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Endothelial cell basal PGI_2 release is stimulated by wine in vitro: One mechanism that may mediate the vasoprotective effects of wine

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Wine consumption is correlated with a reduced incidence of cardiovascular disease. Experimental model systems have demonstrated that wine reduces platelet reactivity, thrombosis, and vasoconstriction. The objective of this investigation was to determine if a single mechanism could mediate these cardioprotective effects. Prostacyclin and nitric oxide are cell signaling molecules that have been described as inhibitors of vasoconstriction, platelet reactivity, and thrombosis. Endothelial cell release of these molecules was investigated because blood-borne phytochemicals can come in contact with endothelial cells. Cabernet Sauvignon, *alcoholized and dealcholized, stimulated bovine aortic endothelial cell release of prostacyclin but not nitric oxide. In addition, concentrations that significantly increased prostacyclin release were equivalent to those previously published as inducers of vasorelaxation. Prostacyclin release seemed to be dependent on basal or subbasal protein kinase C activity and occurred in the presence of the calcium ionophore ionomycin. The conclusion from this study is that if wine acts in vivo as we observed it to in vitro, the ability of wine to inhibit platelet reactivity, thrombosis, and vasoconstriction could be mediated through the single mechanism of wine-induced prostacyclin release.* (J. Nutr. Biochem. 8:647–651, 1997) © *Elsevier Science Inc. 1997*

Keywords: wine; flavonoids; prostacyclin; nitric oxide; cardiovascular disease; protein kinase C

Introduction

Currently, there is much interest in determining mechanisms to explain the observation that wine consumption is correlated with a reduced incidence of cardiovascular disease.[1–8] Experimental model systems have demonstrated that wine reduces platelet reactivity, thrombosis, and vasoconstriction.[4–8] The objective of this investigation was to determine if a single mechanism could mediate these cardioprotective effects.

Possible mechanisms through which wine may reduce platelet reactivity, thrombosis, and vasoconstriction include increased nitric oxide (NO°) release by endothelial cells (ECs) and increased prostacyclin (PGI_2) release by ECs or smooth muscle cells (SMCs).[7,9,10] These effects could also be mediated by inhibition of EC and platelet thromboxane release or increased concentrations of platelet and SMC cyclic nucleotides.[4,11]

Phytochemicals such as flavonoids are hypothesized to be important in wine-promoted vascular protection.[4,7,8] Although interaction between wine phytochemicals and SMCs may alter SMC physiology, the extent to which phytochemicals penetrate into the subendothelium and interact with smooth muscle cells is unknown. In contrast, ECs are in direct contact with blood-borne phytochemicals. Because either NO° or PGI_2 alone could mediate inhibition of vasoconstriction, platelet reactivity, and thrombosis,[11,12] and in vitro cell systems allow the examination of isolated tissue components, wine-induced EC NO° and PGI_2 release was investigated.

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Methods and materials

Materials

Bovine aortic endothelial cells (BAECs) were a gift from M.E. O'Donnell, University of California, Davis. Cells were cultured in Eagle's minimum essential medium (EMEM), purchased from Mediatech (Herndon, VA USA). Remaining cell culture materials were purchased from Sigma Scientific (Orlando, FL USA). Indomethacin, 1,2-dioctanoyl-sn-glycerol (1,2-DOG), 6-keto-prostaglandin $F_{1\alpha}$ immunoassay kits, and nitrate/nitrite fluorometric assay kits were purchased from Cayman (Ann Arbor, MI USA). Adenosine triphosphate, ionomycin, and phorbol-12-myristate-13-acetate (PMA) were purchased from CALBIOCHEM (La Jolla, CA USA). One-year-old Oakville *Cabernet Sauvignon* was donated by the Department of Viticulture and Enology, University of California, Davis.

Dealcoholization of wine

Wine containing 12% alcohol was dealcoholized at 30°C to one-half its original volume in a Brinkman (Fullerton, CA USA) rotary evaporator. After dealcoholization, concentrated wine was diluted to its original volume with Millipore-filtered water (Bedford, MA USA).

