Dipetalodipin, a Novel Multifunctional Salivary Lipocalin That Inhibits Platelet Aggregation, Vasoconstriction, and Angiogenesis through Unique Binding Specificity for TXA₂, PGF₂α, and 15(S)-HETE*□

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Dipetalodipin (DPTL) is an 18 kDa protein cloned from salivary glands of the triatomine Dipetalogaster maxima. DPTL belongs to the lipocalin superfamily and has strong sequence similarity to pallidipin, a salivary inhibitor of collagen-induced platelet aggregation. DPTL expressed in Escherichia coli was found to inhibit platelet aggregation by collagen, U-46619, or arachidonic acid without affecting aggregation induced by ADP, convulxin, PMA, and ristocetin. An assay based on incubation of DPTL with small molecules (e.g. prostanoids, leukotrienes, lipids, biogenic amines) followed by chromatography, mass spectrometry, and isothermal titration calorimetry showed that DPTL binds with high affinity to carboxyclic TXA₂, TXA₂ mimetic (U-46619), TXB₂, PGH₂ mimetic (U-51605), PGD₂, PGJ₂, and PGF₂α. It also interacts with 15(S)-HETE, being the first lipocalin described to date to bind to a derivative of 15-Lipoxygenase. Binding was not observed to other prostaglandins (e.g. PGE₁, PGE₂, 8-iso-PGF₂α, prostacyclin), leukotrienes (e.g. LTB₄, LTC₄, LTD₄, LTE₄), HETEs (e.g. 5(S)-HETE, 12(S)-HETE, 20-HETE), lipids (e.g. arachidonic acid, PAF), and biogenic amines (e.g. ADP, serotonin, epinephrine, norepinephrine, histamine). Consistent with its binding specificity, DPTL prevents contraction of rat uterus stimulated by PGF₂α and induces relaxation of aorta previously contracted with U-46619. Moreover, it inhibits angiogenesis mediated by 15(S)-HETE and did not enhance inhibition of collagen-induced platelet aggregation by SQ29548 (TXA₂ antagonist) and indomethacin. A 3-D model for DPTL and pallidipin is presented that indicates the presence of a conserved Arg₃⁹ and Gln₁₃⁵ in the binding pocket of both lipocalins. Results suggest that DPTL blocks platelet aggregation, vasoconstriction, and angiogenesis through binding to distinct eicosanoids involved in inflammation.

The hemostatic process, a host defense mechanism to preserve the integrity of the circulatory system, remains inactive until vascular injury occurs, leading to activation of hemostasis. The first step in this cascade of events is platelet interaction with the exposed extracellular matrix (ECM), which contains a large number of adhesive macromolecules such as collagen. Under conditions of high shear, initial tethering of platelets to the ECM is mediated by interaction between the platelet receptor glycoprotein (GP)Ⅱb/Ⅲa and vWF bound to collagen (1). This interaction allows platelet receptor GPVI to bind to collagen, triggering release of the so-called secondary mediators TXA₂ and ADP that are necessary for integrins α₂β₁ and α₁bβ₃ activation and completion of platelet aggregation (2). Vasoconstriction is another critical step triggered by injury and mediated by biogenic amines produced by adrenergic fibers or vasoactive components such as TXA₂ and serotonin released by platelets in an attempt to decrease blood flow at sites of injury and therefore prevent blood loss (3).

