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Protein disulfide isomerase as an antithrombotic target

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Abstract

Protein disulfide isomerase (PDI) is a ubiquitously expressed oxidoreductase required for proper protein folding. It is highly concentrated in the endoplasmic reticulum, but can also be released into the extracellular environment. Several *in vivo* thrombosis models have demonstrated that vascular PDI secreted by platelets and endothelial cells is essential for normal thrombus formation. Inhibition of extracellular PDI thus represents a potential strategy for antithrombotic therapy. Yet this approach requires the discovery of well-tolerated PDI inhibitors. A recent high throughput screen identified the commonly ingested flavonoid, quercetin-3-rutinoside, as an inhibitor of PDI. Quercetin-3-rutinoside blocked thrombus formation at concentrations that are commonly ingested as nutritional supplements. The observation that a compound with Generally Recognized As Safe status inhibits PDI and blocks thrombosis in animal models forms a rationale for clinical trials evaluating PDI inhibitors as a new class of antithrombotics.

PDI in thrombus formation

Protein disulfide isomerase (PDI, EC 5.3.4.1) is the founding member of a large family of thiol isomerases that facilitate the folding of newly transcribed proteins (Appenzeller-Herzog et al., 2008). These enzymes possess both reductase and oxidase activities that enable them to rearrange intramolecular disulfide bonds. The active sites of these enzymes consist of a CXXC motif that mediates thiol-disulfide exchange. PDI includes two a-type thioredoxin domains each containing one CXXC motif and two b-type thioredoxin domains thought to contribute to its chaperone function (Fig. 1). PDI is ubiquitously expressed and highly concentrated in the oxidizing environment of the endoplasmic reticulum, where it ensures proper disulfide bond formation of newly formed proteins. Yet despite having a C-terminal endoplasmic reticulum retention sequence, PDI has been identified in diverse subcellular locations and even outside of the cell. Within the vasculature, extracellular PDI has been found on the surface of platelets, endothelial cells, and leukocytes (Essex et al., 1995; Lame et al., 2005; Santos et al., 2009).

Extracellular PDI has a critical role in thrombus formation. An initial indication that PDI functions in thrombosis was born out of studies in platelets showing that activated platelets release PDI (Chen et al., 1992) and that inhibition of PDI blocks agonist-induced platelet aggregation (Essex et al., 1999). In 2008, two separate groups demonstrated that anti-PDI antibodies inhibit thrombus formation in murine thrombosis models. Reinhardt et al.

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demonstrated inhibition of thrombus formation by an anti-PDI antibody in both a carotid artery ligation model and a model of wire-induced endothelial disruption (Reinhardt et al., 2008). Cho et al. used intravital microscopy to show that PDI accumulates at the site of thrombus formation immediately following laser-induced injury (Cho et al., 2008) and that neutralizing anti-PDI antibodies inhibit thrombus formation in this model. Inhibition of PDI also prevents thrombus formation induced by FeCl_3 (Jasuja et al., 2012). The fact that inhibition of PDI blocks thrombus formation in several injury models (e.g., ligation, ferric chloride exposure, laser injury, mechanical injury) and in several vascular beds (e.g., carotid arteries, cremaster arterioles, mesenteric arterioles) indicates that PDI serves a function that is fundamental to thrombus formation *in vivo*.

The observation that PDI is critical for thrombus formation raises the question of how it is secreted and accumulates in thrombi. In endothelium, PDI co-localizes with markers of the endoplasmic reticulum and partially colocalizes with GRO- α in small secretory vessels (Jasuja et al., 2010). In platelets, PDI colocalizes with TLR9 in a specialized platelet granule termed the T-granule (Thon et al., 2012). However, the storage granules for PDI are not well characterized and the mechanisms of its secretion are presently unknown.

Once secreted, PDI must remain bound to the thrombus under conditions of high shear. Yet PDI does not have a transmembrane domain or obvious membrane binding sequence. To address the question of how PDI is maintained at the site of vascular injury, Cho et al. evaluated PDI accumulation in mice lacking the integrin α_3 . PDI failed to accumulate in thrombi formed in $\alpha_3^{-/-}$ mice (Cho et al., 2012). Reciprocal bone marrow transplant studies showed that α_3 from both the hematopoietic compartment (e.g. IIb_3 on platelets) and from the vascular wall (e.g., ν_3 on endothelial cells) are required for normal accumulation of PDI following vascular injury. From a functional standpoint, the most important source of PDI for thrombus formation may be the endothelium, since inhibition of platelet accumulation following vascular injury did not prevent accumulation of PDI or fibrin generation at sites of laser-induced injury (Jasuja et al., 2010).