Determination of gallic acid equivalents

Gallic acid equivalents were determined as described previously.[13] A 200-μL sample was added to 10% Folin-Ciocalteu's reagent (1 mL) plus 7.5% Na_2CO_3 (800 μL). Sample absorbance was read at 765 nm after incubation for 60 min at room temperature. Gallic acid equivalents were calculated from a gallic acid standard curve.

Cell cultures and treatments

BAECs (passage 9) were seeded onto 24-well plates with EMEM containing 20 mmol/L L-glutamine, 10% fetal bovine serum, 100 units penicillin/mL, and 0.1 mg streptomycin/mL. Confluent cells were washed with sterile PBS and incubated in 500 μL phenol red-free EMEM containing 20 mmol/L L-glutamine, 100 units penicillin/mL, 0.1 mg streptomycin/mL, and treatment compound(s) when applicable. Incubation proceeded for 20 min before 200 μL medium was removed and frozen at −30°C until analyzed. Sample ethanol concentration at 0.05%, 0.1%, and 0.6% wine was 39 mg/dL, 78 mg/dL, and 468 mg/dL, respectively.

Endothelial cell integrity was monitored by trypan blue exclusion and cell morphology.[14] Cell morphology was monitored with phase contrast microscopy for altered cell contact, cell adhesion, and alterations in the polygonal endothelial cell shape. In addition to monitoring cell morphology before and after the experimental period, wells from each treatment group were observed after a 6-hr incubation.

Measurement of NO° and PGI_2

The PGI_2 metabolite 6-keto prostaglandin $F_{1\alpha}$ was measured in cell medium with Cayman enzyme immunoassay No. 515211 following the immunoassay principles described previously.[15] Incubation of sample with antibody for 18 hr prevented altered readings because of varied rates in prostacyclin breakdown and the specificity of the antibodies prevented altered readings because of changes in other eicosanoids, cell medium containing serum, or organ tissue.[16,17] NO° metabolites NO_2^- and NO_3^- were measured in cell medium with Cayman fluorometric assay No. 780051 following principles described previously.[18] In addition to conducting standard curves for these assays as instructed in the Cayman manuals, standard curves were conducted in medium and

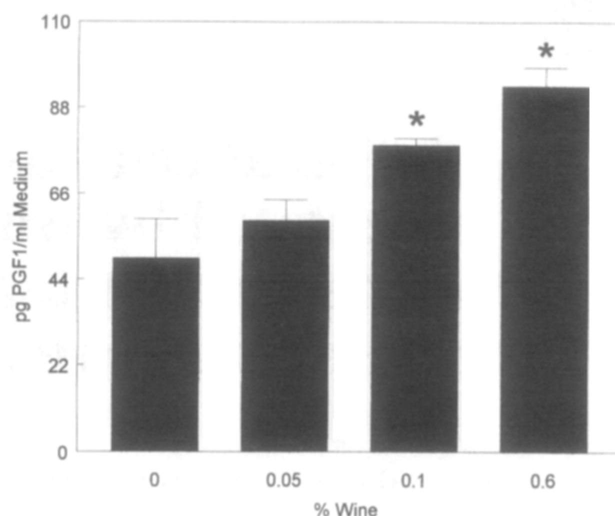

Figure 1 Culture medium was replaced with new medium before experimentation and supplemented with wine at the percents shown. Incubation proceeded for 20 min and medium was collected and analyzed by immunoassay for 6-keto-PGF1α. Eight confluent wells were used for each group. *, significantly different from control at $P < 0.05$.

in medium with wine. No interference from medium or medium with wine was observed.

Statistical analysis

Experimental data were compared by one-way analysis of variance (ANOVA) and F-test with significance assigned at a level of $P < 0.05$.

Results

Control groups averaged 95 ± 3% viability, red wine treatment groups averaged 92 ± 4% viability, and dealcoholized red wine treatment groups averaged 94 ± 2% viability. No significant differences in endothelial cell viability existed. In addition, no morphological abnormalities were observed in ECs after either 20-min or 6-hr exposure to wine.