Because of the interface encountered by vectors upon interaction with their host, salivary glands from bloodsucking arthropods have evolved different mechanisms that counteract hemostasis and inflammation (4). At least thirteen different mechanisms for inhibition of platelet function are reported to explain how these molecules affect platelet function, thus assisting hematophagous animals to acquire a blood meal (5). These inhibitors have been classified as enzymes, small ligand binders, enzymes or enzyme inhibitors, nitric oxide (NO)-releasing molecules, and integrin antagonists. Among members of the lipocalin family (6), inhibitors have been reported to bind to ADP (7), biogenic amines (8–9), and leukotrienes (10). Other salivary components interfere with hemostasis by targeting vasoconstriction, such as tachikinin-like peptides from Aedes aegypti (11) or peptides such as sandfly maxadilan, which specifically activates PAC1, the type 1 receptor for pituitary adenylate cyclase-activating peptide (PACAP) (12). Vasodilation is also mediated through release of NO by NO-carrying nitrophorins from Rhodnius prolixus (13). In this report, we have cloned, expressed, and studied the mechanism of action of a novel lipocalin, herein named dipetalodipin (DPTL). DPTL binds to TXA₂, PGF₂α, 15(S)-HETE, and other prostanoids, and
was found to block platelet aggregation, vasoconstriction, and angiogenesis. The antiinflammatory and antithrombotic properties of DPTL may assist triatomines to successfully feed on blood and counteract host pro-inflammatory mechanisms triggered upon injury.

**EXPERIMENTAL PROCEDURES**

**Materials**—Horse tendon insoluble Horm fibrillar collagen (quaternary, polymeric structure) composed of collagen types I (95%) and III (5%), and Chrono-Lume were from Chrono-Log Corp. (Haverstown, PA). Soluble (tertiary, triple helical) collagen type I was from BD Biosciences (Franklin Lakes, NJ). Calcine-AM was from EMD Chemicals (San Diego, CA). PGD₂, PGE₁, PGE₂, PGE₃, 8-iso-PGF₂α, PGH₂ endoperoxide mimetic (U-51605), PGI₂ analog (iloprost), PGJ₂, cTXA₂, TXA₂-mimetic (U-46619), TXB₂, arachidonic acid, SQ 29548, LTB₄, LTC₄, LTD₄, LTE₄, 5(S)-HETE, 12(S)-HETE, 15(S)-HETE, and 20-HETE were purchased from Cayman Chemicals (Ann Arbor, MI). PAF, ADP, norepinephrine, epinephrine, serotonin, histamine, and indomethacin were from Sigma. Molecular biology reagents were from Invitrogen (Carlsbad, CA). Convulxin was purified as described (14).

**Dipetalogaster maxima Salivary Gland cDNA Construction**—This was done as described before (15) and in the supplemental data. Sequencing of cDNA indicate that DPTL is an abundant secreted lipocalin (data not shown).

**Sequence Analysis**—Sequence similarity searches were performed using BLAST. Cleavage site predictions of the mature proteins used the SignalP program. The molar extinction coefficient (ε₂₈₀ nm) of mature DPTL at 280 nm was obtained at Exasy Proteomics server, yielding for mature DPTL a value of ε₂₈₀ nm = 20315 M⁻¹ cm⁻¹; A₂₈₀ nm/cm 0.1% (1 mg/ml) = 1.132, molecular weight 17,951.1 (165 aa), and pl 8.49.

**Expression of DPTL in Escherichia coli**—Synthetic cDNA for DPTL was produced by Biobasics (Ontario, Canada). The sequence displays an N-terminal NdeI and a C-terminal XhoI restriction sites. The NdeI site adds a 5’-methionine codon to all sequences that acts as start codon in the bacterial expression system, whereas the XhoI site was incorporated after the stop codon. pET 17b constructs were confirmed before transformation of E. coli strain BL21(DE3)pLysS cells. Detailed description of expression of recombinant DPTL is available online in the supplemental data.

**Protein Purification, PAGE, and Edman Degradation**—These steps were performed as described in detail in the supplemental data available online.

