), thereby dissociating a p38MAPK/cPLA2 pathway from low dose collagen-induced arachidonic acid release.
A second potential arachidonic acid–liberating process is by the action of DGL on DG. Moriyama and colleagues (1999) have purified and characterized DGL in platelets. Of particular note, DGL is active at basal Ca\(^{2+}\) and therefore would not require a PLC-mediated generation of IP\(_3\) and subsequent elevation of intracellular Ca\(^{2+}\) for activity. In the same study, RHC-80267 was shown to inhibit DGL (Moriyama et al., 1999); however, other studies have shown that, due to its relatively nonselective activity, RHC-80267 cannot be used to determine the role of DGL in intact platelets (Bross et al., 1983; Oglesby and Gorman, 1984).

More recently, novel tetrahydrolipstatin analogs with inhibitory effects on DGL have been synthesized (Ortar et al., 2008; Min et al., 2010). In the current study one of these analogs, OMDM-188, inhibited low dose collagen-induced aggregation of human platelets and this was associated with an inhibition of both arachidonic acid release and TxB\(_2\) formation. At similar concentrations OMDM-188 had significantly less of an inhibitory effect on arachidonic acid–induced aggregation and had no effect on arachidonic acid–induced TxB\(_2\) formation. Taken together these data demonstrate that OMDM-188 has an inhibitory action on collagen-induced platelet activation prior to the conversion of arachidonic acid to TxA\(_2\) by COX, likely by an action on DGL. This would suggest that DGL is a key enzyme in the pathway underlying platelet activation in response to low doses of collagen.

This supports the study of Moriyama et al. (1994), who postulated that the action of DGL distal to PLC, at resting intracellular Ca\(^{2+}\), to release arachidonic acid was a critical early step in collagen-induced platelet activation. Similarly, a role for a PLC, but not for cPLA\(_2\), in collagen-induced platelet arachidonic acid release, via DGL, was postulated in a study utilizing a battery of pharmacological inhibitors (Lockhart et al., 2001) (Fig. 6).

A second potential DGL-mediated pathway of liberating arachidonic acid in response to low-doses of collagen is by the sequential actions of PLD and DGL, as suggested by Chiang (1994). There are two isoforms of PLD in platelets, PLD1 and PLD2, both of which hydrolyze phosphatidylcholine to generate phosphatidic acid, which, by the action of phosphatidic acid phosphohydrolase, synthesizes DG (Vorland and Holmsen, 2008; Vorland et al., 2008). As reviewed by Vorland et al. (2008), these two PLD isoforms are stimulated by collagen and inhibited by protein kinase A, suggesting a role in the signaling pathway, are found in different locations in the platelet, and likely play differing roles during activation. These functions...
may include lysosomal secretion and actin polymerization (Vorland et al., 2008); whether the DG generated provides a source of arachidonic acid via the action of DGL is possible but has not been addressed (Fig. 6). Interestingly, however, a recent study using a novel PLD inhibitor, FIPI, as well as Pld
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/2 murine platelets has suggested that PLD plays a negative role in platelet-signaling in response to a variety of agonists, including low dose collagen (Elvers et al., 2012). Therefore it appears that a PLC/DLG, rather than a PLD/phosphatidic acid phosphohydrolase/DLG is the more likely pathway engaged (Fig. 6).

The identity of the collagen receptor involved in PLC activation is open to speculation and further investigation. It is clear that a role for GPVI/FcR
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\gamma
, as occurs at higher collagen concentrations (Li et al., 2010), is a possibility. An intriguing potential alternative is CD148, the only receptor-like protein tyrosine phosphatase on the platelet surface (Senis et al., 2009). Engagement of CD148 may remove the restraints on the phosphorylated FcR
\gamma
 ITAM, leading to the activation of Syk and subsequent downstream regulators, including PLC\gamma
2
. In both cases however the levels of DG formed would have to provide sufficient substrate for DGL to initiate the arachidonic acid/TxA2 cascade without liberating enough IP3 to trigger the Ca2+-mediated cPLA2 pathway. The α2β1 integrin is also critical to the interaction of platelets with collagen; however, it is likely that it plays a role as an adhesive receptor that requires an initial wave of internal signaling (Stalker et al., 2012).

The effects of OMDM-188 on MAPK activity in response to collagen were assessed to address any relationship between these enzymes and DGL function. Previous studies have shown that inhibition of the MEK/ERK pathway had no effect on platelet aggregation in response to a variety of agonists, including low doses of collagen (McNicol and Jackson, 2003). Therefore, it is unlikely that this pathway is engaged in response to collagen, leading to DGL activity, arachidonic acid release, and aggregation. Indeed the observed inhibition of aggregation by OMDM-188 combined with the lack of a corresponding effect on ERK phosphorylation supports the dissociation between these two events.

In the current study, OMDM-188, at concentrations that blocked aggregation, inhibited collagen-induced p38MAPK phosphorylation. Studies by Saklatvala et al. (1996) demonstrated that the effects of collagen at concentrations that were minimally required to induce aggregation of platelets were sensitive to inhibition of p38MAPK, suggesting that p38MAPK precedes arachidonic acid release under these conditions. Taken together these observations potentially implicate p38MAPK in the DGL-activating pathway, however this proposition needs to be addressed further (Fig. 6).
Therapeutically inhibition of DGL is an attractive anti-thrombotic target as it would affect the release of arachidonic acid/TxA₂ without a corresponding action on endothelial COX-2-mediated arachidonic acid/prostacyclin release. However, the required concentrations of OMDM-18 [the most potent and selective DGL inhibitor reported to date (Ortar et al., 2008)] are high for this to be a viable agent. In addition tetrahydrolipstatin has a low permeability through the gastrointestinal tract, which limits its oral bioavailability, and it is probable that this will also affect OMDM118 (and other tetrahydrolipstatin analogs). Hence, it is necessary for DGL inhibitors of other chemical classes to be developed.

In conclusion, the current study is consistent with a role for a pathway involving the liberation of DG from membrane phospholipids in response to minimally activating concentrations of collagen. The DG serves as a substrate for DGL, potentially under the regulation of P38 MAPK, to release arachidonic acid that is subsequently converted to TxA₂, which mediates the final platelet response.

Authorship Contributions

**Participated in research design:** McNicol.

**Conducted experiments:** Jackson.

**Contributed new reagents or analytic tools:** Ortar.

**Performed data analysis:** McNicol.

**Wrote or contributed to the writing of the manuscript:** McNicol.

References


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