Mechanism of PDI activity during thrombus formation

PDI functions in both platelet accumulation and fibrin generation during thrombus formation (Cho et al., 2008). This observation raises the possibility that PDI acts at the top of the coagulation cascade, preventing both downstream pathways. Alternatively, PDI may act on more than one substrate during thrombus formation. Several substrates have been proposed based on *in vitro* studies. Tissue factor activation (deencryption) has been postulated to involve the formation of an allosteric disulfide bond between cysteine 186 and cysteine 200 (Chen et al., 2006). PDI may participate in thiol pathways leading to decryption of tissue factor (Ruf, 2012). Yet the details of how this pathway functions *in vivo* during thrombus formation have not been directly assessed. Platelet adhesion molecules have also been proposed as PDI substrates relevant for thrombus formation. Antibodies directed at PDI inhibit platelet aggregation *in vitro* (Essex et al., 1999), and this effect has been attributed in part to the influence of PDI on IIb_3 conformation (Essex and Li, 1999; Essex et al., 2001). PDI-mediated thiol exchange enhances adhesion of collagen to $\alpha_2\beta_1$ (Lahav et al., 2003). Glycoprotein 1b contains free thiols and is modified by PDI (Burgess et al., 2000). *In vitro* studies have also shown that factor XI is a substrate for PDI, enhancing its conversion to factor XIa, but not its enzymatic activity (Giannakopoulos et al., 2012). The relevance of any of these *in vitro* findings to thrombus formation *in vivo* remains to be determined and the critical substrates of PDI during thrombus formation *in vivo* are presently unknown.

Identification of quercetin-3-rutinoside as a PDI inhibitor

Given the observation that PDI is required for thrombus formation *in vivo*, we set out to identify small molecule inhibitors of PDI. The most widely used small molecule inhibitor of PDI has been the antibiotic bacitracin. Bacitracin is a 1423 Da cyclic dodecapeptide that blocks PDI activity with an IC_{50} of approximately 150-200 μM (Lovat et al., 2008; Mandel et al., 1993; Smith et al., 2004). In addition to poor potency, bacitracin inhibits many other thiol isomerases and may contain proteases, depending on the preparation (Rogelj et al., 2000). To identify more potent and selective inhibitors, we initially screened a ~5000 compound small molecule library for compounds capable of inhibiting PDI. The library consisted of a collection of bioactive compounds with known activities. Compounds were selected based on their ability to inhibit the insulin reductase activity of PDI in a turbidimetric-based assay (Jasuja et al., 2012). The most potent inhibitor was a compound, quercetin-3-rutinoside, also as known as rutin.

Quercetin-3-rutinoside inhibited purified PDI with an IC_{50} of ~6 μM and bound PDI under flow with a K_d of ~3 μM (Jasuja et al., 2012). Among thiol isomerases, quercetin-3-rutinoside was selective for PDI, not inhibiting ERp5, ERp57, ERp72, thioredoxin, or thioredoxin reductase. We performed structure activity relationships to identify the chemical moieties within quercetin-3-rutinoside responsible for its antithrombotic activity. This analysis demonstrated that only flavonoids with a 3-O-glycosidic linkage in the 3 position of the C ring blocked PDI activity (Fig. 2). Active analogs included quercetin-3-glucuronide, a metabolite that is abundant in plasma following quercetin-3-rutinoside ingestion (Walle, 2004). The fact that only glycosylated quercetins are active against PDI is important when considering the potential toxicity of these compounds. This glycoside reduces the cell permeability of the quercetin. Thus, the same chemical moiety that renders a quercetin analog active against PDI inhibits its entry into cells. This property could account for the low toxicity of these compounds.