**High-throughput Ligand Binding Assay**—To investigate putative ligands of DPTL, 50 μl of 100 mM ammonium acetate, pH 7.4 (AA buffer) containing 1 μM DPTL and 2 μM each of arachidonic acid, 15(S)-HETE, PGE₂, PGD₂, PGF₂α, TXA₂, U-46619, U-51605, leukotriene B₄, and carbocyclic TXA₂ were injected into a 3.2 × 250-mm Superdex peptide column (GE Healthcare) equilibrated with 100 mM AA buffer. A flow rate of 50 μl/min was maintained with a P4000 SpectraSystem pump (Thermo Scientific, Rockford, IL). The absorbance at 280 nm was monitored using an ABI 785 detector (Applied Biosystems, Foster City, CA). Fractions were collected into a 96-well plate every minute using a Probit apparatus (Dionex, Sunnyvale, CA). Selected fractions (20 μl) were mixed with 1 μl of methanol containing 1 M HCl, centrifuged at 14,000 × g for 10 min, and the supernatant injected into a 0.3 × 150-mm C18 reverse phase column (Magic C18 200 Å; Michrom BioResources, Inc, Auburn CA) equilibrated with 10% methanol/water containing 0.1% acetic acid at a flow rate of 3 μl/min maintained by an ABI 140D pump (Applied Biosystems). After 15 min, the methanol concentration was raised linearly to 90% in the course of 30 min. The column effluent was mixed with pure methanol at a rate of 4 μl/min (to facilitate electrospray) using a syringe pump attached to a LCQ Deca XP Max mass spectrometer (Thermo Scientific). Mass spectrometry was performed in negative-ion mode to detect ligand masses. A similar protocol was used to detect positively charged agonists: PAF acether, leukotrienes C₄, D₄, and E₄, histamine, serotonin, norepinephrine, epinephrine, and adenosine diphosphate, with the mass spectrometer running in positive-ion capture mode.

**Isothermal Titration Calorimetry (ITC)**—Prostanoids (in ethanol or methyl acetate) were placed in glass vials and the vehicle evaporated under nitrogen atmosphere; the dried material was then resuspended in appropriate concentrations in 20 mM Tris-HCl, 0.15 M NaCl, pH 7.4, sonicated, and vortexed. Calorimetric assays for measuring DPTL binding to a number of ligands were performed using a VP-ITC microcalorimeter (Microcal, Northampton, MA) at 35 °C. Titration experiments were performed by making successive injections of 10 μl each of 40 μM ligand into the 1.34-ml sample cell containing 4 μM DPTL until near-saturation was achieved. Prior to the run, the proteins were dialyzed against 20 mM Tris-HCl, 0.15 M NaCl, pH 7.4, for binding experiments. The calorimetric enthalpy (ΔH calor) for each injection was calculated after correction for the heat of DPTL dilution obtained in control experiments performed by titrating DPTL into buffer.

The binding isotherms were fitted according to a model for a single set of identical binding sites by nonlinear squares analysis using Microcal Origin software. Enthalpy change (ΔH), and stoichiometry (n) were determined according to Equation 1,

\[ Q = n\theta M \Delta H v_o \]  
(1)

where Q is total heat content of the solution contained in the cell volume (V_o), at fractional saturation θ, ΔH is the molar heat of ligand binding, n is the number of sites, and Mᵢ is the bulk concentration of macromolecules in V_o. The binding constant, K_s, is described as Equation 2.

\[ K_s = \frac{\theta}{1 - \theta}[X] \]  
(2)

where [X] is the free concentration of ligand.

Free-energy (∆G) and entropy term (−TΔS) of association were calculated according to Equations 3 and 4.

\[ ∆G = -RTln(K_s) \]  
(3)

\[ ∆G = ΔH - TΔS \]  
(4)

**Platelet Aggregation and ATP Release Assays**—Platelet-rich plasma was obtained by plateletpheresis from medication-free platelet donors at the DTM/NIH blood bank. Aggregation and
ATP release were performed as described (16) and in the supplemental data.

Platelet Adhesion Assay under Static Conditions—Inhibition of platelet adhesion to immobilized collagen was examined by fluorometry. Microfluor black microtiter 96-well plates (ThermoLabsystems, Franklin, MA) were coated with 2 μg of fibrillar (Horm) or soluble collagen overnight at 4 °C in PBS, pH 7.2, essentially as described (16) and in the supplemental data.