Effect of wine on BAEC PGI_2 and NO° release

Medium incubated with BAECs plus either alcoholized or dealcoholized wine had a higher concentration of 6-keto-$PGF_{1\alpha}$ than medium incubated with BAECs alone (*Figures 1* and 2). Alcoholized wine increased medium 6-keto-$PGF_{1\alpha}$ concentration more effectively than dealcoholized wine at concentrations of 0.05 and 0.1% ($P < 0.05$). Increased cellular production of prostacyclin (PGI_2) and nitric oxide (NO°) may in some instances be coupled,[12] however, in this study, wine did not increase NO_2^- concentration (*Figure 3*).

Modulation of wine-induced BAEC PGI_2 release

The ability of wine to stimulate BAEC PGI_2 release in cells treated with high concentrations of PGI_2 release modulators is shown in *Table 1*. High concentrations of adenosine triphosphate (ATP) and ionomycin increased BAEC me-

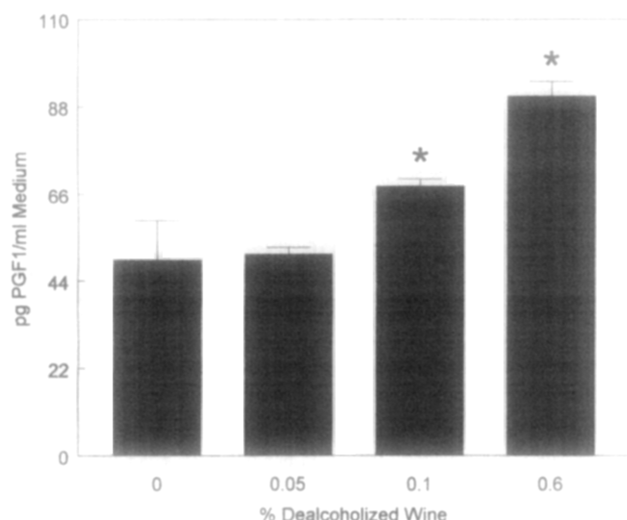

Figure 2 Culture medium was replaced with new medium before experimentation and supplemented with dealcoholized wine at the percents shown. Incubation proceeded for 20 min and medium was collected and analyzed by immunoassay for 6-keto-PGF1α. Eight confluent wells were used for each group. *, significantly different from control at $P < 0.05$.

dium 6-keto-$PGF_{1\alpha}$ concentration ($P < 0.05$), whereas high concentrations of phorbol-12-myristate-1 3-acetate (PMA) and 1,2-dioctanoyl-sn-glycerol (1,2-DOG) reduced basal medium 6-keto-$PGF_{1\alpha}$ concentration ($P < 0.05$). Wine (0.6%) increased cell medium 6-keto-$PGF_{1\alpha}$ concentration in control cells by 104%, in cells concurrently incubated with ionomycin by 107%, and in cells concurrently incubated with ATP by 149%. In contrast, wine did not significantly increase cell medium 6-keto-$PGF_{1\alpha}$ concentration in cells incubated with either PMA or 1,2-DOG.

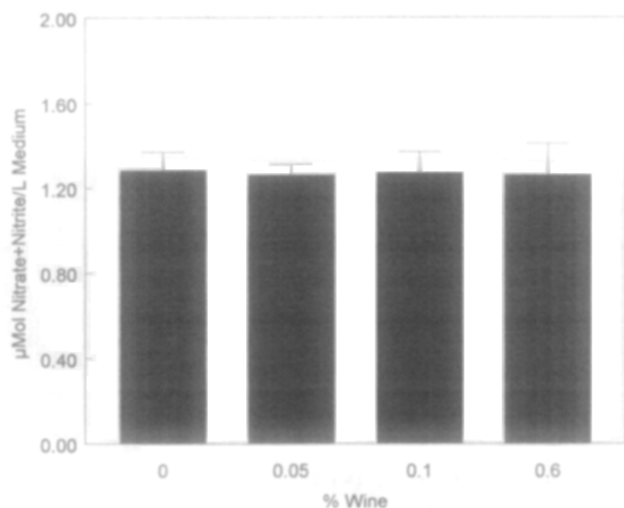

Figure 3 Culture medium was replaced with new medium before experimentation and supplemented with wine at the percents shown. Incubation proceeded for 20 min and medium was collected and analyzed by fluorometric assay for nitrate plus nitrite. Eight confluent wells were used for each group. No significant difference between groups was observed.

