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Revisiting the mechanistic basis of the French Paradox: Red wine inhibits the activity of protein disulfide isomerase *in vitro*

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ABSTRACT

Introduction: Although epidemiologic evidence points to cardioprotective activity of red wine, the mechanistic basis for antithrombotic activity has not been established. Quercetin and related flavonoids are present in high concentrations in red but not white wine. Quercetin-glycosides were recently shown to prevent thrombosis in animal models through the inhibition of extracellular protein disulfide isomerase (PDI). We evaluated whether red or white wine inhibited PDI activity *in vitro*.
Methods: Quercetin levels in red and white wines were measured by HPLC analysis. Inhibition of PDI activity by red and white wines was assessed by an insulin reduction turbidity assay at various concentrations of wine. PDI inhibition was confirmed using a reduced peptide that contained a disulfide containing peptide as a substrate. The inhibition of PDI related thiol isomerases ERp5 and ERp57 was also assessed.
Results: We observed a dose-dependent decrease of PDI activity for a variety of red but not white wines. Red wine diluted to 3% final concentration resulted in over 80% inhibition of PDI activity by insulin reductase assay for all varieties tested. This inhibition was also observed in the peptide based assay. Red grape juice yielded similar results but ethanol alone did not affect PDI activity. Interestingly, red wine also inhibited the PDI related thiol isomerases ERp5 and ERp57, albeit to a lesser degree than PDI.
Conclusions: PDI activity is inhibited by red wine and grape juice, identifying a potentially novel mechanism underlying the cardiovascular benefits attributed to wine consumption.

1. Introduction

The rate of cardiovascular mortality is approximately two-fold lower in France than in the United States, despite the French consuming roughly three times as much animal fat [1]. This phenomena is known as the French Paradox and considerable effort has been made to identify the nutritional/epidemiologic basis for this seemingly incongruous association. A common link among hypothesis is that the cardioprotective activity of red wine is mediated through anti-platelet activity [1–4]. Red wine can inhibit collagen induced, ADP-stimulated or epinephrine-stimulated platelet aggregation, [1,5,6] as well as thrombus formation *in vivo* [7]. However, the mechanism by which red wine inhibits platelet aggregation and thrombus formation is unclear.

Unlike white wine, red wines are produced using the entire grape, included the grape seed and skin. There are a wide variety of phytochemicals found in grape skin and seeds that have been implicated in providing the cardioprotective benefits observed with red wine, including quercetin and its derivatives, which are most associated with the anti-thrombotic actions of wine [8–11]. Quercetin has been previously been associated with the attenuation of reactive oxygen species generation, enhancement of nitric oxide production, and blocking both surface platelet and thromboxane A_2 receptors [9,10,12], as well as inhibition of P-selectin expression, $\alpha_{iib}\beta_3$ activation, and collagen induced ATP release [13]. Subsequently, platelet cell signaling through PI3K, Akt, and MAPK activation was inhibited [13], as well as platelet aggregation, calcium mobilization and thrombus formation [14–16]. Epidemiologic studies have shown that individuals who consume diets high in quercetin have a significantly lower incidence of cardiovascular-related mortality than those who consume less, even when adjusted for standard cardiovascular risk factors [17].

Recently, several related flavonoids (*i.e.* quercetin-3-glycosides) were identified through a high-throughput screen of small molecules as potent inhibitors of protein disulfide isomerase (PDI) activity [18].

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Thiol isomerases such as PDI regulate protein activity through different mechanisms including modification of disulfide bond formation, and while the exact target of PDI in the coagulation cascade is unknown, proteins including tissue factor, VWF and $\alpha_{iib}\beta_3$, are known to be regulated by disulfide bond cleavage [19–21]. PDI plays a central role in thrombus formation and platelet aggregation *in vivo* [8] and in animal studies, quercetin-3-glycosides inhibit thrombus formation *via* a PDI-dependent mechanism [18]. As quercetin 3-glycosides are abundantly present in red but not white grapes [22], we evaluated whether red *versus* white wines variably inhibit PDI activity *in vitro*.

2. Materials and methods

All of the wine varieties were purchased from Sutter Home Vineyards (St. Helena, CA) and diluted as indicated. Red and white grape were purchased from Welch Food Inc. (Concord, MA). Recombinant PDI and all other remaining chemicals were purchased from Sigma-Aldrich (St. Louis, MO) except for ERp5 and ERp57, which were purchased from AbCam (Cambridge, MA).

Quercetin levels were determined by HPLC analysis as described previously [23]. The PDI-catalyzed reduction of insulin was assayed by measuring the increase in turbidity as described previously [24]. The PDI-catalyzed oxidation of a disulfide bond was determined with a 9-mer peptide (H3N-VTWCGACKM-NH_2) containing a disulfide bond. The peptide was synthesized and analyzed as previously described [25].