Contraction of Rat Aorta—Contraction of rat aortic ring preparations by U-46619 was measured isometrically and recorded with transducers from Harvard Apparatus Inc. (Holliston, MA). A modified Tyrode solution (with 10 mM HEPES buffer) that was oxygenated by continuous bubbling of air was used in the assays (17). In the first assay, aortic rings were suspended in a 0.5-ml bath kept at 30°C and were pre-constricted by 100 nM U-46619 before addition of proteins to give final concentrations of 1 μM. In the second assay, aortic ring preparations were preincubated with 100 nM of DPTL, and increments of 100 nM U-46619 were added until maximum contraction was reached. Additions to the bath were never greater than 5% of the volume of the bath.

Contraction of Rat Uterus—Wistar female rats were injected intraperitoneally with 0.1 mg of estradiol in 1 ml of phosphate-buffered saline. 24 h later, they were killed, and the uterus removed into a modified De Jalon solution (NaCl 154 mM, KCl 5.6 mM, D-glucose 2.8 mM, NaHCO3 6 mM, CaCl2 0.4 mM, Hepes 5 mM, 0.1 μM dexamethasone, final pH 7.4). About 1.5-cm pieces of the uterus were attached to a 1-ml bath kept at 35 °C, and their contractions recorded isotonically (Harvard Apparatus Inc.) under a 2 g load. Rhythmic contractions were induced by addition of PGF2α at the indicated concentrations.

Human Dermal Microvascular Endothelial Cell (HMVEC) Culture—HMVEC (CC-2643) were purchased from Clonetech (San Diego, CA) and grown at 37 °C, 5% CO2 in T-25 flasks in the presence of EBM-2 Plus as described (17) and in the supplemental data.

Tube Formation Assay—Tube formation assay was done as described with modifications (18). Costar culture plates (96-well; Corning, NY) were coated with 30 μl of growth factor-reduced Matrigel (BD Biosciences) and allowed to solidify at room temperature. 100 μl of MVEC suspension (5 × 10^5/ml) were added to each well in the presence of vehicle, or 15(S)-HETE, or DPTL, or DPTL plus 15(S)-HETE at the concentrations indicated in the figure legends. Plates were incubated at 37 °C, 5% CO2 for 5–6 h, and formation was observed under an inverted microscope coupled to a digital camera (Axiovert 200; Carl Zeiss, Inc., Thornwood, NJ). Images were captured with AxioCamHR color camera (model 412-312) attached to the microscope. Tube length was measured by outlining the tubes (and converted to pixels) using AxioVision 4.6.3 software.

Modeling of DPTL and Pallidipin—Structures of DPTL and pallidipin (gi388359) were modeled using the alignment mode in SWISS-MODEL (19). The template structure was the ammonium complex of nitrophorin 2 (PDB accession number 1EUO).
manner ATP release triggered by collagen (Fig. 2). Notably, no effect on shape change was observed, suggesting that DPTL did not target collagen itself, nor collagen receptors integrin α2β1 or GPVI. This was confirmed through platelet adhesion assays carried out with calcein-labeled platelets incubated with immobilized soluble (integrin α2β1-mediated) or fibrillar (GPVI and integrin α2β1-dependent) collagen (25–26). Results reported in Table 1 show that adhesion of platelets to fibrillar or soluble collagen was not inhibited by DPTL (1–10 μM). As a positive control, EDTA prevented platelet adhesion to fibrillar collagen ~60% and abolished adhesion to soluble collagen (26). These results, in addition to the lack of inhibition of platelet shape change, demonstrated that DPTL is not a specific collagen inhibitor and suggested that secondary mediators might be the target of the molecule.