Indomethacin inhibited wine-induced prostacyclin release by 93 ± 3% at a concentration of 30 μmol/L.

Discussion

NO° and PGI_2 are EC cell-signaling molecules that participate in the dynamic communication system that regulates both steady state and pathologic vascular tone. In addition, they reduce platelet reactivity, thrombosis, and vasoconstriction.[11,12] Concentrations of both signal molecules in vivo are the result of a cascade of reactions but are ultimately closely associated with the activity of their respective terminal enzymes. NO° is synthesized by nitric oxide synthase, and PGI_2 by prostacyclin synthase. NO° and PGI_2 are conspicuously short lived in vivo and in vitro. Upon reaction with oxygen, NO° forms NO_3^- or NO_2^-.[18] Water reacts with PGI_2, producing 6-keto-$PGF_{1\alpha}$.[15] In this investigation, NO° was measured as NO_2^- after conversion of NO_3^- to NO_2^-, and PGI_2 as 6-keto-$PGF_{1\alpha}$. Wine and dealcoholized wine stimulated BAEC PGI_2 release; neither stimulated BAEC NO° release (*Figures 1, 2, 3*).

The major class of phenolics in wine are the flavonoids.[7,8] Flavonoids, independent of wine, demonstrate the ability to induce vasorelaxation and stimulate EC PGI_2 release.[5,6,15] Therefore, flavonoids in wine may mediate wine-induced EC PGI_2 release. Wine and wine flavonoids may affect EC PGI_2 release by directly activating PGI_2 synthase or through altering signal transduction pathways that stimulate PGI_2 production, such as increasing the activity of phospholipase C and phospholipase A2, reducing the activity of protein kinase C (PKC), increasing intracellular hydrogen ion concentration, and increasing intracellular calcium concentration.[19,20] Data from other studies suggest, however, that phospholipase (PL) activity and ATPase-dependent proton and calcium transport can be inhibited by flavonoids.[21,22] These alterations would inhibit, not stimulate, EC PGI_2 release. In contrast, because flavonoids inhibit PKC activity,[23] inhibition of PKC activity may mediate wine-induced PGI_2 release.

With the objective of gathering data that would suggest a mechanism by which wine induces PGI_2 release, the ability of wine to stimulate EC PGI_2 release in cells pretreated with modulators of PGI_2 synthesis was determined (*Table 1*). Ionomycin stimulates EC PGI_2 release by increasing intracellular calcium.[24] Cells incubated with a high ionomycin concentration (30 μmol/L) released significantly more PGI_2 (249% of the control value) than control cells. The addition of wine to ionomycin-treated cells increased PGI_2 release by 107%, to 356% of the control value. Because cells treated with wine alone increased PGI_2 release by 104%, to 204% of the control value, ionomycin-induced calcium flooding did not alter the ability of wine to increase BAEC PGI_2 release. Thus, wine may act through a mechanism that is not affected by compounds that can increase intracellular calcium.

PMA and 1,2-DOG inhibit PGI_2 release by stimulating the activity of PKC.[24,25] In agreement with results of Dava et al.,[25] the results of this investigation show a reduction in basal EC PGI_2 release by PKC activators (*Table 1*). Wine-induced PGI_2 release may be dependent on either basal PKC activity or on the ability of wine to decrease PKC activity