The fact that quercetin-3-rutinoside inhibited PDI was intriguing to us. Quercetin-3-rutinoside is a flavonoid that is abundant in teas, buckwheat, and certain fruits and vegetables. Like other quercetin flavonoids, it is widely consumed as a nutritional supplement. Prospective cohort studies have shown an inverse correlation with flavonoid intake and cardiovascular disease mortality (Geleijnse et al., 1999; Geleijnse et al., 2002; McCullough et al., 2012). The fact that quercetin-3-rutinoside is both well-tolerated when ingested and inhibits PDI relatively potently indicates that inhibition of PDI may be tolerated in the context of antithrombotic therapy. To evaluate this possibility, we tested the ability of quercetin-3-rutinoside to inhibit thrombus formation *in vivo*. Quercetin-3-rutinoside inhibited both platelet accumulation and fibrin formation following laser-induced vascular injury with an IC_{50} of <0.1 mg/kg for platelet accumulation and <0.3 mg/kg for fibrin generation (Fig. 3) (Jasuja et al., 2012). Quercetin-3-rutinoside also inhibited thrombus formation following $FeCl_3$ -induced injury of arterioles. Quercetin-3-rutinoside was inhibitory in the *in vivo* thrombosis model regardless of whether it was administered intravenously or by gastric lavage (Jasuja et al., 2012). The antithrombotic effect of quercetin-3-rutinoside in mice was totally reversed by infusion of recombinant PDI (Jasuja et al., 2012). This experiment confirmed that quercetin-3-rutinoside inhibited thrombus formation by blocking PDI. These preclinical studies demonstrate that inhibition of PDI is a tractable approach for blocking thrombus formation.

PDI as an antithrombotic target

Despite the widespread use of antiplatelet and anticoagulant therapies, thrombosis remains a major cause of morbidity and mortality in the United States. Limitations of current therapies

for thrombotic disorders are evidenced by the high incidence of recurrent thrombosis, approximately 500,000 cases in the U.S. annually, including both recurrent arterial and venous thrombosis (Kyrle et al., 2004; Nagarakanti et al., 2008). PDI represents a new target for antithrombotic therapy distinct from proteins of the coagulation cascade, platelet receptors, or platelet signaling proteins targeted by current therapies. The observation that a PDI inhibitor is present in commonly ingested food and is inhibitory at concentrations that can be achieved by oral ingestion of a flavonoid quercetin speaks to the feasibility of inhibiting PDI for thromboprophylaxis. Since PDI inhibitors interfere with both platelet accumulation and fibrin formation, therapeutics targeting of PDI could be used in the prevention and or treatment of either arterial or venous thrombosis.

Quercetin flavonoids may represent a first generation of PDI inhibitors. An advantage of developing quercetin flavonoids as anticoagulants is that these compounds have been granted Generally Recognized As Safe (GRAS) status by the FDA. They can therefore be used for new indications without having to undergo typical regulatory filings and extensive preclinical studies. This designation has enabled us to quickly take these compounds into clinical studies, which are presently underway. Initial studies are directed at developing appropriate laboratory tests to detect PDI inhibitory activity in the plasma of patients receiving flavonoid quercetins. Such studies will enable us to determine the appropriate analog and dose for larger studies. Although these compounds have GRAS status, their effect on hemostasis has not been directly evaluated and could have been overlooked in previous clinical studies and in their popular use as nutraceuticals. Assessment of bleeding risk will be a critical component of evaluating the safety of quercetin flavonoids as antithrombotics in larger studies. Several potential indications for quercetin flavonoid-based anticoagulation could be envisioned. There are special circumstances of refractory thrombosis in which flavonoid quercetins could be added to existing antithrombotic regimens. This class of compound could also be used as prophylaxis in settings in which thrombosis prophylaxis is not standard of care (e.g., thrombosis of malignancy).

While quercetin flavonoids could be introduced into clinical trials relatively quickly and will be helpful in identifying scenarios in which PDI inhibition is useful in antithrombotic therapy, they are not without liabilities. These compounds are poorly absorbed and must be taken in high concentrations to achieve adequate oral availability. Although they are selective for PDI among thiol isomerases, alternative targets for flavonoid quercetins have been described. More potent and selective PDI inhibitors that are easier to monitor and demonstrate more favorable pharmacological profiles will serve as a second generation of PDI inhibitors. We and others (Khan et al., 2011; Xu et al., 2012) have already begun screening small molecule libraries to identify more potent and selective PDI inhibitors. We are presently characterizing these leads to assess their antithrombotic activity.