TXA2 and ADP are two important mediators of platelet aggregation that are, respectively, generated and released by platelets upon stimulation by collagen (2). In an attempt to verify the inhibitory profile of DPTL toward other agonists that activate platelets independently of secondary mediators, it was tested as an inhibitor for U-46619 (TXA2 mimetic) and AA-induced platelet aggregation. Fig. 2 shows that DPTL dose-dependently inhibits U-46619 and AA-induced platelet aggregation in a dose-dependent manner, corroborating the notion that DPTL targets TXA2 (or ADP) mediated platelet responses. Because collagen, TXA2, and AA-induced aggregation is particularly sensitive to 5'-nucleotidases (27), ADP receptor antagonists (28), or ADP-binding proteins (7), it was of interest to exclude ADP as a potential target for DPTL. Fig. 2 shows that DPTL was ineffective as an inhibitor when ADP was employed at low or moderate concentrations, excluding this agonist as a target for the inhibitor. In addition, DPTL did not affect platelet aggregation triggered by collagen (Fig. 2). Notably, no effect on shape change was observed, suggesting that DPTL did not target collagen itself, nor collagen receptors integrin α2β1 or GPVI. This was confirmed through platelet adhesion assays carried out with calcein-labeled platelets incubated with immobilized soluble (integrin α2β1-mediated) or fibrillar (GPVI and integrin α2β1-dependent) collagen (25–26). Results reported in Table 1 show that adhesion of platelets to fibrillar or soluble collagen was not inhibited by DPTL (1–10 μM). As a positive control, EDTA prevented platelet adhesion to fibrillar collagen ~60% and abolished adhesion to soluble collagen (26). These results, in addition to the lack of inhibition of platelet shape change, demonstrated that DPTL is not a specific collagen inhibitor and suggested that secondary mediators might be the target of the molecule.

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by strong agonists such as PMA (PKC activator), convulxin (GPVI agonist), and ristocetin (vWF-dependent platelet agglutinatin), which characteristically induce platelet aggregation/agglutination independently of ADP or TXA$_2$.

Whereas results suggested that DPTL targets secondary mediators of platelet aggregation, they did not formally identify TXA$_2$ as the (sole) ligand or establish whether other ligands involved in pro-hemostatic events unrelated to platelet function were targets for DPTL. Therefore, an experiment was optimized to broaden our search, where the inhibitor was incubated with small compounds (e.g. biogenic amines, prostaglandins and endoperoxides, leukotrienes, HETEs, epoxides and lipids) that may affect platelet function, vessel tonus, angiogenesis, or neutrophil function. The mixture was loaded into a gel-filtration column that excludes protein with mol wt higher than 20 kDa but retains small ligands. If DPTL binds to a given ligand(s), complex formation will occur and elute in the void (>20 kDa), while free, unbound ligands remain in the column. Accordingly, Fig. 3A shows a peak eluted at 20 min that represents DPTL and potentially bound ligands. This fraction was acidified to precipitate DPTL. The sample was centrifuged, and the supernatant containing ligands was applied to a RP-HPLC column followed by elution using a gradient of methanol. Each fraction was collected and submitted to reverse phase/mass spectral experiments. Reverse-phase chromatography monitored by mass spectrometry that scanned for different masses compatible with the test mixture. A negative mass detection of 351 was found when fraction 20 was sprayed (Fig. 3C), this mass was compatible with PGD$_2$, or PGJ$_2$, which have masses of 352.4. As a control, fractions 17 (Fig. 3B) and 24 (Fig. 3D), which eluted before and after a peak corresponding to DPTL, were devoid of ligands.