3. Results

To examine the ability of red and white wines to inhibit PDI, we utilized the insulin turbidity assay to measure PDI reductase activity and

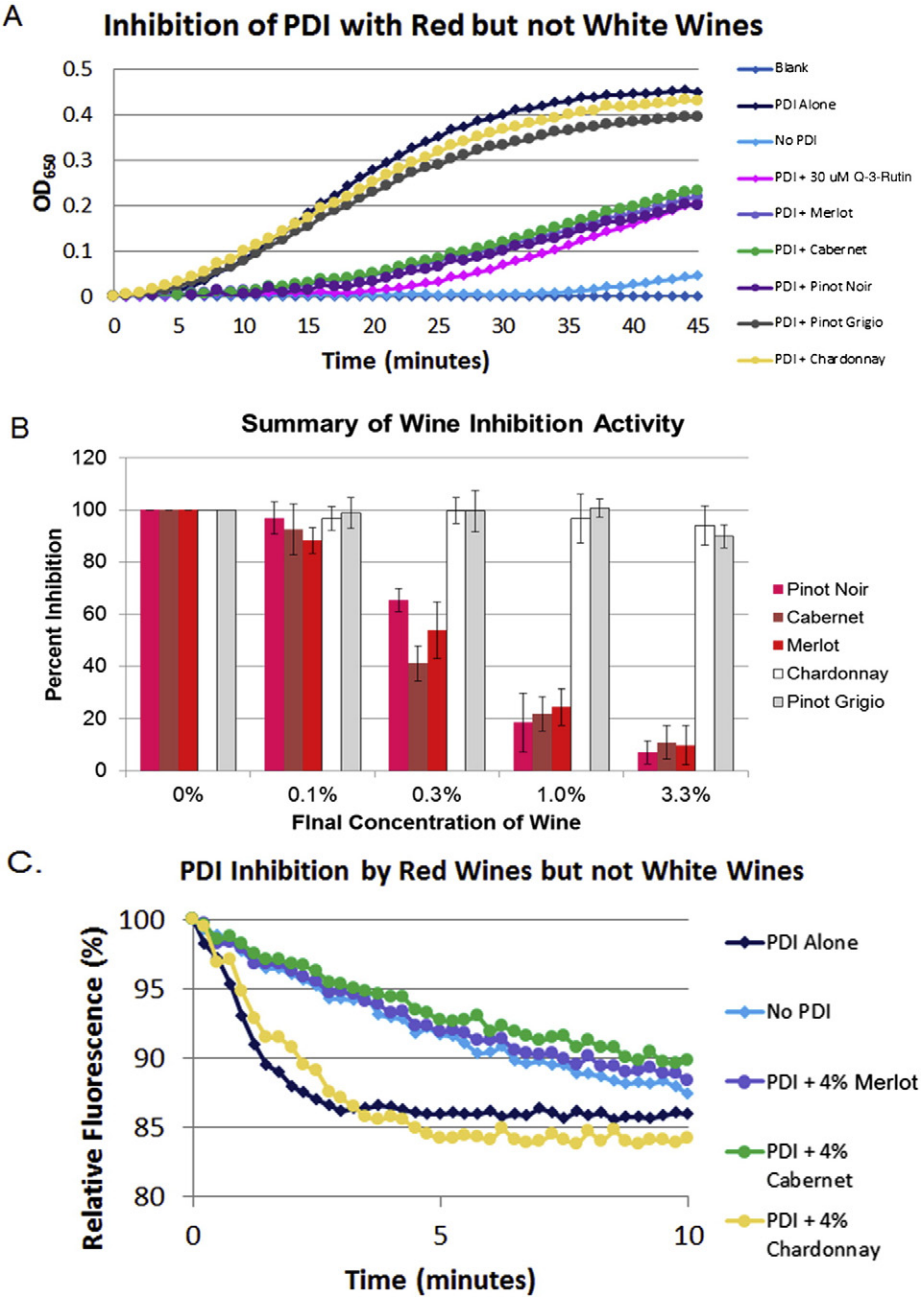

Fig. 1. Inhibition of PDI activity by red but not white wine. (A) The red wines Merlot, Cabernet and Pinot Noir and the white wines Pinot Grigio and Chardonnay were added at a final volume of 3.3% before PDI activity was assessed by the insulin turbidity assay. Q-3-Rutin = Quercetin-3-rutinoside (B) The PDI-catalyzed reduction of insulin was run for 20 min and normalized to a percentage of the control. The 20 min timepoint is displayed for each of the 5 wines examined over a range of concentrations ranging from 0.1% to 3.3%. (C) Merlot, Cabernet, and Chardonnay were added at a final concentration of 4% and the rate of tryptophan fluorescence change was observed in a disulfide bond containing peptide.