Prospective

The discovery that a commonly ingested flavonoid blocks PDI and is profoundly antithrombotic *in vivo* provides proof-of-principle for targeting PDI in the development of novel antithrombotics. Other vascular thiol isomerases may also represent valid targets. Inhibition of ERp57 blocks thrombus formation *in vivo* in mice (Holbrook et al., 2012; Wu et al., 2012). ERp72 and ERp5 are released from platelets and ERp5 appears to function in platelet activation (Holbrook et al., 2010; Jordan et al., 2005). Thiol isomerases inhibitors could represent a new class of antithrombotic agents used in combination with or instead of current antithrombotics.

Yet even as we pursue clinical trials with quercetin flavonoids as PDI inhibitors, our understanding of how PDI contributes to thrombus formation remains rudimentary.

Although PDI has been shown to block thrombus formation in several varied in vivo models, it is not clear whether it is an essential component of thrombus formation required for all forms of thrombosis. The relevant intravascular substrates of PDI are not known. Whether the chaperone activity or the oxidoreductase activities of PDI are required during thrombus formation is not certain. We have little understanding of where within cells PDI is stored and how it is released. The relationship of the different thiol isomerases that appear to function in thrombus formation is essentially unstudied. Researchers have yet to place PDI or other thiol isomerases in the coagulation cascade or understand how it contributes to platelet activation. Many of these questions will need to be addressed for optimal use of this new class of antithrombotics.

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References

- Appenzeller-Herzog C, Ellgaard L. The human PDI family: versatility packed into a single fold. *Biochim Biophys Acta*. 2008; 1783(4):535–548. [PubMed: 18093543]
- Burgess JK, Hotchkiss KA, Suter C, Dudman NP, Szollosi J, Chesterman CN, et al. Physical proximity and functional association of glycoprotein 1balpha and protein-disulfide isomerase on the platelet plasma membrane. *J Biol Chem*. 2000; 275(13):9758–9766. [PubMed: 10734129]
- Chen K, Lin Y, Detwiler TC. Protein disulfide isomerase activity is released by activated platelets. *Blood*. 1992; 79(9):2226–2228. [PubMed: 1571538]
- Chen VM, Ahamed J, Versteeg HH, Berndt MC, Ruf W, Hogg PJ. Evidence for activation of tissue factor by an allosteric disulfide bond. *Biochemistry*. 2006; 45(39):12020–12028. [PubMed: 17002301]
- Cho J, Furie BC, Coughlin SR, Furie B. A critical role for extracellular protein disulfide isomerase during thrombus formation in mice. *J Clin Invest*. 2008; 118(3):1123–1131. [PubMed: 18292814]
- Cho J, Kennedy DR, Lin L, Huang M, Merrill-Skoloff G, Furie BC, et al. Protein disulfide isomerase capture during thrombus formation in vivo depends on the presence of beta3 integrins. *Blood*. 2012; 120(3):647–655. [PubMed: 22653978]
- Essex DW, Chen K, Swiatkowska M. Localization of protein disulfide isomerase to the external surface of the platelet plasma membrane. *Blood*. 1995; 86(6):2168–2173. [PubMed: 7662965]
- Essex DW, Li M. A polyclonal antibody to protein disulfide isomerase induces platelet aggregation and secretion. *Thromb Res*. 1999; 96(6):445–450. [PubMed: 10632467]
- Essex DW, Li M. Protein disulphide isomerase mediates platelet aggregation and secretion. *Br J Haematol*. 1999; 104(3):448–454. [PubMed: 10086777]
- Essex DW, Li M, Miller A, Feinman RD. Protein disulfide isomerase and sulfhydryl-dependent pathways in platelet activation. *Biochemistry*. 2001; 40(20):6070–6075. [PubMed: 11352743]
- Geleijnse JM, Launer LJ, Hofman A, Pols HA, Witteman JC. Tea flavonoids may protect against atherosclerosis: the Rotterdam Study. *Arch Intern Med*. 1999; 159(18):2170–2174. [PubMed: 10527294]
- Geleijnse JM, Launer LJ, Van der Kuip DA, Hofman A, Witteman JC. Inverse association of tea and flavonoid intakes with incident myocardial infarction: the Rotterdam Study. *Am J Clin Nutr*. 2002; 75(5):880–886. [PubMed: 11976162]
- Giannakopoulos B, Gao L, Qi M, Wong JW, Yu DM, Vlachoyiannopoulos PG, et al. Factor XI is a substrate for oxidoreductases: enhanced activation of reduced FXI and its role in antiphospholipid syndrome thrombosis. *J Autoimmun*. 2012; 39(3):121–129. [PubMed: 22704541]
- Holbrook LM, Sasikumar P, Stanley RG, Simmonds AD, Bicknell AB, Gibbins JM. The platelet-surface thiol isomerase enzyme ERp57 modulates platelet function. *J Thromb Haemost*. 2012; 10(2):278–288. [PubMed: 22168334]