ITC experiments were then carried out where separate ligands were added to a solution containing DPTL as described under “Experimental Procedures.” Fig. 4 shows the ITC profiles that represent binding in solution of DPTL and U-46619, PGF$_{2\alpha}$, and 15(S)-HETE. Binding was also seen for cTXA$_2$, PGH$_2$, mimetic (U-51605), TXB$_2$, PGD$_2$, and PGJ$_2$ (supplemental data). Analysis of the results according to a single set of identical binding sites yields a dissociation constant ($K_d$) of 100–200 nm for DPTL binding to most ligands. No interaction was observed when DPTL was added to PGE$_2$ (supplemental data), PGE$_1$, LTB$_4$, LTC$_4$, LTD$_4$, LTE$_4$, 5(S)-HETE, 12(S)-HETE, 20-HETE, PAF, NE, EPI, histamine, 5-HT, ADP, AA, and PAF (calorimetry not shown). Table 2 summarizes our findings including enthalpy ($\Delta H$), calculated free energy ($\Delta G$), and entropy ($\Delta S$) for binding of each compound to DPTL.

Calorimetry results therefore indicated that DPTL binds TXA$_2$, PGF$_{2\alpha}$, or 15(S)-HETE among other ligands. This was also tested through additional pharmacologic assays. Fig. 5A shows that inhibition of collagen (5.2 $\mu$g/ml)-induced platelet aggregation by DPTL was identical to inhibition by SQ 29548, a TXA$_2$ antagonist or by indomethacin which blocks TXA$_2$ production. Further, when DPTL was added to platelets incubated with SQ 29548 and indomethacin, no additional inhibition was observed. These results indicated a common target, i.e. TXA$_2$ pathway. Additionally, Fig. 5B shows that DPTL suppressed rhythmic contractions of the rat uterus induced by 0.2 $\mu$M PGF$_{2\alpha}$, being the inhibitory effect surmounted by high concentrations of the prostaglandin. Moreover, DPTL induces relaxation of aorta previously contracted with U-46619 (Fig. 5C).
Finally, DPTL was found to inhibit by >85% tube formation evoked by 15(S)-HETE (18), suggesting that it could negatively modulate angiogenesis (Fig. 5D).

Because DPTL is a lipocalin with sequence homology to nitrophorin 2 (NP2), a NO-binding protein from another triatomine species whose structure has been determined (13), we constructed a molecular model for the inhibitor using the NP2 structure as a template. Fig. 6A shows that DPTL displays structural features typical of the lipocalin family of proteins, whose structure consists of eight-stranded antiparallel β-barrel forming a central hydrophobic cavity; ligands are normally bound at a site located in the center of the β-barrel. Fig. 6B shows a comparison of this putative binding pocket in the models of DPTL and pallidipin. Many of the
residues predicted to lie in this pocket are shared by the two proteins and may play a role in stabilizing the bound ligand. Among these are a number of hydrophobic and aromatic residues that could be important in interactions with the hydrocarbon chain of eicosanoid ligands. Hydrogen bonding interactions with polar functional groups on the ligand are also normally essential, and their presence is suggested by the highly favorable enthalpies measured in ITC experiments. A number of residues that could potentially form hydrogen bonding or ionic interactions with bound ligands are also present in the binding pocket. Most notable among these are the conserved residues Arg<sup>39</sup> and Gln<sup>135</sup> in both (mature) DPTL and pallidipin. Remarkably high sequence similarity for the full-length sequences of both lipocalins is also depicted by the CLUSTAL alignment presented in Fig. 6C.

FIGURE 5. DPTL pharmacologic properties are compatible with its binding specificity. A, platelet-rich plasma (2 × 10<sup>9</sup>/μl) was incubated with 50 μM indomethacin, or 0.2 μM SQ29548 for 3 min, with or without DPTL (1 μM) followed by addition of collagen (5.2 μg/ml). B, DPTL (1 μM) inhibits rat uterus contraction elicited by PGF<sub>2α</sub> at 0.2 μM, but its effect is surmounted by 1 μM PGF<sub>2α</sub>. C, DPTL (1 μM) inhibits aorta contraction induced by U-46619 (0.2 μM). D, MVEC were seeded onto a 96-well plate coated with growth factor-reduced Matrigel. Cells were then treated with vehicle or 0.2 μM of 15(S)-HETE, or 15(S)-HETE plus DPTL (3 μM), or DPTL alone (3 μM) for 5–6 h at 37 °C. Addition of DPTL alone was comparable to addition of buffer. Tube formation was observed under an inverted microscope, images were captured with a color camera, and tube length was measured using AxioVision 4 software.