Table 1 Effect of PGI_2 release modulators on medium 6-keto-PGF1α concentration

Wine (%)	ATP (μmol/L)	Ionomycin (μmol/L)	PMA (μmol/L)	1,2-DOG (μmol/L)	6-Keto-PGF1α Mean (pg/mL)	6-Keto-PGF1α Range (pg/mL)	Percent (%) control	*P*
0	0	0	0	0	49.4	39.7–55.7	100	
0.6	0	0	0	0	100.8	96.3–111.6	204	<0.001
0	200	0	0	0	143.4	130.7–160.8	291	<0.001
0.6	200	0	0	0	217.1	172.3–293.1	440	<0.001
0	0	30	0	0	122.7	82.9–245.1	249	<0.001
0.6	0	30	0	0	175.5	146.3–283.5	356	<0.001
0	0	0	1	0	43.8	36.2–47.2	89	0.02
0.6	0	0	1	0	45.5	34.6–48.5	92	NS
0	0	0	0	100	40.2	31.3–53.3	82	0.03
0.6	0	0	0	100	45.1	39.2–47.7	91	0.05

Culture medium was replaced with new medium before experimentation, some of which was supplemented, as displayed above, with wine, PGI_2 release modulators, or combinations of both. Incubation proceeded for 20 min and medium fractions were collected and analyzed by immunoassay for 6-keto-PGF1α. Eight confluent wells were used for each group. NS, not statistically significant.

because wine did not stimulate PGI_2 release in the presence of high concentrations of PKC stimulators (30 μmol/L 1,2-DOG or 1 μmol/L PMA).

Flavonoid inhibition of PKC has been shown to be phospholipase and calcium independent.[5,23] Likewise, data presented within suggest that wine-induced PGI_2 release may be PKC dependent but calcium independent. In addition, the ability of flavonoids to inhibit vasoconstriction correlates with their ability to inhibit PKC,[23] and the more effective flavonoid inhibitors of PKC activity and vasoconstriction contained both 4-carbonyl and 3′-hydroxyl group structures.[5] Therefore, wine-induced PGI_2 release in vitro may be mediated by flavonoid inhibition of PKC activity.

Investigation of dietary factors that reduce the frequency and severity of vascular disease is important because diet is easily altered and vascular disease causes over 500,000 deaths annually.[26] Wine consumption is believed to reduce the frequency and severity of vascular disease in humans,[1–3] effects that may be mediated by reducing platelet reactivity, thrombosis, and vasoconstriction.[4,8,7] Because little evidence is available that documents significant subendothelial passage of phytochemicals in vivo, endothelial cell alterations, not SMC alterations, may mediate the vasoprotective properties of wine. Wine concentrations observed to stimulate PGI_2 synthesis in this investigation are similar to those observed by Fitzpatrick et al. to be necessary for vasorelaxation.[7] Furthermore, only a small portion of the effect of wine was alcohol dependent, an observation supported by previous investigation.[27] Increased PGI_2 production may mediate the cardioprotective properties of wine if wine acts in vivo as we observed it to in vitro because PGI_2 release was stimulated at 0.1% wine and because PGI_2 reduces platelet aggregation, thrombosis, vasoconstriction, smooth muscle cell proliferation, and the entry of low-density lipoproteins into the arterial wall.[11]

Investigations into the effects of wine on intracellular calcium concentration, protein kinase enzyme activity, intracellular hydrogen ion concentration, and cell PL activity will provide insight into how wine alters BAEC signal transduction to increase PGI_2 release. Preliminary evidence suggests that BAEC alterations occur almost immediately because a significant increase in BAEC PGI_2 release was observed after the incubation of cells with wine for 4 min (data not shown).

Identification of the active compounds in wine is of importance because this will ultimately determine the most effective dietary vehicle for the vasoprotective molecules. Once active compounds are identified, absorption and metabolism studies will need to be conducted. Currently, little is known concerning the absorption of compounds from plant extracts such as wine. Nevertheless, if 200 mL of wine with a phenol concentration of 4.2 mmol/L was ingested, absorbed, and present in 5 L of human blood, then blood phenol concentration would increase by 168 μmol/L. In this investigation, cell medium that was 0.1% wine increased cell prostacyclin release compared with cell medium without wine. The cell medium that was 0.1% wine had a phenol concentration that was 6.7 μmol/L higher than cell medium without wine. For wine to increase blood phenol concentration by 6.7 μmol/L, 4% of the phenolic phytochemicals from 200 mL of wine would need to be present in blood at a given time. This concentration may be attainable after wine consumption.

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