- Holbrook LM, Watkins NA, Simmonds AD, Jones CI, Ouwehand WH, Gibbins JM. Platelets release novel thiol isomerase enzymes which are recruited to the cell surface following activation. *Br J Haematol.* 2010; 148(4):627–637. [PubMed: 19995400]
- Jasuja R, Furie B, Furie BC. Endothelium-derived but not platelet-derived protein disulfide isomerase is required for thrombus formation in vivo. *Blood.* 2010; 116(22):4665–4674. [PubMed: 20668226]
- Jasuja R, Passam FH, Kennedy DR, Kim SH, van Hessem L, Lin L, et al. Protein disulfide isomerase inhibitors constitute a new class of antithrombotic agents. *J Clin Invest.* 2012; 122(6):2104–2113. [PubMed: 22565308]
- Jordan PA, Stevens JM, Hubbard GP, Barrett NE, Sage T, Authi KS, et al. A role for the thiol isomerase protein ERP5 in platelet function. *Blood.* 2005; 105(4):1500–1507. [PubMed: 15466936]
- Khan MM, Simizu S, Lai NS, Kawatani M, Shimizu T, Osada H. Discovery of a small molecule PDI inhibitor that inhibits reduction of HIV-1 envelope glycoprotein gp120. *ACS Chem Biol.* 2011; 6(3):245–251. [PubMed: 21121641]
- Kyrle PA, Minar E, Bialonczyk C, Hirschl M, Weltermann A, Eichinger S. The risk of recurrent venous thromboembolism in men and women. *N Engl J Med.* 2004; 350(25):2558–2563. [PubMed: 15201412]
- Lahav J, Wijnen EM, Hess O, Hamaia SW, Griffiths D, Makris M, et al. Enzymatically catalyzed disulfide exchange is required for platelet adhesion to collagen via integrin $\alpha 2\beta 1$. *Blood.* 2003; 102(6):2085–2092. [PubMed: 12791669]
- Lame MW, Jones AD, Wilson DW, Segall HJ. Monocrotaline pyrrole targets proteins with and without cysteine residues in the cytosol and membranes of human pulmonary artery endothelial cells. *Proteomics.* 2005; 5(17):4398–4413. [PubMed: 16222722]
- Lovat PE, Corazzari M, Armstrong JL, Martin S, Pagliarini V, Hill D, et al. Increasing melanoma cell death using inhibitors of protein disulfide isomerases to abrogate survival responses to endoplasmic reticulum stress. *Cancer Res.* 2008; 68(13):5363–5369. [PubMed: 18593938]
- Mandel R, Ryser HJ, Ghani F, Wu M, Peak D. Inhibition of a reductive function of the plasma membrane by bacitracin and antibodies against protein disulfide-isomerase. *Proc Natl Acad Sci U S A.* 1993; 90(9):4112–4116. [PubMed: 8387210]
- McCullough ML, Peterson JJ, Patel R, Jacques PF, Shah R, Dwyer JT. Flavonoid intake and cardiovascular disease mortality in a prospective cohort of US adults. *Am J Clin Nutr.* 2012; 95(2):454–464. [PubMed: 22218162]
- Nagarakanti R, Sodhi S, Lee R, Ezekowitz M. Chronic antithrombotic therapy in post-myocardial infarction patients. *Cardiol Clin.* 2008; 26(2):277–288. vii. [PubMed: 18407000]
- Reinhardt C, von Bruhl ML, Manukyan D, Grahl L, Lorenz M, Altmann B, et al. Protein disulfide isomerase acts as an injury response signal that enhances fibrin generation via tissue factor activation. *J Clin Invest.* 2008; 118(3):1110–1122. [PubMed: 18274674]
- Rogelj S, Reiter KJ, Kesner L, Li M, Essex D. Enzyme destruction by a protease contaminant in bacitracin. *Biochem Biophys Res Commun.* 2000; 273(3):829–832. [PubMed: 10891332]
- Ruf W. Role of thiol pathways in TF procoagulant regulation. *Thromb Res.* 2012; 129(Suppl 2):S11–12. [PubMed: 22401798]
- Santos CX, Stolf BS, Takemoto PV, Amanso AM, Lopes LR, Souza EB, et al. Protein disulfide isomerase (PDI) associates with NADPH oxidase and is required for phagocytosis of *Leishmania chagasi* promastigotes by macrophages. *J Leukoc Biol.* 2009; 86(4):989–998. [PubMed: 19564574]
- Smith AM, Chan J, Oksenberg D, Urfer R, Wexler DS, Ow A, et al. A high-throughput turbidometric assay for screening inhibitors of protein disulfide isomerase activity. *J Biomol Screen.* 2004; 9(7):614–620. [PubMed: 15475481]
- Thon JN, Peters CG, Machlus KR, Aslam R, Rowley J, Macleod H, et al. T granules in human platelets function in TLR9 organization and signaling. *J Cell Biol.* 2012; 198(4):561–574. [PubMed: 22908309]
- Walle T. Absorption and metabolism of flavonoids. *Free Radic Biol Med.* 2004; 36(7):829–837. [PubMed: 15019968]