15(S)-HETE 15(S)-HETE + DPTL Buffer
Figure 6. Molecular modeling. A, DPTL displays structural features typical of the lipocalin family of proteins, whose structure consists of eight-stranded antiparallel β-barrel forming a central hydrophobic cavity (left panel; frontal view; right panel; side view). B, putative binding pocket of DPTL and C, pallidipin. Secondary structural elements of the β-barrel are shown in ribbon format, while side chains of residues lining the pocket are shown as stick diagrams. Carbon atoms are colored white, oxygen is colored red, and nitrogen is colored blue. D, CLUSTAL alignment of DPTL and pallidipin (gi 388359) displays high sequence homology (2e-56).
**DISCUSSION**

Salivary secretions are rich sources of bioactive molecules that counteract host defenses in distinct ways. Many of these molecules have turned out to display unique and specific pharmacologic properties that in several instances contribute to our understanding of vertebrate biology (4). In this report, we have identified DPTL as a novel salivary lipocalin that binds to distinct prostanoids. Accordingly, ITC experiments demonstrated that DPTL binds to cTXA<sub>2</sub>, U-46619 (stable TXA<sub>2</sub> mimetic), and TXB<sub>2</sub>, which is the metabolic end product of TXA<sub>2</sub>. Binding occurred with a K<sub>d</sub> ~100–200 nM and was compatible with 1:1 stoichiometry. Binding to TXA<sub>2</sub> was consistent with the inhibitory profile for platelet aggregation observed in the presence of the inhibitor and suggests that native TXA<sub>2</sub> generated by platelets is a target for the inhibitor. Accordingly, DPTL affected only platelet responses induced by low concentrations of collagen or by AA and U-46619, which are TXA<sub>2</sub>-dependent. A similar inhibitory profile is observed for aggregation of platelets from a patient with a mutation of the TXA<sub>2</sub> receptor (29), or for mice with a gene deletion of G<sub>q</sub> (30). Targeting of the TXA<sub>2</sub> pathway by DPTL is also corroborated by a lack of additional effects of the inhibitor on collagen-induced platelet aggregation in the presence of SQ29548 and indomethacin (Fig. 5A).

In contrast, DPTL did not interfere with aggregation triggered by high doses of collagen, which occurs via tyrosine kinase-dependent PLC<sub>y</sub>2 activation (2, 31), or by strong agonists such as convulxin and PMA or vWF-dependent agglutinating agent ristocetin.

Targeting TXA<sub>2</sub> has several implications, as it is the major contributor of platelet aggregation by collagen, which is the most atherogenic protein of the vessel wall (32). Upon platelet adhesion to collagen, TXA<sub>2</sub> is generated and activates platelets through the TP receptors that are coupled to G<sub>q</sub> and G<sub>12</sub>/13 (33). This promotes shape change, and activation of an intracellular pathway that leads to granule secretion, and ADP release. ADP is critical for completion of platelet aggregation by TXA<sub>2</sub> pathway that leads to granule secretion, and ADP release. ADP promotes shape change, and activation of an intracellular

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**TXA<sub>2</sub>/PGF<sub>2α</sub>/15(S)-HETE-binding Protein**

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**Interference with angiogenesis**

Interference with angiogenesis may potentially attenuate inflammation and granulation tissue formation at site of bite, as was reported previously for tick saliva (17).