- Wu Y, Ahmad SS, Zhou J, Wang L, Cully MP, Essex DW. The disulfide isomerase ERp57 mediates platelet aggregation, hemostasis, and thrombosis. *Blood*. 2012; 119(7):1737–1746. [PubMed: 22207737]
- Xu S, Butkevich AN, Yamada R, Zhou Y, Debnath B, Duncan R, et al. Discovery of an orally active small-molecule irreversible inhibitor of protein disulfide isomerase for ovarian cancer treatment. *Proc Natl Acad Sci U S A*. 2012; 109(40):16348–16353. [PubMed: 22988091]

Figure 1. Domain structure of PDI

The thioredoxin-like domains a and a' (light gray) contain the catalytic motif (CGCH) that mediates thiol-disulfide exchange. The b and b' domains (dark gray) have no catalytic activity. A large hydrophobic pocket in the b' domain is thought to participate in substrate binding. A sequence connecting the b' and a' domains is termed the x-linker (x in schematic). The C-terminal extension consists of an acidic sequence (c in schematic) and contains a KDEL endoplasmic reticulum retention sequence.

Sugar on position 3 of C ring:

Rutin	IC ₅₀ 6.1
Hyperoside	IC ₅₀ 5.9
Isoquercetin	IC ₅₀ 7.1
Quercetin glucuronide	IC ₅₀ 5.9

No sugar on position 3 of C ring:

Quercetin	IC ₅₀ >100
Tamarixetin	IC ₅₀ >100
Isorhamnetin	IC ₅₀ >100
Diosmetin	IC ₅₀ >100

Figure 2. Inhibition of PDI by quercetin-3-rutinoside and analogs

High throughput screening identified quercetin-3-rutinoside as an inhibitor of PDI as monitored in an insulin-based turbidimetric assay. Evaluation of structure activity relationships demonstrated that a sugar at 3 position in the C ring of quercetin-3-rutinoside is critical for its ability to inhibit PDI. All analogs tested with a sugar in this position inhibited PDI, while analogs lacking this sugar failed to demonstrate inhibition.

Figure 3. Quercetin-3-rutinoside inhibits platelet accumulation and fibrin generation *in vivo* Platelet accumulation (*red*) and fibrin formation (*green*) were monitored over 180 seconds after laser-induced vessel wall injury following infusion of vehicle only (A) or quercetin-3-rutinoside at 0.1 mg/kg body weight (B), 0.3 mg/kg (C), or 0.5 mg/kg (D). Median integrated platelet fluorescence intensity (E) and median integrated fibrin fluorescence intensity (F) at the injury site are plotted versus time. Vehicle only (*black*); quercetin-3-rutinoside at 0.1 mg/kg body weight (*green*); 0.3 mg/kg (*blue*); 0.5 mg/kg (*red*). Data are from 30 thrombi in 3 mice for each condition. (Figure adapted from Jasuja et al. 2012. J. Clin. Invest. 122:2104-13).