Notably, DPTL did not interact with PGE<sub>2</sub>, a prostaglandin, which displays structural similarities with other prostaglandins tested here. This is not entirely surprising, as PGE<sub>2</sub> displays diverse and complex biologic effects depending on the amount of prostaglandin available in the microenvironment of diverse tissues (e.g. vasodilator versus vasoconstrictor) and on the subtype of receptors expressed on target cells (38, 45). The lack of DPTL interaction with PGE<sub>1</sub>, PGE<sub>2</sub>, and 8-isoprostaglandin D<sub>2</sub> and its high-affinity for PGD<sub>2</sub> indicates a remarkable specificity to prostanoids involved in pro-inflammatory events at sites relevant to vector-host interaction (e.g. skin) and suggest that subtle structural changes are important determinants for binding. In contrast, DPTL did not interact with leukotrienes (e.g. LTB<sub>4</sub>), cysteinyl leukotrienes (e.g. LTC<sub>4</sub>, LTD<sub>4</sub>, LTE<sub>4</sub>), biogenic amines (e.g. ADP, 5-HT, EPI, and NE), 5- and 12-LOX-derived HETEs, epoxide produced by cytochrome P450 (e.g. 20-HETE), or lipids (e.g. AA and PAF). The unique binding properties of DPTL therefore distinguish it from other salivary lipocalins (specificity in parenthesis) such as RPAI-1 (e.g. LTD<sub>4</sub>, LTD<sub>3</sub>, LTE<sub>4</sub>), bioactive lipids (e.g. ADP, 5-HT, EPI, and NE), 5- and 12-LOX-derived HETEs, epoxide produced by cytochrome P450 (e.g. 20-HETE), or lipids (e.g. AA and PAF). The unique binding properties of DPTL therefore distinguish it from other salivary lipocalins (specificity in parenthesis) such as RPAI-1 (e.g. ADP) (7), ADP (e.g. 5-HT/NE/EPI) (8), short D7s (e.g. 5HT/NE) or long D7s (e.g. LTD<sub>4</sub>/LTE<sub>4</sub>), e.g. 5-HT (47), SHBP (e.g. 5-HT) (48), moubatin and TSGP3 (e.g. TXA<sub>2</sub>/LTB<sub>4</sub>) (23).

These results suggest that DPTL potentially displays multi-functional antihemostatic properties, through attenuation of platelet aggregation mediated by TXA<sub>2</sub>, negative modulation of vessel tonus by PGF<sub>2α</sub>, and blockade of angiogenesis by 15(S)-HETE (Fig. 5). It is important to recognize that DPTL is a very
abundant lipocalin in the salivary gland, accounting for at least 30% of total salivary lipocalins estimated by SDS/PAGE (Fig. 1C). Assuming a molecular mass of ~20 kDa for DPTL and release of 50% of the salivary contents (~1 µg/salivary gland pair) upon feeding being ~30% DPTL, a concentration of at least 1 mM of the inhibitor could exist in the feeding environment (~15 µl); this concentration is clearly above the Kd for TXA2 and other prostanoids. In addition, D. maxima expresses an apyrase and several other uncharacterized salivary proteins that may interfere with hemostasis in a distinct yet redundant manner.

Results obtained by pharmacologic assays were compatible with DPTL being a lipocalin that evolved with a binding pocket adapted to accommodate small eicosanoids. To get further insights into the mechanism of binding of DPTL to TXA2, a molecular model based on NP2 was constructed (13). The putative binding pocket of the model exhibits a generally hydrophobic structure that would be consistent in the pallidipin, which has been claimed as a specific inhibitor of collagen-mediated platelet aggregation, suggesting that the two proteins may have similar functions; however, it is clear that DPTL (and pallidipin) are not specific collagen-binding proteins or receptor antagonists because they do not affect platelet shape change or platelet adhesion. Accordingly, it is plausible that pallidipin exerts its antiplatelet activity through a similar mechanism characterized here for DPTL as binding to TXA2 interaction with other eicosanoids is also likely. In conclusion, DPTL displays unique ligand specificities that may assist the triatamine D. maxima to successfully feed on blood. DPTL is a potentially useful activity through a similar mechanism characterized here for affecting platelet shape change or platelet adhesion. Accord-

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